SYSTEMIC RESPONSE STIMULATED BY *BACILLUS* SPP. CAN MANAGE *MELOIDOGYNE INCOGNITA* POPULATION DENSITY IN *GOSSYPIUM HIRSUTUM*

Kaitlin Gattoni
Ni Xiang
Bisho R. Lawaju
Kathy S. Lawrence
Joseph W. Kloepper
Auburn University
Auburn, AL

Abstract

Meloidogyne incognita is a plant parasitic nematode that causes economic losses in upland cotton. Management of the nematode includes nematicides, cultural control and biological control. Due to the inexpensive cost and low environmental impact, biological control agents are becoming more popular as a management method for *M. incognita*. Biological control agents can work by direct or indirect antagonism of their target pathogen. Indirect antagonism includes the upregulation of plant defense pathways. This includes the induced systemic resistance pathway, which utilizes jasmonic acid and is stimulated by plant-growth promoting rhizobacteria, and the systemic acquired resistance pathway, which utilizes salicylic acid. Determining which pathway a biological control agent, with an indirect method of antagonism, stimulates is essential to the integration of the biological control agent into a successful pest management program. This research examined five selected *Bacillus* species and their ability to stimulate a systemic response to *M. incognita* within cotton. Experiments included a greenhouse test, an *in vitro* assay, a split root assay and a RT-qPCR assay. Results of the greenhouse test determined the efficacy of the *Bacillus* spp., while results of the *in vitro* assay and split root assay determined whether the mechanism of action is direct or indirect for each bacterial species. The RT-qPCR assay further elucidated whether systemic resistance occurred within the cotton plant. The findings of this research will help implement these biological control agents in an integrated pest management program for nematodes, specifically *M. incognita*.

Introduction

Meloidogyne incognita (Kofoid &White, 1919) Chitwood, or the southern root-knot nematode, is an endoparasitic nematode that causes economic losses in cotton. In 2018, the nematode caused losses of 2.78% of total cotton production in the United States, equating to approximately $183 million (Lawrence et al., 2018). There are various management strategies available to decrease nematode population density including nematicides, crop rotation and biological control. Biological control agents have been growing in popularity because they are more environmentally friendly (Bale et al., 2008).

Biological control typically works by two mechanisms; direct or indirect antagonism. Direct antagonism by biological control agents often refers to the release of metabolite or predation of the target pathogen (Pal et al., 2006). Indirect antagonism is often accomplished by induced systemic resistance (ISR) or systemic acquired resistance (SAR). ISR refers to the activation of plant defenses by plant growth-promoting rhizobacteria (PGPR) and typically jasmonic acid and ethylene (van Loon et al., 1998). SAR refers to the broad spectrum activation of pathogenesis related genes by salicylic acid (Durant et al., 2004). Previously, various *Bacillus* spp. have shown the ability to stimulate systemic resistance within a plant (Kloepper et al., 2004). Determining the mechanism of each biological control agent is important to optimize its functioning.

To determine if five selected *Bacillus* spp. can stimulate systemic resistance in cotton five assays were performed. A greenhouse pot test determined the ability of the *Bacillus* spp. to decrease *M. incognita* population density *in planta*. An in vitro assay was utilized to determine any direct antagonistic capabilities of the *Bacillus* spp. A split root assay was done to determine systemic capabilities, followed by an RT-qPCR to determine the regulations of genes related to jasmonic acid and salicylic acid synthesis and functioning.

Materials and Methods

Greenhouse Pot Test

Four cotton seeds (Phytogen 333) were planted in 500 cm^3 polystyrene pots with a 2:1 soil to sand mixture and standard amounts of lime and fertilizer as recommended by the Auburn University Soil Lab. The seeds were inoculated with one mL containing appoximately 5,000 *M. incognita* eggs, obtained from stock pots in the greenhouse at Auburn University, and one of the following treatments: 1) control, 2) 0.5 µL of fluopyram followed by one mL of water, 3) 1 mL of *B. firmus* I-1582, 4) 1 mL of *B. amyloliquedfaciens* QST713, 5) 1 mL of *B. pumilus* GB34, 6) 1 mL of *B. velenzensis* strain 2, and 7) *B. mojavensis* strain 3. The *Bacillus* sp. for treatments 5, 6 and 7 were obtained from Dr. Joseph W. Kloepper's lab at Auburn University, Auburn AL. The *Bacillus* spp. for treatments 3 and 4 were obtained from Bayer CropScience and are the active ingredients of the products VOTiVO and Serenade, respectively. The five *Bacillus* treatments were applied at a rate of 1 $x10^6$ cfu/mL. Thirty days after inoculation (DAI) the plant height, shoot fresh weight and root fresh weight were measured. The roots were added to a 0.625% NaOCl solution and placed on rotary shaker set to 120 rpm for 4 minutes. The roots were washed with tap water over a 200 and 500 µm sieve. *Meloidogyne incognita* eggs collected during the NaOCl extraction were combined with a 45.4% sucrose solution and centrifuged for 1 minute at 1,400 rpm. The sucrose solution containing the *M. incognita* eggs was run through the sieves again. *Meloidogyne incognita* eggs were washed from the bottom sieve and counted using the 40× magnification of an inverted TS100 Nikon microscope. Statistical analysis was performed in SAS 9.4 (SAS Institute, Cary, NC) using the PROC Glimmix procedure with *P* d0.1. This experiment was repeated once.

in vitro Assay

The five selected *Bacillus* spp. were grown on tryptic soy agar plates for 6 days in an incubator set to35°C. The metabolites were extracted following the methodology of Apotroaie-Constantin et al., 2009. *Meloidogyne incognita* eggs were extracted from stock pots maintained in the greenhouse using NaOCl extraction and sucrose centrifugation as previously described. The eggs were placed in a modified Baermann funnel on a slide warmer set to 30 degrees Celsius. After one week, the *M. incognita* juveniles that have migrated though the funnel were run through a 200 and a 500 µm sieve to collect the second stage juveniles. To each well of a 96 well plate, 10 µL of 20-30 *M. incognita* second stage juveniles and 90 µL of a water control or one of the *Bacillus* treatments at a concentration of 1 $x10^6$ cfu/mL were added. The live and dead *M. incognita* juveniles were counted at 0 and 48 hours. Using the methodology of Xiang et al., 2016, the percent mortality was calculated and statistics were analyzed in SAS 9.4 (SAS Institute, Cary, NC) using the PROC Glimmix procedure with *P* d 0.05. This assay was repeated twice.

Split Root Assay

Cotton seeds (Phytogen 333) were germinated in moist germination paper on a hot plate set to 30.0°C for 4-6 days until short, fine lateral roots began to develop. One mm was cut from the tip of the root and the seed was placed back in the germination paper. After 6 hours in the germination paper, each cotton seedling was planted in 500 cm^3 polystyrene pots filled with sand, fertilizer and lime as recommended by the Auburn University Soil Lab. The cotton roots were carefully monitored in the greenhouse under standard conditions for one to two weeks after planting to determine if the lateral root system was large enough to be planted in the split root set up. The split root set ups consisted of two 150 cm^3 conetainers positioned directly next to each other with a small cup, that had the bottom cut off and was positioned so that half was above each conetainer. The conetainers and cup were filled with 3:1 soil to sand along with lime and fertilizer as recommended by the Auburn University Soil Lab. When the cotton plants developed long enough lateral roots, the seedling was planted so that half of the lateral roots were directed towards one conetainer and the other half directed towards the second conetainer. After a two day adjustment period, the root halves were inoculated. There were five distinct inoculation patterns for each treatment. These patterns were: 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 (Fig 1). The treatments were inoculated at the same concentrations and volumes as described in greenhouse pot test. Thirty DAI, plant height, shoot fresh weight, and root fresh weight were measured. *Meloidogyne incognita* eggs were extracted in NaOCl and centrifuged in 45.4% sucrose solution as previously described. Statistics were analyzed in SAS 9.4 (SAS Institute, Cary, NC) using the PROC Glimmix procedure with pd 0.1. This assay was repeated once.

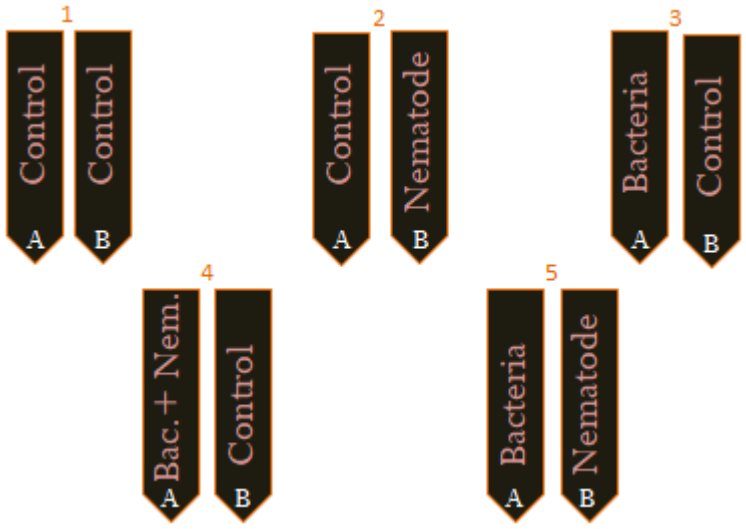

Figure 1: Model of 5 different split root set ups 1) no inoculation on either root half, 2) no inoculation on root half A and nematode inoculation on root half B, 3) bacterial or chemical inoculation on root half A and no inoculation on root half B, 4) Bacterial or chemical inoculation plus nematode inoculation on root half A and no inoculation on root half B, 5) bacterial or chemical inoculation on root half A and nematode inoculation on root half B.

RT-qPCR to Determine JA and SA Concentrations

Cotton seeds (Phytogen 333) were planted in 150 cm^3 conetainers in the greenhouse under standard conditions. The cotton was grown for about 2-3 weeks or until the second true leaf stage. The plants were inoculated with the following treatments: 1) Control, 2) *M.* incognita second stage juveniles, 3) 1 mL of *B. firmus* I-1582, and 4) 1 mL of *B. amyloliquefaciens* QST713. The *Bacillus* treatments were applied at a rate of 1 $x10^6$ cfu/mL and the M. incognita juveniles were at a concentration of 1,000 juveniles/mL. Samples of the roots were taken and immediately frozen in liquid nitrogen at 0 hours (h), 1 h, 3 h, 12 h, 96 h, and 1 week after inoculation. The samples were stored in a -80 °C freezer until ready for use. The samples were ground in liquid nitrogen into a fine powder. RNA was extracted from this powder using the Spectrum™ Plant Total RNA Kit (Sigma Aldrich, St. Louis, MO, USA) according the manufacturer's instruction. The concentration and purity of the RNA was determined using the NanoDropTM Spectrophotometer ND 2000 (Thermo Scientific, Wilmington, USA) and a 1% agarose gel was used to determine the integrity of the RNA. The RNA was converted to cDNA using the GoscrpitTM reverse transcription system Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.Rt-q-PCR was performed using 12.5 ¼L of PerfeCTA® SYBR® Green Fastmix®, ROX qPCR Master Mix (Qunita Biosciences, Inc, Gaithersburg, MD, USA), 0.5 ¼L of cDNA and 100 nM of primers obtained from Invitrogen (ThermoFisher Scientific; Waltham, MA), for a total of 25 µL. An ABI 7500 Real time PCR system (Life Technologies, Carlsbad, CA, USA) with a 96 well rotor was used to carry out the RT-qPCR. The level of stable housekeeping gene, histone (H_3) ribosomal mRNA was used to calibrate and normalize relative RNA levels.

Table 1: Primers for RT-qPCR to determine levels of genes related to jasmonic acid (OPR3) and salicylic acid(GLU) levels

Gene	Forward (5'-3')	Reverse (3'-5')	Reference
Histone (H_3)	GAAGCCTCATCGATACCGT	CTACCACTACCATCATGGC	Zebelo 2015
12-Oxophytodienoate reductase 3 (OPR3)	ATGTGACGCAACCTCGTTATC	CCGCCACTACACATGAAAGTT	Zebelo 2015
' -1,3-glucanase (GLU)	AATGCGCTCTATGATCCG	GATGATTTATCAATAGCAGCG	Zhang 2011

qPCR to Determine Concentration of *Bacillus* sp.

Cotton seeds (Phytogen 333) were planted in 150 cm^3 conetainers filled with 2:1 soil to sand and standard fertilizer and lime and were inoculated at planting with 1 mL of *B. firmus* I-1582 or 1 mL of *B. amyloliquefaciens* QST713 at a rate of 1x 10^8 cfu/mL. Samples were taken at 24 DAI and kept in a -80 °C freezer until ready for use. The concentration of the *Bacillus* spp. was determined following the procedure outlined in Mendis et al., 2018.

Results and Discussion

First, three parts of this experiment; a greenhouse pot test, an *in vitro* assay and a split root assay. The greenhouse pot test was performed to verify that each selected *Bacillus* strain could decrease *M. incognita* reproduction as measured by number of eggs per gram of root. After 30 days, the plant parameters were not different between the treatments but the number of eggs per gram of root was decreased by two treatments, *B. firmus* I-1582 and *B. amyloliquefacien* QST713, compared to the control (Fig 2). These two treatments decreased the number of eggs similarly to the chemical control of Fluopyram (Fig 2).

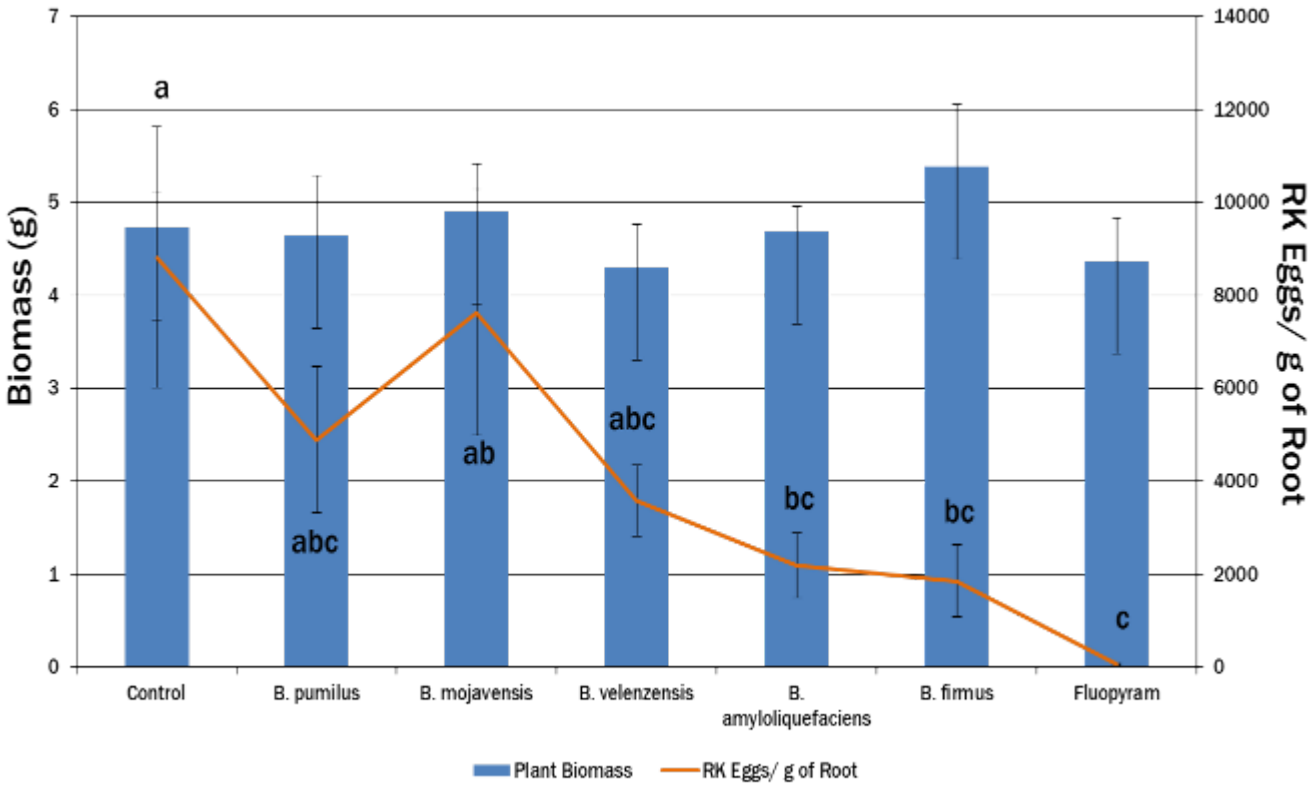

Figure 2: Biomass (g), calculated by adding the shoot fresh weight and root fresh weight, and *M. incognita* eggs per gram of root of cotton during the green house pot test (pd0.1).

The *in vitro* assay determined the percent mortality of *M. incognita* second stage juveniles caused by the select *Bacillus* spp. and their metabolites. Fluopyram was not used in this assay because it is a thick, opaque white liquid that made the percent mortality difficult to determine. After 48 hours the, *B. firmus* I-1582, the *B. firmus* I-1582 metabolites and the *B. amyloliquefaciens* QST713 metabolites increased the percent mortality compared to the water control (Fig 3). This indicated that *B. firmus* I-1582 can directly antagonize the nematode potentially through the release of a metabolite(s). It is unknown whether *B. amyloliqeufaciens* QST713 can release the metabolites that caused an increase in percent mortality (Fig 3), therefore it cannot be concluded that this *Bacillus* sp. works by direct antagonism

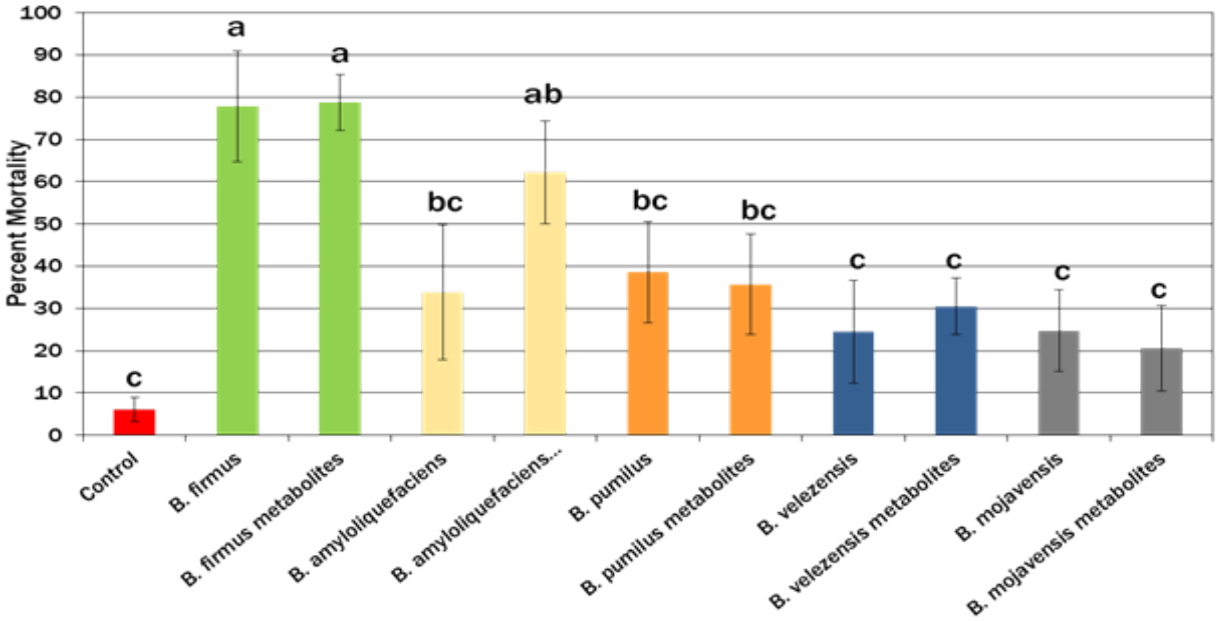

Figure 3: Effect of *Bacillus* sp. and metabolites on mortality percentage of *M. incognita* second stage juveniles (pd0.05).

The split root assay was used to determine the potential of each *Bacillus* sp. to cause a systemic response. The bacteria species that did not show a systemic effect were omitted from the results. There was no difference in plant parameters between any of the treatments. Two of the five *Bacillus* sp. showed systemic capabilities by decreasing the number of nematode eggs per gram of root when the bacteria were in contact with the nematode and when it is not in contact with the nematode (Fig 4). The mechanism by which the *Bacillus* spp. would decrease the nematode eggs when not in direct contact could be a potential systemic response from the cotton plant, stimulated by the *Bacillus* sp. These potential systemic capabilities will be further analyzed and confirmed in an RT-qPCR of genes correlating to jasmonic acid and salicylic acid levels.

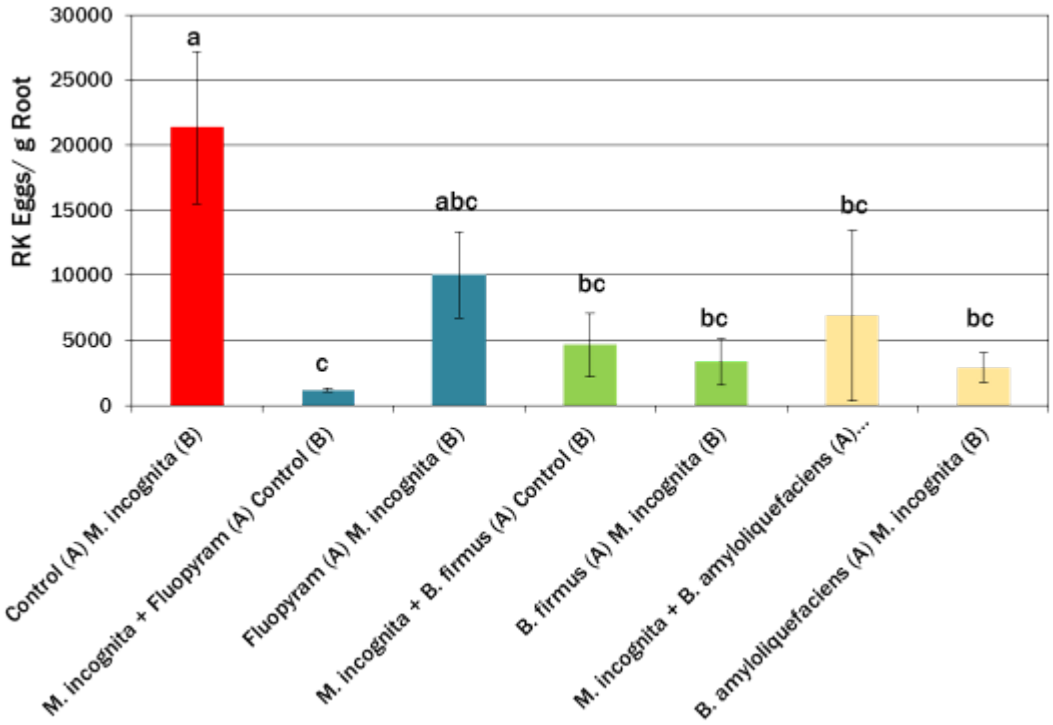

Figure 4: Results *Meloidogyne incognita* eggs per gram of root counts for fluopyram, *B. firmus* I-1582 and *B. amyloliquefaciens* QST713 during the split root assay (pd0.1).

To further determine systemic resistance, a RT-qPCR assay was used to determine the level of genes that correlate to jasmonic acid and salicylic acid levels. When *M. incognita* was inoculated alone, the genes corresponding to both jasmonic acid (OPR3) and salicylic acid (² -1,3-glucanase) were upregulated at 1 hour (Fig 5A). After 3 hours, OPR3 was downregulated until sometime between 12 and 96 hours when it switched to being upregulated (Fig 5A). Oppositely, ² -1,3-glucanase was upregulated until between 3 and 12 hour when it was downregulated (Fig 5A). *Bacillus amyloliquefaciens* QST713 stimulated an upregulation of ² -1,3-glucanase for between 12 and 96 hours, at which point it become downregulated (Fig 5B). The OPR3 gene was downregulated initially by *B. amyloliquefaciens* QST713 and switched to being upregulated between 12 and 96 hours (Fig 5B). *Bacillus firmus* I-1582 caused a similar reaction with ² -1,3-glucanase, initially upregulated until between 96 hours and 1 week (Fig 5C). OPR3 was downregulated until between 12 and 96 hours when it was upregulated (Fig 5C). These results are similar to what Martinez-Medina et al., 2017 saw with *Trichoderma* sp. and *M. incognita* in tomatoes. Initially the biological control agent, *Bacillus* sp. or *Trichoderma* sp., upregulated salicylic acid which was downregulated after a short period of time followed by the upregulation of jasmonic acid defenses. It has also been shown that *M. incognita* has effectors that can suppress both salicylic acid and jasmonic acid (Shi et al., 2018, Wang 2018). In this study, *Bacillus* sp. maintained salicylic acid functioning longer than the plant affected by *M. incognita,* indicating that the *Bacillus* sp. could counteract the effectors released by *M. incognita* through systemic resistance.

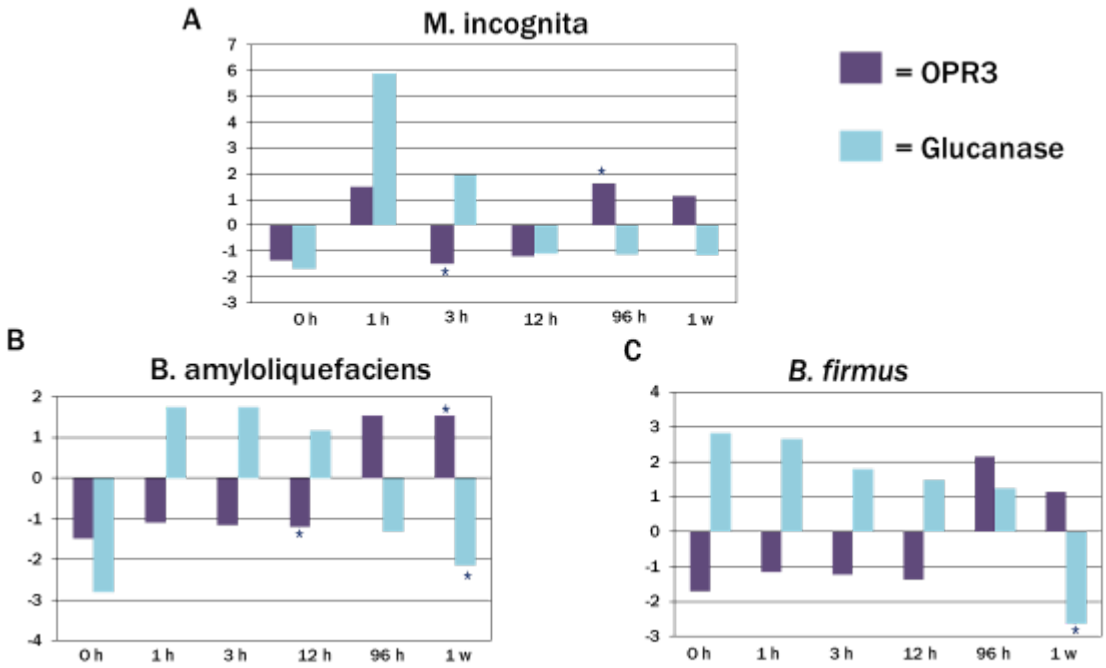

Figure 5:RT-qPCR results to determine levels of genes correlated to jasmonic acid levels (OPR3, purple bar) and salicylic acid levels (Glucanase, blue bar) of plants inoculated with *M. incognita* second stage juveniles (A), *B. amyloliquefaciens* QST713 (B), and *B. firmus* I-1582 (C). * = compared to control and housekeeping gene.

The final assay performed was a qPCr to determine the concentration of the *Bacillus* sp. on the cotton roots 24 days after inoculation. The initial inoculation of the bacteria was 10^8 cfu/mL. The standards calculated by plotting known cocentrations of bacteria against CQ values obtained from the qPCR of the known concentrations of bacteria were y=-1.9421x + 34.227; R^2 = 0.9371 for *B. amyloliquefaciens* QST713 and y= -2.2036x + 44.362; R^2 = 0.8217 for *B. firmus* I-1582. After 24 days, the bacterial population decreased to $10^{4.66}$ cfu/mL for *B. amyloliquefaciens* QST713 and $10^{3.93}$ cfu/mL for *B. firmus* I-1582 (Fig 6). This is similar to results seen by Mendis et al., 2018 who performed the protocol on corn. This indicates that the bacteria were able to colonize the cotton roots well.

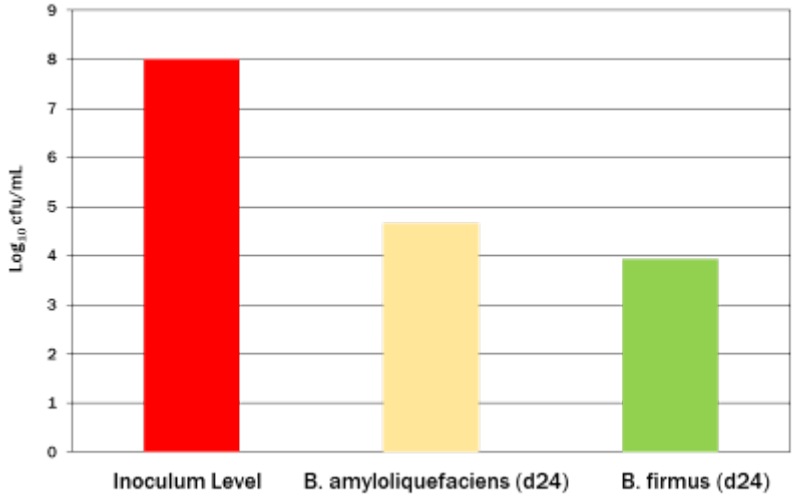

Figure 6: qPCR results determining concentration of *Bacillus* sp. on roots 24 days after inoculation, with an initial inoculation population of 10^8 cfu/m

Summary

Throughout the five assays performed, *B. firmus* I-1582 and *B. amyloliquefaciens* QST713 showed systemic capabilities in cotton against *M. incognita*. During the *in vitro* assay, *B. firmus* I-1582 and its metabolites directly antagonized the nematode. The *B. amyloliquefaciens* QST712 metabolites increased percent mortality of the nematode as well; however, the intact bacteria did not. This indicates that *B. firmus* I-1582 was the only bacteria able to directly affect the *M. incognita* second stage juveniles. During the split root assay, *B. firmus* I-1592 and B. *amyloliquefacien* QST713 showed systemic capabilities. This was confirmed in the RT-qPCR where these bacteria stimulated salicylic acid to maintain high levels for a longer period of times. In contrast, *M. incognita* juveniles decreased levels of salicylic acid rapidly. Another study by Martinez-Mendoza et al., 2017, saw that this extended response of salicylic acid related genes stimulated by a biological control product, can defend the plant against *M. incognita*. The systemic response and decrease in *M. incognita* eggs in the split root assay and greenhouse pot test seemed to be due to the increase in initial salicylic acid levels as indicated by previous research and the RT-qPCR. The final assay, a qPCR, determined that *B. firmus* I-1582 and *B. amyloliquefaciens* QST713 were able to successfully colonize the roots. In conclusion, two of the five *Bacillus* spp. screened could produce a systemic response.

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