Resistance to *Meloidogyne incognita* Induced by *Bacillus* spp.

by

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Abstract

Meloidogyne incognita race 3 is a major pest of hundreds of susceptible plant hosts around the world. Biological control agents are one management strategy that can be employed against this nematode. The goal of this research was to determine the mechanism of action by which five *Bacillus* spp. can manage *M. incognita* population density in cotton. The overall objectives were to 1) determine the efficacy and direct antagonistic capabilities of the *Bacillus* spp. and 2) determine the systemic capabilities of the *Bacillus* spp. The greenhouse *in planta* assay indicated *B. amyloliquefaciens* QST713 and *B. firmus* I-1582 could manage *M. incognita* similarly to the chemical standard fluopyram. An *in vitro* assay determined that *B. firmus* I-1582 and its extracted metabolites were able to directly manage *M. incognita* second stage juveniles by increasing mortality rate above 75%. A split root assay, used to determine systemic capabilities of the bacteria, indicated *B. amyloliquefaciens* QST713 and *B. firmus* I-1582 could indirectly decrease the nematode population density. Another species, *B. mojavensis* strain 2, also demonstrated systemic capabilities but was ruled out as a successful biological control agent because it had the second highest population density behind the control when in contact with the nematode in greenhouse *in planta* assay and the split root assay compared to any other treatment. A RT-qPCR assay was used to evaluate systemic activity observed in the split root assay. At 24 hours, both *B. amyloliquefaciens* QST713 and *B. firmus* I-1582 upregulated one gene involved in the initial stages of jasmonic acid (JA) synthesis pathway but not another gene involved in the later stages of JA synthesis. These results point to a JA intermediate molecule, most likely 12-

oxo-phytodienoci acid (OPDA), stimulated by the bacteria rather than JA in a short-term systemic response. After 1 week, the *Bacillus* spp. stimulated a salicylic acid (SA) responsive defense related gene. The long-term systemic response to the *Bacillus* spp. indicates SA also plays a role in defense conferred by these bacteria. The final assay used qPCR to determine the concentration of the bacteria on the cotton roots after 24 days. *Bacillus amyloliquefaciens* QST713 and *B. firmus* I-1582 were able to colonize the root successfully, with the concentration after 24 days not significantly differing from the concentration at inoculation. This study identifies two bacteria that induce systemic resistance and will help aid in implementing these species in an integrated management system.

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Chapter 1: Introduction and Review of Literature

Introduction:

Meloidogyne spp., including *M. incognita* (Kofoid and White, 1919) Chitwood, are the most important plant-parasitic nematodes worldwide (Jones et al., 2013). They cause economic damage on many crops including cotton, corn and soybeans, a few of the main row crops in Alabama. Management strategies for *M. incognita* include the application of chemical nematicides, resistant varieties and cultural control. Nematicides are not always an environmentally friendly option and can be costly if a nematode problem is wide spread across a field (Starr et al., 2007). Cultural control is not always feasible and resistant varieties are better used in an integrative management situation (Kirkpatrick and Sasser, 1984). Biological control agents can provide a more environmentally friendly, cost effective method of nematode management for producers.

Biological control agents, typically fungi or bacteria, can work by direct or indirect antagonism. Direct antagonism can occur through predation, the release of metabolites or competition (Kerry, 2000). Indirect antagonism can occur through induced systemic resistance (ISR) or systemic acquired resistance (SAR). ISR is defined as the induction of plant defense by typically jasmonic acid and ethylene upon stimulation with a plant growth promoting rhizobacteria (PGPR) (van Loon et. al., 1998). SAR is defined as the systemic upregulation of disease resistance induced by salicylic acid following pathogen infections (Durrant and Dong 2004). While ISR is specifically stimulated by PGPR, there have been PGPR strains that stimulate salicylic acid and SAR rather than jasmonic acid and ISR (Chen et al., 1999; Syamala and Sivaji, 2017; Beris et al., 2018).

Biological control agents can be very effective against nematodes, both through direct and indirect antagonism. Often biological control agents that exhibit direct antagonism are more readily recognized while biological control agents that work by indirect antagonism can be overlooked. Biological control agents should be screened for capabilities to indirectly manage as well as to directly manage nematodes.

***Meloidogyne incognita*:**

M. incognita on cotton:

Cotton, *Gossypium* spp., is the largest textile fiber crop in the world. Currently, *G. hirsutum*, or upland cotton, accounts for 95% of the world's cotton production, making it the most economically important cotton species (Tyagi et al., 2014). In 2017, there was a total of 12,613,000 acres of *G. hirsutum* planted in the USA equating to about $1.8 billion (National Cotton Council of America, 2017a). In Alabama, specifically, there were 706,000 bales of cotton produced equating to about $325 million in the same time period (National Cotton Council of America, 2017b). One major yield-limiting pathogen of *G. hirsutum* is the southern root-knot nematode, *M. incognita*. The nematode is found in all parts of the southern US where cotton is grown, including Alabama (Thomas and Kirkpatrick 2001). In 2017, *M. incognita* was responsible for the estimated loss of 49,800 bales of cotton in Alabama and 628,600 bales nationwide (Lawrence et al., 2018). With the current price of cotton at $0.93, this equates to a loss of $23 million in Alabama and $282.8 million nationwide.

M. incognita life cycle and symptoms:

Meloidogyne incognita is a plant-pathogenic sedentary endoparasitic nematode. This nematode has a large plant host range with over 3000 host plant species (Sasser 1987). *Meloidogyne incognita* is found in 30 of the 50 states nationwide. The nematode is small, with an

average length of 609 micrometers as a vermiform, or worm-like, juvenile (Whitehead 1967). As an adult female the nematode becomes an apple shape as an egg mass develops (Mitkowski and Abawi, 2003).

The life cycle of *M. incognita* begins when a female nematode lays eggs into an egg mass produced by the female (Moens et al., 2010). The egg mass is composed of glycoproteins that protect the eggs from the environment and predators, and provides some antimicrobial properties (Orion and Kritzman, 1991; Moens et al., 2010). The egg mass is laid on the outside of the female's body and can be on the outside of the roots or within the roots. The first stage juvenile (J1) is formed when embryogenesis occurs within the egg (Moens et al., 2010). After the J1 stage, the nematode will molt into a second stage infectious juvenile (J2), which will hatch from the egg when environmental conditions, specifically temperature, root exudates and level of moisture, are favorable (Moens et al., 2010). The J2 pierces the plant cell wall with the stylet and the nematode's feeding tubes begin to form (Abad et al., 2003). The nematode releases effector proteins which cause the formation of a multinucleated giant cell that the nematode will feed from (Abad et al., 2003). The nematode will go through three more molts within the root to become an adult. If males are present, they will mate with females then leave the roots, but males are only present when condition are unfavorable. Females will most often undergo mitotic parthenogenesis to produce an egg mass which will continue the lineage (Moens et al., 2010). When parthenogenesis occurs, the resulting nematodes will be clones of the mother nematode. If mating occurs with the males, there is a genetic mixing. The life cycle of *M. incognita* takes 25 days at 27 °C.

The most distinctive symptom of *M. incognita* is galling on the roots (Shepherd and Huck, 1989). Galls are the multinucleated cells that develop when the *M. incognita* J2 pierces the

root cells and inserts salivary gland substances which stimulates the formation of multiple giant cells though hypertrophy and hyperplasia (Moens et al., 2010). Symptoms visible above the soil's surface include yellowing and stunting of the plant, which typically occurs in a wave-like pattern across an infested field. The nematode significantly decrease yield.

M. incognita management:

The most commonly used management strategy for dealing with *M. incognita* is the application of chemical nematicides. Though this method of management has been an effective management strategy, chemical nematicides can be harmful to the environment, dangerous to apply and very costly (Starr et al., 2007). Crop rotation can be the most effective cultural practice to control *M. incognita* population density (Kirkpatrick and Sasser, 1984). However, crop rotation may not be a feasible option for some farmers. For example, in Alabama one of the most efficient crop rotations schemes for *M. incognita* infected fields include the non-host crop of peanut (Kirkpatrick and Sasser, 1984; Johnson et al., 1998). However, peanuts require different harvesting equipment than cotton, soybeans and corn, the most prominent row crops in Alabama. This harvesting equipment can range into the hundreds of thousands of dollars, of which many farmers do not have available for these different harvesting tools. Resistant varieties are available, as well. These are often resistant or tolerant to the nematode. Resistant varieties will keep nematodes from colonizing the roots successfully; however, this is often accompanies by decreased yield (Vyska et al., 2016). A tolerant variety will not decrease nematode colonization of the roots but will have a high yield despite high nematode numbers (Trudgill, 1991). There are variations on resistant and tolerant varieties, for example resistant varieties with differing levels of resistance. These are the most successful when used in an integrated management system. Alternative strategies must be looked at for effective, sustainable management of *M. incognita*.

One option is biological control. Biological control, encompasses microbes, typically fungi and bacteria, that can reduce the impact of a pathogen.

Potential noncultural management strategies have different mechanisms of action. Each nematicidal compound has a mechanism of action, often a way to directly kill the nematode. For example, some nematicides, such as aldicarb and oxamyl, inhibit cholinesterase to cause acute toxicity within the nematode (Rhoads 1981; Chitwood 2003). The mechanisms of action of nematicidal products utilizing biological control agents are often less well understood (Belanger et al., 2012). They encompass direct and indirect antagonism. The most efficient strategies use a more integrated management strategy, which implies using different cultural techniques and using nematicides with different mechanisms of action to manage the nematode problem.

Biologic control and direct antagonism:

The widely accepted definition of biological control in plant pathology describes it as a control option that reduces disease by utilization of one or more organism, not including men (Cook and Baker, 1983). Fungi and bacteria are the common organisms studied as biological control agents, especially against nematodes (Meyer and Roberts, 2002).

Biological control agents typically work indirectly or directly against the pathogen upon colonization of the host plant. Direct antagonism of a pathogen encompasses the release of metabolites, predation or competition (Pal and Gardener, 2006). Certain species of bacteria can also decrease the impact of nematodes by increasing plant health and biomass. There are various bacteria shown to control nematodes through a direct mechanism. An example of predation of nematodes is *Pasteuria penetrans*, a bacterium which infects *Meloidogyne* spp. and colonizes the body cavity of the nematodes (Mankau at al., 1976). A few other species of *Pasteuria*, a highly host specific biological control agent, that have similar level of nematode predation have been

made into commercially available products, including Clariva, Naviva ST and NewPro. A recent study looked at sugarcane where *P. penetrans* treatment and was able to decrease *M. javanica* by an average of 97.5%, indicating that *Pasteuria* can be a good option for biocontrol against this particular nematode (Bhuiyan et al., 2018). While *Pasteuria* spp. have been developed into successful commercial products, the extreme host specificity limits the use of this biological control agent to very specific situations. Farmers often look for a product with a broader spectrum of nematodes and diseases that can be controlled by the biological control agent.

Direct antagonism also occurs through the release of metabolites. For example, *P. fluorescens* strain F113 is able to control the potato cyst nematode, *Globodera* spp., *in vitro* and in soil conditions by releasing the metabolite 2,4-diacetylphloroglucinol (Cronin et al., 1997). The bacteria decreased juvenile motility by 85% *in vitro* and in soil compared to a mutant with 2,4-diacetylphloroglucinol functionality silenced (Cronin et al., 1997). Another strain, *Pseudomonas flourescens* CHA0, was shown to release both 2,4-diacetylephloroglucinol and pyoluteorin, two secondary metabolites that can significantly decreased population density of *M. javanica in vitro* and in a soil setting (Hamid et al., 2003).

While competition is not often employed as a control strategy, there is evidence that it can decrease the number of individual species of plant-parasitic nematodes in soil. When there are a large number of diverse nematodes in the soil, no one nematode species, including plant-parasites, could rapidly multiply and cause significant damage to the plant. This is compared to an environment where few or only one species of plant-parasitic nematode were present due to competition between the nematodes for plant resources (Piskiewic et al., 2007). Other studies have seen similar results, including a study done by Brinkman et al. (2005) that showed competition between *M. maritima*, *Heterodera arenaria*, and *Pratylenchus penetrans* in a grass

system. The damage to the plants was decreased when all three species of nematodes were present in comparison to when only one of the nematodes was present.

Direct antagonism of nematodes by biological control agents is observed frequently. Predation, the release of metabolites and competition are good control mechanisms for a biological control agent to employ against nematodes and can be easily identified. Another control strategy, using an indirect control mechanism, can be overlooked when analyzing biological control agents. Often the first test employed to determine a bacteria's ability to control a disease is an *in vitro* assay. These assays are successful because they allow the researcher to visualize the impact of the biological agent on the nematode or pathogen. As well, *in vitro* tests are quick, typically taking no more than a week, and are inexpensive. Determining indirect antagonism is often much more difficult and time consuming.

Indirect antagonism through activation of plant defense pathways:

Indirect antagonism of plant pathogens occurs by systemic resistance which most often encompasses two different pathways that stimulate plant defenses. The two pathways for indirect antagonism are ISR and SAR. ISR is activated via the stimulation of jasmonic acid (JA) and ethylene by PGPRs. SAR is developed by salicylic acid, often paralleled with the systemic expression of pathogenesis related (PR) genes. Classical definitions of the two types of resistance often differentiate them based on the classification of the pathogen or pest involved, with SAR activated in response to a biotrophic pathogen and ISR activated in response to an herbivorous pest or a necrotrophic pathogen (Caarls 2015). However, using these more traditional classification guidelines excludes *Bacillus* spp. and other biocontrol agents that are neither necrotrophic nor biotrophic and are not known to be pathogenic. This section will review each of

the pathways and the hormones involved to get a clearer understanding of their definitions and functions.

Induced systemic resistance and jasmonic acid:

One of the most accepted definitions of ISR comes from van Loon et al. (1998) who referred to ISR as the elicitation of plant defenses by PGPR, indicated by a reduced disease incidence or severity, when the disease causing pathogen is not in contact with the PGPR. While this definition is more accepting of biocontrol agents in systemic resistance, it does not include any reference to which defense pathways may be stimulated. The most commonly referred to defense pathway stimulated during ISR is the JA and ethylene pathways, with a larger emphasis on JA defenses than ethylene defenses. While JA is involved in ISR, it is not limited to this role in defense. JA can be stimulated by many different pathogens as well as PGPRs.

JA is synthesized from a fatty acid, α- linolenic acid, released by the galactolipids on the chloroplast membrane (Wasternack and Hause, 2013). Upon wounding or other stimulation, lipoxygenase (LOX) homologues initiate the oxygenation of α- linolenic acid in the chloroplast (Bannenberg et al., 2009), and produce an unstable 13(S)-hydroperoxylinolinic acid (13-HPOT). This is then cyclized by 13-allene oxide synthase (AOS), and subsequently stabilized by allene oxide cyclase (AOC) to form cis-(+)-OPDA (Stenzel et al., 2012). OPDA is then further reduced by the OPDA reductase, OPR3, in the peroxisome (Breithaupt et al., 2001), and undergoes three beta-oxidation reactions to become (+)-7-iso-JA (Stenzel et al., 2012). After initial formation of (+)-7-iso-JA, it can continue being derivertized to the bioactive jasmonate, JA-Ile, created through a reaction with L-isoleucine (Fonseca et al., 2009). The intermediate molecules, primarily OPDA, are thought to potentially play a separate role in defense as well (Gleason et al. 2016).

JA and its derivatives function as signaling molecules. Not all of the receptors JA interacts with have been identified, but one is well characterized (Kazan and Manners, 2008). It is known that the coronatine-insensitive1 (COI1) protein acts as one key part of the receptor for JA-Ile (Chini et al., 2007). Studies involving mutant *coi1* observed all JA-dependent responses are defective without *coi1* functionality and the plant was very susceptible to pathogen attack (Xie et al., 1998). Following synthesis, JA-Ile binds COI1, the Skip-Cullin-F-box protein of the E3 ubiquitin-ligase (SCF^{COI1}; Hickman et al., 2017). SCF^{COI1} will target and ubiquitinate (degrade) jasmonate ZIM-domain (JAZ) proteins, inhibitors to the transcription of JA responsive genes (Sheard et al., 2010; Hickman et al., 2017).

After interaction with a receptor, JA-dependent defenses begin. Many studies that determine the JA-defense response use exogenous application of methyl jasmonate. JA biosynthesis genes are upregulated by JA, creating a positive feedback loop (Kazan and Manners 2008, Bonaventure et al., 2007). One of the major defense-related proteins correlated with JA signaling is PDF1.2 (Penninckx et al., 1996). *PDF1.2*, the gene responsible for PDF1.2 accumulation, is a gene encoding a plant defensin that protects the plant against pathogens. Another gene activated by JA is *HEL*, which encodes a hevien-like protein, a PR4 protein (Bertini et al., 2012). Other defense-related JA-responsive genes are the thionin gene *Thi2.1* and basic chitinase *ChiB* (Pierterse and van Loon 1999, Thomma et al., 1998). These genes are all involved in the defense against pathogens or have been correlated to JA signaling.

Jasmonic acid is thought to be important in plant defense against nematodes. Fujimoto et al. (2011) observed a decrease in *M. incognita* population density in tomatoes after application of exogenous methyl JA and used RT-qPCR to confirm the stimulation of JA and that JA may be important in root-knot nematode defense. Another study used a JA silenced mutant rice to

determine that JA was the hormone pathway most responsible for defense against *M. gramnicola* in rice (Nahar et al., 2011). Similarly, biological control agents activated JA defenses to manage nematodes. JA was activated about a week after *Trichoderma* spp. application, as determined by RT-qPCR, which correlated with a decrease in *M. incognita* parasitism (Martinez-Medina et al., 2017a). The researchers concluded that JA was most effective at the feeding and developmental stage of the nematode after it invaded the root system. Further studies by this researcher using transgenic tomato plants corroborated the claim that it is JA upregulated by *Trichoderma* spp. that can decrease nematode numbers (Martinez-Medina et al., 2017b).

In response to JA, there are effectors released by nematodes to combat this defense pathway. Specifically in *M. incognita*, there are a few effectors that target JA. Examples of these effectors include *MilSE5* which is a *M. incognita* effector that activates genes related to the suppression of JA, *MiCRT* which suppresses a defense gene down stream of JA, and *Misp12* which downregulates genes related to JA biosynthesis (Shi et al., 2018; Jaouannet et al., 2013; Xie, et al., 2016). Effectors released by *M. incognita* to target JA defense signaling will make the JA defense pathway ineffective upon nematode attack. A biological control agent which can increase JA levels at an equivalent or higher rate than the nematode effectors can suppress JA levels would be a good option for managing these nematodes.

Systemic acquired resistance and salicylic acid:

Development of SAR, a state of heightened defense that is activated throughout the plants following a primary infection, requires the systemic induction of SA (Durrant and Dong, 2004). SA is a phenolic compound found in plants that functions as a phytohormone. SA plays a role in plant growth, development and defense. Production of SA is stimulated by the recognition of pathogens by cell surface receptors (Macho and Zipfel 2015).

The synthesis process for SA is not very well understood. There are two proposed pathways for SA synthesis within the plant. Both of these require the primary metabolite chorismate (Dempsey et al., 2011; Dempsey and Klessig 2017). The first is the phenylalanine ammonia-lysate (PAL) pathway. In the PAL pathway, the first step is conversion of phenylalanine to *trans*-cinnamic acid (*t*-CA) (Dempsey et al., 2011). Following the conversion to *t*-CA, multiple biochemical reactions result in the formation of one of two intermediate products, ortho-coumaric acid or benzoic acid (Klämbt, 1962; Chadha and Brown, 1974). The formation of benzoic acid from *t*-CA occurs through three different possible pathways. It is unknown why one pathway to form benzoic or ortho-coumaric acid may be utilized; however, upon formation of benzoic acid or ortho-coumaric acid, further reactions occur to form SA (Dempsey et al., 2011). The second pathway for synthesis of SA is the isochorismate (IC) pathway. While many studies have proven that SA synthesis can occur through the IC pathway, there is not a definitive mechanism by which isochorismate is converted to SA (Dempsey et al., 2011). When the IC pathway was hindered in an *Arabidopsis* mutant, accumulation of pathogen induced SA decreased by 90-95% (Wildermuth et al., 2001).

Following synthesis, SA can be active or modified to become inactive. The three main modifications are glucosylation, methylation and amino acid conjugation. Glucosylation enhances storage capacity of SA, methylation increases membrane permeability which allows easier transport of SA, and amino acid conjugation is thought to fine-tune SA catabolism (Dempsey et al., 2011).

The regulation of SA is also not well understood. There is evidence that calcium plays a role in activating and inactivating genes used in the formation and maintenance of SA (Seyfferth

and Tsuda, 2014). It is known that there are other regulatory components of SA accumulation within the plant. However, it is not known what those are (Seyfferth and Tsuda, 2014).

Upon biosynthesis, SA triggers thioredoxin (TRX)-mediated reduction (activation) of transcriptional regulators, non-expressor of pathogenesis-related genes *(NPR*) 1, or conveys suppression of *NPR2*, *NPR3* and *NPR4* (Thomma et al., 1998, Fu et al., 2012, Wu et al., 2012). *NPR1* is known as a positive transcriptional regulator of *PR* genes, whereas *NPR2*, *NPR3* and *NPR4* are considered as a suppressor of *PR* gene expression (Maier et al., 2010). Most PR proteins are cell-wall degrading enzymes and their expressions are highly correlated with defense activation in plants. The function of PR1 is not well understood; however, recent studies link this protein to sterol-binding (Gamir et al., 2016). PR2, β-1,3 glucanase, can hydrolyze glucans (Hong and Hwang 2005). PR3, or chitinases, act similarly hydrolyzing chitin, which is a structural component of nematode eggs among other pathogenic structures for nematodes and other pests (Hong and Hwang 2005). PR5, or thaumatin-like proteins, are noted to have anti-fungal properties; however, the exact mechanism of action is not well understood (Zhai et al., 2018).

SA is often stimulated by pathogens, but there are a few examples of PGPR species stimulating SA signaling. Examples of this include *Pseudomonas aurofaciens* and *P. corrugata*, both of which were seen to stimulate SA activity after inoculation as determined by HPLC (Chen et al., 1999). Another prominent study used qRT-PCR to determine that *Bacillus amyloliquefaciens* can activate SA-dependent defenses to defend tomato plants against two viruses (Beris et al., 2018).

SA can have an impact on nematodes; however, this is studied less often than JA's impact on nematodes. The study by Martinez-Medina et al. (2017a) saw an increased JA

signaling 7 days after *Trichoderma* spp. stimulation and saw an increase in SA activity upon initial inoculation. The researchers concluded that SA activity was initially stimulated and was responsible for defense against the root-knot nematode's initial invasion (Martinez-Medina et al., 2017a). Another study found that *Mi-1* resistance gene, the most commonly used gene to confer resistance against *M. incognita*, relies on SA signaling (Branch et al., 2004). This also indicates that SA is involved in defense against initial nematode invasion.

Similarly to JA, there are effectors released by *M. incognita* that will disrupt SA biosynthesis (Wang et al., 2018). These effectors are not as well characterized as the JA effectors, however. Regardless, the literature seems to point to both JA and SA being involved in defense against nematodes and stimulation of defenses by PGPR. It is important to consider both phytohormones when analyzing systemic resistance.

Salicylic acid and jasmonic acid interactions:

It is often implied that JA and SA cross talk with each other. This interaction can be either antagonistic or synergistic. One of the first investigations into interactions between SA and JA demonstrated an antagonistic relationship between the phytohormones in tomatoes (Pena-Cortes et al., 1993). They observed that aspirin, formulated SA, significantly suppressed the expression of JA related genes in tomato (Pena-Cortes et al., 1993). Similarly, genes related to SA expression in tomato were inhibited upon stimulation of JA by *Pseudomonas syringae* (Uppalapati et al., 2007). Applications of exogenous JA also correlated with a decrease in SA activity and an increase in JA activity (Tamaoki et al., 2013). A more in depth look into the antagonism between SA and JA demonstrated that SA targets downstream JA responsive promotors and inhibits any further JA activity (van der Does et al., 2013). It is thought the

antagonism between SA and JA helps the plant conserve energy and optimize the defense pathways in the presence of a single attacker (Gimenez-Ibanez and Solano, 2013).

In contrast, there is evidence that indicates JA and SA can work in a synergistic manner rather than antagonistically. It has been noted a few different pathogens can stimulate both JA and SA at the same time. Examples of those pathogens are *Botrytis cinerea* on tomatoes and tobacco mosaic virus (Mitter et al., 1998; Clarke et al., 2000). One study discovered that the concentration of the hormones determined the interaction between them (Mur et al., 2006). If the level of JA or SA is too high there was an antagonistic relationship; however, if the levels of each hormone were lower, the two hormones acted synergistically (Mur et al., 2006). The exact concentration where synergism between the two hormones turns to antagonism is yet to be determined.

The goal of biological control research is to optimize the biological control agent to be as effective and efficient as possible against the target pathogen(s). Determining any manipulation of the JA and SA pathways by the target pathogen is an essential part of this type of research. This can, in turn, determine the best pathway to provide the most effective protection and defense against the target pathogen. This is important to keep in mind when selecting the best biological control agent in each management strategy.

Bacillus *spp. as biological control agents:*

One genus of bacteria often used in biological control is *Bacillus* spp. These are a good options because of their ability to replicate and colonize quickly, tolerate harsher environments, and easily form endospores. They are documented to affect a broad spectrum of pathogens (Shafi et al., 2017).

Many *Bacillus* spp. fall into the category of plant growth-promoting rhizobacteria (PGPR). These are bacteria that will promote growth in the plant by various mechanisms, including nitrogen fixation, production of phytohormones and siderophore production (Kloepper et al., 1989; Vacheron et al., 2013). *Bacillus* spp. are the main species of commercialized PGPR due to their hardiness, as described above, compared to other effective PGPR bacteria such as *Pseudomonas* spp. (Kloepper et al., 2004).

Direct antagonism:

Many *Bacillus* spp. are effective in biological control against different pathogens. Some *Bacillus* spp. use secondary metabolites and anti-microbial properties as a form of direct antagonism to reduce disease and pests. An example of this is the synthesis of a bacillomycin D by *B. subtilis* which can inhibit spore germination and sporulation of *Aspergillus* spp. (Gong et al., 2014). Similarly, a strain of B. *amyliloquefaciens* caused abnormal germination of various fungi, including *Fusarium* and *Aspergillus* spp., because it produces secondary metabolite such as iturins-like and fengycin-like peptides (Benitez et al., 2010). There are many other examples of different *Bacillus* spp., mainly strains of *B. subtilis*, which reduce bacterial or fungal diseases by release of metabolites and or exhibiting anti-microbial properties. Fewer examples of *Bacillus* spp. that release metabolites that impact nematodes are documented. In one study, *M. incognita* was decreased *in vitro* by secondary metabolites extracted from *B. firmus* (Mendoza et al., 2008). Another study looked at over 600 *Bacillus* strains and demonstrated that 33% of those, from various species, were able to increase the percent mortality of *M. incognita in vitro* (Xiang et al., 2017). This was attributed to direct antagonism against the nematode, which is predicted to be by the release of metabolites. A similar study evaluated *B. cereus* strain S2 *in vitro* and determined

it released sphingosine to cause reactive oxygen (ROS) response in *M. incognita* which resulted in cell necrosis and injury in nematodes (Gao et al., 2016).

Indirect antagonism:

There have also been various examples of JA induced defenses and ISR. A study with *B. subtilis* UMAF6639 used a JA-deficient plant in comparison to a functional plant and determined that this bacterium utilized the JA pathway in *Arabidopsis* to defend against the powdery mildew pathogen *Podosphaera fusca* (Garcia-Gutierrez et al., 2013). Another study used a transgenic JA deficient tomato to determine the mechanism of action of the PGPRs *B. cereus* and *B. pumilus* (Yan at al., 2002). This particular study used transgenic tomatoes with low levels of JA and found that the *Bacillus* spp. was unable to defend the transgenic plant against *Phytophthora infestans* compared to a tomato with intact JA defenses (Yan et al., 2002). The reduction of symptoms in the control plant with normal levels of JA compared to the transgenic plant indicates that JA is stimulated by these *Bacillus* spp.

SA and SAR are less frequently seen to be correlated with *Bacillus* spp.; however, there are some examples of this. Li et al. (2015) found that *B. amyloliquefaciens* LJ02 stimulated SA activity and the activation of PR11 in cucumber. This species was tested previously to be effective in managing *Fusarium oxysporum*, *Botrytis cinerea* and *Alternaria* spp. in cucumber (Li et al., 2015). Another strain, *B. amyloliquefaciens* strain MBI600 from the commercial product Serifel® (BASF SE) induced salicylic acid to reduce the disease severity of tomato spotted wilt virus in tomatoes (Beris et al., 2018).

While there are fewer examples of SA acid stimulated by *Bacillus* spp. then there are examples of JA stimulation. Few studies; however, look at both hormone pathways when analyzing systemic resistance. It is likely that both pathways are involved in some form of plant

defense response to *M. incognita*, therefore it is important to analyze both pathways that could possibly be stimulated by a *Bacillus* spp. biological control agent.

Conclusion:

Biological control agents have been successful in managing nematode population density. However, there are many potential biological control agents that are overlooked due to their use of indirect antagonism rather than direct antagonism. Potential biological control agents need to be screened for systemic resistance. Full understanding of the mechanisms of action employed by each biological control agent aids in the implementation of the biological control agent in a commercial integrated pest management strategy.

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Chapter 2: Evaluation of the mechanism of action of *Bacillus* spp. to manage *Meloidogyne incognita* through a greenhouse *in planta* assay, *in vitro* assay, split root assay, RT-qPCR and qPCR.

Abstract:

Meloidogyne incognita race 3 is a major pest of hundreds of susceptible plant hosts around the world. Biological control agents are one management strategy that can be employed against this nematode. The goal of this research was to determine the mechanism of action by which five *Bacillus* spp. can manage *M. incognita* population density in cotton. The overall objectives were to 1) determine the efficacy and direct antagonistic capabilities of the *Bacillus* spp. and 2) determine the systemic capabilities of the *Bacillus* spp. The greenhouse *in planta* assay indicated *B. amyloliquefaciens* QST713 and *B. firmus* I-1582 could manage *M. incognita* similarly to the chemical standard fluopyram. An *in vitro* assay determined that *B. firmus* I-1582 and its extracted metabolites were able to directly manage *M. incognita* second stage juveniles by increasing mortality rate above 75%. A split root assay, used to determine systemic capabilities of the bacteria, indicated *B. amyloliquefaciens* QST713 and *B. firmus* I-1582 could indirectly decrease the nematode population density. Another species, *B. mojavensis* strain 2, also demonstrated systemic capabilities but was ruled out as a successful biological control agent because it had the second highest population density behind the control when in contact with the nematode in greenhouse *in planta* assay and the split root assay compared to any other treatment. A RT-qPCR assay was used to evaluate systemic activity observed in the split root assay. At 24 hours, both *B. amyloliquefaciens* QST713 and *B. firmus* I-1582 upregulated one gene involved in the initial stages of jasmonic acid (JA) synthesis pathway but not another gene involved in the later stages of JA synthesis. These results point to a JA intermediate molecule, most likely 12-

oxo-phytodienoci acid (OPDA), stimulated by the bacteria rather than JA in a short-term systemic response. After 1 week, the *Bacillus* spp. stimulated a salicylic acid (SA) responsive defense related gene. The long-term systemic response to the *Bacillus* spp. indicates SA also plays a role in defense conferred by these bacteria. The final assay used qPCR to determine the concentration of the bacteria on the cotton roots after 24 days. *Bacillus amyloliquefaciens* QST713 and *B. firmus* I-1582 were able to colonize the root successfully, with the concentration after 24 days not significantly differing from the concentration at inoculation. This study identifies two bacteria that induce systemic resistance and will help aid in implementing these species in an integrated management system.

Introduction:

Meloidogyne incognita (Kofoid and White, 1919) Chitwood, the southern root-knot nematode, is an endoparasitic nematode that feeds on hundreds of susceptible plant hosts. The nematode is a major yield-limiting pathogen of cotton, *Gossypium hirsutum* L. *Meloidogyne incognita* caused an estimated 628,600 bale yield reduction in 2017 and a 483,300 bale yield reduction in 2018 across the United States (Lawrence et al., 2018, 2019). There are various management options available, the most common being chemical nematicides. However, chemical nematicides can be harmful to the environment and very costly if a nematode problem is widespread in a field (Starr et al., 2007). Crop rotation can be an effective management strategy, but it is not always feasible due to the different expensive equipment needed to harvest and maintain different crops (Kirkpatrick and Sasser, 1984). Biological control has been explored for its ability to manage *M. incognita* in cotton due to the low manufacturing cost and expected environmentally friendliness (Bale et al., 2008).

Biological control agents are one or more organisms, typically fungi or bacteria, which reduce the severity or incidence of a plant disease (Cook and Baker, 1993). There are two proposed mechanisms of action for biological control agents; direct or indirect antagonism. Direct antagonism often involves the release of metabolites, predation or competition (Kerry 2000). The most common of which is the release of metabolites and predation; competition is very rarely seen or used as biological control strategy. *Pseudomonas flourescens* CHA0, for example, releases two metabolites, 2,4-diacetylephloroglucinol and pyoluteorin, that can significantly decrease *M. javanica* population density (Hamid et al., 2003). A biological control agent that works via predation is *Pastueria penetrans* which infects *Meloidogyne* spp., feeds and reproduces within the nematode (Bhuiyan et al., 2018). Determining the direct antagonistic abilities of biological control agents to manage nematodes is often fairly simple. Bacteria and fungi can be screened by an *in vitro* assay that will quickly determine any direct antagonism of nematodes (Xiang et al., 2017). The other biological control strategy, indirect resistance, is not as easily observed and organisms that work by this strategy can be overlooked.

Indirect antagonism occurs by systemic resistance, which encompasses induced systemic resistance and systemic acquired resistance. Induced systemic resistance (ISR) is the enhanced disease resistance stimulated typically via jasmonic acid (JA) that is produced upon plants' encounter with plant growth promoting rhizobacteria (PGPR). Plants induce JA upon infections of pathogens, PGPR or herbivores (Seo et al., 1995). Following JA biosynthesis, bioactive JA-Ile binds a SCF ubiquitin E3 ligase, CORONATINE INSENSITIVE1 (COI1), to recruit and ubiquitinate JASMONATE JIM-domain (JAZ) proteins, transcription inhibitors of JA-responsive defense genes (Sheard et al., 2010; Hickman et al., 2017). JA-responsive defense genes include *PDF1.2*, *HEL*, *Thi2.1* and *Chib*, which encode defense proteins, plant defensin 1.2, hevien-like

protein, thionin and basic chitinase, respectively (Penninckx et al., 1996; Thomma et al., 1998; Pierterse and van Loon 1999; Bertini et al., 2012). Systemic acquired resistance (SAR) is a state of heightened defense that is activated through salicylic acid (SA) signaling. SA signaling is stimulated by the recognition of pathogens on the cell surface (Macho and Zipfel 2015). SA is well known to activate a NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEIN 1 (NPR1) which are transcriptional regulators of SA-responsive defense genes (Maier et al., 2010; Fu et al., 2012; Wu et al., 2012).

Both of these defense hormones and systemic resistance pathways play a role in plant defense against nematodes. Exogenous application of methyl jasmonate, which creates a positive feedback loop for JA, was able to decrease *M. incognita* population density (Fujimoto et al., 2011). When JA biosynthetic pathway was silenced in rice and tomatoes, plant defenses against *Meloidogyne* spp. were unsuccessful (Nahar et al., 2011; Martinez-Medina et al., 2017). SA is known to be heavily involved in *Mi-1* resistance, the main form of plant resistance to *M. incognita* (Branch et al., 2004). As well as being involved in nematode defense, both of these hormones can be stimulated by PGPR. There are more examples of JA stimulation by PGPRs, including strains of *B. subtilis*, *B. cereus*, *B. pumilus*, and *B. amyloliquefaciens* and species of *Pseudomonas*, than there are examples of SA stimulation (Yan at al., 2002; Murphy et al., 2003; Beneduzi et al., 2012; Garcia-Gutierrez et al., 2013) However, there are a few instances where SA is upregulated by PGPR, including *B. amyloliquefaciens* LJ02, *B. amyloliquefaciens* strain MBI600, *Pseudomonas aurofaciens* and *P. corrugata* (Chen et al., 1999; Li et al., 2015; Beris et al., 2018). Stimulation of SA or JA by PGPRs can be a successful indirect management strategy of nematodes.

The goal of integrated pest management (IPM) is to efficiently and effectively manage diseases and pests in crops using an environmentally sensitive approach. Biological control agents are an integral part of IPM. Development of commercial biological control agents requires extensive knowledge of the bacteria or fungi being considered, including the mechanism of action and any interactions with the pest. The goal of this study is to determine the mechanism of action, direct or indirect, of *Bacillus* spp. to help successfully implement them in an IPM setting that also involves cultural control, resistant varieties, and limited use of chemical nematicides. The objectives of this research are to determine the efficacy of select *Bacillus* spp. and their direct effect on *M. incognita* and to determine the systemic capabilities of the *Bacillus* spp. and potentially explain the mechanism of action of the *Bacillus* spp.

Material and Methods:

Nematode inoculum preparations:

Meloidogyne incognita race 3 was used in the greenhouse *in planta* assay, the *in vitro* assay, the split root assay and the RT-qPCR. Stock pots of the nematode were grown on corn, Mycogen 2H723 (Dow AgroScience, Indianapolis, IN), in 500 cm^3 polystyrene pots in the greenhouses at the Plant Science Research Center (PSRC) in Auburn, AL. Eggs were extracted by placing the corn roots in a 0.625% NaOCl solution and shaking them for 4 minutes at 1 g-force on a Barnstead Lab Line Max Q 5000 E Class shaker (Conquer Scientific, San Diego, CA). The roots were rinsed with tap water and the eggs were collected on a 25-µm pore sieve. The collected eggs were washed into 50mL centrifuge tubes and processed by sucrose centrifugation at 427 g-forces for 1 minute (Jenkins 1964). The eggs were recollected on a 25-µm pore sieve. For the greenhouse *in planta* assay and the split root assay, the nematodes were quantified using an inverted TS100 Nikon microscope at 40x magnification. The nematode eggs were

standardized to 5,000 eggs per mL. For the *in vitro* assay and RT-qPCR, the eggs were hatched to second stage juveniles (J2) using a modified Baermann funnel (Castillo et al., 2013). The modified Baermann funnel was placed on a slide warmer (Model 77; Marshall Scientific, Brentwood, NH) set to 30°C and left to incubate for 5 to 10 days, dependent on outside temperature and time of the year (Xiang et al., 2016). The J2s in the Baermann funnel were washed onto a 25-µm pore sieve and collected in a beaker using minimal water. The number of J2s was quantified under an inverted TS100 Nikon microscope at 40x magnification. For the *in vitro* assay the number of J2s was standardized to approximately 30 J2s per 10 µL of water. For the RT-qPCR the number of J2s was standardized to 1,000 juveniles per 1 mL of water.

Bacterial inoculum preparations:

Five *Bacillus* spp. were used in all of the assays. Three species, *B. mojavensis* strain 3, *B. velenzensis* strain 2, and *B. pumilis* GB 34 were originally isolated, identified and stored by Dr. J. W. Kloepper at Auburn University, Auburn, AL. Two species, *B. firmus* I-1582 and *B. amyloliquefaciens* QST713, are the active ingredients of Bayer CropScience products, VOTiVO® and Serenade®, respectively. The *Bacillus* spp. were stored in 30% glycerol at -80°C. Prior to utilization, the *Bacillus* spp. were transferred to tryptic soy agar (VWR, Radnor, PA) plates and incubated at 35°C for 24 hours. The vegetative cells were washed into beakers and standardized. For the greenhouse *in planta* assay, *in vitro* assay, split root assay and RT-qPCR, the bacteria were standardized to 1 x 10^6 CFU/mL. For the qPCR, the bacteria were standardized to 1 x10^8 CFU/mL.

Greenhouse in planta *assay:*

Soil used in all experiments was a Kalmia loamy sand (80% sand, 10% silt, and 10% clay) obtained from the Plant Breeding Unit located at the E.V Smith Research Center near

Tallassee, AL. This soil was brought to the PSRC and pasteurized at 88°C for 12 hours then cooled for 24 hours before the pasteurization was repeated. Four cotton seed (Phytogen 333 WRF) were planted in 500 cm^3 polystyrene pots filled with 2:1 pasteurized soil to sand mixed with fertilizer and lime as recommended by the Auburn University Soil Lab. The seeds were inoculated at planting with *M. incognita* and a control of 1 mL of water per seed, 1 mL of each of the *Bacillus* spp., or the chemical control of fluopyram (Velum, Bayer CropScience) at a rate of 0.5 µL followed by 1 mL of water per seed. Natural light in the greenhouse at PSRC was supplemented with light from 1,000-watt halide bulbs producing 110,000 lumens to provide 14 hours of light. Temperatures ranged from 22ºC to 34ºC and the tests were watered twice a day. Thirty days after inoculation, plant data [plant height (cm), shoot fresh weight, (g) and root fresh weight (g)] were measured. Biomass (g) was determined by adding the root fresh weight to the shoot fresh weight. The nematodes were extracted from the roots of the cotton plant as previously described. Nematode eggs were quantified under the inverted TS100 Nikon microscope at 40x magnification. This test was a randomized complete block design (RCBD) with 5 replicates of each treatment per assay and the entire experiment was repeated two times for a total of 70 experimental units.

in vitro *assay*:

The *in vitro* assay measured the direct response of the J2 to each bacterial isolate. Bacteria, grown as described above, were incubated for 6 days, after which they were carefully washed into 2 separate 1.5 µL microcentrifuge tubes. The metabolites were extracted from one of the tubes by rotating between a hot water bath and ice bath for 15 minutes. The tubes were then centrifuged for 1 minute following the methodology of Apotroaie-Constantin et al. (2008). The supernatant containing the metabolites was collected after centrifugation. Following

metabolite extraction, 10 µL of J2s along with 90 µL of a water control, the extracted metabolites, or the bacterial inoculum was added to each well of a 96 well plate. The fluopyram chemical control was not utilized as it is an opaque white liquid that made determining mortality of nematode under microscopy difficult. At 0 hours and 48 hours the number of live and dead nematodes was quantified using stimulation with sodium hydroxide Xiang et al. (2016), under the inverted TS100 Nikon microscope at 40x magnification. The percent mortality was calculated [(live J2s at 0 hours – live J2s at 48 hours)/ live J2s at 0 hours] x 100. The treatments were replicated 5 times per assay and the assays were repeated three times for a total of 90 experimental units.

Split root assay:

Cotton seeds (Phytogen 333 WRF) were germinated in germination paper on a slide warmer for 4-6 days or until a small taproot had formed. The roots were cut horizontally from the root tip with a sterile razor blade approximately 1 mm from the end. The seedlings were planted in sand supplemented with fertilizer at a rate of four seedlings per pot. The plants were allowed to grow in the greenhouse for 1-2 weeks or until two equivalent root halves were produced (Figure 1A). The cotton plants were gently excavated from the soil and the roots were washed very carefully with tap water to remove excess sand. The two root halves of each cotton plant were planted in two 150 cm^3 conetainers (Stuewe & Sons Inc.; Tangent, Oregon) positioned immediately next to each other filled with 2:1 sand to soil with fertilizer and lime as recommended by the Auburn University Soil Lab. The shoots were positioned in small plastic cups with the bottoms cut off that were positioned equally over the two conetainers containing the two root halves (Figure 1B). The root halves were inoculated with the bacteria, nematodes and fluopyram two days after the cotton seedling was transplanted in the split root system. For

each bacterial or chemical treatment there were five distinct split root inoculation patterns: 1) a control with no inoculation on either root half (control), 2) bacteria or fluopyram inoculated on root half A and no inoculation on root half B (bacteria control), 3) no inoculation on root half A and *M. incognita* eggs inoculated on root half B (nematode control), 4) bacteria or fluopyram and *M. incognita* eggs inoculated on root half A and no inoculation on root half B, and 5) bacteria or fluopyram inoculated on root half A and *M. incognita* eggs inoculated on root half B (Figure 2; Martinez-Medina et al., 2017). Thirty days after inoculation, the plant parameters were measured as previously described and the nematode eggs were quantified. The split root test was designed in a RCBD. The patterns for each treatment were replicated 5 times per assay and the assay was repeated 3 times for a total of 900 experiment units.

RT-qPCR:

Quantitative real time PCR was used to determine the transcript level of genes related to JA expression (*GhLOX, GhOPR3*; Zebelo et al., 2016) and SA activity (*β-1,3-glucanase*; Zhang et al., 2011). Cotton seeds, as described previously, were planted in containers filled with 2:1 sand to soil with fertilizer and lime as previously described. The cotton was grown in the greenhouse for 2 to 3 weeks, or until the second true leaf stage. Once the cotton plants reached this growth stage the cotton plants were inoculated with *B. amyloliqufaciens* QST713, *B. firmus* I-1582 or *M. incognita* J2s. At 0 hours (h), 1h, 24h, and 1 week, approximately 2 grams of roots were placed in a 1.5 mL microcentrifuge tube and immediately frozen in liquid nitrogen. The samples were kept in -80°C until ready for use. The samples were ground in liquid nitrogen into a fine powder using a mortal and pestle. RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich, St. Louis, MO, USA) according to manufacturer instructions. Concentration and purity of the RNA was determined using the NanoDrop™ Spectrophotometer

ND-2000 (A_{260}/A_{280} > 1.8 and A_{260}/A_{230} > 2.0; Thermo Scientific, Wilmington, USA). RT reactions were carried out using the GoScript™ reverse transcription system Kit (Promega, Madison, WI, USA), and qPCR was performed with the PerfeCTA® SYBR® Green Fastmix® qPCR Master Mix (Qunita Biosciences, Inc, Gaithersburg, MD, USA) in a CFX96 RealTime System (Bio-Rad) cycled 40 times using gene specific primer sets (Tables 1; Invitrogen, ThermoFisher Scientific; Waltham, MA). The annealing temperatures for the primer pairs were 60 ^{o}C. Relative RNA levels were calibrated and normalized with a housekeeping gene, *HISTONE H3*. Relative fold change was calculated by normalizing the average threshold cycles (Ct) of target genes to that of *H3* as $2^{-\Delta CT}$, where $-\Delta CT = (C_{t'gene}-C_{t'H3})$ (Livak and Schmittgen, 2001). There were 6 biological replicates per treatment, for a total of 96 experimental units, and 3 technical replicates for each biological sample.

qPCR for bacterial concentration:

The protocol to determine the concentration of the bacteria on the roots was adapted for cotton from Mendis et al. (2018). Cotton seeds were planted in conetainers filled with 2:1 sand to soil with fertilizer and lime as previously described. The plants were inoculated at planting with *B. firmus* I-1582 and *B. amyloliquefaciens* QST713. After 24 days of growth, the roots were gently shaken to remove most excess soil retaining the rhizosphere soil on the roots. An amount of 1.5 g of sampled roots and rhizospheric soil was added to a 7mL plastic vial (BioSpec Products Inc, Bartlesville, OK) filled with approximately 1.75 g of 2 mm Zirconia beads (BioSpec Products Inc, Bartlesville, OK). A volume of 2 mL of sterile water was added to each vial. The vials were beadbeated in a Mini-BeadBeater-96 (BioSpec Products Inc, Bartlesville, OK) at 2,400 oscillations/min for 5 minutes. The vials were then centrifuged at 427 g-forces for 1 second. A volume of 200 µL was taken from the supernatant and added to the ZR BashingBead

Lysis Tube from the ZR Soil Microbe DNA miniprep kit (Zymo Research Corporation, Irvine, CA). The DNA was extracted according to the manufacturer's instructions.

qPCR was carried out on a CFX96 RealTime System (Bio-Rad) using primers and TaqMan® probes developed by Mendis et al. (2018). qPCR was done with PerfeCTa Multiplex qPCR ToughMix Low ROX (Quantabio, Beverly, MA), and the annealing temperatures were 95° C for 39 cycles. There were 15 biological samples per treatment, for a total of 30 experimental units, and 3 technical replicates of each biological sample.

DNA was extracted from samples with a known concentration of bacteria to create a standard curve used to calculate the concentration of the experimental samples based on the Cq values. A serial dilution of bacteria, 10^2 to 10^9 cfu/mL, was created. A volume of 1 mL of each serial dilution was added to 1.5 g of cotton roots in 7mL plastic vial filled with approximately 1.75 g of 2 mm Zirconia beads, as previously described. A volume of 1 mL of sterile water was added to this before the samples were processed and the DNA was extracted as previously described. qPCR was performed as previously described. The Cq value was plotted against the log value for the serial dilution to get a standard curve. The experimental concentrations were calculated with the slope of the standard curve and the experimental Cq values.

Statistical Analysis:

Data collected from the greenhouse *in planta* assay, *in vitro* assay, split root assay and qPCR for bacterial concentration were analyzed in SAS 9.4 (SAS Institute, Cary, NC) using the Glimmix procedure with means separated by use of the Tukey-Kramer method with a significance level of $P \leq 0.05$ or $P \leq 0.10$. For the RT-qPCR, the data were statistically analyzed in SAS 9.4 (SAS Institute, Cary, NC) by two-way ANOVA with a level of significance of $P \leq 0.001$, $P \leq 0.01$, or $P \leq 0.05$.

Results:

Greenhouse in planta *assay:*

In the greenhouse *in planta* assay, two of the five *Bacillus* spp. reduced *M. incognita* population density. *Bacillus firmus* I-1582 and *B. amyloliquefaciens* QST713, decreased the nematode eggs per gram of root compared to the control, similarly to fluopyram ($P \leq 0.05$; Table 2). The number of nematode eggs per gram of root was also decreased by the chemical standard of fluopyram compared to the control. The plant parameters, including plant height, shoot fresh weight, root fresh weight and biomass, did not differ between any of the treatments 30 days after inoculation.

in vitro *assay:*

The percent mortality ranged from 6.1% to 78.7% with the lowest mortality rate occurring in the water control (Table 3). *Bacillus firmus* I-1582 and the *B. firmus* I-1582 metabolites increased percent mortality significantly, by 77.8% and 78.7% respectively, compared to the water control ($P \leq 0.05$; Table 3). The *B. amyloliquefaciens* QST713 metabolites also increased percent mortality, by 62.2%, compared to the control; however, the *B. amyloliquefaciens* QST713 intact bacteria did not, only increasing percent mortality by 33.8% ($P \leq 0.05$; Table 3).

Split root assay:

There was no difference in the plant parameters between the split root treatments; however, there was a difference in the eggs per gram of root. Fluopyram inoculated concomitantly on the same root half as the nematode decreased *M. incognita* numbers by 94% compared to the nematode control ($P \leq 0.05$; Table 4). *Bacillus firmus* I-1582 decreased nematode numbers by 78% when the bacteria and nematode were inoculated concomitantly on

the same root half and by 84% when inoculated on the opposite root half, as compared to the nematode control ($P \leq 0.05$; Table 4). Similarly, *B. amyloliquefaciens* QST 713 decreased nematode numbers by 68% when the bacteria and nematode were inoculated concomitantly on the same root half and by 86% when inoculated on the opposite root half, as compared to the nematode control ($P \leq 0.05$; Table 4). *Bacillus mojavensis* strain 3 decreased nematode numbers by 82% when the bacteria and nematode were inoculated on the opposite root half, as compared to the nematode control ($P \leq 0.05$; Table 4). This species did not decrease nematode numbers when inoculated on the same root half as the nematode, having the largest number of nematode eggs of all the *Bacillus* spp. and chemical treatments ($P \leq 0.05$; Table 4). This indicates that *B. mojavensis* strain 3 is not a good control option for *M. incognita* and was not be used in the RT-qPCR. The two bacteria that exhibited systemic responses, *B. amyloliquefaciens* QST 713 and *B. firmus* I-1582, were analyzed further with RT-qPCR.

RT-qPCR:

The RT-qPCR was conducted to analyze genes correlated to systemic resistance. *GhLOX1* and *GhOPR3* typically correlate to JA regulation or regulation of an intermediate jasmonate defense molecule. *β-1,3-glucanase* correlates with SA activity. *GhLOX1* expression displayed significant upregulation at 24 hour post *Bacillus* spp. inoculation, whereas *GhOPR3* expression was upregulated at 24 hours after stimulation by the nematode but not the *Bacillus* spp. (Figures 3 and 4). At 24 hours, *β-1,3-glucanase* was also significantly upregulated by the nematode. At all other time points, the nematode downregulated *β-1,3-glucanase* (Figure 5). In contrast, stimulation by the *Bacillus* spp. lead to a steady increase of *β-1,3-glucanase* expression. At one week, both *Bacillus* spp. significantly upregulated *β-1,3-glucanase* compared to the nematode.

qPCR for bacterial concentrations:

Using a serial dilution of *B. amyloliquefaciens* QST713 and *B. firmus* I-1582, two standard curves were created. The equation for the *B. amyloliquefaciens* QST713 was y = -1.9421x + 34.227; R^2 = 09371. The equation for the *B. firmus* QST713 I-1582 was y = -2.2036x + 44.362; R^2 = 08217. The inoculum level of the bacteria at day 1 was 10^8 cfu/mL of both *B. amyloliquefaciens* QST713 and *B. firmus* I-1582. After 24 days, qPCR determined that concentration for *B. amyloliquefaciens* QST713 was $10^{4.52}$ cfu/mL and *B. firmus* I-1582 was at a concentration of $10^{5.79}$ cfu/mL (Figure 6). Indicating these bacteria can successfully colonize the plant roots.

Discussion:

Biological control agents that utilize unique mechanisms of action are a good option for management of *M. incognita* and can be readily implemented into an integrated pest management strategy. *Bacillus* spp. are popular bacteria to consider as biological control agents because they can tolerate harsh environments, easily form endospores, effect a wide range of pathogens, and replicate and colonize quickly (Shafi et al., 2018).

Five *Bacillus* spp. were tested in this study for the ability to manage *M. incognita* population density. In a greenhouse *in planta* test, *B. firmus* I-1582 and *B. amyloliquefaciens* QST713 showed potential for decreasing nematode numbers. An *in vitro* assay determined that one of these species, *B. firmus* I-1582 and its metabolites, was able to directly antagonize the nematode. The percent mortality 48 hour after exposure to the extracted metabolites and the percent mortality of the intact bacteria were equivalent, indicating the bacteria can potentially release the extracted metabolites to antagonize the nematode. This is different from a previous study that looked at the percent mortality of Poncho/VOTiVO®, a formulated mixture of *B.*

firmus I-1582 and the insecticide clothianidin, in the same style of *in vitro* assay and observed only a 24.4% increase in mortality and no significant difference compared to the control (Xiang et al., 2017). Contradictory to this study and in agreement with our study another researcher found that a similar strain of *B. firmus* was able to manage *M. incognita,* the burrowing nematode *Radopholus similis* and the stem nematode *Ditylenchus dipsaci in vitro* (Mendoza et al., 2007). This may indicate that the bacteria's ability to directly manage nematodes can be impeded when mixed with other products, in this case an insecticide. On the other hand, *B. amyloliquefaciens* QST713 metabolites also increased percent mortality but the intact bacteria did not, indicating the intact bacteria cannot readily release the metabolites that could result in direct antagonism. There have previously been no examples of this specific strain of *B. amyloliquefaciens* managing nematodes *in vitro* or *in planta*. However, another strain of *B. amyloliquefaciens* was seen to reduce galling in tomatoes (Dickson et al., 2003). As well, in an *in vitro* screening four strains of *B. amyloliquefaciens* increased percent mortality by over 50% (Xiang et al., 2014). There are no studies that look at metabolites released by this bacteria and their effect on nematodes, however. Further studies should determine metabolite identification for both species to determine if they can be utilized in a commercial product.

The split root technique is fairly common in determining systemic resistance, pathogen-pathogen interactions and rhizobium formation on legumes (Selim et al., 2014). Split root assays involve observing the reaction of the responder, *M. incognita* in this case, on one root half to the inducer, *Bacillus* spp., on the other root half. Three species showed potential systemic responses in the split root assay. *Bacillus firmus* I-1582 and *B. amyloliquefaciens* QST713 decreased nematode numbers when inoculated on the same root half as the nematode and when inoculated on the opposite root half as compared to the nematode control. The other bacteria that exhibited

systemic resistance, *B. mojavensis* strain 3, only decreased nematode numbers when on the opposite root half as the nematode. When this bacterium was in contact with the nematode in both the split root and greenhouse *in planta* assay, the population density of eggs per gram of root was equivalent to the nematode control, indicating this bacterium will not be a good biological control agent. These results from the split root assay are useful for determining systemic capabilities; however, this assay opens questions as to the specific pathway being stimulated by the *Bacillus* spp.

Meloidogyne incognita resistant varieties of tomato often use *Mi* resistance, the most common of which is *Mi-1* resistance (Branch et al., 2004; Bhattarai et al., 2007). *Mi-1* resistance relies on SA, one of the major defense hormones that can be stimulated by biological control agents (Bhattarai et al., 2007). The other major defense hormone involved in pathogen defense is JA, which can inhibit SA production and can be inhibited by SA (Pena-Cortes et al., 1993; Uppalapati et al., 2007). This antagonistic relationship between the hormones is typically based on the concentration of each hormone; the higher the concentration of one, the more antagonistic the relationship (Mur et al., 2006). The use of a *Mi-1* resistant plants along with a biological control agent that significantly stimulates JA may result in less protection against *M. incognita* than expected dependent on the interaction between SA and JA (Gutjahr and Paszkowski, 2009; Fujimoto et al., 2011). The source of a major resistant strain of cotton, Auburn 623 RNR, and other sources of resistance in many different varieties of cotton to *M. incognita* have not been identified yet (Kumar et al., 2016;). It is possible that, similarly to tomato *Mi-1* resistance, the major form of resistance in cotton involves one of these two major defense hormones.

To confirm the split root assay results and determine the specific pathway stimulated, a RT-qPCR analyzed the expression level of genes correlating to levels of JA, SA, and a possible

intermediate defense molecule. *GhLOX1* is involved in initial JA synthesis within the chloroplast, *GhOPR3* is involved with JA synthesis within the peroxisome and *β-1,3-glucanase* is a SA responsive gene (Breithaupt et al., 2001; Zhang et al. 2011; Zebelo et al, 2016). In our RT-qPCR, *GhLOX1* was upregulated by the *Bacillus* spp. but not the nematode at 24 hours after inoculation. In contrast, *GhOPR3* was upregulated by the nematode but not the *Bacillus* spp. at 24 hours. At all other time points, *GhOPR3* and *GhLOX1* were not upregulated indicating a short-term local response by the plant to the stimulants. The upregulation of *GhOPR3* by the nematode at 24 hours is thought to be the plant's natural response to the nematode. It is known that the soybean cyst nematode, a nematode with a similar pathogenic strategy to *M. incognita*, penetrates the roots of the target plant within a day of being inoculated as a juvenile (Lauritis et al., 1983). Our results, of upregulated *GhOPR3*, fall within that timeline and make it likely that this is when the plant defenses will be triggered by the nematode.

If JA was induced by the bacteria in this assay then both *GhLOX1* and *GhOPR3* would have been upregulated upon stimulation by the *Bacillus* spp. rather than just *GhLOX1*. The upregulation of only *GhLOX1* by the *Bacillus* spp. suggests an intermediate jasmonate is responsible for the local and temporal resistance response stimulated by the bacteria. Gleason et al. (2016) determined that OPDA was most likely responsible for defense against *M. hapla* in *Arabidopsis*, rather than JA, and may be more important than JA in defense against nematodes. Other studies also implied that OPDA, rather than another intermediate molecule, is important in defense (Stintzi et al., 2001; Bosch et al, 2014; Gua et al., 2014; Varsani et al., 2019).

In contrast, *β-1,3-glucanase* was upregulated steadily by the *Bacillus* spp. until there was an almost 2-fold increase in expression stimulated by both *Bacillus* spp. It's hypothesized that if the experiment was continued for a longer time period, this upregulation may increase to larger

than 2-fold. The slight increase in *β-1,3- glucanase* expression could indicate an increased activity level of SA as a late term defense response (Li et al., 2003; Zhang et al., 2011). The nematode also upregulated expression of this gene at 24 hours thought to be a result of the plant natural response to the nematode (Hoysted et al., 2017). This would be similar to the upregulation of *GhOPR3* by the nematode after 24 hours.

This particular dynamic between SA and JA after stimulation with a biological control agent has not previously been documented. One of the few other studies that looks at both SA and JA after stimulation with a biological control agent, *Trichoderma* spp. specifically saw a very different interaction between the hormones in tomatoes (Martinez-Medina et al., 2017). In this study, SA was initially stimulated while genes related to JA were only upregulated after about 7 days. These results were very different from our own study which saw no JA response after *Bacillus* spp. stimulation and a long term response of SA. Another study by Garcia-Gutierrez et al. 2013, observed both SA and JA dependent defenses stimulated by *B. subtilis* UMAF6639 in melon at approximately the same time, which is also very different than our results. Many of these studies have not been repeated using the same strain of bacteria on other crops or pathogens. Though systemic resistance by a bacterium can be seen in many different crops against a variety of pathogens, the results are highly variable. Further studies are needed to fully understand indirect control strategies of biological control agents, especially regarding their interactions with the plant and pathogen, in order to successfully implement them in a management strategy.

Our results, in cotton, indicate two *Bacillus* spp. are successful control options that work via systemic resistance to manage *M. incognita*. As mentioned previously, it is important that a biological control agent is fully understood to successfully integrate it into an integrated

management strategy. Neither species activates JA; rather they activate an intermediate defense molecule, thought to be OPDA, and a potential long term SA response within the plant. While our results identified the mechanism of action of these bacteria, microplot and field studies are required before trying to implement any biological control agent as a successful control strategy for a nematode in a commercial setting.

Conclusion:

Bacillus amyloliquefaciens QST713 and *B. firmus* I-1582 can cause systemic resistance, as indicated by the split root assay and confirmed in the RT-qPCR. Both of these bacteria increased signaling of an intermediate jasmonate, most likely OPDA, for a short-term defense response and slightly increased SA activity for a long-term defense response. *Bacillus firmus I-1582* may even have two mechanism of action by which it manages the nematode including the release of metabolites and systemic resistance. Our results also indicate that the techniques used, an *in vitro* assay, a split root assay, and RT-qPCR, can successfully determine systemic resistance. We can also conclude that these two biological control agents are successful in systemically managing *M. incognita*.

Tables:

Table 1: Primers for *GhLOX1*, *GhOPR3* and *β-1,3, glucanase*.

Gene	Forward (5' to 3')	Reverse (5' to 3')	Reference
Histone (H3)	GAAGCCTCATCGATACCGT	CTACCACTACCATCATGGC	Zebelo et al. 2016
GhLOX1	GCCAAGGAGAGCTTCAAGAAT	TAGGGGTACTTGGCAGAACCT	Zebelo et al. 2016
GhOPR3	ATGTGACGCAACCTCGTTATC	CCGCCACTACACATGAAAGTT	Zebelo et al, 2016
β-1,3, glucanase	AATGCGCTCTATGATCCG	GATGATTTATCAATAGCAGCG	Zhang et al. 2011

Table 2: Greenhouse *in planta* test to evaluate five *Bacillus* spp. as biological control agents of *Meloidogyne incognita* on cotton as measured by plant height, shoot fresh weight (SFW), root fresh weight (RFW), biomass and *M. incognita* eggs/ gram of root 30 days after inoculation[y].

Treatment	Plant Height (cm)	SFW (g)	RFW (g)	Biomass (SFW+RFW)	*M. incognita* eggs/ g of root
Control	15.26	2.79	1.52	4.31	4561 a[z]
Fluopyram	15.40	2.96	1.54	4.50	40 c
B. firmus I-1582	14.16	2.52	1.19	3.71	1135 bc
B. amyloliquefaciens QST713	15.26	3.24	1.59	4.83	951 bc
B. pumilus GB34	15.29	3.01	1.35	4.36	2520 abc
B. velenzensis strain 2	14.75	2.66	1.34	4.00	1930 abc
B. mojavensis strain 3	14.49	2.82	1.36	4.18	3853 ab

[y]Randomized complete block design (RCBD) with five replicates of each treatment per assay and two replicates of the assay was used

[z]Data were statistically analyzed in SAS 9.4 using the Glimmix procedure with means separated utilizing Tukey-Kramer's method $P \leq 0.05$. Values in the same column followed by the same letter do not significantly differ

Table 3: *in vitro* assay to determine the percent mortality of *Meloidogyne incognita* J2s 48 hours after exposure to five *Bacillus* spp.

Treatment	Percent of J2 mortality[x]
Water control[y]	6.1 c[z]
B. firmus I-1582	77.8 a
B. firmus I-1582 metabolites	78.7 a
B. amyloliquefaciens QST713	33.8 bc
B. amyloliquefaciens QST713 metabolites	62.2 ab
B. pumilus GB34	38.6 bc
B. pumilus GB34 metabolites	35.7 bc
B. velenzensis strain 2	24.5 c
B. velenzensis strain 2metabolites	30.5 c
B. mojavensis strain 3	24.7 c
B. mojavensis strain 3 metabolites	20.6 c

[X]Assays were performed in 96 well plates

[y]Percent mortality calculated using this formula: ([(live J2s at 0 hours – live J2s at 48 hours)/ live J2s at 0 hours] x 100)

[z]All treatments were done in a replicate of 5 per assay and the assay was repeated 3 times. Data were statistically analyzed in SAS 9.4 using the Glimmix procedure with means separated utilizing Tukey-Kramer's method $P \leq 0.1$. Values in the same column followed by the same letter do not significantly differ

Table 4: Split root assay to measure the effect of the *Bacillus* spp. on *Meloidogyne incognita* on cotton as measured by plant height, shoot fresh weight (SFW), root fresh weight (RFW), biomass and *M. incognita* eggs/ gram of root 30 days after inoculation[x].

Treatment[y]	Plant height (cm)	SFW (g)	RFW (g)	Biomass (SFW +RFW)	*M. incognita* eggs/ g of root
Control (A) Control (B)	16.79	3.82	1.44	5.26	NA
Control (A) *M. incognita* (B)	16.24	3.40	2.14	5.55	21335 a[z]
B. firmus I-1582 (A) Control (B)	18.46	4.35	1.70	6.06	NA
B. firmus I-1582 + *M. incognita* (A) Control (B)	17.24	4.76	2.09	6.85	4648 bc
B. firmus I-1582 (A) *M. incognita* (B)	15.24	4.35	1.80	6.47	3386 bc
B. amyloliquefaciens QST713 (A) Control (B)	16.46	4.13	1.94	6.08	NA
B. amyloliquefaciens QST713 + *M. incognita* (A) Control (B)	15.68	4.03	1.56	5.60	6912 bc
B. amyloliqeufaciens QST713 (A) *M. incognita* (B)	16.88	4.10	1.47	5.58	2922 bc
B. pumilus GB34 (A) Control (B)	16.91	4.23	1.77	6.16	NA
B. pumilus GB34 + *M. incognita* (A) Control (B)	15.91	3.82	1.98	5.80	11033 abc
B. pumilus GB34 (A) *M. incognita* (B)	16.46	3.84	1.62	5.47	12498 ab
B. velenzensis strain 2 (A) Control (B)	14.57	3.44	1.96	5.41	NA
B. velenzensis strain 2 + *M. incognita* (A) Control (B)	16.02	3.51	1.38	4.90	15522 ab
B. velenzensis strain 2 (A) *M. incognita* (B)	15.68	3.55	1.79	5.34	9104 abc
B. mojavensis strain 3 (A) Control (B)	15.68	4.25	1.82	6.07	NA
B. mojavensis strain 3 + *M. incognita* (A) Control (B)	17.02	4.00	1.80	5.80	18904a b
B. mojavensis (A) *M. incognita* (B)	15.62	3.46	1.54	5.00	3848 bc
Fluopyram (A) Control (B)	15.91	3.49	1.69	5.18	NA
Fluopyram + *M. incognita* (A) Control (B)	14.91	3.39	1.47	5.06	1190 c
Fluopyram (A) *M. incognita* (B)	17.46	3.90	1.74	5.65	10020 abc

[x]Randomized complete block design (RCBD) with five replicates of each treatment per assay and three replicates of the assay was used

[y]Split root inoculation patterns in Figure 2.

[z]Data were statistically analyzed in SAS 9.4 using the Glimmix procedure with means separated utilizing Tukey-Kramer's method $P \leq 0.1$. Values in the same column followed by the same letter do not significantly differ.

Figures:

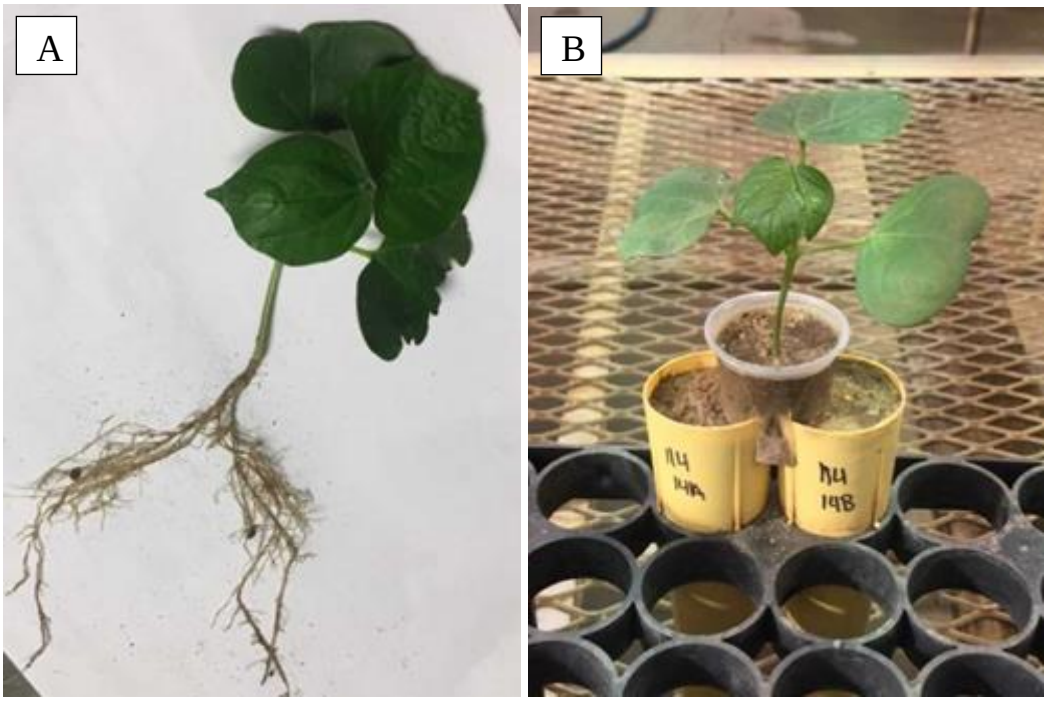

Figure 1: Example of a split root system; A) two equivalent cotton root systems before planting in the split root set up, B) after planting the cotton in the split root set up.

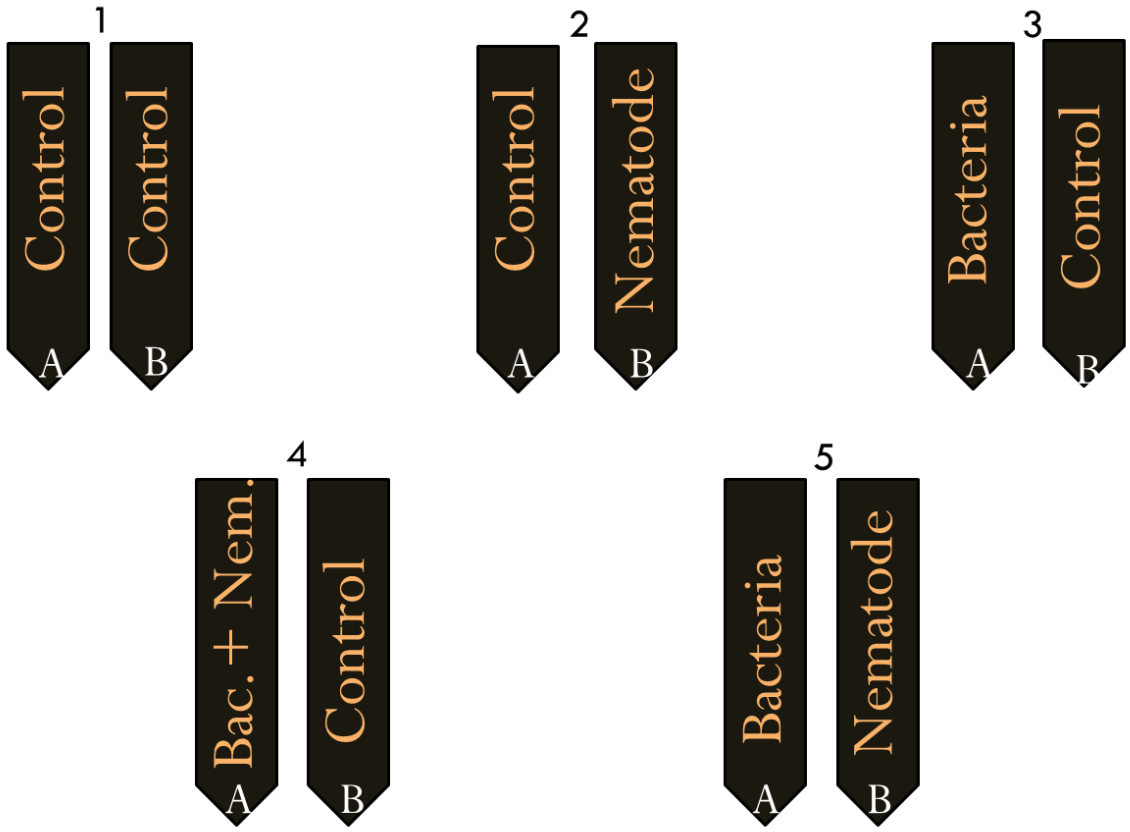

Figure 2: Inoculation pattern for the split root assay with 1) a control on root half A and B (control), 2) bacteria or fluopyram inoculated on root half A and control on root half B (bacteria control), 3) control on root half A and *Meloidogyne incognita* eggs inoculated on root half B (nematode control), 4) bacteria or fluopyram and *M. incognita* eggs inoculated on root half A and control on root half B, and 5) bacteria or fluopyram inoculated on root half A and *M. incognita* eggs inoculated on root half

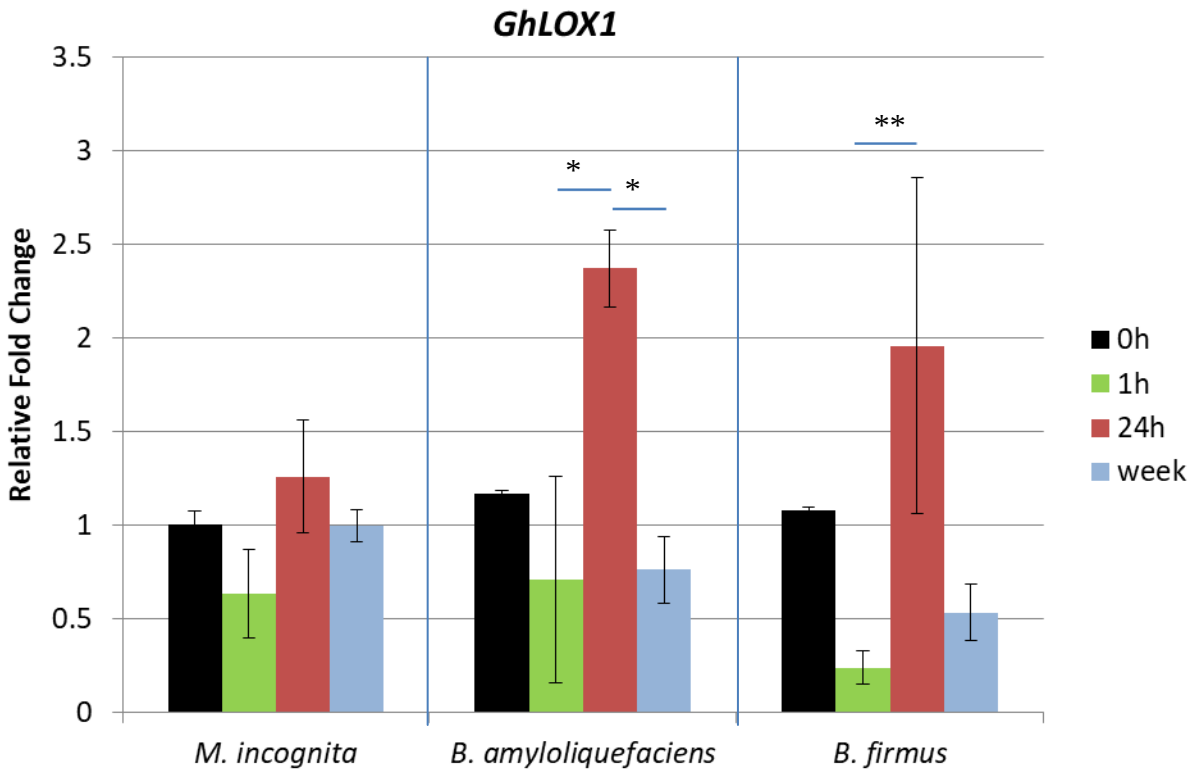

Figure 3: Results from the RT-qPCR depicting the relative fold change of *GhLOX1* in cotton at 0 hours (h), 1h, 24h and 1 week after inoculation of *Meloidogyne incognita* J2s, *Bacillus amyloliquefaciens* QST713 and *B. firmus* I-1582. Fold change was calculated 2−ΔΔC T (Livak and Schmittgen, 2001) where the treated samples were compared to a negative control group and normalized with the reference gene histone H3. Asterisks represent significance as determined by a two way ANOVA performed in SAS 9.4 (*$P \leq 0.05$; ** $P \leq 0.01$).

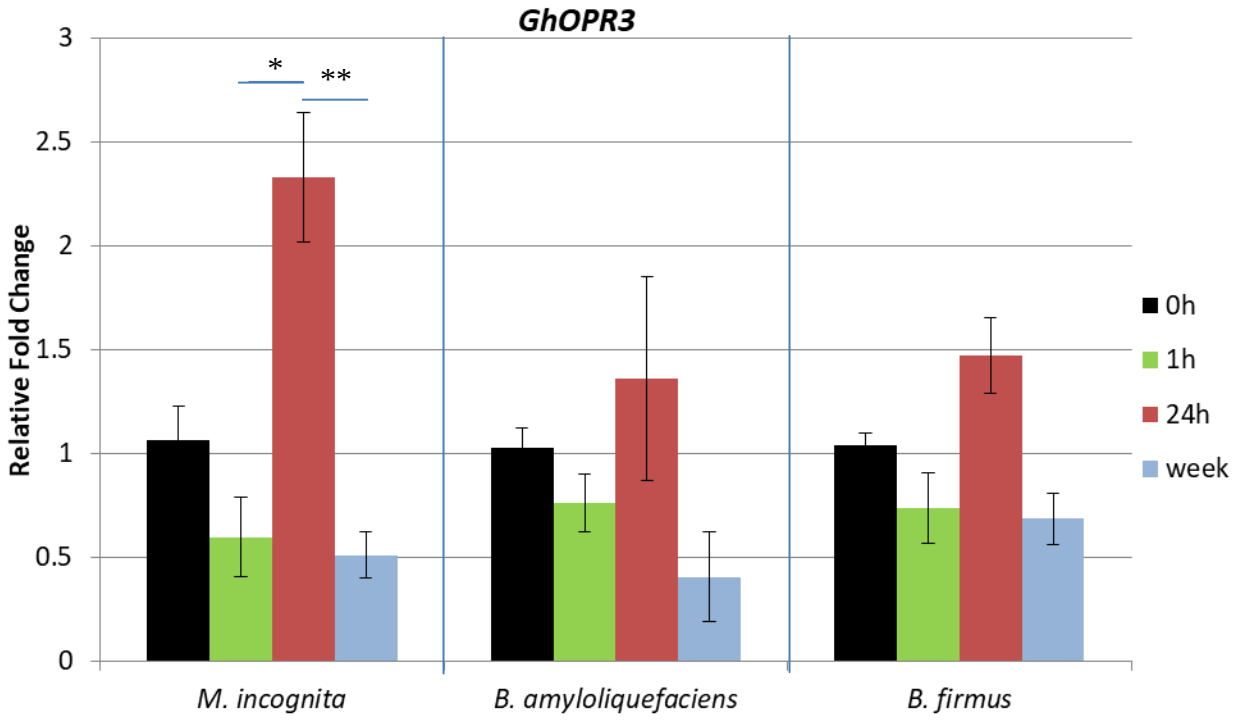

Figure 4: Results from the RT-qPCR depicting the relative fold change of *GhOPR3* in cotton at 0 hours (h), 1h, 24h and 1 week after inoculation of *Meloidogyne incognita* J2s, *Bacillus amyloliquefaciens* QST713 and *B. firmus* I-1582. Fold change was calculated $2^{-\Delta\Delta C}$ T (Livak and Schmittgen, 2001) where the treated samples were compared to a negative control group and normalized with the reference gene histone H3. Asterisks represent significance as determined by a two way ANOVA performed in SAS 9.4 (*$P \leq 0.01$; ** $P \leq 0.001$).

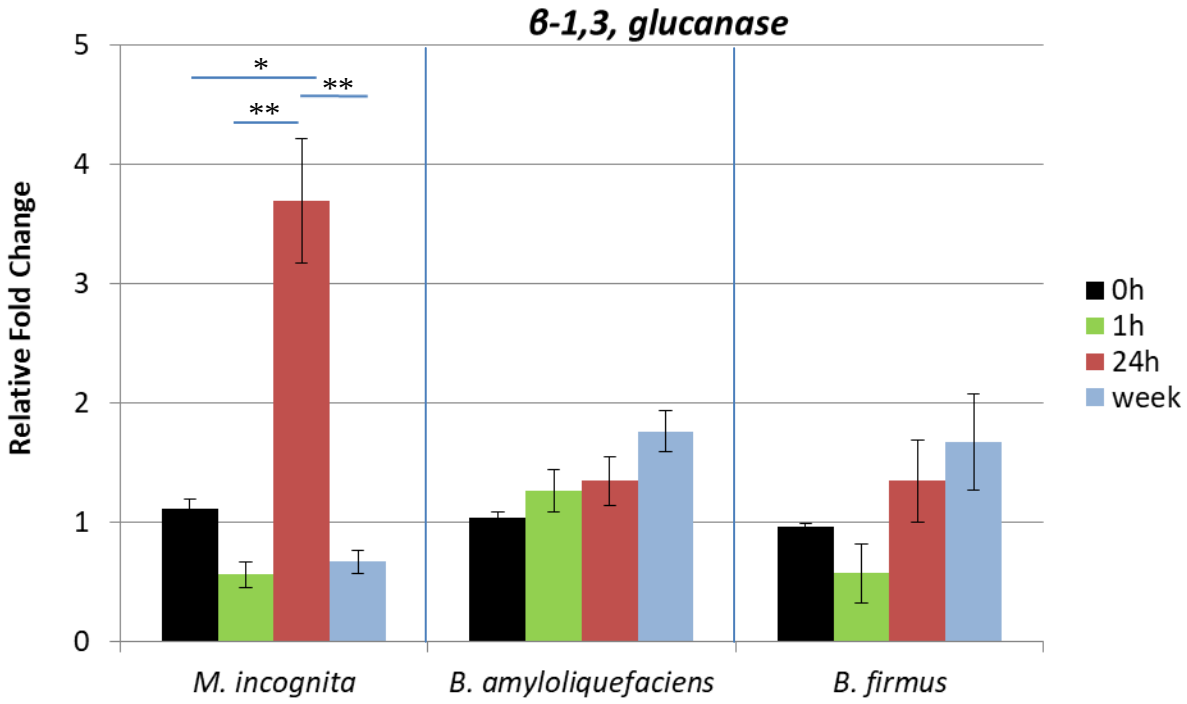

Figure 5: Results from the RT-qPCR depicting the relative fold change of *β-1,3, glucanase* in cotton at 0 hours (h), 1h, 24h and 1 week after inoculation of *Meloidogyne incognita* J2s, *Bacillus amyloliquefaciens* QST713 and *B. firmus* I-1582. Fold change was calculated $2^{-\Delta\Delta C}$ T (Livak and Schmittgen, 2001) where the treated samples were compared to a negative control group and normalized with the reference gene histone H3. Asterisks represent significance as determined by a two way ANOVA performed in SAS 9.4 (*$P \leq 0.01$; ** $P \leq 0.001$).

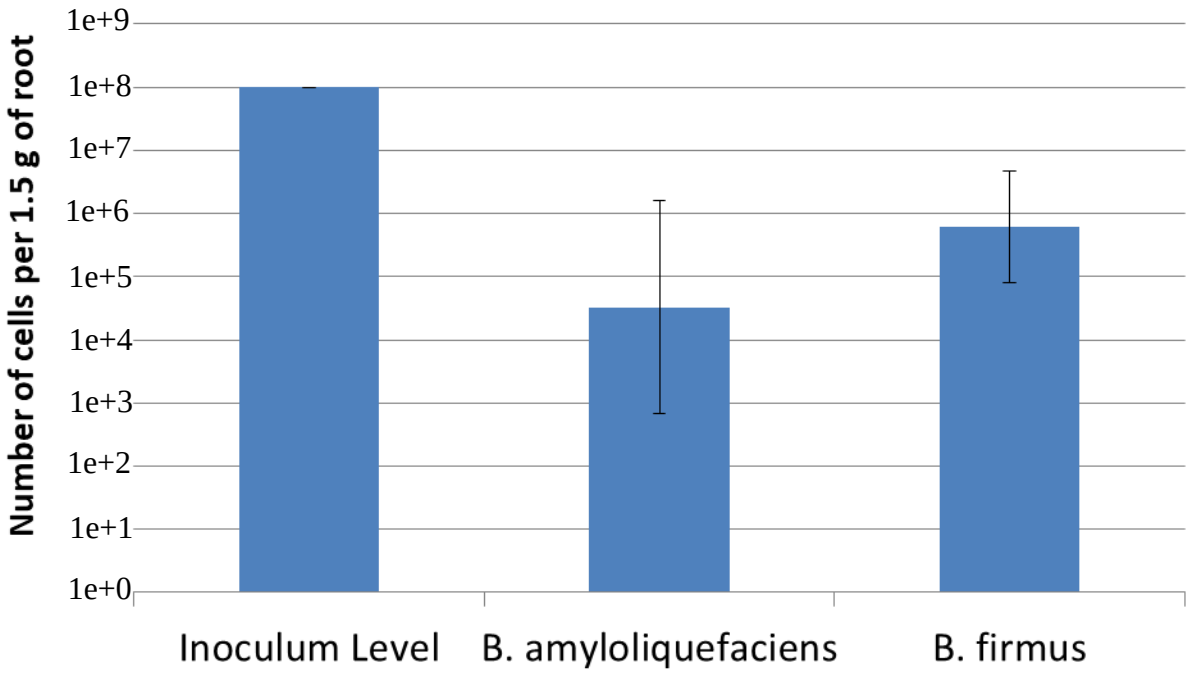

Figure 6: Quantification of *Bacillus amyloliquefaciens* QST713 and *B. firmus* I-1582 after 24 days on cotton in the greenhouse using qPCR. Cotton seeds were inoculated with 10^8 cfu/mL of each of the bacteria at day 0. The concentration was calculated using the standard curves for each bacteria; *B. amyloliquefaciens* QST713: y = -1.9421x + 34.227; R^2 = 09371; *B. firmus* QST713 I-1582: y = -2.2036x + 44.362; R^2 = 08217. The treatments were done in a replicate of 5 per assay and the assay was repeated 3 times. Data were statistically analyzed in SAS 9.4 using the Glimmix procedure with $P \leq 0.1$. The treatments did not significantly differ from each other.

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