SYSTEMIC INDUCED RESISTANCE TO THE ROOT-KNOT NEMATODE CAUSE BY *BACILLUS* SPP.

K. Gattoni
N. Xiang
K. Lawrenace
J. W. Kloepper
Auburn University
Auburn, AL

Abstract

Biological control agents are one management strategy that can be utilized to manage population density of the southern root-knot nematode, *Meloidogyne incognita*, in upland cotton, *Gossypium hirsutum*. Successful implementation of biological control agents requires knowledge of how each agent affects its target pathogen. Biological control agents can directly or indirectly antagonize a pathogen. Indirect antagonism of a pathogen by a biological control agent is called systemic induced resistance (SIR). The objective of this research was to determine if three selected *Bacillus* strains could induce SIR in a cotton system as measured by a decrease in *M. incognita* population density. A greenhouse pot test, an *in vitro* assay and split root assay were used to test our hypothesis. The greenhouse pot test indicated that all three *Bacillus* strains had potential as biocontrol agents of *M. incognita*. The results of the split root assay showed that *B. firmus* I-1582 and *B. subtilis* QST713 may induce SIR to *M. incognita* in cotton. Further testing will be necessary to confirm these results. If the results are confirmed, the method of application of these *Bacillus* strains to cotton can be adjusted to best fit the mechanism of action.

Introduction

The southern root-knot nematode, *M. incognita*, is an endoparasitic nematode that parasitizes hundreds of susceptible host plants including economically important crops such as cotton, corn, soybean, tomato and pepper. The root-knot nematode accounts for 2.2% of loss in total cotton production in the United States (Lawrence 2017). There are several management strategies available for *M. incognita*. These include nematicides, crop rotation, resistant varieties and biological control agents.

Biological control agents, particularly, can work by a direct or an indirect mechanism (Raupach 1998). Biological control agents directly antagonize *M. incognita* by releasing secondary metabolites and proteins which are toxic to the nematode. For example, *B. firmus* I-1582 has been reported to reduce *M. incognita* population density by producing ammonia that is toxic to all nematodes (Mendoza 2007). Biological control agents can also act by an indirect mechanism where they stimulate a plant defense response. This is termed systemic induced resistance (SIR). There are two main pathways by which SIR can occur. The SIR pathways both increase plant defenses but one is through the jasmonic acid and ethylene signaling pathway, termed induced systemic resistance, and the other is through the salicylic acid signaling pathway, termed systemic induced resistance. Various biological control agents have shown they can produce SIR within a plant. One of the first reports of a biological control agent causing SIR was tobacco inoculated with *Pseudomonas flourescens* strain CHA0 (Maurhofer 1993). Since then, many species of bacteria, including various *Bacillus* species, have been identified to systemically induce resistance in different crops (Kloepper 2004). Determining the mode of action by which each biological control agent works can help successfully implement them in the field. We tested whether three selected *Bacillus* strains induced SIR in the presence of *M. incognita* in an upland cotton system using a greenhouse pot test, an *in vitro* assay and split root assay. *Bacillus* species were analyzed to determine their potential to decrease root-knot nematode eggs per gram of root. Through the *in vitro* and split root assays, two *Bacillus* strains were identified as potential inducers of SIR in cotton.

Materials and Methods

Greenhouse Pot Test

In the Auburn University Plant Science Greenhouse located on the Auburn University campus, four cotton seeds (Phytogen 333 with standard fungicide and insecticide treatment) were planted 2 cm deep in 500 cm^3 polystyrene pots containing 3 parts pasteurized soil and 1 part sand. The seeds were inoculated with one mL of 5,000 *M. incognita* eggs, obtained from stock pots in the greenhouse at Auburn University, and one of the following treatments was applied per pot: 1) control of water, 2) 0.5 µL of fluopyram (Bayer Crop Science) followed by one

mL of water in accordance with the labeled rate, 3) 1 mL of *B. firmus* I-1582, 4) 1 mL of *B. subtilis* QST713, and 5) 1 mL of *B. pumilus* GB34. The three selected *Bacillus* treatments were applied at a rate of 1 x10^6 cfu/mL. Thirty days after inoculation (DAI), plant height, shoot fresh weight and root fresh weight were measured. The roots were added to a 0.625% NaCl 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 sucrose (454 g in 1 L of water) solution and centrifuged at 1,200 rpm for 1 minute. The supernatant was run through the sieves again. *Meloidogyne incognita* eggs were collected 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 $p \leq 0.1$.

***in vitro* Assay**

Meloidogyne incognita eggs were extracted from stock pots obtained from the greenhouse using NaOCl extraction and sucrose centrifugation as previously described. The eggs were placed in a modified Baermann funnel on a hot plate at 30 degrees C. After one week, the contents of the funnel were run over a stacked 200 and 500 µm sieves 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 selected *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. Juveniles were considered alive if they had shape to their bodies and showed any movement. Juveniles were considered dead if they did not move and their bodies were pin straight. The percent mortality was calculated and statistics were analyzed in SAS 9.4 (SAS Institute, Cary, NC) using the PROC Glimmix procedure with $p \leq 0.05$.

Split Root Assay

Cotton seeds (Phytogen 333 as previously described) were germinated on moistened germination paper placed in a plastic bag half open for 4-6 days on a hot plate set to 30° C until short, fine lateral roots began to develop. One mm was cut from the tip of the root with a scalpel and the modified seedling was placed back in the germination paper. After 6 hours in the germination paper, the cotton seedling was planted in 500 cm^3 polystyrene pots filled with sand, 120 mL per bucket of granular fertilizer and 120 mL per bucket of lime. The cotton roots were carefully monitored 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 containers positioned directly next to each other. A small cup that had the bottom removed was positioned with half of the cup above each container. The containers and cup were filled with 3:1 pasteurized soil to sand along with 60 mL per bucket of lime and 60 mL per bucket of granular fertilizer. When the cotton seedling had developed long enough lateral root, the seed was planted so that half of the lateral roots were directed towards one container and the other half directed towards the second container. 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. The treatments were inoculated at the same concentrations and volumes as described in greenhouse pot test. Thirty DAI, the plant height, shoot fresh weight, and root fresh weight were measured. *Meloidogyne incognita* eggs were extracted in NaOCl and centrifuged in sucrose solution as previously described. Statistics were analyzed in SAS 9.4 (SAS Institute, Cary, NC) using the PROC Glimmix procedure with $p \leq 0.1$.

Results and Discussion

There were 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 the number of *M. incognita* eggs. After 30 days, the number of nematode eggs per gram of root was decreased by fluopyram, *B. pumilus* GB 34, *B. subtilis* QST 713, and *B. firmus* I- 1582 compared to the control (Fig. 1). *Bacillus firmus* I-1582 and *B. subtilis* QST713, specifically, reduced nematode eggs per gram of root similarly to the chemical standard of fluopyram (Fig 1). Though *B. pumilus* GB34 only decreased the number of eggs per gram of root numerically compared to the control, it was included in the *in vitro* assay and split root assay because previous work with this *Bacillus* strain showed stimulation of the systemic induced resistance, specifically upregulating jasmonic acid, in a cotton system (Zebelo 2016).

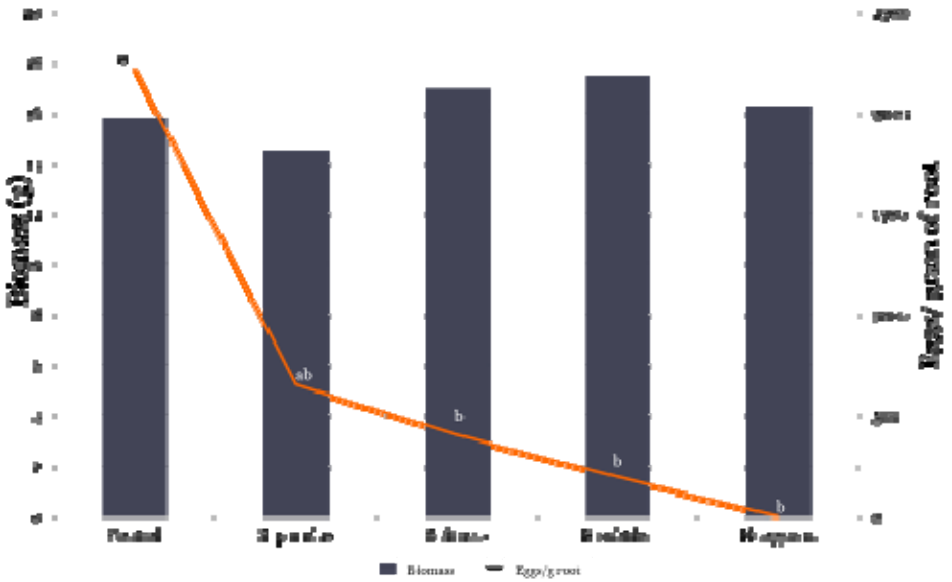

Figure 1: Effect of *Bacillus strains* on biomass (g), which included the root and shoot fresh weights, and *M. incognita* eggs per gram of root for cotton plants in the greenhouse pot test 30 DAP (p≤0.1).

The *in vitro* assay was used to measure the ability of each *Bacillus* strain to directly antagonize *M. incognita* second stage juveniles. Fluopyram was not evaluated in this assay as it is an opaque white color that made it too difficult to visualize the living and dead juveniles in each well. After 48 hours the percent mortality was only increased, compared to the water control, by *B. firmus* I-1582, which was able to kill 100% of the juveniles after 48 hours (Fig. 2). This result was expected, as *B. firmus* I-1582 was previously reported to release ammonia which is toxic to *M. incognita* (Mendoza 2007). Neither *B. pumilus* GB34 or *B. subtilis* QST713 increased mortality compared to the water control (Fig. 2). This result and the results of the greenhouse pot test indicate that these two *Bacillus* species may produce SIR in the cotton plant to control *M. incognita* population density because they were able to decrease population size when in contact with the plant and the nematode but not when just in contact with the nematode.

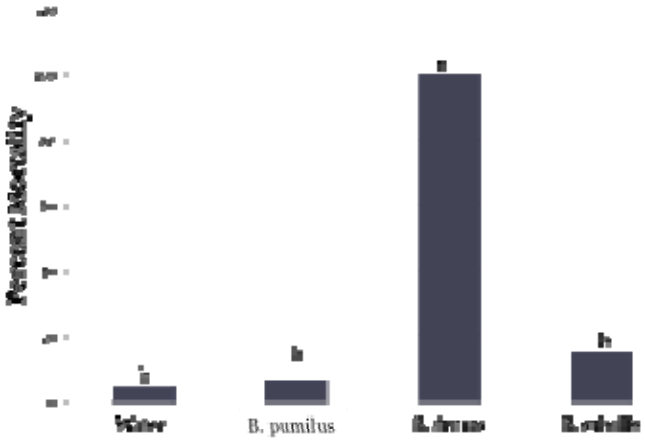

Figure 2: Effect of *Bacillus* strain on mortality percentage of *M. incognita* second stage juveniles 30 DAP (p≤0.05).

A split root assay was used to determine if the *Bacillus* strains induced SIR. Specifically, if the treatment could reduce the number *of M. incognita* eggs per gram of root when the treatment was on opposite root half as the *M. incognita eggs*, then the treatment can induce a systemic response in cotton. The biomass and other plant parameters did not differ between the inoculation patterns within the treatments. In the chemical control of fluopyram, there was only a decrease in the number of *M. incognita* eggs per gram of root (99%) compared to the nematode control when fluopyram was inoculated on the same root half as the nematode (Fig. 3), indicating this chemical works by direct antagonism rather than SIR. *Bacillus pumilus* GB34 was unable to decrease the *M. incognita* eggs per gram of root

when applied directly to the root half with *M. incognita* eggs or on the opposite side of the *M. incognita* eggs (Fig. 4). This was not expected as *B. pumilus* GB34 was able to produce SIR in a cotton system against a different pest (Zebelo 2016). *Bacillus firmus* I-1582 decreased nematode eggs per gram of root when on the same root half (63%) and the opposite root half (92%) as the *M. incognita* inoculation (Fig. 5). The decrease in eggs when *B. firmus* I-1582 was not in direct contact with the nematode indicates a systemic response. The results from the *in vitro* assay and the split root assay indicate that *B. firmus* I-1582 may antagonize *M. incognita* directly and by SIR. *Bacillus subtilis* QST713 also decreased the nematode eggs per gram of root when on the same root half (83%) and the opposite root half (68%) as the *M. incognita* inoculation (Fig. 6). Due to the results of the *in vitro* test and the split root assay, it is thought that *B. subtilis* QST713 can produce SIR when in contact with and when not in contact with *M. incognita*, rather than directly impact the nematode.

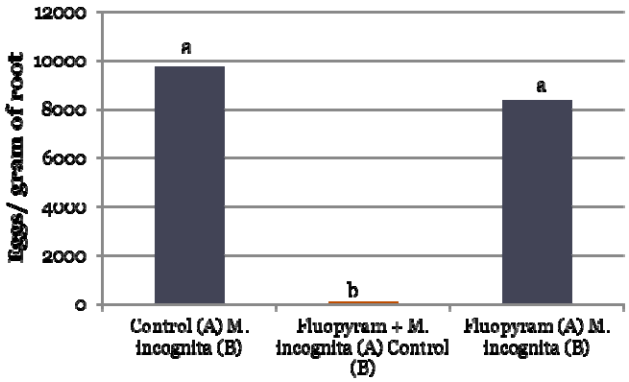

Figure 3: M*eloidogyne incognita* eggs per gram of root in the split root assay with fluopyram 30 DAP (p≤0.1)

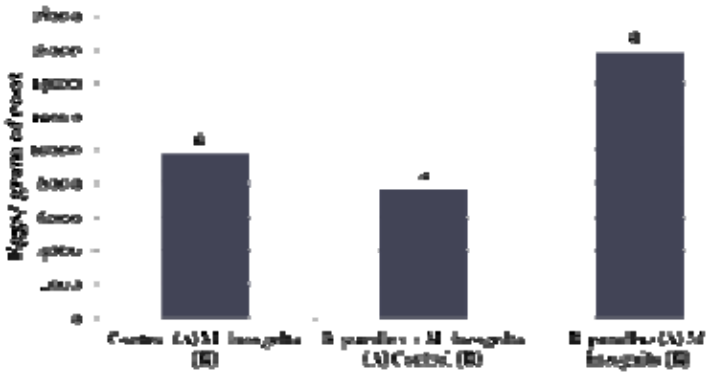

Figure 4: *Meloidogyne incognita* eggs per gram of root in the split root assay with *B. pumilus* GB34 30 DAP (p≤0.1)

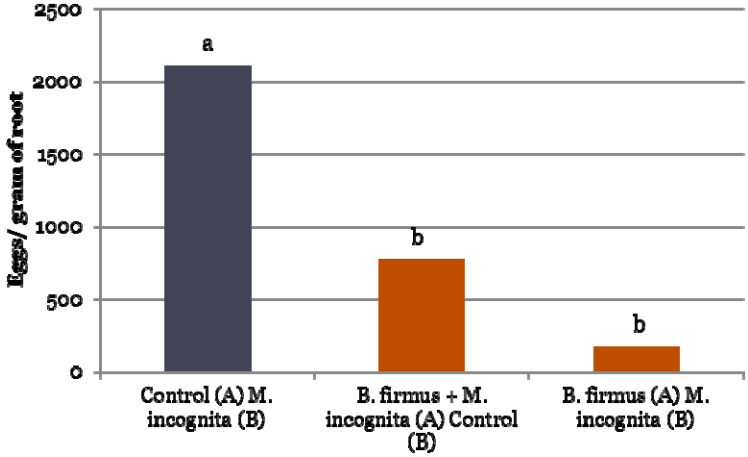

Figure 5: *Meloidogyne incognita* eggs per gram of root in the split root assay with *B. firmus* I-1582 (p≤0.1)

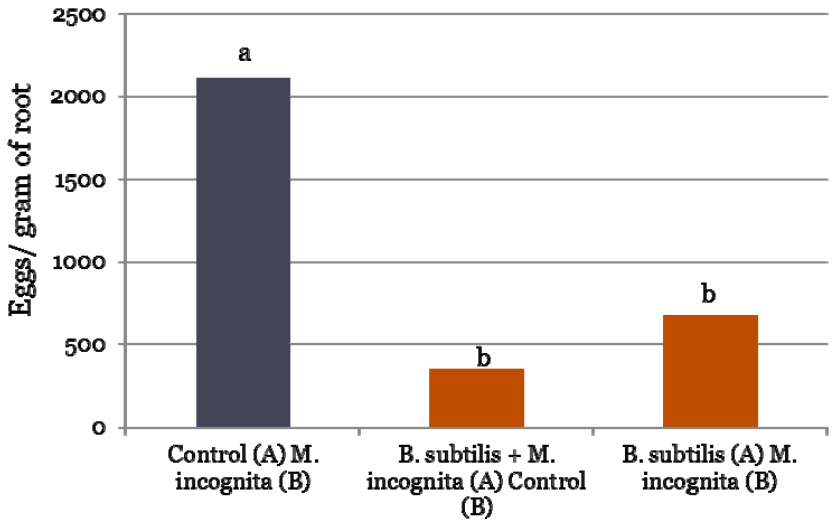

Figure 6: *Meloidogyne incognita* eggs per gram of root in the split root assay with *B. subtilis* QST713 30 DAP ($p \leq 0.1$)

Summary

Through a greenhouse pot test, *in vitro* assay and split root assay, *B. firmus* I-1582 and *B. subtilis* GST713 were identified as biological control agents that induce SIR against *M. incognita* in cotton. Fluopyram and *B. firmus* I-1582 were also seen to directly antagonize *M. incognita*. This agrees with previous research using *B. firmus* showing the bacteria produces secondary metabolites, such as ammonia, that are toxic to *M. incognita* (Mendoza 2017). *Bacillus pumilus* GB34 did not decrease the eggs per gram of root in the split root assay or have an increased mortality rate of second stage juveniles during the *in vitro* assay. This was unexpected as *B. pumilus* GB34 was previously reported to stimulate SIR in a cotton system (Zebelo 2016). However, both *B. firmus* I-1582 and *B. subtilis* QST713 did induce systemic resistance within the cotton plant. For *B. firmus* I-1582, it is possible given the results of the *in vitro* assay and split root assay that this bacteria controls *M. incognita* population density directly and indirectly. Further research will be needed on all three bacterial strains to confirm that they induce a SIR response in cotton. Specifically, real time PCR will be performed to look at genes related to the upregulation of SIR products.

References

Kloepper, J.W., C.M. Ryu, and S. Zhang. 2004 Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94:1259-1266.

Lawrence, K., A. Hagan, R. Norton, T. Faske, R. Hutmacher, J. Mueller, D. Wright, I. Small, R. Kemerait, C. Overstreet, P. Price, G. Lawrence, T. Allen, S. Atwell, A. Jones, S. Thomas, N. Goldberg, R. Bowman, J. Goodson, H. Young, J. Woodward, and H. Mehl. 2017. Cotton disease loss estimate committee report, 2016. Proceedings of the 2017 Beltwide Cotton Conference Vol. 1:150-152. National Cotton Council of America, Memphis, TN.

Mendoza, A. R., K. Sebastian, and R. A. Sikora. 2008. *In vitro* activity of *Bacillus firmus* against the burrowing nematode *Radopholus similis*, the root-knot nematode *Meloidogyne incognita* and the stem nematode *Ditylenchus dipsaci*. Biocontrol Science and Technology 18:377-389.

Raupach, G. S. and J.W. Kloepper. 1998. Mixtures of plant growth promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology 88:1158-1164

Zebelo, S., Y. Song, J.W. Kloepper, and H. Fadamiro. 2016. Rhizobacteris activates (+)-δ-cadinene synthase genes and induces systemic resistance in cotton against beet armyworm (Spodoptera exigua). Plant, Cell & Environment 38:935-43.