

The Studies of Plant Host Resistance to the Reniform Nematode in Upland Cotton and the Effects of *Bacillus firmus* GB-126 on Plant-Parasitic Nematodes.

by

Drew Waddell Schrimsher

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
May 4, 2013

Copyright © 2013 by Drew Waddell Schrimsher

Approved by

Kathy S. Lawrence, Chair, Associate Professor of Plant Pathology
David B. Weaver, Professor of Agronomy
David W. Held, Associate Professor of Entomology
Charles H. Burmester, Extension Agronomist

Abstract

Germplasm lines LONREN-1 and LONREN-2 were released in 2007 for cotton breeders to incorporate reniform nematode resistance into breeding efforts with desirable cultivars to establish nematode resistant high yielding cultivars. Previous screenings for reniform resistance in the LONREN-1 X FM966 breeding lines developed at Auburn University have demonstrated that the reniform resistance is accompanied by severe stunting and limited plant growth followed by low yields. The objectives of this study were to evaluate the effects that applying nematicides to selected LONREN breeding lines have on reniform nematode populations, early seedling plant stunting, yield, and fiber quality. Three resistant breeding lines from the LONREN-1xFM966 cross, one susceptible line from the LONREN-1xFM966 cross, and the susceptible cultivar DP393 were treated with nematicides and their performances evaluated. In the greenhouse, nematicides increased plant heights in the resistant lines. Nematicides further reduced reniform populations in the resistant lines 45 days after planting (DAP). Reniform populations were 50% lower in the resistant lines compared to the susceptible lines by the end of the growing period. In microplot and field trials, the phenotypic stunting response of the resistant lines was reduced by nematicides with increases in plant heights at 30 and 75 DAP. Increases in yields were also evident in the resistant breeding lines that were treated with nematicides.

Bacillus firmus strain GB-126 was evaluated for the capacity to reduce mobility of juveniles, inhibit egg production, and induce systemic resistance when used as a control against *Heterodera glycines* and *Meloidogyne incognita*. Experiments were established *in vitro* to examine egg hatching and mobility and paralysis of J-2s in 96 well plates containing 100µl of

GB-126 cells at 1×10^7 and 1×10^6 cfu/ml and cell-free extracts at 100%, 50%, and 25% concentrations. Split-root assays were established to evaluate induced systemic resistance. GB-126 cells at both concentrations significantly reduced mobility of *H. glycines* J2s compared to Tryptic Soy Broth (TSB) and sterilized tap water (STW) controls at 36 h after treatment. Cell-free extracts of GB-126 reduced mobility significantly at 12 h after treatment in both 100% and 50% concentrations compared to TSB and STW controls. GB-126 cells and cell-free extracts also significantly reduced egg hatching of *H. glycines* and *M. incognita* at 9 and 4 days after inoculation, respectively. Induced systemic resistance was evident in the *H. glycines* split-root assay but not in the *M. incognita* split-root assay. The results of these experiments indicate that both cells and cell-free extracts of GB-126 can have antagonistic effects on *H. glycines* and *M. incognita*.

Acknowledgments

I would like to extend my expression of gratitude to everyone that helped me complete this project. I want to extend a very special thank you to my major professor, Dr. Kathy Lawrence, for her guidance, encouragement, and endless support throughout this project. Thank you to the sponsors, Cotton Incorporated and Bayer CropScience, for their contributions to the funding of this project. Thank you to my committee members Dr. David B. Weaver, Dr. David W. Held, and Charlie Burmester for their contributions of time and guidance of this project. Thank you to the graduate student and undergraduate students, Scott Moore, Juan David Castillo, Ni Xiang, David Bailey, Austin White, Andrew Sikkens, Justin Laungkhot, Caroline Land, Amber Smith, Cale Patterson, Hilliary Patterson, Presley Townsend, and Brian Simpson, for their countless hours of labor and support that helped make this project possible. Thank you to colleague and collaborator Roelof Sikkens who helped with many different aspects of this project. Thank you to the staff of the Tennessee Valley Research and Extension Center and the Plant Science Research Center whose extensive time and care was put into the experimental trials at each location.

I would also like to extend a very special thank you to my wife, Danielle, her never ending support and devotion was a blessing to have through this whole challenging experience. Thanks also to each and every member of my family for their support, encouragement, and prayers they continued to provide me with along the way.

Table of Contents

Abstract	ii
Acknowledgments	iv
List of Tables	vii
List of Figures	viii
Chapter I: Introduction and Review of Literature.....	1
Introduction	1
<i>Rotylenchulus reniformis</i> life cycle	2
<i>Rotylenchulus reniformis</i> resistance in upland cotton	3
Resistance to <i>Rotylenchulus reniformis</i> in “Wild Type” cotton species	4
Current nematode management practices.....	8
Literature cited	10
Chapter II: Nematicide enhancements of <i>Rotylenchulus reniformis</i> resistant genotypes.....	14
Abstract	14
Introduction.....	15
Materials and Methods.....	17
Results	23
Discussion.....	28
Literature Cited	32
Appendix.....	35

Chapter III: Evaluation of the Effects That <i>Bacillus firmus</i> GB-126 Have on Plant-parasitic Nematodes <i>in Vitro</i> and Split-Root Experiments	45
Abstract	45
Introduction	46
Materials and Methods	48
Results	50
Discussion	52
Literature Cited	55
Appendix	58
Overall Conclusion	64

List of Tables

Table 1. Mean <i>Rotylenchulus reniformis</i> egg population densities, eggs per gram of root, and plant heights at 45 DAP in the initial greenhouse trial.	48
Table 2. Mean <i>Rotylenchulus reniformis</i> egg population densities, eggs per gram of root, and plant heights at 45 DAP in the second greenhouse trial.	49
Table 3. Mean <i>Rotylenchulus reniformis</i> vermiform life stages per 150 cm ³ at 30 and 60 DAP, plant heights at 45 and 75, and seed cotton yields in the initial microplot trial.....	50
Table 4. Mean <i>Rotylenchulus reniformis</i> vermiform life stages per 150 cm ³ at 30 and 150 DAP, plant heights at 45 and 75, and seed cotton yields in the second microplot trial.....	51
Table 5. Mean <i>Rotylenchulus reniformis</i> vermiform life stages per 150 cm ³ at 30 and 60 DAP, plant heights at 45 and 75, and seed cotton yields in the first year's field trial.....	52
Table 6. Mean <i>Rotylenchulus reniformis</i> vermiform life stages per 150 cm ³ at 30 and 60 DAP, plant heights at 45 and 79, and seed cotton yields in the second year's field trial.	53

List of Figures

- Figure 1. Infective stage *Rotylenchulus reniformis* in a cotton root: (A) Two infective stage females that have entered a LONREN genotype root, swollen to a kidney shape but are not producing eggs. (B) An infective stage female that has entered a susceptible genotype root and was able to produce eggs. 14
- Figure 2. When the LONREN genotypes were introduced to a field with high levels of *Rotylenchulus reniformis* populations, severe stunting occurred (A). When these same LONREN genotypes were introduced to a field that was absent of *R. reniformis*, no stunting was observed resulting in a more uniform stand at the seedling stage (B). 15
- Figure 3. Phenotypic response of LONREN derived resistant lines A107 (A) and B103 (B) at 45 DAP in greenhouse conditions. Nematicide treatments provided significant increases in plant heights over the untreated controls. 44
- Figure 4. Phenotypic response of reduced stunting of LONREN derived genotype B103 at 45 DAP in response to nematicides. Aldicarb or Abamectin/thiodicarb + Aldicarb provided significant increases in early season plant growth parameters. 45
- Figure 5. *Rotylenchulus reniformis* egg population means at 100 DAP in the 2012 field trial. Standard errors are shown to separate statistical difference of egg populations supported by each genotype. All resistant genotypes supported significantly fewer egg populations per gram of root compared to the cultivar DP393 at the $P \leq 0.10$ significance level. 46
- Figure 6. The phenotypic response of the LONREN derived resistant breeding line B103 was vivid throughout all greenhouse (A) and field trials (B)..... 47
- Figure 7. The radical from germinated soybean seeds were split evenly with a fine razor blade and separate halves were placed in two different conetainers (A). Plants were given 10 days to establish two separate root systems into each conetainer (B)..... 67
- Figure 8. Split Root Corn Assay. Corn seeds were planted in polystyrene cups with the bottom removed and positioned equally over two separate plastic growth pots. The corn plant's fibrous root system allows two independent root halves to naturally form into each of the two plastic pots..... 68
- Figure 9. *Bacillus firmus* GB-126 mobility assay. (A) Live cell effects on *Heterodera glycines* second stage juvenile mobility over a period of 48 h and (B) cell-free extract effects on *Heterodera glycines* second stage juvenile mobility over a period of 48 h..... 69

Figure 10. Means of percentages and standard errors for the effects that *Bacillus firmus* GB-126 live cells had on egg hatching of *Meloidogyne incognita* at 96 hours after inoculation (A) and the effects that *Bacillus firmus* GB-126 cell-free extracts had on egg hatching *Meloidogyne incognita* at 96 hours after inoculation (B). 70

Figure 11. Means of percentages and standard errors for the effects that *Bacillus firmus* GB-126 live cells had on egg hatching of *Heterodera glycines* at 9 days after inoculation (A) and the effects that *Bacillus firmus* GB-126 cell-free extracts had on egg hatching *Heterodera glycines* at 9 days after inoculation (B)..... 71

Figure 12. Least square means for the effects when applying *Heterodera glycines* and *Bacillus firmus* to opposite root halves, a translocatable effect was evident resulting in a significant decrease ($P < 0.033$) in second stage juveniles (A). No translocatable effect was observed in the *Meloidogyne incognita* split root assay on corn (B). 72

Chapter I: Introduction and Review of Literature

Introduction

The reniform nematode, *Rotylenchulus reniformis* Linford and Olivia 1940, is one of the most damaging pests to cotton crops grown in the southeastern region of the United States. The reniform nematode is a semi-endoparasitic nematode that is widely distributed in subtropical and tropical regions of the world (Robinson, 2002). The first report of the reniform nematode as a pest in cotton was in 1940 (Smith, 1940). In the southeast region of the U.S., the reniform nematode has become a yield limiting problem in cotton production (Koenning et al., 2004). In 2010, an estimated 213,627 bales were lost in the United States due to damage caused by the reniform nematode, with Alabama, Louisiana, and Mississippi having the largest yield reduction impacts (Blasingame et al., 2011). The in-furrow use of the insecticide/nematicide, aldicarb known as Aldicarb 15 G, at planting has been the primary management practice of early season insects and reniform nematodes for several years. Significant yield increases have been observed when aldicarb has been applied at planting (Burmester et al., 1996; Lawrence et al., 1990). The recent call for the phase out process of aldicarb has increased the efforts to find alternative management strategies for reniform nematodes. Host plant resistance within cotton would be the optimal alternative for management of reniform nematodes. However, suitable sources of resistance have not been found in upland cotton (*Gossypium hirsutum*) cultivars that are available to cotton producers (Robinson *et al.*, 1999; Usery et al., 2005). Robinson et al. (1999) reported that out of 55 upland cotton species tested, all of them were highly susceptible. Similarly, Usery et al. (2005) reported that out of 52 commercial cultivars of upland cotton

tested, none were resistant to *R. reniformis*. Weaver et al. (2007) also reported that out of 1973 accessions screened only seven supported lower populations than the control PM 1218, none of these seven were considered to have any significant levels of resistance. This study also indicated that to find useful resistant in cotton improvement strategies, introgression of reniform resistant genes will be needed for long term breeding goals.

***Rotylenchulus reniformis* life cycle**

The *Rotylenchulus reniformis* life cycle starts with the development of a one-celled egg that has been produced by a fully mature female. The first stage juvenile (J1) is formed in this egg and undergoes the first molt (Nakasono, 1973). The second stage juvenile (J2) hatches from the egg at 1 to 3 days after formation (Sivakumar and Seshadri, 1971). At this stage the nematode has taken a vermiform shape and a stylet can be observed. The J2 completes two more molts which produce a fourth stage juvenile (J4). The J4 stage is the stage of sexual differentiation in which half of the nematodes will become infective females and the other half develop into non-infective males (Dasgupta and Raski, 1968). The females infect plants by penetrating the root cortex of the host plant intracellular (Rebois, 1980). Once the females have penetrated the roots they establish a feeding site in the stele of the plant (Rebois, 1980). The female develops into a kidney shape form 6 to 15 days after initial infection. Once the female has developed into a kidney shape, she is considered to be mature and can attract males by secreting a gelatinous substance from the vaginal area (Robinson et al., 1997; Sivakumar and Seshadri, 1971). Males are attracted to the female and carry out sexual reproduction and fertilize the female. Mature females lay their eggs in a gelatinous matrix that is formed on the posterior portion of her body (Robinson et al., 1997). The female generally produces up to 60 eggs within these gelatinous egg matrixes but in some cases up to 200 eggs have been observed (Sivakumar

and Seshadri, 1971). The complete life cycle of *R. reniformis* is carried out in 17 to 23 days depending on temperature and other environmental factors such as moisture availability (Birchfield, 1962; Robinson et al., 1997).

***Rotylenchulus reniformis* resistance in upland cotton**

There are currently many different management strategies for plant parasitic nematodes being practiced in cotton production today. These management strategies include chemical control, biological control, crop rotation, weed management, and host plant resistance (Robinson 2007). Host plant resistance is an important management practice that has potential and needs to be efficiently exploited into nematode management (Starr et al., 2002). Plant resistance to nematodes is described as the plant's capacity to inhibit nematode reproduction (Roberts, 2002). Long term benefits from plant resistance to nematodes include greater yield potential, higher profits for producers, and reduced cost for consumers (Boerma and Hussey, 1992). Utilization of resistant cultivars can reduce nematode populations in infested fields to a level that minimal damage can be found in the following crop (Roberts, 2002). As enticing as these benefits of plant resistance sound, a grower's ability to implement this tactic is limited due to a lack of developed resistant cultivars.

Previous research efforts to find resistance in primitive accessions of *G. hirsutum* have yielded minimal success. Yik and Birchfield (1984) screened 110 *G. hirsutum* accessions and found three accessions that supported significantly fewer reniform nematodes than the susceptible check 'Deltapine 16'. Robinson and Percival (1997) screened forty-six *G. hirsutum* accessions and reported that no appreciable resistance was found in any of the *G. hirsutum* accessions that were tested when compared to the susceptible check 'Deltapine 16'. This test also included the three *G. hirsutum* accessions that Yik and Birchfield (1984) reported as resistant.

Robinson et al., (2004) screened 1866 primitive accessions of *G. hirsutum* for resistance to the reniform nematode and found only six accession that were considered moderately resistance as compared to the susceptible check ‘Deltapine 16’. These six *G. hirsutum* accessions had a range of less than 10% to greater than 34% of *R. reniformis* when compared to the susceptible check ‘Deltapine 16’.

Resistance to *Rotylenchulus reniformis* in “Wild Type” cotton species

Gossypium longicalyx Hutch. and Lee is an exotic cotton species that has been determined to be resistant to reniform nematodes. Yik and Birchfield (1984) tested four different *G. longicalyx* accessions for resistance and reported that all females that entered the root of these accessions never took the kidney shape form and no eggs were produced by the females. This source of resistance has been described as the absence of egg production once the female has established a feeding site within the roots of *G. longicalyx* (Agudelo et al., 2005). In 2007, Robinson et al. was able to introgress resistance to reniform nematode from *G. longicalyx* into upland cotton *G. hirsutum* in the attempt to provide the cotton industry a source of resistance to use in management strategies. *G. armourianum* Kearney (a hybrid cross from *hirsutum* + *longicalyx* + *armourianum*) and *G. herbaceum* L. (a hybrid cross from *hirsutum* + *herbaceum* + *longicalyx*) were used as bridges to perform backcrosses with *G. hirsutum*. These two hybrid species were previous developed by Bell and Robinson (2004). In April 2007, the United States Department of Agriculture (USDA) released two germplasm lines LONREN-1 and LONREN-2 (Starr et al., 2007).

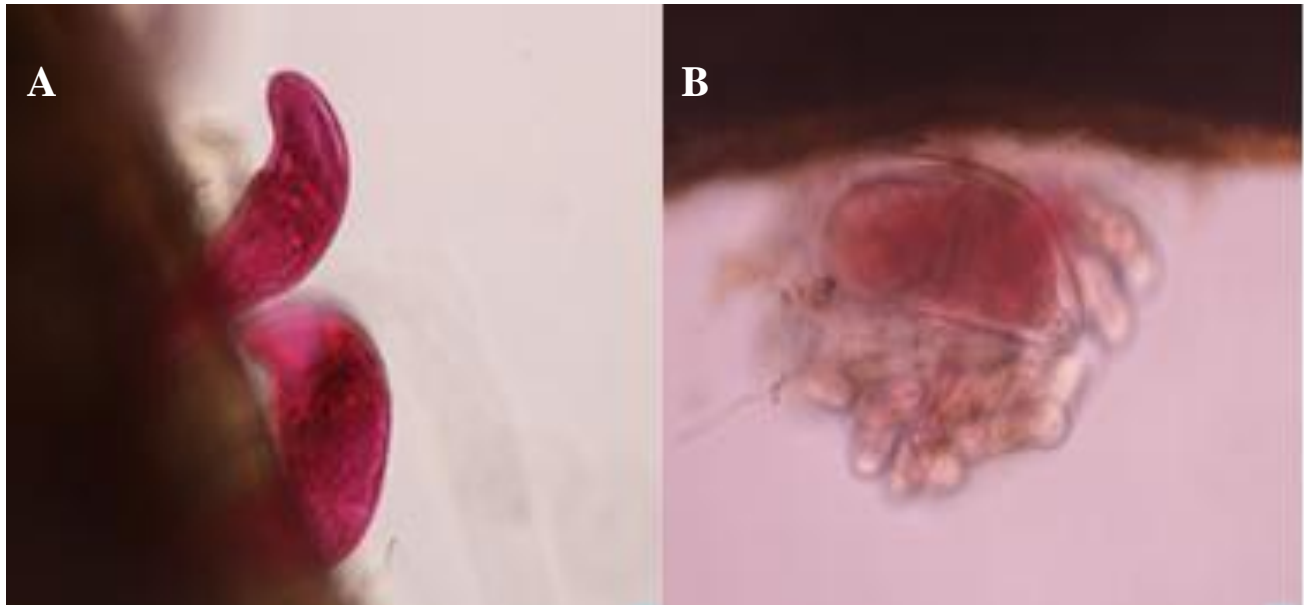


Figure 1. Infective stage *Rotylenchulus reniformis* in a cotton root: (A) Two infective stage females (80x) that have entered a LONREN genotype root, swollen to a kidney shape but are not producing eggs. (B) An infective stage female (80x) that has entered a susceptible genotype root and was able to produce eggs.

The ability to reduce reniform populations and increase fiber strength have been observed thus far from the LONREN breeding lines. Since released in April 2007, LONREN lines have decreased reniform populations in greenhouse and field trial experiments (Bell et al., 2009; Weaver et al. 2011). Bell et al. (2009) evaluated forty-one LONREN lines for resistance to reniform nematodes and found that all 41 lines reduced populations to < 5% as compared to susceptible checks. Reniform populations in field trial experiments also were reduced by 50% – 90% when comparing populations at planting to populations in the following fall. Similar results were found when Weaver et al. (2011) evaluated 20 resistant LONREN-1 X Fibermax 966 breeding lines for their capacity to reduce reniform populations. In this study, reniform populations were greatly reduced in the LONREN-1 X Fibermax 966 breeding lines as compared

to susceptible checks. Fiber quality from LONREN lines was also found to be desirable with an increase in fiber strength (Bell et al., 2009; Weaver et al., 2011). Furthermore, when LONREN resistant lines were planted in field trials with high reniform nematode populations, severe stunting (Figure 2), delayed physiological development, and significant yield loss were evident when compared to susceptible lines (Bell et al., 2009; Weaver et al. 2011). In corresponding field trials that was absent of reniform nematodes (Figure 2), no significant yield loss was observed in the LONREN lines when compared to susceptible lines (Weaver et al., 2011).



Figure 2: When the LONREN genotypes were introduced to a field with high levels of *Rotylenchulus reniformis* populations severe stunting occurred (A). When these same LONREN genotypes were introduced to a field that was absent of *R. reniformis*, no stunting was observed resulting in a more uniform stand at the seedling stage (B).

The cotton plant stunting phenomenon that first appears early in the growing season with LONREN lines has generated different hypotheses by researchers working within this project.

Bell et al. (2009) found that the use of PSII herbicides presented a positive correlation with the early season stunting of LONREN lines in a growth chamber experiment. It was later determined that stunting had occurred in field trial experiments where PSII herbicides were not used (Nichols et al., 2010). Sikkens et al. (2011) hypothesized three different circumstances for the stunting phenomenon. The three different hypotheses were that LONREN resistant lines were sensitive to the photosynthesis inhibition of PSII herbicides; LONREN lines had a heightened hypersensitive reaction when high levels of nematodes are present, and LONREN lines are more sensitive to other seedling pathogens. The results of Sikkens et al. study indicated that a hypersensitive reaction of the plant may be the primary reason for the early season stunting associated with LONREN lines. The hypersensitive reaction is best described as a defense mechanism that the plant uses to fight off attacks from pathogens (Goodman and Novacky, 1994). In nematology, the hypersensitive reaction occurs when the host plant kills off cells surrounding the feeding site that has been established by the nematode. Agudelo et al. (2005) found that death and collapse of cells surrounding the area where female nematodes enter the roots of *G. longicalyx* plants indicate that there is a plant hypersensitive reaction to nematodes within this species.

Gossypium barbadense Linn. is another wild type species of cotton that has shown to suppress reniform reproduction to levels that would classify them as resistant. Yik and Birchfield (1984) reported that out of six *G. barbadense* germplasm lines tested, the germplasm line TX-110 was classified as highly resistant and supported only 8% of *R. reniformis* egg production found on the susceptible check Deltapine 16. Robinson and Percival (1997) reported that two *G. barbadense* accessions, TX-1347 and TX-1348, supported significantly fewer *R. reniformis* eggs than the susceptible check Deltapine 16. Later studies by Robinson et al. (2004) found that five

G. barbadense accessions, GB-49, GB-13, GB-264, GB-171, and GB-713, supported less than 11% of the egg production found on susceptible check Deltapine 16 thus they classified these as resistant to *R. reniformis*. Furthermore, in this study only 3% of *R. reniformis* egg production was observed on the *G. barbadense* accession GB-713. Due to the high levels of *R. reniformis* resistance found in the *G. barbadense* accession GB-713, scientists are focusing their research efforts on the introgression of the resistant genes from this species into the upland cotton species *Gossypium hirsutum*.

Current nematode management practices

Current nematode management in cotton production is primarily focused around non-host crop rotations and the use of chemical nematicides such as abamectin (Avicta), aldicarb (Aldicarb 15G), oxamyl (Vydate), thiodicarb (Aeris), and 1,3dichloropropene (Telone). The use of aldicarb as an in furrow application at planting has been the most predominant control of the reniform nematode in cotton for the past two decades (Koenning et al. 2004). Numerous research studies throughout the southeast have indicated that using aldicarb at the rates of 1.4 to 3.2 kg/ha suppress nematode pressure in the early season leading to an increase in yields (Baird et al., 2000; Burmester et al., 1996; Erwin et al., 2002, Lawrence et al., 1990; Rich and Kinloch, 2000). In 2006 and 2007, both seed treatments abamectin and thiodicarb were introduced to the market for nematode management in cotton. These new seed treatments allowed growers to incorporate a safer and more environmentally sound management options into their productions systems. Lawrence and Lawrence (2007) reported that over a series of field trials conducted by University and Extension scientists, the seed treatments abamectin and thiodicarb produced statistically similar increase in cotton yields as compared to the in-furrow treatment of aldicarb. Over the years results from scientists evaluating the efficacy of chemical nematicides have been variable

and somewhat non-conclusive (Koenning et al., 2004). One of the most common trends with the research efforts with chemical nematicides is that the suppression of early season nematode pressure often leads to an increase in yields at harvest.

The overall hypothesis of this study is that nematicides applied to selected LONREN breeding lines will suppress nematode populations and reduce damage in the cotton seedling stage, diminishing the hypersensitive reaction of our resistant lines. The supporting objectives of this hypothesis are: 1.) Evaluate reniform nematode populations on LONREN derived breeding lines with and without nematicides; 2.) Evaluate the effects that applying nematicides have on early seedling stunting of LONREN breeding lines; and 3.) Evaluate overall yield performances when nematicides are applied to LONREN breeding lines. The overall goal of this project is to provide protection from *R. reniformis* to our newly developed reniform resistant breeding lines. This protection provided by the nematicides will increase plant health allowing these lines to produce high yields and desirable lint, especially in fiber strength, and allow them to perform in a way that is significant to U.S. cotton production in areas where reniform nematodes create problems. Cotton production in the mid-south and southeastern U.S. is in desperate need of reniform resistant cultivars.

Literature Cited

- Agudelo, P., Robbins, R.T., Stewart, J.M., Bell, A., and Robinson, A.F. 2005. Histological observations of *Rotylenchulus reniformis* on *Gossypium longicalyx* and interspecific cotton hybrids. *Journal of Nematology* 37:444–447.
- Bell, A.A., Starr, J.L., Jones, J.E., Lemon, R., Nichols, R.L., Overstreet, C., and Stelly, D.M.. 2009. Nematode resistant and agronomic performance of LONREN and NEMSTACK lines. *Proceedings of the Beltwide Cotton Conferences* 1:178. National Cotton Council of America, Memphis, TN.
- Birchfield, W. 1962. Host-parasite relations of *Rotylenchulus reniformis* on *Gossypium hirsutum*. *Phytopathology* 52:862–865.
- Blasingame, D., Patel, M. V., Lawrence, K., Olsen, M., Kirkpatrick, T., Davis, M., Marios, J., Kemerait, B., Colyer, P., Scuibato, G., Wrather, A., Goldberg, N., Koenning, S., Pitts, J.T., Mueller, J., Newman, M., Woodward, J., Phipps, P. 2011. 2010 Cotton disease loss estimate committee report. *Proceedings of the Beltwide Cotton Conferences* 1:306–308. National Cotton Council of America, Memphis, TN.
- Boerma, H.R. and Hussey, R.S. 1992. Breeding Plants for Resistance to Nematodes. *Journal of Nematology* 24:242–252.
- Burmester, C.H., Gazaway, W.S., Potter, D.J., Ingram E.G. 1996. Rates and timing of nematicides for control of reniform nematodes in northern Alabama. *Proceedings of the Beltwide Cotton Conferences* 1:306–308. National Cotton Council of America, Memphis, TN.

- Dasgupta, D.R., and Raski, D.J. 1968. The biology of *Rotylenchulus parvus*.
Nematologica 14:429–440.
- Koenning, S.R., Kirkpatrick, T.L., Starr, J.L., Walker, N.A., Wrather, J.A., and Mueller, J.D.
2004. Plant-parasitic nematodes attacking cotton in the United States: Old and emerging
production challenges. Plant Disease 88: 100–113.
- Lawrence, G.W., Mclean, K.S., Batson, W.E., Miller, D., and Borbon, J.C., 1990. Response of
Rotylenchulus reniformis to nematicide applications on cotton. Journal of Nematology
22:707–711.
- Rebois, R.V. 1980. Ultrastructure of a feeding peg and tube associated with *Rotylenchulus*
reniformis in cotton. Nematologica 26:396–405.
- Roberts, P.A. 2002. Concepts and consequences of resistance. Pages 23-41 in: Plant resistance to
parasitic nematodes. J.L. Starr, R. Cook, and J. Bridge, eds. CABI Publishings,
Wallingford, UK.
- Robinson, A.F., Inserra, R.N., Caswell-Chen, E.P., Vovlas, N., and Troccoli, A. 1997.
Rotylenchulus species: Identification, distribution, host ranges, and crop plant resistance.
Nematropica 27:127–180.
- Robinson, A.F. 2002. Reniform Nematodes: *Rotylenchulus* Species. Pages 153–174 in: Plant
resistance to plant-parasitic nematodes. J.L. Starr, R. Cook, and J. Bridge, eds. CABI
Publishings, Wallingford, UK.
- Robinson, A.F. 2007. Reniform in U.S. Cotton: When, Where, Why, and Some Remedies.
Annual Review of Phytopathology. 45:263–288.

- Robinson, A.F., Bell, A.A., Dighe, N.C., Menz, M.A., Nichols, R.L., and Stelly, D.M., 2007. Introgression of resistance to nematode *Rotylenchulus reniformis* into upland cotton (*Gossypium hirsutum*) from *Gossypium longicalyx*. *Crop Science* 47:1865–1877.
- Robinson, A.F., and Percival, A.E. 1997. Resistance to *Meloidogyne incognita* race 3 and *Rotylenchulus reniformis* in wild accessions of *Gossypium hirsutum* and *G. barbadense* from Mexico. *Journal of Nematology* 29:746–755.
- Sikkens, R.B., Wu, T., Weaver, D.B., Moore, S.R., and Lawrence, K.S. LONREN germplasm response to reniform nematode population levels. *Proceeding of the Beltwide Cotton Conferences* 1:728–736. National Cotton Council of America, Memphis, TN.
- Sivakumar, C.V. and Seshadri, A.R. 1971. Life history of the reniform nematode *Rotylenchulus reniformis* Linford and Oliveira, 1940. *Indian Journal of Nematology*. 1:7–20.
- Smith, A.L. 1940. Distribution and relation of meadow nematode, *Pratylenchulus pratensis*, to Fusarium of cotton in Georgia. *Phytopathology* 30:710.
- Starr, J.L., Bridge, J., and Cook, R. 2002. Resistance to Plant-parasitic Nematodes: History, Current Use, and Future Potential. Pages 1–22 in: *Plant Resistance to Parasitic Nematodes*. J.L. Starr, R. Cook, and J. Bridge, eds. CABI Publishing, Wallingford, UK.
- Starr, J.L., Koenning, S.R., Kirkpatrick, T.L., Robinson, A.F., Roberts, P.A., and Nichols R.L. 2007. The future of nematode management in cotton. *Journal of Nematology* 39:283–294.
- Usery, S.R., Lawrence, K.S., Lawrence, G.W., and Burmester, C.H. 2005. Evaluation of cotton cultivars for resistance and tolerance to *Rotylenchulus reniformis*. *Nematropica* 35:121–133.

Weaver, D.B., Sikkens, R.B., Sahrpe, R.R., Moore, S.R., and Lawrence, K.S. 2011. LONREN X FM966 progeny evaluation in a field infested with reniform nematodes. Proceedings of the Beltwide Cotton Conferences 1:737–741.

Yik, C. P. and Birchfield, W. 1984. Resistant germplasm in *Gossypium* species and related plants to *Rotylenchulus reniformis*. Journal of Nematology 16:146–153.

Chapter II. Nematicide enhancements of *Rotylenchulus reniformis* resistant cotton genotypes

Abstract

Germplasm lines LONREN-1 and LONREN-2 were released in 2007 for cotton breeders to incorporate *R. reniformis* nematode resistance into breeding efforts with desirable cultivars to establish nematode resistant high yielding cultivars. Previous screenings for *R. reniformis* resistance in the LONREN-1 X FM966 breeding lines developed at Auburn University have demonstrated that the nematode resistance is accompanied by severe stunting and limited plant growth followed by low yields. The objectives of this study were to evaluate the effects that applying nematicides to selected LONREN breeding lines have on *R. reniformis* nematode populations, early seedling plant stunting, yield, and fiber quality. Three resistant breeding lines from the LONREN-1xFM966 cross, one susceptible line from the LONREN-1xFM966 cross, and the susceptible cultivar DP393 were treated with nematicides and their performances evaluated. In the greenhouse, nematicides increased plant heights in the resistant lines. Nematicides further reduced reniform populations in the resistant lines 45 days after planting (DAP). *Rotylenchulus reniformis* populations were 50% lower in the resistant lines compared to the susceptible lines by the end of the growing period. In microplot and field trials, the phenotypic stunting response of the resistant lines was reduced by nematicides with increases in plant heights at 30 and 75 DAP. Increases in yields were also evident in the resistant breeding lines that were treated with nematicides.

Introduction

The reniform nematode (*Rotylenchulus reniformis*) is considered the most damaging pests to cotton grown in Alabama and causes significant yield losses in many southeastern states of the United States. Resistant cultivars to this pathogen have not been available to growers and research efforts are looking into wild cotton relatives to establish a source (Usery et al. 2004). Breeding efforts have turned to LONREN and BARBREN germplasm lines to use in crosses with upland cotton cultivars. The LONREN genetic material was developed from an exotic cotton species, *Gossypium longicalyx* Hutch. and Lee, that is resistant to reniform nematodes. Yik and Birchfield (1984) tested four different *G. longicalyx* accessions for resistance and reported that all females that entered the root of these accessions never took the kidney shape form and no eggs were produced by the females. This type of resistance has been described as the absence of egg production once the female has established a feeding site within the roots of *G. longicalyx* (Agudelo et al., 2005). Robinson et al. (2007) was able to introgress resistance to reniform nematodes from *G. longicalyx* into upland cotton *G. hirsutum* in the attempt to provide the cotton industry a source of resistance to use in management strategies. The BARBREN genetic material comes from the exotic cotton species *Gossypium barbadense*, another wild type species of cotton that has shown to suppress reniform reproduction to levels that would classify them as resistant. Yik and Birchfield (1984) reported that out of six *G. barbadense* germplasm lines tested the germplasm line TX-110 was classified as highly resistant and supported only 8% of *R. reniformis* egg production found on the susceptible check Deltapine 16. Robinson and Percival (1997) reported that two *G. barbadense* accessions, TX-1347 and TX-1348, supported significantly fewer *R. reniformis* eggs than the susceptible check Deltapine 16. Later studies by

Robinson et al. (2004) found that five *G. barbadense* accessions, GB-49, GB-13, GB-264, GB-171, and GB-713, supported less than 11% of the egg production found on susceptible check Deltapine 16 thus they classified these as resistant to *R. reniformis*. Furthermore, in this study only 3% of *R. reniformis* egg production was observed on the *G. barbadense* accession GB-713. Due to the high levels of *R. reniformis* resistance found in the *G. barbadense* accession GB-713, scientists are focusing their research efforts on the introgression of the resistant genes from this species into the upland cotton species *Gossypium hirsutum*.

In April of 2007, the United States Department of Agriculture (USDA) Texas AgriLife, and Cotton Incorporated released two germplasm lines LONREN-1 and LONREN-2 (Starr et al., 2007). LONREN derived breeding lines have shown the potential to reduce reniform nematode populations in previous greenhouse and field trials (Bell et al., 2009; Weaver et al. 2011). The LONREN derived lines have also had superb fiber quality in previous field trials (Bell et al., 2009; Weaver et al., 2011). It was later reported that LONREN derived lines were introduced to high levels of reniform populations, early season stunting occurred indicating that these lines were intolerant to the initial attack from the reniform nematode (Nichols et al., 2010, Sikkens et al., 2011). The common response to nematode attack in the host plants carrying a resistance gene is an early hypersensitive reaction (HR) which results in cell death around the nematode feeding site preventing nematode feeding and inducing nematode death (Sayan Das et al, 2008). Intolerance describes this nematode resistant reaction when the cell death is also damaging to the host plant. Visually plants are stunted, root systems are smaller and plant yields are compromised.

The BARBREN germplasm line (BARBREN-713) was released in 2012 to cotton breeders to use in crosses with upland cotton as well. In 2012, Sikkens et al. reported that this

line did not display any intolerant responses to the reniform nematode, supported lower populations of reniform and had comparable yields to the susceptible lines. The BARBREN-713 germplasm line may be a very good alternative source for reniform resistance but due to the novelty of this genetic materials response to reniform nematodes more evaluations are needed.

The overall hypothesis of this study is that nematicides applied to selected LONREN breeding lines will suppress nematode populations and reduce the intolerant response and subsequent damage to cotton seedlings, diminishing the hypersensitive reaction of our resistant lines. The supporting objectives of this hypothesis are: 1.) Evaluate reniform nematode populations on LONREN derived breeding lines with and without nematicides; 2.) Evaluate the effects that applying nematicides have on early seedling stunting of LONREN breeding lines; and 3.) Evaluate overall yield performances when nematicides are applied to LONREN breeding lines. The overall goal of this project is to provide protection from *R. reniformis* to our newly developed LONREN derived reniform resistant breeding lines. This protection provided by the nematicides will increase plant health allowing these lines to produce high yields and desirable lint, especially in fiber strength, and allow them to perform in a way that is significant to U.S. cotton production in areas where reniform nematodes create problems. Cotton production in the mid-south and southeastern United States is in desperate need of reniform resistant cultivars.

Materials and Methods

Experimental Design

In 2011 and 2012, trials were conducted in the greenhouse, microplots, and field to determine if the application of a nematicide would benefit the growth and yield of *R. reniformis* resistant intolerant cotton genotypes. In the first year's trials; 6 genotypes were evaluated in a

6x2 factorial design with genotypes being the main factor and the second factor was the addition of a nematicide or nothing. The genotypes entered in this study included the *R. reniformis* resistant germplasm line LONREN-1, resistant breeding lines A107, A122, and B219 that were derived from the cross LONREN-1 x FM966. These resistant lines were compared to the *R. reniformis* susceptible cultivar Deltapine 393 (DP393) and susceptible breeding line B211, also derived from the LONREN-1 x FM966 cross. The resistant lines selected for this study reduced nematode populations in the 2009 field trial screenings and appeared highly resistant to *R. reniformis* in previous greenhouse screenings (Sikkens et al., unpublished data). The selected lines produced superior yields with excellent fiber qualities, especially in fiber strength. The second factor in this test was the addition of 2 nematicide seed treatments, abamectin applied at 0.15 mg ai/seed and thiodicarb applied at 0.375 mg ai/seed or no nematicide application at all. All seed were treated with the standard fungicides thiram at .002 mg ai/seed, metalaxyl at 0.0003 mg ai/seed, and ipconazole at 0.0001 mg ai/seed to manage seedling disease, and the insecticide imidacloprid at 0.34 mg ai/seed to reduce thrip damage in the first 4 weeks after planting. The 12 total genotype treatment combinations tested in 2011 were arranged in a randomized split block design with 5 replications and each test was repeated for a total of 120 experimental units. Identical tests were conducted in the greenhouse house, microplots, and field.

In the second year's trials, the same experimental design was repeated and expanded to an 8x4 factorial to include the *R. reniformis* resistant LONREN-1 X FM966 derived breeding line B103 and the resistant Barbren germplasm line BARBREN-713 as well as two additional nematicide treatments. The 4 nematicide treatments for 2012 were 1) An in furrow application at planting of aldicarb at the rate of 840 g ai/ ha; 2) The nematicide seed treatments abamectin and thiodicarb previously described combined with an in furrow application at planting of aldicarb of

840 g ai/ ha; 3) The nematicide seed treatments abamectin and thiodicarb alone, and 4) an untreated control. The 32 total genotype and treatment combinations were arranged in a 4 row split plot design, with 5 replications and repeated twice for a total of 320 experimental units

Rotylenchulus reniformis inoculum preparation

The cultures of *Rotylenchulus reniformis* used to inoculate the greenhouse and microplot experimental units in this study were extracted from 500 cm³ polystyrene stock cultures maintained in the greenhouse. These stock pots of *R. reniformis* infested cotton plants were allowed to grow in greenhouse conditions for 60 days in order to increase inoculum levels to desired quantities. *Rotylenchulus reniformis* juvenile and vermiform adult stages were extracted from the soil using gravitational sieving followed by sucrose centrifugation 1.14 sp. g. Eggs were collected from the roots of the cotton plants by shaking the roots on a rotary shaker for four minutes in a 0.625 % NaOCl. Eggs were washed with water over nested 75 µm and 25 µm sieves. The *R. reniformis* eggs and vermiform adult and juvenile stages were enumerated at 40 X with a Nikon TSX inverted microscope. The nematode inoculum was combined and added to naturally infested soils used in greenhouse and microplot trials to standardized population averages similar to those found in the field at the Tennessee Valley Research and Experiment (TVREC) located near Belle Mina, Alabama.

Greenhouse Trials

The performance of *R. reniformis* resistant genotypes, with and without nematicides, was evaluated for their ability to subdue early season nematode pressure and prevent seedling stunting in a controlled greenhouse environment. Two separate greenhouse trials were established at the Plant Science Research Center located in Auburn, Alabama. The previous described 6x2 and 8x4 factorial design were planted in 500 cm³ polystyrene pots containing a

Decatur silt loam soil (% sand, silt, clay of 23-49-28) collected from the TVREC field location. This Decatur silt loam soil contained a population of *R. reniformis* within its microflora estimated to be 3,750 vermiform adult and juvenile life stages per 500 cm³ of soil. Before the cotton seeds were planted, a mixture of 1,250 *R. reniformis* vermiform and egg life stages were pipetted into each polystyrene pot to standardize the nematode population to 5,000 *R. reniformis* per 500 cm³ of soil. One seed from each of the genotypes was planted per polystyrene pot. The plants were allowed to grow for forty-five days. Plant heights were recorded at 45 days after planting (DAP). *Rotylenchulus reniformis* egg populations were extracted from the roots as previously described. Numbers of eggs per gram of root were determined.

Microplot Trials

In the first year's trial the performance of the 6 genotypes, with and without nematicides, was evaluated for their ability to subdue early season nematode pressure, prevent seedling stunting, and increase overall yields. This microplot experiment allowed the genotypes to be evaluated in an outside micro-managed environment that will be somewhat similar to field type conditions, thus allowing the genotypes to reach full maturity. These microplot trials were also established at the Plant Science Research Center located on the campus of Auburn University in Auburn, AL. The same 6x2 factorial design previously described for year one was set up in 4,500 cm³ containers filled with Decatur silt loam (% sand, silt, clay of 23-49-28) natural field soil collected from the TVREC. Nematode samples were taken to quantify the initial *R. reniformis* population. An additional 3,500 *R. reniformis* egg and vermiform mixed life stages were added to each microplot to standardize the population to 5,000 per 500 cm³ of soil. Four seed from each of the six genotypes were hand planted per container. Parameters measured in the microplot study included *R. reniformis* population counts, plant heights, and seed cotton yields.

Soil samples were taken at 30 and 150 DAP to determine *R. reniformis* vermiform life stage populations. Four soil cores, 15 cm deep, were taken from the interior portion of each microplot using a soil probe. *Rotylenchulus reniformis* juvenile and vermiform adult stages were extracted from the soil using gravitational sieving followed by sucrose centrifugation 1.14 sp. g. Plant heights were also measured at 45 and 75 DAP. At cotton plant maturity, near 150 DAP, each microplot was handpicked and cotton yield was recorded as grams of seed cotton per microplot.

In the second year's microplot trial the experimental design was repeated and expanded to include the B103 and BARBREN 713 lines as previously described. However, the combination of abamectin/thiodicarb + aldicarb treatment was omitted from the second year's microplot trials due to the lack of available space to include all four treatments. The parameters measured were identical to the first year's trial.

Field Trials

In the first year's field trial, the performance of the six genotypes, with and without nematicides, were evaluated for their ability to subdue early season nematode pressure, prevent seedling stunting, and increase overall yield. This field trial experiment allowed the genotypes to be evaluated in an environment similar to what is experienced in actual cotton production. The field is located at the TVREC and has a high reniform nematode population. The same 6x2 factorial design previously described was set up in one row plots that were 7.6 m long on 1.02 m centers. One hundred seed per row from each of the treatment combinations was planted with an Almaco plot planter. Soil samples were taken at planting, 30 DAP, and at harvest to determine *R. reniformis* vermiform life stage populations in the soil. The sampling method consisted of collecting ten 20 cm deep cores at the base of the plants from each one row plot. The 10 cores from each plot were combined to make up a composite sample for each plot and placed in plastic

zippered bags. The samples were transported to a laboratory and a 150cm³ subsample was taken from each sample for extraction using the gravity sieving and sucrose centrifugation methods. Plant heights for each plot were also recorded at 45 DAP and 75 DAP to determine what effects nematicides had on early season stunting. Four plant heights were measured in each of the one row plots and an average plant height was calculated and recorded in centimeters. The plants were measured with a one meter ruler starting at the soil line and measuring to the apical meristem. All plots were machine harvested with a modified Case IH 2022 plot picker and seed cotton yields for each plot were recorded.

The second year's field trials were repeated and expanded to include the B103 and BARBREN 713 line in the 8x4 factorial design previously described. This 8x4 factorial was repeated in two different locations in the nematode infested field at TVREC in 2012. The in furrow application of aldicarb at the rate of 840 g ai/ha was applied the second year at planting with granular applicators that were attached to the planter. All the parameters measured in the second year's trial were conducted as described in the first year with the addition of collecting roots samples to quantify egg numbers. At 100 DAP, one plant including its root system, were dug from each sub-plot to determine levels of resistance for each genotype entry. Each of the root systems were cut from the shoot and combined to make a composite sample for the whole plot (four rows). The root samples were transported to a lab where 1 gm subsamples consisting of small fibrous roots were cut from each composite sample and the eggs were extracted using the 0.625 % NaOCl agitation method. Egg populations were recorded as eggs per gram of root for the whole plot.

Statistical Analysis

Data collected in all trials were analyzed in SAS 9.2 (SAS Institute, Inc.) using the GLIMMIX procedure. Student panel graphs generated were evaluated to determine the normality assumption of the residuals. The plant heights were analyzed using a normal distribution. The periodical *R. reniformis* nematode vermiform life stage populations extracted from the soil and the eggs collected from roots required a lognormal distribution transformation to satisfy the normality assumption. In the LSMEANS command, the PDIFF option was used to differentiate treatments at the $P \leq 0.10$ significance level. The LSMEANS estimates for the lognormal distribution function was transformed back to the original value using PROC MEANS. The original mean values are presented in the tables with P values to determine statistical differences.

Results

Greenhouse trials

In the initial greenhouse trial, no interactions of genotypes by nematicides were evident for total egg population densities, eggs per gram of root, or plant heights (Table 1). The abamectin/thiodicarb seed treatment reduced both total egg population densities and eggs per gram of root by 51 and 50%, respectively. Both the susceptible cultivar DP393 and B211 genotype supported significantly higher total egg densities ($P \leq 0.001$) and eggs per gram of root ($P \leq 0.001$) than their resistant counterparts A107, A122, B219 and LONREN-1. Overall resistant genotypes supported 82% fewer eggs and 74% fewer eggs per gram of root than the susceptible genotypes (Table 1). Significant increases ($P \leq 0.001$) in plant heights were observed when the abamectin/thiodicarb seed treatment was applied to genotypes. The resistant genotypes A107, A122, B219, and LONREN-1 exhibited plant heights that were similar to the susceptible check

B211 indicating a reduction in early season stunting was influenced by the abamectin/thiodicarb seed treatment. Furthermore, the resistant genotype A122 was similar to the cultivar DP393, thus indicating that the abamectin/thiodicarb seed treatment provided protection against early season stunting.

A second greenhouse trial was conducted and expanded to include an aldicarb treatment to further enhance the LONREN derived resistant genotypes. Two additional highly resistance genotypes, B103 and BARBREN 713, were also included to evaluate their responses to nematicide treatments. In this trial no interactions of genotypes by nematicides were evident for total egg population densities, egg per gram of root, or plant heights (Table 2). Significant differences were evident for total egg populations ($P \leq 0.001$) and eggs per gram of root ($P \leq 0.001$) among all genotypes. The susceptible genotypes DP393 and B211 produced total egg populations that were 74% and 71% greater than the resistant genotypes. Nematicides did provide a significant reduction in both total eggs ($P \leq 0.001$) and eggs per gram of root ($P \leq 0.001$). Aldicarb alone and the combination of aldicarb + abamectin/thiodicarb provided significant reductions in total egg densities ($P \leq 0.001$) and eggs per gram of root ($P \leq 0.001$) compared to the untreated controls, respectively. All three nematicide treatments influenced significant increases in plant heights compared to the untreated control ($P \leq 0.006$). The LONREN derived genotypes A107, A122, B219, and LONREN-1 had similar plant heights to the susceptible checks DP393 and B211 indicating that the nematicide treatments provided similar protection to all cotton lines. The BARBREN 713 line exhibited the highest plant heights among all genotypes. The phenotypic response of the LONREN derived genotypes was a visual trend of increasing plant heights and overall biomass where the nematicide options were applied (Figure 3).

Microplot trials

In the first year's microplots *R. reniformis* initial population densities were extremely low, ranging from 39 to 93 vermiforms per 150 cm³ at the 30 DAP sampling period. There was no genotype by nematicide interaction for *R. reniformis* population densities, plant heights, or seed cotton yields (Table 3). *Rotylenchulus reniformis* population densities were similar among all genotypes at 30 DAP. However, by 150 DAP population densities had increased to levels that were 74% higher in the susceptible checks than those found on the resistant genotypes. The abamectin/thiodicarb seed treatments had no effect on *R. reniformis* population densities in the early season sampling period at 30 DAP. Plant heights recorded at 45 DAP and 79 DAP were not affected by abamectin/thiodicarb seed treatments and all genotypes had similar plant heights at these time periods. Seed cotton yields were not affected by nematicides and all genotypes had similar yields. The results of this study supported the findings of Weaver *et al.*, 2011 in which intolerant responses of these LONREN derived genotypes did not occur where high levels of *R. reniformis* were not present.

The experimental design for the second year's microplot trial was expanded to replicate and further investigate the findings of the second greenhouse trial. In this microplot trial, no genotype by nematicide interaction for *R. reniformis* population densities, plant heights, or seed cotton yields occurred (Table 4). At 30 DAP, the resistant genotypes supported 42% lower *R. reniformis* population densities than the susceptible checks. By 150 DAP, *R. reniformis* population densities had increased to levels that were significantly higher ($P \leq 0.001$) in the susceptible checks compared to populations found in the resistant genotypes. Aldicarb applied as an in-furrow treatment provided a significant reduction ($P \leq 0.001$) in *R. reniformis* population

densities in the early season at 30 DAP. Plant heights at 45 DAP for the resistant lines A107, A122, LONREN-1, and BARBREN 713 were similar to the susceptible checks indicating a reduction of early season stunting. Nematicides effects were observed at 45 and 79 DAP (Table 4). A significant increase in plant heights was evident in the aldicarb treatment ($P \leq 0.079$) at 45 DAP and the abamectin/thiodicarb seed treatment and aldicarb treatment both provided significant increases ($P \leq 0.001$) in plant heights at 79 DAP. The resistant genotypes A107, A122, LONREN-1 and BARBREN 713 all had plant heights that were similar to the susceptible check DP393 at 45 DAP indicating that the early season stunting of the LONREN genotypes was reduced. At 79 DAP; plant heights of these same resistant genotypes were still similar to the susceptible check. Seed cotton yields were affected by nematicide treatments (Table 4). The aldicarb treatment significantly enhanced seed cotton yields ($P \leq 0.062$) that were 21% higher than the untreated control. All resistant genotypes exhibited seed cotton yields that were similar to the susceptible cultivar DP393.

Field Trials

In the first year's field trials, no genotype by nematicide interaction was observed for *R. reniformis* population densities, plant heights, or seed cotton yields (Table 5). *Rotylenchulus reniformis* populations among all genotypes were similar in the early season sampling period at 30 DAP. A reduction of 48% of *R. reniformis* population densities was evident for the resistant genotypes by 150 DAP while the susceptible checks exhibited a slight increase in populations. Furthermore, *R. reniformis* population densities were significantly lower ($P \leq 0.005$) in all the resistant genotypes compared to the susceptible cultivar DP393. No nematicide effects were observed on *R. reniformis* population densities at 30 DAP or 150 DAP (Table 5). Early season plant heights at 45 DAP for the resistant genotypes A122 and B219 were similar to the

susceptible checks indicating that early season stunting in the field were reduced. The abamectin/thiodicarb seed treatment provided significant increases in plant heights ($P \leq 0.005$) at 45 DAP compared to the untreated control. Plant heights at 79 DAP were variable among genotypes but nematicide enhancements were still evident in the resistant lines A107 and A122 in which both exhibited heights that were similar to susceptible checks DP393 and B211. Seed cotton yields followed a similar pattern of variability with A107 and A122 exhibiting comparable yields to the susceptible checks.

The 2012 field trials were conducted to replicate the second greenhouse trial that was expanded to include the aldicarb nematicide treatments. The highly resistant genotypes BARBREN 713 and B103 were also included in these field trials. As observed in all greenhouse and microplot trials, no genotype by nematicide interaction occurred in this field trial (Table 6). *Rotylenchulus reniformis* population densities were variable among the LONREN genotypes at 30 DAP, however BARBREN 713 had significantly lower population density than the susceptible cultivar DP393. No nematicide effects on *R. reniformis* population densities were observed at 30 DAP. However, all nematicide options provided numerical data that were lower in *R. reniformis* population densities than the untreated control. By 150 DAP, resistant genotypes supported 52% lower populations than the susceptible genotypes. All resistant genotypes supported significantly fewer ($P \leq 0.001$) *R. reniformis* than the susceptible cultivar DP393 at 150 DAP. At 100 DAP, egg populations for each genotype were evaluated to determine levels of resistance among genotypes. All resistant genotype entries supported significantly fewer eggs at the ($P \leq 0.10$) than the susceptible cultivar DP393 (Figure 5). The lowest egg populations were observed on the LONREN derived genotype B103 and BARBREN 713, and supported 98% and 94% less egg production than the susceptible cultivar DP393. Early season plant heights at 45

DAP were similar in the resistant lines A107, A122, and B219 compared to the susceptible checks. BARBREN 713 exhibited significantly taller plants ($P \leq 0.001$) at 45 DAP than all other genotypes, resistant or susceptible. Similar trends among genotypes were evident in the midseason at 79 DAP with the A122 and BARBREN 713 line both exhibiting significantly taller plants than the susceptible checks. All three nematicides options provided significant increases ($P \leq 0.001$) in plant heights at 45 DAP and the aldicarb alone and aldicarb + abamectin/thiodicarb treatment provided significant increases in plant heights that were still evident at 79 DAP (Figure 4). Yields were similar to the susceptible checks in the resistant lines A107, A122, and LONREN-1. The BARBREN 713 resistant line exhibited significantly higher yields ($P \leq 0.001$) than all other genotypes, resistant or susceptible. The nematicide options influenced significant increases in seed cotton yields, primarily where aldicarb was applied. Aldicarb alone or in combination with abamectin/thiodicarb enhanced yields that were significantly greater ($P \leq 0.001$) than the untreated controls.

Discussion

The primary goal of this study was to determine if we could protect the LONREN derived resistant genotypes from the intolerant response of phenotypic stunting and subsequent yield losses that occur when these genotypes are planted in soils with high population densities of the reniform nematode. Results of our greenhouse, microplot, and field trials indicate that that the resistant lines do support significantly lower reniform population densities not only in the greenhouse and microplots trails but also in the natural field environment as well. The field trials indicated the LONREN derived *R. reniformis* resistant lines do lower nematode population densities at the end of the season and could reduce this pest population numbers to below damage levels. Our results confirm suppression of *R. reniformis* populations observed by Bell et

al., (2009) and Weaver et al. (2011). In initial nematode resistance and agronomic performance trials, LONREN lines greatly suppressed *R. reniformis* populations while yielding superior cotton compared to susceptible lines (Bell *et al.*, 2009). Bell *et al.*, (2009) also reported that gene segments from *G. longicalyx* were responsible for an increase in strength of fiber quality. Weaver *et al.* (2011) reported that the LONREN derived resistant genotypes produced similar amounts of seed cotton to the susceptible industry cultivars, DP 393 and FM 966 in a field without reniform nematodes. These LONREN derived genotypes also had excellent fiber quality in which fiber strength was greater than non-resistant sister lines. Thus these LONREN derived resistant lines could reduce *R. reniformis* populations while producing optimum cotton yields with high quality fiber.

Results of our greenhouse, microplot, and field trials indicate applying aldicarb or aldicarb + abamectin/thiodicarb nematicides at planting to the LONREN derived resistant lines suppressed initial nematode intolerance expressed by the cotton seedling and reduced the phenotypic early season plant stunting. Applying aldicarb at planting for nematode management in cotton production systems has been the industry standard for nematode management for many years (Koenning *et al.*, 2004). Previous research has found early season plant growth stimulation when aldicarb was applied to cotton even in the absence of nematode and insect pests (Reddy et al. 1997). Similar studies have reported plant growth promotion from applying aldicarb at planting to other crops including tobacco and soybeans (Barker and Powell, 1988; Barker et al., 1988). The seed treatments abamectin and thiodicarb were introduced to the market in 2006 and 2007, respectively. These nematicides have provided early season nematode management for the susceptible cotton cultivars across the cotton belt. These nematicides also protected the LONREN derived resistant lines similarly reducing the intolerant phenotypic stunting. This early season

protection would allow these resistant cotton lines to be part of nematode management in the cotton industry in areas where *R. reniformis* is a yield limiting factor.

Individual performances of the LONREN derived breeding lines in this study suggest that the level of resistance was variable across resistant genotypes. The LONREN derived resistant line A122 consistently, across all trials, supported *R. reniformis* population densities that were the highest among the LONREN genotypes. However, these population densities were considerably lower than those found on the susceptible cotton cultivars. Considering the egg data that was collected from the field trial at 100 DAP, this genotype would still be classified as moderately resistant when compared to the susceptible cultivar DP393. This A122 line was consistently the highest yielding LONREN genotype throughout these studies. In contrast, the LONREN derived genotype B103 consistently supported the fewest *R. reniformis* and exhibited the lowest yields. The phenotypic response of B103 was always the shortest, less vigorous plant throughout greenhouse, microplot and field trial evaluations. Due to the severe stunting associated with this B103 line in the presence of *R. reniformis*, the significant increases in plant heights provided from the nematicide treatments were the most vivid in the phenotypic response throughout this study (Figure 6).

The future practicality of the LONREN source of resistance is dependent on initial reniform populations in which this genetic material is introduced to. In our research study, aldicarb suppressed the initial nematode pressure thus reducing the amount of damage that occurs when high populations of *R. reniformis* are present in the field. However, other management practices that reduce initial populations could be recommended before planting a LONREN derived genotype such as non-host crop rotations. Non-host crop rotations with corn

and peanut have been reported to significantly reduce *R. reniformis* populations to a level that would be low enough the following season that the LONREN derived genotypes could tolerate (Gazaway et al., 2000; Moore et al., 2010). In our 2011 microplot study, the *R. reniformis* population levels remained low all season and the LONREN genotypes that contain the *R. reniformis* resistant gene gave no indication of the intolerant response of stunted plants. In Sikkens *et al.* (2011), it was reported that as *R. reniformis* population levels increased from 0 to 50,000, plant growth parameters of shoot and root dry mass declined and severe stunting of the plant occurred. Sikkens *et al.* (2011) also reported that plant height was comparable to susceptible checks when reniform levels were below 5,000 vermiform per pint of soil. The findings from the Sikkens *et al.*, 2011 study and our study indicate that if *R. reniformis* initial populations are low, generally below 1000 vermiform per pint of soil, selected LONREN resistance genotypes could be incorporated as preventative measure in a management practice.

The BARBREN germplasm line (BARBREN-713) that was included in the 2012 experimental design allowed a new source of *R. reniformis* resistant to be compared to the LONREN derived genotypes. In our microplot and field trials, there was no evidence of any intolerant response to the *R. reniformis* nematode with this genetic material. This line performed well with or without nematicide treatments and produced the highest seed cotton yields out of all the genotypes entered including the resistant and susceptible lines. *Rotylenchulus reniformis* population densities at harvest were much lower for the BARBREN-713 line than the susceptible entries. Similar results were also reported by Sikkens *et al.* (2012) in which BARBREN-713 supported much lower nematodes than susceptible checks while exhibiting the highest seed cotton yields. The results of this study and our study indicate that the BARBREN source of resistance will replace the LONREN source of resistant in future *R. reniformis* resistant research.

Literature Cited

- Agudelo, P., Robbins, R.T., Stewart, J.M., Bell, A., and Robinson, A.F. 2005. Histological observations of *Rotylenchulus reniformis* on *Gossypium longicalyx* and interspecific cotton hybrids. *Journal of Nematology* 37:444–447.
- Barker, K.R., Koenning, S.R., Bostain, A.L. and Ayers, A.R., 1988. Growth and yield responses of soybeans to aldicarb. *Journal of Nematology* 20:421–431.
- Barker, K.R., and Powell, N.T., 1988. Influence of aldicarb on the growth and yield of tobacco. *Journal of Nematology*. 20(3):432–438.
- Bell, A.A., Starr, J.L., Jones, J.E., Lemon, R., Nichols, R.L., Overstreet, C., and Stelly, D.M.. 2009. Nematode resistant and agronomic performance of LONREN and NEMSTACK lines. *Proceedings of the Beltwide Cotton Conferences* 1:178. National Cotton Council of America, Memphis, TN.
- Burmester, C.H., Gazaway, W.S., Potter, D.J., Ingram E.G. 1996. Rates and timing of nematicides for control of reniform nematodes in northern Alabama. *Proceedings of the Beltwide Cotton Conferences* 1:306-308. National Cotton Council of America, Memphis, TN.
- Erwin, T.L., Overstreet C., McGawley, E.C. 2002. Nematicide against Reniform nematode in northeast Louisiana. *Proceedings of the Beltwide Cotton Conferences* 1:306–308. National Cotton Council of America, Memphis, TN.
- Gazaway, W. S., J. R. Akridge and K. McLean. 2000. Impact of various crop rotations and various winter cover crops on reniform nematode in cotton. *Proceedings of the Beltwide Cotton Conferences* 1:162–163/ National Cotton Council of America, Memphis, TN.

- Koenning, S.R., Kirkpatrick, T.L., Starr, J.L., Walker, N.A., Wrather, J.A., and Mueller, J.D. 2004. Plant-parasitic nematodes attacking cotton in the United States: Old and emerging production challenges. *Plant Disease* 88: 100–113.
- Lawrence, G.W., Mclean, K.S., Batson, W.E., Miller, D., and Borbon, J.C., 1990. Response of *Rotylenchulus reniformis* to nematicide applications on cotton. *Journal of Nematology* 22:707–711.
- Moore, S.R., Gazaway, W. S., Lawrence, K.S., Goodman, B., Akridge, J. R. 2010. Value of rotational crops for profit increase and reniform nematode suppression with and without nematicide in Alabama. *Proceedings of the Beltwide Cotton Conferences* 1:260–168. National Cotton Council of America, Memphis, TN.
- Nichols, R.L., Bell, A., Stelly, D., Dighe, N., Robinson, F., Menz, M., Starr, J., Agudelo, P., Jones, J., Overstreet, C., Burris, E., Cook, C., Lemon, R., and Fang, D., 2010. Phenotypic and genetic evaluation of LONREN germplasm. *Proceedings of the Beltwide Cotton Conferences* 1:798–799. National Cotton Council of America, Memphis, TN.
- Reddy, V.R., Wang, Z., and Reddy, K.R. 1997. Growth responses of cotton to aldicarb and temperature. *Environmental and Experimental Botany* 38:39–48.
- Rich, J.R., and Kinloch, R.A. 2000. Influence of aldicarb and 1,3-dichloropropene applications on cotton yield and *Rotylenchulus reniformis* post-harvest populations. *Nematropica* 30(1):47:51.
- Robinson, A.F. 2007. Reniform in U.S. Cotton: When, Where, Why, and Some Remedies. *Annual Review of Phytopathology*. 45:263–288.

- Robinson, A.F., Bell, A.A., Dighe, N.C., Menz, M.A., Nichols, R.L., and Stelly, D.M., 2007. Introgression of resistance to nematode *Rotylenchulus reniformis* into upland cotton (*Gossypium hirsutum*) from *Gossypium longicalyx*. *Crop Science* 47:1865–1877.
- Robinson, A.F., and Percival, A.E. 1997. Resistance to *Meloidogyne incognita* race 3 and *Rotylenchulus reniformis* in wild accessions of *Gossypium hirsutum* and *G. barbadense* from Mexico. *Journal of Nematology* 29:746–755.
- Sikkens, R.B., Weaver, D.B., Lawrence, K.S., Moore, S.R., and van Santen, E., 2011. LONREN upland cotton germplasm response to *Rotylenchulus reniformis* inoculum level. *Nematropica* 41:68–74.
- Sikkens, R.B., Wu, T., Weaver, D.B., Moore, S.R., and Lawrence, K.S. 2011. LONREN germplasm response to reniform nematode population levels. *Proceeding of the Beltwide Cotton Conferences* 1:728–736. National Cotton Council of America, Memphis, TN.
- Starr, J.L., Koenning, S.R., Kirkpatrick, T.L., Robinson, A.F., Roberts, P.A., and Nichols R.L. 2007. The future of nematode management in cotton. *Journal of Nematology* 39:283–294.
- Usery, S.R., Lawrence, K.S., Lawrence, G.W., and Burmester, C.H. 2005. Evaluation of cotton cultivars for resistance and tolerance to *Rotylenchulus reniformis*. *Nematropica* 35:121–133.
- Weaver, D.B., Sikkens, R.B., Sahrpe, R.R., Moore, S.R., and Lawrence, K.S. 2011. LONREN X FM966 progeny evaluation in a field infested with reniform nematodes. *Proceedings of the Beltwide Cotton Conferences* 1:737–741.
- Yik, C. P. and Birchfield, W. 1984. Resistant germplasm in *Gossypium* species and related plants to *Rotylenchulus reniformis*. *Journal of Nematology* 16:146–153.

Appendix

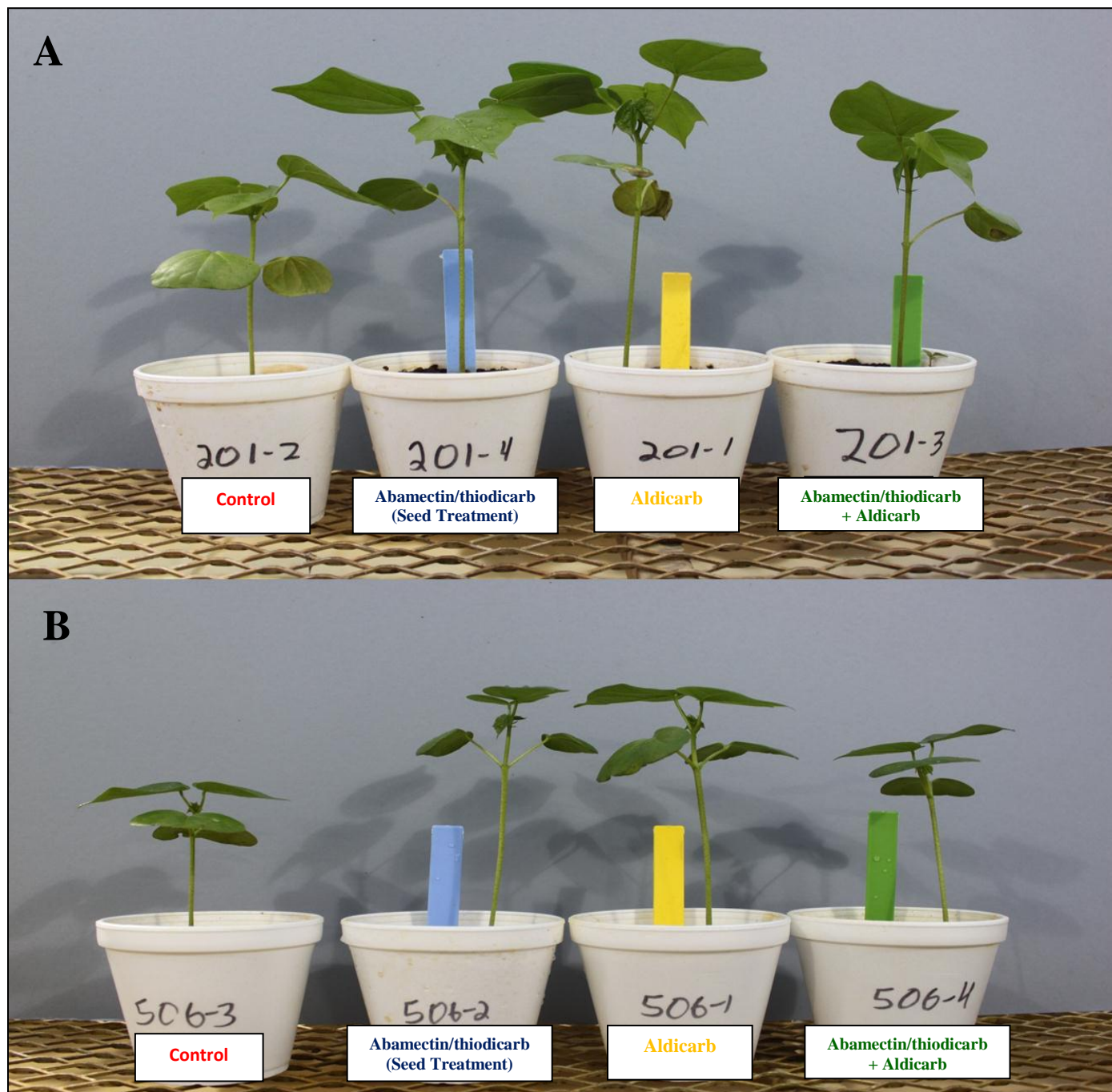


Figure 3. Phenotypic response of LONREN derived resistant lines A107 (A) and B103 (B) at 45 DAP in greenhouse conditions. Nematicide treatments provided significant increases in plant heights over the untreated controls.

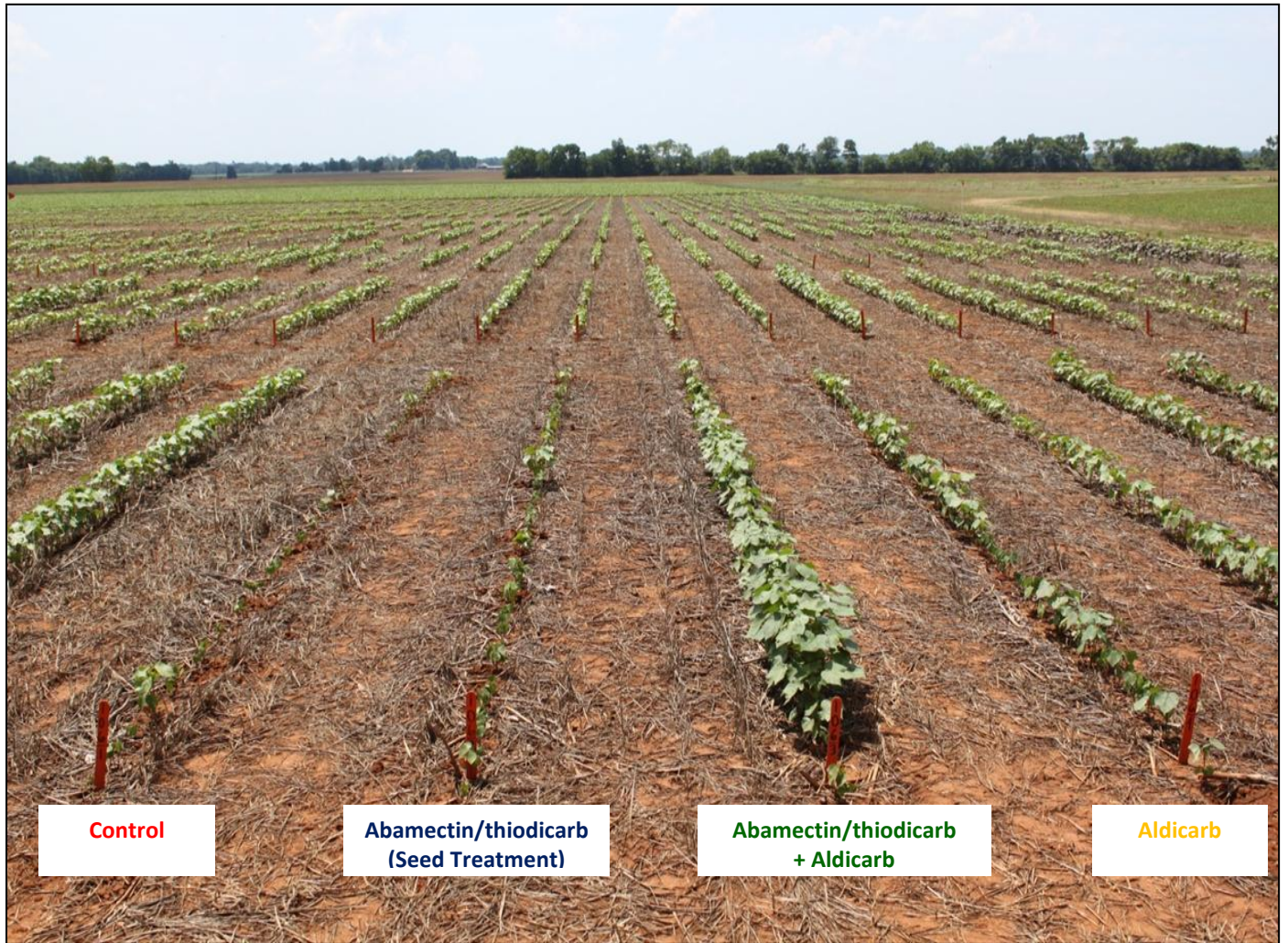


Figure 4. Phenotypic response of reduced stunting of LONREN derived genotype B103 at 45 DAP in response to nematicides. Aldicarb or Abamectin/thiodicarb + Aldicarb provided significant increases in early season plant growth parameters.

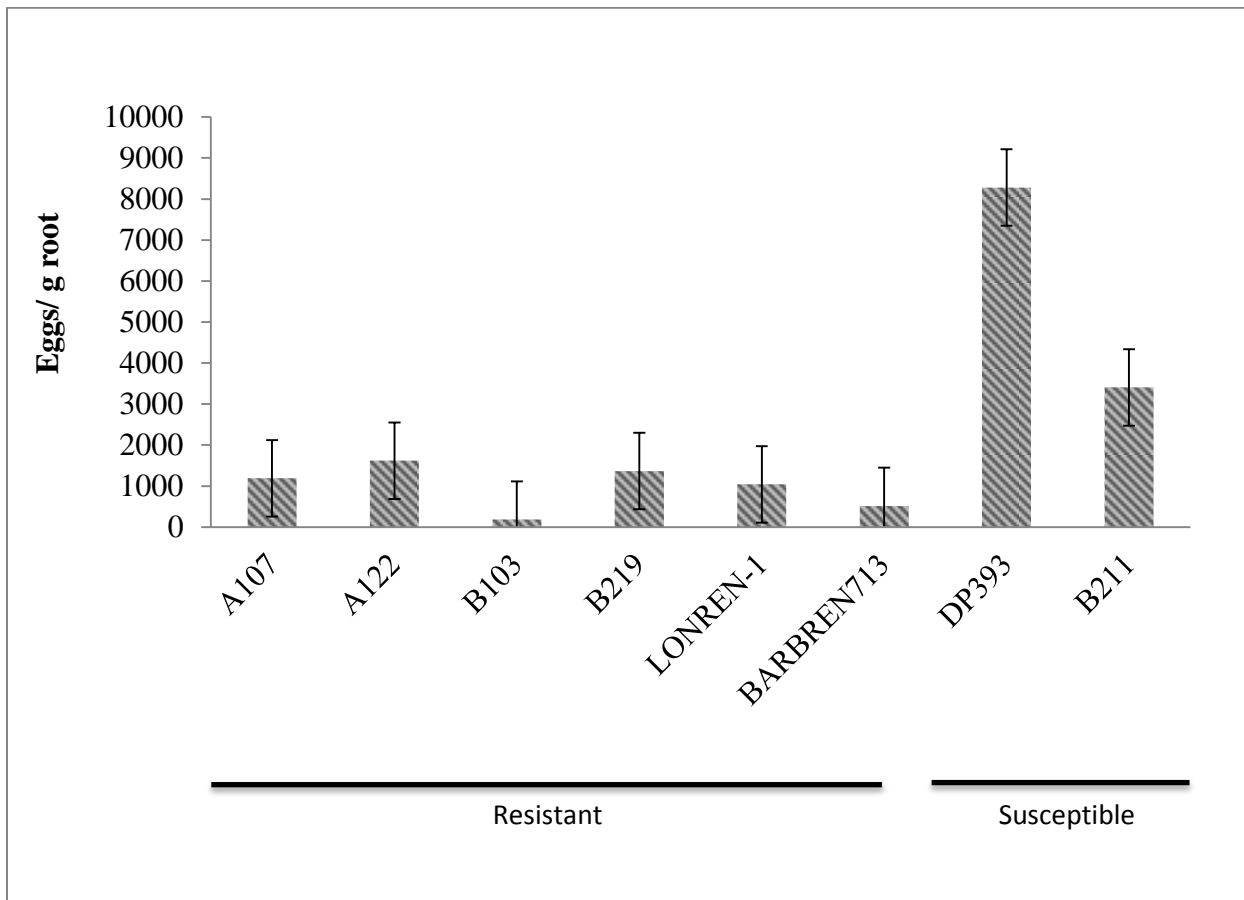


Figure 5. *Rotylenchulus reniformis* egg populations means at 100 DAP in the 2012 field trial. Standard errors are shown to separate statistical difference of egg populations supported by each genotype. All resistant genotypes supported significantly fewer egg populations per gram of root compared to the cultivar DP393 at the $P \leq 0.10$ significance level.

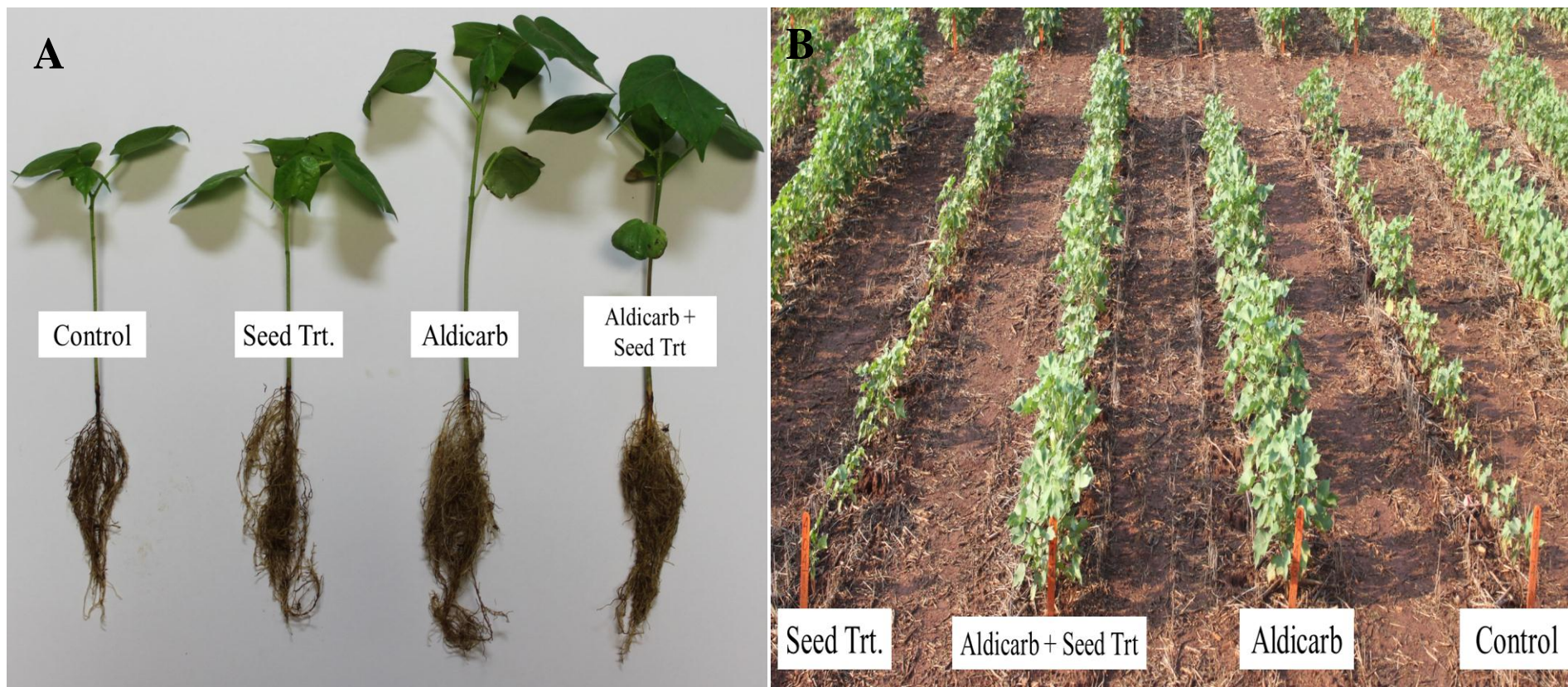


Figure 6. The phenotypic response of the LONREN derived resistant breeding line B103 was vivid throughout all greenhouse (A) and field trials (B).

Table 1. Mean *Rotylenchulus reniformis* egg population densities, eggs per gram of root, and plant heights at 45 DAP in the initial greenhouse trial.

Group	Genotype/Nematicide	<i>Rotylenchulus reniformis</i>		Plant Heights
		Total Eggs	Eggs/g root	Avg PH (cm)
Resistant	A107	227 b ^y	325 b	8.20 bc
	A122	456 b	458 b	9.00 ab
	B219	147 b	363 b	8.23 bc
	LONREN-1	112 b	272 b	7.35 c
Susceptible	DP393	1742 a	1362 a	9.60 a
	B211	1259 a	1400 a	7.93 bc
Nematicide	Control	872 a ^z	931 a	7.20 b
	Seed Trt ^x	443 b	463 b	9.57 a
P-value	Genotypes	0.0001	0.0001	0.0154
	Nematicides	0.0063	0.0005	0.0001
	Genotypes*Nematicides	0.8518	0.1971	0.7064

^x abamectin and thiodicarb

^y Means for the genotype group in the same column followed by the same letter do not differ significantly ($P \leq 0.10$) according to differences in LS MEANS.

^z Means for the nematicide group in the same column followed by the same letter do not differ significantly ($P \leq 0.10$) according to differences in LS MEANS.

Table 2: Mean *Rotylenchulus reniformis* egg population densities, eggs per gram of root, and plant heights at 45 DAP in the second greenhouse trial.

Group	Genotype/Nematicide	<i>Rotylenchulus reniformis</i>		Plant Heights
		Total Eggs	Eggs/g root	Avg PH (cm)
Resistant	A107	6056 b ^y	7156 a	12.7 ab
	A122	5565 b	2798 b	13.23 a
	B103	335 d	170 c	10.75 c
	B219	6848 b	5883 a	12.03 b
	LONREN-1	1447 c	737 bc	11.18 b
	BARBREN 713	1853 c	1221 b	13.58 a
Susceptible	DP393	14415 a	4067 a	13.08 ab
	B211	12648 a	6520 a	12.7 ab
Nematicide	Control	12485 a ^z	8742 a	10.73 b
	Seed Trt ^x	5190 ab	2993 ab	12.63 a
	Aldicarb	3728 bc	976 b	12.87 a
	Aldicarb + ST	2323 c	637 b	13.51 a
P-value	Genotypes	0.0001	0.0001	0.0002
	Nematicides	0.0001	0.0001	0.0006
	Genotypes*Nematicides	0.2562	0.5587	0.5660

^x abamectin and thiodicarb

^y Means for the genotype group in the same column followed by the same letter do not differ significantly ($P \leq 0.10$) according to differences in LS MEANS.

^z Means for the nematicide group in the same column followed by the same letter do not differ significantly ($P \leq 0.10$) according to differences in LS MEANS.

Table 3: Mean *Rotylenchulus reniformis* vermiform life stages per 150 cm³ at 30 and 60 DAP, plant heights at 45 and 75, and seed cotton yields in the initial microplot trial.

Group	Genotype/Nematicide	<i>R. reniformis</i> / 150 cm ³ soil		Plant Heights		Seed Cotton (g)
		30 DAP	150 DAP	45 DAP	75 DAP	Yield
Resistant	A107	54 a ^y	348 ab	30.60 a	33.26 a	27 a
	A122	39 a	124 b	29.88 a	33.15 a	26 a
	B219	39 a	100 b	29.26 a	32.24 a	24 a
	LONREN-1	93 a	100 b	27.66 a	32.43 a	28 a
Susceptible	DP393	62 a	610 a	29.04 a	32.68 a	27 a
	B211	54 a	680 a	26.21 a	28.19 a	28 a
Nematicide	Control	61 a ^z	381 a	27.59 a	32.41 a	26 a
	Seed Trt ^x	51 a	272 a	29.96 a	31.58 a	27 a
P-value	Genotypes	0.4259	0.0001	0.4276	0.7195	0.7147
	Nematicides	0.2331	0.1048	0.1158	0.7364	0.8382
	Genotypes*Nematicides	0.3381	0.8530	0.8163	0.6704	0.5754

^x abamectin and thiodicarb

^y Means for the genotype group in the same column followed by the same letter do not differ significantly ($P \leq 0.10$) according to differences in LS MEANS.

^z Means for the nematicide group in the same column followed by the same letter do not differ significantly ($P \leq 0.10$) according to differences in LS MEANS.

Table 4: Mean *Rotylenchulus reniformis* vermiform life stages per 150 cm³ at 30 and 150 DAP, plant heights at 45 and 75, and seed cotton yields in the second microplot trial.

Group	Genotype/Nematicide	<i>R. reniformis</i> / 150 cm ³ soil		Plant Height (cm)		Seed Cotton (g)
		30 DAP	150 DAP	45 DAP	75 DAP	Yield
Resistant	A107	618 bc ^y	8266 b	21.13 ab	41.93 a	46.85 a
	A122	742 abc	7150 b	22.54 ab	41.08 a	37.33 a
	B103	1020 ab	2029 c	18.08 b	34.75 b	39.97 a
	B219	567 bc	7184 b	20.63 ab	40.50 a	43.01 a
	LONREN-1	664 bc	6175 b	23.71 a	41.25 a	40.09 a
	BARBREN 713	443 c	6139 b	23.21 ab	42.33 a	42.50 a
Susceptible	DP393	1344 a	13503 a	23.63 a	43.08 a	43.03 a
	B211	994 ab	12860 a	22.79 ab	41.67 a	36.87 a
Nematicide	Control	1097 a ^z	8395 a	20.73 b	38.34 c	37.08 b
	Seed Trt ^x	848 a	7590 a	22.17 ab	40.59 b	40.68 ab
	Aldicarb	452 b	7758 a	22.98 a	43.53 a	45.65 a
P-value	Genotypes	0.1178	0.0001	0.0020	0.0001	0.7709
	Nematicides	0.0001	0.4077	0.0799	0.0001	0.0619
	Genotypes*Nematicides	0.3198	0.7141	0.4022	0.1901	0.7565

^x abamectin and thiodicarb

^y Means for the genotype group in the same column followed by the same letter do not differ significantly ($P \leq 0.10$) according to differences in LS MEANS.

^z Means for the nematicide group in the same column followed by the same letter do not differ significantly ($P \leq 0.10$) according to differences in LS MEANS.

Table 5: Mean *Rotylenchulus reniformis* vermiform life stages per 150 cm³ at 30 and 60 DAP, plant heights at 45 and 75, and seed cotton yields in the first year's field trial.

Group	Genotype/Nematicide	<i>R. reniformis</i> / 150 cm ³ soil		Plant Height (cm)		Seed Cotton
		30 DAP	150 DAP	45 DAP	75 DAP	Yield (lb/A)
Resistant	A107	3206 a ^y	1383 cd	11.78 c	38.67 ab	1901 abc
	A122	2766 a	1985 bc	12.33 abc	42.23 ab	2038 ab
	B219	2665 a	1159 d	12.48 abc	32.00 b	1576 bc
	LONREN-1	3605 a	1456 cd	10.89 c	34.62 a	1472 c
Susceptible	DP393	3183 a	4017 a	15.05 a	47.75 a	2779 a
	B211	3115 a	2523 ab	14.22 ab	42.43 ab	2556 ab
Nematicide	Control	3923 a ^z	1972 a	11.93 b	39.58 a	2172 a
	Seed Trt ^x	2848 a	2202 a	13.72 a	39.96 a	1972 a
P-value	Genotypes	0.9162	0.0005	0.0027	0.0633	0.0155
	Nematicides	0.4543	0.7516	0.0047	0.8370	0.5410
	Genotypes*Nematicides	0.4521	0.2450	0.6928	0.9561	0.8237

^x abamectin and thiodicarb

^y Means for the genotype group in the same column followed by the same letter do not differ significantly ($P \leq 0.10$) according to differences in LS MEANS.

^z Means for the nematicide group in the same column followed by the same letter do not differ significantly ($P \leq 0.10$) according to differences in LS MEANS.

Table 6: Mean *Rotylenchulus reniformis* vermiform life stages per 150 cm³ at 30 and 60 DAP, plant heights at 45 and 79, and seed cotton yields in the second year's field trial.

Group	Genotype/Nematicide	<i>R. reniformis</i> / 150 cm ³		Plant Height (cm)		Seed Cotton
		30 DAP	150 DAP	45 DAP	79 DAP	Yield (lb/A)
Resistant	A107	7771 a ^y	6219 c	14.89 cd	53.84 cd	2247 cd
	A122	6022 ab	7864 bc	16.04 b	62.28 b	2465 cb
	B103	6653 ab	2036 e	10.60 e	33.75 e	1592 e
	B219	6327 ab	5091 cd	14.52 cd	51.22 d	2158 d
	LONREN-1	8314 a	5720 c	14.18 d	53.19 cd	2460 cbd
	BARBREN 713	5054 b	3160 d	17.45 a	71.59 a	3416 a
Susceptible	DP393	6790 a	11746 a	15.34 cb	54.97 c	2632 b
	B211	6248 ab	8857 b	15.06 cbd	50.78 d	2320 cd
Nematicide	Control	7612 a ^z	5697 a	13.05 c	50.58 b	2139 b
	Seed Trt (ST) ^x	6551 ab	6989 a	14.16 b	52.34 b	2297 b
	Aldicarb	6021 b	6535 a	15.82 a	56.30 a	2574 a
	Aldicarb + ST	6406 ab	6124 a	16.02 a	56.58 a	2635 a
P-value	Genotypes	0.0380	0.0001	0.0001	0.0001	0.0001
	Nematicides	0.5007	0.5513	0.0001	0.0001	0.0001
	Genotypes*Nematicides	0.9811	0.7954	0.7702	0.3998	0.9664

^x abamectin and thiodicarb

^y Means for the genotype group in the same column followed by the same letter do not differ significantly ($P \leq 0.10$) according to differences in LS MEANS.

^z Means for the nematicide group in the same column followed by the same letter do not differ significantly ($P \leq 0.10$) according to differences in LS MEANS.

Chapter III. Evaluation of the Effects That *Bacillus firmus* GB-126 Have on Plant-parasitic Nematodes *in Vitro* and Split-Root Experiments

Abstract

Bacillus firmus strain GB-126 was evaluated for the capacity to reduce mobility of juveniles, inhibit egg production, and induce systemic resistance when used as a control against *Heterodera glycines* and *Meloidogyne incognita*. Experiments were established *in vitro* to examine egg hatching and mobility and paralysis of J-2s in 96 well plates containing 100 μ l of GB-126 cells at 1×10^7 and 1×10^6 cfu/ml and cell-free extracts at 100%, 50%, and 25% concentrations. Split-root assays were established to evaluate induced systemic resistance. GB-126 cells at both concentrations significantly reduced mobility of *H. glycines* J2s compared to Tryptic Soy Broth (TSB) and sterilized tap water (STW) controls at 36 h after treatment. Cell-free extracts of GB-126 reduced mobility significantly at 12 h after treatment in both 100% and 50% concentrations compared to TSB and STW controls. GB-126 cells and cell-free extracts also significantly reduced egg hatching of *H. glycines* and *M. incognita* at 9 and 4 days after inoculation, respectively. Induced systemic resistance was evident in the *H. glycines* split-root assay but not in the *M. incognita* split-root assay. The results of these experiments indicate that both cells and cell-free extracts of GB-126 can have antagonistic effects on *H. glycines* and *M. incognita*.

Introduction

In plant pathology, biocontrol is considered to be one or more organisms that have the capacity to reduce inoculum densities of disease causing pathogens (Baker and Cook, 1974). Living organisms such as bacteria, fungi, and other nematodes can be used as biocontrol agents to antagonize and reduce plant-parasitic nematode populations (Stirling, 1991). The use of bacteria as biocontrol agents for plant parasitic nematode control has yielded promising results in previous studies. Sayre and Starr, (1988) reported that *Pasteuria spp.* produce endospores that attach to plant parasitic nematodes and set up parasitism of the nematodes. These spores can increase through propagation of the bacteria once the nematodes become infected (Preston *et al.*, 2003). Once a mature female that has been colonized by *Pasteuria spp.* ruptures, millions of endospores will be released into the soil (Preston *et al.*, 2003) The endospores that are released attach to other nematodes that are in the soil and cause further parasitism eventually leading to suppression of nematode populations (Sayre and Starr, 1988). As little as 40 spores attached to one infective stage plant parasitic nematode can inhibit root infection (Stirling, 1984). Another species of bacteria, *Pseudomonas fluorescens* has also displayed antagonistic interactions of plant-parasitic nematodes. Isolates that were collected from the root zones of cotton plants in India had the capacity to reduce *Rotylenchulus reniformis* populations in the soil (Jayakumar *et al.*, 2003). *Pseudomonas fluorescens* inhibits host recognition of plant parasitic by colonizing on the root surface (Jayakumar *et al.*, 2003).

Specific plant host responses to bacteria can stimulate important defense mechanisms to pathogens that initiate infection. These defense mechanisms include production of toxins and

ammonia compounds, altered root exudate production, and induced systemic resistance (Kloepper, *et al.*, 1992; Aatlen *et al.*, 1998; Martinez-Ochoa, 2000). The induced systemic resistance (ISR) defense mechanism is best described as a process in which rhizobacteria or similar microorganisms can produce metabolites that increase the plant's resistance to pathogens (Kloepper and Ryu, 2006). Hasky and Sikora (1995) reported that ISR activities from the bacterium *Bacillus sphaericus* were observed when this bacterium had been applied to potato for the control of *Globodera pallida*. Similarly, ISR interactions were observed when the bacterium *Rhizobium etli* was applied to tomato for the control of *Meloidogyne incognita* (Schafer *et al.*, 2006).

Biological control agents for plant parasitic nematodes offer environmentally sound management strategies for the agriculture industry. The industry has relied heavily on chemical nematicides to reduce nematode populations the past few decades. The bacterium *Bacillus firmus* is a bio-control agent that could be an environmentally safe control alternative to reduce nematode populations. This bacterium has shown the capacity to reduce Root-knot (*Meloidogyne incognita*) in tomatoes (Terefe *et al.*, 2008). *Bacillus firmus* has also shown the capacity to reduce other plant parasitic nematode populations such as *Radopholus similis* in bananas, (Mendoza *et al.*, 2008). *Bacillus firmus* GB-126 was originally isolated in Israel and later it became commercially formulated by Agrogreen, Ashdod, Israel (Terefe *et al.*, 2008). Bayer CropScience purchased *Bacillus firmus* GB-126 from Agrogreen and have acquired registration labels for commercially formulated wettable powders in the turfgrass industry and as seed treatments on soybeans. Castillo *et al.* (2013) reported that seeds treated with *B. firmus* GB-126 reduced *Rotylenchulus reniformis* populations on cotton in greenhouse, microplot, and field

conditions. Although *Bacillus firmus* GB-126 has shown antagonistic characteristics in the previous mentioned studies, little is known about the mode of action of this agent.

The objectives of this study were to 1) evaluate the effects that *B. firmus* GB-126 cells and cell-free extracts have on egg hatching and second stage juvenile mobility of *M. incognita*, and *H. glycines in vitro* and to 2) determine if *B. firmus* has systemic capabilities when used as a control against *M. incognita* and *H. glycines*.

Materials and Methods

Two *in vitro* assays were developed in 96 well plates to determine mobility and paralysis of SCN J2. The first assay contained treatments of GB-126 cells at 1×10^7 and 1×10^6 cfu/ml as well as Tryptic Soy Broth (TSB), Sterilized Tap Water (STW) and. A volume of 100 μ l of each treatment was added to each well in the 96 well plate and replicated five times. A range of 30 to 50 J2s were added to each well and incubated for 48 hours at 27°C. The juveniles were evaluated for mobility and paralysis with a Nikon TS100 microscope at 0, 12, 24, and 48 hours. Before each counting period, the assay was placed on a rotary shaker for one hour to stimulate movement of mobile J2s. Any juveniles that displayed a serpentine shape and demonstrated movement after shaken were considered mobile. Any juveniles that displayed a linear shape and did not show signs of movement after shaken were considered paralyzed.

The second assay contained treatments of GB-126 cell free extracts at concentrations of 100% and 50% as well as TSB, and STW. A volume of 100 μ l of each treatment was added to the 96 well plate and replicated five times. A range of 30 to 50 J2s were added to each well and incubated for 48 hours at 27°C. The juveniles were evaluated for mobility and paralysis with a

Nikon TS100 microscope at 0, 12, 24, and 48 hours. Before each counting period, the assay was placed on a rotary shaker for 1 hour to stimulate movement of mobile J2s. Mobility and paralysis were evaluated as previously described.

Egg Hatch Assay

Using the same in vitro experimental design, the effects that *B. firmus* GB-126 cells and cell-free extracts have on egg hatching of *M. incognita* and *H. glycines* were evaluated. The treatments used in these evaluations consisted of bacteria cells at a concentration of 1×10^7 cfu/ml or either cell-free extracts at a concentration of 100% and 50% as well as TSB and STW for controls. The percentages of eggs hatched were observed under a Nikon TS100 microscope every 24 hours for four consecutive days in the *M. incognita* assays. In the *H. glycines* assay the percentage of eggs hatched were observed under a Nikon TS100 microscope every 24 hours for nine consecutive days. Each of these assays had five replications and was repeated twice.

Split Root Assay: Soybean Cyst Nematode on Soybeans

Hutcheson soybean seed were germinated 5 days before planting to ensure a root radical length of 2.54 cm. Each radical was evenly split (longitudinally) with a fine razor blade and planted into two separate cone-tainers (Figure 6). Two independent root halves were established by 10 days after planting (DAP) and inoculated with either GB-126 at a concentration of 1×10^7 cfu/ml or soybean cyst nematodes (SCN) at a concentration of 2000 J2s/5 ml or a combination of both organisms. There were a total of four different split root treatment combinations: GB-126 and SCN on opposite root halves, both organisms on one root half, SCN alone, or a control with neither one. The soybean plants were allowed to grow for 60 days in a greenhouse environment. The number of SCN J2s and cysts/150cm³ of soil, shoot and root fresh weights, and plant heights were measured.

Split Root Assay: Root Knot Nematode on Corn

Corn seeds were planted in 88.72 ml polystyrene cups with the bottom removed and positioned equally over two separate 1000 cm³ plastic growth pots (Figure 7). The corn plant's fibrous root system allows two independent root halves to naturally form. The two separate root halves were allowed to grow for 10 DAP and inoculated with either GB-126 at a concentration of 1x10⁷ cfu/ml, root-knot nematodes (RK) at a concentration of 2000 J2s/5 ml or a combination of both organisms. There were a total of four different treatment combinations: GB-126 and RK on opposite root halves, both organism on one root halve, RK alone on one root halve, and control with neither organism. The corn plants were allowed to grow for 60 days in a greenhouse environment. The number of RK J2s/150cm³ of soil, RK females per gram of root, root and shoot fresh weights, and plant heights were measured.

Results

Mobility Assay

GB-126 cells at both concentrations significantly reduced mobility of *H. glycines* compared to Tryptic Soy Broth (TSB) and Sterilized Tap Water (STW) controls at 36 h after treatment. Mobility was reduced to 61% and 67%, respectively, in the 1x10⁷ and 1x10⁶ cfu/ml cell suspensions at 48 h (Figure 8). With cell-free extracts, mobility was significantly reduced 12 h after treatment with 100% and 50% concentrations compared to TSB and STW controls. Mobility of SCN J2s ceased completely to a paralytic form in the 100% cell-free extract concentration at 48 h. The 50% and cell-free extract concentration reduced mobility of SCN J2s by 95% at 48 h. The results of the experiments indicate that both cells and cell-free extracts of GB-126 can a direct effect on the mobility of *H. glycines*.

Egg Hatch Assay

Egg hatching of *M. incognita* was also significantly reduced when introduced to bacteria cells and cell-free extracts of *B. firmus* GB-126. Bacteria cell concentrations of 1×10^7 and 1×10^6 reduced egg hatching to 12% and 15%, where 21% of eggs were hatched in the STW treatment at 96 hours after inoculation (Figure 10). Cell-free extracts of 100% and 50% reduced egg hatching of *M. incognita* to 7% and 7.5%, respectively, where 19.3 % of eggs were hatched in the STW treatment at 96 hours after inoculation (Figure 10). Similar results were also found in the *H. glycines* egg hatching assay. After 9 days of initial inoculation, bacteria cell concentrations of 1×10^7 and 1×10^6 reduced egg hatching to 6% and 11%, respectively (Figure 11). The two cell-free extract concentrations of 100% and 50% reduced *H. glycine* egg hatching to 1.5% and 3%, respectively, after 9 days of being exposed to the treatments (Figure 11).

Split Root Assay: Soybean Cyst Nematode on Soybeans

The soybean cyst nematode split root experiment found GB-126 has both systemic and localized effects on soybean cyst nematodes. A decrease in SCN cyst populations was observed when GB-126 was present. A translocatable effect of GB-126 was observed in the treatment SCN/GB-126 on opposite root halves. The number of cyst per 150cm^3 was reduced by 27% when compared to the SCN/Check split root system. The concomitant treatment reduced populations of cysts per 150cm^3 by 84% when compared to the SCN/Check split root systems. A significant decrease in SCN J2 populations was observed in both treatments where GB-126 was present (Figure 11). The SCN/GB-126 treatment on opposite root halves significantly reduced the number of J2s per 150cm^3 by 43%, while the concomitant SCN+GB-126/Check reduced J2s per 150cm^3 of soil by 91% when compared to the SCN/Check treatment ($P \leq 0.0327$).

Split Root Assay: Root Knot Nematode on Corn

The root-knot nematode split root experiment on corn indicated that GB-126 has a localized effect on root-knot nematodes. A decrease in root-knot females per gram of root was only evident in the concomitant treatment with RK+GB126/Check on one root system. The concomitant treatment reduced females per gram of root by 48% when compared to the RK/Check split root system. No translocatable results were found when comparing the RK/GB-126 on opposite root systems to the RK/Check. Similar results were found when comparing the means from root-knot J2s per 150cm³ of soil (Figure 11). The concomitant treatment RK+GB126/Check significantly reduced the number of RK J2s by 33% when compared to the RK/Check treatment. No systemic effects on *M. incognita* numbers were evident when RK/GB-126 was on opposite root halves.

Discussion

The results of these experiments indicate that both cells and cell-free extracts of GB-126 can have direct effects on J2 mobility of *H. glycines*. The common trend with the two mobility assays was that over time inhibition of movement of second stage juveniles did occur. In each of the assays conducted, mobility was decreased and the J2s would take on a paralytic form over the time period of 48 hours. The loss of mobility could effectively kill the SCN J2 since the nematode would not be able to move toward and penetrate the soybean root. Similar results were reported by Terefe *et al.* (2008) in which BioNem MP, a product derived from *Bacillus firmus*, caused a 100 % reduction in second-stage juvenile mobility of *M. incognita*. Mendoza *et al.* (2008) also reported that secondary metabolites produced by *B. firmus* were responsible for paralysis and mortality of *M. incognita* and *R. similis*.

Egg hatching of *H. glycines* and *M. incognita* was also influenced by cells and cell-free extracts of *B. firmus* GB-126 in our *in vitro* assays. Even though the time periods were different the common trend was that overtime a significant reduction occurred for both nematode species. The reduction of egg hatching will ultimately reduce the amount of infective stage juveniles, thus reducing disease severity caused by either of these plant parasitic nematodes. Terefe et al (2008) also reported similar results in egg hatching experiments that were conducted with BioNem MP. Furthermore, Mendoza *et al.* (2008) reported that cell free extracts used in their study also significantly reduced egg hatching of *M. incognita*. Our results also had greater evidence that cell-free extracts was primarily responsible for egg hatch inhibition of *H. glycines* and *M. incognita*.

In this study, systemic effects were evident when *B. firmus* GB-126 was applied to the roots of soybeans for control against the soybean cyst nematode. However, no systemic effects were evident when GB-126 was applied to the roots of corn for control against the root-knot nematode. The specific interrelationships that plant species have with plant parasitic nematodes could be altered or stimulated differently by biocontrol agents. Similar results have been reported in which the same strain of rhizobacteria, *Bacillus subtilis* A-13, had the capacity to reduce *M. incognita* in sugar beet but no reductions occurred when evaluated against *M. incognita* in cotton (Sikora, 1988).

In summary, the evaluation of *Bacillus firmus* GB-126 *in vitro* demonstrated antagonistic effects against *H. glycines* and *M. incognita*. The primary antagonistic effects were paralysis of second stage juveniles and inhibition of egg hatching in which both will lead to a reduction in inoculum densities, further validating the biocontrol potential of *Bacillus firmus* GB-126. The split root experiments conducted in this study also indicate that ISR could be an antagonistic

effect for *H. glycines* on soybeans. However, further research is needed to confirm this ISR phenomenon.

Literature Cited

- Baker, K.F., Cook, R.J. 1974. Biological control of plant pathogens. W.H. Freeman and Company. San Francisco, CA.
- Aatlen, P.M., Vitour, D., Blanvillain, D., Gowen, S.R., Sutra, L. 1998. Effect of the rhizosphere fluorescent *Pseudomonas* strains on plant-parasitic nematodes *Radopholus similis* and *Meloidogyne* spp. Letters in Applied Microbiology 27:357–361.
- Castillo, J.D., Lawrence, K.S., Kloepper, J.W., 2013. Biocontrol of the Reniform nematode by *Bacillus firmus* GB-126 and *Paecilomyces lilacinus* 251 on cotton. Plant Disease. In Press.
- Hasky, K, Sikora, R. 1995. Induced resistance – a mechanism induced systemically through the root system by rhizosphere bacteria towards the potato cyst nematode *Globodera pallida*. Nematologica 41:306.
- Jayakumar, J., Ramakrishnan, S., Rajendran, G. 2003. Bio-efficacy of fluorescent Pseudomonads isolates against reniform nematode, *Rotylenchulus reniformis* infecting cotton. Indian Journal of Nematology 33:13–15.
- Kloepper, J.W., Ryu, C.M. 2006. Bacterial endophytes as elicitors of induced systemic resistance. Soil Biology 9: 33–52.
- Kloepper, J.W., Wei, G., Tuzun, S. 1992. Rhizosphere population dynamics and internal colonization of cucumber by plant growth-promoting rhizobacteria which induce systemic resistance to *Colletotrichum orbiculare*. In: Tjamos ES, Papavizas GC, Cook RJ (eds).

- Martinez–Ochoa, N. 2000. Biological control of the root-knot nematode with rhizobacterial and organic amendments. Doctor of Philosophy Dissertation. Auburn University.
- Mendoza, A.R., Kiewnick, S., Sikora, R. 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.
- Preston, J. F., Dickson, D. W., Maruniak, J. E., Nong, G., Brito, J. A., Schmidt, L. M., and Giblin-Davis, R. M. 2003. *Pasteuria* spp.: Systematics and Phylogeny of These Bacterial Parasites of Phytopathogenic Nematodes. *Journal of Nematology* 35(2):198–207.
- Sayre, R.M., Starr, M.P. 1988. Bacterial disease and antagonisms of nematodes. Pp. 69–101. *In: Poinar, G.O. and Jansson, H.B. (eds). Diseases of nematodes Vol. I. CRC Press Inc. Boca Raton, Florida.*
- Schäfer, K., Silva Fabry, C., Sikora, R. A., and Hauschild, R. 2006. Molecular investigations of rhizobacteria-induced systemic resistance towards the root-knot nematode *Meloidogyne incognita* in tomato. *Multitrophic interactions in soil. IOBC/WPRS Bull.* 29:135–140.
- Stirling, G.R., 1984. Biological control of *Meloidogyne javanica* with *Bacillus penetrans*. *Phytopathology* 74:55–60.
- Stirling, G. R. 1991. Biological control of plant parasitic nematodes. Wallingford. CAB International.

Sikora, R.A. 1988. Interrelationship between plant health promoting rhizobacteria, plant parasitic nematodes and soil microorganisms. Med. Fac. Landbouww. Rijksuniv. Gent 53/2b:867–878.

Terefe, M., Tefera, T., Sakhuja, P.K. 2009. Effect of a formulation of *Bacillus firmus* on root-knot nematode *Meloidogyne incognita* infestation and the growth of tomato plants in the greenhouse and nursery. *Journal of Invertebrate Pathology* 100:94–99.

Appendix

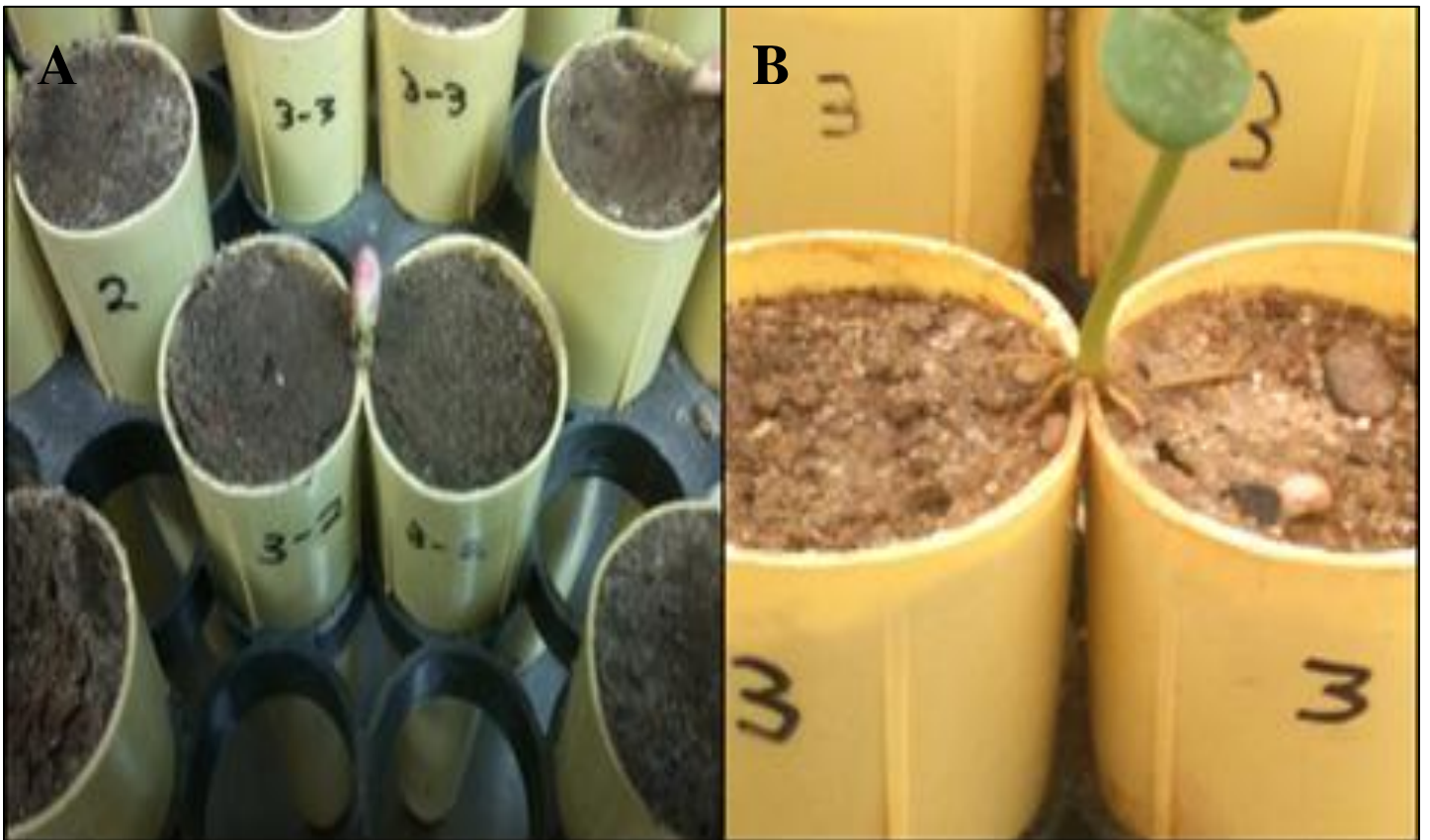
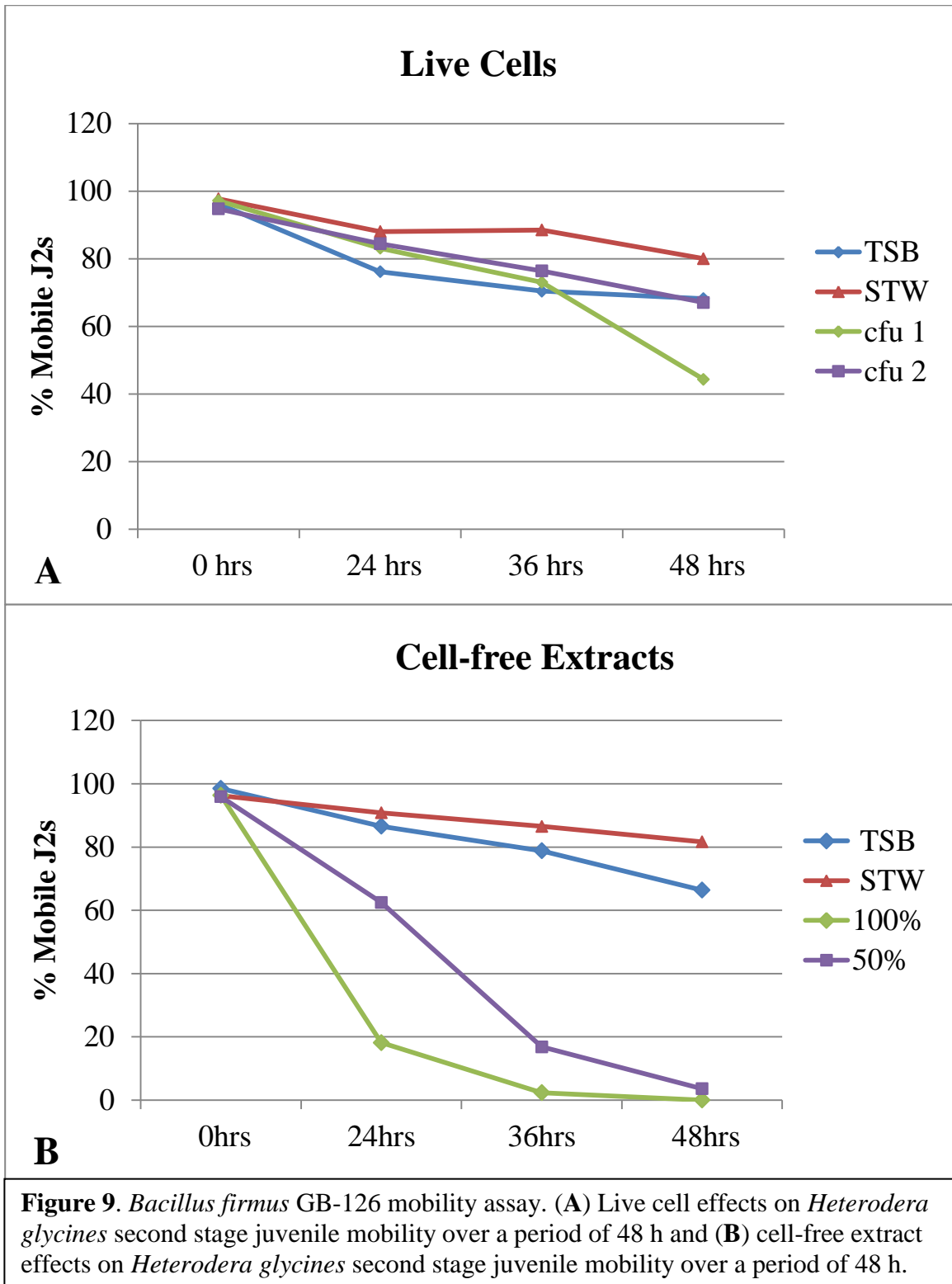


Figure 7: The radical from germinated soybean seeds were split evenly with a fine razor blade and separate halves were placed in two different containers (A). Plants were given 10 days to establish two separate root systems into each container (B).



Figure 8. Split Root Corn Assay. Corn seeds were planted in polystyrene cups with the bottom removed and positioned equally over two separate plastic growth pots. The corn plant's fibrous root system allows two independent root halves to naturally form into each of the two plastic pots.



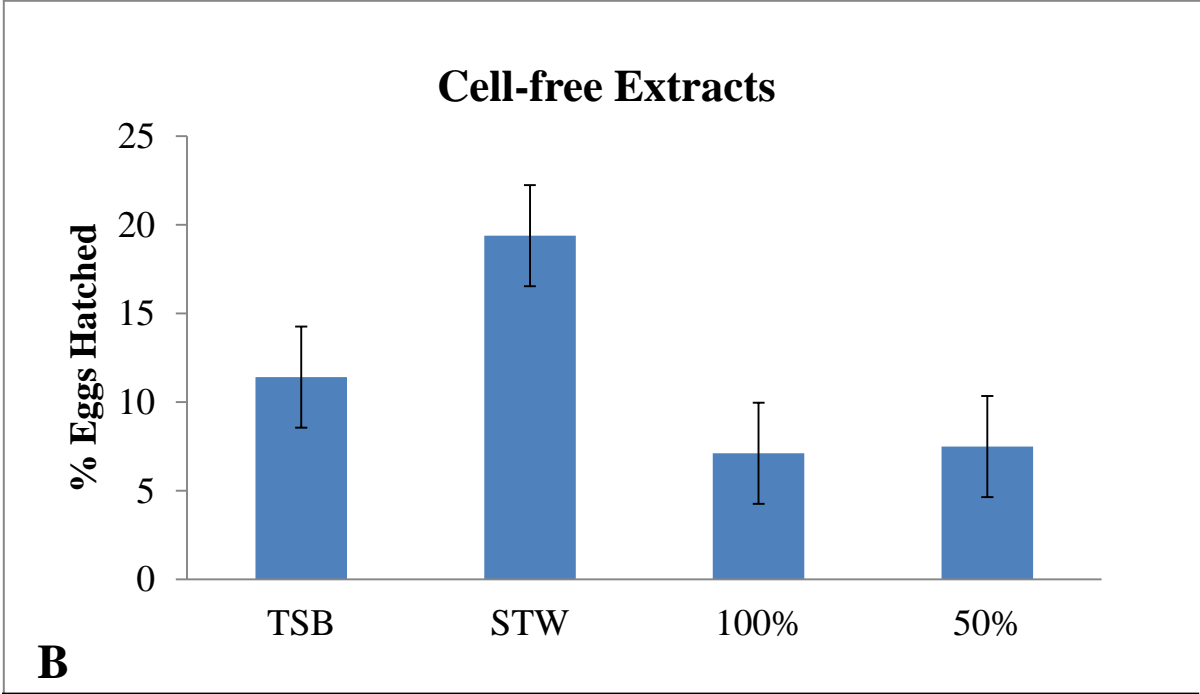
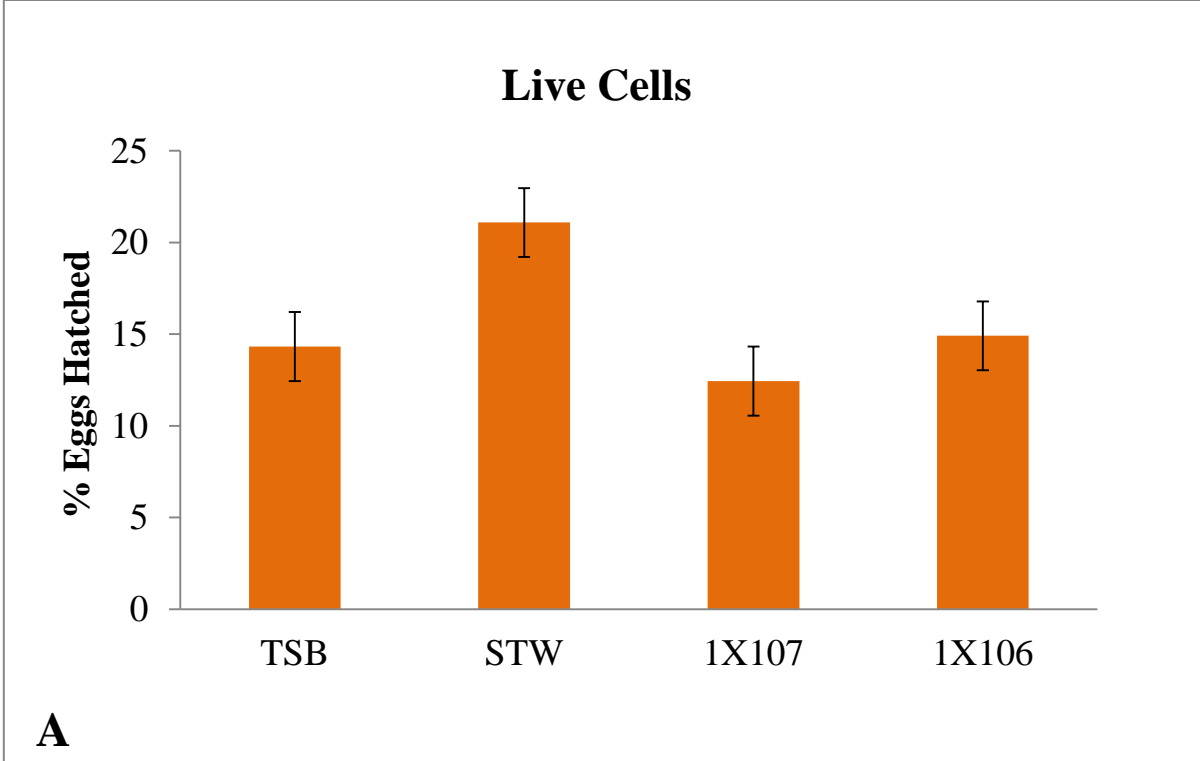
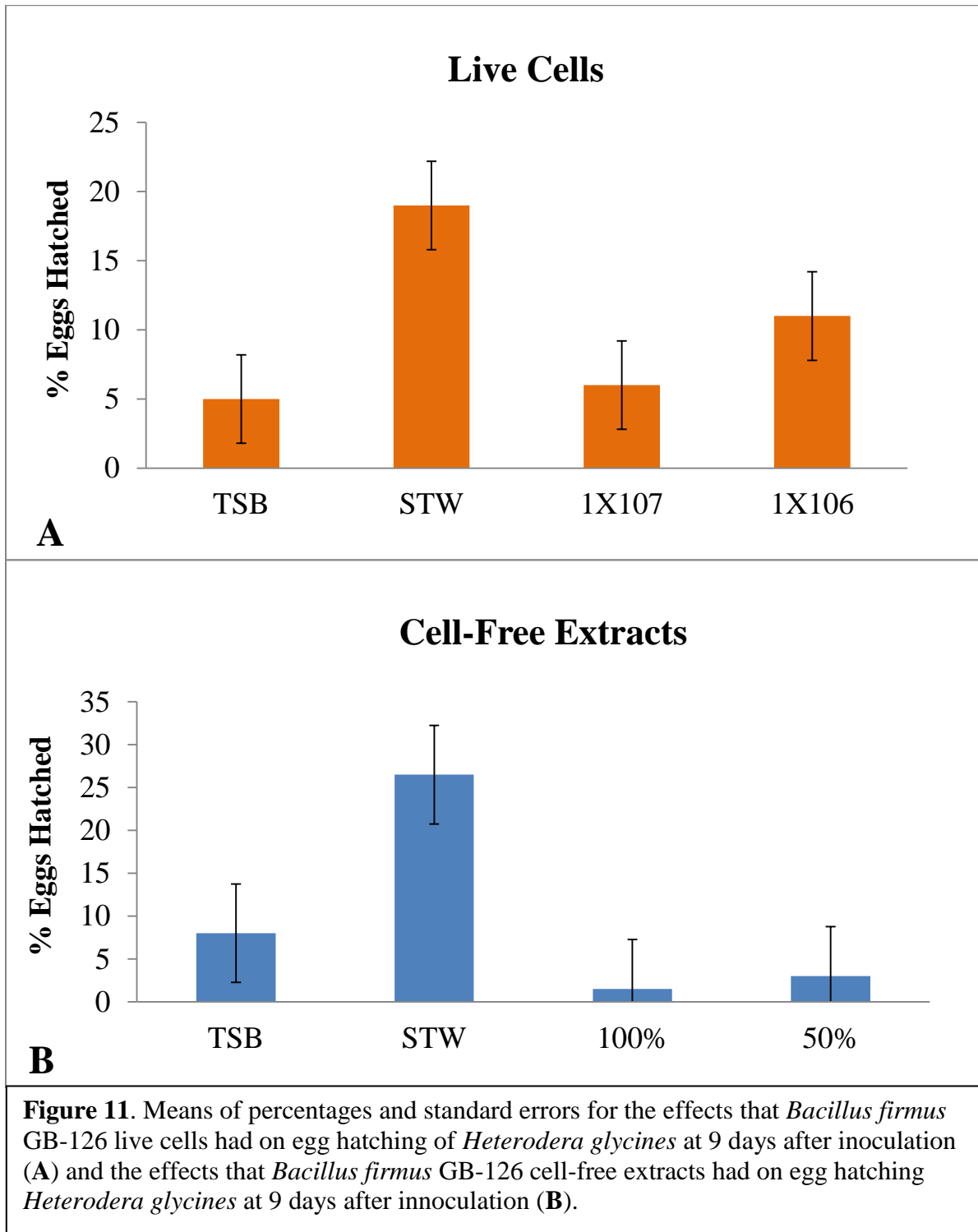


Figure 10. Means of percentages and standard errors for the effects that *Bacillus firmus* GB-126 live cells had on egg hatching of *Meloidogyne incognita* at 96 hours after inoculation (**A**) and the effects that *Bacillus firmus* GB-126 cell-free extracts had on egg hatching *Meloidogyne incognita* at 96 hours after inoculation (**B**).



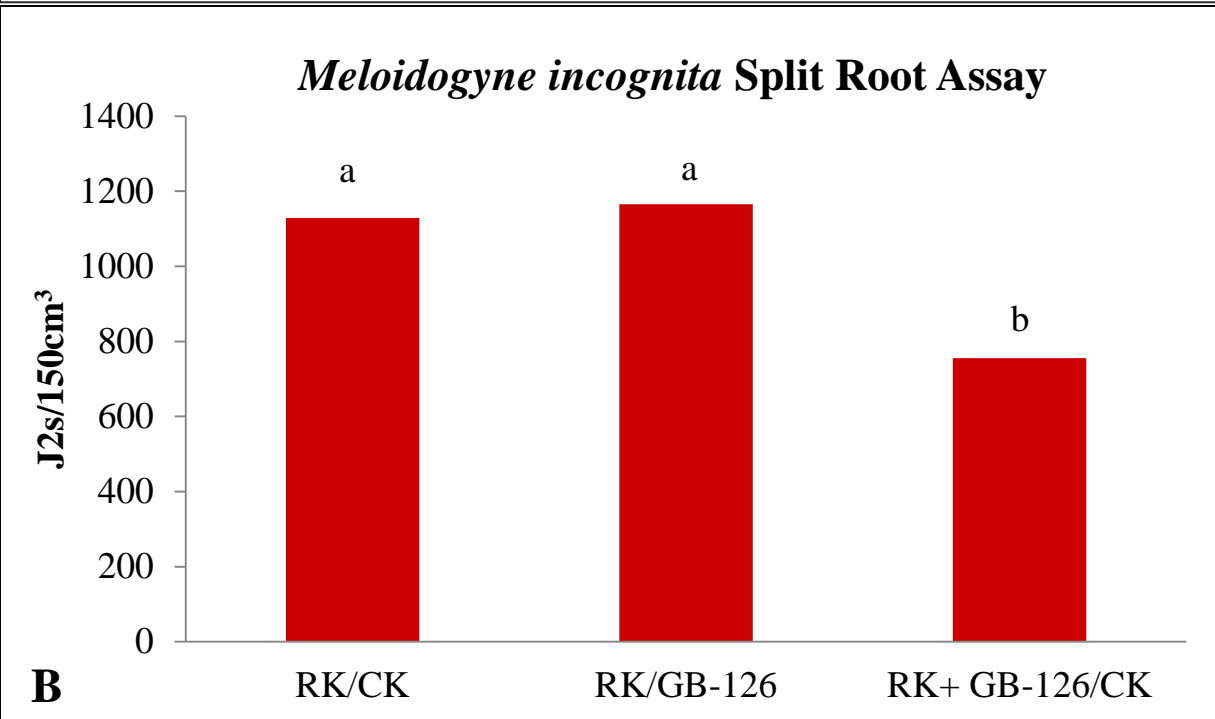
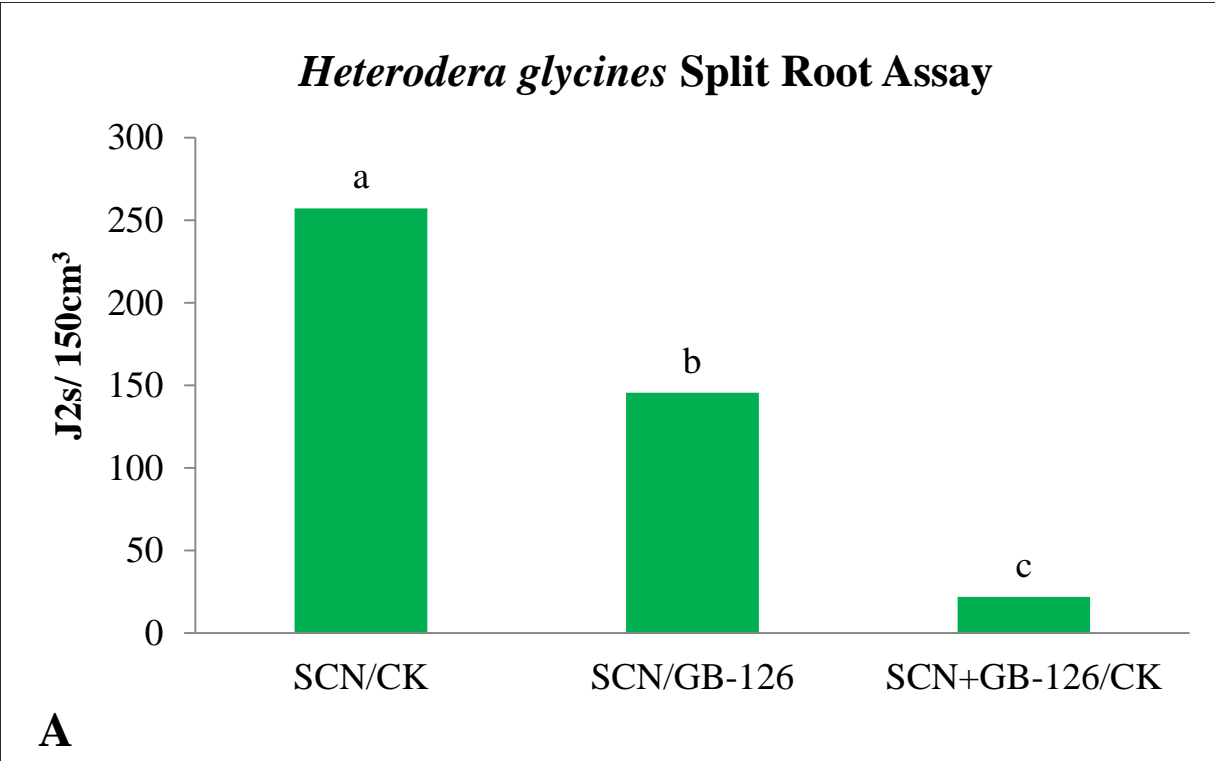


Figure 12. Least square means for the effects when applying *Heterodera glycines* and *B. firmus* to opposite root halves, a translocatable effect was evident resulting in a significant decrease ($P \leq 0.033$) in second stage juveniles (A). No translocatable effect was observed in the *Meloidogyne incognita* split root assay on corn (B).

Overall Conclusion

In conclusion, the studies conducted in this thesis project provide results that can impact future management decisions of plant-parasitic nematodes. The resistant LONREN genotypes evaluated in this study could be utilized in areas where initial populations of *R. reniformis* are low. These resistant genotypes exhibited the ability to reduce reniform populations throughout the growing seasons. Increases in plant heights and yields were evident when nematicides were applied at planting in trials that had moderate to high populations. In trials where populations were low, no intolerant responses were evident and yields were similar to, or superior to susceptible checks. If combined in a non-host crop rotation scheme, *R. reniformis* populations could be lowered to levels that would be suitable for the use of these LONREN genotypes. The biological control agent *Bacillus firmus* GB-126 that was evaluated in this thesis project could be used in a non-host rotation scheme to provide protection during early season seedling establishment. The results of our *in vitro* experiments indicate that GB-126 has the capacity to antagonize plant-parasitic nematodes by causing paralysis and inhibition of egg hatch. This biocontrol agent is registered by Bayer CropScience as a seed treatment in agronomic crop production under the trade name Votivo®. The combination of this seed treatment with a resistant LONREN genotype would allow an environmentally sound management tactic to be utilized in an Integrated Pest Management system for *Rotylenchulus reniformis*.