

FUNGAL ENDOPHYTES AND THEIR ECOLOGICAL ROLES IN COTTON:
EVALUATION OF POTENTIAL ANTAGONISTIC ACTIVITY AGAINST PLANT
PARASITIC NEMATODES AND INSECT HERBIVORES

A Dissertation

by

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ABSTRACT

The goal of integrated pest management (IPM) is to reduce the threats posed to crops by pests and reduce the use of synthetic chemicals by applying knowledge of the biology of each pest and its interactions within the environment. This project aimed at developing the use of beneficial fungal endophytes as a biological control method to enhance plant resistance against insect herbivores and plant parasitic nematodes. Although cotton was used as a model agroecosystem, an improved understanding of the ability to manipulate plant-endophyte interactions as a component of IPM strategies will help facilitate the application of this approach to enhance plant resistance against biotic and/or abiotic stressors across a wider range of crop plants.

The objectives for this project were to: (1) test for potential effects of endophytic *Purpureocillium lilacinum* in cotton on root-knot nematodes (RKN) under greenhouse conditions; (2) test for effects of endophytic *Chaetomium globosum* in cotton on root-knot nematodes, cotton aphids, and beet armyworms in cotton plants under greenhouse conditions; and (3) evaluate the efficacy of both endophytes against root-knot and reniform nematodes in the field under typical agronomic conditions, along with their effects on yield.

Results showed that both endophytes could negatively affect root-knot nematode infection and reproduction in cotton under greenhouse conditions. Further, endophytic *C.*

globosum was also shown to negatively affect the fecundity of both cotton aphids and beet armyworms in greenhouse trials. *C. globosum* as an endophyte in cotton also affected the development rates and growth of beet armyworm larvae. This was the first study to demonstrate the negative effects of a since fungal endophyte, *C. globosum*, on insect herbivores feeding above-ground as well as plant parasitic nematodes feeding below-ground, using the same host plant species.

Across two years of field trials evaluating efficacy of the endophytes against nematodes, no significant effects of either *P. lilacinum* or *C. globosum* were detected on root-knot or reniform nematode populations. However, positive effects on cotton plant growth and yields were observed in some treatments combinations of endophyte genotype, seed treatment and plant genotype at some sites, indicating the importance of context-dependency in determining the outcome of cotton-endophyte-nematode interactions in the field.

The results of this study indicate that the presence of fungal endophytes in crops can be manipulated and many have the potential to be incorporated as part of an IPM strategy to protect plants against both insect herbivores and plant parasitic nematodes. This novel approach may help provide an environmentally-sound and sustainable tool for pest management in agricultural systems in which the application of pesticides is currently the most commonly utilized control tactic.

DEDICATION

This dissertation is gratefully dedicated to my parents for their unwavering support along the way. I also hope that this achievement will complete the dream that my grandfather had for me many years ago. May his soul rest in peace.

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NOMENCLATURE

BAW	Beet armyworm
DAI	Day after inoculation
J2	Second-stage juvenile
PHY	PhytoGen
RKN	Root-knot nematode
TX	Texas
WAI	Week after inoculation

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

In the past three decades, a large number of studies on endophytic microbes have been conducted, examining their biological and ecological functions in both natural and agricultural systems. When present inside plant tissues, endophytes can directly or indirectly interact with other plant-associated organisms (i.e. invertebrate and vertebrate herbivores, phytopathogens, plant parasitic nematodes, and soil microbes). This dissertation examines the defensive mutualism between cotton plants and fungal endophytes in relation to insect herbivores and plant parasitic nematodes, and also addresses the application of fungal endophytes in cotton agroecosystems as a novel biological control practice for integrated pest management.

Plant-microbe symbioses include many different groups of fungi and bacteria that interact with host plants. The genetic interactions between endosymbiosis of plant-fungi and plant-bacteria associations suggests that bacteria and fungi co-evolved with each other within their host (Gherbi et al., 2008). The fossil record supports the establishment of plant-fungus symbioses during early colonization of land by plants (Redecker et al., 2000). The first land plants had endophytic associations resembling arbuscular mycorrhiza even before roots evolved (Brundrett, 2002). This interaction gradually led to coevolution of both plants and fungi, in the form of an obligatory life style for the

microbes and a morphological adaptation that favored nutrient uptake by fungi from the soil environment for use by the plants.

Definition of endophyte

Plant-associated fungi are generally grouped according to their functional characteristics: mycorrhizal, pathogenic, epiphytic, endophytic, and saprotrophic fungi (Porras-Alfaro and Bayman, 2011a). The term “endophyte” was first introduced by Anton de Bary and refers to the microbes that reside internally in plant tissue (de Bary, 1886). Several researchers have since attempted to specify more accurate definitions for endophytes (Carroll, 1988, Wilson, 1995, Stone et al., 2004, Rodriguez et al., 2009). Porras-Alfaro and Bayman (2011a) defined endophytes as “microorganisms that live within plant tissues without causing symptoms of disease.” Unlike mycorrhizal fungi, which colonize plant roots and also rhizosphere, endophytes only live inside plant tissues including leaves, fruits, stems, and roots. Functional mycorrhizal fungi are phylogenetically different from most endophyte lineages, as they are specialized for nutrient uptake from rhizosphere to plant roots (Brundrett, 2002, Arnold et al., 2007, Porras-Alfaro et al., 2008).

Due to the increasing recognition of endophytes as an important type of plant-fungi symbiosis, it is critical to correctly distinguish endophytes from mycorrhizas. Brundrett (2006) summarized the major differences between endophytes and mycorrhizas, such as

the lack of massive nutrient transfer from fungi to plants and non-synchronized plant-fungi development in endophyte associations. The mycorrhizal fungi are dual soil-plant inhabitants that can absorb nutrients from both soil and plants (Brundrett, 2002).

Although mycorrhizas can occasionally colonize roots endophytically, the nutrients acquired from the plant would not be sufficient to maintain mycorrhizal fungi in soil (Brundrett, 1991). Moreover, mycorrhizal formation is restricted to mostly to young roots because the morphological association between the plant and fungi depends on root growth. Active growth is not necessarily for plant colonization by endophytes (Brundrett, 2004, 2006).

Ecological roles of endophytes

Endophytes are ubiquitous in plants. Successful colonization of endophytes depends on plant genotype, tissue type, endophytic microbial community composition, rhizospheric microbial community, and other biotic/abiotic conditions (Hardoim et al., 2015). The endophyte community composition is also influenced by geographic latitude and location (Gange et al., 2007, Higgins et al., 2007, Hoffman and Arnold, 2008). Wearn et al. (2012) described that endophyte communities can differ across growing seasons within the same plant species at the same location, with tissue specificity within a single plant. Primarily, plant genotype, developmental stage, and originating environment of the endophyte (i.e. soil, air, or water) are the main sources for variation microbial community structure (Rasche et al., 2006). Plants may benefit from endophyte

associations via indirect effects such as endophyte-mediated plant resistance against herbivores, pathogens, other biotic or abiotic stressors (Saikkonen et al., 1998). For examples, a number of studies have shown endophyte-associated changes in plant physiology in which the host plants benefit with enhanced tolerance against drought, heat, cold, metal contamination, and salt (Arachevaleta et al., 1989, Malinowski and Belesky, 2000, Waller et al., 2005, Redman et al., 2011, Khan et al., 2012). In most of these cases, increased tolerance enhanced plant performance in measures such as root and/or leaf growth, and endophyte-mediated stomata closure (Elbersen and West, 1996, Swarthout et al., 2009). Due to their beneficial effects on plant performance and fitness, the endophytes may also impact other ecological processes including plant community species composition and nutrient cycling (Saikkonen et al., 2015).

Endophyte and invertebrate herbivore interactions

Fungal endophytes have been shown to affect plant-herbivore interactions by producing defense-related compounds that are associated with inhibition activity against plant pathogens and/or herbivores (Van der Putten et al., 2001, Faeth and Fagan, 2002, Hartley and Gange, 2009) as well as altering nutrition quality in plant (Bernays, 1994). To date, most studies conducted on plant-endophyte-insect complexes have been focused on above-ground foliar endophytes. Chemical changes within a plant can protect the plants in many ways such as deterring insect herbivores, reducing herbivory, changing developmental rate, reducing fecundity, or increasing mortality of various herbivores

(Raman et al., 2012). Some entomopathogenic fungi such as *Beauveria*, *Metarhizium*, *Lecanicillium*, and *Aspergillus* have also been identified as endophytes, and can be inoculated successfully in the field to colonize various plants (Gimenez et al., 2007, Ownley et al., 2010, Lopez et al., 2014, Kaur et al., 2015, Mantzoukas et al., 2015). Raps and Vidal (1998) suggested the insect feeding biology could, to some extent, affect entomopathogenic endophyte activity. According to Gange et al. (2012), the host range of insect herbivores and insect-feeding guild are critical factors that affect the outcome of endophyte-insect relationships above-ground.

Alternatively, it has been suggested that root-feeding invertebrate herbivores may not encounter the same level of endophyte-secreted alkaloids as foliar-feeders do (Richmond et al., 2004). Given that the soil is the habitat for both root-feeding insects and plant parasitic nematodes, interactions between endophytes and the invertebrates in a different ecological guilds may possibly lead to diverse effects among herbivores feeding above-versus below-ground (Faeth, 2002). In particular, relatively few studies have focused on the ecology of plant-endophyte-nematode complexes. Certain beneficial *Fusarium* strains, *Pochonia chlamydosporia*, *Purpureocillium lilacinum*, and *Piriformospora indica*, have all been reported to have antagonistic effects on nematodes while being present as endophytes (Yan et al., 2011, Bajaj et al., 2015, Larriba et al., 2015, Martinuz et al., 2015). Most of these studies were observational bioassays, focused on measuring reductions in nematode infection or reproduction with an emphasis on the biological control aspects (Sikora et al., 2008). Only a few studies were mechanism-related,

addressing possibilities such as endophyte-mediated secondary metabolite production or plant induced systemic resistance (Hallmann and Sikora, 1996, Vu et al., 2006, Dababat and Sikora, 2007, Sikora et al., 2007).

Biology of cotton herbivores

Root-knot nematodes

The root-knot nematodes, *Meloidogyne* spp., are economically important polyphagous obligate plant parasites. They are distributed worldwide and parasitize almost all higher plants. They feed and reproduce on living plant cells in roots, and induce formation of giant cells and galls, which leads to disrupted plant water and nutrient uptakes, severe crop damage and reduced crop yield. External symptoms due to nematode infection include various degrees of stunting and wilting. Secondary infection by other pathogens may lead to decay of nematode-infected tissues (Karssen et al., 2006).

Root-knot nematode females produce eggs into gelatinous egg masses, which protect the eggs from environmental stressors and predation. The egg masses are generally deposited on the surface of galled roots, sometimes inside root tissues. Hatching of the root-knot nematode eggs is temperature and moisture driven. The first juvenile molt occurs following embryogenesis, and then to the infective second-stage juveniles (J2). After hatching, J2 leave the egg masses, and move towards plant roots. The infective J2s accumulate at the cell elongation region of lateral roots and penetrate behind the root tip.

An invaded J2 moves intercellularly to initiate a permanent feeding site. Giant cells of the plant are induced by nematode feeding, and the nematode becomes sedentary and enlarges into a “sausage” shape. The J2s then molt twice through J4 stage, and then reach the adult stage (Perry et al., 2009). Males are not necessary in completing the entire life cycle and females can produce viable eggs in the absence of males. The root-knot nematodes have a life cycle for approximately 30 days (Perry et al., 2009). The generation time and reproduction of *M. incognita* were shown to be related with soil temperature (Ploeg and Maris, 1999).

Reniform nematodes

Reniform nematodes, *Rotylenchulus spp.*, are semi-endoparasites of plant roots. Host range for reniform nematodes is relatively limited compared with root-knot nematodes. Juveniles have four vermiform stages, from J1 to J4, with the J2 hatching from eggs after the second molt (Robinson, 2002). Females embed in the roots along the taproot or/and lateral roots by penetrating the root cortex into the root axis. Females feed permanently on a single cell in the endodermis to deep cortex, and induce the formation of a syncytium at the feeding site (Robinson, 2002). Males remain outside of roots and can mate with females before females reach sexual maturity. Some reniform species can reproduce parthenogenetically. Females lay eggs in a gelatinous matrix on the surface of roots. *R. reniformis* has a life cycle in approximately 27 – 36 days, with the minimum of 17 days under optimal conditions (Dasgupta and Raski, 1968, Sivakumar and Seshadri, 1971, Gaur and Perry, 1991, Robinson et al., 1997).

Cotton aphids

The cotton aphid, *Aphis gossypii*, is considered as a highly polyphagous species with primarily parthenogenetic reproduction in the southern US. Reproduction is mostly asexual with alate or apterous females giving birth to live young. Offspring are clones of the female without gene recombination (Sarwar et al., 2013). An anholocyclic life cycle is exhibited when the aphids are exposed to warm environments; while under cooler environments, a heteroecious or autoecious holocyclic life cycle will be exhibited (Slosser et al., 1989, Zhang and Zhong, 1990). Reproductive rates are generally determined by temperature and host species attributes (Ebert and Cartwright, 1997). Aphids and other homopterans feed on phloem sieve elements by penetrating their piercing mouthparts (stylets) through plant cuticle, epidermis, and mesophyll (Tjallingii, 2006, Walling, 2008). Aphids secrete salivary flange (gelling saliva) before stylet insertion and continuously produce salivary flange during the penetration process. The saliva acts as the salivary sheath to envelop the stylets during insertion. During feeding, the aphids then inject salivary chemicals and/or proteins into the plants through their stylets to prevent coagulation of phloem proteins (referred to phloem wound response), inside the sieve elements and influence defense signaling pathways and volatile emissions (Tjallingii, 2006, Walling, 2008). Adults and nymphs are mobile and can feed on several feeding sites during their lifetime.

Beet armyworm

Spodoptera exigua has been recorded to attack more than 90 plant species from at least 18 families in North America (Pearson, 1982). Larval development is considered to be temperature dependent, and a reduced number of developmental stages was found under cooler environment (Tingle and Mitchell, 1977). There are typically five instars, with fewer or more instars reported on occasion. Larvae feed on both plant foliage and fruit. Young larvae feed gregariously, but will become solitary later in life. Mating occurs after emergence of the moth, and oviposition begins in the following two to three days. Females lay eggs on the lower surface of the leaf in clusters (Pearson, 1982). Larval developmental time from egg to pupation on cotton may range from 10 to 24 days (Huffman et al., 1996).

Study system and research objectives

Interactions between soil organisms (i.e. insects, nematodes, and microbes) and roots can lead to direct (phytotoxin production) and indirect (volatiles secretion) host defense responses above-ground, which can then affect above-ground herbivores and their multitrophic interactions (Bezemer and van Dam (2005). In plant-nematode complexes, both decreased and increased levels of above-ground defense compounds have been detected (van Dam et al., 2003, Van Dam et al., 2005). As to plant indirect defensive response, studies also found that mycorrhizal fungi can alter plant-secreted volatiles and lead to the plants being more attractive to aphid parasitoids than the non-mycorrhizal

colonized plants (Guerrieri et al., 2004). However, no study to date has yet considered the multitrophic-level interactions of insect and nematodes associated with the same plant-endophyte symbiosis. The major goal of this dissertation is to explore the biological and ecological effects of fungal endophytes in herbivore-endophyte-plant complexes. In particular, the ecological effects of plant-endophyte associations in herbivores with different modes of feeding will be considered separately in different spatial contexts, namely (1) a plant parasitic nematode feeding below-ground, (2) a phloem-feeding insect feeding above-ground, and, (3) a foliar-feeding insect feeding above-ground. In addition to greenhouse studies, field experiments were conducted to further explore the ecological effects of manipulating the presence of fungal endophytes on nematode population dynamics and plant growth in cotton agroecosystems.

The work described in this dissertation addresses the potential of using fungal endophytes as a biological control agent in the management of insect and nematode pests in agriculture. Biological control of arthropod pests is an essential component of the IPM concept. The application of entomopathogenic fungi as biopesticides for insect control continues to be widely studied. Over 750 fungal species have been identified as effective against insect pests (Butt et al., 2001). Managing natural enemies such as predators, parasites, and pathogens in agro-ecosystems provides a solid ecological foundation for sustainable cotton production. Importantly, although the use of fungi as pathogens to directly infect and kill insects has been intensively studied, the potential for their use in insect management as endophytes has been largely overlooked. Furthermore, among all

the fungi that have been recorded being antagonistic to insects or nematodes, none have been reported to negatively affect both insects and nematodes on the same host plant, as I demonstrate can be the case when the fungi are present as an endophyte.

In the following chapters, endophyte-cotton relationships as a defensive mutualism will be discussed from both ecological and agricultural perspectives. In the second chapter, endophytic *Purpureocillium lilacinum* strain 490 was tested against root-knot nematode in a series of greenhouse experiments. Effects of the endophyte on plant performance were also evaluated. In the third chapter, endophytic *Chaetomium globosum* strain 520 was evaluated for its potential as an antagonist against cotton aphid (*Aphis gossypii*) and beet armyworm (*Spodoptera exigua*) feeding on cotton above-ground, as well against root-knot nematode feeding below-ground. Plant performance was again measured to test for an effect of the endophyte on plant growth. In the fourth chapter, both endophytes, *P. lilacinum* strain 490 and *C. globosum* strain 520, were evaluated in the field under typical agricultural conditions for efficacy against populations of both root-knot nematode (*Meloidogyne incognita*) and reniform nematode (*Rotylenchulus reniformis*). Cotton plant performance and yield data were collected and compared. Possible interactions among endophyte genotype, plant genotype, and different seed treatment methods were examined.

CHAPTER II

A FUNGAL ENDOPHYTE DEFENSIVE SYMBIOSIS AFFECTS PLANT- NEMATODE INTERACTIONS IN COTTON

Introduction

Microorganisms residing in the rhizosphere, particularly arbuscular mycorrhizal fungi, have evolved mutualistic symbiotic associations with most terrestrial plants (Tedersoo et al., 2010, Kohler et al., 2015, Venturini and Delledonne, 2015). The organic nutrients released from roots, mainly amino acids, organic acids, sugars, proteins, and secondary metabolites, are rapidly assimilated by rhizosphere microbes as their major carbon and nitrogen sources (Jones et al., 2009, Saikkonen et al., 2015). In turn, microbes such as mycorrhizal fungi affect the plants by mediating rhizospheric microbial community structure (Pérez-Jaramillo et al., 2015, Urbanová et al., 2015), regulating plant growth (Smith et al., 2010, Smith and Smith, 2011), and inducing plant defensive reactions against herbivores or pathogens (Chapelle et al., 2015, Johnson and Rasmann, 2015). The effects of another major fungal assemblage, fungal endophytes, have received considerable attention in the past two decades (Wilson, 1995, Saikkonen et al., 1998, Rodriguez et al., 2009, Porras-Alfaro and Bayman, 2011b, Saikkonen et al., 2015).

Endophytes, as defined by Porras-Alfaro and Bayman (2011b), are microorganisms that live within plant tissues without causing symptoms of disease. Some endophytic

microbes, predominantly fungi and bacteria, have been shown to confer protection to host plants against multiple stressors including drought (Hubbard et al., 2012), pathogens (Arnold et al., 2003, Flor-Peregrín et al., 2014), and insect herbivores (Thakur et al., 2013), as well as enhance plant growth (Ren et al., 2011, Lopez and Sword, 2015). Fungal endophytes are ubiquitous in most terrestrial plants (Arnold et al., 2000) and plant tissues (Rodriguez et al., 2009), but with distinct community composition and abundance in different plant tissues and plant species (Kumar and Hyde, 2004, Addy et al., 2005, Massimo et al., 2015). Additionally, the diversity of endophyte communities in plants can also be influenced by biotic and/or abiotic factors (i.e. plant species, environmental conditions, presence of herbivores or pathogens, and soil environment (Clay et al., 2005, Compant et al., 2010, Knief et al., 2010, Brosi et al., 2011, Peršoh, 2013, Muller and Hilger, 2015).

Several important fungal genera that occur in the rhizosphere are well known to have negative effects on plant-parasitic nematodes either through parasitism, predation or antagonism, for instance *Penicillium* (Martinez - Beringola et al., 2013), *Verticillium* (Bourne et al., 1996, Kerry, 2001), *Chaetomium* (Yan et al., 2011), *Fusarium* (Waweru et al., 2013), *Arthrobotrys* (Stirling et al., 1998), and *Trichoderma* (Szabó et al., 2012). There are multiple examples of the application of fungi acting as nematode parasites to the soil for suppression of various plant-parasitic nematodes (Siddiqui and Mahmood, 1996). One classical example is the fungus *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*)(Luangsa-ard et al., 2011). This fungus was first known to be a

soil-borne fungus, but was later found in root-knot nematode eggs and egg masses (occasionally from females) and cyst nematodes (*Globodera* spp. and *Heterodera* spp.) (Jatala and Kaltenbach, 1979, Esser and El-Gholl, 1993). It was used for the first time as a biological control agent applied as a soil treatment for nematodes in 1979 (Jatala and Kaltenbach, 1979). The commercialized *P. lilacinum* Bioact 251 was originally isolated from nematode eggs in Philippines (Davide and Zorilla, 1983), and *P. lilacinum* is currently one of the best-known bio-nematicides in the U.S. having been evaluated for use against nematodes in several crops including corn, cotton, sweet potato, cucumber, tomato and turf grasses (Cabanillas et al., 1988, Khan and Saxena, 1997, Lawrence et al., 2008, Lawrence et al., 2010, Yan et al., 2011, Castillo et al., 2013, Crow, 2013).

A number of studies have considered the effects of nematode pathogenic fungi as beneficial endophytes for use against nematodes (Mendoza and Sikora, 2009, Yan et al., 2011, Jia et al., 2013, Martinez - Beringola et al., 2013, Waweru et al., 2013, Tian et al., 2014a, Bajaj et al., 2015). However, none of these studies attempted to differentiate between the potential effects of these fungi as endophytes within the plant versus their presence in the soil as an artifact (i.e., contaminant) of inoculating the plants with the fungi via soil or root drenching (Yan et al., 2011). As such, negative effects on nematodes due to the target fungi living as an endophyte within the plant can potentially be confounded by the fungi also occurring and interacting with nematodes or their eggs outside the plant, either in the soil or as an epiphyte. Holland et al. (2003) screened eight crop species with *P. lilacinum* Bioact 251 and concluded that this strain is not an

endophyte, but lives in the rhizosphere, with occasional colonization of the root surface. In a study of the nematophagous fungus *Pochonia chlamydosporia* conducted by Escudero and Lopez-Llorca (2012), the fungus was shown to be an endophyte in tomato roots by a quantitative PCR assay and a stable GFP transformant. However, it was also reported to be soil-colonizing fungus. A similar study of *Piriformospora indica*-nematode interactions concluded that the fungus negatively affected nematodes as an endophyte (Bajaj et al., 2015), but did not attempt to control for the possible effects of co-existing rhizospheric fungi.

Gossypium hirsutum, known as American Upland cotton or Mexican cotton, is the most commonly cultivated cotton species across the U.S. and plant-parasitic nematodes can be a major constraint to cotton production. The root-knot nematodes, *Meloidogyne* spp., generally have a broad host range that includes almost all higher plant species (Moens et al., 2009). The Southern root-knot nematode, *Meloidogyne incognita*, is the most widely distributed species found in every state in the southern U.S. (Walters and Barker, 1994). A recent survey of fungal endophyte communities in cultivated cotton in the U.S. isolated a number of endophytic strains identified by morphological and genetic characters (Ek-Ramos et al., 2013). A *P. lilacinum* (strain 490) isolated from cotton leaf tissue has recently been shown to confer resistance to insects when present as an endophyte in cotton (Lopez et al., 2014, Lopez and Sword, 2015). Here, we evaluated its potential effects on *M. incognita* when present as an endophyte in cotton, and explicitly

address the distinction between soil-borne and endophytic effects. We conducted a series of experiments to answer the following questions:

1. Does endophytic *P. lilacinum* confer resistance to cotton against *M. incognita*?
2. Are the effects of *P. lilacinum* on nematodes due to its presence in the soil or as an endophyte in the plant?
3. Does colonization of cotton by *P. lilacinum* affect plant growth?
4. Is this plant-endophyte association a defensive mutualism?

Materials and methods

Plants and fungi

G. hirsutum (var. LA122, All-Tex Seed Inc., TX), which is susceptible to *M. incognita*, was used for four experiments conducted in a greenhouse at College Station, TX, USA. An independent experiment was conducted in a greenhouse in Lubbock, TX using another susceptible *G. hirsutum* cultivar (var. FM1740B2RF, Bayer CropScience). Seeds were surface-sterilized by submerging in 2% sodium hypochloride (NaOCl) solution for 2 min and 70% ethanol for 2 min. Seeds were then rinsed with sterilized water at least four times (Posada et al., 2007). The fungal endophyte *P. lilacinum* was originally isolated from surface-sterilized cotton leaves collected from Texas, USA (Ek-Ramos et al., 2013), and stored in mineral oil suspension at -80°C. Fungal inoculum for experiments was cultured in petri dishes (100 × 15 mm) on potato dextrose agar (PDA) media at 25°C in the dark. Fungal conidia were harvested when mature by flooding the

petri dish with sterile water, scraping the plate with a sterile razor blade, and spores were filtered through autoclaved cheesecloth. Conidia concentrations of the resulting suspension were quantified using a hemacytometer (Thomas Scientific, Philadelphia, PA, USA). Concentrations of spore suspension for seed treatments were finalized to 10^5 (Low), 10^6 (Medium), or 10^7 (High) conidia/ml. A preliminary petri dish assay was conducted using this strain to test its potential pathogenicity to root-knot nematode eggs on PDA agar plates (a modified method based on Castillo et al. (2010). Successful egg parasitism by *P. lilacinum* mycelium was observed (data not shown).

Seed inoculation

Surface-sterilized cotton seeds were soaked in conidial suspensions overnight (approximately 200 seeds/25ml). Seeds for the control treatment were treated with sterile water under the same conditions. All seeds were planted in pasteurized sand (heated for eight hours at 72°C), and germinated in seed starter trays (4cm top diameter × 6cm deep) placed in growth chambers at 30°C/ 22°C day/night (12L: 12D photoperiod) until first true-leaf stage. All plants were then transplanted into 15 cm diameter × 11 cm deep pots filled with pasteurized sand 24 hrs by carefully removing soil without causing damage to the roots. Transplanted plants were allowed recover for one week in the greenhouse before use in experiments. Plants were fertilized regularly every two weeks using Botanicare® CNS17 Grow Formula 3-2-4 (Botanicare, LLC, Chandler, AZ, USA) and Spray-N-Grow® Micronutrients (Spray-N-Grow, Inc., Rockport, TX, USA).

Confirmation of endophyte colonization

Endophyte colonization efficacy was examined by surface-sterilizing plant tissues with 70% ethanol for 2 min, followed by 2% NaClO (for roots) or 0.5% NaClO (for leaves and stems) for 3 min, and four rinses in sterile water. Tissues pieces of 0.5 cm × 0.5 cm were placed onto PDA media at 25±1°C in the dark, and fungi recovered were examined one week later to confirm target endophyte inoculation efficiency (Arnold and Engelbrecht, 2007). All samples were processed via imprinting before transferred to PDA culturing plates, in case of insufficient surface sterilization. Because of relatively low colonization detected by plating plant tissues, we also conducted diagnostic PCR assays to confirm colonization of the plants by the target endophyte. Surface-sterilized plant tissues from the same plants used for plating were ground in liquid nitrogen. Fungal DNA was extracted by a modified chloroform isoamyl alcohol (24:1) extraction protocol (Sambrook et al., 1989). A species-specific primer set for *P. lilacinum* (PaeF: 5' CTC AGT TGC CTC GGC GGG AA 3' and PaeR: 5' GTG CAA CTC AGA GAA GAA ATT CCG 3') was used for diagnostic PCR assays (Atkins et al. 2005). PCR products were separated and visualized on 2% agarose gels.

Fungicide efficacy tests in vitro

To test for an endophytic versus soil-borne effects of *P. lilacinum* on nematodes as described below, we first tested two contact fungicides for efficacy against *P. lilacinum in vitro*, - Bonide® Liquid Copper Fungicide Concentrate and Bonide® Mancozeb Flowable Zinc Fungicide 861 (Bonide Products Inc., 6301 Sutliff Rd., Oriskany, NY,

USA). Fungicides were mixed with autoclaved PDA solution and prepared in 100 × 15 mm petri dishes at 15.63 ml/L for Liquid Copper Fungicide and at 19.53 ml/L for Mancozeb (manufacturer recommended concentration). To test for fungicide inhibition of germination, *P. lilacinum* spore suspensions were adjusted to 1×10^7 conidia/ml and 100 µl of the suspension was applied to PDA plates with or without fungicides. Drigalski spatulas were used to distribute spores equally on to the medium. To test for inhibition of mycelial growth, a 4mm diameter agar disc containing fungal mycelia was placed onto fungicide or non-fungicide amended PDA plates. Each treatment was replicated 10 times. The effects on *P. lilacinum* spore germination were assessed microscopically by counting germinated spores per 100 spores observed per field of view, at 24 hrs, 72 hrs, 7 days, and 14 days, with four views per plate and ten replicate plates per treatment. Mycelia growth was evaluated by measuring mycelial colony diameters in four perpendicular directions at 7 and 14 days, with ten replicate plates per treatment.

Nematode preparation and infection

M. incognita eggs were extracted from infected tomato plants by agitating the roots in 0.6% NaClO for 4 min, and collected on a 25µm pore sieve (Hussey and Barker, 1973). Three concentrations of nematode inoculants, 1,000 eggs/plant, 2,000 eggs/plant, and 10,000 eggs/plant, were utilized depending on the particular experiment described below. Cotton seedlings were inoculated after transplanting by pipetting an appropriate volume of egg suspensions directly to the soil at the base of the plant.

Evaluation of nematode root penetration, galling and egg production

Plants were maintained in the greenhouse for 12 days after nematode inoculation (DAI), then carefully removed from pots and washed free of soil from the roots (Starr et al., 2002, Atamian et al., 2012). Roots were agitated in 1.5% NaClO solution for 4 min, and then rinsed for 30 seconds under running water. The roots were submerged in water for 15 min to remove all traces of Cl and boiled in 12.5% (v/v) McCormick® Red Food Color solution for 30 seconds to stain the nematodes inside the roots (Thies et al., 2002). Samples were cooled to room temperature and rinsed twice in water. Roots were then examined microscopically to estimate numbers of juveniles that had penetrated into the roots.

Nematode infection was also estimated at 6 weeks after inoculation by counting galls per root systems and egg masses per root system. To facilitate counting the egg masses, the roots were treated with Phloxine B to stain the egg masses (Fenner, 1962, Holbrook et al., 1983). Finally, nematode reproduction was estimated based on eggs produced per gram of roots by extraction of the eggs with 0.6% NaClO for 4 min and collecting the eggs on a 25µm pore sieve. Numbers of eggs in 1ml aliquots of the extraction solution were estimated microscopically.

Plant growth evaluation

Plant performance was evaluated by measuring fresh shoot weights and heights, together with roots weights and lengths at each time when plants were sampled for nematode bioassays.

Experimental design

Four independent efficacy assays were conducted using different concentrations of fungal inoculants and nematode inoculants. An additional fungicide experiment was conducted to test the hypothesis that the observed effects on RNK were due to *P.*

lilacinum as an endophyte in cotton as opposed to its presence in the soil. All plants were maintained in greenhouses at $25 \pm 3^\circ\text{C}$.

Efficacy assay 1. Cotton (var. LA122) seeds were treated as described above with either 10^5 (Low) or 10^6 (Medium) conidia/ml concentrations of *P. lilacinum* conidial suspension, or sterile water as a control. After germination, plants at first true-leaf stage were inoculated with 10,000 nematode eggs/plant or equal volume of sterile water (Control group). Plants from all six treatments were harvested 6 weeks after nematode inoculation, with 10 replicates per treatment group. Number of galls and egg masses per gram root tissue per plant and total number of eggs were quantified for all nematode treatment groups.

Efficacy assay 2. Similar to efficacy assay 1, but cotton seeds were treated with either 10^6 (Medium) or 10^7 (High) conidia/ml concentrations of *P. lilacinum* conidial suspension, or sterile water as a control. A suspension containing 10,000 eggs or equal volume of sterile water (Control group) was inoculated to each plant at the first true-leaf stage. Plants from all six treatments were harvested and processed 6 weeks after nematode inoculation. Total number of galls and eggs were collected for all nematode treated plants, with 15 replicates per treatment group.

Efficacy assay 3. Similar to efficacy assay 2, but plants were inoculated with 2,000 eggs or equal volume of sterile water (Control group). Plants from all six treatments were harvested and processed 6 weeks after nematode inoculation. Total number of galls and eggs were collected for all nematode treated plants, with 15 replicates per treatment group.

Efficacy assay 4. To confirm the results of assays 1-3, an independent assay was conducted in a separate laboratory using a different variety of cotton (var. FM1740B2RF) and different source of nematodes, but the same *P. lilacinum* strain. Cotton seeds were treated with either 10^6 or 10^7 conidia/ml concentrations of *P. lilacinum* conidial suspension, or sterile water as a control. A nematode inoculum of 1,000 eggs/plant or equal volume of sterile water (Control group) was applied to plants at first true-leaf stage. Soil used for this experiment was Amarillo sandy loam (a mixture of 81% sand, 8% silt, 11% clay, 0.4% organic matter and pH = 8.0) autoclaved twice at

134°C before use. Number of eggs per plant and number of galls per gram root tissue per plant were quantified. In total, six treatments (+/- endophyte and +/- nematodes) were assessed with 10 replicates for each treatment group.

Endophyte versus soil effect experiment. Cotton seeds were treated with either 10^6 (Medium) or 10^7 (High) concentrations of *P. lilacinum* conidial suspension, or sterile water as a control. To test whether the results in efficacy assays 1-4 could be due to the effects of *P. lilacinum* as an endophyte versus negatively affecting nematodes in the soil, we included a fungicide treatment to kill *P. lilacinum* in the soil and on the surface of the plant prior to infection with nematodes. This resulted in a total of 12 treatments groups (+/- endophyte, +/- fungicide and +/- nematodes). A volume of 50ml of Bonide® Liquid Copper Fungicide Concentrate (15.63 ml/L, the highest dosage recommended by the manufacturer's label) was applied to each pot in seed starter trays, when plants were at first true-leaf stage. All plants were transplanted as described above, and were allowed to sit for one week for recovery. 2,000 eggs/plant or equal volume of sterile water (Control group) was applied to plants of nematode treatment groups. Ten plants of each treatment (12 treatments in total) were randomly collected at 12 DAI and processed for juvenile root penetration staining and quantification of early gall formation.

Statistical analyses

All data were tested for normality using Shapiro-Wilk tests and Normal Quantile Plots, then analyzed using either one-way ANOVA tests, or Kruskal-Wallis and Wilcoxon

Signed Rank tests (when data were not normally distributed) ($\alpha = 0.05$). JMP® Pro, Version 11.2.0 (SAS Institute Inc., Cary, NC, 1989-2013) was used for all analyses. Box plots are used to plot all data regardless of distribution. In the fungicide assay, a general linear model (GLM) was used to examine main effects for the endophyte and fungicide treatments as well as their interaction on juvenile root infection and galling.

Results

Endophyte colonization

Positive endophytic colonization of cotton plants by *P. lilacinum* was detected across all the efficacy assays at 20%, 20% and 26% of the sampled plants in the Low, Medium and High spore concentration treatment groups, respectively. In the fungicide assay, the average endophyte colonization frequencies of two fungicide-included treatments were 20% and 15% of the sampled plants in the Medium and High treatment groups, respectively. The estimates were based on the combined confirmation of the presence of target fungus from both the tissue plating and PCR assays. No target fungus was observed on any plate following a tissue imprint, which indicated that the surface sterilization was effective and all the re-isolated targeted fungi had colonized endophytically.

Nematode bioassays

Nematode reproduction. Significant suppression of *M. incognita* egg production were observed in cotton plants treated with *P. lilacinum* spores as a seed treatment in all four efficacy assays. In the first efficacy assay when plants were inoculated with 10,000 nematode eggs/plant, there was a significant overall effect of endophyte treatment (Kruskal-Wallis $\chi^2 = 10.33$, $df = 2$, $p = 0.0057$)(Fig. 2.1a). However, significantly more eggs were produced in plants treated with a low (10^5 spores/ml) concentration of *P. lilacinum* compared to either the control or Medium (10^6 spores/ml) endophyte treatment group (Table 2.1). In the second efficacy experiment, the number of nematodes was kept the same, but plants were treated as seeds with either a Medium (10^6) or High (10^7) concentration of *P. lilacinum* conidia. In this case, there was a significant overall effect of endophyte treatment (Kruskal-Wallis $\chi^2 = 12.19$, $df = 2$, $p = 0.0023$), with significantly lower numbers of eggs produced in the Medium (Wilcoxon rank sum test, $p = 0.014$) and High (Wilcoxon rank sum test, $p = 0.0004$) treatment groups relative the control plants (Table 2.1). We also tested for the effects of two endophyte seed treatment concentrations (Medium, 10^6 conidia/ml and High, 10^7 conidia/ml) at two lower, more ecologically realistic, nematode infestation levels (2,000 eggs/plant and 1,000 eggs/plant). When plants received 2,000 eggs/plant, there was a significant overall effect of endophyte treatment (Kruskal-Wallis $\chi^2 = 35.69$, $df = 2$, $p < 0.0001$). Significant reductions in nematode egg production were observed relative to the control plants in both the Medium and High endophyte treatments (Wilcoxon rank sum test, $p < 0.0001$ and $p < 0.0001$, respectively; Fig. 2.1c and Table 2.1), with the Medium concentration

group exhibiting a significantly greater reduction in egg numbers compared to the High concentration treatment (Wilcoxon rank sum test $p < 0.0001$; Fig. 2.1c and Table 2.1). When plants were challenged by 1,000 eggs/plant, there was again a significant overall effect of endophyte treatment (Kruskal-Wallis $\chi^2 = 6.57$, $df = 2$, $p = 0.037$) with fewer eggs produced relative to the control plants in both the Medium (Wilcoxon rank sum test, $p = 0.02$; Fig. 2.1d and Table 2.1), and High endophyte treatments (Wilcoxon rank sum test, $p = 0.08$; Fig. 2.1d and Table 2.1).

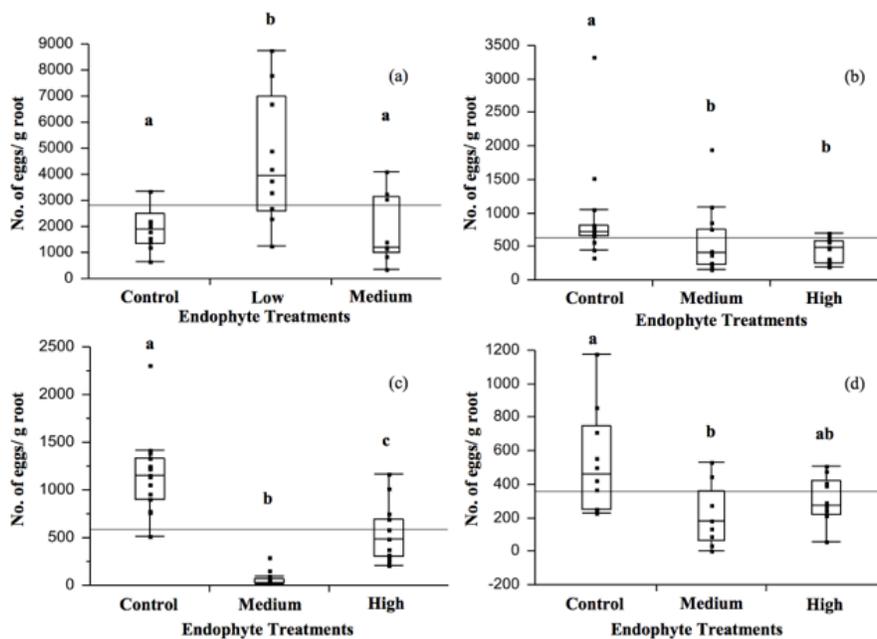


Fig. 2.1 Number of root-knot nematode eggs per gram of root six weeks after nematode inoculation. (a) Each plant received 10,000 nematode eggs. (b) Each plant received 10,000 nematode eggs. (c) Each plant received 2,000 nematode eggs. (d) Each plant received 1,000 nematode eggs; plants were grown using Amarillo sandy loam. The mid-line represents the grand mean of entire sample set. Each dot represents one original data collection.

Table 2.1 Statistical analyses of the first four efficacy assays on *M. incognita* galling, fecundity, and egg masses (conducted only in assay 1) at 6 WAI (Wilcoxon-Mann-Whitney test, $\alpha = 0.05$).

Experiment	Treatment Pairs	#Galls/g root		#Eggs/g root		#Egg masses/g root	
		Z	P	Z	P	Z	P
Efficacy assay 1 (10,000 eggs)	Low vs. Control	0.26	0.79	2.68	0.0073	-0.26	0.79
	Medium vs. Control	-0.61	0.54	-0.77	0.44	-2.33	0.020
	Medium vs. Low	-0.61	0.54	-2.65	0.008	-2.33	0.020
Efficacy assay 2 (10,000 eggs)	Medium vs. Control	-1.97	0.049	-2.45	0.014	-	-
	High vs. Control	-1.54	0.12	-3.53	0.0004	-	-
	High vs. Medium	0.64	0.52	0.50	0.62	-	-
Efficacy assay 3 (2,000 eggs)	Medium vs. Control	-4.65	<0.0001	-4.65	<0.0001	-	-
	High vs. Control	-4.02	<0.0001	-3.90	<0.0001	-	-
	High vs. Medium	4.65	<0.0001	4.52	<0.0001	-	-
Efficacy assay 4 (1,000 eggs)	Medium vs. Control	-1.02	0.31	-2.25	0.025	-	-
	High vs. Control	0	1	-1.78	0.076	-	-
	High vs. Medium	0.86	0.39	1.10	0.27	-	-

For plants that were inoculated with 10,000 eggs/plant in the first efficacy assay, there was a significant overall endophyte effect on the production of egg masses (Kruskal-Wallis $\chi^2 = 7.52$, $df = 2$, $p = 0.023$; Fig. 2.2). Number of egg masses produced per gram of root tissues (wet weight) was significantly reduced on plants inoculated with *P. lilacinum* 10^6 conidia/ml (Wilcoxon rank sum test $p = 0.02$; Fig. 2.2 and Table 2.1); but no significant effect was observed on the plants from Low treatment (10^5 conidia/ml)

(Wilcoxon rank sum test $p = 0.79$; Fig. 2.2 and Table 2.1). Egg masses were not quantified in the other efficacy trials.

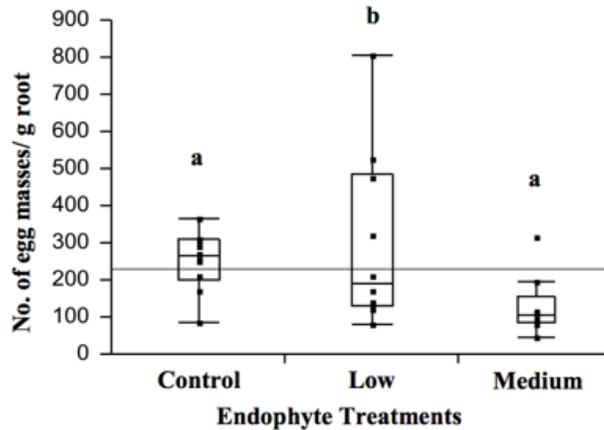


Fig. 2.2 Numbers of egg masses per gram of root produced by female root-knot nematode six weeks after nematode infection, with initial inoculant of 10,000 eggs/plant. The mid-line represents the grand mean of entire sample set. Each dot represents one original data collection.

Nematode infection. The impact of endophytic *P. lilacinum* on nematode root infection was examined by quantifying the number of galls on roots 6 weeks after plants were inoculated with nematode eggs (Fig. 2.3). For the two assays conducted using 10,000 eggs/plant, there was no overall significant difference in gall numbers among all endophyte treatments (Kruskal-Wallis test, Assay 1: $\chi^2 = 0.61$, $df = 2$, $p = 0.74$; Fig. 2.3a and Assay 2: $\chi^2 = 4.59$, $df = 2$, $p = 0.10$; Fig. 2.3b). Nor was there a significant effect on galling when plants were challenged by 1,000 eggs/plant, (Kruskal-Wallis test, Assay 3:

$\chi^2 = 1.29$, $df = 2$, $p = 0.53$; Fig. 2.3d). The only assay in which a significant effect on gall numbers was observed was the third assay where plants challenged with 2,000 eggs/plant had a reduced number of galls in both endophyte treated groups (Medium and High treatments) (Wilcoxon rank sum test, Medium: $p < 0.0001$ and High: $p < 0.0001$; Fig. 2.3c and Table 2.1).

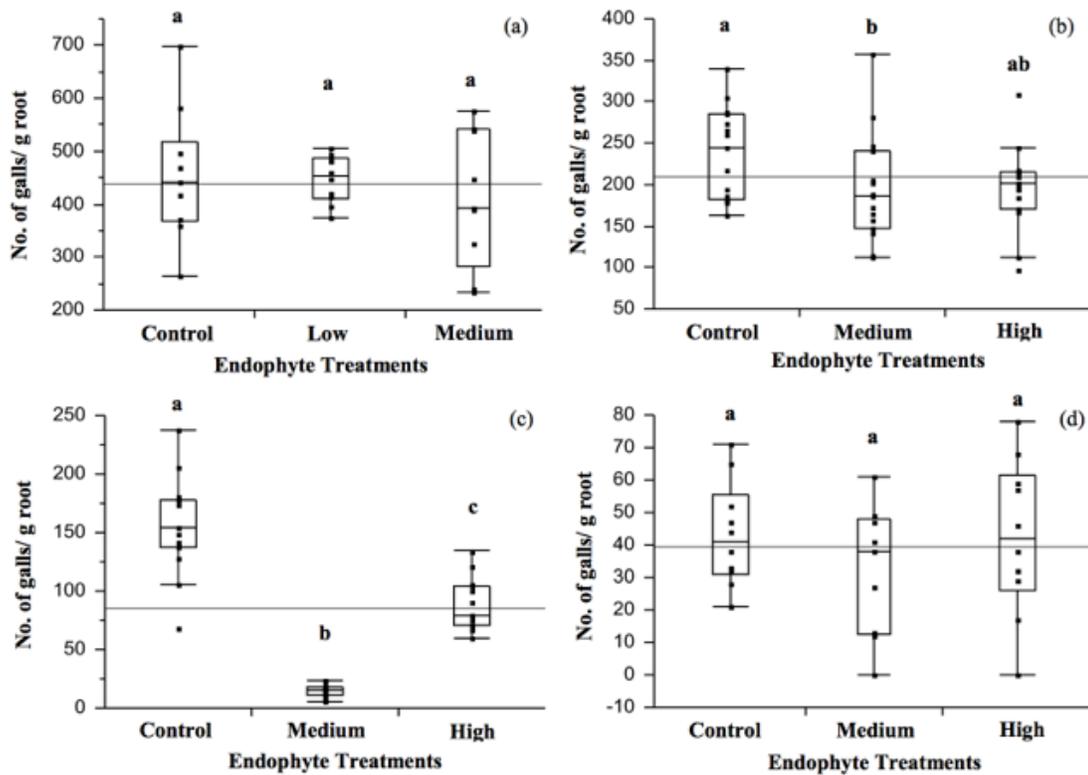


Fig. 2.3 Number of nematode produced galls per gram of root six weeks after infected. Plants were inoculated with nematode inoculant at (a) Each plant received 10,000 nematode eggs. (b) Each plant received 10,000 nematode eggs. (c) Each plant received 2,000 nematode eggs. (d) Each plant received 1,000 nematode eggs; plants were grown using Amarillo sandy loam. The mid-line represents the grand mean of entire sample set. Each dot represents one original data collection.

Fungicide efficacy test in vitro and in planta. The germination of *P. lilacinum* spores was completely inhibited on both the Liquid Copper Fungicide–PDA medium and Mancozeb–PDA medium. Both fungicides also completely inhibited the growth of *P. lilacinum* mycelia at Day 7 ($F_{2, 27} = 2348.80, p < 0.0001$) and Day 14 ($F_{2, 27} = 18947.23, p < 0.0001$).

Endophyte versus soil effect experiment. We selected the Liquid Copper Fungicide for the soil drenching treatment to control for effects of *P. lilacinum* present in the soil. The endophyte treatment significantly influenced the J2 infection (GLM, endophyte: $F_{2, 54} = 9.83, p = 0.0002$), whereas the fungicide treatment showed no effect (GLM, fungicide: $F_{1, 54} = 2.63, p = 0.11$), with no interaction between the two factors (GLM, endophyte×fungicide: $F_{2, 54} = 1.13, p = 0.33$). Similar to the pattern of J2 infection, nematode galling was also significantly affected by the endophyte (GLM, endophyte: $F_{2, 54} = 90.04, p < 0.0001$), but not the fungicide treatment (GLM, fungicide: $F_{2, 54} = 1.45, p = 0.23$) or the interaction between the two factors (GLM, endophyte×fungicide: $F_{2, 54} = 1.88, p = 0.16$). Post fungicide soil treatment, the number of J2 that penetrated the roots per gram root tissue was significantly reduced when plants were previously treated with 10^6 conidia/ml (Student's t test, $p = 0.0011$) versus the corresponding Control treatment (Fig. 2.4a and Table 2.2). Additionally, both endophyte concentrations (Medium and High treatments) significantly suppressed gall formation at 12 DAI (Student's t test,

Medium: $p = 0.024$ and High: $p = 0.0041$; Fig. 2.4b and Table 2.2). Meanwhile, a significant reduction in root penetration by juvenile nematodes was observed on both Medium and High treatments (plants not previously treated with fungicide) (Student's t test, Medium: $p = 0.024$ and High: $p = 0.0041$; Fig. 2.4a and Table 2.2). Additionally, the nematode galling of roots at 12 DAI was significantly suppressed in both Medium and High endophyte treatments (plants not previously treated with fungicide) (Student's t test, Medium: $p < 0.0001$ and High: $p < 0.0001$; Fig. 2.4b and Table 2.2)

Plant performance

There was no significant overall effect of the endophyte treatment on plant growth (see Appendix, Fig. A1- A5).

Table 2.2 Statistical analyses on *M. incognita* juveniles early galling and penetration at 12 DAI with fungicide treatment included (Student's t test, $\alpha = 0.05$).

Fungicide	Treatment Pairs	#Galls/g root		#Juveniles/g root	
		Mean difference	p-Value	Mean difference	p-Value
Control	Control vs. Medium	18.05	<0.0001	6.67	0.024
	Control vs. High	18.39	<0.0001	8.59	0.0041
	Medium vs. High	0.35	0.86	1.92	0.51
Treated	F-Control vs. F-Medium	-3.67	0.0002	9.87	0.0011
	F-Control vs. F-High	-3.67	0.0002	5.71	0.051
	F-High vs. F-Medium	4.16	0.29	4.16	0.15
	F-Control vs. Control	-0.57	0.57	2.79	0.33

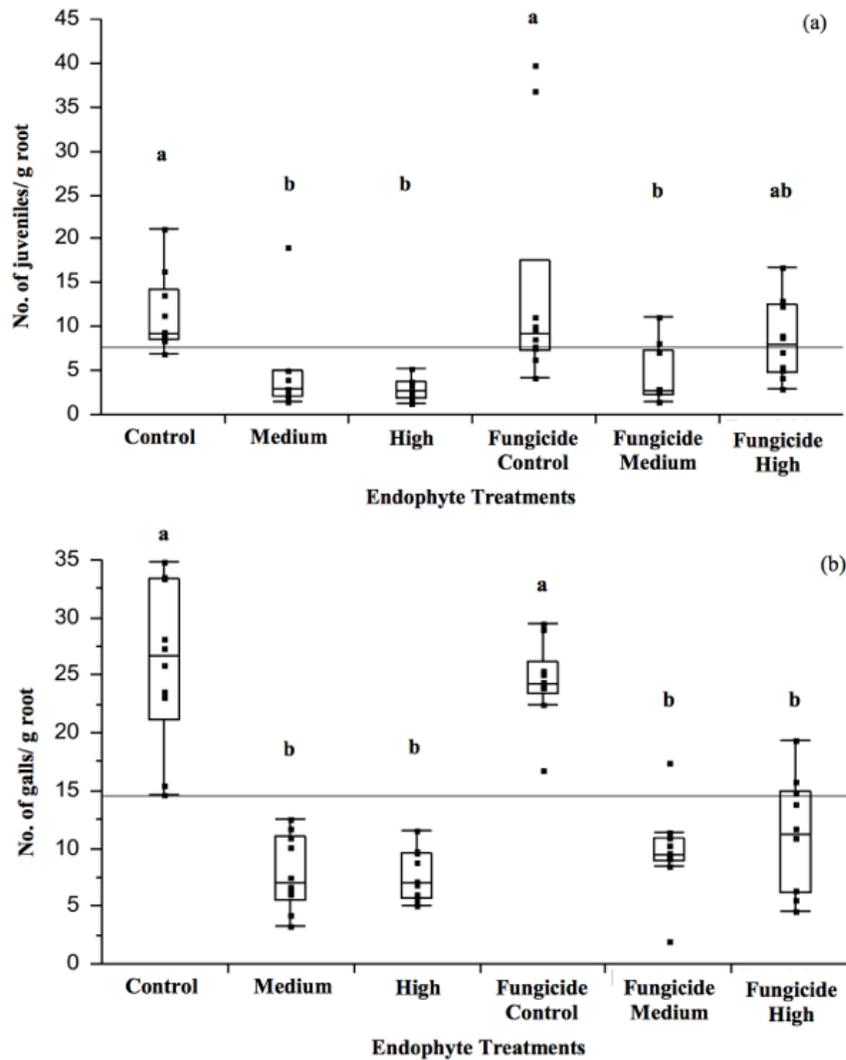


Fig. 2.4 Evaluation of nematode infection after each plant received 2,000 nematode eggs. (a) Number of juveniles penetrated per gram of root 12 days after nematode inoculation. (b) Number of galls per gram of root induced by nematode 12 days after nematode inoculation. The mid-line represents the grand mean of entire sample set. Each dot represents one original data collection.

Discussion

A persistent endophyte-mediated suppression of root-knot nematode galling and reproduction in cotton was demonstrated across multiple levels of nematode infection in this study. Six weeks after nematode inoculation, plants from Medium and High endophyte inoculation seed treatments suppressed *M. incognita* fecundity on average by 65.3% and 50.1%, and nematode galling on average by 44.8% and 20.6%, respectively (Fig. 2.1d and Fig. 2.3d). An endophyte-associated inhibition of J2 root infection was observed at 12 DAI in endophyte-treated plants (Fig. 2.4). Linford et al. (1938) initially proposed the hypothesis that nematophagous fungi are dependent on nematodes for nutrition, and fungal activity is positively correlated with nematode density. Our results indicate that the interactions between fungi and nematodes may be more complicated than the simple correlation between nematophagous fungi and nematodes in the soil, particularly considering that some nematophagous fungi can colonize plants as an endophyte.

In order to distinguish the endophytic effect of *P. lilacinum* strain 490 in cotton from a rhizospheric effect, we designed a fungicide experiment to test the prediction that the fungus was present as an endophyte and capable of negatively affecting nematodes after its elimination in soil. Previous studies using *P. lilacinum* Bioact 251 as a soil treatment for root-knot nematode reported 20 ~ 80% reduction in nematode population density and

galling on various host plants (Kiewnick and Sikora, 2003, Kiewnick and Sikora, 2006, Anastasiadis et al., 2008), but considered its effects to be due to activity in the soil rather than the plant (Holland et al., 2003). We used a broad-spectrum liquid copper fungicide that inhibits *P. lilacinum* spore germination and mycelial growth followed by transplantation to remove viable *P. lilacinum* from the soil and surface of experimental plants prior to nematode infestation. Successful systemic endophytic establishment of *P. lilacinum* in leaves and root tissue was detected at both the transplant stage and final harvest six weeks after nematode inoculation in both fungicide and non-fungicide treatments. Significant suppression of juvenile penetration and early galling was maintained in the *P. lilacinum* treatment groups even after the fungicide treatment (Fig. 2.4). Although the number of juveniles infecting roots of plants in the High endophyte inoculation group at 12 DAI was lower than its corresponding control in the fungicide treatment, the difference was not statistically significant (Fig. 2.4a). However, the same plants did exhibit significantly fewer galls, consistent with the suppression of early nematode infection (Fig. 2.4b). These results indicate that *P. lilacinum* strain 490 can colonize plant tissues and have negative effects on nematodes in addition to being a nematode egg parasite in the rhizosphere as is typically assumed. Given that *P. lilacinum* strain 490 was originally isolated from foliar tissue (Ek-Ramos et al., 2013), it may be better adapted for endophytic colonization relative to other *P. lilacinum* strains for which evidence for an endophytic lifestyle is lacking (Holland et al., 2003). To our knowledge, this is the first study to apply a foliar-isolated *P. lilacinum* strain to control *M. incognita* in cotton or any other plant as a seed-delivered endophyte.

A methodological constraint to studying the effects of facultative endophytic fungi is the difficulty in confirming endophytic colonization by the target fungi in treated plants, even when seemingly strong phenotypic effects consistent with endophytic colonization are observed at the treatment group level (Ownley et al., 2008, Powell et al., 2009, Lopez et al., 2014, Lohse et al., 2015, Lopez and Sword, 2015). Colonization frequencies are potentially underestimated because the ability to re-isolate the target fungi from surface-sterilized plant tissues after attempted inoculation can be negatively affected by several factors. This process typically involves immersion in diluted sodium hypochlorite and ethanol; and the time required for surface-sterilization in general depends on the concentration of solutions, and also plant tissue type, age, sensitivity and thickness (Fröhlich et al., 2000, Schulz and Boyle, 2005, Hyde and Soyong, 2008, Greenfield et al., 2015). The process of sterilization may kill the fungus within the plant (Schulz et al., 1993), the target fungus may be outcompeted by other fungi when plated on non-selective media, or the target fungus might not be present in the particular tissue fragments sampled (Ownley et al., 2008, Behie et al., 2015, Lohse et al., 2015). The latter factor can also complicate diagnostic PCR-based DNA detection assays to confirm successful endophytic colonization of the plant (Lohse et al., 2015). Moreover, false negatives due to unsuccessful reactions or false positives due to contamination can also be a challenge in PCR assays (Shadrach and Warshawsky, 2004, Gonçalves-de-Albuquerque et al., 2014). We attempted to optimize our surface sterilization protocol by separating roots and green tissues, with a lower concentration of NaClO used for the

green tissue. Nevertheless, the colonization frequencies were obtained were relatively low considering the extent and consistency of the phenotypic effects on nematode performance that we repeatedly observed throughout our study. As an alternative to direct detection assays, we also performed a manipulative fungicide treatment experiment (Fig. 2.4) as an indirect test of endophytic colonization that provided additional support for the colonization of cotton by *P. lilacinum* strain 490 as an endophyte.

To test of the generality of our results, our fourth greenhouse efficacy assay was conducted independently of the previous three trials by a different investigator in a separate laboratory using a different plant genotype, nematode colony, and soil type (more soil organic matter and greater variation of soil physical structure). The results were largely consistent with the other assays. A significant reduction in nematode fecundity was observed, however, there was no effect on galling. Differences in plant species and soil composition can alter plant-nematode-fungi interactions (Rumbos and Kiewnick (2006). Differences in available soil nutrients (particularly organic matter and mineral fraction), soil physical structure, microbial community structure, and root exudates components are can all affect plant-nematode-fungus complexes (Stirling, 2014). Despite the range of variables that may have varied between these assays, the *P. lilacinum* endophyte treatment was the most likely common causal factor in the reductions in nematode fecundity observed across all of the assays.

Plants and many rhizosphere microbes have co-evolved and are dependent on each other for survival. Plants can produce signals for microbes to locate roots, and microbes elicit physiological changes in the plant to create appropriate niches (Bais et al., 2004, Bais et al., 2006, Gorzelak et al., 2015). Fungal endophytes have also been shown to affect plant growth (Rodriguez et al., 2009). However, few if any studies conducted so far have examined endophyte-mediated effects on plant performance in the presence of plant-parasitic nematodes. Holland et al. (2003) suggested that a fungus colonizing plant tissue may lead to declined plant vigor. Recent studies have concluded that *P. lilacinum*, applied via soil treatment, can produce plant growth regulating substances that promote plant growth (Hashem and Abo-Elyousr, 2011, Hardoim et al., 2015, Yu et al., 2015). Also, the root-knot nematode is known to regulate plant metabolism and nutrient transport by forming giant cells which acts a nutrient sink for the nematode to feed upon and ultimately leads to gall formation that can negatively affect plant growth (Gheysen and Fenoll, 2002, Escobar et al., 2015, Favery et al., 2015, Truong et al., 2015). Given that *P. lilacinum* can be an endophyte as well as a nematode parasite in rhizosphere, how does the plant perform in the presence of both the fungus and nematodes? We compared plant growth using a factorial design involving plants that were untreated and treated with *P. lilacinum* and infected or not infected with nematodes at each time the plants were sampled in the nematode assays. Significantly enhanced plant growth was observed in some cases, but the effects were inconsistent (see Appendix A). Notably, we did not observed any negative effects of *P. lilacinum* treatment on plant growth, which does not

support the idea of a cost in terms of growth associated with endophytic colonization (Holland et al., 2003).

Beneficial endophytes are known to interact with plants in plant defensive reactions against pathogens and herbivores via antibiosis or induced resistance (Hardoim et al., 2015). Carroll (1988) suggested that the endophytes are actively evolving with their host plants, experiencing selection for the expression of antagonistic traits that lead to resistance against short-living pathogens and herbivores. Endophytes may also interact with the plant defense system, affecting induced systemic resistance that results in increased tolerance of pathogens or defense against herbivores (Robert-Seilaniantz et al., 2011, Zamioudis and Pieterse, 2011, Navarro-Meléndez and Heil, 2014). Tanaka et al. (2006) reported that endophyte-produced reactive oxygen species bursts are required to inactivate plant defense responses against the endophyte; and it may also be associated with the mechanisms of host's systemic acquired resistance responses. Some fungal endophytes are known to secrete compounds including alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols, phenols, and chlorinated compounds (Gunatilaka, 2006, Higginbotham et al., 2013, Tian et al., 2014b). These endophyte produced secondary metabolites can contribute directly to defense (Bush et al., 1997, Wilkinson et al., 2000, McGee, 2002) or to signaling and genetic regulation of symbiosis establishment (Schulz and Boyle, 2005), as well as mediate the production of plant secondary metabolites (Zhang et al., 2006). Comparative genomics studies also suggest that lateral gene transfer has also played a major role in the establishment of endophyte-

plant symbioses (Taghavi et al., 2010, Mitter et al., 2013, Tisserant et al., 2013, Wright et al., 2013).

Several studies have investigated *P. lilacinum* – nematode interactions outside the plant, but not in the context of its effects on nematodes as a fungal endophyte. For example, *P. lilacinum* secreted extracellular protease and chitinases have been shown to significantly reduced hatching of *M. javanica* juveniles (Khan et al., 2004). Bonants et al. (1995) suggested that a serine protease produced by *P. lilacinum* could possibly be involved in fungal penetration through nematode egg-shell as egg parasite. Some *P. lilacinum*-secreted secondary metabolites are thought to be involved in the parasitism of *M. javanica* as well (Park et al., 2004). The specific mechanisms by which *P. lilacinum* establishes as an endophyte in the plant and results in negative effects on nematode infection and fecundity as observed in our study remain to be determined.

CHAPTER III

A FUNGAL ENDOPHYTE AFFECTS BOTH ABOVE- AND BELOW-GROUND

HERBIVORES IN COTTON

Introduction

Endophytes are microorganisms that live within plant tissues without causing any symptoms of disease (Porrás-Alfaro and Bayman, 2011b). The endophytic fungi may also include latent pathogens, opportunistic pathogens with a short endophytic period, decomposers, and other obligate fungi (Carroll, 1988, Saikkonen et al., 1998). As to their ecological functions within the host plant, the most common endophytes are commensals, with less common ones being neutral, mutualistic, or detrimental (Hardoim et al., 2015). Endophytes, primarily fungal and bacterial, have been found in all species of plants sampled to date, with the best understood systems being the obligate fungal endophyte-grass symbioses (Saikkonen et al., 1998). Fungal endophytes, in particular, have been found in all woody plants that have been examined (Petrini, 1991, Bills, 1996, Kowalski et al., 1996, Redlin and Carris, 1996). It is known that some fungal endophytes can enhance plant resistance or tolerance to biotic and/or abiotic stresses (such as insect herbivores, plant parasitic nematodes, drought, heat, etc.), as well as enhance plant growth and performance (Elmi et al., 2000, Redman et al., 2002, Vega et al., 2008, Guo et al., 2015, Pandya et al., 2015). Successful colonization and establishment of endophytes inside plant tissues also depend on both biotic and abiotic factors. For

example, variation in host and endophyte genotypes, environmental conditions (e.g., humidity, temperature, etc.), plant developmental stage, and plant tissue types can all have major impacts on endophyte community assemblage (Hardoim et al., 2015).

While living within in plant tissues, endophytes can interact with invertebrate herbivores either directly or indirectly. The consequences of such interactions could be neutral, beneficial, or disadvantageous to the herbivores. For example, endophytic fungi can produce fungal metabolites that directly affect the behavior or performance of herbivores (Wilkinson et al., 2000, McGee, 2002). Additionally, the endophytes may also indirectly influence higher trophic level interactions in the ecological community (e.g., the natural enemies of some insect herbivores, such as parasitoids and predators)(Goggin, 2007). In addition to affecting herbivores, endophytes can also positively affect plant growth and increase host resistance to plant pathogens (Hardoim et al., 2015). Therefore, under the proper ecological conditions, many endophyte-plant associations could readily be considered as mutualisms that enhance plant fitness (Clay et al., 1993, Omacini et al., 2001).

Although endophytes are ubiquitous across leaf, stem, and root, the endophytic community in each tissue type may differ significantly in terms of both community composition and ecological functions (Porrás-Alfaro and Bayman, 2011b). The effects of endophytes on aboveground insect herbivores and their natural enemies have been intensively studied during the last two decades. Hartley and Gange (2009) summarized

that Lepidoptera, Coleoptera, and Hemiptera are the three major groups of insect herbivores that have been most intensively studied regarding the effects of plant symbiotic fungi on herbivore growth. As a general pattern of insect-endophyte interactions, it has been suggested that mycorrhiza typically have negative effects on generalist chewing insects, but positively affect most sucking insects and specialized chewing insects (Gange et al., 1999, Hartley and Gange, 2009). On the contrary, little is known about the interactions between endophytic microorganisms and belowground herbivores (Hartley and Gange, 2009, Porrás-Alfaro and Bayman, 2011b). So far, only a few studies have examined belowground endophyte-herbivore interactions involving root-feeding nematodes and insects (Richmond et al., 2004, Gera Hol et al., 2007, Yan et al., 2015). Additionally, no study has investigated the ecological effects of a single endophyte on both above- and belowground herbivores attacking the same host plant.

Gossypium hirsutum, also known as Upland cotton, is susceptible to a wide range of herbivores feeding both above- and below-ground including insects, spider mites, and plant parasitic nematodes. In this study, the impact of a plant-endophyte symbiosis on the performance of three ecologically-distinct invertebrate herbivores feeding on cotton was evaluated. The three herbivores tested in this study, the southern root-knot nematode (RKN) *Meloidogyne incognita*, cotton aphid *Aphis gossypii*, and beet armyworm (BAW) *Spodoptera exigua*, are all economically important pests with distinct modes of feeding on different tissues.

The root-knot nematodes, *Meloidogyne* spp., are primarily tropical to sub-tropical obligate plant parasites of roots in soil. The second-stage juveniles (J2) penetrate host roots, move intercellularly between cortical cells, and then enter into the vascular cylinder. Once the mobile stage is completed, the nematodes become sedentary and begin feeding at a permanent feeding site. Females remain sedentary, but males move out of the roots (Abad et al., 2003). The cotton aphid is a piercing-sucking hemipteran insect that feeds above-ground on photosynthate after inserting its needle-like mouthparts into phloem. Their asexual reproduction can lead to severe infestations in a short period of time. Life cycle is reported to range from 10.4 days at 30°C to 24.5 days as 15°C, as the optimal temperature range for population growth on cotton, *G. hirsutum* (Kersting et al., 1999). Beet armyworm larvae feed on plant foliage, flowers and fruits. As pest of cotton, BAWs may feed on bolls (the developing fruit), but more frequently consume foliage, squares (developing flowers) and blooms. Young larvae feed gregariously and become solitary in later stages. Adult females are highly fecund and produce an average number of eggs per individual ranging from 604.7 to 1724.7 (Wilson, 1934, Hogg and Gutierrez, 1980, Chu and Wu, 1992).

An earlier study of fungal endophytes in cotton recovered thousands of isolates that were grouped into a total of 69 unique taxa (Ek-Ramos et al., 2013). Among these were multiple endophytic isolates of *Chaetomium globosum*, from which strain 520 was randomly selected for further study. *C. globosum* is known for producing a number of bioactive metabolites (Sekita et al., 1981). Some of these compounds have been found to

provide host resistance to several diseases caused by seed-borne and soil-borne plant pathogens (Istifadah and McGee, 2006, Kumar et al., 2010). However, only a few studies have considered the effects of its metabolites on insects or nematodes (Wicklow et al., 1999, Nitao et al., 2002, Istifadah and McGee, 2006, Hu et al., 2012). In terms of the functional significance of *C. globosum* as an endophyte, Yan et al. (2011) indicated that its presence as an endophyte in cucumber reduced galling by the root-knot nematode, but did not control for potential effects of the fungus on nematodes that may have occurred outside of the plant in the rhizosphere.

Here we tested the following hypotheses about the effects of endophytic *C. globosum* in cotton:

1. *C. globosum* can be inoculated to colonize cotton systemically as an endophyte using a seed treatment;
2. Endophytic *C. globosum* negatively affects root-knot nematode infection of roots and subsequent reproduction below-ground;
3. Negative effects on root-knot nematodes are due to the presence of *C. globosum* as an endophyte as opposed to soil-borne effects;
4. Endophytic *C. globosum* negatively affects cotton aphid reproduction above-ground;
5. Endophytic *C. globosum* negatively affects beet armyworm development and fecundity above-ground;
6. Endophytic *C. globosum* enhances plant growth.

Materials and methods

Fungal inoculation of host plants

The fungal endophyte, *C. globosum* strain 520, was originally isolated from surface-sterilized cotton squares (developing flowers) collected in Texas, USA (Ek-Ramos et al., 2013). Fungal inoculum for experiments was cultured on potato dextrose agar (PDA) media in 100 × 15 mm petri dishes at 25°C in the dark. Fungal spores were harvested from PDA plates when mature by flooding the petri dish with sterile water and scraping the plate with a sterile razor blade. Spores were filtered through autoclaved cheesecloth and collected for determining spore concentration. Spore concentration was quantified using a hemacytometer (Thomas Scientific, Philadelphia, PA, USA), and finalized to 10⁶ spores/ml and 10⁷ spores/ml for seed treatment.

Cotton, *Gossypium hirsutum* (All-Tex LA122, All-Tex Seed Inc., TX), seeds were surface-sterilized by first submerging in 2% sodium hypochloride (NaOCl) solution for 2 min, followed by 70% ethanol for 2 min. All seeds were then rinsed with sterilized water four times (Posada et al., 2007). Surface-sterilized seeds were soaked in spore suspensions overnight (10⁶ spores/ml and/ or 10⁷ spores/ml, approximately 200 seeds/25ml). Seeds for the Control treatment were treated using an equal volume of sterile water under the same conditions.

For the nematode assays, seeds were planted in pasteurized sand (heated for eight hours at 72°C), and germinated in seed starter trays (each cell pot measured 4 cm top diameter × 6 cm deep) in plant growth chambers at 30°C/ 22°C day/night (12L: 12D photoperiod) until first true-leaf stage. Plants were then transplanted into 6-inch Azalea pots (15.24 cm diameter × 11 cm deep) using pasteurized sand, and kept in a greenhouse at 25±3°C. All plants were fertilized regularly every three weeks using Botanicare® CNS17 Grow Formula 3-2-4 (Botanicare, LLC, Chandler, AZ, USA) and Spray-N-Grow® Micronutrients (Spray-N-Grow, Inc., Rockport, TX, USA).

For all insect assays, seeds were planted in unsterilized Metro-Mix® 900 soil (Sun Gro Horticulture, Agawam, MA; ingredients: bark, vermiculite, peat moss, perlite, dolomitic limestone), and kept in growth chambers at 30°C/ 22°C day/night (12L: 12D photoperiod) until first true-leaf stage. Plants were then transplanted into 6-inch Azalea pots (15.24 cm diameter × 11 cm deep) using unsterilized Metro-Mix® 900 soil, and kept in the greenhouse at 25 ± 3°C.

C. globosum - nematode interactions

Two replicate experiments using two different concentrations of endophyte spore suspensions were conducted to test for the effects of endophyte treatment on root-knot nematode, *M. incognita*, infection and plant performance. Seeds were treated with two concentrations of *C. globosum* spore suspension, Low (10⁶ spores/ml) and High (10⁷ spores/ml) along with an untreated Control (sterile water). *M. incognita* eggs came from

a nematode colony maintained in the greenhouse and were extracted from infected tomato plants by agitating the roots in 0.6% NaClO for 4 min, and collected on a 25 μ m pore sieve (Hussey & Barker 1973). All nematode inoculations were applied after the plant transplantation.

Plants at the first true-leaf stage were inoculated with 2,000 eggs/plant or equal volume of sterile water (Control group) after transplantation. Plants from all treatments were harvested at 12 days after nematode inoculation (DAI) and 6 weeks after nematode inoculation (WAI), respectively, with 10 replicates per treatment group. Number of penetrated J2 stage juveniles, number of galls per gram root tissue, and total number of eggs were quantified for all nematode treated plants. Plant size and fresh weight were also measured at the time plants were harvested.

To quantify the early infection of roots by J2 larvae, plants were maintained in the greenhouse for 12 DAI, carefully removed from the pots, and the soil was washed from the roots (Starr et al., 2002, Atamian et al., 2012). Roots were agitated in 1.5% NaClO solution for 4 min, and then rinsed for 30 seconds under running water. Roots were submerged in water for 15 min to remove all traces of Cl and boiled in 12.5% (v/v) McCormick® Red Food Color solution for 30 seconds to stain the nematodes inside the roots (Thies et al., 2002). Samples were cooled to room temperature and rinsed twice in water. Roots were then examined microscopically to count numbers of juveniles that had penetrated into the roots. Nematode infection at 6 weeks after inoculation was estimated

by counting galls per root system. Nematode reproduction was estimated based on eggs produced per gram of roots by extraction of the eggs with 0.6% NaClO for 4 min and collecting the eggs on a 25 µm pore sieve (Hussey and Barker, 1973). Numbers of eggs in 1ml aliquots of egg extraction were estimated microscopically to determine egg production from each sampled plant.

Test of endophyte versus rhizosphere effects on nematodes

Given that *C. globosum* was inoculated to the plant as a seed treatment, the fungus could potentially interact with and negatively affect nematodes in rhizosphere soil or epiphytically on the root surface rather than due to its presence as an endophyte. Therefore, to test for the effect of the fungus as an endophyte and control for potentially confounding effects outside the plant, a fungicide treatment was applied to eliminate the target fungus in the rhizosphere and root surface prior to infecting the plants with nematodes.

We first tested the efficacy of two broad-spectrum contact fungicides against *C. globosum in vitro*, – Bonide® Liquid Copper Fungicide Concentrate and Bonide® Mancozeb Flowable Zinc Fungicide 861 (Bonide Products Inc., 6301 Sutliff Rd., Oriskany, NY, USA). Fungicides were mixed with autoclaved PDA solution and prepared in 100 × 15 mm petri dishes at 15.63 ml/L for Liquid Copper Fungicide and at 19.53 ml/L for Mancozeb. Fungicide concentrations used were the highest dose recommended by the manufacturer. *C. globosum* spores were harvested from lab cultures

in PDA media. Spore suspensions were adjusted to 1×10^7 spores/ml and 100 μ l of the suspension was applied to PDA plates with or without fungicides. Autoclaved Drigalski spatulas were used to distribute spores equally across the medium. For the mycelial growth test, a 4 mm diameter agar disc containing fungal mycelia was placed onto fungicide or non-fungicide amended PDA plates. The effects on *C. globosum* spore germination were assessed microscopically by counting germinated spores per 100 spores observed per field of view, at 24 hours, 72 hours, 7 days, and 14 days. Mycelia growth was evaluated by measuring mycelial colony diameters in four directions at 7 and 14 days. Ten replicated plates per treatment were randomly selected to quantify the spore germination or mycelial growth at each time interval.

Having demonstrated the efficacy of Bonide® Liquid Copper Fungicide Concentrate against *C. globosum* (see Results section), we then conducted an experiment to test for the endophytic effects of *C. globosum* against nematodes while controlling for the presence of the fungi outside the plant. Seeds were treated and germinated to the first true leaf stage as described above. 50 ml of diluted Bonide® Liquid Copper Fungicide Concentrate (15.63 ml/L) was then applied to each pot in seed starter trays to saturate the soil and left for 24 hours. All plants were then transplanted into 6-inch Azalea pots (15.24 cm diameter \times 11 cm deep) using pasteurized sand by carefully removing soil without causing damage to the roots. Plants were allowed to sit for one week for recovery. 2,000 nematode eggs / plant or equal volume of sterile water (Control group) was then applied to plants of the nematode treatment groups. The experiment was fully

factorial (3 endophyte treatments [High, Low & Control] × 2 fungicide treatments [+/-] × 2 nematode treatments [+/-]) with 10 replicate plants for each treatment combination and sampling time. Number of galls and penetrated juveniles were quantified for plants at 12 DAI (Thies et al., 2002). An additional 10 replicate plants from each treatment were harvested and processed 6 weeks after nematode inoculation. Total number of galls and eggs were quantified for all nematode treated plants. Plant size and fresh biomass were also measured at the time plants were harvested.

C. globosum - aphid interactions

Cotton aphids, *A. gossypii*, were collected in cotton fields at the Texas A&M AgriLife Field Station, Burleson, Co., TX (30°31'21.20"N 96°24'0.72"W) and maintained for multiple generations in cages on conventional cotton variety LA122 in the greenhouse at 25 ± 3°C (12 L: 12 D photoperiod).

Two no-choice experiments were conducted to test for the effects of endophytic *C. globosum* on aphid survival and reproduction. For all the experiments, cotton seeds were previously surface sterilized and treated with two fungal spore suspensions (10⁶ spores/ml for Low treatment and 10⁷ spores/ml for High treatment) and an untreated Control (water only) by soaking overnight. Seeds were planted individually in unsterilized Metro-Mix[®] 900 soil. In the first experiment, plants were grown to the 8th - 9th true-leaf stage in a greenhouse with a scheduled contact insecticide spray (Worry Free[®] brand Vegol[™] Year-Round Pesticidal Oil, containing 96% canola oil as the

major active ingredient) every two weeks prior to experiment setup. Frameless clip cages (BioQuip Products, Rancho Dominguez, CA) consisting of no-thrip screen bonded to two foam rings (2.54 cm inner diameter) were used with one 2nd instar aphid nymph placed on the lower leaf surface of 20 replicate plants for each treatment. The number of aphids in each cage was counted at Day 7 and Day 14 after initial infestation.

A second similar experiment was conducted following the same protocol as above, but included only the Control and Low (10^6 spores/ml) endophyte treatments, with 15 replicate plants per treatment group.

C. globosum - beet armyworm interactions

Three separate experiments were conducted to test for the effects of endophytic *C. globosum* on beet armyworm, *S. exigua*, growth, fecundity and sex specific differences. For all experiments, plants were individually caged immediately after the seedlings emerged to prevent infestation by other insects, and grown in the greenhouse at 25-30°C (12 L: 12 D photoperiod). *S. exigua* eggs were obtained from Benzon Research Inc., Carlisle, PA.

To test for effects of endophytic *C. globosum* on beet armyworm larval growth, seeds were treated with one of two concentrations of endophyte spore suspensions, 10^6 spores/ml (Low) or 10^7 spores/ml (High), along with untreated Controls (sterile water only). Twenty replicate plants for each treatment were used. Ten *S. exigua* eggs were

placed onto each caged plant at the 6th – 7th true-leaf stage. The developmental stage of the caterpillars in all endophyte treatments was recorded at two weeks and three weeks after hatching based on larval morphology (Capinera, 1999).

The second experiment used the same endophyte plant treatments as described above (High, Low and Control), but utilized a different rearing protocol and included an assessment of adult female fecundity. In this experiment, the *S. exigua* eggs were first hatched indoors (under $25 \pm 3^\circ\text{C}$ with 12 L: 12 D photoperiod) on freshly cut cotton leaves from each corresponding treatment to ensure that viable larvae were used to set up the experimental treatment groups. One day after hatching, five 1st instar individuals were transferred onto each caged plant for 15 replicate plants per treatment and maintained in the greenhouse. Plants were at the squaring stage (e.g., early flower development) at the time of infestation and remained completely caged until completion of the experiment. Individual body mass and head capsule width were measured two weeks after hatching for caterpillars in each treatment group. Individual developmental stage was recorded at the same time when caterpillars were measured for body size. Pupae were collected and maintained in small plastic cups until adult emergence at $25 \pm 3^\circ\text{C}$ with 12 L: 12 D photoperiod and $30 \pm 10\%$ RH. Number of days for each individual to develop to adult stage was calculated for all three treatments. Lifetime fecundity was evaluated by allowing the unmated females to oviposit until death. All moths were kept individually in a 44 ml clear plastic vial (No. 55-12, Thornton Plastics Co., Salt Lake City, UT) with seven 1-mm-diameter holes drilled through the cap to allow air flow. A

piece of tissue paper was placed in each vial to collect the eggs that females produced. No food was provided after adult emergence.

The third experiment was conducted to test for sex-specific effects of *C. globosum* as an endophyte on pupal mass. Based on the effects observed in the previous two experiments, the endophyte treatments consisted only of plants grown from seeds treated with the Low spore concentration (10^6 spores/ml) and Controls (sterile water only). Five 1st instar larvae were placed onto each caged plant (8-9 weeks after planting), with 16 replicate plants for each treatment group. Larval weight and head capsule width were measured two weeks after hatching, and individual developmental stage was recorded. Pupae were collected, labeled, weighed and sexed before being placed into individual 44 ml clear plastic vials (modified as in the previous experiment), and maintained in an insect rearing room at $25 \pm 3^\circ\text{C}$ with a 12 L: 12 D photoperiod and $30 \pm 10\%$ RH. A piece of tissue paper was placed in each vial to collect the eggs that females produced. Lifetime fecundity was evaluated by allowing the unmated females to oviposit until death. No diet was provided after adult emergence.

Confirmation of endophyte colonization

Endophyte colonization efficiency was examined by surface sterilizing plant tissues with 70% ethanol for 2 min, 2% NaClO (for roots) or 0.5% NaClO (for green tissues) for 3 min, followed by four rinses in sterile water. Tissue cuttings of $0.5\text{cm} \times 0.5\text{cm}$ were placed onto PDA media and fungi recovered were examined one week later for

confirmation of target endophyte presence (Arnold and Engelbrecht, 2007). All tissue samples were separately imprinted onto PDA plates to test the efficacy of surface sterilization. The imprinting plates were kept together with the tissue culturing plates. Plants from nematode experiments were processed for endophyte re-isolation when samples were collected for nematode quantification, with ten plants per treatment. In the aphid and beet armyworm experiments, 10 plants from each treatment at first true leaf stage were collected to confirm endophytic colonization. Successful colonization was recorded by tissue type.

Plant performance evaluation

Endophyte treatment effects on plant growth were evaluated by measuring plant shoot fresh weight and height, together with root fresh weight and length at each time of sampling during both nematode experiments.

Statistical analyses

All statistical analyses were performed using JMP® Pro, Version 11.2.0 (SAS Institute Inc., Cary, NC, 1989-2013). All data were tested for normality and equality of variances. One-way ANOVA was performed to analyse the impact of endophyte treatment on nematode fitness ($\alpha = 0.05$). If a significant overall treatment effect was detected, the post-hoc comparisons of means were performed using the Tukey-Kramer HSD (honestly significant difference) test ($\alpha = 0.05$).

To investigate whether the nematode fitness differed due to endophyte or rhizosphere effects following fungicide treatment, a generalized linear model (GLM) was performed with a normal distribution and identity link function, with the endophyte and fungicide and their interactions as fixed factors. If there was a significant treatment effect, the post-hoc Tukey-Kramer HSD test was performed to compare means between treatments ($\alpha = 0.05$).

A log-likelihood ratio test was conducted to examine endophyte effects on beet armyworm larval developmental rate ($\alpha = 0.05$). A factorial ANOVA was performed to detect major impact factor between Endophyte and insect sex on pupal weight.

Relationships between beet armyworm pupal weight and fecundity were investigated by performing a simple linear regression for each treatment.

Results

Fungicide efficacy tests

The germination of *C. globosum* spores was completely inhibited on both Liquid Copper Fungicide-PDA plates and Mancozeb-PDA plates. Both fungicide treatments also significantly suppressed mycelial growth at Day 7 (one-way ANOVA $F_{2, 27} = 602.5608$, $P < 0.001$). Mycelial growth at Day 7 was completely inhibited by the Liquid Copper Fungicide treatment (Tukey's HSD, $P < 0.001$), but some mycelial growth was observed in the Mancozeb treatment, although it was still significantly reduced in size compared

to the control (Tukey's HSD, $P < 0.001$). At Day 14, mycelial growth continued to be significantly affected overall by fungicide treatment (one-way ANOVA $F_{2,27} = 337.5195$, $P < 0.001$). Mycelial growth was completely inhibited by the Liquid Copper Fungicide treatment (Tukey's HSD, $P < 0.001$), but was not significantly suppressed relative to the control in Mancozeb treatment (Tukey's HSD, $P = 0.9094$).

C. globosum - nematode interactions

Nematode infection. Nematode infection was determined by quantifying root penetration by second-stage juveniles (J2) and early gall formation at 12 DAI, and followed by root galling at 6 WAI (Fig. 3.1 a-c). The endophyte treatment significantly affected early galling (one-way ANOVA $F_{2,27} = 40.4287$, $P < 0.0001$; Fig. 3.1a) and J2 root penetration (one-way ANOVA $F_{2,27} = 36.4119$, $P < 0.0001$; Fig. 3.1b) at 12 DAI. Both Low and High endophyte treatments significantly reduced root penetration (Tukey's HSD, Low: $P < 0.0001$; High: $P < 0.0001$) and early galling (Tukey's HSD, Low: $P < 0.0001$; High: $P < 0.0001$) compared with the Control. There was no dose-dependent effect on either root penetration or early galling between Low and High treatments (Tukey's HSD, root penetration: $P = 0.9034$; early galling: $P = 0.3327$). The endophyte treatment also significantly affected the nematode root galling at 6 WAI (one-way ANOVA $F_{2,27} = 40.1207$, $P < 0.0001$; Fig. 3.1c). Both Low and High treatments significantly reduced root galling compared with the Control (Tukey's HSD, Low: $P < 0.0001$; High: $P < 0.0001$). No difference was observed between the Low and High treatments (Tukey's HSD, $P = 0.8078$).

Nematode reproduction. The endophyte treatment significantly affected *M. incognita* reproduction at 6 WAI (one-way ANOVA $F_{2,27} = 15.7610$, $P < 0.0001$; Fig. 3.1d). Reproduction was significantly reduced relative to the Control in both the Low and High treatments (Tukey's HSD, Low: $P = 0.0002$; High: $P < 0.0001$). There was no difference between the High and Low treatments (Tukey's HSD, $P = 0.9505$).

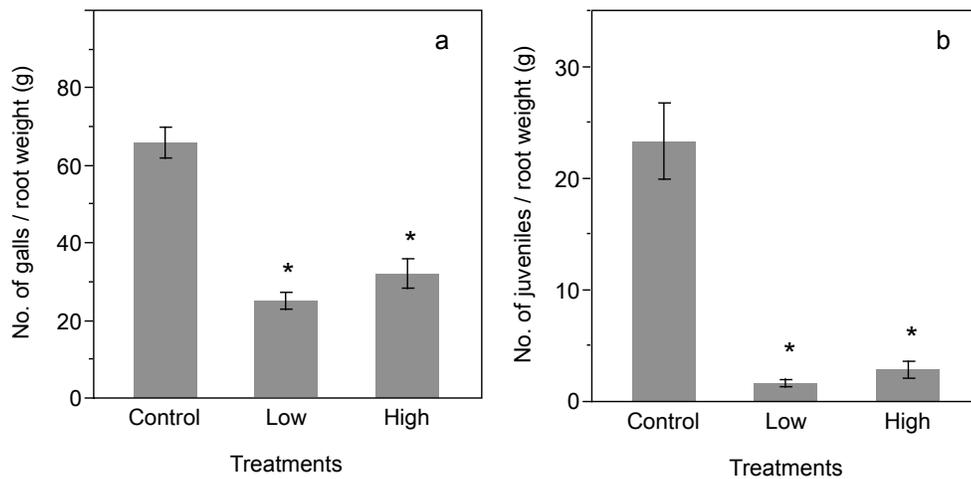


Fig. 3.1 Effects of endophytic *C. globosum* in cotton on root-knot nematodes. Data are presented as the average (a) number of early galls at 12 DAI, (b) number of juveniles penetrating roots at 12 DAI, (c) number of galls at 6 WAI, and (d) number of eggs at 6 WAI in the first nematode. Error bar represents ± 1 standard error of the mean. Symbol on each bar indicates a significant difference from the control treatment, $*P < 0.05$.

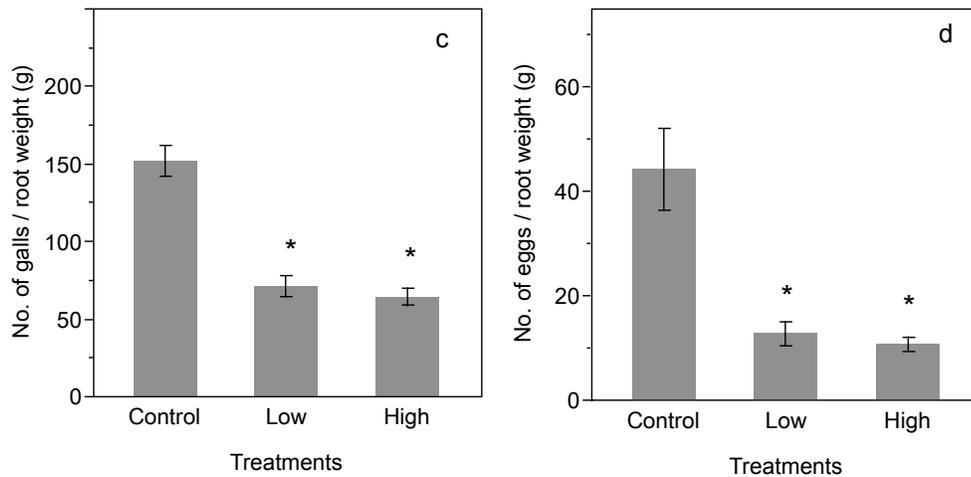


Fig. 3.1 Continued.

Endophyte vs. soil effects on nematode infection and reproduction. The endophyte and fungicide treatments both affected early galling at 12 DAI (GLM, Endophyte: $F_{2, 54} = 37.1418$, $P < 0.0001$; Fungicide: $F_{1, 54} = 16.5220$, $P = 0.0002$; Fig. 3.2a); but there was no significant interaction between the two factors (GLM, Endophyte \times Fungicide: $F_{2, 54} = 0.1709$, $P = 0.8433$). Overall, both Low and High treatments negatively affected early galling compared with the corresponding Control (Tukey's HSD, Low: $P < 0.0001$; High: $P < 0.0001$) and no difference between the two treatments was detected (Tukey's HSD, $P = 0.9015$). In the fungicide treatment group, both Low and High endophyte treatments significantly suppressed early galling compared with the Control treatment (Tukey's HSD, Fungicide-Low: $P < 0.0001$; Fungicide-High: $P = 0.0004$), and no difference was found between the High and Low endophyte treatments (Tukey's HSD, $P = 0.9871$). There was no difference in early galling between the no-endophyte Control

treatment in the absence of fungicide as compared to the no-endophyte Control in the Fungicide treatment group at 12 DAI (Tukey's HSD, $P = 0.3966$; Fig. 3.2a).

Root penetration by juvenile nematodes at 12 DAI was significantly affected by the endophyte treatment (GLM, Endophyte: $F_{2,54} = 52.1681$, $P < 0.0001$; Fig. 3.2b). There was no significant fungicide effect on nematode infection at 12 DAI and the interaction between the endophyte and fungicide treatments was marginally significant (GLM, Fungicide: $F_{1,54} = 0.3823$, $P = 0.5390$; Endophyte \times Fungicide: $F_{2,54} = 3.0921$, $P = 0.0535$). Overall, significantly reduced root penetration was found in both Low and High endophyte treatments compared with the Control (Tukey's HSD, Low: $P < 0.0001$; High: $P < 0.0001$). Within the fungicide treatment group, the Low and High treatments both negatively affected J2 penetration at 12 DAI compared to the Control (Tukey's HSD, Fungicide-Low: $P = 0.0001$; Fungicide-High: $P = 0.0004$). There was no significant difference in J2 penetration between the High and Low treatments in the fungicide treatment group (Tukey's HSD, $P = 0.9073$).

The endophyte and fungicide treatments both significantly affected galling at 6 WAI (GLM, Endophyte: $F_{2,54} = 96.3924$, $P < 0.0001$; Fungicide: $F_{1,54} = 29.7076$, $P < 0.0001$; Fig. 3.2c), with a significant interaction between the two (GLM, Endophyte \times Fungicide: $F_{2,54} = 3.4703$, $P = 0.0382$). Within the non-fungicide treatment group, both Low and High treatments significantly suppressed galling at 6 WAI (Tukey's HSD, Low: $P < 0.0001$; High: $P < 0.0001$); but no endophyte dosage effect was found (Tukey's HSD, P

= 0.1547). In the fungicide treatment group, both the Low and High dosages negatively affected galling (Tukey's HSD, Fungicide-Low: $P < 0.0001$; Fungicide-High: $P < 0.0001$), but there was no significant endophyte dosage effect (Tukey's HSD, $P = 0.9585$). Galling was significantly lower in no-endophyte Control treatment in the absence of fungicide as compared to the no-endophyte Control in the Fungicide treatment group (Tukey's HSD, $P < 0.0001$).

As with galling, nematode reproduction at 6 WAI was also significantly affected by both the endophyte and fungicide treatments (GLM, Endophyte: $F_{2, 54} = 94.8560$, $P < 0.0001$; Fungicide: $F_{2, 54} = 94.8560$, $P = 0.0062$; Fig. 3.2d), with a marginal interaction effect (GLM, Endophyte \times Fungicide: $F_{2, 54} = 3.1176$, $P = 0.0523$). In the non-fungicide treatment group, both of the endophyte dosages reduced egg production at 6 WAI (Tukey's HSD, Low: $P < 0.0001$; High: $P < 0.0001$), with no difference between the two (Tukey's HSD, $P = 0.9518$). In the fungicide treatment group, nematode reproduction was significantly reduced by both endophyte dosage treatments relative to the Control (Tukey's HSD, Fungicide-Low: $P < 0.0001$; Fungicide-High: $P < 0.0001$), with no endophyte dosage effect (Tukey's HSD, $P = 0.9585$). The fungicide treatment did have an effect on *M. incognita* reproduction in the absence of any endophyte treatments, with egg production being significantly lower in the no-endophyte Control treatment in the absence of fungicide as compared to the no-endophyte Control in the Fungicide treatment group (Tukey's HSD, $P = 0.0073$).

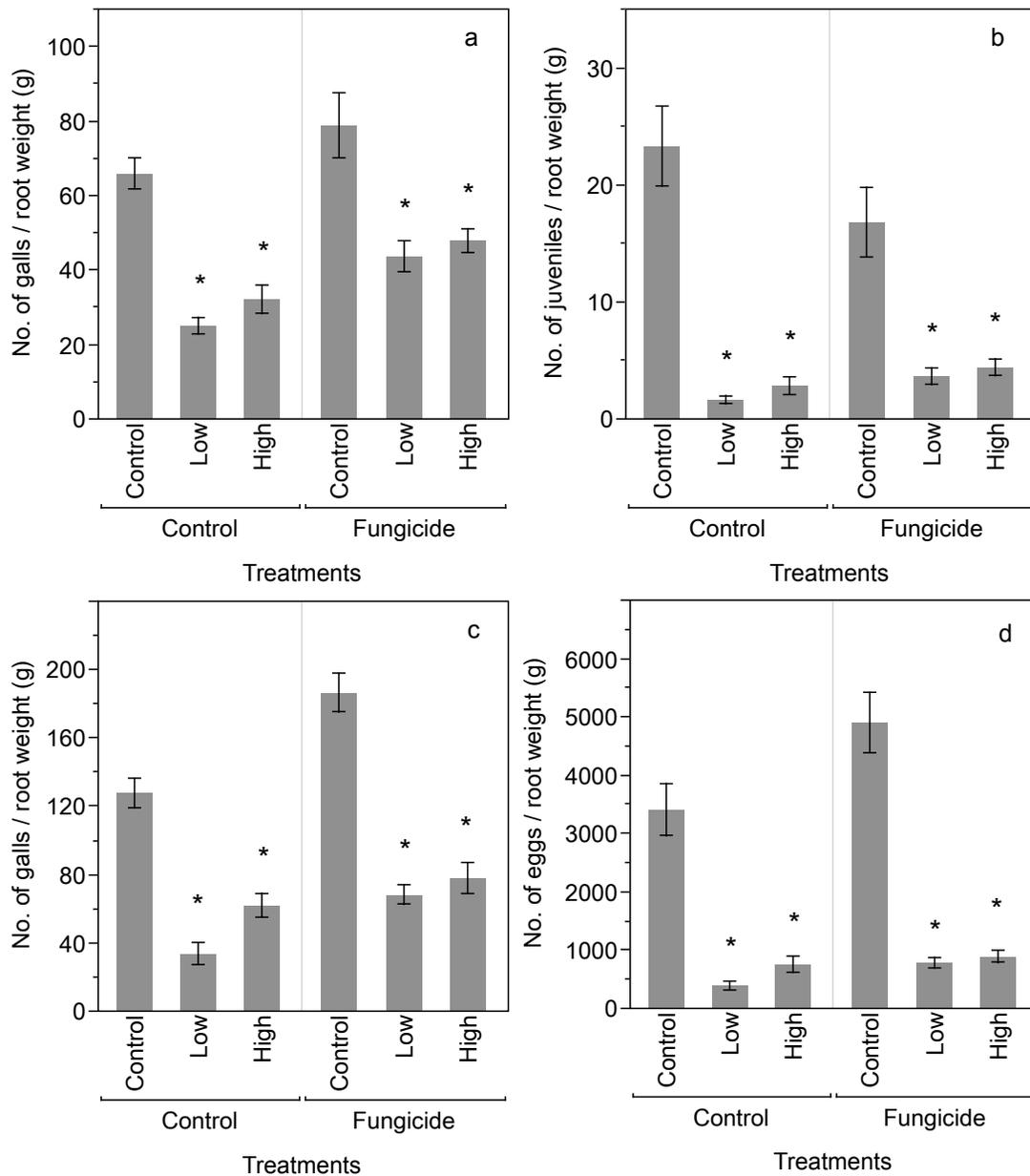


Fig. 3.2 *C. globosum* negatively affects nematodes as an endophyte in cotton when its presence in soil is controlled for with a fungicide treatment. (a) Number of galls at 12 DAI, (b) juvenile penetration at 12 DAI; (c) number of galls at 6 WAI, and (d) number of eggs at 6 WAI. Error bar represents ± 1 standard error of the mean. Symbol on each bar indicates a significant difference from the corresponding control treatment, $*P < 0.05$.

C. globosum - aphid interactions

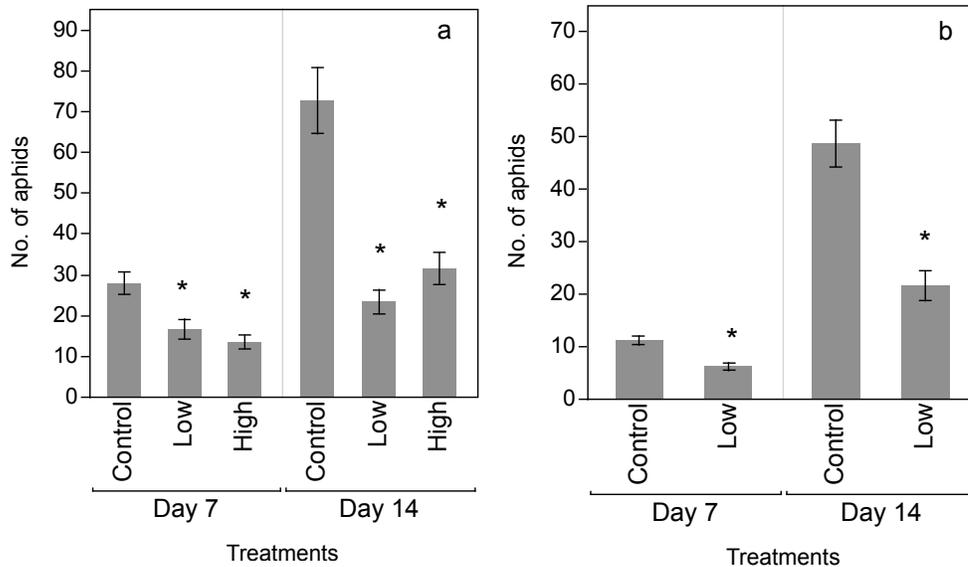


Fig. 3.3 Endophytic *C. globosum* in cotton negatively affected cotton aphid reproduction. Average number of aphids per plant after 7 and 14 days in two experiments. The experiments tested untreated control plants versus (a) both Low and High seed treatment concentrations, and (b) only a single Low seed treatment concentration. Error bar represents ± 1 standard error of the mean. Symbol on each bar indicates a significant difference from the corresponding control treatment, * $P < 0.05$.

The endophyte treatment had a significant effect on aphid population density per plant at Day 7 (one-way ANOVA, $F_{2, 56} = 10.6069$, $P = 0.0001$; Fig. 3.3a). Aphid populations were significantly reduced in both Low and High treatments (Tukey's HSD, Low: $P = 0.0032$; High: $P = 0.0001$) relative to the Control, but did not differ between the two dosage treatments (Tukey's HSD, $P = 0.5899$). At Day 14, a significant overall

endophyte effect on aphid population density was maintained (one-way ANOVA, $F_{2,56} = 23.9863$, $P < 0.0001$), with significant reductions in aphid population in both the Low and High treatment groups (Tukey's HSD, Low: $P < 0.0001$; High: $P < 0.0001$), with no endophyte dosage effect (Tukey's HSD, $P = 0.5298$).

Given the lack of a significant dosage effect on aphid population density, only one endophyte dosage was used in the second experiment. Aphid population size in the endophyte treatment was again significantly reduced versus the Control at both Day 7 (Student's t-test, $t_{29} = -4.4182$, $P = 0.0001$) and Day 14 (Student's t-test, $t_{29} = -5.1115$, $P < 0.0001$; Fig. 3.3b).

C. globosum - armyworm interactions

C. globosum as an endophyte in cotton affected caterpillar development. Caterpillars reared on endophyte treated plants developed significantly faster than those reared on untreated plants. At Week 2, only 13.08% of the individuals in the Control treatment had developed to the 4th and 5th instars compared to 28.8% of the Low and 23.93% of High treatment individuals (Chi-square, $\chi^2 = 14.532$, $df = 4$, $N = 372$, $P = 0.0058$; Fig. 3.4a). By Week 3 in the same experiment, a similar significant endophyte effect on caterpillar development remained with a higher proportion of individuals in the Low and High endophyte treatment groups reaching the pre-pupation and pupal stages, compared to the Control insects (Chi-square, $\chi^2 = 28.181$, $df = 6$, $N = 253$, $P < 0.0001$; Fig. 3.4b). In the Control treatment, only 28.26% individuals started pupation by Week 3, as opposed to

55.68% in Low and 42.47% in High endophyte treatments. Consistent effects on caterpillar developmental rate were found also in the second and third experiments at Week 2. In the second experiment, caterpillars in Low and High treatments developed faster than the ones in the Control (Chi-square, $\chi^2 = 19.074$, $df = 6$, $N = 210$, $P = 0.0045$; Fig. 3.4c). In the Control treatment, 36.24% individuals had pupated compared with 60.87% in the Low and 59.73% in the High treatment groups. Caterpillars in the third experiment exhibited a similar pattern of faster development in the endophyte treatment than the Control (Chi-square, $\chi^2 = 22.407$, $df = 3$, $N = 131$, $P < 0.0001$; Fig. 3.4d). Two weeks after hatching, 30.16% individuals in the Low endophyte treatment started pupating comparing with 4.41% individuals in the Control treatment.

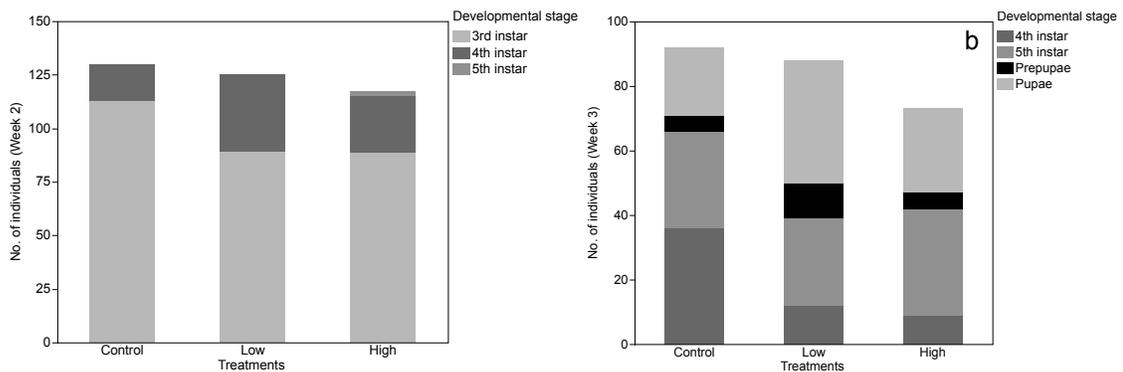


Fig. 3.4 *C. globosum* as an endophyte in cotton affects beet armyworm developmental rate. Number of beet armyworms at different developmental stages in each endophyte treatment group for (a) Experiment 1 sampled at week 2, (b) Experiment 1 sampled at week 3, (c) Experiment 1 sampled at week 2, and (d) Experiment 3 sampled at week 2.

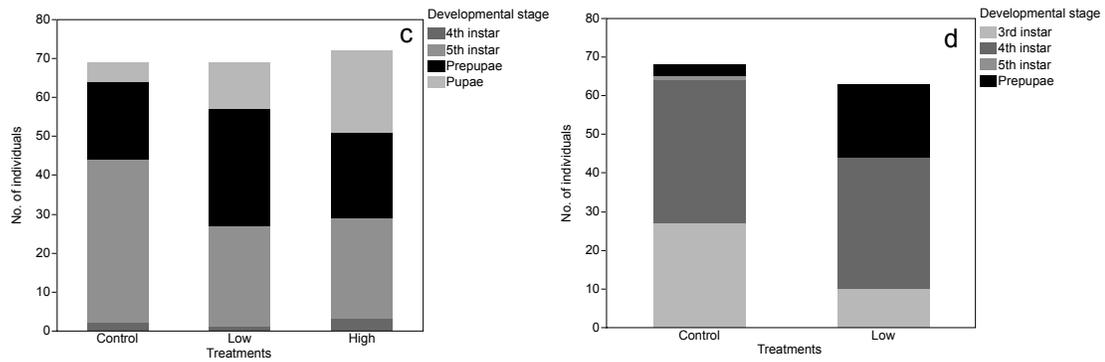


Fig. 3.4 Continued.

No endophyte effect was found on individual body weight at the larval (one-way ANOVA, $F_{2, 93} = 0.3976$, $P = 0.6731$), prepupal (one-way ANOVA, $F_{2, 72} = 0.4036$, $P = 0.6694$), or pupal stages (one-way ANOVA, $F_{2, 34} = 0.81$, $P = 0.4532$; Fig. 3.5a) (measurements were collected by Week 2 when individuals were at different developmental stages). However, a negative effect of the endophyte on growth was observed as evidenced by reduced head capsule widths in the endophyte treatment caterpillars two weeks after hatching (one-way ANOVA, $F_{2, 92} = 39.2601$, $P < 0.0001$; Fig. 3.5b). Caterpillars in both Low and High treatment had smaller head sizes (Tukey's HSD, Low: $P < 0.0001$; High: $P < 0.0001$), but no difference was found between the two dosage treatments (Tukey's HSD, $P = 0.7853$). Despite having smaller head capsules during the larval stage, insects in the endophyte treatments still had the same final pupal mass as the Control insects (Fig. 3.5a). Individuals in both the Low and High *C. globosum* treatments also reached the pupal stage significantly faster than Control insects (one-way ANOVA, $F_{2, 192} = 9.6056$, $P = 0.0001$; Fig. 3.5c) and there was no

difference in developmental time between the two dosage treatments (Tukey's HSD, $P = 0.6107$). Even though pupal weights did not vary, adult female fecundity was significantly reduced due to the endophyte treatments (one-way ANOVA, $F_{2, 69} = 7.2686$, $P = 0.0014$; Fig. 3.5d). Individuals from both the Low and High treatments produced significantly fewer eggs than the Control group insects (Tukey's HSD, Low: $P = 0.0046$; High: $P = 0.0051$), with no difference between the High and Low endophyte treatments (Tukey's HSD, $P = 0.9422$).

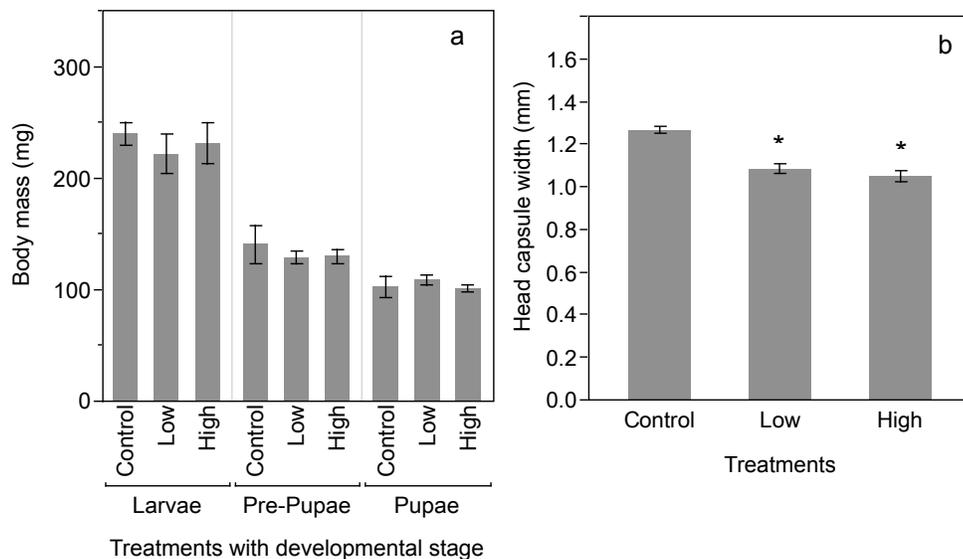


Fig. 3.5 *C. globosum* as an endophyte in cotton affected beet armyworm larval development and adult female fecundity. Average (a) body mass at Week 2, (b) larval head capsule width, (c) number of days to adult stage, and (d) female fecundity from three endophyte treatments in the second experiment. Error bar represents ± 1 standard error of the mean. Symbol on each bar indicates a significant difference from the corresponding control treatment, $*P < 0.05$.

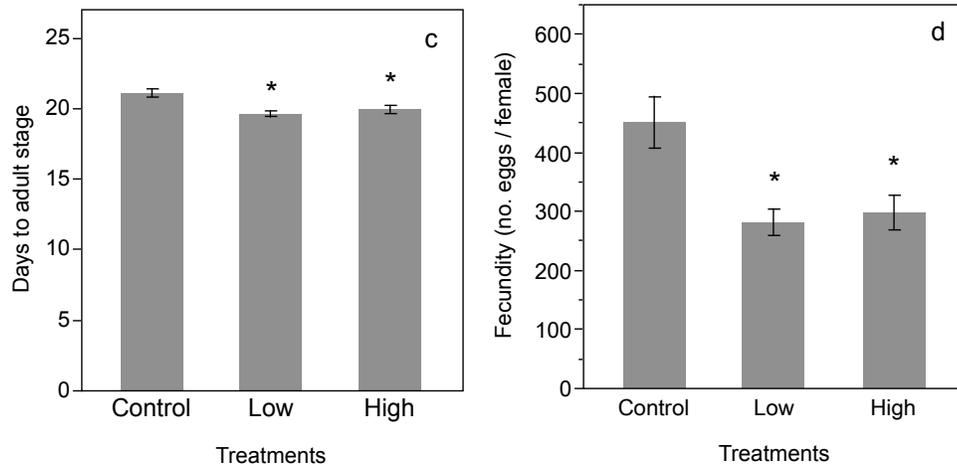


Fig. 3.5 Continued.

Given the lack of a dosage effect observed in the previous two *S. exigua* experiments, only one endophyte treatment concentration was used in the follow-up experiment testing for sex-specific endophyte effects on pupal mass and fecundity. Among the caterpillars (note that sex cannot be determined until the pupal stage), there was no effect of endophyte treatment on larval weights at Week 2 (Student's t-test, $t_{129} = -0.9309$, $P = 0.3537$; Fig. 3.6a), but larval head capsule width was smaller in the endophyte treatment versus the control (Student's t-test, $t_{129} = -5.4495$, $P < 0.0001$; Fig. 3.6b), consistent with results from the previous experiment (Fig. 3.5). Sex had major effect on pupal weights (2×2 factorial ANOVA, $F_{2, 113} = 2.5478$, $P = 0.059$; Fig. 3.6a; sex effect: $df = 1$, $F = 5.1023$, $P = 0.0258$), but neither the endophyte treatment (endophyte effect: $df = 1$, $F = 1.4666$, $P = 0.2284$) nor the interaction between endophyte and sex (sex \times endophyte effect: $df = 1$, $F = 1.3836$, $P = 0.242$) was significant, indicating no sex-

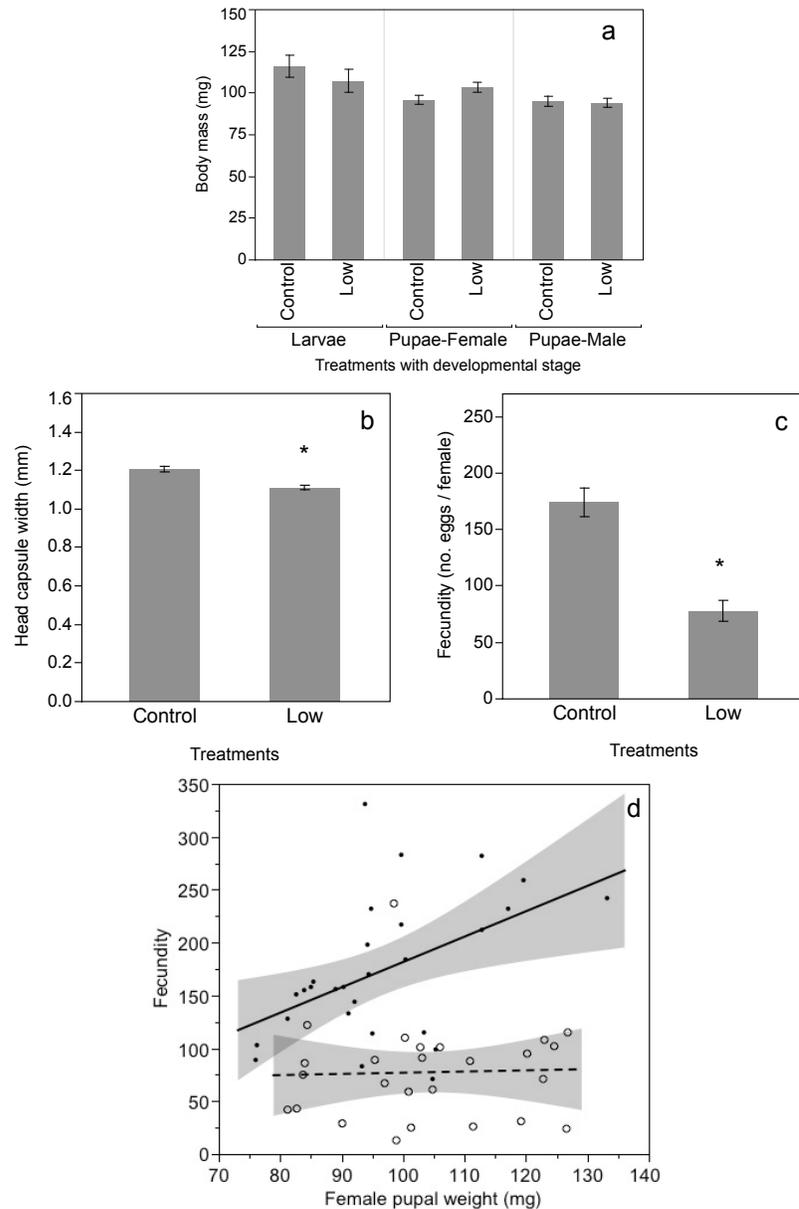


Fig. 3.6 Sex specific effects of *C. globosum* as an endophyte in cotton on beet armyworm development and female fecundity. Average beet armyworm (a) larval and pupal body weights, (b) larval head capsule width, and (c) female fecundity. Error bar represents ± 1 standard error of the mean. Significance level was at $\alpha = 0.05$. (d) Relationships between female pupal weight and fecundity of beet armyworms fed on cotton endophytically colonized by *C. globosum* (dashed line) or untreated control plants (solid line). Filled circles and open circles denote individuals from Control and endophyte treatments, respectively. Lines are regression lines. The shaded area represents the 95% confidence interval from the mean value.

specific effect of the endophyte on pupal weights. As in the previous experiment, the endophyte treatment significantly reduced adult female fecundity (Student's t-test, $t_{52} = -6.1010$, $P < 0.0001$; Fig. 3.6c). The endophyte treatment also had contrasting effects on the relationship between body size and female fecundity. Fecundity and female pupal weight showed a clear positive correlation in the Control treatment (Pearson correlation coefficient $r = 0.4832$, $n = 28$, 95% CI = [0.1344, 0.7255], $P = 0.0092$; Fig. 3.6d). However, no correlation was found between fecundity and female pupal weight among the endophyte treatment insects (Pearson correlation coefficient $r = 0.0355$, $n = 26$, 95% CI = [-0.3567, 0.4172], $P = 0.8631$; Fig. 3.6d).

Confirmation of endophyte colonization efficacy

Endophytic colonization frequencies of cotton by *C. globosum* were high across all the experiments, ranging from 90-96.7% of the sampled individuals. In the nematode experiments, endophytic colonization of cotton by *C. globosum* in the Low and High treatment groups was confirmed in 90% and 92.5% of the sampled plants, respectively. In the fungicide experiments, endophytic colonization was confirmed in 90% and 92.5% of the Fungicide-Low and Fungicide-High treatment group individuals, respectively. In the aphid and beet armyworm experiments, colonization frequencies in the Low and High treatments were 94% and 96.7%, respectively. Successful endophyte colonization was detected in both leaf and root tissues. No fungal contamination was found in the

imprinting plates, indicating successful surface sterilization. Detailed colonization efficacy data by tissue type for each experiment are provided in Table 3.1.

Table 3.1 *C. globosum* endophytic colonization efficacy in cotton. Data shown as the percentage of leaf (Leaf), root (Root), and whole plant samples (All) from which *C. globosum* was isolated from surface-sterilized tissue samples at 12 DAI (12D) and 6 WAI (6W). Colonization was defined as at least one piece of tissue with positive colonization by the target fungus. Colonization results are reported for each of the experiments conducted (CK: Control; RKN: root knot nematode treatments; F-CK: Control plants treated with fungicide, but lacking nematodes; F-RKN: plants treated with fungicide and nematodes). Treatments not included in that particular experiment are marked as "--".

Experimental System			Endophyte colonization efficacy (%)								
			Control			Low			High		
			Leaf	Root	All	Leaf	Root	All	Leaf	Root	All
			12D/6W	12D/6W	12D/6W	12D/6W	12D/6W	12D/6W	12D/6W	12D/6W	12D/6W
<i>M. incognita</i>	Ex. 1	CK	0/0	0/0	0/0	70/60	100/60	100/60	60/50	90/100	90/100
		RKN	0/0	0/0	0/0	70/ 80	100/90	100/90	50/70	100/90	100/90
	Ex. 2	CK	0/0	0/0	0/0	60/50	90/100	90/100	50/80	90/80	90/80
		RKN	0/0	0/0	0/0	70/50	80/100	80/100	60/70	90/100	90/100
		F- CK	0/0	0/0	0/0	60/70	90/90	90/90	60/50	80/100	80/100
		F-RKN	0/0	0/0	0/0	50/60	80/90	80/90	70/50	90/100	90/100
<i>A. gossypii</i>	Ex. 1	0	0	0	70	100	100	80	90	90	
	Ex. 2	0	0	0	80	90	90	--	--	--	
<i>S. exigua</i>	Ex. 1	0	0	0	70	100	100	70	100	100	
	Ex. 2	0	0	0	80	90	90	60	100	100	
	Ex. 3	0	0	0	60	90	90	--	--	--	

Plant growth enhancement

No consistent positive or negative effects on plant growth were observed in either of the nematode experiments (Appendix B).

Discussion

C. globosum strain 520 can be effectively inoculated to occur as an endophyte in cotton using a seed treatment, with positive colonization of a very high proportion of all the treated plants. Endophytic *C. globosum* was associated with a range of negative effects on both above- and below-ground herbivores with very different feeding modes.

Previous studies have reported endophytic establishment of *Chaetomium* sp. in various host plants (Istifadah and McGee, 2006, Istifadah et al., 2006, Jiao et al., 2006, Borges et al., 2011). Importantly, this is the first study to provide evidence for negative effects of any naturally-occurring *Chaetomium* fungi on insect herbivores when present in a plant as an endophyte.

Anti-nematode activity

Two previous studies reported the anti-nematode activity of endophytic *Chaetomium* fungi in plants (Sikora et al., 2003, Yan et al., 2011). Yan et al. (2011) reported establishment of endophytic *Chaetomium* strain Ch1001 both in both above- and belowground tissues of cucumber, as well as endophyte-mediated negative effects against *M. incognita*. It is known that *Chaetomium* sp. fungi can live in the soil (Tiedje and Hagedorn, 1975, Brewer and Taylor, 1978, Rodríguez et al., 2002). However, previous investigations of endophytic *Chaetomium* against nematodes did not differentiate between effects mediated by its presence as an endophyte within the plant versus the potential for antagonistic effects due to its presence in the soil arising as a

result of the inoculation process. We controlled for the potential rhizospheric effects of *C. globosum* on nematode experiments by applying a fungicide soil treatment prior to infesting the plants with nematodes. The fungicide soil treatment did not affect the endophytic colonization of the plant, nor did it affect the anti-nematode activity of the fungus as an endophyte. These results provide strong support for the role of endophytic *C. globosum* in conferring resistance to root-knot nematodes in colonized plants.

In a previous study of the effects of *Purpureocillium lilacinum* as a fungal endophyte in cotton against the root-knot nematode (Chapter II), the application of a similar fungicide in the absence of any endophyte treatment did not affect nematode penetration or galling as it did in this study. In the current study, the fungicide treatment itself had slight effects on galling at 12 DAI and 6 WAI, as well as on egg production (Fig. 3.2 a, c & d). Regardless, the same pattern of strong negative effects of the endophyte treatments on nematode infection and reproduction were present regardless of the fungicide application (Fig. 3.2). The underlying reason for variation in the fungicide effect across experiments is unknown, but could be due a range of factors including, but not limited to, variation in environmental conditions, plant condition, or indirect effects of *C. globosum* in the soil such as alteration of microbial communities prior to the fungicide treatment that did not occur with *P. lilacinum* used as seed treatment.

A few studies have reported pathogenicity of *Chaetomium* fungi against plant parasitic nematodes (Nitao et al., 2002, Yuan et al., 2011, Hu et al., 2012), but only two studies

considered its effects as an endophyte (Sikora et al., 2003, Yan et al., 2011). In our study, endophytic *C. globosum* inhibited J2 penetration, galling, and female reproduction at both early and late infection stages. Two dosage levels of endophyte spores applied as seed treatments were compared, but dose-dependent effects on efficacy against nematodes were not observed. The negative effects of endophytic *C. globosum* on nematodes were robust across multiple experiments, including following exposure of the plant to a fungicide treatment (Fig. 3.1 & Fig. 3.2).

Root-knot nematodes can secrete a number of signaling molecules that serve as the major effectors involved in nematode parasitism of host plants (Jasmer et al., 2003, Neveu et al., 2003, Davis et al., 2004). The infective J2 larvae are attracted to root exudates, but the surface of larvae is also modified in response to the root exudates (Lopez et al., 2000, Akhkha et al., 2002). Once the larvae penetrate the root, the host plant responds to nematode attack by producing reactive oxygen species (Melillo et al., 2006). The nematode may also secrete a number of proteins that interact with plant signaling pathways and suppress host defense (Doyle and Lambert, 2003, Jaubert et al., 2004). Nothing is known to date regarding how the presence of *C. globosum* as an endophyte might affect these processes within the plant to alter the outcome of nematode-host plant interactions. Previous studies have shown that endophytic fungi alter the expression patterns of phytohormone levels in host plants (Bunyard, 1990, Tudzynski, 1997, Waqas et al., 2012, Navarro-Meléndez and Heil, 2014), which can potentially affect induced defensive responses against nematode attack. Alternatively,

Chaetomium fungi are also known to secrete a number of bioactive secondary metabolites, some having anti-nematode activity (Nitao et al., 2002, Hu et al., 2012), potentially providing a means for direct protection of the plant against nematode parasitism.

Anti-aphid activity

Consistent reductions in aphid reproduction were observed on cotton plants in the presence of endophytic *C. globosum* at 7 and 14 days after infestation across replicate experiments. Both Low and High concentration seed treatment groups exhibited significantly lower levels of aphid reproduction than the untreated control plants (Fig. 3.3).

Several studies have shown negative effects of fungal endophytes on aphid fitness on various host plants (Omacini et al., 2001, Meister et al., 2006, Lopez et al., 2014). This is the first study to show a negative endophytic effect of a naturally-occurring strain of *C. globosum* on aphid reproduction. Qi et al. (2011) worked with a strain of *C. globosum* that could endophytically colonize several host plants, but did not find an antagonistic effect against aphids in rape seedlings. These results highlight the importance of variation in genotypes among different strains of endophytic fungi in affecting plant-herbivore interactions. Notably, a lab-generated strain of *C. globosum* genetically-modified to express the *Pinellia ternata* agglutinin gene was shown to endophytically colonize oilseed rape seedlings and reduce both the survival and reproduction of green

peach aphids, *Myzus persicae*, relative to the untransformed wildtype strain (Qi et al., 2011). Although the effects on aphids of the recombinant *C. globosum* strain were more extreme than those we observed for endophytic *C. globosum* strain 520, our results clearly illustrate that genetic modification of *C. globosum* is not required for all *C. globosum* genotypes in order to endophytically-confer resistance against insects to plants.

The mechanisms underlying *C. globosum*-mediated resistance to aphids in cotton remain unknown. Previous study of aphid feeding on *Arabidopsis* suggested that the feeding process leads to stimulation of plant defense pathways related to both pathogen infection and wounding (Moran and Thompson, 2001). Endophyte mediated antagonistic effects against insect herbivores may be due to a number of microbe produced compounds that act as elicitors of the host defense reaction (Tripathi et al., 2008, Van Wees et al., 2008). Endophytes may also produce secondary metabolites that can directly inhibit insect herbivory (Harman et al., 2004, Ownley et al., 2010). Endophyte associated resistance against insect herbivory due to the production of fungal alkaloids has been well established (Rowan et al., 1990, Siegel et al., 1990, Breen, 1993b, Salminen et al., 2005). For example, Wilkinson et al. (2000) found that loline alkaloid production by an obligate fungal endophyte was associated with protecting their grass hosts from certain aphids. Similar examples of endophyte-produced secondary metabolites reducing aphid fitness have been reported in other grass-obligate endophyte systems (Siegel et al., 1990, Shymanovich et al., 2015). However, the role, if any, of secondary metabolite

production by facultative fungal endophytes such as *C. globosum* in deterring herbivory in other plant systems remains to be determined.

Anti-beet armyworm activity

According to Hartley and Gange (2009), 61% of the studies examining grass fungal endophyte effects on lepidopterans were conducted using fall armyworms, *Spodoptera frugiperda*. The endophyte-related effects on caterpillars observed in these studies included lower larval weight (Hardy et al., 1985, Boning and Bultman, 1996), reduced survivorship, reduced plant consumption (Breen, 1993a), and accelerated development (Bultman and Conard, 1998). This study utilized a different armyworm species, the beet armyworm, *S. exigua*, in the context of a different plant-endophyte system involving a facultative endophyte colonizing a dicot host plant. Endophytic *C. globosum* in cotton significantly affected caterpillar developmental speed. Individuals fed on endophyte-plants grew more rapidly than those fed untreated Control plants and the effect of accelerated development was consistently found across three different experiments (Fig. 3.4). The effect of accelerated development was also apparent in the shorter time to reach the adult stage observed among the caterpillars fed *C. globosum* colonized plants (Fig. 3.5c).

Contrary to the *S. frugiperda* results reviewed by Hartley and Gange (2009), reduced *S. exigua* larval weights were not observed when *C. globosum* was present as an endophyte in cotton. Although larval weights did not differ, reduced head widths were found when

caterpillars were fed on endophyte-colonized plants, with a consistent effect across both experiments in which head widths were measured (Fig. 3.5b & Fig. 3.6b). These results suggest that insects fed the endophyte treated plants were developing faster (Fig. 3.4), with smaller head capsules (Fig. 3.5b & Fig. 3.6d), but not at the expense of body mass (Fig. 3.5a & Fig. 3.6a).

The mechanisms that regulate body size and developmental characteristics are reasonably well known in insects. Insect body size and growth are controlled by levels of two hormones, insulin and ecdysone. The critical weight is defined as the threshold weight a larvae must achieve in order to undergo metamorphosis. It reflects the size of a larvae when the uptake of nutrients is no longer necessary for metamorphosis to occur (Johnston et al., 1971). Nijhout and Callier (2015) summarized that the nutrition and growth speed both largely affect larval critical weight. The threshold size at which molting will occur can be determined by either larval weight at molting or the head-capsule width at beginning of each instar (Nijhout and Callier, 2015). In this study, how the presence of *C. globosum* as an endophyte affected beet armyworm larvae in determining their critical weight or head size for molting is not known. However, given that head capsule sizes were smaller among larvae fed endophyte-treated plants, but their body weights did not differ from the Controls, our results suggest that larval weight took precedence over head capsule width as a proxy for body size as a determining factor for critical weights in this system. The fact that final pupal weights were the same across endophyte treatment groups that previously differed in developmental rate and head

capsule sizes further supports the idea that a threshold size for metamorphosis based on body weight was the key determinant of pupal weights.

Although pupal weights did not differ in this study, endophytic *C. globosum* negatively affected the fecundity of the resulting adult females. Similar results of reduced herbivore fecundity after feeding on endophyte-colonized plants have also been found in other lepidopterans (Jallow et al., 2004, Lopez and Sword, 2015), as well as insect herbivores in other orders (Barker et al., 1984, Mathias et al., 1990, Van Bael et al., 2009, Akello and Sikora, 2012). Several studies have also shown a positive relationship between pupal weight and lifetime fecundity in *S. exigua* (Greenberg et al., 2001, Azidah and Sofian-Azirun, 2006). In this study, a positive correlation between pupal weight and fecundity was only established in the absence of endophytic *C. globosum*; there was no correlation between pupal size and fecundity among females reared on cotton colonized by *C. globosum* as an endophyte (Fig. 3.6d). Thus, not only did *C. globosum* as an endophyte reduce female fecundity, it also eliminated the often-observed correlation between body size and female fecundity. Given that adult females reared on either *C. globosum*-colonized or uncolonized plants all had similar pupal weights, the fact that egg production did not scale with body size among the females reared on endophyte-colonized plants suggests that something limited the absolute number of eggs they could produce independent of their body size. Kaur et al. (2013) suggested that the ethyl acetate extract from a fungal endophyte effectively reduced fecundity of *S. litura*, indicating that fungal metabolites could play a role in reducing fecundity on endophyte-

colonized plants. *C. globosum* is known to produce a number of bioactive metabolites against plant pathogens (Sekita et al., 1981, Dipietro et al., 1992, Aggarwal et al., 2004, Park et al., 2005, Istifadah and McGee, 2006). Whether *C. globosum* metabolite production when present as an endophyte in cotton is responsible for the reduction in *S. exigua* fecundity observed here remains to be seen.

Ecological context of endophytic *C. globosum* in cotton

This study demonstrated for the first time that a single endophyte, *C. globosum* strain 520, can affect the ecological interactions between a host plant and a suite of its above- and below-ground herbivores with three different modes of feeding: sucking insects, leaf chewing insects, and plant parasitic nematodes. A number of studies primarily considering the ecological effects of mycorrhizal fungi suggest that they can affect the performance of herbivores feeding above-ground, but that their effects depend of the mode of herbivory (Hartley and Gange, 2009, Schausberger et al., 2012). It is commonly suggested that the arbuscular mycorrhizal fungi will negatively affect generalist chewing insects, but often have contrasting positive effects on specialist chewing and sucking insects (Gange et al., 2005, Bennett and Bever, 2007). Tintjer and Rudgers (2006) found similar results with fungal endophytes in *Elymus hystrix* grasses, as generalized chewing insects were more sensitive to the presence of endophytes than more specialized herbivores. In a study by Crawford et al. (2010), endophyte-infected grasses were more attractive to aphids in the laboratory, but received less damage in the field, contrary to a marginally negative effect on *S. frugiperda* larval developmental rate in the presence of

endophyte. Other work suggested a lack of an endophyte effect on *S. frugiperda* larval or pupal weight in grass-endophyte symbiosis (Afkhami and Rudgers, 2009), a finding that is contrary to the previously noted negative effects on *S. frugiperda* survival, mass gain, and development rate in other endophyte-*Festuca* species complex (Cheplick and Clay, 1988). As a generalist leaf chewing herbivore, *S. exigua* is known to have a broad host range including more than 50 plant species from over ten families globally (Wilson, 1932, Smits et al., 1987) and it was accordingly negatively affected by endophytic *C. globosum* in our study. As such, our *S. exigua* results largely support the idea of negative impacts of certain endophytes on generalist chewing insects (Crawford et al., 2010).

A. gossypii is a sucking insect and considered one of the most polyphagous aphid species (Van Emden and Harrington, 2007). However, a recent analysis of the population genetic structure of *A. gossypii* suggested that the aphid lineage on cotton is a specialized clone specifically adapted to cotton plants (Carletto et al., 2009). In so far as the cotton-associated lineage of *A. gossypii* can be considered a specialist sucking insect, the negative effects of *C. globosum* on aphid performance observed in this study are opposite to the hypotheses that fungal endophytes should have positive effects on specialist sucking insects (Gange et al., 2005, Bennett and Bever, 2007).

Although no consistent effect of endophytic *C. globosum* on plant growth was observed in this greenhouse study, some differences in plant performance in the presence of endophytic *C. globosum* in cotton have been observed under field conditions (Chapter

IV). *C. globosum* is known to affect plant performance in other systems, including as an endophyte in other plants. Tarafdar and Gharu (2006) described application of *C. globosum* as soil treatment that significantly enhanced plant growth of wheat and pearl millet crops, with positive effects attributed to production of phosphatases and phytases by the fungus. Enhanced plant growth by endophytic *C. globosum* and *C. funicola* in barley were also demonstrated when applied as a seed treatment (Vilich et al., 1998).

Plant microbiomes are populated with numerous microorganisms that may potentially compete with endophytic *C. globosum* for nutrients and other resources. The microbial endophyte community is largely affected by plant genotype (Whipps et al., 2008) and its diversity can vary greatly among tissues types within the same individual plant (Kumar and Hyde, 2004). Additionally, intentionally inoculating plants with a particular target endophyte may lead to correlated changes in microbiome community structure due to either facilitation or inhibition of other microbes in the plant (Andreote et al., 2009, Andreote et al., 2010, Bullington and Larkin, 2015). In such cases where inoculating plants leads to an observed phenotypic or ecological effect, it is difficult to distinguish between the direct effects of the target endophyte versus correlated responses in the other microbiome community members. Although the negative effects observed here on aphids, caterpillars and nematodes are associated with the endophytic colonization of cotton by *C. globosum*, future work is required to establish direct causal relationships for these effects and to determine the underlying mechanisms.

CHAPTER IV
APPLYING ENDOPHYTIC *PURPUREOCILLIUM LILACINUM* AND
CHAETOMIUM GLOBOSUM TO MANAGE NEMATODES IN COTTON
AGROECOSYSTEMS

Introduction

Plant parasitic nematodes can cause great economic losses in crop production systems. In particular, sedentary endoparasitic nematodes such as the root-knot (*Meloidogyne* spp.) and reniform nematodes (*Rotylenchulus* spp.) have a complex interaction with their host plants, causing the most damaging on a wide range of major agricultural crops (Tytgat et al., 2000). Both root-knot and reniform nematodes are among the major nematodes pests of cotton (*Gossypium hirsutum*) (Robinson, 2007). In root-knot nematodes, the females entirely penetrate root tissue and induce the formation of specialized feeding structures known as giant cells in plant roots (Perry et al., 2009). Heavy infection and massive gall formation due to root-knot nematodes may lead to a smaller root system and reduced root efficiency due to deficient water and nutrient translocation (Taylor and Sasser, 1978). The *Meloidogyne* spp. can survive in the soil, overwintering for months if soil temperature and moisture are favorable (Daulton and Nusbaum, 1961, Sayre, 1963). Reniform nematode females penetrate the root cortex with only the anterior part of their body and establish a permanent feeding site involving syncytial cells, while the posterior body remains in the soil. Damage caused by reniform

nematodes in cotton includes dwarfed and chlorotic plants, with fewer secondary roots and higher plant mortality (Lambe and Horne, 1963). Some species such as *R. reniformis* can survive at least two years in dry soil, even when host plants are absent (Radewald and Takeshita, 1964).

Current nematode control practices include chemical and cultural control with some use of host plant resistance. Beginning in late 1970s, the increasing awareness of environmental and human health safety has greatly reduced the amount of chemical usage or number of new nematicides approved for use (Nyczepir & Thomas 2009). Studies using nematophagous microbes as biological control agents for nematode management have received more attention as the withdrawal of several nematicides (e.g. methyl bromide, dichloropropene, aldicarb and phenamiphos) from the pest control market increases the need for new strategies to control nematode damage.

One major group of potential biological control agents is nematophagous fungi. The most intensively studied fungal agents, *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinus*, by Luangsa-ard et al. (2011)), *P. chlamydosporia*, and *Trichoderma* spp., are soil borne fungi that are parasites of sedentary stages of plant parasitic nematodes (Siddiqui and Mahmood, 1996, Sharon et al., 2001, Sharon et al., 2007). An alternative to the application of fungal biological control agents to the soil for nematode control is the manipulation of fungal endophytes within the plant (Latch, 1993, Hallmann and Sikora, 1996). Endophytes, according to Porras-Alfaro and Bayman

(2011b), are microorganisms that colonize plants without showing any symptoms of disease. Several studies have considered the use of endophytes against plant parasitic nematodes (Cabanillas et al., 1988, Mendoza and Sikora, 2009, Yan et al., 2011, Martinez-Beringola et al., 2013, Waweru et al., 2013, Tian et al., 2014a, Bajaj et al., 2015). However, few studies attempting to use endophytes to specifically control reniform nematodes have been reported (Sitaramaiah and Sikora, 1982, Sikora, 1992).

In addition to providing plants enhanced resistance against nematodes, beneficial endophytes have been shown to promote plant growth and resistance against other biotic or abiotic stressors (Hardoim et al., 2015). For example, Arnold et al. (2003) reported that horizontally transmitted endophytes were involved in host defense activity and limited pathogen damage in a tropical tree. Several studies have shown the anti-insect effects of the endophytic insect pathogen *Beauveria bassiana* (Vega et al., 2008, Lopez et al., 2014), as well as its effective suppression of plant disease (Ownley et al., 2008, Gómez-Vidal et al., 2009) and plant growth enhancing effects (Lopez and Sword, 2015, Murphy et al., 2015, Waqas et al., 2015). Fungal endophytes have also been shown to increase tolerance towards drought stress (Malinowski and Belesky, 2000, Bae et al., 2009), nitrogen deficiency (Ravel et al., 1997), salt stress (Baltruschat et al., 2008), and temperature extremes (Redman et al., 2011, Hubbard et al., 2012).

The fungi, *P. lilacinum* and *Chaetomium globosum*, have been shown to negatively affect the colonization of roots and the subsequent fecundity of *M. incognita* when

present as an endophyte in cotton in multiple greenhouse trials (Chapters II & III; Zhou, Wheeler, Starr & Sword, unpublished manuscripts). Although *P. lilacinum* is well known as a nematophagous fungus, fewer studies have considered *C. globosum* for potential antagonistic activity against plant parasitic nematodes (Maosong, 1989, Nitao et al., 2002, Yan et al., 2011). In this study, six field trials were conducted in 2013 and 2014, testing for efficacy of *P. lilacinum* and *C. globosum* against both root-knot nematode and reniform nematodes in cotton. Both nematode populations and plant growth were quantified to address the following questions about the efficacy of fungal endophytes for nematode management under field conditions in cotton agro-ecosystems:

1. Can fungal endophytes be inoculated to cotton and survive in the field?
2. Do endophytes provide the plants with enhanced resistance against root-knot and reniform nematodes?
3. Do the endophytes benefit host plants by promoting plant growth and/or increasing yield?

Materials and methods

Fungal culture preparation

Fungal endophytes were originally isolated from surface-sterilized cotton leaves (*Purpureocillium lilacinum* strain 490) or squares (*Chaetomium globosum* strain 520) collected in Texas, USA (Ek-Ramos et al., 2013), and stored in mineral oil suspension at -80°C. Fungal inoculum for experiments was cultured in petri dishes (100 × 15 mm,

VWR® disposable petri dish, sterile, VWR International) on potato dextrose agar (PDA) media at 25°C in the dark. Conidia were harvested when mature by flooding the petri dish with sterile water, scraping the plate with a sterile razor blade, and spores were filtered out through autoclaved cheesecloth. Conidia concentrations of the resulting suspension were quantified using a hemacytometer (Thomas Scientific, Philadelphia, PA, USA). Concentrations of spore suspension for seed treatments were finalized to 10⁶ (Low) or 10⁷ (High) conidia/ml. In 2013, only *P. lilacinum* was evaluated in the field, whereas both *P. lilacinum* and *C. globosum* were tested in the field in 2014.

Plant genotype

In 2013, cotton cultivar FiberMax FM2484B2F (Bayer CropScience, Lubbock, TX) was used. In 2014, Phytogen PHY499WRF and PHY367WRF (Dow AgroSciences, Indianapolis, IN) were planted. FM2484B2F and PHY499WRF are susceptible to the southern root-knot nematode (*M. incognita*) (Reid et al., 2012, McPherson, 2014) whereas PHY367WRF has partial resistance to the southern root-knot nematode (Aryal, 2011).

Seed inoculation

Two methods of inoculating seed with the candidate endophytic fungi for the field experiments were tested, soaking versus seed coating. In 2013, only the seed soaking treatment was used whereas in 2014, both seed soaking and seed coating were tested. For the soaking treatment, seeds (delinted black seed without fungicides or insecticides)

were soaked in conidial suspensions overnight (approximately 200 seeds/10ml). Seeds for the Control treatment were treated using sterile water under the same conditions. In 2013, three soaking treatments were compared: Control (sterile water), Low (10^6 conidia/ml), and High (10^7 conidia/ml). In 2014, only two seed soaking treatments were tested, Control (sterile water) and High (10^7 conidia/ml). All soaked seeds were then planted in the field trials the next day. For the seed coating treatment in 2014, a sticker solution containing 1% Methyl cellulose (MC) (Sigma-Aldrich[®], M7140-250G, 15cP viscosity) and fungal endophyte spores were combined and applied to cotton seeds with an average of 10^5 spores delivered to each seed (Bardin and Huang, 2003, Kumar et al., 2007). Seeds coated using the sticker solution mixed with either water (Control) or the High concentration spore suspension were then dried at room temperature and coated with talc powder (Sigma-Aldrich[®], Prod. No.18654) to prevent sticking together. All methylcellulose treated seeds were planted within one week after seed treatment was completed.

Root-knot nematode egg extraction

Eggs were extracted from 500 cm³ soil samples surrounding cotton roots. Roots and soil were mixed in 3 liters of water for 15 seconds and allowed to settle for 15 seconds. The water and organic matter were then poured through a sieve with a pore size of 23 µm. Eggs were extracted from the organic matter caught on the sieve by the NaOCl extraction method (Hussey and Barker, 1973).

Nematode soil extraction

Vermiform nematodes were extracted from 200 cm³ soil using a pie-pan extraction method for 48 hours (Thistlethwayte, 1970).

Confirmation of endophyte colonization

Endophyte colonization efficacy was examined by surface-sterilizing plant tissues with 70% ethanol for 2 min, followed by 2% NaClO (for roots) or 0.5% NaClO (for leaves and stems) for 3 min, and four rinses in sterile water. Surface sterilization efficacy was tested by tissue imprinting on sterile PDA plates before placing tissues onto culture medium for endophyte recovery. Tissues cuttings of 0.5cm × 0.5cm were placed onto PDA media and incubated at 25 ± 1°C in the dark. Fungi recovered were visually examined one week later for confirmation of target endophyte colonization and calculation of inoculation efficiency (Arnold and Engelbrecht, 2007). The number of plants sampled for endophyte colonization varied among field trials; therefore inoculation frequency is reported as the percentage of plants within a treatment group from which at least one tissue fragment was positively colonized by the candidate endophytic fungi.

Field site locations

Field trials were conducted at three sites each year evaluating the effects of endophytes on the reniform (*R. reniformis*) and southern root-knot (*M. incognita*) nematodes. The field site for the reniform experiment in both years was in Lubbock County, TX. Field

locations for root-knot nematode trials in 2013 were in Gaines County and Cochran County, TX. In 2014, the trials were conducted in Dawson County and Cochran County, TX. All the fields were naturally infested with nematodes.

Experimental design

A randomized complete block design was used for all field experiments in 2013. In 2014, a split-plot design was used with endophyte species and cotton variety as the main plot factors, and seed soaking versus sticker treatment as the subplots. All tests contained six replicate plots of each treatment. Plots were 11 m in length, and either four rows wide in 2013 or two rows wide in 2014 for the split plot design. There were 200 seeds per row planted using a tractor-drawn cone planter (Kincaid Equipment manufacturing, Haven, KS).

The measurements below were taken in-season. The sampling dates at each location are provided in Table 4.1.

- 1) Plant samples were collected at the 1st – 2nd true leaf stage to determine endophyte colonization frequency under field conditions.
- 2) Plant density: the number of plants was counted for one entire row within in each plot.
- 3) Gall ratings: 10 plants were removed from the plot, and the numbers of galls present on the roots were counted in the lab.

- 4) Soil sampling for nematodes: soil samples were taken at 10 random locations in a plot with a narrow bladed shovel at a depth of 10 – 20 cm near the taproots of the plants. Both soil and roots were removed at that depth. The soil from the 10 locations was mixed and a subsample of approximately 1,000 cm³ soil was removed and used in the soil assays (described above).
- 5) Yield: two rows were harvested with a cotton stripper modified for plot work. The harvest weight from the plot was obtained by loading cells in the stripper. Harvest weight consists of lint, seed, and trash. Samples were taken from the harvest weight (approximately 1,000 g) and ginned to determine the turnout (lint weight).

Table 4.1 Timing of various field operations conducted as part of 2013 and 2014 endophyte-nematode field trials in Texas.

Location	Nematode	Year	Planting date	Stand counts	Plant samples	Gall samples	Soil samples	Harvest date
Cochran	Root-knot	2013	14 May	11 July	20 June	11 July	12 Sept.	21 Nov.
Gaines	Root-knot	2013	23 May	13 June	10 June	30 July	30 July 21 Aug.	9 Nov.
Lubbock	Reniform	2013	16 May	5 June	30 May 20 June	--	24 June 30 July	4 Nov.
Cochran	Root-knot	2014	20 May	12 June	12 June	23 June	12 Aug.	2 Dec.
Dawson	Root-knot	2014	19 May	18 June	11 June	25 June	22 July	20 Nov.
Lubbock	Reniform	2014	10 May	5 June	5 June	--	5 May 17 June 21 July	19 Nov.

Statistical analysis

All data were tested for normality using Shapiro-Wilk tests and Normal Quantile Plots, and analyzed using one-way ANOVA tests when data were distributed normally. When multiple treatment variables were included in one site, a Generalized Linear Model (GLM) was used to test for main effects of the endophyte, seed treatment method, and variety, and all pairwise interactions on all measurements. A Student's t-test was conducted for pairwise comparisons. If data were not normally distributed, an overall non-parametric Kruskal-Wallis rank sum test was performed, followed by Wilcoxon Signed Rank tests for pairwise comparison. A significance level of $\alpha = 0.10$ was used to infer statistical significance due to the constraint on the number of replicate plots that could be planted and managed for these field trials.

A contour heat map was generated to visualize the spatial distribution of nematodes in our field sites. Accordingly, given that the distribution of nematodes was patchy in the field, all data were analyzed using the original dataset and an adjusted dataset controlled for the spatial factor of nematode distribution. All variables were adjusted by subtracting the measurement of the nearest Control treatment plot from the corresponding treatment plot measurement, yielding a contrast variable that reflects the local effects in both plots.

Within each replicated block, data were transformed using the following equation:

$$N_{adj} = \frac{(N_{trt} - N_{CK})}{N_{CK}}$$
 where N_{adj} = the adjusted value of the focal variable N , N_{trt} = focal

variable in the treatment plots, and N_{CK} = focal variable in the nearest control plot. When a zero value from a Control plot led to a zero in the denominator, data were instead

transformed by: $N_{adj} = N_{trt} - N_{CK}$ for that variable. All pairwise comparisons were conducted using spatially adjusted data. JMP® Pro, Version 11.2.0 (SAS Institute Inc., Cary, NC, 1989-2013) was used for all analyses.

Results

Endophyte colonization efficiency

Due to technical problems, colonization efficacy data were not collected in 2013. In 2014, positive endophytic colonization of cotton by *P. lilacinum* and *C. globosum* was found at all sites. Results are reported in Table 4.2 by treatment.

Table 4.2 Endophyte colonization efficacy in the 2014 field trials. Data reported by tissue types (leaf and root) and overall plants (All) as the percentage of plants from which the target fungi were positively isolated in three endophyte treatments: Control, *C. globosum*, and *P. lilacinum*; two cotton varieties: 499 (PHY499WRF) and 367 (PHY367WRF); and two seed treatment methods: S (soaking) and M (methylcellulose); at three locations: Lubbock (LB), Dawson (DS), Cochran (CR).

Location	Nematode	Var.	Endophyte colonization efficacy (%)																		
			Control						<i>C. globosum</i>						<i>P. lilacinum</i>						
			Root		Leaf		All		Root		Leaf		All		Root		Leaf		All		
			S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	S	M	
Lubbock	Reniform	499	0	0	0	0	0	0	0	58	33	42	58	67	75	25	0	25	17	42	25
		367	0	0	0	0	0	0	0	100	42	83	42	100	75	0	17	42	25	42	42
Dawson	RKN	499	0	0	0	0	0	0	25	8	17	0	42	8	17	17	17	17	33	33	
		367	0	0	0	0	0	0	33	17	33	0	58	17	0	8	17	17	17	17	25
Cochran	RKN	499	0	0	0	0	0	0	8	0	50	25	58	25	25	8	25	33	50	42	

Nematode quantification

Heat maps to visualize nematode population distributions at different stages in the field indicated substantial spatial variation in nematode distributions among the test plots. Examples of the typical distribution patterns at one site are shown in Fig. 4.1. Based on the patchy distribution pattern, we standardized all data by controlling for the spatial factor. Analyses of both the original and spatially-adjusted nematode data are presented below for each location and year.

Gaines County 2013 (root-knot nematode). There was no statistically significant effect of endophyte treatment on root-knot galling (one-way ANOVA, original data: $F_{2,15} = 0.14, P = 0.87$; adjusted data: $F_{2,15} = 0.32, P = 0.73$), juvenile population (one-way ANOVA, original: $F_{2,15} = 1.27, P = 0.31$; adjusted: $F_{2,15} = 0.72, P = 0.50$), or egg number (one-way ANOVA, original: $F_{2,15} = 1.15, P = 0.34$; adjusted: $F_{2,15} = 0.98, P = 0.40$) in July 2013. We also sampled the juveniles and eggs in August, but there was no significant treatment effect on either juvenile population (one-way ANOVA, original: $F_{2,15} = 1.65, P = 0.23$; adjusted: $F_{2,15} = 0.46, P = 0.64$), or egg number (one-way ANOVA, original: $F_{2,15} = 0.063, P = 0.94$; adjusted: $F_{2,15} = 0.32, P = 0.73$).

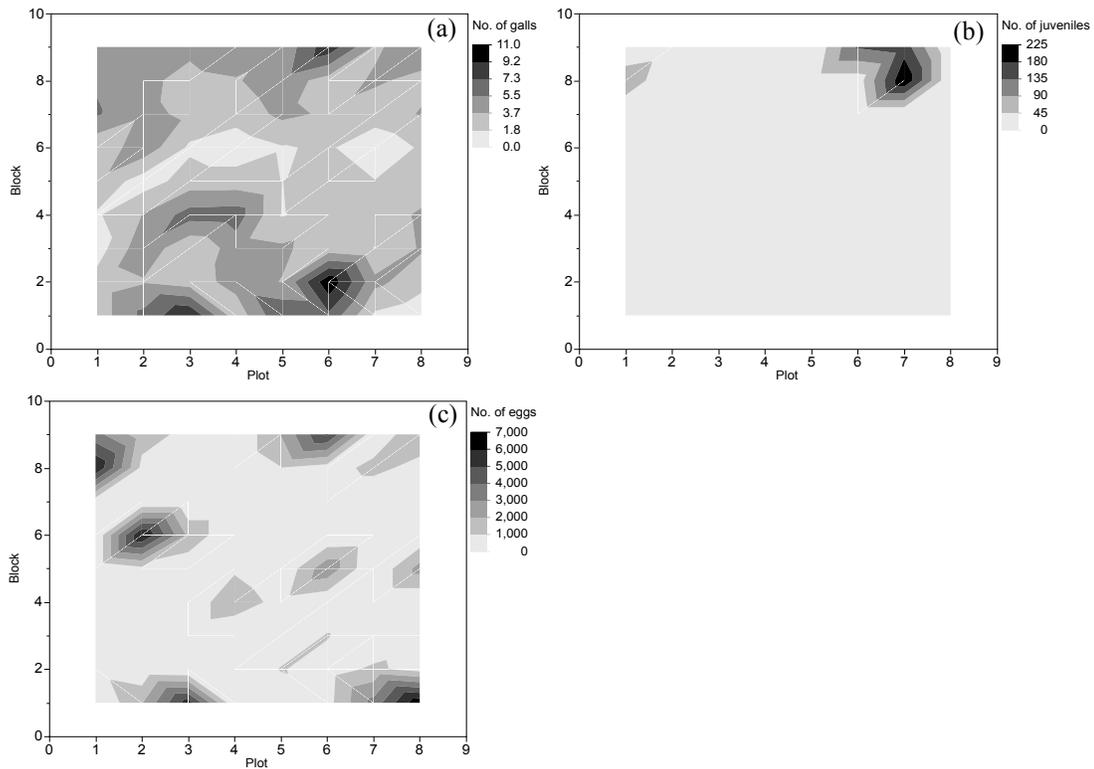


Fig. 4.1 Example of a heat map depicting spatial variation in the field of root-knot nematode variables measured in July 2014 as part of the Dawson County, TX endophyte-nematode field trial. The heat maps illustrate spatial variation in (a) number of galls in each plot, (b) number of juveniles in each plot, and (c) number of eggs in each plot on July 2014 at Dawson County, TX. Y axis shows the plot arrangement and X axis represents the replicated blocks in the field.

Cochran County 2013 (root-knot nematode). There was no significant endophyte treatment effect on root-knot galling (one-way ANOVA, original: $F_{2, 15} = 0.27$, $P = 0.77$; adjusted: $F_{2, 15} = 0.60$, $P = 0.56$), juvenile population (one-way ANOVA, original: $F_{2, 15} = 0.75$, $P = 0.49$; adjusted: $F_{2, 15} = 1.97$, $P = 0.17$), or egg numbers (Kruskal-Wallis, original: $\chi^2 = 2.54$, $df = 2$, $P = 0.28$; adjusted: $\chi^2 = 3.54$, $df = 2$, $P = 0.17$).

Lubbock County 2013 (reniform nematode). We sampled this field twice during the season to quantify reniform populations in the soil. No significant endophyte treatment effect on juvenile population was found in either June (Kruskal-Wallis, original: $\chi^2 = 2.46$, $df = 2$, $P = 0.29$; adjusted: $\chi^2 = 0.92$, $df = 2$, $P = 0.63$) or July (Kruskal-Wallis, original: $\chi^2 = 0.54$, $df = 2$, $P = 0.76$; adjusted: $\chi^2 = 2.79$, $df = 2$, $P = 0.25$). We also compared the quantitative change in reniform population size from July to June. No significant treatment effect was detected, but a trend for a population decline in the endophyte plots was apparent in the spatially adjusted analysis (ANOVA, original: $F_{2, 15} = 0.23$, $P = 0.80$, Fig. 4.2a; Kruskal-Wallis, adjusted: $\chi^2 = 3.93$, $df = 2$, $P = 0.14$, Fig. 4.2b). Follow-up pairwise comparisons indicated that nematode population sizes in the Low treatment plots decreased significantly more relative to the controls (Wilcoxon, $Z = -2.99$, $P = 0.049$; Fig. 4.2b).

Dawson County 2014 (root-knot nematode). No endophyte treatment effect was detected on galling (GLM, original: $F_{9, 62} = 0.59$, $P = 0.80$; adjusted: $F_{9, 62} = 0.76$, $P = 0.65$), egg number (GLM, original: $F_{9, 62} = 1.38$, $P = 0.22$; adjusted: $F_{9, 62} = 0.99$, $P = 0.45$), or juvenile population (GLM, original: $F_{9, 62} = 0.55$, $P = 0.83$; adjusted: $F_{9, 62} = 1.07$, $P = 0.39$).

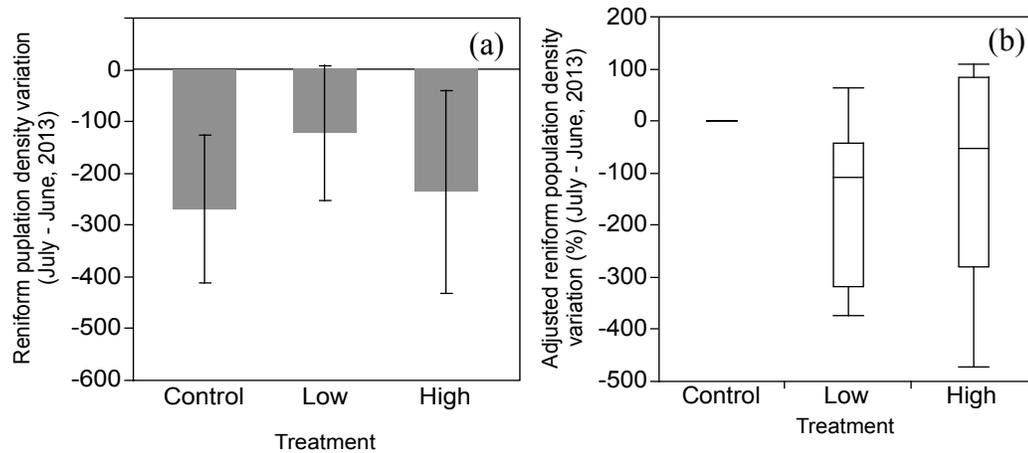


Fig. 4.2 Changes in reniform nematode population density between between July and June 2013 in the Lubbock County, TX endophyte-nematode field trial. Plots depict the difference between July and June in the (a) mean \pm SE absolute number of reniform nematodes sampled in soil, and (b) box plots of the number of nematodes adjusted for spatial position expressed as a percentage.

Cochran County 2014 (root-knot nematode). We only obtained gall samples for this field site in 2014. A number of plants were lost and damaged in this field during the early season due to severe weather (i.e. hail and dust storms) and *Fusarium* wilt infection. A marginal endophyte treatment effect was found in the original data set (GLM, $F_{5,30} = 2.021$, $P = 0.10$, Fig. 4.3a) and the effect was significant when the data was adjusted for spatial factor (GLM, $F_{5,30} = 3.17$, $P = 0.020$, Fig. 4.3b). A significant reduction in galls was detected among *C. globosum* treated plants using MC as a sticker coating versus the Control-MC treatment (Student's t , $P = 0.05$; Fig. 4.3b).

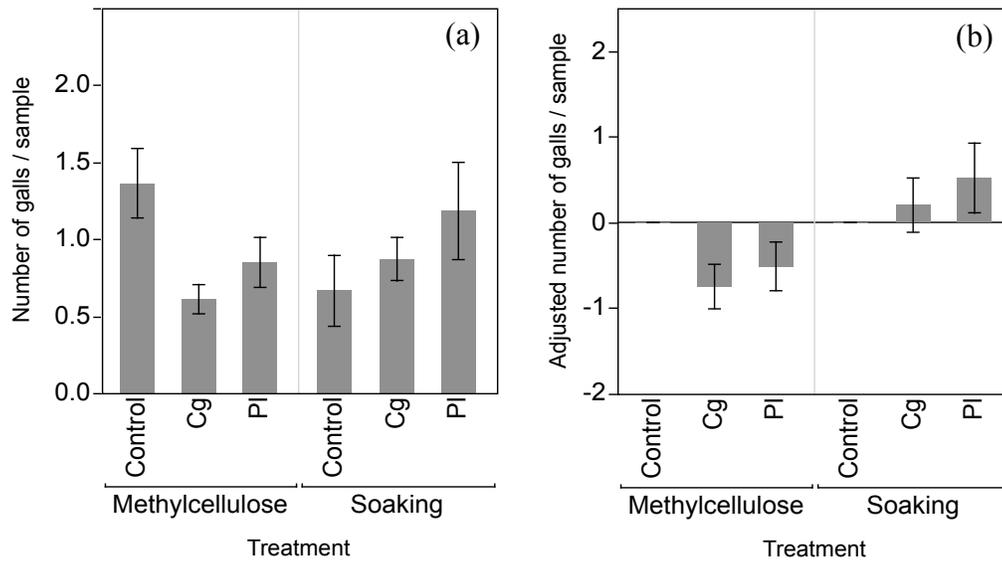


Fig. 4.3 Root-knot galling in the 2014 at Cochran County, TX endophyte-nematode field trial. Plots depict the mean \pm SE (a) number of galls per sample, and (b) difference in the number of galls from plants in treatment plots versus their nearest corresponding control treatments.

Lubbock County 2014 (reniform nematode). We sampled the reniform population in the field before planting in May, and there was no overall significant difference in reniform abundance among treatment plots (GLM, original: $F_{9, 62} = 1.10$, $P = 0.38$; adjusted: $F_{9, 62} = 1.33$, $P = 0.24$). In June, there was significant variation in reniform abundance among the plots (GLM, original: $F_{9, 62} = 1.99$, $P = 0.055$; adjusted: $F_{9, 62} = 3.01$, $P = 0.0048$). Seed treatment method was found to be the only main factor with a significant effect on reniform density (GLM effect test, original: $F = 3.83$, $df = 1$, $P = 0.055$; adjusted: $F = 11.24$, $df = 1$, $P = 0.0014$). In pairwise comparisons among treatments, the PHY367WRF-*C. globosum*-Soaking treatment was significantly different from the PHY367WRF-Control-Soaking treatment, but the endophyte treatment had higher

reniform density (Student's t test, $P = 0.0018$). In July, reniform densities also varied significantly among treatment plots. (GLM, original: $F_{9, 62} = 2.94$, $P = 0.0057$; adjusted: $F_{9, 62} = 2.076$, $P = 0.045$). The main effects of endophyte treatment (GLM effect tests, original: $F = 3.53$, $df = 2$, $P = 0.035$; adjusted: $F = 3.71$, $df = 2$, $P = 0.030$), coating method (GLM effect tests, original: $F = 4.62$, $df = 1$, $P = 0.036$), and plant variety (GLM effect tests, original: $F = 8.68$, $df = 1$, $P = 0.0045$; adjusted: $F = 3.23$, $df = 1$, $P = 0.077$), all had major effects on the reniform density. Coating method did not show any impact on reniform density after data was adjusted by spatial factor (GLM effect tests, $F = 1.39$, $df = 1$, $P = 0.24$). In pairwise comparisons, a significant difference in nematode density was found between the PHY499WRF-*C. globosum*-MC treatment and PHY499-Control-MC treatment, with the endophyte treatment having a higher population density than the Control (Student's t test, $P = 0.018$). There was no significant change in reniform density within treatments between July and May (GLM, original: $F_{9, 62} = 1.0042$, $P = 0.45$; adjusted: $F_{9, 62} = 0.66$, $P = 0.74$).

Plant performance evaluation

We measured plant density for all three field trials during the early season of 2013 (Fig. 4.4). No significant effect of endophyte treatment on plant density was found in any of the trials, Gaines (one-way ANOVA, original: $F_{2, 15} = 0.28$, $P = 0.76$, Fig. 4.4a; adjusted: $F_{2, 15} = 0.092$, $P = 0.91$, Fig. 4.4b), Cochran (one-way ANOVA, original: $F_{2, 15} = 1.32$, $P = 0.30$, Fig. 4.4a; adjusted: $F_{2, 15} = 1.80$, $P = 0.20$, Fig. 4.4b), or Lubbock (one-way ANOVA, original: $F_{2, 15} = 0.24$, $P = 0.79$, Fig. 4.4a; adjusted: $F_{2, 15} = 0.19$, $P = 0.83$, Fig.

4.4b). In the Cochran County trial, there was a non-significant trend for plant density to be higher for both endophyte treatments relative to the control (Mean \pm Std. Dev. for each treatment: Control, 42.83 \pm 30.32; Low, 67.83 \pm 20.14; High, 67.17 \pm 37.97; Fig. 4.4a).

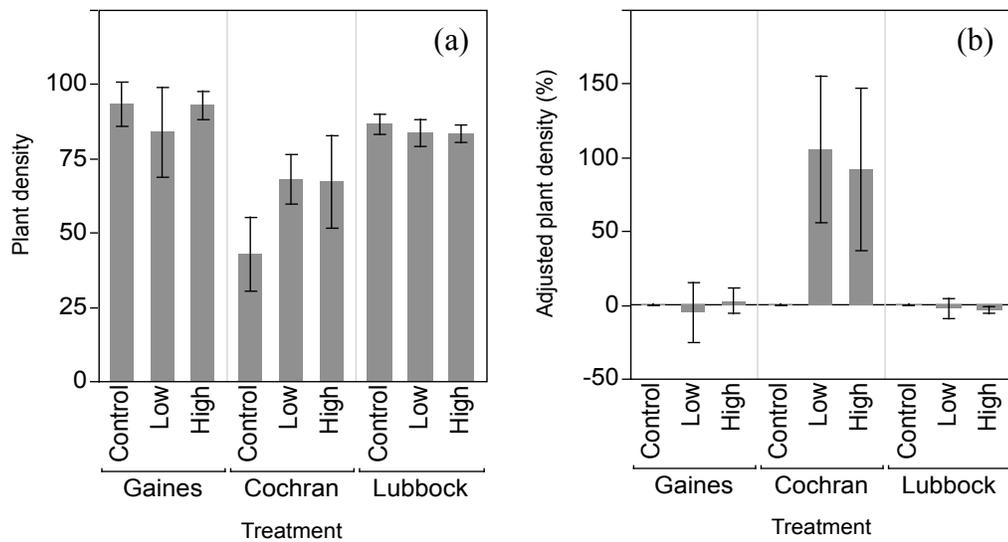


Fig. 4.4 Plant density and spatially adjusted difference in plant density for all three *P. lilacinum* endophyte field trials in 2013. The plots depict the mean \pm SE (a) plant density (average number of plants per row) and (b) adjusted plant density (in percentage).

Plant density data in 2014 were collected for all three fields, with no overall significant treatment effect detected at either the Dawson (GLM, original: $F_{9,62} = 0.69$, $P = 0.71$; adjusted: $F_{9,62} = 0.40$, $P = 0.93$) or Cochran site (GLM, original: $F_{5,30} = 1.66$, $P = 0.18$; adjusted: $F_{5,30} = 0.39$, $P = 0.85$). However, an overall significant treatment effect on

plant density was observed at Lubbock, but it was only marginally significant when the data were adjusted for spatial variation in the field (GLM, original: $F_{9, 62} = 4.72$, $P < 0.0001$; adjusted: $F_{5, 30} = 1.65$, $P = 0.12$; Fig. 4.5). Endophyte treatment was the only significant main factor in this trial (GLM effect test, original: $F = 17.00$, $df = 2$, $P < 0.0001$). Pairwise comparisons revealed that plant densities were higher in all following treatments as opposed to their corresponding no-endophyte Control treatments: PHY367WRF-*C. globosum*-Soaking (Student's t , $P = 0.072$), PHY367WRF- *C. globosum*-MC (Student's t , $P = 0.0067$), PHY499WRF-*C. globosum*-MC (Student's t , $P = 0.023$), PHY499WRF-*C. globosum*-Soaking (Student's t , $P = 0.026$) (Fig. 4.5).

Cotton yields

Gaines 2013 (root-knot nematode). There was no significant effect of endophyte treatment on estimated yields (one-way ANOVA, original: $F_{2, 15} = 0.012$, $P = 0.99$, Fig. 4.6a; adjusted: $F_{2, 15} = 0.065$, $P = 0.94$, Fig. 4.6b).

Cochran 2013 (root-knot nematode). There was no significant treatment effect on estimated yields (one-way ANOVA, $F_{2, 15} = 0.37$, $P = 0.69$, Fig. 4.6a) or the adjusted difference of estimated yields (one-way ANOVA, $F_{2, 15} = 0.74$, $P = 0.49$, Fig. 4.6b).

Although not significant, there was a positive trend for endophyte treatment yields to be higher than the Control (Mean \pm Std. Dev. for each treatment: Control, 536.54 ± 226.63 ; Low, 595.37 ± 144.21 ; High, 635.77 ± 219.42).

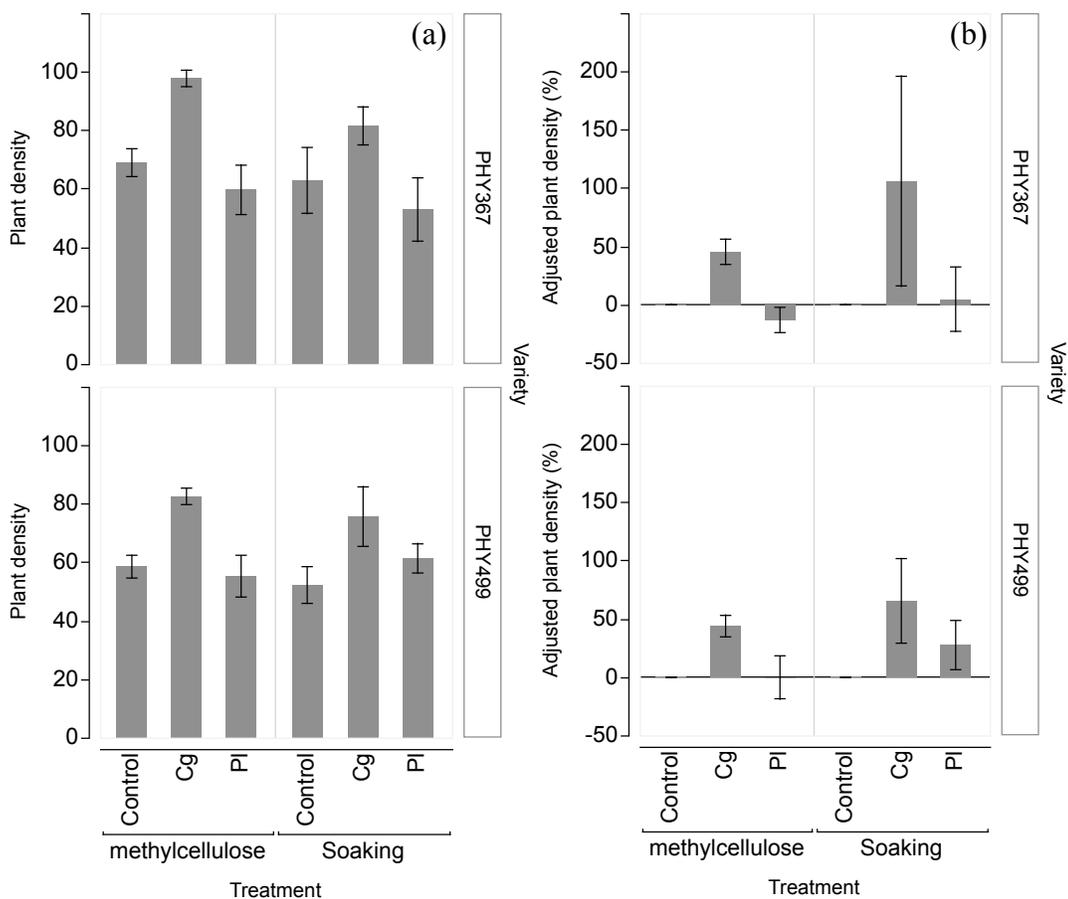


Fig. 4.5 Plant density and spatially adjusted difference in plant density at the Lubbock County, TX endophyte-nematode field trial in 2014. The plots depict the mean \pm SE of (a) plant density (number of plants per row), and (b) spatially adjusted plant density (in percentage).

Lubbock 2013 (reniform nematode). There was no significant treatment effect on estimated yield (one-way ANOVA, $F_{2,15} = 0.085$, $P = 0.92$, Fig. 4.6a) or the adjusted difference of estimated yield (one-way ANOVA, $F_{2,15} = 0.092$, $P = 0.91$, Fig. 4.6b).

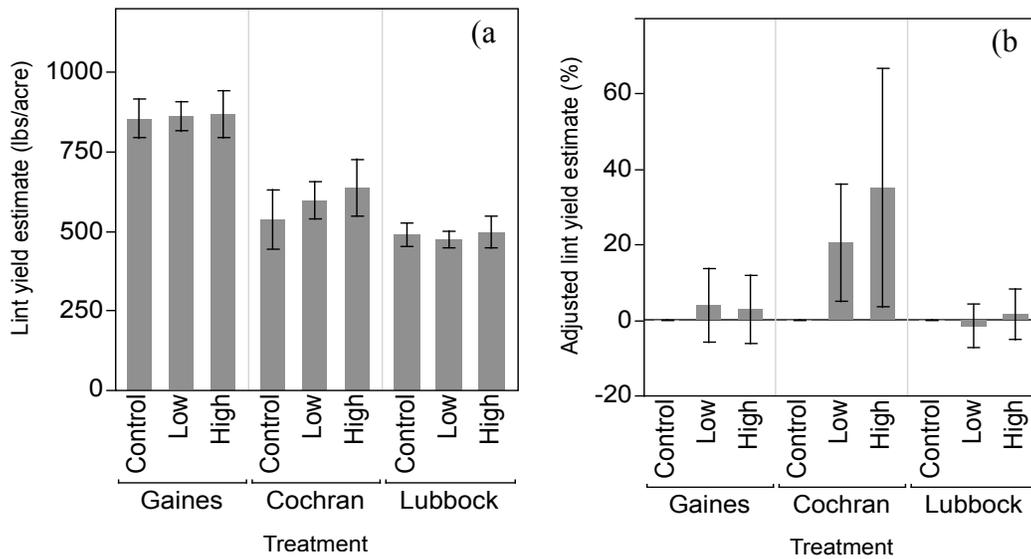


Fig. 4.6 Lint yield estimates across all *P. lilacinum* endophyte-nematode field trials in 2013. The plots depict the mean \pm SE of (a) absolute lint yield estimates (lbs/acre), and (b) spatially adjusted lint yield estimates (in percentage).

Dawson 2014 (root-knot nematode). No significant treatment effect on estimated yield (GLM, $F_{9, 62} = 0.44$, $P = 0.91$, Fig. 4.7a) or adjusted difference of estimated yield (GLM, $F_{9, 62} = 0.87$, $P = 0.56$) was detected (Fig. 4.7b). Although not significant, there was a consistent trend for higher yields in both PHY499WRF and PHY369WRF when either *C. globosum* or *P. lilacinum* was applied using methylcellulose as a sticker (Fig. 4.7 a&b).

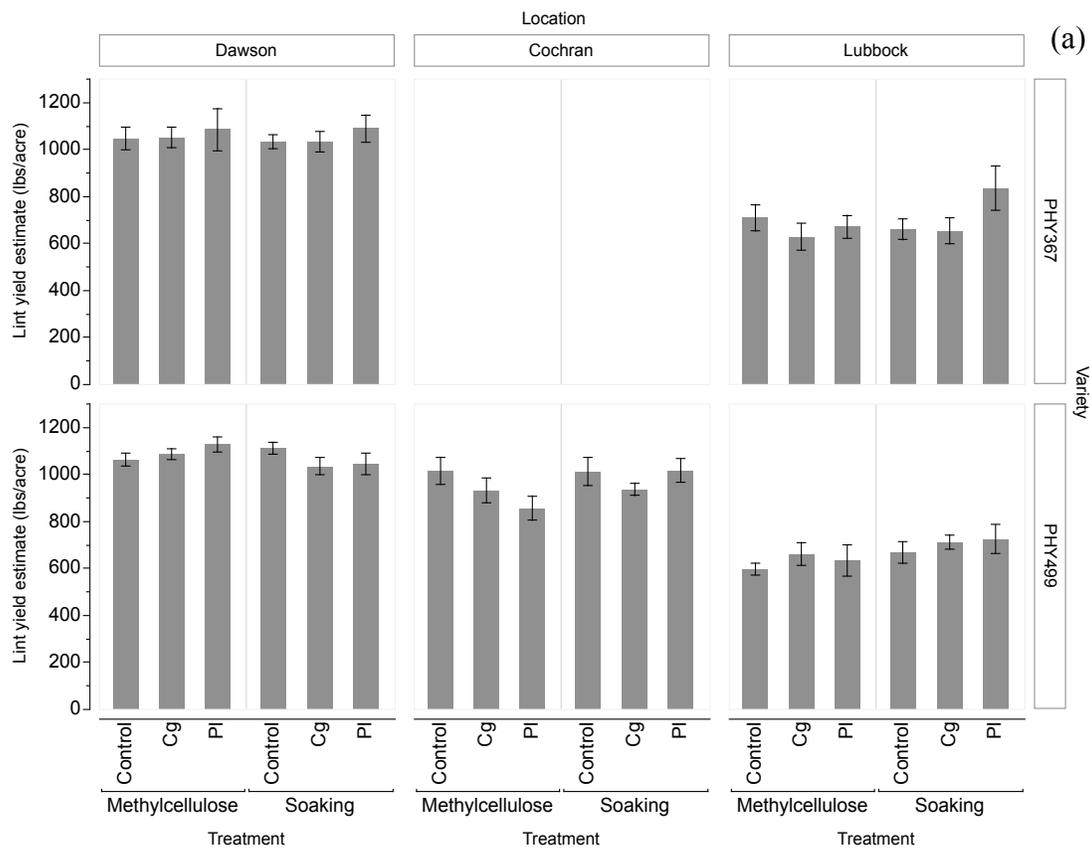


Fig. 4.7 Lint yield estimates across all endophyte-nematode field trials in 2014. The plots depict the mean \pm SE of (a) average lint yield estimates (lbs/ acre), and (b) adjusted lint yield estimates (in percentage).

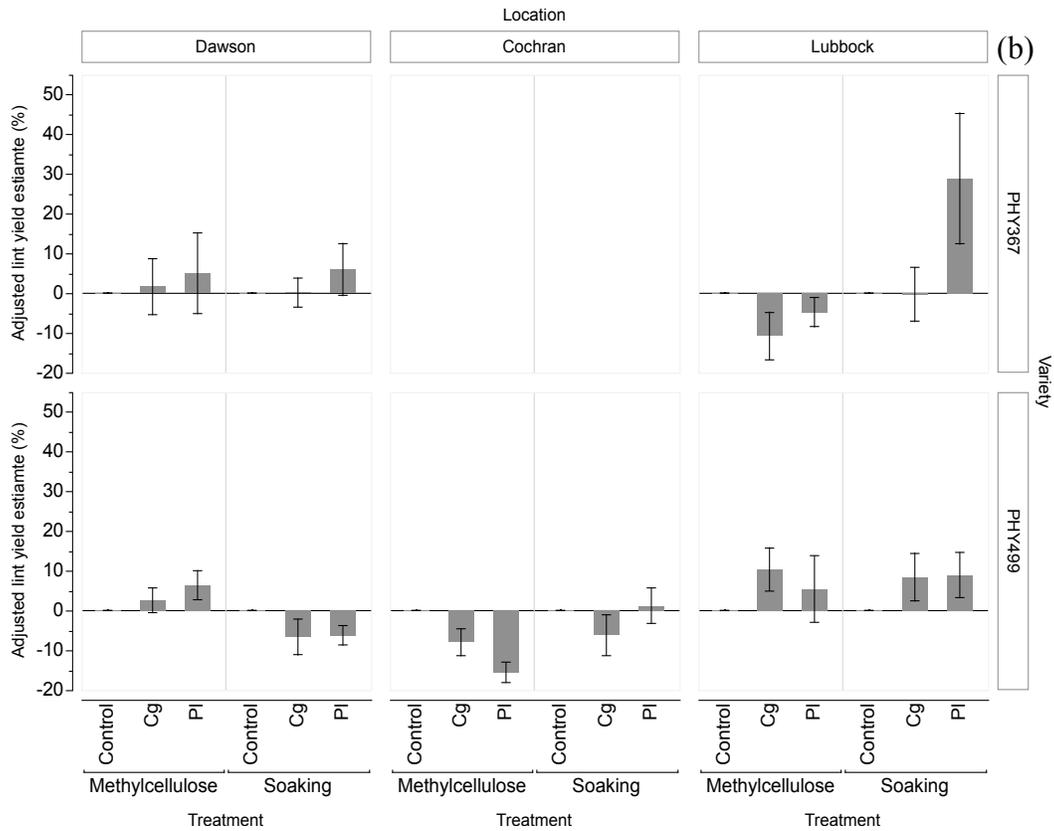


Fig. 4.7 Continued.

Cochran 2014 (root-knot nematode). There was no significant treatment effect on estimated yields (GLM, $F_{5, 30} = 1.08$, $P = 0.39$, Fig. 4.7a). However, a significant treatment effect became apparent when the yield was adjusted for spatial variation in the field (GLM, $F_{5, 30} = 3.99$, $P = 0.0068$, Fig. 4.7b). In pairwise comparisons, yield for the PHY499WRF-*P. lilacinum*-MC treatment was significantly lower than that from the PHY499WRF-Control-MC treatment (Student's t , $P = 0.0020$, Fig. 4.7b).

Lubbock 2014 (reniform nematode). There was no overall treatment effect on estimated lint yield (GLM, $F_{9, 62} = 1.35$, $P = 0.23$, Fig. 4.7a). However, a significant treatment effect became apparent when the yield was adjusted for spatial variation in the field (GLM, $F_{9, 62} = 2.39$, $P = 0.022$, Fig. 4.7b). Both Endophyte and Seed treatment method had significant effects as main factors on the adjusted difference of yield estimates according to GLM effect tests (Endophyte factor: $F = 2.29$, $df = 2$, $P = 0.11$; Seed treatment method factor: $F = 3.84$, $df = 1$, $P = 0.055$). Among all the treatments, PHY367WRF-*P. lilacinum*-Soaking stood out as having significantly higher yields than its corresponding control lacking *P. lilacinum* in the PHY367WRF-Control-Soaking treatment group (Student's *t*, $P = 0.0034$).

Discussion

Several studies have tested the use of *P. lilacinum* as a biological control agent for controlling plant parasitic nematodes (Davide and Zorilla, 1983, Bonants et al., 1995, Anastasiadis et al., 2008, Castillo et al., 2013), but most studies of *C. globosum* as a potential biological control agent to date have only considered its antagonistic effects against fungal and bacterial disease (Marwah et al., 2007). In this study, both *C. globosum* and *P. lilacinum* were tested for efficacy against root-knot nematodes and reniform nematodes when applied using seed treatments that facilitate endophytic colonization of the plant during germination. No overall significant endophyte treatment effect on root-knot nematodes was observed in 2013 and 2014. This lack of an effect in

the field is inconsistent with previous greenhouse assays on root-knot nematodes (Chapter II and Chapter III) in which both endophytes significantly reduced root-knot nematode galling and fecundity across multiple assays. In 2013, the Gaines County site encountered high root-knot pressure in the soil; whereas plants grown in Cochran County received high pressure from *Fusarium* wilt disease with a moderate nematode density during early season (T. Wheeler, personal observations). Based on the greenhouse assay results reported in Chapter II, when susceptible plants were exposed to very high nematode pressure (plants received 10,000 eggs at seedling stage), the negative effects of the endophyte treatment on nematodes can be reduced relative to when the plants experience lower nematode pressure, which might help explain the unsuccessful results.

In addition to testing for the effects of *C. globosum* and *P. lilacinum* as fungal endophytes against root-knot nematodes at multiple sites in 2013 and 2014, we also tested for their effects against reniform nematodes in both years at the same site in Lubbock County, TX. Only one significant effect was found in June 2014 in which seed treatment method (soaking versus seed coating) was the major factor that positively affected reniform density, but such effect was not consistent over time. Mishra and Dwivedi (2008) previously tested one *P. lilacinum* strain as a soil treatment for reniform nematodes on pulse crops in field trials, and observed a mild reduction effect. However, no study to date has attempted to use endophytic *C. globosum* or *P. lilacinum* to control *R. reniformis*. Given the lack of consistent major effects on nematodes observed in both

the *M. incognita* and *R. reniformis* trials, the question of whether manipulating the presence of *C. globosum* and *P. lilacinum* as endophytes in cotton can have same the ecological effects on nematodes in modern agrosystems as they do in greenhouse trials remains problematic. A major difficulty in conducting field trials that can accurately assess the effects of control treatments on nematodes in the field is the extremely patchy distribution of nematodes (e.g., Fig. 4.1). Because of this, experimental treatments are not uniformly exposed to nematode pressure as they are in greenhouse trials, complicating the ability to make accurate comparisons among treatments in plots distributed across heterogeneous nematode pressure.

In spite of ambiguous results in terms of direct effects on nematode numbers in the soil and root infection levels, endophyte treatments did have some detectable effects on cotton plant performance in the field, including final yields. Plant stand densities were significantly higher in the Lubbock County 2014 trial that targeted reniform nematodes for all *C. globosum* treatments, regardless of whether the cotton variety was susceptible or resistant to nematodes (Fig. 4.5). There was a similar trend for higher stand densities associated with *P. lilacinum* treatment at the Cochran County trial in 2013 (Fig. 4.4). With respect to final yields, there were no overall significant effects on lint yield in any of the trials when analyzed using the original data without attempting to control for spatial variation in the field. However, when spatial variation in the field was accounted for, there was a significant treatment effect on adjusted yield differences in both the Cochran County and Lubbock County trials in 2014. At least one endophyte treatment

combination at each location significantly increased adjusted yield relative to the corresponding control treatments (Fig. 4.7b). Endophytic *P. lilacinum* treatments in the 2013 Cochran County field trial also exhibited a strong trend for higher yields (Fig. 4.6) associated with a similar trend in stand density (Fig. 4.4).

Importantly, the direction of yield effects often varied between endophyte treatments and plant genotypes (Fig. 4.7). Yield drag is defined as yield reduction due to addition of foreign genes into a given cultivar (Knezevic and Cassman, 2003). For example, in the 2014 Lubbock field trial, a yield drag was observed for both *C. globosum* and *P. lilacinum* treatments when applied as a methylcellulose seed treatment in the nematode resistant variety PHY367WRF. In contrast, the susceptible variety PHY499WRF treated with both endophytes yielded higher than the corresponding controls at the same site (Fig. 4.7b). A similar contrasting effect was observed at the other site in Dawson County where nematode susceptible and resistant varieties were compared in the same trial. However, in this case, the yield drag was associated with the soaking seed treatment for both endophytes in the susceptible variety PHY499WRF (Fig. 4.7b). These results indicate that despite the patchy distribution of nematode density in the fields, specific endophyte, plant genotype, and seed treatment combinations did affect plant performance and final yields, but their effects are highly influenced by local ecological conditions. The effects of plant genotype on endophyte community composition, as well as specific endophyte performance inside the plant tissues, are well known (Saikkonen et al., 1998, Faeth and Bultman, 2002, Faeth and Fagan, 2002). Although research on

endophyte-plant genotype interactions is relatively rare, Saikkonen et al. (2004) suggested that the plant-fungus interaction is affected by the specific genotype-genotype combination between the plant and fungus. The idea that variation in fungal genotypes, plant genotypes and local environments all interact to affect ecological interactions is well known in studies of endophytes and often referred to as context-dependency (Hartley and Gange, 2009, Davitt et al., 2011).

A variety of environmental factors in addition to nematode density can vary among fields and years in agroecosystems to affect endophyte communities and their performance in plants such as weather conditions, irrigation levels, and soil types (Siciliano et al., 2001) to name just a few. Other microbes including root endophytes and mycorrhizal fungi also reside in the same environment and may be competing to colonize plants, utilize nutrients from plants, or interact with other microbes as part of the endophytic community. In addition to the impact of variation in the factors above, the logistics of conducting field trials under more realistic modern agronomic conditions limits the size and number of replicate plots that can be planted and managed throughout the season. As such, relatively small sample sizes and low statistical power for all six field trials may also explain the lack of significance of many treatment effects. For example, at the Dawson site in 2014 where both positive and negative trends in yields were observed (Fig. 4.7), statistical power based on the observed effect size on yield was $1-\beta = 0.24$ for the original yield, and $1-\beta = 0.39$ for adjusted yield estimates.

Importantly, this study clearly demonstrated that the presence of both *P. lilacinum* and *C. globosum* in cotton as an endophyte can be successfully manipulated in the field. Both fungi survived during planting and endophytically colonize cotton plants in the field under standard agricultural practices. The frequencies of detected colonization were slightly different among treatments in 2014 (Table. 4.1), potentially due to variation in host plant genotypes, seed treatment method, or other environmental factors. Hoffman and Arnold (2008) reported that the abundance, diversity, and composition of endophyte communities are variable, but nevertheless directly associated with host identity and locality. In previous greenhouse assays (Chapters II and III), the overall average colonization frequencies of cotton inoculated with *P. lilacinum* and *C. globosum* by soaking were 26% and 92.5%, respectively, in a different plant genotype. Although the colonization of cotton by *P. lilacinum* did not differ much between the greenhouse experiments and field trials, *C. globosum* colonization efficiency showed greater variation under different environmental conditions. The results may indicate the possibility of seed treatment effects on endophyte colonization host plants. Additionally, the surface-sterilization of plant samples for the detection of endophytic fungi remains a big challenge for accurately determining colonization efficiencies, and likely leads to underestimated colonization efficacy (Schulz et al., 1993, Fröhlich et al., 2000, Schulz and Boyle, 2005, Hyde and Soyong, 2008, Greenfield et al., 2015, Lohse et al., 2015). The most commonly used sterilants generally consist of sodium hypochlorite and ethanol. The concentration and time required for each sterilant can vary greatly depending on the host plant tissue, age, and thickness (Fröhlich et al., 2000, Schulz and

Boyle, 2005, Hyde and Soyong, 2008). In this study, surface-sterilization process has been optimized for cotton tissues specifically, with separating green tissues from roots for different concentration of sterilants and increasing water rinses afterwards. The imprinting process of all sampling tissues was also included to verify removal of epiphytes. Although colonization frequencies of cotton by *C. globosum* were generally very high, even 100% in some trials (Table 1), it is worth noting that detectable positive colonization of cotton by *P. lilacinus* is rarely as high as the level observed for *C. globosum* across multiple studies (Lopez et al., 2014). The fact that strong treatment and repeatable treatment effects of *P. lilacinum* are observed despite lower levels of positive colonization detection suggests that the detection of *P. lilacinum* as an endophyte using these methods is more difficult and likely underestimates actual colonization frequency.

Dubey et al. (2013) suggested that seed carrier formulation for biological seed treatments can affect plant performance, including seed germination rate, plant vigor, and disease resistance. Polymers, acting as film-coating binders, can provide good protection from environmental factors such as UV-light and chemicals (Scher, 1977). The respiration sensitivity of the seed during early germination is greatly affected by O₂ and a number of bioactive metabolites (Shull, 1911, Yentur and Leopold, 1976). The advantage of applying fungal endophyte treatments as a film-coating is that the seeds can remain dormant until being planted, whereas the soaking method stimulates the germination process once soaking process starts. *C. globosum* has previously been utilized for control of soilborne and seedborne diseases when delivered via seed treatment (McQuilken et

al., 1998). Vannacci and Harman (1987) applied a seed treatment using a solution of *C. globosum* spores and methylcellulose to cruciferous seeds for on average $10^4 - 10^5$ spores/ seed. In another study, fungal inoculum using *C. globosum* ascospores were applied in methylcellulose solution to coat sugarbeet seeds, and spores on the seeds were kept viable for more than two years (Walther and Gindrat, 1988). Bora et al. (2015) compared several formulation methods for *Naga chilli* seeds. *P. variotii* inoculum on the seeds remained viable for at least one year when methylcellulose and talc powder was applied as the carrier. However, these previous studies have not considered the impact of seed treatment methods on endophyte or plant performance. Our results suggested a lower colonization frequency of *C. globosum* when seeds were treated with a methylcellulose seed coating as opposed to soaking (Table 4.1). Seed soaking and film-coating as different seed treatment methods were also compared for their potential effects on seed germination and plant growth. Results collected from the Lubbock site in 2014 showed that the *P. lilacinum* had a greater variation in plant density between the soaking and methylcellulose treatment methods (Fig. 4.5b). There were also instances in which the seed treatment methods appear to have differentially affected the level of gall formation (Fig. 4.3b) and final yields (Fig. 4.7b) for the same endophyte tested in the same fields.

In conclusion, treatment of the nematode susceptible and resistant varieties tested here with endophytic *C. globosum* and *P. lilacinum* did not consistently reduce nematode density or root infection in the field. However, there were specific combinations of

endophyte, cotton variety and seed treatment methods that did have detectable positive effects on plant performance and yields, but their performance was context-dependent and strongly affected by the local environmental conditions. Many of the problems faced by these trials are common to all field trials, in particular unpredictable and unreliable levels of pest pressure across years and sites. Larger sample sizes are also needed in order to obtain sufficient statistical power to detect treatment effects. Future studies to better understand how to manipulate plant-endophyte-nematode interactions in the field will require longer-term and larger-scale studies to overcome the challenges due to variation in environmental factors. Variations warrant further consideration. Multiple years of field experiments at the same locations will help ensure that plants are exposed to high nematodes densities at least in some years due to variation in weather conditions. Capture the effects of variable levels of nematode is also suggested, since the location of field sites may also influence the experimental effects with variable environmental factors. A better understanding of the mechanisms underlying endophyte colonization of the plants and their effects on nematodes and plant performance will also help provide insight into specific endophyte genotype, plant genotype, and seed treatment methods that may have potential for nematode management.

CHAPTER V

SUMMARY AND CONCLUSIONS

Microorganisms are ubiquitous. They serve as the hidden bridge to connect all organisms in an ecosystem. Terrestrial plants, in particular, present a spatially and temporally diverse ecological habitat for microbes. Leaves and roots each represent distinct interfaces between plants and their environment. As a result, root fungi are quite different from those in the phyllosphere, having adapted to different moisture conditions, access to organic materials, exposure to light and UV irradiance, germination and penetration interactions with different tissue structures, and vibration (Juniper, 1991). Only a few studies have discussed differences between the rhizosphere and phyllosphere environments, and noted that little or no overlap existed between fungal communities in the roots and shoots (Arnold, 2007).

A major challenge in understanding spatial diversity in fungal communities is the use of appropriate detection methodology. According to microbiologists, approximately 99% of microbial diversity may be unculturable (Arnold, 2007). The predominant methods for detecting endophyte colonization currently consists largely of culturing fungi from plant tissues on nutritive media, as well as complementary environmental PCR assays. For cultured-based diagnostic assays, the detection of colonization is mostly limited by the efficacy of the surface sterilization methods (Greenfield et al., 2015). In this study, we utilized different surface sterilization methods optimized for roots and leaves. Although

the *P. lilacinum* and *C. globosum* strains used here were originally isolated from cotton leaves and squares, systemic colonization of the plant by both fungi was detected in both greenhouse and field assays (Chapters II, II & IV).

This study examined, for the first time, the outcome of ecological interactions between the same endophyte-plant complex, *C. globosum* and cotton, when challenged by above-ground insect herbivores and below-ground plant parasitic nematodes. Previous studies of endophyte species diversity referred to the “mosaic infections” theory to explain foliar endophyte occurrence, which predicted that the both the distribution and effects of foliar endophytes were location specific and restricted to a small-scale (Lodge et al., 1996, Rodriguez et al., 2009). The results presented here clearly indicate that it is possible for an artificially inoculated endophyte to colonize plants systemically, but colonization frequency among tissue types may depend on other biological or environmental factors, particularly the interaction between plant and fungal genotypes.

Fungi in the genus *Purpureocillium* have been known as a nematode egg parasites for a long time. Results presented here indicate that the endophytic *P. lilacinum* strain 490 isolated from cotton is not only a nematode parasite, but also an endophytic fungus that can inhibit nematode infection and reproduction in cotton as well as potentially promote plant growth. Other studies using the same strain have demonstrated that is also an entomopathogen that can directly infect and kill cotton aphids (*A. gossypii*) (Lopez et al., 2014). The same strain has also been shown to negatively affect the performance of both

cotton aphids and the cotton bollworm (*Helicoverpa zea*) when present as endophyte in cotton (Lopez et al., 2014, Lopez and Sword, 2015). These studies proposed an endophyte-mediated priming effect of the plant induced response defense system as a potential mechanism to explain the observed negative effects on insects. Similarly, little was known about the mechanistic interaction between endophytic *P. lilacinum* and plant parasitic nematodes (Bonants et al., 1995, Khan et al., 2004, Park et al., 2004, Yan et al., 2011); and noticeably none had considered the non-mutually exclusive possibility of both rhizospheric and endophytic effects of this fungus on nematodes. The work presented in Chapter II in which *P. lilacinum* was eliminated from the rhizosphere with a fungicide treatment indicates negative effects on root-knot nematodes following seed treatment with *P. lilacinum* are, in fact, due to the presence of the fungus as an endophyte in cotton. A similar demonstration of the endophytic effects of *C. globosum* as an endophyte against root-knot nematodes was also conducted as part of the work presented in Chapter III.

Chaetomium fungi are known to produce a number of bioactive metabolites (Meyer et al., 2004, Qin et al., 2009, Yan et al., 2011, Hu et al., 2012). Gange et al. (2012) suggested that the opposite effects of endophytic *C. cochliodes* in the perennial forb *Cirsium arvense* on two different insect species might be due to endophyte-related chemical changes in host plants. Yan et al. (2011) showed the nematopathogenic activity of an endophytic *Chaetomium* strain Ch1001 against *M. incognita*, and suggested that it produced compounds that affect juvenile motility. For the endophytic *C. globosum* strain

520 tested here, consistent systemic colonization of cotton and ornamental plants has been observed by several researchers (G.A. Sword, unpublished data). Given the fact that antagonistic effects against a piercing-sucking insect, a leaf-chewing insect, and a root parasitic nematode were all observed as part of the cotton-*C. globosum* defensive symbiosis (Chapter III), the defense conferred by the fungus is most likely systemic. However, whether the negative effects against cotton aphids, beet armyworms, and root-knot nematodes are mediated by fungal metabolites, plant induced defenses, or both remains to be determined.

In addition to greenhouse studies, field trials were conducted to examine the possibility of manipulating fungal endophytes in cotton agroecosystems as a tool for nematode management. Our field studies across two years did not observe any endophyte effects on either root-knot or reniform nematode population densities under natural conditions. We also failed to see consistent effects of either plant genotype or seed treatment methods on efficacy of the endophytes. Previous studies suggested that interactions between plant and microbial genomes, as well as microbial and microbial genomes, can have great impacts on the rate and direction of coevolutionary interactions within the plant-endophyte symbioses (Wade, 2007). In 2014, we tested two cotton genotypes, one root-knot nematode susceptible and resistant variety, along with two seed treatment methods inoculated with either *P. lilacinum* or *C. globosum*. The results showed that the lint yield estimates of the susceptible variety PHY499WRF did not differ from those of the resistant variety PHY367WRF. Although the results did not provide evidence for a

consistent significant impact of the endophytes on either plant performance or nematode population density, there were specific instances in which certain plant genotype, seed treatment and endophyte combinations did affect plant performance. Apart from this field evidence, nothing is known about how seed soaking differs from methylcellulose coating in their effects on endophyte colonization, abundance and diversity within the plant, and plant performance.

The different results observed in the greenhouse assays (Chapter II & III) and field experiments (Chapter IV) strongly illustrate how environmental factors can play an important role in plant-endophyte symbioses. Given that under field conditions the soil contains significantly more organic components and likely a richer microbial community than hydroponic-sandy soil used in the greenhouse assays, both the plants and endophytes experienced quite different rhizosphere environments in the greenhouse versus field experiments. Additionally, roots in the field were not restricted in a small space, which would have allowed the plants to develop a more robust root system that could provide microorganisms with greater spatial variation in habitat. Environmental variation due to these and other factors, coupled with spatial variation in nematode pressure in the fields trials, complicated the ability fully assess the differences in efficacy of the endophytes against nematodes in the field in these trials versus the greenhouse assays.

A number of studies have found increased endophyte-mediated plant resistance against herbivores, including pathogen vectoring insects, due to endophyte infection (Clay and Schardl, 2002, Hartley and Gange, 2009). Most of these endophytes, particularly grass endophytes, can produce alkaloids that negatively affect herbivores and benefit the hosts (Bush et al., 1997, Siegel and Bush, 1997). May and Nelson (2014) discussed the defensive symbioses at the community level, and suggested that selection for defensive traits should be influenced by ecological context and may only be dynamically stable. The defensive mutualism may also be due to interactions among other microbes in the plant. Faeth and Saari (2012) argued that specialist herbivores might evolve the ability to detoxify endophytic alkaloids or that natural enemies may be negatively affected more by alkaloids than the herbivores; therefore, herbivore preference, abundance, and species richness may be positively affected on endophyte-colonized grasses. However, most studies investigating the plant-fungal mutualistic symbioses to date have focused on grass-fungal endophyte systems.

The similarities and differences between the relative well-studied grass endophyte systems and those involving colonization of plants by facultative fungal endophytes remained poorly understood, as does their impact of endophytes on higher trophic levels of the ecosystem. Some grass fungal endophytes, such as *Neotyphodium* fungi, are asexual and strictly vertically transmitted by seeds (Faeth and Saari, 2012), and are therefore considered as plant mutualists (Clay and Schardl, 2002). Such endophyte-mediated host resistance against herbivores has been well studied in grasses with seed-

borne endophytes (Cheplick and Faeth, 2009). Increased herbivore resistance, enhanced plant growth, increased tolerance of biotic and abiotic stresses have also been demonstrated due to the systemic colonization of such endophytes (Elbersen and West, 1996, Clay and Holah, 1999, Faeth and Saari, 2012). In comparison, whether the plant acts as a vector for facultative fungal endophyte transmission or is actively selecting the endophytes to co-evolve as defensive mutualists remains unclear (Hardoim et al., 2015). Moreover, it is also assumed that the facultative endophytes should incur a fitness cost to the plant in the absence of a beneficial effect because they consume resources derived from host plants (Hardoim et al., 2015). This study explored endophyte-plant interactions using a dicot woody plant, cotton, and showed that it can be colonized systemically by the facultative endophytic fungi, *P. lilacinum* and *C. globosum* both in greenhouse and natural environments. Contrary to the previously stated hypotheses (Hardoim et al., 2015), no negative impacts on host plant performance and fitness (inferred as yield) were observed either in the greenhouse or field in the presence of the endophytes, with the exception of one particular treatment combination in one field trial. In addition to failing to support the hypothesis of a fitness cost to hosting *P. lilacinum* and *C. globosum* as facultative endophytes, both endophytes can confer resistance to cotton against herbivory, at least under some conditions. As such, the relation between cotton and *P. lilacinum* and *C. globosum* can reasonably be regarded as an example of a defensive mutualism in which both parties benefit from the interaction.

This study provided an important first step in realizing the potential for fungal endophytes to be used as biocontrol approach to protect plants against plant parasitic nematodes and insect herbivores without negatively affecting plant growth. As the concept of IPM is focused on using biological and cultural controls while minimizing the need for synthetic pesticides (Allen and Rajotte, 1990), the manipulation of plant-endophyte symbioses may provide a novel option for biological control of both plant parasitic nematodes and insect pests. This study has clearly illustrated that these interactions can be manipulated in the greenhouse as well as under typical agronomic conditions in cotton agroecosystems, and that positive effects of such manipulations on plant performance are possible. Future work will need to focus on more rigorous field evaluations under consistent nematode pressure along with developing a better understanding of the interactions between the endophytes, plant genotypes, inoculation methods, and local environmental conditions.

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APPENDIX

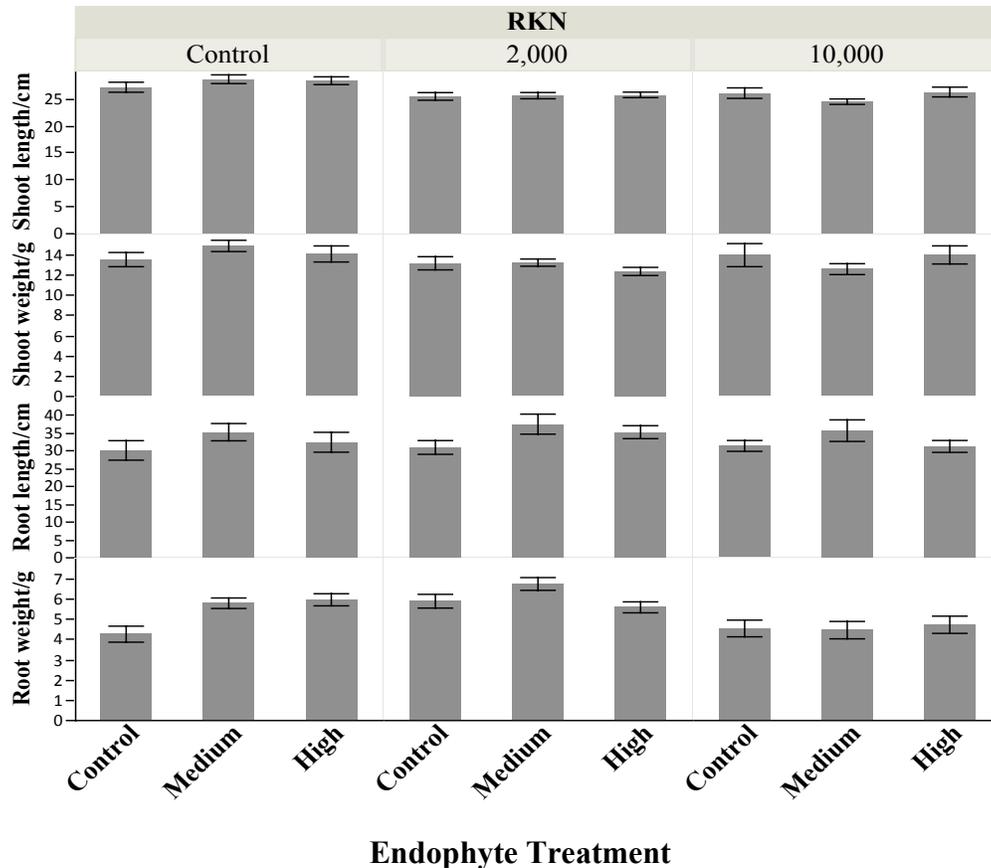


Fig. A1 Plant growth was evaluated six weeks after nematode inoculation by: shoot height (cm), shoot weight (g), root length (cm), root weight(g). Ten plants were sampled for each treatment. Three endophyte treatments were included: Control, Medium, and High. Two nematode inoculants were tested on endophyte treatment by inoculating plants with 2,000 eggs/plant or 10,000 eggs/plant. In most assays, no overall significant treatment effect was detected (ANOVA test, see Table A1 below). Each error bar represents 1 standard error from the mean.

Table A1. Statistical analyses of plant growth six weeks after nematode inoculation (ANOVA test, $\alpha = 0.05$).

Measurements	Nematode inoculum					
	Control		2,000		10,000	
	$F_{2, 42}$	p -value	$F_{2, 42}$	p -value	$F_{2, 42}$	p -value
Shoot length	0.85	0.43	0.056	0.95	1.43	0.25
Shoot weight	0.95	0.40	0.99	0.38	0.81	0.45
Root length	0.91	0.41	2.22	0.12	1.33	0.28
Root weight	8.11	0.0012	3.74	0.032	0.10	0.90

Student's t test:

1. RKN-Control assay: High treatment is significant different from its Control in root weight (Student's t, $p=0.0007$); Medium treatment is significant different from the Control (Student's t, $p=0.0017$).
2. RKN-2,000 assay: Medium treatment is significant different from its Control in root weight (Student's t, $p=0.012$)

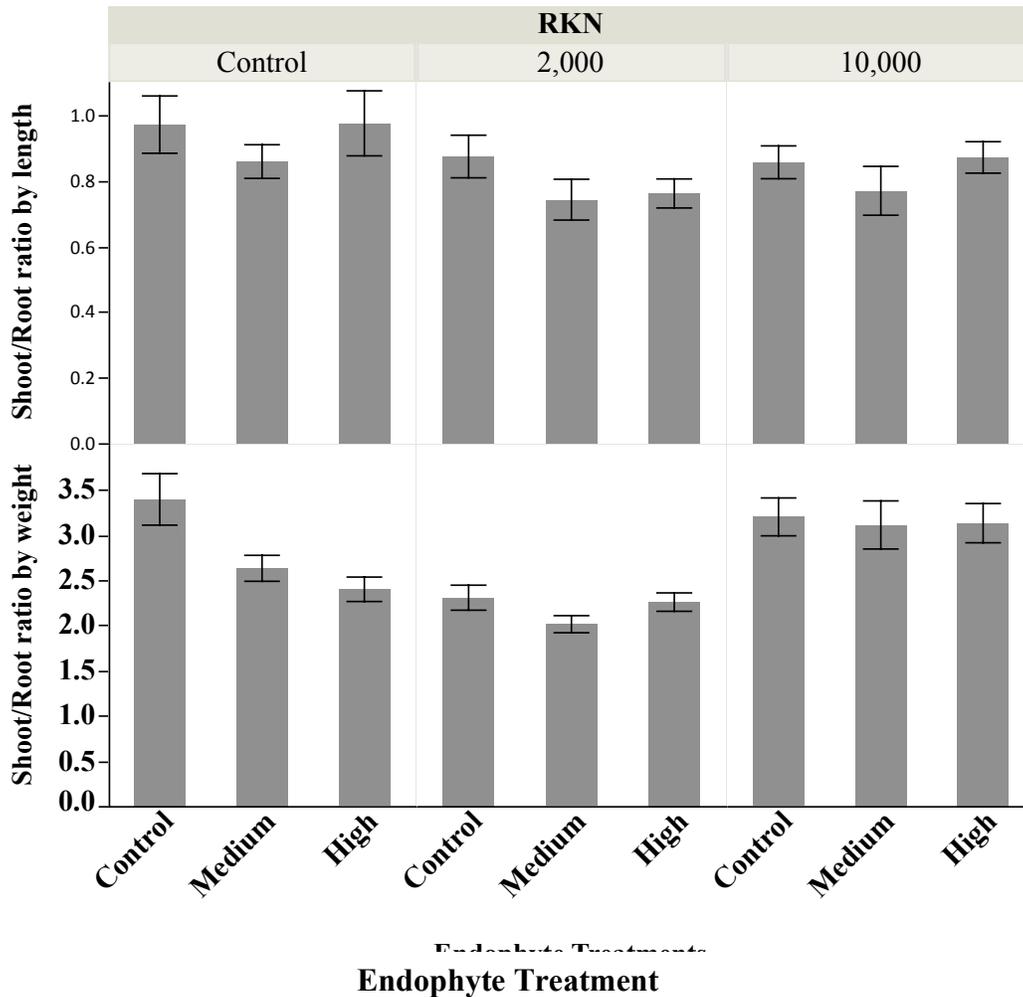


Fig. A2 Plant growth was evaluated six weeks after nematode inoculation by: shoot/root ratio by length, shoot/root ratio by weight. Ten plants were sampled for each treatment. Three endophyte treatments were included: Control, Medium, and High. Two nematode inoculants were tested on endophyte treatment by inoculating plants with 2,000 eggs/plant or 10,000 eggs/plant. For most of the assays, no overall significant treatment effect was detected (ANOVA, see Table A2 below). Each error bar represents 1 standard error from the mean.

Table A2. Statistical analyses of plant growth by shoot/root ratio six weeks after nematode inoculation (ANOVA test, $\alpha = 0.05$).

Measurements	Nematode inoculum					
	Control		2,000		10,000	
	$F_{2,42}$	p -value	$F_{2,42}$	p -value	$F_{2,42}$	p -value
Shoot/Root ratio by length	0.72	0.49	1.51	0.23	0.88	0.42
Shoot/Root ratio by weight	7.25	0.0022	1.93	0.16	0.041	0.96

Student's t test:

1. RKN-Control assay: High treatment is significant different from its Control in the weight ratio (Student's t, $p=0.0007$); Medium treatment is significant different from its Control in the weight ratio (Student's t, $p=0.0067$).

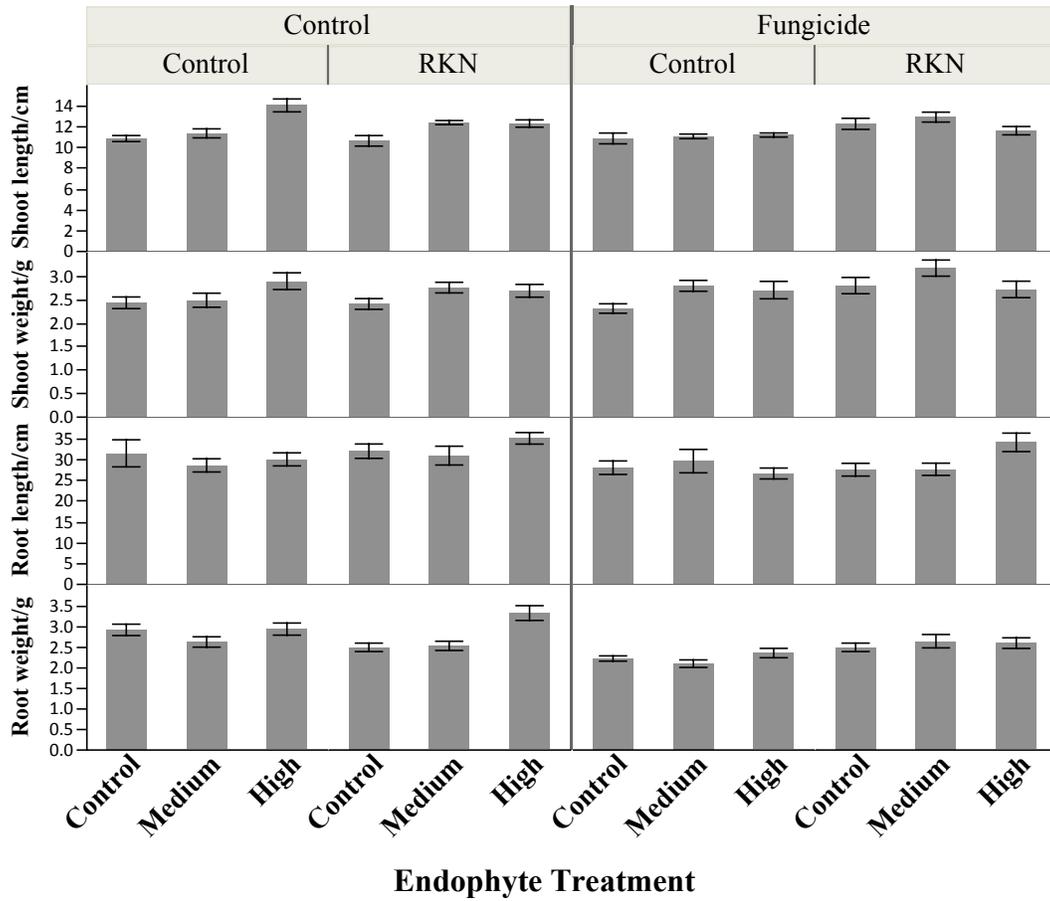


Fig. A3 Plant growth evaluation 12 days after nematode inoculation: shoot height (cm), shoot weight (g), root length (cm), root weight (g). Plants from all treatments were sampled with ten replicates per treatment. A total of 12 treatments were included: RKN (+/-), Endophyte (Control, Medium, High), Fungicide (+/-). In most assays, no overall significant treatment effect was detected (ANOVA test, see Table A3 below). Each error bar represents 1 standard error from the mean.

Table A3. Statistical analyses of plant growth 12 days after nematode inoculation (ANOVA test, $\alpha = 0.05$).

Measurements	Control				Fungicide			
	Control		RKN		Control		RKN	
	$F_{2,27}$	p -vare						
Shoot length	13.15	<0.0001	6.99	0.0036	0.23	0.80	1.90	0.17
Shoot weight	2.73	0.083	2.31	0.12	3.42	0.047	1.95	0.16
Root length	0.40	0.67	1.39	0.27	0.54	0.59	4.51	0.021
Root weight	1.63	0.21	12.05	0.0002	1.96	0.16	0.33	0.72

Student's t test:

1. Control-Control assay: High treatment is significant different from its Control in shoot length (Student's t, $p < 0.0001$).
2. Control-RKN assay: In shoot length measurement, High treatment is significant different from its Control (Student's t, $p = 0.0040$); Medium treatment is significant different from its Control (Student's t, $p = 0.0026$). In root weight measurement, High treatment is significant different from its Control (Student's t, $p = 0.0002$).
3. Fungicide-Control assay: Medium treatment is significant different from its Control in shoot weight (Student's t, $p = 0.021$).
4. Fungicide-RKN assay: High treatment is significant different from its Control in root length (Student's t, $p = 0.021$).

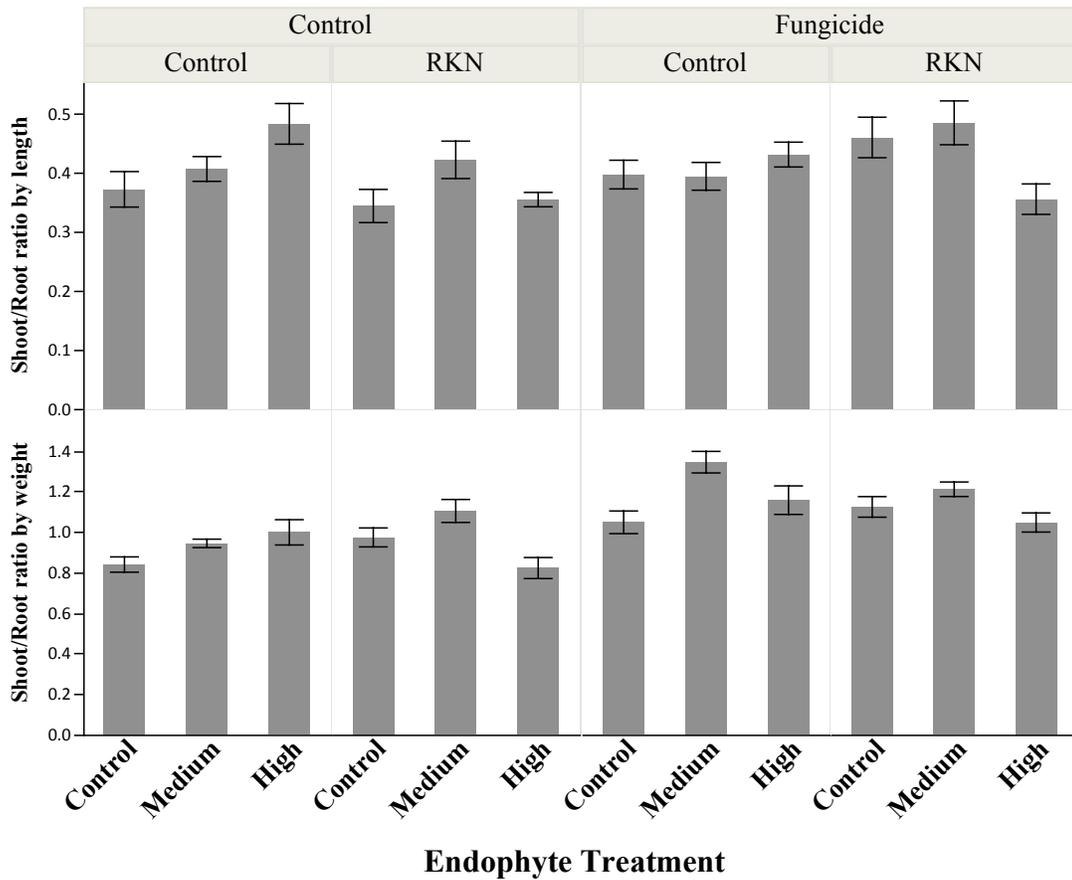


Fig. A4 Plant growth evaluation 12 days after nematode inoculation: shoot/root ratio by length, shoot/root ratio by weight. Plants from all treatments were sampled with ten replicates per treatment. A total of 12 treatments were included: RKN (+/-), Endophyte (Control, Medium, High), Fungicide (+/-). No overall significant treatment effect was detected (ANOVA test, see Table A4 below). Each error bar represents 1 standard error from the mean.

Table A4. Statistical analyses of plant growth by shoot/root ratio 12 days after nematode inoculation (ANOVA test, $\alpha = 0.05$).

Measurements	Control				Fungicide			
	Control		RKN		Control		RKN	
	$F_{2,27}$	p -valeur						
Shoot/Root ratio by length	3.82	0.035	2.77	0.080	0.79	0.46	4.39	0.022
Shoot/Root ratio by weight	3.40	0.048	7.36	0.0028	6.21	0.0061	3.32	0.051

Student's t test:

1. Control-Control assay: High treatment is significant different from its Control in the length ratio (Student's t, $p=0.012$), and in weight ratio (Student's t, $p=0.016$). In the length ratio measurement, High treatment is significant different from its Control (Student's t, $p=0.012$).
2. Control-RKN assay: In the weight ratio measurement, High treatment is significant different from its Control (Student's t, $p=0.050$).
3. Fungicide-Control assay: Medium treatment is significant different from its Control in the weight ratio (Student's t, $p=0.0017$).
4. Fungicide-RKN assay: High treatment is significant different from its Control in the length ratio (Student's t, $p=0.033$).

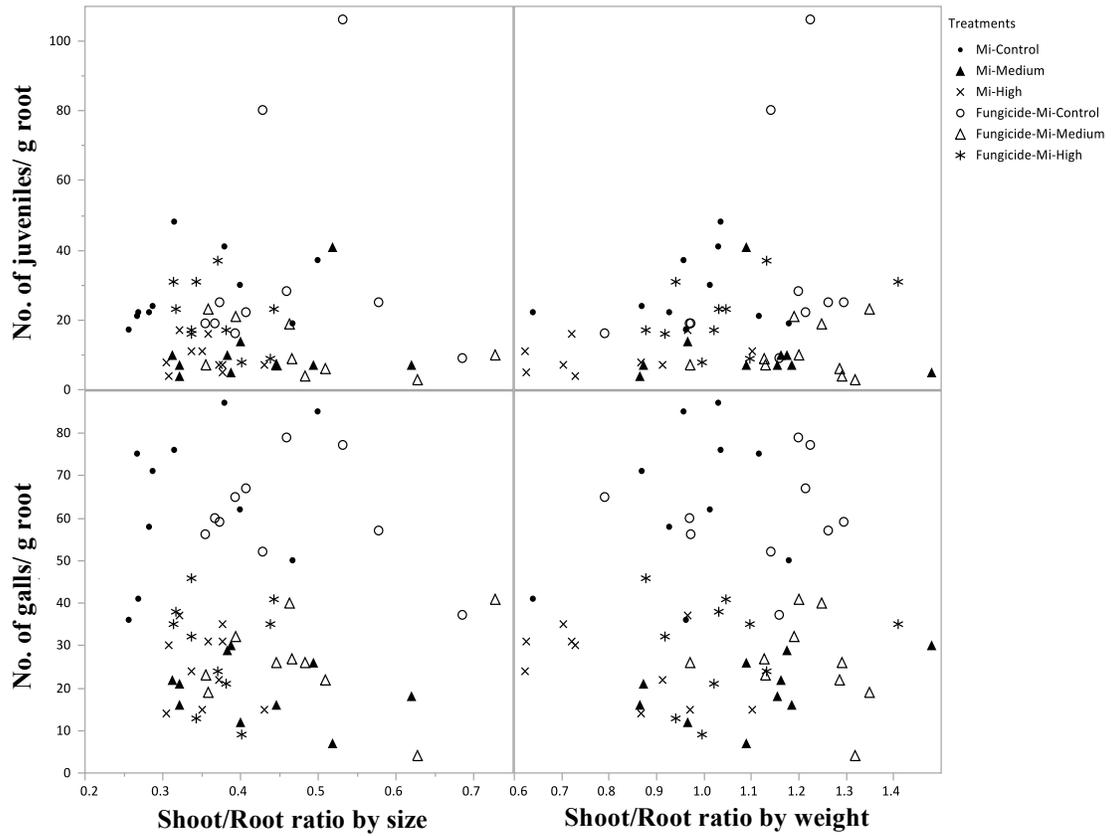


Fig. A5 Scatter plot of interactions between plant performances (in shoot/root ratio, by size and by weight) and nematode infection (numbers of juveniles and number of early galling) at 12 DAI. Each plant received an egg inoculum of 2,000 eggs.

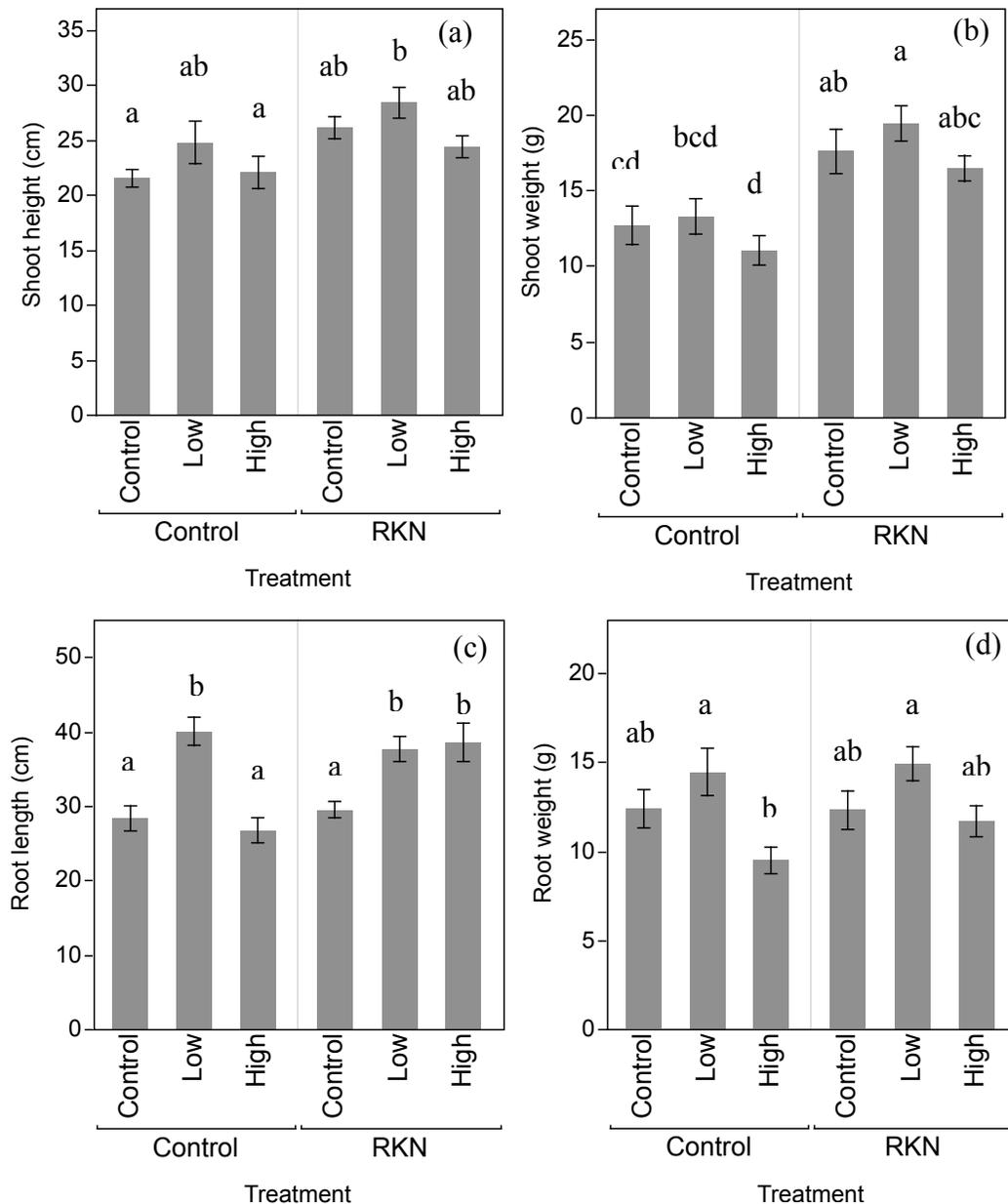


Fig B. Plant performances collected in the first root-knot nematode (RKN) experiment. Plant measurements at 6 WAI were: (a) shoot height (GLM, $F_{5, 54} = 3.8617$, $P = 0.0046$; Endophyte effect: $df = 2$, $F = 3.7771$, $P = 0.0292$; RKN effect: $df = 1$, $F = 11.0046$, $P = 0.0016$), (b) shoot fresh weight (GLM, $F_{5, 54} = 7.8614$, $P < 0.0001$; RKN effect: $df = 1$, $F = 33.9745$, $P < 0.0001$), (c) root length (GLM, $F_{5, 54} = 10.6441$, $P < 0.0001$; Endophyte

effect: $df = 2$, $F = 15.3069$, $P < 0.0001$; RKN effect: $df = 1$, $F = 5.7262$, $P = 0.0202$), (d) root fresh weight (GLM, $F_{5, 54} = 3.7148$, $P = 0.0058$; Endophyte effect: $df = 2$, $F = 8.0626$, $P = 0.0009$), (e) shoot / root ratio by size (GLM, $F_{5, 54} = 3.0467$, $P = 0.0171$; Endophyte \times RKN effect: $df = 2$, $F = 5.0138$, $P = 0.0101$), and (f) shoot / root ratio by weight (GLM, $F_{5, 54} = 6.4326$, $P < 0.0001$; RKN effect: $df = 1$, $F = 27.0477$, $P < 0.0001$). Each error bar represents 1 standard error from the mean. Different letters indicate significant differences among means by a Tukey's HSD test ($\alpha = 0.05$.)

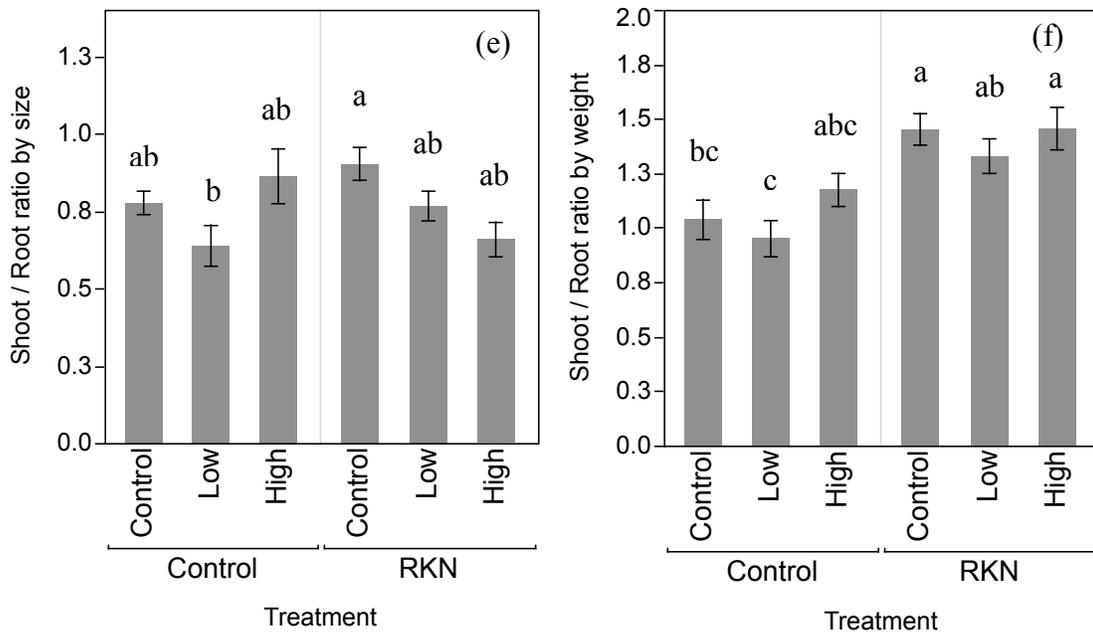


Fig B. Continued.