Evaluation of *Catenaria anguillulae* and its potential use as a biological control agent of

Meloidogyne incognita*, *Heterodera glycines,* and *Rotylenchulus reniformis

by

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Abstract

The overall objectives of this study are 1) to isolate *Catenaria* sp. from nematode samples, grow them in pure culture, and determine the best culture media and incubation temperatures; 2) identify species of *Catenaria* found through morphological and molecular techniques; 3) test pathogenicity of *Catenaria* sp. on *Rotylenchulus reniformis*, *Meloidogyne incognita*, and *Heterodera glycines in vitro* to determine biological control potential; 4) evaluate biological control potential of isolated *Catenaria* sp. in greenhouse, microplot, and field settings. *Catenaria* sp. was isolated from *R. reniformis* and *H. glycines* and increased on 0.4% beef extract agar (BEA) plates. Sequencing of the internal transcribed spacer (ITS1) and ITS4 regions of *Catenaria* sp. DNA indicated that isolates of *Catenaria* sp. obtained from both *H. glycines* and *R. reniformis* shared a 95% identity with *C. anguillulae*. Growth tests were conducted on five different medium and BEA was the only media tested that supported growth of *C. anguillulae*. Six incubation temperatures ranging from 10-40°C indicated that *C. anguillulae* grew at temperatures of 20-35°C. *In vitro* testing found that *C. anguillulae* has the ability to infect all three nematode species tested with highest infection rates occurring on heat-killed *M. incognita* J2. In greenhouse testing, *C. anguillulae* lowered *H. glycines* cyst numbers by an average of 45.6%. *Meloidogyne incognita* population density was reduced when treated with heat-killed *M. incognita* J2 that were colonized by *C. anguillulae* in microplot testing. Field tests with *C. anguillulae* found no reduction in nematode population density at 45 days after planting under the condition used in the testing.

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Chapter 1: Review of Literature

Statement of Purpose

Plant-parasitic nematodes are pests of many crops, with an annual economic loss estimated to be

$173 billion worldwide (Elling, 2013). With many of the standard nematicides used to control

these nematodes being removed from the market, a potential biological agent such as *Catenaria*

anguillulae Sorokin could provide another tool for nematode management.

Catenaria spp.

First reported in 1876, *Catenaria* spp. belong to the small fungal family *Catenariaceae*

(Bessey, 1950). Taxonomy of *Catenaria spp*. has changed over the years due to the unique

characteristics of this genus. They are now classified in the phylum of *Blastocladiomycota*

(James et al., 2006). *Catenaria* spp. fungi are believed to be generalist feeders and have shown

the ability to consume a wide range of microscopic animals and fungi including many plant-

parasitic nematodes (Alexopoulos and Mims, 1979; Bessey, 1950). *Catenaria anguillulae* is an

endoparasitic nematophagous fungus; meaning it is a natural enemy of nematodes and has the

ability to infect and colonize the internal organs and tissues of its host using them as a source of

nutrients (Dijksterhuis et al., 1994). The density of *C. anguillulae in* soils depends on soil type

and environmental conditions. According to Vaish and Singh (2002), C. *anguillulae* is widely

distributed in soils in India, and Costa et al. (2011) stated that it is "the most common

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endoparasitic fungus attacking nematodes". *Catenaria allomycis* Couch was first discovered in

1945 as an obligate parasite of the genus *Allomyces* (Couch, 1945). *Catenaria auxuliaris* (Kuhn)

Tribe was reported to be a parasite of cyst nematodes that is widespread in *Heterodera schachtii*

Schmidt (Tribe, 1977). Castillo and Lawrence (2011) reported *C. auxuliaris* as a parasite of *R.*

reniformis colonizing up to 46% of individuals under greenhouse conditions.

Life cycle and infection process

     Catenaria anguillulae infects nematodes as a motile zoospore. The zoospores measuring

6.1-6.8 µm in length and 3.7 µm wide, have a single whiplash flagellum that measures 19-20 µm

in length (Deacon and Saxena, 1997). *Catenaria anguillulae* zoospores are able to use their

flagellum to move through films of water in the soil to find a nematode host. Sufficient soil

moisture is vital to the infection process and survival of the fungi. It is not known how the

zoospores are able to locate a susceptible host, but it is thought to be due to chemotaxis towards

secretions from the nematode (Barron, 1977). Wyss et al. (1990) state that after a zoospore has

encysted onto a host it releases chemotactic substances that attract other zoospores to the

infection site.

     When a host has been located, the zoospores encyst in clusters around the natural

openings of the nematode including the stoma, excretory pore, and anus (Curtis et al., 2011).

Once the zoospore has attached to the cuticle of a nematode, the flagellum is retracted into the

cell. The zoospore germinates by the formation of a narrow germination tube that penetrates the

nematodes through natural openings or by piercing directly through the cuticle. A swollen

vesicle forms at the tip of the germ tube (Deacon and Saxena, 1997).   Mycelium begins to grow

inside the nematode and leads to the death of the host. Reproductive bodies form on the mycelium that are either thin-walled sporangia or thick-walled resting spores (Bessey, 1950). Sporangia cytoplasm develops into zoospores and an exit tube begins to form on each sporangium (Singh et al., 1993). Once fully developed, zoospores are released one by one through the exit tube and swim off to find a host (Singh et al., 1993). Detailed descriptions of *C. anguillulae* development processes are given by Chong and Barr (1974), Deacon and Saxena (1997), Singh et al. (1993) and Singh et al. (1996).

Factors affecting the growth and pathogenicity of *C. anguillulae*

Sufficient soil moisture is vitally important to the survival and spread of *C. anguillulae*. Temperature is also an important factor that affects the growth of *C. anguillulae*. Two studies by Gupta et al. (2004) and Gupta and Singh (2004) displayed the growth and pathogenicity effects of temperature on 10 isolates of *C. anguillulae* grown on linseed oil-cake agar, beef extract agar (BEA) and in *in vitro* testing. The optimal temperature for growth in these tests varied among the isolates but consistently ranged from 30-35°C (Gupta et al., 2004; Gupta and Singh, 2004). Another influential factor in the growth and pathogenicity of *C. anguillulae* is pH of the media on which it is being grown. Gupta et al. (2004) showed that a pH ranging from 6.0-8.0 resulted in the best growth of *C. anguillulae* on artificial media while maximum pathogenicity occurred at a pH of 7.0. This study also reported that repeated sub-culturing on BEA could reduce the virulence of the pathogen. Sub-culturing *C. anguillulae* 15 times reduced the mortality rate by 33-42%; after 60 sub-cultures the mortality was reduced by 49-100% (Gupta et al., 2004). This effect of sub-culturing is important to remember when working with *C. anguillulae* as a biological control agent.

Since the discovery of *C. anguillulae,* in 1876, there have been many studies done on its use as a biological control agent. Results have led to many opinions as to whether or not *C. anguillulae* is an effective pathogen of nematodes. Some consider *C. anguillulae* to be a weak parasite of nematodes (Stirling, 2014). However, others reported *C. anguillulae* as an effective pathogen that has great potential as a biological agent of a wide range of nematodes. *Catenaria anguillulae* has been reported as a natural biocontrol agent that was able to reduce high populations of *Meloidogyne graminicola* in rice (Singh et al. 2007). It was also shown that *C. anguillulae* was capable of high infection rates of up to 100% on *Heterodera cajani* and *M. javanica* females and *Anguina tritici* juveniles (Singh et al., 1996; Singh at al., 2012).

Meloidogyne incognita

The genus *Meloidogyne* encompasses more than 90 species of root-knot nematodes (Hunt and Handoo, 2009) which are so named because of characteristic galling symptoms they cause on plant roots. Miles Joseph Berkeley made the first observation of these symptoms in 1855 when he reported the galling on cucumbers roots in England (Hunt and Handoo, 2009). Initially, Maxime Cornu placed these nematodes into the genus of *Anguillula* and it was not until 1887 that Emilio Göldi proposed the genus of *Meloidogyne* with the description of *M. exigua* (Hunt and Handoo, 2009). In 1949 the genus of *Meloidogyne* was revised by Chitwood adding four species: *M. exigua, M. incognita, M. javanica, and M. arenaria* (Hunt and Handoo, 2009).

Meloidogyne incognita (Kofoid and White) Chitwood is a detrimental pathogen to hundreds of crops around the world. *M. incognita* is commonly found in the southern part of the

United States where the climate is optimal for the growth and reproduction of the nematode. Vrain et al. (1978) showed that a temperature of at least 10°C is needed for the development of *M. incognita*. Figure 1 is a map of the U.S. indicating where *M. incognita* has been reported (Widely Prevalent Nematodes of the United States, 2017). Second stage juveniles (J2) are the infecting stage of M. incognita. *Meloidogyne incognita* are sedentary endoparasitic nematodes that establish feeding sites inside a plant root and during this J2 stage. Upon entering the root, the nematode induces the differentiation of parenchymatic root cells into giant cells (Adab et al., 2010). The giant cells can enlarge to over 400 times the size of a normal root cell and can contain more than 100 nuclei (Adab et al., 2010) as a result of repeated nuclear division without the occurrence cytokinesis (Jones and Payne, 1977). These giant cells work as a nutrient sink to feed and sustain the nematode throughout its life cycle (Cailluad et al. 2008).

Life cycle and symptoms

The life cycle of *M. incognita* begins as an egg embedded in a gelatinous matrix that is usually found on the surface of infested roots. The eggs are laid at the single cell stage but began cell division within hours (Guiran and Ritter, 1979). This cell division leads to the development of the first stage juvenile which then molts into a J2 while it is still in the egg (Moens et al., 2010). Drawing energy from food reserves in its intestine, the J2 becomes more active in the egg and repeatedly thrusts its stylet through one end of the eggshell to create an opening (Guiran and Ritter, 1979). The J2 hatches from the egg and initiates its search for a host. The J2 penetrate a root through the use of cell-wall-degrading enzymes produced in the oesophageal glands and injected by use of the stylet. After infection, the nematode migrates intercellularly and intracellularly through the root to the root tip where a feeding site is established (Abad et al.

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2003). Once giant cells have been established the nematode begins to swell and then undergoes three more molts which take the nematode thought the third and fourth stage juvenile stages and into the adult form (Moens et al. 2010). Male *M. incognita* nematodes are very rare and are not known to be needed for reproduction which is accomplished by parthenogenesis (Abad et al. 2010). The female nematodes are pear-shaped and sedentary inside the root with the exception of their head region which has the ability to move between the giant cells to feed (Eisenback and Hunt, 2010). Once the adult females reach maturity they begin to lay eggs which are deposited on the root surface in a protective gelatinous matrix (Abad et al. 2010).

The most common symptom associated with *M. incognita* infection is the formation of the galls or knots on plant roots. The process of hypertrophy along with increased cell multiplication known as hyperplasia cause theses characteristic galling symptoms (Taylor and Sasser, 1978). Small galls are approximately one or two millimeters in size and typically contain one female nematode; however, larger galls can contain several females and be one centimeter or larger in diameter (Taylor and Sasser, 1978). The galls can vary in size depending on host susceptibility and level of infection. Aboveground symptoms on infected plants can include stunting, necrosis, chlorosis, reduced yields and overall reduced quality of the crop (Agrios, 2005).

Management of *M. incognita*

Management options for *M. incognita* include cultural practices, planting resistant varieties, application of chemical nematicides or biological controls (Agrios, 2005). Cultural practices include the use of sanitation, crop rotation, cover crops, soil amendments and

solarization.   Sanitation is an important step to minimize the spread on *M. incognita* and includes removal of infected plant material as well as cleaning of farm equipment to remove any soil partials that may contain the nematode after working in infested fields (Nyczepir and Thomas, 2010). Cover crops and soil amendments are used to lessen the impact of *M. incognita* by improving overall soil health such as increasing soil organic matter or lowering soil erosion (Nyczepir and Thomas, 2010). Crop rotation is an effective method used to manage nematode populations but its usefulness for controlling *M. incognita* is limited by the polyphagous nature of the genus (Lamberti, 1979). Planting resistant varieties can be an effective way of controlling *M. incognita* population density in crops such as soybeans, but resistant varieties are not available for many agronomic crops such as corn.

Chemical nematicides are commonly divided into fumigant and non-fumigant groups. Fumigant nematicides are liquids that are injected into the soil where they volatilize to a gaseous phase. Many fumigants not only have nematicidal properties but also are proficient fungal, weed and insect biocides (Nyczepir and Thomas, 2010). Many of these fumigant nematicides such as methyl Bromide have been removed from the market in the U.S. due to environmental and human health concerns (Nyczepir and Thomas, 2010). Non-fumigant nematicides are formulated as either granular materials which are applied to the seed furrow, or liquid materials which can be applied to the soil or as a seed treatment. Unlike fumigants, these forms of nematicides must be dispersed by means of soil moisture to be effective; many have also been removed from the market due to environmental or health concerns (Nyczepir and Thomas, 2010). Due to the removal of nematicidal compounds from the market, and with few sources of

plant resistance, biological control has become more popular as a tool for nematode management in recent years.

Common biological control organisms of *M. incognita* include both bacteria and fungi that are pathogens of the nematode or that have antagonistic activity. Nematode pathogens are organisms that are directly responsible for killing the nematodes and include organisms such as *Pasteuria penetrans* and *C. anguillulae*. Though *C. anguillulae* does have the ability to infect nematode eggs, *M. incognita* are most susceptible to parasitism by the fungus when it is in the J2 stage. This J2 life stage is the only part of the life cycle where the nematode is not protected by the gelatinous matrix produced by the nematode or hosts root tissue (Guiran and Ritter, 1979).

Heterodera glycines

It is believed that *Heterodera glycines* Ichinohe was the causal agent of a disease known since 1881 in Southeast Asia as moon night disease (Riggs, 2004). Originally, this nematode was thought to be the sugar beet cyst nematode and it was not until 1952 that Ichinohe described it as a new species given the name *H. glycines* (Riggs, 2004). The first report of the nematode in the U.S. was in 1954 on soybeans in North Carolina (Winstead et al. 1955). It has now been reported in 30 states in the U.S. (Figure 2). (Tylka and Marett, 2014). *Heterodera glycines* can be found in most countries that produce soybeans.

Heterodera glycines are sedentary endoparasites of soybeans. Populations can vary in their ability to parasitize soybean cultivars. In a response to this variability of nematode populations, a group of nematologists and soybean breeders met in 1970 and developed a race test using four resistant soybean cultivars: Pickett, Peking, PI 88788, and PI 90763 (Golden et

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al., 1970). The nematode was originally classified into four races but the classification was later

expanded by Riggs and Schmitt (1988) to contain 16 races which was the method of

classification until 2002. Niblack et al (2002) developed a new classification scheme that

separated *H. glycines* into HG types based on their ability to feed on some soybean cultivars

known as indicator lines (Table 1). The nematode reproduction on these indicator lines is based

on a female index (FI). The FI is calculated as $\left(\frac{Average\ number\ of\ females\ on\ indicator\ line}{Average\ number\ of\ females\ on\ Lee74}\right) \times$

100. If the FI is greater than 10 the line is said to be positive for host compatibility (Niblack et

al. 2002). For example, a population of *H. glycines* that sustained an FI of greater than 10 on

indicator lines PI 88788, PI 209332, and PI 548316 the HG type would be 2.5.7.

Life cycle and symptoms

 Heterodera glycines reproduces by sexual reproduction. Starting the life cycle when the

egg is fertilized. The eggs may either be in the body of the female (the cyst) or in the gelatinous

matrix that forms on the posterior end of the cyst (Ishibashi et al., 1973). The eggs that are laid

in the gelatinous matrix are quicker to hatch and infect new plant roots than those that are inside

the cyst (Ishibashi et al., 1973). This delayed hatching of the eggs inside the cyst is a result of

hatching inhibitors that are in the cyst contents (Okada, 1972). First stage juveniles go through

the first molt inside the egg and become J2 (Koenning, 2004).   Once hatched, the J2 becomes

the infective stage of *H. glycines.* Soil particle size and aggregation play a large role in the

nematodes ability to move through the soil environment. Soybean leachates that are produced by

the plant and present in the soil can attract the nematode to a new host (Koenning, 2004). The *H.*

glycines J2 locates the host, penetrates the root, and forms a feeding site known as a syncytium

in the stele (Koenning, 2004). Unlike the giant cells associated with *M. incognita,* cell wall breakdown is thought to be the mechanism for the formation of the syncytium (Gipson et al., 1971). If the root that the nematode penetrates is of a resistant variety of soybean, a functional syncytium will not form (Koenning, 2004). Once the nematode has established the syncytium and starts to feed, the nematode molts into a third stage juvenile. This stage only lasts for 48 hours or less under optimal conditions, but during this life stage, the sex of the nematode can be determined (Koenning, 2004).   The body of females continue to swell as a fourth stage juvenile, but males become vermiform and exit the root to mate (Koenning, 2004). The body of adult female ruptures the epidermis of the root and exposes her posterior end for mating (Koenning, 2004). Once mating has occurred, the female begins to lay eggs and then her body begins senescence, turning from white to yellow to brown to form the cyst (Koenning, 2004). The cyst provides protection for up to 600 eggs that are contained within (Lawrence and McLean, 1998). Inagaki and Tsutsumi (1971) showed that the eggs inside the cyst could survive and produce viable nematodes for 9 years. At temperatures of 25°C, *H. glycines* can develop into an egg-laying adult in just 21 days' time, but this can fluctuate depending on environmental conditions (Lauritis et al., 1983).

Heterodera glycines can cause aboveground symptoms that include stunting, chlorosis, fewer seeds per pod, and fewer pods per plant (Noel, 2004). These symptoms often are mistaken for other biotic and abiotic stresses that commonly occur in soybeans. Typically H. glycines damage appears in circular or oval-shaped patterns in an infested soybean field (Tylka, 1995). However, Wang et al. (2003) found that significant yield losses can occur when no aboveground symptoms are visible. Root symptoms can include stunted root growth with a decrease in the

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number of nitrogen-fixing nodules and increased susceptibility to other soil-borne pathogens (Tylka, 1995). If the roots are carefully removed from the soil, the adult female nematodes and cysts will be visible as small lemon-shaped protrusions that are white, yellow, or brown in color (Tylka, 1995).

Management of *H. glycines*

The two most commonly used and effective management strategies for *H. glycines* are rotation with non-host crops and planting of resistant varieties of soybeans. Crop rotation can be an effective management strategy but to be effective, multiple years of a non-host needs to be planted (Francl and Dropkin, 1986). However, Porter et al. (2001) found that even after five years of growing corn (a non-host) *H. glycines* populations could recover within two years of planting susceptible soybeans.

Planting resistant cultivars can have much the same effect as crop rotation in lowering population density and increasing yields. However, due to the fluctuating performance of resistant cultivars to variability in nematode virulence, use of resistant cultivars can be difficult. McCann et al. (1982) demonstrated that it was possible to select populations of *H. glycines* that were able to feed on multiple resistant varieties from a single soil sample. This means that in a single field, nematodes may vary in their ability to feed on resistant varieties, and the continuous planting of the same resistant variety, or different varieties that obtained their resistance from the same source, could result in the loss of their effectiveness in that field. Planting resistant soybeans that obtain their resistance from different sources such as PI 88788 and Peking can prevent this situation (Niblack and Chen, 2004). A rotation scheme that involves both non-host

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crops and resistant varieties can be effective in controlling *H. glycines*. One such rotation scheme that was suggested by Tylka (1995) was year 1) non-host, 2) soybean with PI 88788 source of resistance, 3) non-host, 4) soybean with Peking source of resistance, 5) non-host, and 6) susceptible soybean. However, almost all soybeans that are resistant to *H. glycines* have a source of resistance derived from PI 88788 making it difficult to switch sources of resistance (Tylka and Mullaney, 2016). This could and has already in Iowa, lead to an increased virulence on soybean varieties with a PI88788 source of resistance (McCarville et al., 2017).

Some nematicides are labeled for management of *H. glycines,* however, their effectiveness can depend on environmental factors (Tylka, 1995). Nematicides also do not provide season-long control as nematode population density tends to rebound by the end of the season (Tylka, 1995). Biological agents provide another option for management of *H. glycines*. There are a few times in the *H. glycines* life cycle when the nematode is susceptible to parasitism by biological agents such as *C. anguillulae*. These include the J2 stage and the adult male stage. During these stages the nematode is motile in the soil and therefore susceptible to soil-dwelling organisms (Chen, 2004). Another susceptible stage is when the eggs are contained in the cyst and gelatinous matrix; the close proximity of the eggs provides an opportunity for hundreds of eggs to be destroyed in a small area (Chen, 2004).

Rotylenchulus reniformis

Rotylenchulus reniformis Linford and Oliveira, the reniform nematode, was first described in 1940 from specimens collected from cowpeas in Hawaii (Linford and Oliveira, 1940). The nematode is a semi-endoparasitic pathogen with a very wide host range that includes

at least 314 plant species (Wang, 2013). *Rotylenchulus reniformis* is spread throughout the tropical, subtropical, and warm temperate regions of the world including North America, Central America, South America, Africa, Southern Europe, India, Australasia, China, Japan, and the Philippines just to name a few (Robinson et al. 1997). As a major pathogen of cotton, in the United States, the nematode is widespread throughout a large part of the cotton growing regions of the country (Figure 3). In United States cotton production, it is estimated that more than 205,000 bales of cotton were lost to *R. reniformis* in the 2016 production season (National Cotton Council of America, 2017).

Life cycle and symptoms

The life cycle of *R. reniformis* begins with eggs that undergo the process of embryogenesis and develops the first stage juvenile (Robinson et al, 1997). In the egg, the nematode undergoes its first molt and develops into a J2 and hatches from the egg (Robinson et al. 1997). The nematode remains vermiform and in the soil through two more molts, producing third and fourth stage juveniles; with each molt, the nematode becomes slightly shorter and smaller in volume (Bird, 1983). The nematodes go through one final molt to produce the adult stages of the nematode, which are vermiform in shape. The female nematodes are parasitic but the males do not feed (Robinson et al. 1997). The final molt of some populations of *R. reniformis* produces equal numbers of males and females while other populations can produce few to no males and reproduce parthenogenetically (Nakasono, 1983).

Once they have reached the adult stage, the females will enter the root cortex and establish feeding sites inside the stele; at this point the nematodes become sedentary (Robinson

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et al., 1997). About one-third of the body, the head region, is inside the root while the rest of the body is exposed to the soil and swells into the reniform (kidney) shape for which the nematode is named (Sivakumar and Seshadri, 1971). The adult female starts to produce the gelatinous matrix after it attains the reniform shape and within seven or eight days of entering a root they will begin to lay eggs (Sivakumar and Seshadri, 1971).   A study conducted by Sivakumar and Seshadri (1971) found an average of 45 eggs per egg mass but that number can be much higher. The time it takes to complete a life cycle can vary depending on temperature, soil conditions, and host availability. The typical life cycle will last from 3-6 weeks but can take as long as 2 years, due to delay in the vermiform life stages, if a host is not present and if the soil remains dry (Robinson et al., 1997).

On cotton, the major aboveground symptom caused by *R. reniformis* is yield loss. In newly, infested fields plant stunting can be seen and may be irregular in its appearance, giving the cotton crop a wavy appearance, but as the population density increases, stunted growth symptoms will become uniform across the field (Robinson, 2007). Along with the stunting, the leaves can take on an off-green appearance that can be confused with potassium deficiency (Robinson, 2007). Flowering and fruit set of infected plants can be delayed and if plants are removed from the soil and roots are gently washed, soil particles can be seen adhering to the egg masses that are produced by the nematode (Robinson, 2007). Yield losses in infested fields can be as high as 40-60% (Birchfield and Jones, 1961; Groover et al. 2017; Till et al. 2016).

Management of *R. reniformis*

According to Sasser (1972) "the most expedient and widely used method" for controlling plant-parasitic nematodes in cotton, including *R. reniformis*, is chemical control through the use of nematicides. Chemical control of *R. reniformis* can be effective in lowering population density in the early season and can support an increase in yield, but the results depend on environmental conditions throughout the season. The population density tends to rebound by the end of the season. This may be due to the ability of *R. reniformis* to live deep in the soil below the effects of nematicides (Robinson, 2007; Robinson et al., 2005). Cultural practices, such as crop rotation can be an important tool in the management of *R. reniformis* but can be difficult due to the nematode's wide host range. In cotton production, rotating with non-host crops such as corn, peanuts, or resistant soybeans can lower nematode population density and increase yields in subsequent cotton crops; however, population density tends to rebound during the first year cotton is replanted (Robinson, 2007). There are no cultivars of cotton on the market that are resistant to *R. reniformis* so this is not an option for management (Robinson, 2007).

Many biological agents have been identified and studied for their control of *R. reniformis*. McLean et al. (2001) reported multiple fungi colonized vermiform *R. reniformis* and showed the ability of some to reduce nematode population density. Castillo and Lawrence (2011) reported *C. auxiliaris* parasitizing all life stages of *R. reniformis*. Due to the fact that *R. reniformis* females and the eggs they lay are partly exposed on the root surface, parasites of the females and eggs could be an effective control method (Wang et al., 2004). Biological options are becoming increasingly popular for the control of *R. reniformis* in all affected crops.

Research Objectives

The overall objectives of this study are 1) to isolate *Catenaria* sp. from nematode samples, grow them in pure culture, and determine the best culture media and incubation temperatures; 2) identify species of *Catenaria* found through morphological and molecular techniques.; 3) test pathogenicity of *Catenaria* sp. on *R. reniformis*, *M. incognita*, and *H. glycines in vitro* to determine biological control potential; 4) evaluate biological control potential of isolated *Catenaria* sp. in greenhouse, microplot, and field settings.

Literature Cited

Abad, P., Favery, B., Rosso, M. N., and Castagnone-Sereno, P. 2003. Root-knot nematode parasitism and host response: molecular basis of a sophisticated interaction. Molecular Plant Pathology 4:217-224.

Abad, P., Castagnone-Sereno, P., Rosso, M., Engler, J., and Favery, B. 2010. Invasion, Feeding and Development. Pp 163-181 *in* R. N. Perry, M. Moens, and J. L. Starr, ed. Root-knot Nematodes. Oxfordshire: CAB International.

Agrios, G. R. 2005. Plant Pathology, Fifth edition. Pp 825-874. Burlington: Elsevier Academic Press.

Alexopoulos, C. J. and Mims, C. W. 1979. Introductory Mycology, Third edition. Pp 127. New York: John Wiley & Sons, Inc.

Barron, G. L. 1977. The Nematode-Destroying Fungi. Pp 59-61. Ontario: Canadian Biological Publications.

Bessey, E. A. 1950. Morphology and Taxonomy of Fungi. Pp 78-86. New York: Hafner Press.

Birchfield, W. and Jones, J. E. 1961. Distribution of the reniform nematode in relation to crop failure of cotton in Louisiana. Plant Disease Reporter 45:671-673.

Bird, A. F. 1983. Growth and molting in nematodes: changes in the dimensions and morphology of *Rotylenchulus reniformis* for start to finish of molting. International Journal of Parasitology 13:201-206.

Caillaud, M. C., Dubreuil, G., Quentin, M., Perfus-Barbeoch, L., Lecomte, P., de Almeida Engler, J., Abad, P., Rosso, M. N., and Favery, B. 2008. Root-knot nematodes manipulate plant cell functions during a compatible interaction. Journal of Plant Physiology 165:104-113.

Castillo, J. D. and Lawrence K. S. 2011. First report of *Catenaria auxiliaris* parasitizing the reniform nematode *Rotylenchulus reniformis* on cotton in Alabama. Plant Disease 95:490.

Chen, S. Y. 2004. Management with biological methods. Pp 207-242 *in* D. P. Schmitt, J. A. Wrather, and R. D. Riggs, ed. Biology and Management of Soybean Cyst Nematode, Second edition. Marceline: Schmitt and Associates of Marceline.

Chong, J. and Barr, D. J. 1974. Ultrastructure of zoospores of *Entophlyctis confervae-glomeratae*, *Rhizophydium patellarium* and *Catenaria anguillulae*. Canadian Journal of Botany 52:1197-1204.

Costa, S. R., van der Putten, W. H., and Kerry, B. R. 2011. Microbial ecology and nematode control in natural ecosystems. Pp. 39-64 *in* K. Davies and Y. Spiegel, ed. Biological Control of Plant-Parasitic Nematodes: Building Coherence between Microbial Ecology and Molecular Mechanisms. New York: Springer.

Couch, J. N. 1945. Observations on the Genus *Catenaria*. Mycologia 37:163-193.

Curtis, R. H., Jones, J. T., Davies, K. G., Sharon, E., and Spiegel, Y. 2011. Plant nematode surfaces. Pp 115-144 *in* K. Davies and Y. Spiegel, ed. Biological Control of Plant-Parasitic Nematodes: Building Coherence between Microbial Ecology and Molecular Mechanisms. New York: Springer.

Deacon, J. W. and Saxena, G. 1997. Orientated zoospore attachment and cyst germination in *Catenaria anguillulae*, a facultative endoparasite of nematodes. Mycological Research 101:513-522.

Dijksterhuis, J., Veenhuis, M., Harder, W., and Nordbring-Hertz, B. 1994. Nematophagous fungi: physiological aspects and structure-function relationships. Advances in Microbial Physiology 36:111-143.

Eisenback, J. D. and Hunt, D. J. 2010. General morphology. Pp 18-54 *in* R. N. Perry, M. Moens, and J. L. Starr, ed. Root-knot Nematodes. Oxfordshire: CAB International.

Elling, A. A. 2013. Major emerging problems with minor *Meloidogyne* species. Phytopathology 103:1092-1102.

Francl, L. J. and Dropkin, V. H. 1986. *Heterodera glycines* population dynamics and relation of initial population to soybean yield. Plant Disease 70:791-795.

Gipson, I., Kim, K. S., and Riggs, R. D. 1971. An ultrastructural study of syncytium development in soybean roots infected with *Heterodera glycines*. Phytopathology 61:347-353.

Golden, A. M., Epps, J. M., Riggs, R. D., Duclos, L. A., Fox, J. A., and Bernard, R. L. 1970. Terminology and identity of infraspecific forms of the soybean cyst nematode (*Heterodera glycines*). Plant Disease Reporter 54:544-546.

Groover, W., Lawrence, K., Glass, K., Till, S. R., Hall, M., Dyer, D. R., and Dodge, D. 2017. Yield loss of twelve cotton cultivars due to the reniform nematode and the added benefit of Velum Total. Pp 216-219 *in* Proc. Beltwide Cotton Conference National Cotton Council of America. Dallas, TX.

Guiran, G. and Ritter, M. 1979. Life cycle of *Meloidogyne* species and factors influencing their development. Pp. 173-191 *in* R. Lamberti and C. E. Taylor, ed. Root-knot Nematodes (*Meloidogyne* species) Systematics, Biology and Control. New York: Academic Press.

Gupta, R. C. and Singh K. P. 2004. Effect of temperature on biology of different isolates of *Catenaria anguillulae*. Mycobiology 32:17-23.

Gupta, R C., Vaish, S. S., Singh, R. K., Singh, N. K., and Singh, K. P. 2004. Effects of temperature, pH and sub-culturing on virulence of *Catenaria anguillulae* Sorokin. Current Nematology 15:101-108.

Hunt, D. J. and Handoo, Z. A. 2009. Taxonomy, identification and principal species. Pp. 55-97 *in* R. N. Perry, M. Moens, and J. L. Starr, ed. Root-knot Nematodes. Oxfordshire: CAB International.

Inagaki, H. and Tsutsumi, M. 1971. Survival of the soybean cyst nematode, *Heterodera glycines* Ichinohe (Tylenchida: Heteroderidae) under certain storing conditions. Applied Entomology and Zoology 6:156-162.

Ishibashi, N., Kondo, E., Muraoka, M., and Yokoo, T. 1973. Ecological significance of dormancy in plant-parasitic nematodes I. Ecological differences between eggs in gelatinous matrix and cyst of *Heterodera glycines* Ichinohe. Applied Entomology and Zoology 8:53-63.

James, T. Y., Letcher, P. M., Longcore, J. E., Mozly-Stanbridge, S. E., Porter, D., Powell, M. J., Giffith, G. W., and Vilgalys, R. 2006. A molecular phylogeny of flagellated fungi (*Chytridiomycota*) and description of a new phylum (*Blastocladiomycota*). Mycologia 98:860-871.

Jones M. G. K. and Payne, H. L. 1977. Early stages of nematode-induced giant-cell formation in roots of *Impatiens balsamina.* Journal of Nematology 10:70-84.

Koenning, S. R. 2004. Population biology. Pp 73-88 *in* D. P. Schmitt, J. A. Wrather, and R. D. Riggs, ed. Biology and Management of Soybean Cyst Nematode, Second edition. Marceline: Schmitt and Associates of Marceline.

Lamberti, F. 1979. Chemical and cultural nematodes of control. Pp 405-423 *in* F. Lamberti and C. E. Taylor, ed. Root-knot Nematodes (*Meloidogyne* species) Systematics, Biology and Control. New York: Academic Press.

Lauritis, J. A., Rebois, R. V., and Graney, L. S. 1983. Development of *Heterodera glycines* Ichinohe on soybean, *Glycine max* (L.) Merr., under gnotobiotic conditions. Journal of Nematology 15:272-281.

Lawrence, G. W. and McLean, K. S. 1998. Plant-parasitic nematode pests of soybean Pp 291-310 *in* L. G. Heatherly and H. F. Hodges, ed. Soybean Production in the Midsouth. Boca Raton: CRC Press.

Linford, M. B. and Oliveira, J. M. 1940. *Rotylenchulus reniformis*, nov. gen. n. sp., a nematode parasite of roots. Proceeding of the Helminthological Society of Washington 7: 35–42.

McCann, J., Luedders, V D., and Dropkin, V. H. 1982. Selection and reproduction of soybean cyst nematodes on resistant soybeans. Crop Science 22:78-80.

McCarville, M. T., Marett, C. C., Mullaney, M. P., Gebhart, G. D., and Tylka, G. L. 2017. Increase in soybean cyst nematode virulence and reproduction on resistant soybean varieties in Iowa from 2001 to 2015 and the effects on soybean yield. Plant Health Progress 18:146-155.

McLean, K. S., Lawrence, G. W., Palmateer, A. J., and Morgan-Jones, G. 2001. Endoparasitic fungal colonist of *Rotylenchulus reniformis*. Phytopathology 91:S138 Abstract.

Moens, M., Perry, R. P., and Starr, J. L. 2010. *Meloidogyne* species – a diverse group of novel and important plant parasites. Pp 1-17 *in* R. N. Perry, M. Moens, and J. L. Starr, ed. Root-knot Nematodes. Oxfordshire: CAB International.

Nakasono, K. 1983. Studies on morphological physio-ecological variations of the reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira, 1940 with an emphasis on differential geographical distribution of amphimictic and parthenogenetic populations in Japan. Bulletin of National Institute of Agricultural Sciences of Japan 38:1-67.

National Cotton Council of America. 2017. National Cotton Council disease database: 1952-2016. http://www.cotton.org/tech/pest/nematode/ (accessed September 2017).

Niblack, T. L., Arelli, P. R., Noel, G. R., Opperman, C. H., Orf, J. H., Schmitt, D. P., Shannon, J. G., and Tylka, G. L. 2002. A revised classification scheme for genetically diverse populations of *Heterodera glycines*. Journal of Nematology 34:279-288.

Niblack, T. L. and Chen, S. Y. 2004. Cropping systems and crop management. Pp 182-206 *in* D. P. Schmitt, J. A. Wrather, and R. D. Riggs, ed. Biology and Management of Soybean Cyst Nematode, Second edition. Marceline: Schmitt and Associates of Marceline.

Noel, G. R. 2004. Soybean response to infection. Pp 131-151 *in* D. P. Schmitt, J. A. Wrather, and R. D. Riggs, ed. Biology and Management of Soybean Cyst Nematode, Second edition. Marceline: Schmitt and Associates of Marceline.

Nyczepir, A. P. and Thomas S. H. 2010. Current and future management strategies in intensive crop production systems. Pp 412-443 *in* R. N. Perry, M. Moens, and J. L. Starr, ed. Root-knot Nematodes. Oxfordshire: CAB International.

Okada, T. 1972. Hatching inhibitory factor in the cyst contents of the soybean cyst nematode, *Heterodera glycines* Ichinohe. Applied Entomology and Zoology 7:99-102.

Porter, P M., Chen, S. Y., Reese, C. D., and Klossner, L. D. 2001. Population response of soybean cyst nematode to long term corn-soybean cropping sequences in Minnesota. Agronomy Journal 93:619-626.

Riggs, R. D. 2004. History and distribution. Pp 9-39 *in* D. P. Schmitt, J. A. Wrather, and R. D. Riggs, ed. Biology and Management of Soybean Cyst Nematode, Second edition. Marceline: Schmitt and Associates of Marceline.

Riggs, R. D. and Schmitt, D. P. 1988. Complete characterization of the race scheme for *Heterodera glycines*. Journal of Nematology 20:392-395.

Robinson, A. F. 2007. Reniform in U. S. cotton: when, where, why, and some remedies. Annual Review of Phytopathology 45:263-288.

Robinson, A. F., Inserra, R. N., Caswell-Chen, E. P., Vovlas, N., and Troccoli, A. 1997. *Rotylenchulus* species: identification, distribution, host ranges, and crop plant resistance. Nematropica 27:127-180.

Robinson, A. F., Akridge, G., Bradford, J. M., Cook, C. G., Gazway, W. S., Kirkpatrick, T. L., Lawrence, G W., Lee, G., McGawley, E. C., Overstreet, C., Padgett, B., Rodriguez-Kabana, R., Westphal, A., and Young, L. D. 2005. Vertical distribution of *Rotylenchulus reniformis* in cotton fields. Journal of Nematology 37:265-271.

Sasser, J. N. 1972. Nematode diseases of cotton. Pp 187-214 *in* J. M. Webster, ed. Economic Nematology. New York: Academic Press.

Singh, K. P., Jaiswal, R. K., and Kumar, N. 2007. *Catenaria anguillulae* Sorokin: a biocontrol agent of *Meloidogyne graminicola* causing root-knot disease on rice (*Oryza sativa* L.). World Journal of Microbiology and Biotechnology 23:291-294.

Singh, K. P., Stephen, R. A., and Makeshkumar, T. 1993. Development of *Catenaria anguillulae* in *Heterodera cajani*. Mycological Research 97:957-960.

Singh, K. P., Stephen, R. A., and Vaish, S. S. 1996. Pathogenicity and development of *Catenaria anguillulae* on some nematodes. Mycological Research 100:1204-1206.

Singh, K. P., Vaish, S. S., Kumar, N., Singh, K. D., and Kumari, M. 2012. *Catenaria anguillulae* as an efficient biological control agent of *Anguina tritici in vitro.* Biological Control 61:185-193.

Sivakumar, C. V. and Seshadri, S. R. 1971. Life history of the reniform nematode *Rotylenchulus reniformis* Linford and Oliveira, 1940. Indian Journal of Nematology 1:7-20.

Stirling, G. R. 2014. Biological Control of Plant-Parasitic Nematode, Second edition Soil Ecosystem Management in Sustainable Agriculture. Pp 101-156. Oxfordshire: CAB International.

Taylor, A. L. and Sasser, J. N. 1978. Biology, Identification and Control of Root-knot Nematodes. Pp 17-19. North Carolina: North Carolina State University Graphics.

Till, S. R., Lawrence, K., Glass, K., and Schrimsher, D. 2016. Evaluation of cotton cultivars in the presence and absence of reniform nematode and the efficacy of Velum Total. Pp

590-592 *in* Proc. Beltwide Cotton Conference National Cotton Council of America. New Orleans, LA.

Tribe, H. T. 1977. A parasite of white cysts of *Heterodera*: *Catenaria auxiliaris*. Transactions of the British Mycological Society 69:367-376.

Tylka, G. L. 1995. Soybean cyst nematode. Iowa State University Extension Pm-879.

Tylka, G. L. and Marett, C. C. 2014. Distribution of the soybean cyst nematode, *Heterodera glycines*, in the United States and Canada; 1954 to 2014. Plant Health Progress 15:85-87.

Tylka, G. L. and Mullany, M. 2016. Soybean cyst nematode-resistant soybeans for Iowa. Iowa State University Extension and Outreach PM 1649.

Vaish, S. S. and Singh, K. P. 2002. Distribution of *Catenaria anguillulae* Sorkin, a facultative endoparasite of nematodes in soils from different location of India. World Journal of Microbiology & Biotechnology 18:65-67.

Vrain, T. C., Barker, K. R., and Holtzman, G. I. 1978. Influence of low temperature on rate of development of *Meloidogyne incognita* and *M. hapla* larvae. Journal of Nematology 10:166-171.

Wang, J., Niblack, T. L., Tremain, J. A., Wiebold, W. J., Tylka, G. L., Marett, C. C., Noel, G. R., Myers, O., and Schmidt, M. E. 2003. Soybean cyst nematode reduces soybean yield without causing obvious aboveground symptoms. Plant Disease 87:623-628.

Wang, K. 2013. Reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira (Nematoda: Tylenchida: Tylenchoidea: Hoplolaimida: Rotylenchulinae). University of Florida IFAS Extension EENY-210.

Wang, K., Riggs, R. D., and Crippen, D. 2004. Suppression of *Rotylenchulus reniformis* on cotton by the nematophagous fungus ARF. Journal of Nematology 36:186-191.

Widely Prevalent Nematodes of the United States. 2017. *Meloidogyne incognita* (Kofoid & White) Chitwood distribution. https://www.prevalentnematodes.org/subject.cfm?id=3708 (accessed September 2017.

Widely Prevalent Nematodes of the United States. 2017. *Rotylenchus reniformis Linford & Oliveira* distribution. https://www.prevalentnematodes.org/subject.cfm?id=21271 (accessed September 2017.

Winstead, N. N., Skotland, C. B., and Sasser, J. N. 1955. Soybean cyst nematode in North Carolina. Plant Disease Reporter 39: 9-11.

Wyss, U., Voss, B., and Jansson, H. 1990. *In vitro* observations on the infection of *Meloidogyne incognita* eggs by the zoosporic fungus *Catenaria anguillulae* Sorokin. Fundamental & Applied Nematology 15:133-139.

Figure 1: *Meloidogyne incognita* distribution map of United States by state (Widely Prevalent Nematodes of the United States)
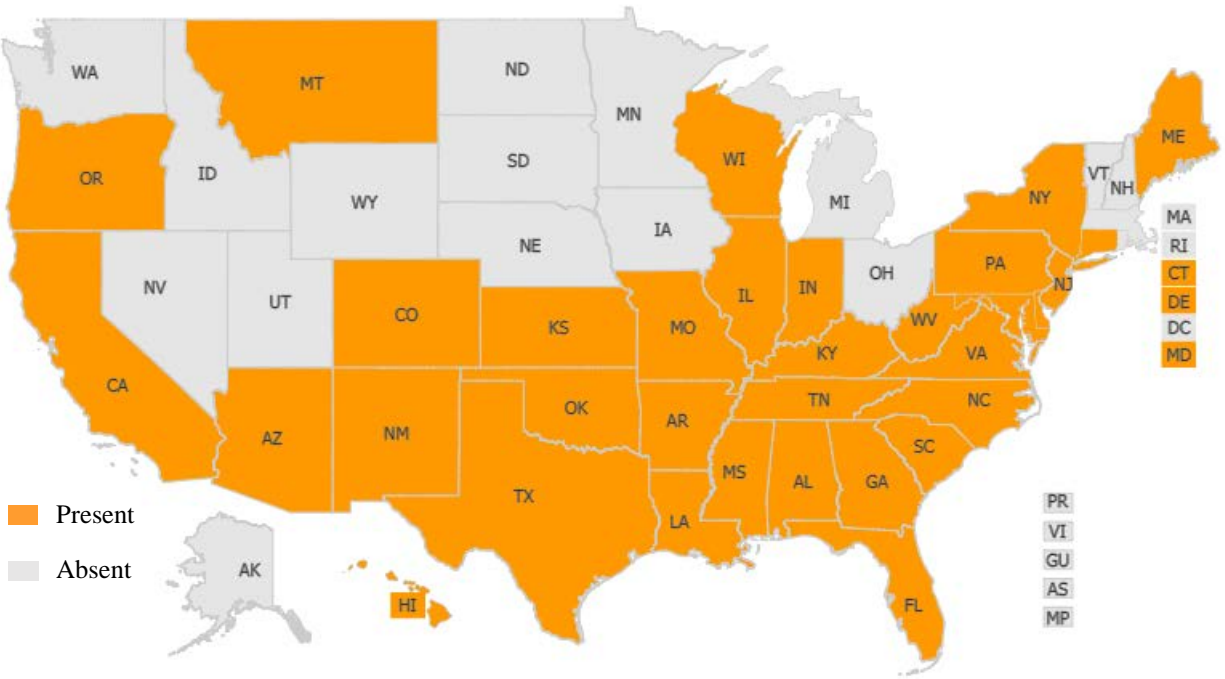
Figure 2: *Heterodera glycines* distribution map of the United States by county (Tylka and Marett, 2014).
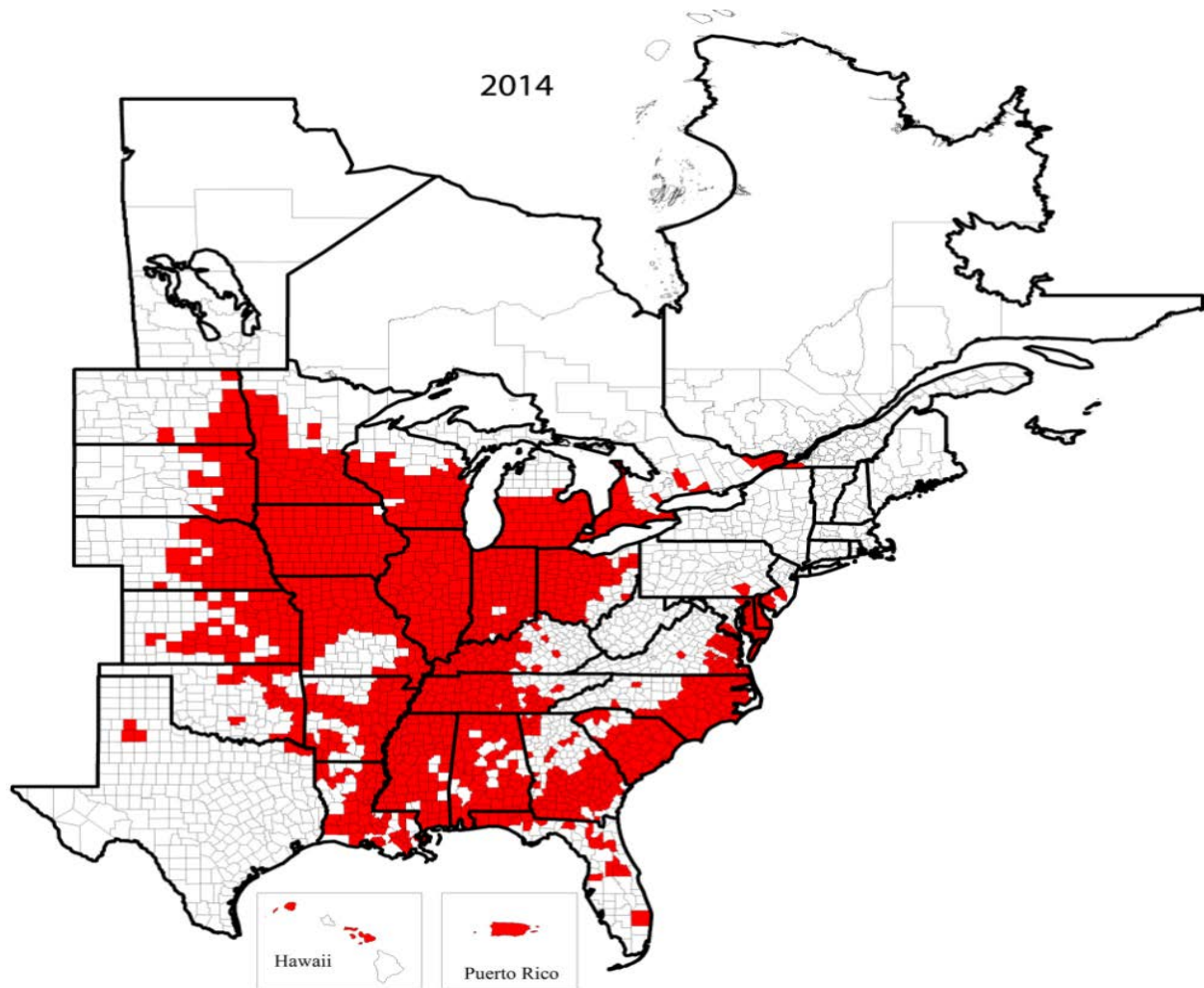
Figure 3: *Rotylenchulus reniformis* distribution map of the United States by state (Widely Prevalent Nematodes of the United States, 2017)
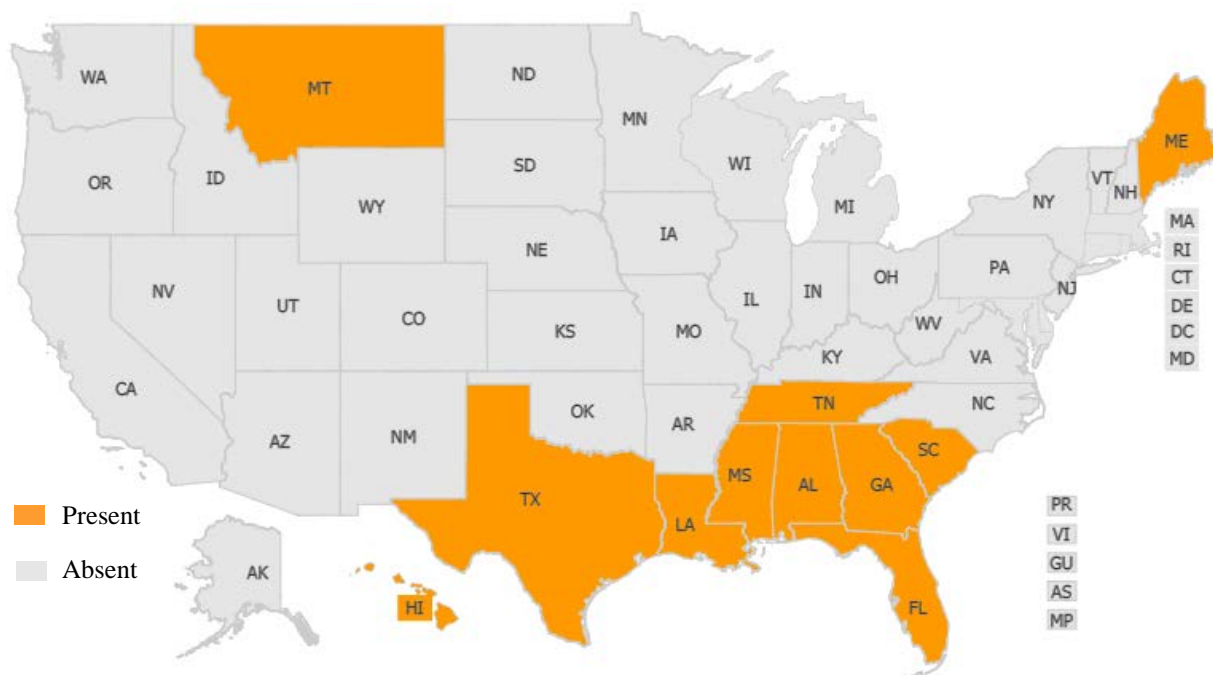
Table 1: Indicator lines for HG type classification of *Heterodera glycines*

HG Type Index Number	HG Type Indicator Line
1	PI 548402 (Peking)
2	PI 88788
3	PI 90763
4	PI 437654
5	PI 209332
6	PI 89772
7	PI 548316 (Cloud)

Chapter 2: Evaluation of *Catenaria* sp. as a potential biological control agent through *in vitro*, greenhouse, microplot and field studies

Abstract

The overall objectives of this study are 1) to isolate *Catenaria* sp. from nematode samples, grow them in pure culture, and determine the best culture media and incubation temperatures; 2) identify species of *Catenaria* found through morphological and molecular techniques; 3) test pathogenicity of *Catenaria* sp. on *Rotylenchulus reniformis*, *Meloidogyne incognita*, and *Heterodera glycines in vitro* to determine biological control potential; 4) evaluate biological control potential of isolated *Catenaria* sp. in greenhouse, microplot, and field settings. *Catenaria* sp. was isolated from *R. reniformis* and *H. glycines* and increased on 0.4% beef extract agar (BEA) plates. Sequencing of the internal transcribed spacer (ITS1) and ITS4 regions of *Catenaria* sp. DNA indicated that isolates of *Catenaria* sp. obtained from both *H. glycines* and *R. reniformis* shared a 95% identity with *C. anguillulae*. Growth tests were conducted on five different media and BEA was the only medium tested that supported growth of *C. anguillulae*. Growth tests at six incubation temperatures ranging from 10-40°C indicated that *C. anguillulae* grew at temperatures of 20-35°C. *In vitro* testing found that *C. anguillulae* has the ability to infect all three nematode species tested with highest infection rates occurring on heat-killed *M. incognita* J2. In greenhouse testing, *C. anguillulae* lowered *H. glycines* cyst numbers by an average of 45.6%. *Meloidogyne incognita* population density was reduced when

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treated with heat-killed *M. incognita* eggs that were colonized by *C. anguillulae* in microplot

testing. Field tests with *C. anguillulae* found no reduction in nematode population density at 45

days after planting under the condition used in the testing.

Introduction

Meloidogyne incognita (Kofoid and White) Chitwood is an economically important

pathogen to hundreds of crops in the United States and around the world. The nematode's

common name, southern root-knot nematode, is derived from its characteristic galling symptom

observed on plant roots when the nematode is present. Miles Joseph Berkeley made the first

observation of these symptoms in 1855 when he reported galling on cucumbers roots in England

(Hunt and Handoo, 2009). Root-knot nematodes are sedentary endoparasites that form giant

cells inside the plant root after the second stage juvenile (J2) establishes a feeding site that serves

as a nutrient sink (Adab et al., 2010). Management of *M. incognita* includes a combination of

cultural practices that include planting of resistant varieties, chemical nematicides and the use of

biological control agents. Many of the chemical nematicides that are used for management have

been removed from the market in the United States due to environmental and human health

concerns (Nyczepir and Thomas, 2010).

Heterodera glycines Ichinohe, the soybean cyst nematode (SCN), was first reported in

1915 and was thought to be the sugar beet cyst nematode. It was not identified as a new species

until the work of Ichinohe in 1952 (Riggs, 2004). The first reporting of the nematode in the U.S.

came in 1954 when it was discovered in North Carolina (Winstead et al. 1955). Above ground

symptoms of *H. glycines* can include stunting, chlorosis, fewer seeds per pod, and fewer pods per

plant that result in yield losses (Noel, 2004). However, Wang et al. (2003) found that significant

yield losses can occur even when no aboveground symptoms such as chlorosis or stunting are

visible. Management of *H. glycines* relies heavily on crop rotation and the planting of resistant

cultivars. The fluctuating performance of resistant cultivars to variability in nematode virulence

makes relying only on resistance an unreliable method of control of H. glycines.

Rotylenchulus reniformis Linford and Oliveira was first described in 1940 from samples

of cowpeas in Hawaii (Linford and Oliveira, 1940). The nematode is a semi-endoparasite

pathogen with a wide host range that includes at least 314 plant species (Wang, 2013). Due to

this wide host range, the nematode is found throughout the world in tropical, subtropical and

warm temperate regions (Robinson et al., 1997). *Rotylenchulus reniformis* is a major pathogen

of cotton in the United States, where yield losses can be as high as 40-60% (Birchfield and

Jones, 1961). With no resistant varieties of cotton or biological control products available in the

United States, management of *R. reniformis* is heavily dependent upon chemical nematicides.

These can be effective in lowering population density early in the season and provide an increase

in yield but the results depend on environmental conditions, including soil moisture and

temperature. Even when used effectively, nematode population density is known to rebound by

the end of the season (Robinson, 2007). Because of the limited number of nematode control

methods available at this time, biological control is a needed and a valuable tool to help in

management efforts.

First reported in 1876, *Catenaria* spp. fungi are believed to be generalist feeders and have

shown the ability to feed on a wide range of soil-dwelling microorganisms, including many

nematodes (Barron, 1977). *Catenaria* spp. can be very common in soils; it has been found in

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90% of all Ontario farmland samples (Barron, 1977) and is also known to be widely distributed in Indian soils (Vaish and Singh, 2002). Classified in the phylum of *Blastocladiomycota*, *Catenaria* spp. begin their infection process as a zoospore (Figure 1C) that is capable of movement by a single whiplash flagellum (Deacon and Saxena, 1997). There have been contradicting reports on the capabilities of *Catenaria* spp. as a biological control agents (Stirling, 2014; Singh et al., 2007; Singh et al., 1996). It has been reported as a natural biocontrol agent able to reduce high populations density of *M. graminicola* in rice (Singh et al., 2007). *Catenaria anguillulae* has also been shown to produce high infection rates and up to 100% parasitism on *H. cajani* and *M. javanica* females and *Anguina tritici* juveniles (Singh et al., 1996). *Catenaria auxuliaris* (Kuhn) Tribe was reported to be a parasite of cyst nematodes and is commonly found in *Heterodera schachtii* Schmidt (Tribe, 1977). Castillo and Lawrence (2011) reported *C. auxuliaris* as a parasite of *R. reniformis* with colonization up to 46% of individuals under greenhouse conditions.

This study looks at the ability of a *Catenaria* sp. to reduce population density of *M. incognita*, *H. glycines* and *R. reniformis*. The overall objectives of this study are 1) to isolate *Catenaria* sp. from nematode samples, grow them in pure culture, and determine the best culture media and incubation temperatures; 2) identify species of *Catenaria* found through morphological and molecular techniques; 3) test pathogenicity of *Catenaria* sp. on *Rotylenchulus reniformis*, *Meloidogyne incognita* and *Heterodera glycines in vitro* to determine biological control potential; and 4) evaluate biological control potential of isolated *Catenaria* sp. in greenhouse, microplot, and field settings.

Materials and Methods

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Catenaria sp. isolation

Catenaria sp. was originally observed colonizing stock cultures of *H. glycines* and *R. reniformis* that were being increased at the Plant Science Research Center (PSRC) located in Auburn, Alabama during 2016. The nematode cultures were grown in 500 cm³ polystyrene pots planted with soybean and cotton. *Heterodera glycines* and *R. reniformis* were extracted from the pots by means of gravity sieving utilizing a 250-µm pore sieve to remove trash and the vermiform nematodes were collected on a 45-µm pore sieve. The sieve contents were collected and sucrose centrifugation was performed following the procedure of Jenkins (1964). After centrifugation, samples were collected on a 25-µm pore sieve and rinsed with water to remove the sucrose solution.

Samples of the vermiform *H. glycines* and *R. reniformis* were then examined under a Nikon TSX 100 inverted microscope (Nikon; Tokyo, Japan) at 40 times magnification. Individuals that appeared to be colonized by *Catenaria* sp. were removed by means of a 0.5-10 µl pipette and placed into separate wells (one per well) of a 96-well plate containing 50µ of sterile water. Colonized vermiform nematodes were observed with sporangia inside the nematode bodies. It was observed that on some sporangia the exit tubes had begun to form (Figure 1A, B). Once all colonized nematodes had been removed from samples, 150-200 heat-killed, vermiform nematodes of the respective species were added to each well of the 96-well plate that contained colonized nematodes. Nematodes were heat-killed by first pouring the solution containing the vermiform nematode over a 25-µm pore sieve to remove excess water. Samples were then washed from the sieve into 1.5 mL microcentrifuge tubes and were centrifuged at 3913 g-forces for one minute in a MiniSpin plus (Eppendorf; Hamburg,

Germany). Excess water was removed from the nematodes collected at the bottom of the tube.
One thousand mL of water was microwaved, about two minutes, in a beaker until water
temperature reached 70°C. The microcentrifuge tubes, containing the nematodes, were then
immersed in the water for 7 minutes. The tubes were removed from the 70°C water, one mL of
sterile room temperature water was added, and tubes were shaken to disperse nematodes.

Plates were incubated at room temperature for 48 hours until the majority of the
nematodes contained in the wells were colonized. At this time, individual nematodes that
showed signs of colonization were removed using a 0.5-10 µl pipette, rinsed in sterile water and
then placed on a 0.4% beef extract agar (BEA) plate. This media was chosen based on the work
of Couch (1945) who was able to grow *Catenaria* on beef liver extract agar. BEA plates were
made by mixing 4 grams of beef extract power (Hardy Diagnostics; Santa Maria, CA) and 15
grams of agar powder (Alfa Aesar; Haverhill, MA) into one liter of water. This was sterilized in
a SET-M Analog Sterilimatic Sterilizer (Market Forge Industries Inc.; Burlingotn, VT). After
sterilization, five mL of medium was poured into 15 mm petri dishes (VWR International;
Radnor, PA) and allowed to cool. After five days of incubation at room temperature, the
mycelium was aseptically transferred to new BEA plates while being examined under a Nikon
TSX 100 inverted microscope at 40 times magnification to remove any contaminants.

DNA extraction

After five days of growth in pure culture, a five mm mycelium plug was transferred to a
new BEA plate that had been covered with a sterilized cellophane sheet to aid in the collection of
mycelium for DNA extraction. These plates were incubated for two weeks at room temperature

to allow mycelial growth. After two weeks the mycelium was collected and prepared for PCR

by the protocol contained within a ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research,

Irvine, CA). PCR amplification was performed in a 50µL reaction. Each reaction contained 4µL

of fungal DNA, 2µL of forward and 2µL of reverse primers, 24µL of JumpStart REDTaq Ready

Mix (Sigma-Aldrich, St. Louis, MO), and 18µL of ddH$_2$O. The mixture was placed in a

MultiGene DNA thermal cycler (Labnet International: Edison, NJ). PCR amplification consisted

of 94°C for five minutes followed by 40 cycles of 94°C for one minute, 55°C for one minute,

and 72°C for two minutes. The 40 cycles were followed by 72°C for ten minutes, ITS 1 (5'-

TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') were

used. PCR products were sent to Eurofines Genomics (Louisville, KY) for sequencing.

Optimal growth medium

Catenaria sp. was propagated on heat-killed *H. glycines* and *R. reniformis* vermiform life

stages, as described previously. After 48 hours of incubation at room temperature, when the

majority of the nematodes had become colonized, individuals were removed from the wells and

placed onto one of five types of agar plates. The media tested were BEA, potato dextrose agar

(HiMedia; West Chester, PA), potato carrot agar (HiMedia; West Chester, PA), oatmeal agar

(Honeywell Research Chemicals; Mexico City, Mexico), and cornmeal agar (Neogen

Corporation; Lansing, MI). Tests were arranged in a randomized complete block design

(RCBD) with five replications and the test was repeated once. After five days of incubation at

room temperature, sections of the mycelium were aseptically transferred to new plates of the

same media. Plates were examined under a Nikon TSX 100 inverted microscope at 40 times

magnification to ensure visibly pure cultures. These plates were then incubated for seven days at room temperature and at that time the diameter of mycelial growth was measured to determine growth on each medium (Table 1).

Optimal growth temperature

All tests for fungal growth were performed with the fungi grown on 0.4% BEA. One 5 mm mycelial plug was removed from an existing pure culture of *Catenaria* sp. and placed in the center of a new BEA plate. Plates were then incubated at one of six temperatures (10, 20, 25, 30, 35 and 40°C). The test was arranged in a RCBD with five replications and repeated once. Mycelium growth diameter measurements were made at 24 hours and at 3, 5, 7, 11 and 15 days after incubation was initiated (Table 2).

Nematode inoculum preparation

Eggs were extracted from stock cultures grown at the PSRC. Eggs of *M. incognita* and *R. reniformis* were extracted by first washing roots in water to remove excess soil. Roots were then placed in a 0.625% NaOCl solution and shaken for four minutes at one g-force on a Barnsted Lab Line Max Q 5000E Class shaker (Conquer Scientific: San Diego, CA) which is a modified methodology initially described by Hussey and Barker (1973). This step was followed by a rinse with water and physical scrubbing of the roots. Nematode eggs were collected on a 25-µm pore sieve and washed into a 50 mL centrifuge tube and sucrose centrifugation was performed following the procedure of Jenkins (1964). After centrifugation, samples were collected on a 25-µm pore sieve and rinsed with water to remove the sucrose solution.

Nematodes were enumerated using a Nikon TSX 100 inverted microscope at 40 times magnification.

Heterodera glycines eggs were obtained by washing roots in a 3.8-liter container to dislodge cysts and females. Water containing the cysts was poured through an 850-µm and 250-µm pore sieve to separate trash from the cysts and females. Cysts and females were collected from the 250-µm pore sieve and ground three times with a mortar and pestle for five minutes to release the eggs. After grinding, H. glycines eggs were collected on a 25-µm pore sieve and washed into a 50 mL centrifuge tube and sucrose centrifugation was performed a previously described. Eggs of M. incognita, H. glycines and R. reniformis were enumerated with a Nikon TSX 100 inverted microscope (Nikon; Tokyo, Japan).

In vitro testing for pathogenicity

Tests to determine pathogenicity of the isolated Catenaria sp. were performed on H. glycines, R. reniformis and M. incognita. Vermiform life stages of H. glycines and R. reniformis were heat-killed separately (as previously described) and one 5 mm plug of Catenaria sp. mycelium was added to each microcentrifuge tube. The tubes were then incubated at room temperature for 48 hours until the nematodes had been colonized. In three different 96-well plates, one of the three species of nematodes was added at a rate of 150-200 per well. The test included: 1) heat-killed vermiform nematodes; 2) live vermiform nematodes; 3) heat-killed nematode eggs; and 4) and live nematode eggs. One individual nematode of the respective species, colonized by Catenaria sp., was added to each well in the 96-well plate. Nematode eggs were extracted as previously described and heat-killed using the same process as applied for

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heat-killing vermiform nematodes. Plates containing *M. incognita* received a 5 mm mycelium plug that had been isolated from colonized *R. reniformis*. This test was arranged in a complete block design that contained five replicates and was repeated once. The plates were monitored for 15 days and percentage of colonization was calculated for each well based on the total number of nematodes contained in that well.

Fungal inoculum preparation

Preparation of *Catenaria* for use in greenhouse testing began by placing 300 mL of 0.4% beef extract broth into a 500 mL Erlenmeyer flasks. To each flask three 5 mm mycelium plugs of *Catenaria* sp. were added and placed on a Lab-Line Orbit Environ-Shaker (Lab-Line Instruments, Inc.; Melrose Park, IL). Flasks were shaken at 85 rpm for 14 days to facilitate fungal growth. After this time, numerous round clumps of mycelium had grown in each flask. The excess broth was decanted leaving the mycelium which was placed into a Waring Commercial model 7011S blender (Conair Corporation; Stamford, CT) and mixed at the high speed for 1 minute to break up to mycelium. Sterile water was added to dilute the mycelial suspension to the desired concentration. The concentration of the fungal suspension was measured with a spectrometer at a 600 nm wavelength to obtain an OD_{600} value. Concentrations with OD_{600} values of 0.182, 0.272, 0.377, 0.566 and 0.754 were prepared for use in subsequent testing.

Greenhouse testing

Greenhouse testing was conducted to determine the biological control potential of the *Catenaria* sp. on *M. incognita*, *H. glycines* and *R. reniformis* as well as compare it with

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commonly used nematicides. All greenhouse testing was conducted at the PSRC. The soil was a

Kalmia loamy sand texture (80% sand, 10% silt, and 10% clay; 1.2% organic matter, pH 5.8).

The soil was collected from the Plant Breeding Unit of Auburn University's E. V. Smith

Research Center located near Tallassee, AL. Before use, the soil was pasteurization at 88°C for

12 hours then allowed to cool for 24 hours before the process was repeated. Fertilizer and lime

were added to the soil at rates recommended by the Auburn University Soil Lab and mixed with

sand at a 2:1 soil to sand ratio. This soil and sand mixture was then used to fill each 150-cm^3

cone-tainer (Stuewe & Sons Inc.; Tangent, Oregon) for the tests (Figure 2). Testing of *M.*

incognita was conducted using Mycogen 2C797 (Dow AgroSciences; Indianapolis, IN) corn and

UniSouth Genetics 75T40 (UniSouth Genetics Inc.; Dickson, TN) soybean as host plants.

Testing of *H. glycines* was conducted using UniSouth Genetics 75T40 soybeans as a host plant,

and testing of *R. reniformis* was conducted using Phytogen 487WRF (Dow AgroSciences;

Indianapolis, IN) cotton as a host. Two seeds were sown into each cone-tainer at a depth of 2.5

cm and thinned to one seedling per cone shortly after emergence.

In each test, five dilutions of *Catenaria* sp. with OD_{600} values of 0.182, 0.272, 0.377,

0.566 and 0.754 were applied at a rate of one mL in a straight line across the diameter of the

cone-tainer. These treatments were compared to an untreated control and Poncho/VOTiVO

(Clothianidin and *Bacillus firmus* I-1582) (Bayer Crop Science; Leverkusen, Germany) seed

treatment at labeled rates. Seeds treatment products were applied as a slurry using a Gustafson

laboratory tabletop seed-treater. After treatment, seeds were allowed to dry for 48 hours before

planting. The control, *Catenaria* sp. and Poncho/VOTiVO treatments were compared to one

other nematicide that varied with the host crop. For testing on corn, Terbufos in the form of

Counter 20G[TM] (AMVAC Chemical Corporation; Los Angeles, CA) was applied in a line across the diameter of the cone-tainer as a granular at a rate of 7.3 kg per hectare. For testing on soybeans, Abamectin in the form of Avicta 500FS [TM] (Syngenta: Basel, Switzerland) was applied as a seed treatment at a rate of 0.1 mg of Abamectin per seed. For testing on cotton, Fluopyram and Imidacloprid in the form of Velum Total[TM] (Bayer Crop Science; Leverkusen, Germany) was applied in a line across the diameter of the cone-tainer at a rate of 1.04 L per hector. All liquid and granular treatments were made at the time of planting. To each cone-tainer, 2,000 nematode eggs were applied at the time of planting. Tests were arranged in an RCBD with five replications and repeated once. Inside the PSRC greenhouses, natural light was supplemented with the light of 1,000-watt halide bulbs producing 110,000 lumens to maintain a day length of 14 hours. Temperatures ranged from 22-34°C and tests were watered twice daily.

Greenhouse tests were terminated after 45 days. Plant parameters were measured and nematodes were extracted and enumerated. Plant shoots were clipped at the soil line and plant height (cm) and SFW (g) were measured. Roots were then gently washed to remove soil, dried with a paper towel, and weighed to obtain the RFW (g). Biomass measurements were calculated by adding the SFW and RFW of each plant. Eggs were enumerated as previously described. In *H. glycines* testing J2 nematodes and cysts were collected as previously described. The cysts were enumerated by use of a Nikon SMZ800 dissecting microscope (Nikon; Tokyo, Japan). *Meloidogyne incognita* and *R. reniformis* nematode numbers were converted to eggs/g of root. *Heterodera glycines* J2 and cyst numbers were converted to J2/150 cm^3 or cysts/150 cm^3 of soil.

Microplot testing

Microplot testing was conducted at two locations at the PSRC during 2017. Tests were conducted to determine the effect of *Catenaria* sp. with and without the addition of a nematicide on *M. incognita* population density using UniSouth Genetics 75T40 soybeans as a host. Microplots consisted of a pot within a pot design with one 25 L plastic tree pot placed inside an identical pot with a brick in the middle of the two pots. Microplots were filled with the same Kalmia loamy sand used in greenhouse tests. A second set of microplots, which were also located at PSRC, were used to duplicate this test. These microplots were made of a clay flue liner that was buried in the ground and contained a Marvyn loamy sand (77% sand, 9% silt, 14% clay; 1.1% organic matter, pH 6.1) soil type. Both sets of microplots representing 0.3 m of row were arranged in the same RCBD with five replications per treatment. Into each microplot, 10 soybean seeds were sown at a depth of 2.5 cm and shortly after emergence plants were thinned to 6 seedlings per microplot. An automated drip irrigation system was set to administer 30 ml of water per minute and was adjusted through the season to run for 15-45 minutes twice a day for a total of 450 – 1350 ml of water per microplot per day.

The treatments for the microplots tests included two forms of *Catenaria* sp. inoculum. The first *C. anguillulae* mycelium inoculum was grown in beef extract broth and prepared in the manner explained previously and was applied with an OD_{600} value of 0.18 at a rate of 0.75 liters per pot. The second form of inoculum was *C. anguillulae* infected nematode inoculum applied as heat-killed J2 *M. incognita* infected with *Catenaria* species. Nematodes were heat-killed and infected with *Catenaria* sp. as described previously and applied at a rate of 8,300 infected nematodes per pot in 0.75 liters of water. The six treatments included an untreated control, Avicta 500FS[TM] (seed treatment at 0.15 mg of active ingredient per seed), *C. anguillulae*

mycelium inoculum, *C. anguillulae* infected nematode inoculum, Avicta 500FS^{TM} + *C. anguillulae* mycelium inoculum, and Avicta 500FS^{TM} + *C. anguillulae* infected nematode inoculum. Tests were arranged in an RCBD and replicated 5 times for a total of 30 microplots at each location. Forty-five days after planting one plant was carefully removed from each microplot, plant parameters were measured and nematode eggs were extracted from the roots as described for greenhouse testing. After the soybeans matured, plants were clipped at the soil line and weighed to determine a final biomass.

Field testing

Field tests were conducted at Auburn University's Brewton Agricultural Research Unit located in Brewton, AL. This field location has a Benndale fine sandy loam (73% sand, 20% silt, and 7% clay; 0.8% organic matter, pH 6.7) soil type that contained an established population of *M. incognita*. Test plots consisted of two rows, 5.5 meters long, with a 0.9-meter row spacing and a 1.8-meter alley between replications. UniSouth Genetics 75T40 variety soybeans were planted at a rate of 302,500 seeds/hectare with a John Deere MaxEmerge ^{TM} planter (John Deere; Moline, IL) and Almaco cone planters (Almaco; Nevada, IA). Treatments for the test were the same six used in microplot testing with the following modifications: *C. anguillulae* mycelium inoculum was applied at a rate of 3.8 liters per plot and *C. anguillulae* infected nematode inoculum was applied at a rate of 50,000 colonized *M. incognita* J2 per plot in 3.8 liters of water. At 47 days after planting, four soybean plants were sampled from each plot and plant parameters were measured and nematode eggs extracted as described previously.

Statistical analysis

Data from temperature growth rates, greenhouse testing, microplot testing, and field trials were analyzed in SAS 9.4 (SAS Institute; Cary, NC). Regression analysis was used to examine the linear relationship between days after inoculation and mycelium growth for the six different incubation temperatures of the *Catenaria* sp. Greenhouse testing, microplot testing, and field trials were analyzed using the PROC GLIMMIX procedure. Dependent variables included mycelial growth, plant height, shoot fresh weight (SFW), root fresh weight (RFW), biomass (SFW+RFW), number of eggs per gram of root (eggs/g of root) or J2 and cysts/150 cm^3 of soil, and final biomass. Student panels were produced to determine normality of the residuals, in the case of eggs/g of root a square root transformation was required to satisfy the normal assumptions. Means were separated by use of the Tukey-Kramer method. A significance value of $P \leq 0.1$ was used.

Results

Catenaria sp. isolation and identification

Catenaria sp. was isolated from colonized *H. glycines* J2 and vermiform life stages of *R. reniformis* nematodes from greenhouse cultures and grown on 0.4% BEA. Of the 11 isolates that were collected, 10 isolates were obtained from infected *R. reniformis* and one was obtained from infected *H. glycines*. The results of sequencing the ITS1 and ITS4 regions of the DNA of 11 isolates of *Catenaria* resulted in 10 of the isolates with a 95% identity of *C. anguillulae* Sorokin and one with a 94% identity of the same species.

Testing for optimal growth medium

Five days after isolation, the only medium tested that supported growth of the fungus was BEA (Table 1). The potato dextrose agar, potato carrot agar, oatmeal agar and corn meal agar media were not able to support growth of *C. anguillulae*.

Testing for optimal growth temperature

Mycelia growth ranged from no visible growth at 10°C to 47 mm at 30°C after 15 days of incubation (Figures 3-4). At an incubation temperature of 40°C, less than 10 mm growth was seen between the 24-hour and three-day period; no further growth was observed after three more days of incubation. Growth was observed for the entire 15-day period for all incubation temperatures ranging from 20-35°C.

In vitro testing for pathogenicity

Through *in vitro* testing we learned that *C. anguillulae* had the ability to colonized J2 stages of *M. incognita* and *H. glycines* that were both heat-killed and living. *C. anguillulae* only showed low rates of colonization for heat-killed vermiform life stages of *R. reniformis* (Table 2). Initial infection of *M. incognita* and *H. glycines* was observed 48 hours after exposure to *C. anguillulae*. By the end of the 15-day period, heat-killed J2 and heat-killed eggs of *M. incognita* were colonized at a rate of 100% and 75%, respectively. Live J2 and live eggs of *M. incognita* were colonized at rates of 3% and 50% during the same time period. Heat-killed J2 and eggs of *H. glycines* exhibited 50% and 5% colonization throughout the 15 day time period. Live *H. glycines* J2 exhibited 3% colonization rate and no colonization was observed on live eggs. No colonization of *R. reniformis* vermiform life stages was observed until nine days after exposure

to *C. anguillulae*, and by the end of 15 days, 25% colonization was seen on the heat-killed vermiform nematodes.

Greenhouse testing

Results of greenhouse pathogenicity tests varied between the species of nematodes that were tested. In *R. reniformis* testing (Table 3), using cotton as a host plant, the two concentrations of *C. anguillulae* (OD_{600} of 0.182 and 0.377) resulted in an increase of plant RFW and biomass when compared to the chemical treatments of Poncho/VOTIVO[TM] and Velum Total[TM]. Plant height was increased with *C. anguillulae* at rates of 0.182 and 0.272 when compared to Poncho/VOTIVO[TM]. These increases in plant growth did not correlate with a decrease in nematode eggs/g of root. The *C. anguillulae* treatment rates did not reduce the number of nematode eggs/g of root. Only the chemical nematicide Velum Total[TM] reduced egg numbers when compared to the control.

Catenaria anguillulae applied to *M. incognita* using corn as the host (Table 4) indicated this biological treatment did not increase plant parameters measured or reduce nematode numbers. Poncho/VOTIVO[TM] increased plant height when compared to the control. Nematode eggs/g of root was significantly reduced by the chemical standard Counter[TM]. In testing of *C. anguillulae* on *M. incognita* using soybeans as a host (Table 5), the lowest concentration of *C. anguillulae* (0.182) resulted in a statistical increase in plant height.

Testing with *H. glycines* using soybeans as the host (Table 6) showed an increase in plant height, SFW, RFW and biomass by all *C. anguillulae* concentrations and the control when compared to Poncho/VOTIVO[TM] and Avicta 500FS[TM]. The number of *H. glycines* J2/150 cm³ of

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soil was only reduced by treatment with Avicta 500FS[TM] when compared to the control. All concentrations of *C. anguillulae*, with the exception of the lowest concentration (OD_{600} of 0.182), reduced the number of *H. glycines* cysts/150 cm³ of soil when compared to the control. These *C. anguillulae* concentrations supported a similar number of *H. glycines* cysts/150 cm³ of soil to that of the Poncho/VOTIVO[TM] treatment. On average, the five concentrations of *C. anguillulae* reduced *H. glycines* cyst numbers by 45.6%.

Microplot and field testing

There was a significant increase in plant growth parameters for the biological or nematicide treatments when compared to the control in microplot tests (Table 7). The *C. anguillulae* treatments with infected nematode inoculum (heat-killed J2 nematodes colonized with *C. anguillulae*) and the *C. anguillulae* mycelium + Avicta significantly reduced the number of *M. incognita* eggs/g of root. However, no significant effect was observed in final plant biomass measurements.   In the field setting (Table 8) no significant increase in plant growth parameters or reduction in nematode population density was detected from any of the treatments 45 days after planting. No effect on yield was observed for any of the treatments in this field test.

Discussion

Isolation and identification

Catenaria anguillulae was observed colonizing nematodes in greenhouse cultures and was isolated from cultures of *H. glycines* and *R. reniformis*. While many samples of *M. incognita* were also analyzed, *C. anguillulae* was not detected in these greenhouse cultures.

However, in our testing, high level of parasitism of *C. anguillulae* was observed on *M. incognita*. This raises the question of why *C. anguillulae* was not found in cultures of *M. incognita*.

We determined that there was a lack of *Catenaria* species variability in samples obtained from the PSRC during 2016 – 2017. However, previously published studies by Castillo and Lawrence (2011) found *C. auxiliaris* in the same location. The lack of this species being found in the present study could be attributed to it being an obligate parasite, which would not have grown on BEA that was used to isolate *C. anguillulae*.

<u>Testing for optimal growth medium and temperatures</u>

Results for testing of the optimal growth media are consistent with the finding of Gupta et al. (2005) who found that different isolates of *C. anguillulae* varied in their growth on different media. In their testing, maximum growth of most of the 10 isolates tested was observed on linseed oil-cake agar. Results for the effects of temperature on the growth of *C. anguillulae* indicated the fungus grew between 20 and 35°C which is similar to previously published research (Gupta and Singh, 2004; Gupta et al., 2004). These two previous studies reported some isolates tested had the ability to grow at temperatures of 40°C and above; however, isolates in this test exhibited little growth at temperatures of 40°C. Gupta and Singh (2004) and Gupta et al. (2004) reported their isolates of C. *anguillulae* had the ability to grow at temperatures as low as 13°C, but in this current research and in both of these previous studies, no growth was observed at temperatures as low as 10°C. Growth at temperatures ranging from 13°C to upward of 40°C indicates *C. anguillulae* should be well suited for growth in Alabama soils. At the Brewton Agricultural Research Unit where field trials were conducted the average monthly soil

temperatures at a depth of 10.2 centimeters ranged from 15.2 to 36.9°C since January of 2010 which should be favorable for growth of *C. anguillulae* throughout the year.

In vitro testing for pathogenicity

Results of the *in vitro* testing of pathogenicity of *C. anguillulae* on the three nematode species determined that *R. reniformis* supported the least parasitism of the fungus. Infection was not observed in living vermiform or eggs of this nematode, which was surprising due to the fact that the isolate used in our studies was originally cultured from *R. reniformis*. One explanation for this could be that *R. reniformis* goes through all four molts before infecting a plant root. This can lead to the nematode being ensheathed in molted cuticles that could make it more difficult for the *C. anguillulae* zoospores to penetrate the nematodes once the nematode has progressed past the J2 life stage (Gaur and Perry, 1991). *Heterodera glycines* also demonstrated low colonization of live J2 during this test; however, in greenhouse studies, *C. anguillulae* was able to reduce population density. This could be due to the ability of *C. anguillulae* to infect the female cyst nematode when they rupture through the root surface which causes egg formation to stop (Askary, 2015). This female stage of the life cycle was not tested during this experiment.

Greenhouse testing

The variation in the nematode populations reductions for the different species of nematodes is believed to be due to the different life cycle and feeding habits of the nematodes. A reduction in *R. reniformis* population density was not observed for any of the concentrations of *C. anguillulae*. This could be again attributed to the nematodes ability to retain molted cuticles (Gaur and Perry, 1991) and also to the fact that only low levels of infection were observed on *R.*

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reniformis eggs during *in vitro* testing. *Meloidogyne incognita* testing resulted in only a small numerical reduction in the population density even though *in vitro* testing indicated the highest pathogenicity levels to this nematode. The cause of this could be the endoparasitic lifestyle of *M. incognita* that spends most of its life protected by either the gelatinous matrix or root tissue, with the J2 stage being the only exception (Guiran and Ritter, 1979). The most effective reductions in nematode population density were observed in greenhouse testing on *H. glycines*. The life cycle of these nematodes includes the J2 and adult stage that are exposed to the soil environment. The adult females swell to a size that ruptures the root epidermis and exposes her posterior end for the purposes of mating (Koenning, 2004). This provides *C. anguillulae* the opportunity to infect the adult nematode and also the chance for hundreds of eggs to be infected and destroyed when they are within the cyst (Chen, 2004).

Microplot and field testing

In microplot testing, when *C. anguillulae* infected nematode were applied a significant decrease in *M. incognita* eggs/g of root was observed. However, this was not observed with the same inoculum was applied with a seed treatment of Avicta 500 FS™. This could be a result of the numerically lower population density observed by treatments of Avicta 500 FS™. Since *C. anguillulae* zoospores only have limited energy reserves that can be used to find a new host (Stirling, 2014). When nematode populations are reduced by chemical treatments it could result in *C. anguillulae* having less of an impact. A similar theory could be used to explain the lack of reduction in population density by any *C. anguillulae* treatments in the field test. *Meloidogyne incognita* eggs/g of root numbers in field test were low in comparison to greenhouse test, a high of 175 eggs/g of root in the field vs 2,763 in the greenhouse, where reductions were observed

and thus could have resulted in a reduced efficiency of the *C. anguillulae* treatments. Singh et al.

(2007) made a similar observation in a study conducted on rice fields infested with *Meloidogyne*

graminicola. They observed fields with higher root-knot gall ratings resulting in greater effects

of *C. anguillulae*.

Overall conclusions

Catenaria anguillulae has a true predator-prey relationship with its nematode host as

described by Stirling (2014). Though *C. anguillulae* has been observed by Singh et al. (2007) to

be a natural biological agent; its predation ability seems to depend on many factors. These

factors include nematode population density, virulence of *C. anguillulae* isolate, soil conditions

and nematode host (Singh et al., 2007; Gupta et al., 2004; Singh et al., 1996). Future studies are

needed to further understand these condition as well as determine how the fungus could best be

utilized in a nematode management plan.

Literature Cited

Abad, P., Castagnon-Sereno, P., Rosso, M., Engler, J., and Favery, B. 2010. Invasion, Feeding and Development. Pp 163-181 *in* R. N. Perry, M. Moens, and J. L. Starr, ed. Root-knot Nematodes. Oxfordshire: CAB International.

Askary, T. H. 2015. Nematophagous fungi as biocontrol agent of phytonematodes. Pp 81-125 *in* T. H. Askary and P. R. P. Martinelli, ed. Biocontrol Agents of Phytonematodes. Oxfordshire: CAB International.

Barron, G. 1977. The Nematode Destroying Fungi. Guelph: Canadian Biological Publications Ltd.

Birchfield, W. and Jones, J. E. 1961. Distribution of the reniform nematode in relation to crop failure of cotton in Louisiana. Plant Disease Reporter 45:671-673.

Castillo, J. D. and Lawrence, K. S. 2011. First report of *Catenaria auxiliaris* parasitizing the reniform nematode *Rotylenchulus reniformis* on cotton in Alabama. Plant Disease 95:490.

Chen, S. Y. 2004. Management with biological methods. Pp 207-242 *in* D. P. Schmitt, J. A. Wrather, and R. D. Riggs, ed. Biology and Management of Soybean Cyst Nematode, Second edition. Marceline: Schmitt and Associates of Marceline.

Couch, J. N. 1945. Observations on the Genus *Catenaria*. Mycologia 37:163-193.

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Deacon, J. W. and Saxena, G. 1997. Orientated zoospore attachment and cyst germination in *Catenaria anguillulae*, a facultative endoparasite of nematodes. Mycological Research 101:513-522.

Gaur, H. S. and Perry, R. N. 1991. The role of the molted cuticles in the desiccation survival of adults of *Rotylenchulus reniformis*. Revue de Nematologie, 14:491-496.

Guiran, G. and Ritter, M. 1979. Life cycle of *Meloidogyne* species and factors influencing their development. Pp. 173-191 *in* R. Lamberti and C. E. Taylor, ed. Root-knot Nematodes (*Meloidogyne* species) Systematics, Biology and Control. New York: Academic Press.

Gupta, R. C. and Singh, K. P. 2004. Effect of temperature on biology of different isolates of *Catenaria anguillulae*. Microbiology 32:17-23.

Gupta, R. C., Vaish, S. S., Singh, R. K., Singh, N. K., and Singh, K. P. 2004. Effect of temperature, pH, and sub-culturing on virulence of *Catenaria anguillulae* Sorokin. Current Nematology 15:101-108.

Gupta, R. C., Vaish, S. S., Singh, R. K., Singh, N. K., and Singh, K. P. 2005. Oil cakes as media for growing *Catenaria anguillulae* Sorokin, a facultative endoparasite of nematodes. World Journal of Microbiology & Biotechnology 21:1181-1185.

Hunt, D. J. and Handoo, Z. A. 2009. Taxonomy, identification and principal species. Pp. 55-97 *in* R. N. Perry, M. Moens, and J. L. Starr, ed. Root-knot Nematodes. Oxfordshire: CAB International.

Hussey, R. S. and Barker, K. B. 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Plant Disease Reporter 57:1025-1028.

Jenkins, W. R. 1964. A rapid centrifugal-flotation technique for separating nematode from soil. Plant Disease Reporter 48:692.

Koenning, S. R. 2004. Population biology. *in* D. P. Schmitt, J. A. Wrather, and R. D. Riggs, ed. Biology and Management of Soybean Cyst Nematode, Second edition. Marceline: Schmitt and Associates of Marceline.

Linford, M. B. and Oliveira, J. M. 1940. *Rotylenchulus reniformis*, nov. gen. n. sp., a nematode parasite of roots. Proceeding of the Helminthological Society of Washington 7: 35–42.

Noel, G. R. 2004. Soybean response to infection. Pp 131-151 *in* D. P. Schmitt, J. A. Wrather, and R. D. Riggs, ed. Biology and Management of Soybean Cyst Nematode, Second edition. Marceline: Schmitt and Associates of Marceline.

Nyczepir, A. P. and Thomas S. H. 2010. Current and future management strategies in intensive crop production systems. Pp 412-443 *in* R. N. Perry, M. Moens, and J. L. Starr, ed. Root-knot Nematodes. Oxfordshire: CAB International.

Riggs, R. D. 2004. History and distribution. Pp 9-39 *in* D. P. Schmitt, J. A. Wrather, and R. D. Riggs, ed. Biology and Management of Soybean Cyst Nematode, Second edition. Marceline: Schmitt and Associates of Marceline.

Robinson, A. F. 2007. Reniform in U. S. cotton: when, where, why, and some remedies. Annual Review of Phytopathology 45:263-288.

Robinson, A. F., Inserra, R. N., Caswell-Chen, E. P., Vovlas, N., and Troccoli, A. 1997. *Rotylenchulus* species: identification, distribution, host ranges, and crop plant resistance. Nematropica 27:127-180.

Singh, K. P., Jaiswal, R. K., and Kumar, N. 2007. *Catenaria anguillulae* Sorokin: a biocontrol agent of *Meloidogyne graminicola* causing root-knot disease on rice (*Oryza sativa* L.). World Journal of Microbiology and Biotechnology 23:291-294.

Singh, K. P., Stephen, R. A., and Vaish, S. S. 1996. Pathogenicity and development of *Catenaria anguillulae* on some nematodes. Mycological Research 100:1204-1206.

Stirling, G. R. 2014. Biological Control of Plant-parasitic Nematode, Second edition Soil Ecosystem Management in Sustainable Agriculture. Oxfordshire: CAB International.

Vaish, S. S. and Singh, K. P. 2002. Distribution of *Catenaria anguillulae* Sorkin, a facultative endoparasite of nematodes in soils from different location of India. World Journal of Microbiology & Biotechnology 18:65-67.

Wang, J., Niblack, T. L., Tremain, J. A., Wiebold, W. J., Tylka, G. L., Marett, C. C., Noel, G. R., Myers, O., and Schmidt, M. E. 2003. Soybean cyst nematode reduces soybean yield without causing obvious aboveground symptoms. Plant Disease 87:623-628.

Wang, K. 2013. Reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira (Nematoda: Tylenchida: Tylenchoidea: Hoplolaimida: Rotylenchulinae). University of Florida IFAS Extension EENY-210.

Winstead, N. N., Skotland, C. B., and Sasser, J. N. 1955. Soybean cyst nematode in North Carolina. Plant Disease Reporter 39:9-11.

Figure 1: A) *Heterodera glycines* J2 colonized with *Catenaria* sp. and arrows are indicating sporangia. B) *Rotylenchulus reniformis* vermiform colonized with *Catenaria* sp. and arrows indicating exit tubes formed from sporangia. C) Zoospore of *Catenaria* sp.

Figure 2: Set up of greenhouse testing conducted in 150 cm^3 cone-tainers.

Figure 3: Plates of *Catenaria anguillulae* after 15 days of incubation at varying temperatures A) incubated at10°C, B) 20°C, C) 25°C, D) 30°C, E) 35°C, F) and 40°C.
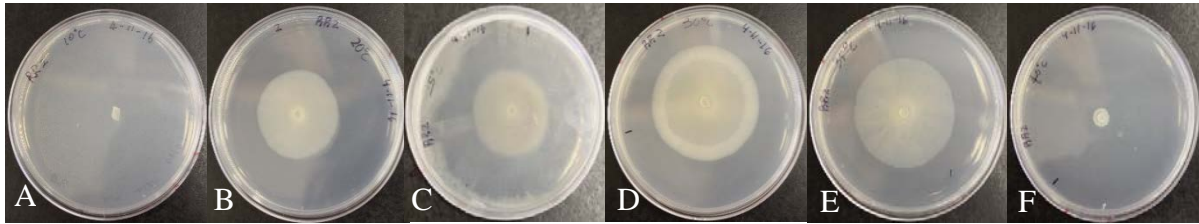
Figure 4: *Catenaria anguillulae* mycelial growth on 0.4% beef extract agar measured in diameter (mm) when subjected to incubation temperatures ranging from 10-40°C.

The figure contains the following regression equations:

10°C) $y = 0$

20°C) $y = -0.461 + 2.513x$ $R^2 = 0.97$

25°C) $y = 1.669 + 2.627x$ $R^2 = 0.92$

30°C) $y = 3.966 + 2.993x$ $R^2 = 0.94$

35°C) $y = 3.939 + 2.793x$ $R^2 = 0.95$

40°C) $y = 2.507 + 0.210x$ $R^2 = 0.05$
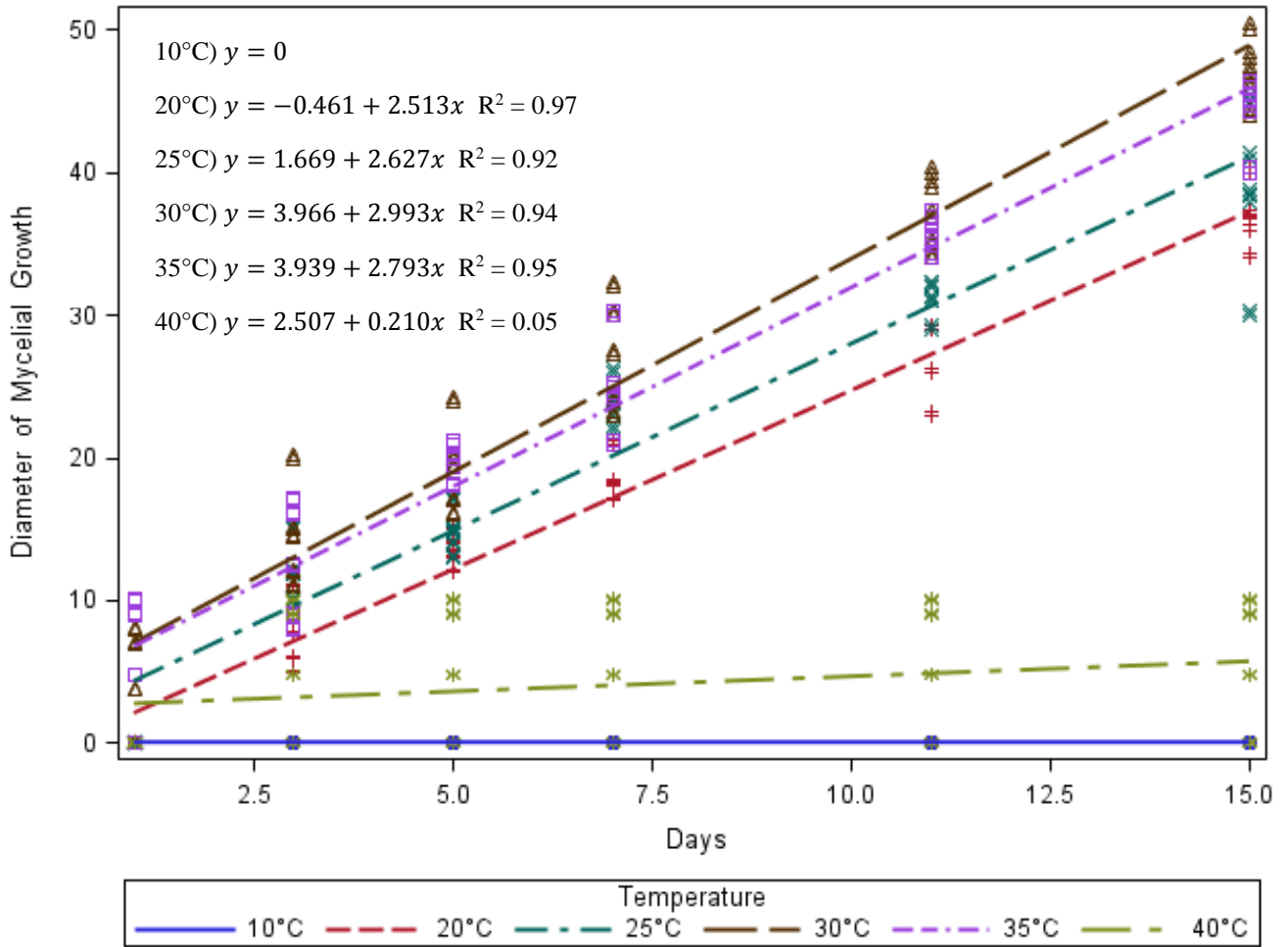
Table 1: Testing for optimal growth medium for *Catenaria anguillulae*.

Growth medium	Diameter of mycelial growth (mm)
0.4% beef extract ager	22^z
2.4% potato dextrose agar	0
2.4% potato carrot agar	0
7.5% oatmeal agar	0
1.7% corn meal agar	0

^{z} Diameter of mycelial growth after seven days of incubation in light at room temperature.

Table 2: *In vitro* testing to determine percent of colonization on heat-killed and live eggs and vermiform life stages of *Rotylenchulus reniformis*, as well as heat-killed and live eggs and J2 of *Heterodera glycines* and *Meloidogyne incognita* 20 days after exposure to *Catenaria anguillulae*.

Nematode life stage	Percent colonization *R. reniformis*	Percent colonization *H. glycines*	Percent colonization *M. incognita*
Live Eggs[y]	0[z]	0	50
Heat-killed Eggs	0	5	75
Live J2	0	3	12
Heat-killed J2	25	50	100

[y] Tests were performed in 96 well plates, with each well containing 150-200 vermiform life stages or eggs.

[z] Percent of vermiform life stages or eggs colonized by *C. anguillulae.*

Table 3: Greenhouse test to evaluate *Catenaria anguillulae* as a biological control agent of *Rotylenchulus reniformis* using cotton (Phytogen 487WRF) as a host and measured by plant height, shoot fresh weight, root fresh weight, biomass, and *R. reniformis* eggs/g of root at 45 DAP[w].

Treatment	N[y]	Plant height (cm)	Shoot fresh weight SFW (g)	Root fresh weight RFW (g)	Biomass (SFW + RFW)	*R. reniformis* eggs/g of root
Control[x]	10	32.0 ab[z]	5.8 ab	4.3 ab	10.1 ab	11052 ab
C. anguillulae $OD_{600} = \mathbf{0.182}$	10	35.8 a	6.7 a	5.2 ab	11.7 a	10165 ab
C. anguillulae $OD_{600} = \mathbf{0.272}$	10	35.6 a	5.9 ab	4.0 abc	9.7 ab	10167 ab
C. anguillulae $OD_{600} = \mathbf{0.377}$	10	30.6 ab	5.7 ab	5.4 a	11.0 a	5901 b
C. anguillulae $OD_{600} = \mathbf{0.566}$	10	31.4 ab	5.3 ab	3.6 bc	8.9 abc	14243 a
C. anguillulae $OD_{600} = \mathbf{0.754}$	10	31.4 ab	5.7 ab	3.9 abc	9.6 ab	12021 a
Poncho/VOTiVO[TM] 0.42 mg ai/seed	10	27.8 b	3.7 ab	2.4 c	6.1 c	11112 ab
Velum Total[TM] 1 L/ha	10	32.0 ab	5.0 b	2.2 c	7.2 bc	508 c

[w] Days after planting (DAP), test contained 5 replications and was repeated once.

[x] At the time of planting all pots were inoculated with 2,000 *R. reniformis* eggs.

[y] Number of observations.

[z] All values present are LS-means separated using the Tukey-Kramer method at $P \leq 0.1$. Values in the same column followed by the same letter do not differ significantly.

Table 4: Greenhouse test to evaluate *Catenaria anguillulae* as a biological control agent of *Meloidogyne incognita* using corn (Mycogen 2C797) as a host and measured by plant height, shoot fresh weight, root fresh weight, biomass, and *M. incognita* eggs/g of root at 45 DAP[w].

Treatment	N[y]	Plant height (cm)	Shoot fresh weight SFW (g)	Root fresh weight RFW (g)	Biomass (SFW + RFW)	*M. incognita* eggs/g of root
Control[x]	10	51.6 b[z]	19.8 ab	16.9 ab	36.7	2764 a
C. anguillulae OD$_{600}$ = **0.182**	10	56.0 ab	19.8 ab	15.1 ab	34.8	2639 a
C. anguillulae OD$_{600}$ = **0.272**	10	59.2 ab	18.6 ab	19.4 a	38.0	2638 a
C. anguillulae OD$_{600}$ = **0.377**	10	59.6 ab	19.8 ab	14.1 b	33.9	1815 ab
C. anguillulae OD$_{600}$ = **0.566**	10	53.8 ab	17.1 b	17.2 ab	34.3	2515 a
C. anguillulae OD$_{600}$ = **0.754**	10	60.8 ab	20.2 ab	18.7 ab	38.9	1730 ab
Poncho/VOTiVO™ 0.25 mg ai/seed	10	62.6 a	20.1 ab	14.0 b	34.1	1887 ab
Counter™ 7.3 kg/ha	10	61.6 ab	21.5 a	13.7 b	35.2	837 b

[w] Days after planting (DAP), test contained 5 replications and was repeated once.

[x] At the time of planting all pots were inoculated with 2,000 *M. incognita* eggs.

[y] Number of observations.

[z] All values present are LS-means separated using the Tukey-Kramer method at $P \leq 0.1$. Values in the same column followed by the same letter, or no letter, do not differ significantly.

Table 5: Greenhouse test to evaluate *Catenaria anguillulae* as a biological control agent of *Meloidogyne incognita* using soybean (UniSouth Genetics 75T40) as a host and measured by plant height, shoot fresh weight, root fresh weight, biomass, and *M. incognita* eggs/g of root at 45 DAP[w].

Treatment	N[y]	Plant height (cm)	Shoot fresh weight SFW (g)	Root fresh weight RFW (g)	Biomass (SFW + RFW)	*M. incognita* eggs/g of root
Control[x]	10	31.3 bc[z]	6.8	5.7	12.6	144
C. anguillulae OD$_{600}$ = **0.182**	10	38.4 a	8.0	7.2	15.1	203
C. anguillulae OD$_{600}$ = **0.272**	10	35.6 ab	9.1	5.4	14.5	196
C. anguillulae OD$_{600}$ = **0.377**	10	29.8 c	8.3	7.1	15.5	176
C. anguillulae OD$_{600}$ = **0.566**	10	36.0 ab	7.5	5.7	13.2	303
C. anguillulae OD$_{600}$ = **0.754**	10	34.4 abc	7.7	4.7	12.4	108
Poncho/VOTiVO^{TM} 0.13 mg ai/seed	10	35.7 ab	8.2	6.2	14.3	216
Avicta 500FS^{TM} 0.1 mg ai/seed	10	35.4 ab	9.7	7.3	17.0	384

[w] Days after planting (DAP), test contained 5 replications and was repeated once.

[x] At the time of planting all pots were inoculated with 2,000 *M. incognita* eggs.

[y] Number of observations.

[z] All values present are LS-means separated using the Tukey-Kramer method at $P \leq 0.1$. Values in the same column followed by the same letter, or no letter, do not differ significantly.

Table 6: Greenhouse test to evaluate *Catenaria anguillulae* as a biological control agent of *Heterodera glycines* using soybean (UniSouth Genetics 75T40) as a host and measured by plant height, shoot fresh weight, root fresh weight, biomass, and *H. glycines* eggs/g of root at 45 DAP[w].

Treatment	N[y]	Plant height (cm)	Shoot fresh weight SFW (g)	Root fresh weight RFW (g)	Biomass (SFW + RFW)	*H. glycines* J2 /150 cm^3 soil	*H. glycines* cyst /150 cm^3 soil
Control[x]	10	67.0 a[z]	11.0 a	9.1 a	20.1 a	4017 ab	818 a
C. anguillulae OD$_{600}$ = **0.182**	10	62.6 a	11.0 a	7.6 b	18.6 a	2920 ab	527 ab
C. anguillulae OD$_{600}$ = **0.272**	10	65.6 a	12.0 a	9.5 a	21.5 a	4218 ab	449 b
C. anguillulae OD$_{600}$ = **0.377**	10	65.2 a	12.2 a	9.2 a	21.4 a	5052 a	457 b
C. anguillulae OD$_{600}$ = **0.566**	10	72.6 a	10.6 a	9.3 a	19.9 a	2287 bc	488 b
C. anguillulae OD$_{600}$ = **0.754**	10	64.4 a	11.0 a	9.0 ab	20.0 a	3415 ab	394 b
Poncho/VOTiVO^{TM} 0.13 mg ai/seed	10	50.2 b	7.8 b	5.7 c	13.5 b	2024 bc	376 b
Avicta 500FS^{TM} 0.1 mg ai/seed	10	42.8 b	7.3 b	3.5 d	10.8 b	108 c	2 c

[w] Days after planting (DAP), test contained 5 replications and was repeated once.

[x] At the time of planting all pots were inoculated with 2,000 *H. glycines* eggs.

[y] Number of observations.

[z] All values present are LS-means separated using the Tukey-Kramer method at $P \leq 0.1$. Values in the same column followed by the same letter do not differ significantly.

Table 7: Microplot tests to evaluate *Catenaria anguillulae* as a biological control agent of *Meloidogyne incognita* using soybean (UniSouth Genetics 75T40) as a host and measured by plant height, shoot fresh weight, root fresh weight, biomass, and *M. incognita* eggs/g of root, and final biomass at 45 DAP[w].

Treatment	N[y]	Plant height (cm)	Shoot fresh weight SFW (g)	Root fresh weight RFW (g)	Biomass (SFW + RFW)	*M. incognita* eggs/g root	Final Biomass (g)
Control	10	45.3[z]	47.4 ab	6.8 ab	54.2 ab	320 a	230.0
C. anguillulae mycelium inoculum[x]	10	52.5	46.2 ab	6.3 ab	52.5 ab	279 ab	224.2
C. anguillulae infected nematode inoculum[x]	10	50.0	42.3 b	5.4 b	47.7 b	127 ab	210.9
Avicta 500FS[TM] 0.1 mg ai/seed	10	53.5	67.5 a	8.5 a	76.0 a	109 ab	265.2
C. anguillulae mycelium inoculum + Avicta 500FS[TM]	10	47.7	56.9 ab	7.1 ab	64.0 ab	76 b	231.1
C. anguillulae infected nematode inoculum + Avicta 500FS[TM]	10	47.9	38.0 b	5.0 b	43.0 b	478 a	217.7

[w] Days after planting, tests contained 5 replications.

[x] Mycelium inoculum consisted of *C. anguillulae* mycelium slurry ($OD_{600} = 0.18$) that was applied as a soil drench at the time of planting. Infected nematode inoculum consisted of heat-killed *M. incognita* J2 that had been infected with *C. anguillulae* applied as a soil drench at a rate of 8,300 nematodes per plot.

[y] Number of observations; table contains data from two microplot tests that had statistically similar results.

[z] All values present are LS-means separated using the Tukey-Kramer method at $P \leq 0.1$. Values in the same column followed by the same letter, or no letter, do not differ significantly.

Table 8: Field test to evaluate *Catenaria anguillulae* as a biological control agent of *Meloidogyne incognita* using soybean (UniSouth Genetics 75T40) as a host and measured by plant height, shoot fresh weight, root fresh weight, biomass, *M. incognita* eggs/g of root at 45 DAP[w], and yield.

Treatment	N[y]	Plant height (cm)	Shoot fresh weight SFW (g)	Root fresh weight RFW (g)	Biomass (SFW + RFW)	*M. incognita* eggs/g root	Yield (kg/ha)
Control	20	31.3[z]	36.4	6.7	43.1	135	1515.0
C. anguillulae mycelium inoculum[x]	20	30.9	29.8	5.6	35.4	165	1692.5
C. anguillulae infected nematode inoculum[x]	20	31.1	30.8	5.8	36.6	85	1392.4
Avicta 500FS[TM] 0.1 mg ai/seed	20	31.9	32.6	5.9	38.6	117	1352.1
C. anguillulae mycelium inoculum + Avicta 500FS[TM]	20	31.2	31.3	5.9	37.2	160	1826.4
C. anguillulae infected nematode inoculum + Avicta 500FS[TM]	20	31.5	30.4	6.3	36.7	176	1806.0

[w] Days after planting, tests contained 5 replications.

[x] Mycelium inoculum consisted of *C. anguillulae* mycelium slurry ($OD_{600} = 0.18$) that was applied as a soil drench at the time of planting. Infected nematode inoculum consisted of heat-killed *M. incognita* J2 that had been infected with *C. anguillulae* applied as a soil drench at a rate of 50,000 nematodes per plot.

[y] Number of observations.

[z] All values present are LS-means separated using the Tukey-Kramer method at P≤0.1; no statically significant differences were observed in any measurements taken during this testing.

Chapter 3: First report of *Catenaria anguillulae* infecting *Rotylenchulus reniformis* and *Heterodera glycines* in Alabama

Rotylenchulus reniformis and *Heterodera glycines* are plant pathogenic nematodes that affect a wide variety of commercially grown crops worldwide. Infection of vermiform life stages of *R. reniformis* and *H. glycines* was first observed in stock cultures of the nematodes at the Plant Science Research Center in Auburn, Alabama. Symptoms of the infection begin with sporangia forming inside the body of the nematodes. The sporangia produce zoospores that are released outside the body of the nematode through the formation of an exit tube. This differs from the *Catenaria auxiliaris* reported by (Castillo and Lawrence, 2011); *C. auxiliaris* does not form an exit tube, but sporangia erupt through the cuticle to release zoospores. *R. reniformis* and *H. glycines* were extracted from stock cultures; infected vermiform nematodes were separated by hand, washed in sterile water, and placed onto 0.4% beef extract agar (BEA) and allowed to grow for 5 days. A sample of fungal mycelium was aseptically transferred from the 5-day-old cultures using a compound microscope to ensure fungal isolate purity and transferred onto a new BEA plate. Cultures were then incubated at room temperature for 14 days to increase mycelium mass for molecular species identification. DNA was extracted and prepared for PCR using a ZR Fungal/Bacterial DNA MiniPrep kit. ITS1 and ITS4 primers were used to amplify the internal transcribed spacer (ITS) region and then sequenced by Eurofins Genomics (Louisville, KY). The results of sequencing were aligned using BioEdit, BLAST showed a 95% shared identity with *C.*

anguillulae (KY606231 and KY606232). Koch's postulates were performed through *in vitro* testing; infected nematodes were placed onto 0.4% BEA plates to grow fungal cultures that were morphology identified as *C. anguillulae*. Healthy, uninfected vermiform *R. reniformis* and *H. glycines* J2 nematodes were exposed to one *C. anguillulae* infected nematode of the respective species in 96 well plates. The plates were incubated at room temperature. The first infected nematodes were observed after 3 days, and colonized nematodes were evaluated by light microscopy to confirm *C. anguillulae* infestation. To confirm Koch's postulates, the infected nematodes were cultured on BEA plates and the resulting fungal growth was morphologically identified as *C. anguillulae*. Greenhouse tests were also conducted by inoculating *R. reniformis* and *H. glycines* cultures growing on cotton and soybean with 2 ml of a *C. anguillulae* slurry. Three slurries of the *C. anguillulae* were used, which had OD_{600} values of 0.377, 0.566, or 0.754, to compare different rates of inoculation, and they were compared with two chemical controls (Poncho/VOTiVO and ILeVO applied as seed treatments) and an untreated control. This test contained five replications and was arranged in an RCBD; data were analyzed in SAS 9.4 using PROC GLIMMIX and means were compared using the Tukey-Kramer's method with a significance level of $P \leq 0.1$. The *C. anguillulae* at any of the inoculation rates did not reduce *R. reniformis* eggs per gram of root compared with the untreated control. However, low to high inoculation rates of *C. anguillulae* significantly ($P \leq 0.1$) reduced *H. glycines* cyst numbers by 44, 40, and 52%, respectively, compared with the control. Population reductions were similar to those induced by the Poncho/VOTiVO chemical control, which reduced cyst numbers by 54% as compared to the control. *C. anguillulae* has been

observed in some Alabama soils and it is important to consider that biological interaction

between nematodes, such as *R. reniformis* and *H. glycines*, and *C. anguillulae* may occur.

Literature Cited

Castillo, J. D**.** and Lawrence, K. S. 2011. First report of *Catenaria auxiliaris* parasitizing the reniform nematode *Rotylenchulus reniformis* on cotton in Alabama. Plant Dis. 95:490. http://apsjournals.apsnet.org/doi/10.1094/PDIS-07-10-0524 (Abstract).

Appendix I: Use of a fertilizer by-product to increase plant growth and reduce *Rotylenchulus reniformis* population density on cotton in Alabama

Abstract

The overall objective of this study was to evaluate the effects of a fertilizer by-product on the population density of *Rotylenchulus reniformis*. The fertilizer by-product is obtained from the process of making the chicken feed additive methionine. Tests included comparisons of the fertilizer by-product with a commonly used fertilizer, nematicide, and fertilizer nematicide combinations. Tests compared an untreated control, the fertilizer by-product alone, 10-34-0 N-P-K fertilizer, Velum Total^{TM}, the fertilizer by-product + Velum Total^{TM}, and 10-34-0 fertilizer + Velum Total^{TM}. Evaluations were conducted in greenhouse, microplot and field settings using Phytogen 487WRF cotton as a host plant. Greenhouse and field tests found all treatments that included the nematicide Velum Total^{TM} significantly ($P \leq 0.1$) reduced the number of *R. reniformis* eggs/g of root when compared to the standard fertilizer 10-34-0 alone. Treatments of the fertilizer by-product in a field setting resulted in a reduced number of *R. reniformis* eggs/g of root when compared to the untreated control, showing that the product may have an effect on *R. reniformis* population density.

Introduction

Since it was first described in 1940, *Rotylenchulus reniformis* Linford and Oliveira, the reniform nematode, has become a very problematic nematode for many producers and in multiple crops around the world. This nematode has a wide host range that includes at least 314 plant species (Wang, 2013). In the United States, the nematode is a major pathogen of cotton and is widespread throughout midsouth and southeast parts of the cotton-growing region of the U.S. It is estimated that more than 205,000 bales of cotton were lost to *R. reniformis* in the 2016 production season (National Cotton Council of America, 2017).

It has been reported that *R. reniformis* becomes slightly shorter and smaller in volume with each molt (Bird, 1983). Due to this reduction in size, the nematode has the ability to retain its molted cuticles and these extra cuticles can aid in the protection of the nematode (Gaur and Perry, 1991). When female *R. reniformis* reaches the infective adult stage she enters the root, establishes a feeding site in the stele, and becomes sedentary (Robinson et al., 1997). Males of the species do not feed and remain vermiform in the soil. About one-third of the female's body (the head region) is embedded within the root. The posterior portion of the female's body which swells into the reniform (kidney) shape for which the nematode is named, is exposed to the soil environment (Sivakumar and Seshadri, 1971).

It has been recognized for some time that soil amendments such as fertilizers can have nematicidal effects (Rodriguez-Kabana, 1986). Badra and Khattab (1980) found that when some forms of nitrogen fertilizers are applied to the soil they can suppress *R. reniformis* levels by as much as 31%. McLean et al. (2003) found no reduction in population density of *R. reniformis* when plots were treated with anhydrous ammonia. Potassium fertilizers have also been studied for their effects on nematode population density. Minton and Ebelhar (1991) found that pre-

plant fertilization with muriate of potash, potassium chloride, reduced *Meloidogyne incognita* (Kofoid and White) Chitwood galling. However, when this fertilizer was studied for its effect on *R. reniformis*, there was an increase in population density in treated plots (Pettigrew et al., 2005). Usery (2005) found that the use of poultry litter as an organic amendment in cotton production lowered *R. reniformis* populations in greenhouse settings, but no reduction was observed in the field.

The product being tested in our study is a low-chloride nitrogen and potassium fertilizer. The product contains approximately 20, 130, and 30 grams per kilogram of nitrogen, potassium, and sulfur, respectively. The fertilizer is a liquid by-product obtained from an aqueous solution of potassium bicarbonate ($KHCO_3$) that is used to precipitate methionine. Methionine is used as a feed additive primarily in monogastric diets (Mitchell et al., 1994). The fertility effects of the product applied to pearl millet, wheat, soybeans and corn were studied by Mitchell et al. (1994). The results indicated that the fertilizer provides a suitable source of potassium and can be applied at relatively high rates in the field without harmful effects to the crop (Mitchell et al., 1994). However, a yield reduction was observed in the greenhouse when the fertilizer was applied immediately prior to planting as a result of excess salts in the soil (Mitchell et al., 1994).   The present study was initiated to look at the effects of this by-product as a fertilizer on the population density of *R. reniformis* in greenhouse, microplot and field studies.

Materials and Methods

<u>Greenhouse testing</u>

Greenhouse testing was performed at the Auburn University Plant Science Research Center (PSRC) located in Auburn, AL. The soil was a Kalmia loamy sand (80% sand, 10% silt, 10% clay with 1.2% organic matter, pH of 5.8) which was collected from the Plant Breeding Unit of Auburn University's E. V. Smith Research Center located near Tallassee, AL. Before use, the soil was pasteurized at 88°C for 12 hours then allowed to cool for 24 hours before the process was repeated. The soil was mixed with sand at a 2:1 soil to sand ratio and this mixture was then used to fill each of the 150-cm³ cone-tainers (Stuewe & Sons Inc., Tangent, Oregon) that were used for the test.

Rotylenchulus reniformis stock cultures were increased in the PSRC greenhouse. Eggs were extracted from these cultures by first washing roots in water to remove excess soil, then roots were placed in a 0.625% NaOCl solution, and shaken for four minutes at 1 g-force on a Barnsted Lab Line Max Q 5000E Class shaker (Conquer Scientific: San Diego, CA). This was followed by a rinse with water and physical scrubbing of the roots which is a modified methodology initially described by Hussey and Barker (1973). After scrubbing, nematode eggs were collected on a 25-µm pore sieve and washed into a 50 mL centrifuge tube and sucrose centrifugation was performed following the methodology of Jenkins (1964). After centrifugation, samples were collected on a 25-µm pore sieve and rinsed with water to remove the sucrose solution. Eggs were enumerated with a Nikon TSX 100 inverted microscope (Nikon; Tokyo, Japan) and diluted with water to a rate of 2,000 *R. reniformis* eggs per 1 mL of water.

Tests were conducted in the greenhouse using Phytogen 487WRF (Dow AgroSciences; Indianapolis, IN) cotton as a host. Two seeds were sown per cone-tainer at a depth of 2.5 cm, seeds were thinned to one seedling per cone-tainer shortly after seedling emergence. The six

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treatments included an untreated control, the fertilizer by-product, the nematicide Velum Total[TM] (active ingredients fluopyram and Imidacloprid) (Bayer Crop Science; Leverkusen, Germany), ammonium polyphosphate (10-34-0) liquid fertilizer (Pennington Seed, Inc.; Madison, GA), fertilizer by-product + Velum Total[TM], and 10-34-0 + Velum Total[TM]. The fertilizer by-product was pipetted onto the soil surface 10 days before planting at a rate of 1871 liters per hectare (L/ha). The 10-34-0 liquid fertilizer was pipetted beside the seed at the time of planting at a rate of 47 L/ha. Velum Total[TM] was pipetted into each cone-tainer at the time of planting at a rate of 1 L/ha. Tests were arranged in a randomized complete block design (RCBD) and run for 35 days and repeated once. Inside the PSRC greenhouse natural light was supplemented with the light of 1,000-watt halide bulbs producing 110,000 lumens to maintain a day length of 14 hours. Temperatures ranged from 22-34°C and tests were watered twice daily.

At the completion of the 35-day growing period, plants were removed from the greenhouse. Plant parameters were measured and *R. reniformis* eggs extracted and enumerated. The plant parameters measured included plant height, shoot fresh weight (SFW), root fresh weight (RFW) and plant biomass (SFW+RFW). Plant shoots were clipped at the soil line prior to measurement of plant height (cm) and SFW (g). Roots were gently washed to remove soil, dried with a paper towel, and weighed to obtain the RFW (g). *Rotylenchulus reniformis* eggs were extracted as previously described, enumerated, and numbers were converted to eggs per gram of root (eggs/g of root).

Microplot testing

Microplot testing was conducted at the PSRC during 2017 to judge the effects of the fertilizer by-product on the population density of *R. reniformis*. Microplots represent 0.3 m of row in the field and are a pot within a pot design with one 25 L plastic tree pot placed inside an identical pot with a brick separating the two pots. The microplots were filled with the same soil used for greenhouse testing. Tests in the microplots received the same six treatments that were used in greenhouse tests and were arranged in an RCBD with five replications. The fertilizer by-product was pipetted onto soil 10 days before planting at a rate of 1871 L/ha. The 10-34-0 liquid fertilizer was pipetted beside the seed at the time of planting at a rate of 47 L/ha. Velum Total[TM] was pipetted in a straight line simulating an in-furrow spray into each seed furrow at the time of planting at a rate of 1 L/ha. Into each microplot, 10 cotton seeds were sown at a depth of 2.5 cm and shortly after germination plants were thinned to six seedlings per microplot. An automated drip irrigation system was set to administer 30 ml of water per minute and was adjusted through the season to run for 15-45 minutes twice a day, for a total of 450 – 1350 ml of water per microplot per day. The test was planted on June 20, 2017.

Forty-six days after planting, one plant was carefully removed from each microplot and plant parameters were measured and nematode eggs were extracted from roots as described for greenhouse testing. Cotton was harvested at maturity to determine yield.

<u>Field testing</u>

Field testing was conducted at Auburn University's Tennessee Valley Research and Extension Center located near Belle Mina, AL. This location has a Decatur silt loam (24% sand, 49% silt, and 28% clay) soil type. At the time of planting, the field contained an average of 799

vermiform *R. reniformis* per 100 cm^3 of soil. Test plots were arranged in an RCBD with five

replication and consisted of two row plots that were 7.6 meters long with a 0.9-meter row

spacing and a 1.8-meter alley between replications. Phytogen 487WRF cotton seeds were

planted at a rate of 107,600 seeds per hectare. Seeds were planted using a John Deere

MaxEmerge ^{TM} planter (John Deere; Moline, IL) with Almaco cone planters (Almaco; Nevada,

IA). Treatments for the test were the same six used in greenhouse and microplot testing with the

following modifications. The liquid by-product was applied as a soil drench at a rate of 1871

L/ha 14 days before planting. Velum Total^{TM} was applied as an in-furrow spray 1 L/ha at the

time of planting.   Treatments of 10-34-0 were applied 5 cm below and 5 cm beside the seed

using a G2 fertilizer disk (Schaffert Manufactur8ing & Sales; Indianola, NE) at a rate of 47L/ha.

The test was planted on May 9, 2017. Forty-four days after planting, shovels were used to

remove the roots and shoots of four plants from each plot, plant parameters were measured, and

nematode eggs were extracted from roots as described for greenhouse testing. Once the cotton

reached maturity it was harvested to determine yield responses to the treatments.

Statistical analysis

Data from greenhouse, microplot, and field trials were analyzed in SAS 9.4 (SAS

Institute; Cary, NC) using the PROC GLIMMIX procedure. Dependent variables included plant

height, SFW, RFW, biomass, and eggs/g of root.   Student panels were produced to determine

normality of the residuals, in the case of eggs/g of root a square root transformation was required

to satisfy the normal assumptions. Means were separated by use of the Tukey-Kramer method

with a significance value of $P \leq 0.1$.

Results

Greenhouse testing

In greenhouse testing, (Table 1) the fertilizer by-product in combination with Velum Total^{TM} resulted in an ($P \leq 0.1$) increase in plant height when compared to the control. Neither the fertilizer by-product or Velum Total^{TM} alone produced this plant height effect. The SFW was not affected by any of the fertilizer or nematicide treatments; however, the 10-34-0 fertilizer resulted in a larger RFW then that of the fertilizer by-product + Velum Total^{TM} combination treatment. The larger root mass of the 10-34-0 also supported a higher population density of *R. reniformis* then all other treatments except the fertilizer by-product alone.

Microplot testing

In microplot testing (Table 2), the fertilizer by-product + Velum Total^{TM} resulted in an increase of plant height, SFW, RFW and biomass when compared to the standard 10-34-0 fertilizer and 10-34-0 + Velum Total^{TM}. The cotton plant height was increased by the fertilizer by-product + Velum Total^{TM} and was ($P \leq 0.1$) taller than Velum Total^{TM} alone. The fertilizer by-product + Velum Total^{TM} had significantly more *R. reniformis* eggs/g of root than treatments of the fertilizer by-product, Velum Total^{TM}, and 10-34-0 + Velum Total^{TM}.

Field testing

At 44 days after planting plant height, SFW and biomass measurements indicated that plots treated with the fertilizer by-product + Velum Total^{TM} had increased compared to all other treatments (Figure 1 and Table 3). Treatments of the fertilizer by-product + Velum Total^{TM} and

10-34-0 + Velum Total[TM] had similar RFW and both ($P \leq 0.1$) were greater than the control and identical treatments without Velum Total[TM]. The population density of *R. reniformis* eggs/g of root were lower ($P \leq 0.1$) in plots treated with the fertilizer by-product alone, Velum Total[TM] alone, the combination of the fertilizer by-product + Velum Total[TM] and the 10-34-0 fertilizer + Velum Total[TM] compared to that of the control and 10-34-0 fertilizer alone. Treatment with the fertilizer by-product also displayed a significant reduction in eggs/g of root from the control plots.

Discussion

The overall objective of this study was to evaluate the effects of a fertilizer by-product on the population density of *R. reniformis*. In general, the largest root mass was observed with standard fertilizer treatments of 10-34-0 followed closely by the addition of Velum Total[TM] to the standard fertilizer in greenhouse testing. As can be anticipated with an increased root mass, the *R. reniformis* population increased with treatments of 10-34-0 fertilizer. However, this increase was not observed when Velum Total[TM] was added to the treatment. In microplot, testing this increase in root growth was not observed with treatments of 10-34-0. Plots treated with 10-34-0 and 10-34-0 + Velum Total[TM] produced smaller plants than the control plots. Suggesting that the slightly higher nematode numbered that were found on plots treated with 10-34-0 were enough to cause significant plant damage.

Field testing demonstrated that the fertilizer by-product alone resulted in nematode eggs/g of root that were similar to treatments of Velum Total[TM] alone and in combination with the two fertilizers. Previous research has indicated that potassium fertilization increased *R. reniformis* population levels (Pettigrew et al. 2005). Low amounts of nitrogen are contained in

the fertilizer by-product and Badra and Khattab (1980) showed that nitrogen fertilizers can reduce *R. reniformis* population density when applied to cotton. However, McLean et al. (2003) found no reduction in population density of *R. reniformis* when plots were treated with anhydrous ammonia. This could be due to the need of these fertilizers to be applied at far higher rates than those required for crop fertilization to be effective at lowering the nematode population density (Rodriguez-Kabana, 1986). This suggests that other aspects of the fertilizer may act upon *R. reniformis*.

When the fertilizer by-product was applied with the addition of Velum Total™ a 45.4% reduction in *R. reniformis* eggs/g of root was observed when compared to Velum Total™ alone. This also supports the claim of this fertilizer by-product may have a nematicidal effect. This effect was enough to produce a larger plant at 45 days after planting. Continued studies are needed to determine the long-term effects of the application of this fertilizer by-product to *R. reniformis* population density and overall soil health.

Literature Cited

Badra, T. and Khattab, M. M. 1980. The effect of nitrogen fertilizers on the growth of olive and in relation to infestations of *Rotylenchulus reniformis*. Nematologia Mediterranea 8:67-72.

Bird, A. F. 1983. Growth and molting in nematodes: changes in the dimensions and morphology of *Rotylenchulus reniformis* for start to finish of molting. International Journal of Parasitology 13:201-206.

Gaur, H. S. and Perry, R. N. 1991. The role of the molted cuticles in the desiccation survival of adults of *Rotylenchulus reniformis*. Revue de Nematologie, 14:491-496.

Hussey, R. S. and Barker, K. B. 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. Plant Disease Reporter 57:1025-1028.

Jenkins, W. R. 1964. A rapid centrifugal-flotation technique for separating nematode from soil. Plant Disease Reporter 48:692.

McLean, K. S., Lawrence, G. W., Overstreet, C., and Young, L. D. 2003. Efficacy of anhydrous ammonia on reniform nematode in cotton. Pp 282-283 *in* Proc. Beltwide Cotton Conference National Cotton Council of America. Nashville, TN.

Minton, E. B. and Ebelhar, M. W. 1991. Potassium and aldicarb-disulfoton on verticillium wilt, yield, and quality of cotton. Crop Science 31:209-212.

Mitchell, C. C., Hiltbold, A. E., and Hue, H. V. 1994. Evaluation of a liquid potassium bicarbonate/amino acid co-product as a source of potassium, nitrogen, and sulfur. Journal of Plant Nutrition 17:2119-2134.

National Cotton Council of America. 2017. National Cotton Council disease database: 1952-2016. http://www.cotton.org/tech/pest/nematode/ (accessed September 2017).

Pettigrew, W. T., Meredith, W. R., and Young, L. D. 2005. Potassium fertilization on cotton lint yield, yield components, and reniform nematode populations. Agronomy Journal 97:1245-1251.

Robinson, A. F., Inserra, R. N., Caswell-Chen, E. P., Vovlas, N., and Troccoli, A. 1997. *Rotylenchulus* species: identification, distribution, host ranges, and crop plant resistance. Nematropica 27:127-180.

Rodriguez-Kabana, R. 1986. Organic and inorganic nitrogen amendments to soil as nematode suppressants. Journal of Nematology 18:129-135.

Sivakumar, C. V. and Seshadri, S. R. 1971. Life history of the reniform nematode *Rotylenchulus reniformis* Linford and Oliveira, 1940. Indian Journal of Nematology 1:7-20.

Usery, S. R. 2005.Managment of *Rotylenchulus reniformis* using poultry litter as a soil amendment. Pp 46-64 *in* S. R. Usery, C. Burmseter, K. S. Lawrence, and J. Kloepper, ed. Evaluations of cultural practices in cotton production for management of *Rotylenchulus reniformis.* Auburn: Auburn University.

Wang, K. 2013. Reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira
(Nematoda: Tylenchida: Tylenchoidea: Hoplolaimida: Rotylenchulinae). University of Florida
IFAS Extension EENY-210.

Figure 1: Field plots at Auburn University's Tennessee Valley Research and Extension Center showing plant growth differences ate 44 days after planting
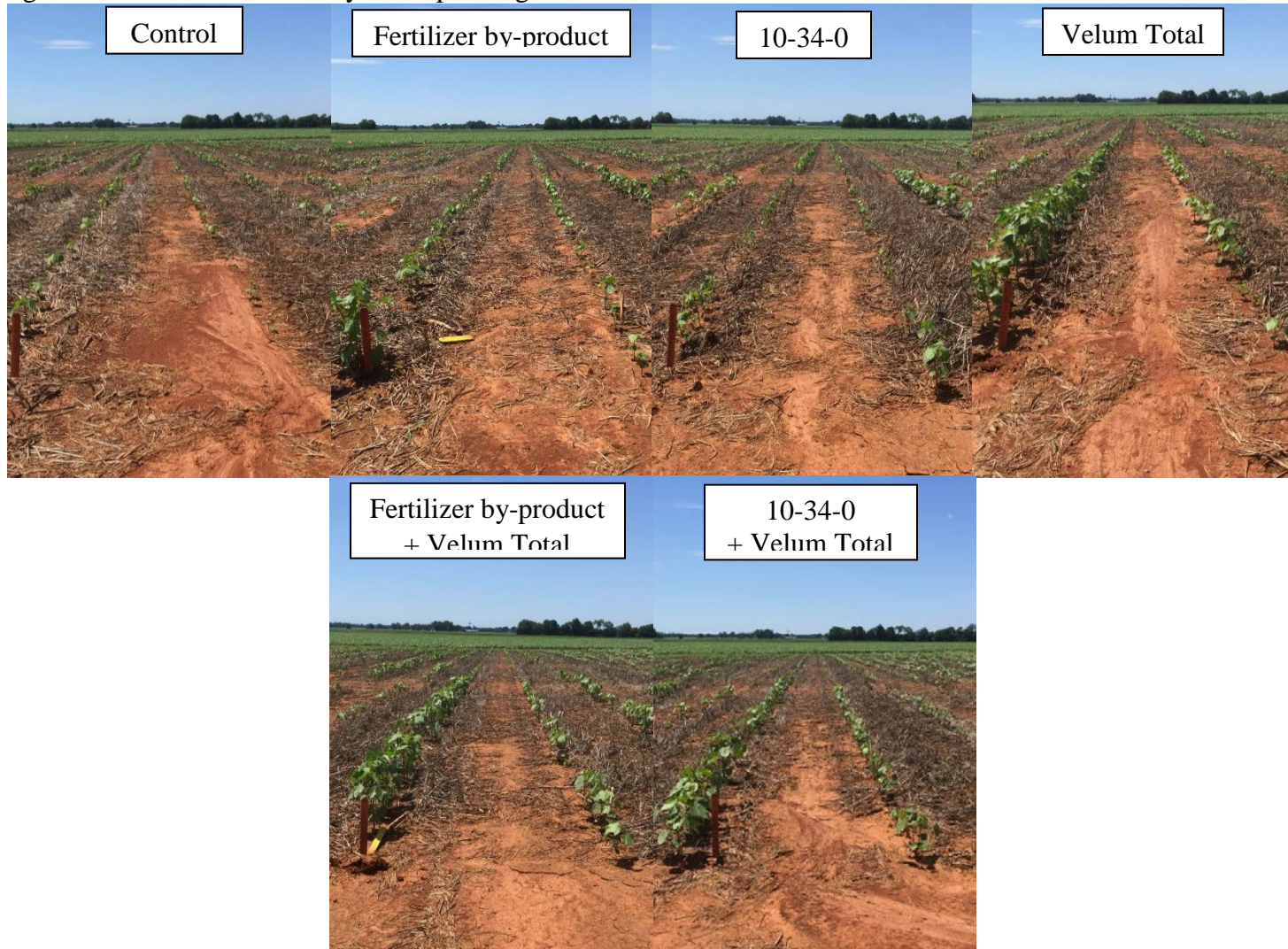
Table 1: Greenhouse evaluation of a fertilizer by-product effects on *Rotylenchulus reniformis* population density using cotton (Phytogen 487WRF) as a host and measured by plant height, shoot fresh weight, root fresh weight, biomass, and *R. reniformis* eggs/g of root at 35 DAP[w].

Treatment		N[y]	Plant height (cm)	Shoot fresh weight SFW (g)	Root fresh weight RFW (g)	Biomass (SFW + RFW)	*R. reniformis* eggs/g of root
Control[x]		10	15.7 b[z]	3.1	2.7 ab	5.9	436 ab
Fertilizer by-product	1871 L/ha	10	16.7 ab	2.9	2.4 ab	5.2	516 ab
10-34-0	46 L/ha	10	16.7 ab	3.2	2.8 a	5.7	884 a
Velum Total™	1 L/ha	10	16.7 ab	3.2	2.5 ab	5.7	169 d
Fertilizer by-product + Velum Total™	1871 L/ha 1 L/ha	10	18.0 a	3.0	2.3 b	5.2	339 cd
10-34-0 + Velum Total™	46 L/ha 1 L/ha	10	17.5 ab	3.4	2.7 ab	6.1	204 cd

[w] Days after planting (DAP), the test contained 5 replications and was repeated once.

[x] At the time of planting all pots were inoculated with 2,000 *R. reniformis* eggs.

[y] Number of observations.

[z] All values present are LS-means separated using the Tukey-Kramer method at $P \leq 0.1$. Values in the same column followed by the same letter, or no letter, do not differ significantly.

Table 2: Microplot evaluation of a fertilizer by-product effects on *Rotylenchulus reniformis* population density using cotton (Phytogen 487WRF) as a host and measured by plant height, shoot fresh weight, root fresh weight, biomass, and *R. reniformis* eggs/g of root at 46 DAP[x].

Treatment		N[y]	Plant height (cm)	Shoot fresh weight SFW (g)	Root fresh weight RFW (g)	Biomass (SFW + RFW)	*R. reniformis* eggs/g of root
Control		5	33.2 ab[z]	20.8 a	3.6 ab	24.4 a	80 ab
Fertilizer by-product	1871 L/ha	5	34.0 ab	17.7 ab	3.5 ab	21.3 ab	49 b
10-34-0	46 L/ha	5	24.6 c	11.9 b	2.3 bc	14.2 b	101 ab
Velum Total[TM]	1 L/ha	5	28.0 bc	15.7 ab	2.8 abc	18.5 ab	72 b
Fertilizer by-product + Velum Total[TM]	1871 L/ha 1 L/ha	5	35.2 a	20.9 a	4.0 a	24.8 a	554 a
10-34-0 + Velum Total[TM]	46 L/ha 1 L/ha	5	28.2 bc	10.9 b	1.9 c	12.8 b	49 b

[x] Days after planting (DAP), the test contained five replications.
[y] Number of observations
[z] All values present are LS-means separated using the Tukey-Kramer method at P≤0.1. Values in the same column followed by the same letter do not differ significantly.

Table 3: Field evaluation of a fertilizer by-product effects on *Rotylenchulus reniformis* population density using cotton (Phytogen 487WRF) as a host and measured by plant height, shoot fresh weight, root fresh weight, biomass, and *R. reniformis* eggs/g of root at 44 DAP[w].

Treatment		N[y]	Plant height (cm)	Shoot fresh weight SFW (g)	Root fresh weight RFW (g)	Biomass (SFW + RFW)	*R. reniformis* eggs/g of root
Control[x]		20	10.8 c[z]	4.9 c	0.5 d	5.4 c	14122 a
Fertilizer by-product	1871 L/ha	20	11.1 bc	6.4 c	0.6 dc	7.0 c	3110 bc
10-34-0	46 L/ha	20	11.7 bc	6.7 c	0.8 bc	7.5 c	9189 ab
Velum Total[TM]	1 L/ha	20	12.1 bc	9.0 b	0.9 ab	9.9 b	1075 c
Fertilizer by-product + Velum Total[TM]	1871 L/ha 1 L/ha	20	14.4 a	11.7 a	1.1 a	12.8 a	587 c
10-34-0 + Velum Total[TM]	46 L/ha 1 L/ha	20	12.7 b	9.3 b	1.1 a	10.4 b	1888 c

[w] Days after planting (DAP), the test contained five replications.

[x] At the time of planting all plots contained an average of 799 *R. reniformis* J2 per 100cm^3 of soil.

[y] Number of observations.

[z] All values present are LS-means separated using the Tukey-Kramer method at $P \leq 0.1$. Values in the same column followed by the same letter do not differ significantly.