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I am submitting herewith a dissertation written by Mary Ruth Griffin entitled "*Beauveria bassiana*, a cotton endophyte with biocontrol activity against seedling disease." I have examined the final electronic copy of this dissertation for form and content and recommend that it by accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plants, Soils and Insects.

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Beauveria bassiana, a cotton endophyte with biocontrol activity against seedling disease

A Dissertation

presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Mary Ruth Griffin
May 2007

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Dedication

To the One, in my heart, I never could have done this without your help. Thank you.

To my son, Daniel, you draw the best from me because I desire to be more for you. You are my greatest gift. Thank you.

In Memory

This work is dedicated to the memory of my mother, Patricia Faye Brewer, who viewed her children as the work of a life time and did everything she could to help me become a better person. Thank you.

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Lastly I wish to thank my family and friends who have supported me all along. Thank you all so very much.

Abstract

Beauveria bassiana isolate 11-98 is entomopathogenic, endophytic in tomato, and has biocontrol activity against *Rhizoctonia solani* on tomato. The objectives of this study were to determine 1) if *B. bassiana* is endophytic in cotton, following seed treatment; 2) if *B. bassiana* can protect cotton against seedling pathogens; 3) if different conidial rates, applied to seed, alter effectiveness of *B. bassiana*; and 4) mechanisms of biocontrol used by *B. bassiana* against plant pathogens. Cotton 'Delta Pine 436' seed was inoculated with isolate 11-98 conidia, sown in a gnotobiotic system, and then isolated from surface-sterilized seedlings on selective medium. Using scanning electron microscopy, hyphal penetration of epithelial cells was observed. Using ITS primers, polymerase chain reaction (PCR), and electrophoresis of PCR products, 11-98 was detected (single 421-bp band) in a dilution series of fungal and plant DNA, and from cotton seedlings endophytically colonized by 11-98. Biocontrol of *B. bassiana* against *R. solani*, *Pythium myriotylum*, and *Thielaviopsis basicola* was examined using several seed treatment rates (log 5 to log 11 CFU). Disease was suppressed and plant growth was increased in potting mix with *B. bassiana* at log 7 and log 9 CFU/seed, and in sandy loam soil at log 7 CFU/seed. With low disease pressure from *P. myriotylum,* seed treated with 11-98 or BotaniGard isolate GHA increased plant growth. Assays with *T. basicola* were inconclusive. *Beauveria bassiana* was evaluated for induced systemic resistance in cotton against *Xanthomonas axonopodis* pv. *malvacearum*. Root drench treatments were *B. bassiana* at log 5, log 7, and log 9 CFU/ml, untreated, 2,6-dichloro-isonicotinic acid (INA), and untreated without *Xanthomonas*. After 13 days, primary leaves were wounded and challenge-inoculated with *Xanthomonas*. Treatment with *B. bassiana* (log 7

CFU/seed) had less disease than untreated controls and was as effective as INA. In antibiosis assays, *Beauveria bassiana* out-competed *T. basicola* on cotton agar, however no clear zone of inhibition was observed; *B. bassiana* was outcompeted by *R. solani* and *P. myriotylum,* however it maintained its original colony diameter. *Beauveria bassiana* hyphae coiled around *P. myriotylum* hyphae in parasitism assays; no coiling was observed with *R. solani;* results for *T. basicola* were inconclusive.

Extended Abstract

Beauveria bassiana, an entomopathogenic fungus, is endophytic in some agronomically important plants, including tomato and corn. Although known for its entomopathogenic traits, *B. bassiana* isolate 11-98 has biocontrol activity against *Rhizoctonia solani* on tomato. The objectives of this study were: 1) to determine if *B. bassiana* could be endophytic in cotton, *Gossypium hirsutum*, following seed treatment; 2) to determine if *B. bassiana* is capable of providing protection for cotton as a seed treatment against soilborne pathogens responsible for cotton seedling disease complex; 3) to determine if different rates of conidia, applied to seed, alter the effectiveness of *B. bassiana*; and 4) to determine mechanisms of biocontrol used by *B. bassiana* against plant pathogens. Seed from 'Delta Pine 436' cotton was inoculated with conidia in 2% methylcellulose and sown in a gnotobiotic system. *Beauveria bassiana* isolate 11-98C (Bb 11-98C) was endophytic in cotton based on standard isolation plating techniques onto selective medium. Scanning electron microscopy was used to determine the mode of penetration by the fungus into cotton. Hyphal penetration points through epithelial cells were observed; however, not all developing hyphae on the leaf surface penetrated the cuticle. Detection of *B. bassiana* in a dilution series of fungal and plant DNA, and from cotton seedling tissue endophytically colonized by Bb 11-98C was determined using polymerase chain reaction (PCR) with ITS primers, and gel electrophoresis of PCR products. Detection was evidenced by the production of a single 421 bp band for Bb 11-98C. The biocontrol efficacy of *B. bassiana* against soilborne cotton pathogens, *R. solani*, *Pythium myriotylum*, and *Thielaviopsis basicola* was determined using a range of rates (1×10^5 to 1×10^{11} CFU) of *B. bassiana* conidia as seed treatments. Experimental designs were

factorials (pathogen level and seed treatment) in randomized complete blocks with 7 to 14 replicates and two seeds per replicate. Plant height, weight, disease severity (1 to 6) and percent survival were determined after 21 days. *Beauveria bassiana* (1×10^7 and 1×10^9 CFU/seed) provided significant control of *R. solani* in three trials in potting mix. *Beauveria bassiana* at 1×10^7 CFU/seed suppressed disease in a sandy loam soil infested with *R. solani*. With low disease pressure from *P. myriotylum* applied 7 days after sowing with a 48 hr flood period in a silt loam soil, seed treated with Bb 11-98 or BotaniGard isolate GHA increased plant growth. Assays with *T. basicola* in sandy loam were inconclusive as pathogen effects were not significant. *Beauveria bassiana* was evaluated for its ability to induce systemic resistance (ISR) in cotton against *Xanthomonas axonopodis* pv. *malvacearum* using conidia of Bb 11-98C applied to seedling roots in a soilless system. Treatments were three rates of *B. bassiana* 11-98C conidia (1×10^5, 1×10^7, 1×10^9) and three controls [untreated, 2,6-dichloro-isonicotinic acid (INA), and untreated without *Xanthomonas* challenge]. Treatments were replicated four to 10 times with four seedlings per replication. Conidial and INA treatments were applied to roots 5 cm below radicle emergence. After 13 days, one primary leaf of each plant was pricked with a needle and challenge-inoculated with *X. axonopodis* (1×10^9 or 1×10^{10} CFU/ml). After which, leaves were rated for bacterial leaf blight daily for 1 week. Treatment with *B. bassiana* 1×10^7 CFU/seed resulted in significantly lower disease ratings than the untreated control and was as effective as INA. *Beauveria bassiana* was examined on host based medium for its ability to produce antimicrobials that would inhibit growth of *R. solani*, *P. myriotylum* or *T. basicola*, and parasitism assays were performed to determine if *B. bassiana* could parasitize these pathogens. In

the antibiosis assays, *B. bassiana* out-competed *T. basicola* on cotton agar, however no clear zone of inhibition was observed. In the assay with *R. solani* and *P. myriotylum*, *B. bassiana* was out-competed however; it maintained its original colony diameter. In the parasitism assay no parasitism was observed with *R. solani* and results for *T. basicola* were inconclusive as the fungus did not produce mycelia in the assay. Coiling of *B. bassiana* hyphae around *P. myriotylum* hyphae was observed.

Table of Contents

Part
1. Literature Review....1
Introduction....2
Literature Cited....22

2. *Beauveria bassiana*: Evidence for endophytic activity in cotton following seed treatment with conidia....28
Abstract....29
Introduction....30
Materials and Methods....31
Results....40
Discussion....55
Literature Cited....58

3. *Beauveria bassiana*: Effect of conidial rate and soil type on biocontrol of *Pythium*, *Rhizoctonia*, and *Thielaivopsis*....60
Abstract....61
Introduction....62
Materials and Methods....64
Results....69
Discussion....109
Literature Cited....115

4. Mechanisms of action, including induced systemic resistance, employed by *Beauveria bassiana* as a biocontrol against cotton pathogens....118
Abstract....119
Introduction....120
Materials and Methods....125
Results....133
Discussion....145
Literature Cited....151

5. Potential of *Beauveria bassiana* as a biocontrol for plant pathogens....155
Research Possibilities....156
Literature Cited....162

Vita....163

List of Tables

Table 2-1. Primer sequences 36

Table 3-1. Seed treatment differences with *Beauveria bassiana* in potting mix infested with 1% *Rhizoctonia solani*, Trial 1 75

Table 3-2 Effect of pathogen on cotton seedling variables, Trial 3 80

Table 3-3. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C CFU/seed on cotton seedling variables, Trial 3 81

Table 3-4. Effect of pathogen on cotton seedling variables, Staser Trial 1 88

Table 3-5. Effect of pathogen on cotton seedling variables, Staser Trial 2 95

Table 3-6. Effect of pathogen on cotton seedling variables in potting mix with *Pythium* pre-emergence application, Trial 2 105

Table 3-7. Effect of pathogen on cotton seedling variables in Staser sandy loam soil with *Thielaviopsis basicola*, Trial 1 108

List of Figures

Figure 1-1. Monomer of chitin molecule.....11

Figure 1-2. Molecular structures of toxins produced by *Beauveria bassiana* 13

Figure 2-1. Cotton seedlings in gnotobiotic assay.....33

Figure 2-2. Violet colored endophytic *Beauveria bassiana* 41

Figure 2-3. Endophytic *Beauveria bassiana* 11-98C was re-isolated from 'DP 436 RR.'.....41

Figure 2-4. Conidium of *Beauveria bassiana* 11-98C germinating on cotton 'DP 436 RR' cotyledon surface 43

Figure 2-5. Conidia of *Beauveria bassiana* 11-98C germinating and growing towards two trichomes 43

Figure 2-6. Mycelia of *Beauveria bassiana* 11-98C spreading out in a seemingly random pattern, penetrating, and exiting epithelial cells 44

Figure 2-7. *Beauveria bassiana* 11-98C conidia germinating outside of stomate.....44

Figure 2-8. Cotton leaf cross section showing the palisade parenchyma and spongy mesophyll.....45

Figure 2-9. Conidia and hyphae of *Beauveria bassiana* 11-98C pushed forward on root tip of cotton radicle three to four days after germination from conidia coated seed 45

Figure 2-10. Dilution series with *Beauveria bassiana* isolate 11-98C and cotton 'DP 436 RR' DNA using ITS 1 and ITS 4 primers 47

Figure 2-11. DNA from original fungal isolate *Beauveria bassiana* 11-98 was isolated and subjected to PCR using new ITS primers 49

Figure 2-12. DNA from *Beauveria bassiana* 11-98C was isolated and subjected to PCR using new ITS primers 50

Figure 2-13. DNA from the violet-colored putative *Beauveria bassiana* 11-98 isolate....51

Figure 2-14. Sequence and comparison of PCR products....52

Figure 2-15. Dilution series with *Beauveria bassiana* 11-98C and cotton 'DP 436 RR' DNA using new ITS primers designed by Dr. J. K. Moulton (University of Tennessee)....53

Figure 2-16. Detection of *Beauveria bassiana* 11-98 DNA extracted from cotton 'DP 436 RR' DNA from plants grown from Bb 11-98 treated seed using new ITS primers designed by Dr. J. K. Moulton (University of Tennessee)....54

Figure 3-1. Germination assay....70

Figure 3-2. Effect of seed treatment rate of *Beauveria bassiana* 11-98C conidia on seedling shoot height, radicle length and seedling survival in a soilless germination assay....71

Figure 3-3. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia and pathogen in potting mix amended with 1% (w/w) *Rhizoctonia solani* on cotton seedling height, Trial 1....73

Figure 3-4. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia and pathogen in potting mix amended with 1% (w/w) *Rhizoctonia solani* on cotton seedling hypocotyl disease index, Trial1....74

Figure 3-5. Effect of different seed treatment rates of *Beauveria bassiana* (Bb) 11-98C conidia on cotton seedling height in potting mix amended with 1% *Rhizoctonia solani,* Trial 2....77

Figure 3-6. Effect of different seed treatment rates of *Beauveria bassiana* (Bb) 11-98C conidia on percentage cotton seedling survival in potting mix amended with 1% *Rhizoctonia solani*, Trial 2....78

Figure 3-7. Effect of different seed treatment rates of *Beauveria bassiana* (Bb) 11-98C conidia on cotton seedling root disease index in potting mix amended with 1% *Rhizoctonia solani,* Trial 2. 79

Figure 3-8. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C on seedling weight in potting mix amended with 1% *Rhizoctonia solani*, Trial 4 83

Figure 3-9. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C on shoot weight in potting mix amended with 1% *Rhizoctonia solani,* Trial 4....84

Figure 3-10. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C on seedling height in potting mix amended with 1% *Rhizoctonia solani,* Trial 4....85

Figure 3-11. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C on hypocotyl disease index in potting mix amended with 1% *Rhizoctonia solani,* Trial 4....86

Figure 3-12. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C on root disease index in potting mix amended with 1% *Rhizoctonia solani*, Trial 4....87

Figure 3-13. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia on seedling weight in a Staser sandy loam soil amended with 1% *Rhizoctonia solani,* Trial 1 90

Figure 3-14. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia on seedling root weight in Staser sandy loam soil amended with 1% *Rhizoctonia solani,* Trial 1 91

Figure 3-15. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia on percent seedling survival in Staser sandy loam soil amended with 1% *Rhizoctonia solani,* Trial 1 92

Figure 3-16. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia on hypocotyl disease index in Staser sandy loam soil amended with 1% *Rhizoctonia solani,* Trial 1 93

Figure 3-17. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia on root disease index in Staser sandy loam soil amended with 1% *Rhizoctonia solani,* Trial 1 94

Figure 3-18. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C conidia on seedling height in Staser sandy loam soil amended with 1% *Rhizoctonia solani,* Trial 2 96

Figure 3-19. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C conidia on percent seedling survival in Staser sandy loam soil amended with 1% *Rhizoctonia solani,* Trial 2 97

Figure 3-20. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on seedling weight in Collins silt loam amended with *Pythium myriotylum* (post-emergence), Trial 2 99

Figure 3-21. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on root weight in Collins silt loam amended with *Pythium myriotylum* (post-emergence), Trial 2 100

Figure 3-22. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on percent seedling survival in Collins silt loam amended with *Pythium myriotylum* (post-emergence), Trial 2.....101

Figure 3-23. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on seedling height in Collins silt loam amended with *Pythium myriotylum* (post-emergence), Trial 2 102

Figure 3-24. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on hypocotyl disease index in Collins silt loam amended with *Pythium myriotylum* (post-emergence), Trial 2 103

Figure 3-25. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on hypocotyl disease index in potting mix infested with *Pythium myriotylum* added immediately after seed sowing (pre-emergence).....106

Figure 3-26. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on root disease index in potting mix infested with *Pythium myriotylum* added immediately after seed sowing (pre-emergence).....107

Figure 4-1. *Beauveria bassiana* after growing on cotton agar for approximately 7 days 131

Figure 4-2. Evidence of induced systemic resistance in cotton caused by *Beauveria bassiana* (Bb) 11-98C applied as a root drench 13 days prior to pathogen challenge.....134

Figure 4-3. Effect of *Beauveria bassiana* (Bb) 11-98C applied as a root drench to cotton seedlings 13 days prior to pathogen challenge on disease progress caused by *Xanthomonas axonopodis* pv. *malvacearum* 137

Figure 4-4. Antibiosis assay with *Beauveria bassiana* (Bb) 11-98C and *Thielaviopsis basicola* 139

Figure 4-5 Antibiosis assay with *Beauveria bassiana* (Bb) 11-98C and *Rhizoctonia solani* 139

Figure 4-6. Antibiosis assay with *Beauveria bassiana* (Bb) 11-98C and *Pythium myriotylum* 139

Figure 4-7. Small hyphae of *Beauveria bassiana* isolate 11-98C (B) coiling around larger hyphae of *Pythium myriotylum* (Pm) 141

Figure 4-8. Endoconidia of *Thielaviopsis basicola* 141

Figure 4-9. Chitin hydrolysis assay 144

Part 1. Literature Review

Introduction

Cotton production

Gossypium hirsutum L. upland cotton has become the dominant species in commercial cotton production. It is a member of Malvaceae, which contains about 95 genera of herbs, shrubs, and small trees. Economically cotton is the most important member of the family (Anonymous 2006b). It is currently grown in over 90 countries, the majority of which are developing countries (Jabaji-Hare and Neate 2005). In the U.S., the Cotton Belt extends through North and South Carolina, Georgia, Alabama, Mississippi, western Tennessee, eastern Arkansas, Louisiana, eastern Texas, to southern Oklahoma (Anonymous 2006a).

In the U.S., more than 13 million acres are planted with cotton, the majority being genetically engineered cultivars (Brooks 2001). Since introduction of transgenics, in 1995, a number of cultivars have been introduced; some with multiple or "stacked" characteristics. Three genes are commonly inserted into cotton: *Bacillus thuringiensis* Berlinger (Bt) toxin, 5-enolpyruvylshikimate-3-phospate (EPSP) synthase (RR- Roundup Ready), and bromoxynil nitrilase (BXN).

Bollgard cotton cultivars are genetically engineered for resistance to tobacco budworm and pink and cotton bollworm. The Bt gene in cotton was isolated from *B. thuringiensis*, a common soil bacterium. Certain strains of the bacterium are capable of producing toxins that are lethal to target insects. The Bt toxin is a crystal protein that kills the cells lining the insect gut in certain insects. When these insects ingest this toxin, it disrupts the function of their digestive systems causing the insects to stop feeding and die (Clark and Russell 2000a). Plants with the RR gene have increased amounts of the

monomeric EPSP. This enzyme is pivotal for the production of essential amino acids (phenylalanine, tyrosine and tryptophan) and is inhibited by glyphosate, the active ingredient in the commercial herbicide Roundup (Clark and Russell 2000b). The gene product ultimately protects RR plants and direct application of the herbicide is possible. The BXN system consists of cultivars genetically engineered for leaf applications of oxynil-containing herbicides, such as bromoxynil. The oxynil family of herbicides is active against dicotyledonous plants by blocking electron flow during the light reaction of photosynthesis. BXN, a gene from the soil bacterium *Klebsiella pneumoniae* (Schroeter) Trevisan, detoxifies bromoxynil in genetically engineered plants by hydrolysis into non-phytotoxic compounds. The modified cotton permits farmers to use oxynil-containing herbicides for weed control in the cultivation of cotton (Hagedorn 2005).

Cotton Diseases

Disease losses in cotton are highly variable within a country or region. Seedling diseases result in the largest yield losses of cotton crops in the U.S., and are among the most important of those diseases that limit cotton lint and seed production (Howell 2001, Wang and Davis 1997). In 2001, the estimated loss in bales of cotton due to seedling diseases was 2.43% or approximately $169,820,640 (Howell 2001). In severely infested fields planted with susceptible cultivars, yield losses can be high and in some cases, complete loss may occur. According to the National Cotton Disease Council, disease losses were estimated at over 109,000 bales (227 kg or 500 lb) in 2004 (Blasingame and Patel 2005).

The seedling disease complex of cotton is caused by *Rhizoctonia solani* Kühn,

Fusarium spp., *Pythium* spp., and *Thielaviopsis basicola* (Berk. & Broome) Ferraris. These soilborne pathogens may occur separately or in combination. They are capable of surviving and maintaining high inoculum loads in fields for multiple years (Watkins 1981). Symptoms include the decay of the seed before germination, decay of the seedling before emergence, girdling of the emerged seedling at or near the soil surface and rotting of the root tips (Blasingame 1993).

Options for control of cotton seedling disease

Several crop management practices reduce losses to seedling disease including crop rotation, planting quality seed, timely planting, and the use of fungicides. Increased disease control is expected as more control practices are utilized.

Rotation is the first line of defense against seedling disease, since following cotton with cotton will increase populations of seedling disease fungi in the soil. Fields should be rotated out of cotton for at least two years. Problem fields should be rotated out of cotton for a longer period of time. The second line of defense is seed quality. High quality seed will emerge from the soil more quickly and develop secondary roots faster, therefore being vulnerable to fungal infection for a shorter period of time. Early plantings, which have the greatest potential for seedling disease, should be planted with seed rated excellent or good. Timely planting is the third step in reducing seedling disease loss. Usually warm weather early in the season entices growers to plant earlier than they should. Even high quality seed should not be planted until the 10-day average soil temperature at the 20-cm depth is 18°C. Unfortunately this control measure is not always viable in areas where the growing season is short (Brown et al. 2002).

Another seedling disease control practice is use of fungicides to protect seed and seedling. Cotton seed is typically treated with fungicides by the supplier. Many different fungicides are used as seed treatments for seedling disease control (Davis 1997), including captan, carboxin, pentachloronitrobenzene (PCNB), metalaxyl, myclobutanil, chloroneb and thiram.

The highest level of input for seedling disease control involves in-furrow fungicides. Cotton growers must also consider that fungicides that are effective against one pathogen may not be effective against others. For example, PCNB is active against *Rhizoctonia*, but not *Pythium*. Metalaxyl, mefenoxam and etridiazole are active against *Pythium* spp., but not *Rhizoctonia* (Brown et al. 2002). Using combination treatments of some of the new systemic fungicides will provide a higher level of disease control (Watkins 1981, Brown et al. 2002).

Many growers may not be fully aware of the price that they are currently paying to reduce disease. For example the cost of fungicide seed treatments is included with the price of seed, and growers often plant at an increased seeding rate, in part to offset potential losses from a poor stand due to seedling disease.

Biocontrol of plant diseases

Public concern over potential health risks caused by chemical pesticides has helped energize interest in pest management alternatives such as biocontrols, which involve the use of a living organism to control another. The use of biocontrols is an important component of efforts to reduce reliance on chemical pesticides and increase agriculture sustainability. Plant pathogens can become tolerant or resistant to individual

or combinations of pesticides. Pesticide resistance often requires farmers to rotate to a less economically valuable crop or use higher amounts of chemical pesticides to produce the same crop. Soilborne fungi generally can exist in the soil for years without a host plant. They are especially difficult to combat since their life cycles are adapted to endure long periods of time in the soil in the form of survival propagules. Mechanisms through which biocontrol agents can antagonize soilborne pathogens are generally divided into four categories: antibiosis, competition for niches and nutrients (niche exclusion), parasitism/predation, and induction of a plant defense response (Chin-A-Woeng et al. 2003).

***Trichoderma* spp.**

Trichoderma spp. have been used as biocontrols against a number of soilborne pathogens and are also known for their ability to enhance plant growth. *Trichoderma* spp. are well known mycoparasites that have been used successfully as effective biocontrols on many crop plants, including cotton. Typically, *Trichoderma* has been applied on plant parts where the disease symptoms are present and direct antagonism with the pathogen is involved. Different mechanisms have been reported to explain this antagonism including cell lysis, antibiosis, competition and mycoparasitism with concomitant production of enzymes that degrade fungal cell walls (Bigirimana et al. 1997).

The role of extracellular chitinase in the biocontrol activity of *T. virens* has been examined using genetically manipulated strains of this fungus. The *T. virens* strains in which the chitinase gene was disrupted or constitutively overexpressed were constructed through genetic transformation. The biocontrol activity of the disrupted chitinase isolate

was decreased significantly while the overexpressed isolate had significantly enhanced biocontrol activity against *R. solani* (Baek et al. 1999).

Trichoderma asperellum Samuels, Lieckf. & Nirenberg. [stat. anam.] can penetrate the roots of cucumber seedlings and colonize the epidermis and root cortex. These interactions induce host plant resistance to pathogens, even in the upper part of the plant. *Trichoderma* root inoculation is effective against different types of pathogens in a wide variety of plants including cucumber (Shoresh et al. 2005).

Trichoderma spp. can also affect host plant physiology and induce plant defense reactions such as the hypersensitive response and production of phytoalexin. When seedlings were treated with *T. harzianum* Rifai and *T. viride* Pers.:Fr, before *Sclerotinia sclerotiorum* (Lib.) de Bary challenge, there was significant reduction in stalk rot severity in cauliflower and tomato compared to treatments that did not receive *Trichoderma* (Sharma and Sain 2004). Development of infection was consistently reduced after prior treatment with *T. harzianum* and *T. viride* on plant parts spatially separated from the site of *S. sclerotiorum* inoculation.

On cotton, *Trichoderma* has been reported to be active against *R. solani*, *Fusarium oxysporum* f. sp. *vasinfectum* (Atk.) W.C. Snyder & H.N. Hansen and *Verticillium dahlia* Kleb. Cotton seedlings treated with an effective biocontrol strain of *T. virens* (J.H. Mill., Giddens & A.A. Foster) Arx have higher levels of defense-related compounds such as terpenoids and peroxidase activity in the roots than seedlings treated with ineffective isolates (Hanson and Howell 2004).

In effect, *Trichoderma* spp. have demonstrated multiple mechanisms of biocontrol. Similarly, the entomopathogenic fungus *Beauveria bassiana* (Bals-Criv.)

Vuill. may use these same biocontrol mechanisms against plant pathogens.

History of *Beauveria bassiana*

The occurrence of mummified silkworm (*Bombyx mori* L.) cadavers was documented in Japan as early as 900 CE. Mummified caterpillars were used medicinally in China for sore throats and as an antiseptic for wounds. They also were pulverized and used in mixtures with other compounds for inciting milk lactation in mammals, to treat abscesses, or as a remedy for toothaches. These uses have been associated with Chinese herbal medicine for about 2,000 years (Boucias and Pendland 1998).

In 1834, an Italian scientist, Agostino Bassi determined that the muscardine disease, causing great economic losses in the silkworm industry, was caused by a living organism (Porter 1973). Because of his recognition of the origin of the disease, Bassi is credited with formulating the parasite theory of disease. Bassi also developed and demonstrated the use of sanitation techniques which made it possible to control the disease (Porter 1973).

Beauveria bassiana is an entomopathogen that has been recognized, utilized and studied for many years. The entomopathogenic characteristics of the organism have probably changed relatively little over time. The identification of a worker ant covered in a fungus, morphologically similar to modern *B. bassiana* isolates was discovered in amber and estimated to be 25 million years old (Poinar and Thomas 1984).

The initial identification of *B. bassiana* was done by Balsamo-Crivelis, who named it *Botrytis bassiana* in honor of Bassi. Later Veillmuin transferred it to *Beauveria* (Porter 1973). Because the sexual stage was not known at the time, *B. bassiana* was

originally grouped in the artificial phylum Deuteromycota. In 2001, the teleomorph of *B. bassiana* was determined to be a *Cordyceps*. The current taxonomy of the sexual stage of the fungus is phylum Ascomycota, class Pyrenomycetes (perithecia), order Hypocreales, family Clavicipitaceae, *Cordyceps bassiana* (teleomorph) and *Beauveria bassiana* (anamorph) (Yokoyama et al. 2004). For the sake of literary continuity, the organism will be referred to as *B. bassiana* throughout this dissertation.

Morphology and nutrition of *Beauveria bassiana*

The asexual structures of *B. bassiana* are conidia, blastospores and mycelia. The asexual stage of *B. bassiana* is well understood and involves the production of septate hyphae, which produce conidiophores from which hyaline (colorless) conidia form singly on a geniculate rachis. Conidia are globose to oval. *Beauveria bassiana* produces aerial conidia on the surface of mummified insect cadavers; submersed conidia are formed in liquid culture. Blastospores are sometimes referred to as the yeast-like phase of the fungus. Blastospores arise from the hypha once penetration of the insect cuticle has occurred. They are capable of reproductive budding. Blastospores inside insect cadavers do not have cell walls, but those in liquid culture suspensions do have walls (Hegedus et al. 1992). These structures are the obligatory parasitic phase of the fungus, since *B. bassiana* in its hyphal mode can sporulate on a dead insect (Boucias and Pendland 1988).

Beauveria bassiana as an entomopathogen

Historically *B. bassiana* is best known as an entomopathogen. Fungal entomopathogens are unique among insect pathogens in that instead of depending on

being eaten by the insect or by opportunity entering through an opening (wound or natural), they are capable of entering the insect directly through the cuticle by production of enzymes (Boucias and Pendland 1988).

The spore(s) can be wind-blown or picked up by the insect as it moves through its environment. The spores are hydrophobic, which enables them to adhere to the insect's cuticle. *Beauveria bassiana* spores require only a carbon source in order to germinate, and germination can occur after only a few hours. However, a nitrogen source is required for continued growth (Boucias and Pendland 1988).

Spore germination is induced on the surface of the insect cuticle which is composed of up to 40% chitin and serves as a source of carbon and nitrogen. The chitin molecule (Fig. 1-1.) is composed of alternating N-acetylglucosamine residues, linked by β-(1-4)-glycosidic bonds. Chitin is always associated with cuticle proteins that determine the mechanical properties of the cuticle (Merzendorfer and Zimoch 2003).

Upon spore germination, a hyphal tube is extended and proteases, chitinases and lipidases are released. Proteases are released first, suggesting that the chitin fibrils are coated with protein, followed by the release of chitinases. The enzymatic action of *B. bassiana* is thought to help the fungus adhere to the insect cuticle (Boucias and Pendland 1988). The hyphae use mechanical pressure (exerting force in one concentrated area) and enzymes to penetrate the cuticle and enter the hemocoel (Steinhaus 1949). If the insect is in the process of a molt, it may discard the exoskeleton before *B. bassiana* can penetrate into the new integument, thereby escaping infection. Infection may appear as dark brown spots on the insect cuticle, as melanization may be part of the insect immune response. Infection can also occur via the mouth or anus; it has also been speculated that infection

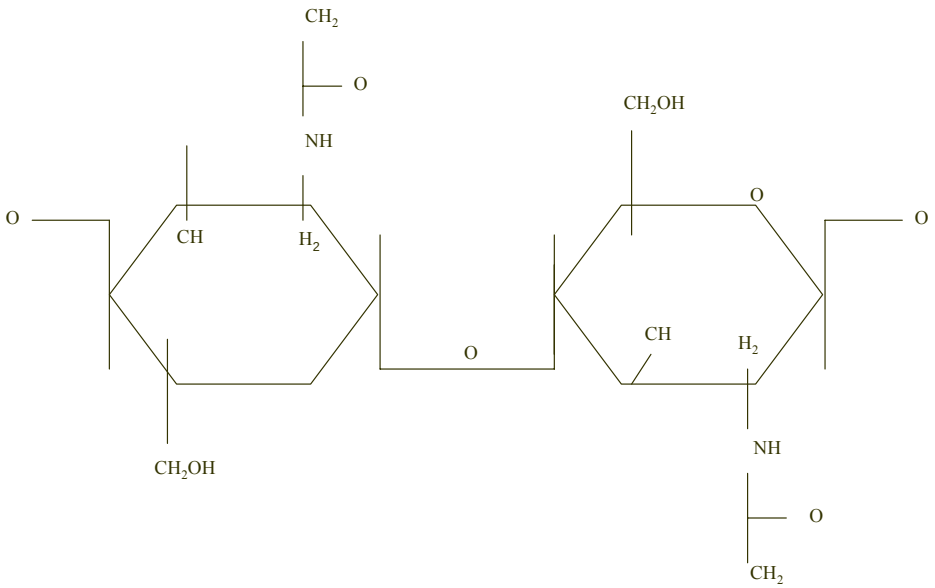

Fig. 1-1. Monomer of chitin molecule.

occurs via spiracles (Boucias and Pendland 1988).

Once penetration of the hemocoel by hyphae has occurred, *B. bassiana* switches into a yeast-like phase, with the production of blastospores. Blastospores will continue to multiply within the insect by budding. Blastospores do not appear to induce an immune response within the insect. Depending on the insect, virulence of the *B. bassiana* strain, internal environmental factors and host immune response, it may take from 2 to 8 days to actually kill the insect. During this time the insect will continue to eat and move; with time, insect activity will slow and a type of confusion or paralysis will occur (Boucias and Pendland 1988). When the insect food source has been utilized, *B. bassiana* enters into the hyphal stage again and sporulation occurs. Aerial conidia are produced and the infection cycle begins again. Sometimes the sexual stage, with production of perithecia, occurs.

Since its initial recognition, the ability of *Beauveria* to feed upon insects has made it a natural alternative to chemical pesticide control. *Beauveria bassiana* is commercially available in the U.S. under several brand names such as, Mycotrol, BotaniGard, and Naturalis. Active ingredients are isolate GHA for Mycotrol and BotaniGard, and TNO isolate F-7744 from ATCC-74040 of *B. bassiana* for Naturalis.

Secondary metabolites

Beauveria bassiana produces an array of relatively low molecular weight secondary metabolites, some of which have antibiotic properties while others are important virulence determinants. These metabolites aid in parasitism of insects but are not required, as some entomopathogenic isolates do not produce all metabolites. Although these metabolites are collectively referred to as toxins, little is known about their properties, production and spatial distribution. They may act as virulence factors by facilitating the ability of the fungus to colonize and use an insect as a food source and preventing subsequent invasion by secondary invaders (Stasser et al. 2000). Toxins produced by isolates of *B. bassiana* include beauvericin (Fig. 1-2A), bassinolide (Fig. 1-2B), beauvolide, beauvirolide (Fig. 1-2C), oosporein (Fig. 1-2D), bassianin (Fig. 1-2E) and tellinin (Fig. 1-2F).

Beauvericin, a cyclohexadepsipeptide, was first isolated from *B. bassiana* on the basis of assays of toxicity to brine shrimp. It exhibits antibacterial activity and has moderate insecticidal properties (Gupta et al. 1991, Stasser et al. 2000). It is structurally similar to enniatins, a class of N-methylated cyclohexadepsipeptides produced by *Fusarium* species with manifold biological activities (Weckwerth et al. 2000).

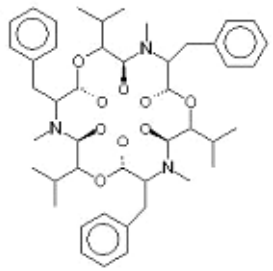

Fig. 1-2A. Beauvericin.
(Google images European Mycotoxin Awareness Network).

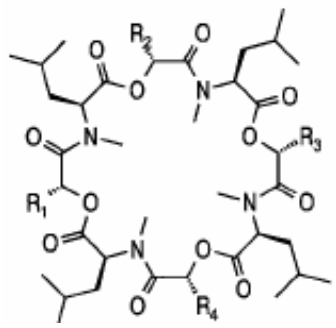

Fig. 1-2B. Bassinolide.
{$R^{1-4} = CH(CH_3)_2$}
(Weckwerth et al. 2000).

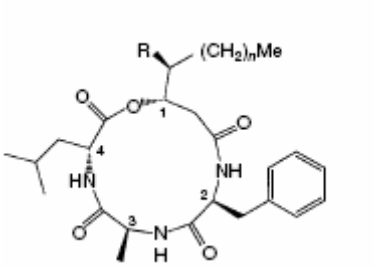

Fig.1-2C. Beauverolides.
(Kuzma et al. 2004).

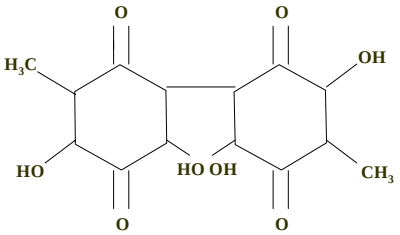

Fig. 1-2D. Oosporein.

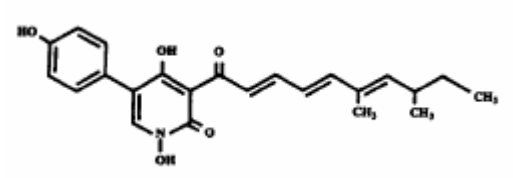

Fig. 1-2E. Bassianin.
(Jeffs and Khachatourians 1997).

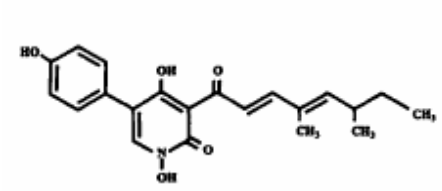

Fig. 1-2F. Tellinin.
(Jeffs and Khachatourians 1997).

Fig. 1-2. Molecular structures of toxins produced by *Beauveria bassiana*. (A) Beauvericin; (B) Bassinolide; (C) Beauverolide; (D) Oosporein; (E) Bassianin; (F) Tellinin.

Beauvericin is an ionophore capable of inserting into the plasma membrane disrupting transmembrane potential. Abnormal ion transport and disrupted cells and membrane bound organelles may result (Plattner and Nelson 1994, Žižka and Weiser 1993). Less is known about bassinolide which, like beauvericin, is structurally similar to enniatins. Bassinolide is toxic to lepidopteran species (Kanaoka et al. 1978).

Beauvolide and beauvirolide are structurally similar to beauvericin and bassinolide, but their toxic effects have not been fully demonstrated against target insects. Biological tests indicate that beauverolides do not exhibit bactericidal, fungicidal, or direct insecticidal effects. However they are involved in insect immunomodulation, a change in the body's immune system, caused by agents that activate or suppress its function (Kuzma et al. 2001).

Oosporein is a red pigmented dibenzoquinone that is a widespread secondary metabolite of soil-dwelling fungi (El Basyouni and Vining 1966, Michelitsch et al. 2004). It is active against several bacteria, particularly Gram-positive species [*Staphylococcus aureus* Rosenbach, *Bacillus subtilis* (Ehrenberg) Cohn, *Proteus vulgaris* Hauser]; however, oosporein has little effect against Gram-negative bacteria (Brewer et al. 1984, Taniguchi et al. 1984, Vining et al. 1962, Wainwright et al. 1986). It has no obvious effect on fungi and plants (Cole et al. 1974). Oosporein was shown to have strong inhibitory effects on radial growth of *Phytophthora infestans* (Mont.) de Bary (minimum inhibitory concentration = 16 μM), but *Alternaria solani* Sorauer and *F. oxysporum* Schltdl.:Fr. were insensitive (Nagaoka et al. 2004). Oosporein oxidizes proteins and amino acids by changing the SH-groups, resulting in enzyme malfunction. The production of this compound may enable *B. bassiana* to compete against the natural

bacteria microflora located within the insect gut. The presence of this compound is generally observed after infection has occurred and *B. bassiana* is within its proliferation phase; the insect sometimes exhibits a pink or reddish color. Oosporein is thought to inhibit opportunistic organisms found in the insect digestive tract thereby enabling *B. bassiana* to sporulate on the cadaver without competition (Stasser et al. 2000).

Bassinin (Fig. 1-2E) and tellinin (Fig. 1-2F), yellow pigments from mycelial extracts, are collectively referred to as bassianins (El Basyouni et al. 1968). These compounds are capable of inhibiting total ATP*ase* activity in erythrocyte membranes and of promoting cell lysis (Jeffs and Khachatourians 1997). All of these toxins are thought to act as virulence factors that aid in the colonization and utilization of a food substrate until fungal propagules are produced.

Effect of environment on control of insects by *B. bassiana*

The use of microbials in pest management is not new in entomology, which has relied on microbials as part of integrated pest management programs. However, their use against plant pathogens in plant production is still somewhat novel. In some cases, plants have formed symbiotic relationships with microbial organisms to enhance their survival. The establishment of these symbiotic relationships may also require specific environmental conditions.

Beauveria bassiana requires certain environmental conditions in order to operate effectively and optimally as a biocontrol against insects. The optimal temperature range for insect control with *B. bassiana* is between 24 and 26°C. Optimal humidity is between 75 to 100%. However, if a humidity of 100% occurs for greater than 24 hours, growth of

B. bassiana is somewhat reduced. These conditions can be met in microenvironments on leaf surfaces, within soil or even on the insect cuticle that enable the conidia to germinate (Luz and Fargues 1999).

The main concern in the use of *B. bassiana* for control of insects is the need for an extended shelf-life. Eighteen months is considered a good shelf-life for many microbials. There is strong evidence that *B. bassiana* isolates retain their entomopathogenic activity over long periods of time. Frequent transfer was found to lower its virulence somewhat, thus it is better to keep a single culture for a long period of time (Steinhaus 1949).

Timing of application affects efficacy of *B. bassiana* (Feng et al. 1994). *Beauveria bassiana* is not effective against Colorado potato beetle as an overwintering control because the winters are too cool and dry for fungal development. Thus an effective treatment schedule would be to spray in the early spring, when moisture levels are high (Boucias and Pendland 1998).

Plant colonization by *B. bassiana*

It has been traditionally accepted that a *B. bassiana* infection cycle begins with conidia landing directly on the host cuticle, then attaching and producing an infection tube before it acts as a microbial pesticide against an insect (Boucias et al.1995). However this accepted paradigm was challenged when Bing and Lewis (1991) conducted a 2-year study to analyze the distribution and persistence of *B. bassiana* within corn plants when injected at the whorl stage, and to determine the effects on population levels of the European corn borer, *Ostrinia nubilalis* Hübner. Hypodermic needles and foliar applications were used to inoculate corn plants with *B. bassiana* conidia. *Ostrinia*

nubilalis was used to infest corn in the field at anthesis and plant samples were collected at harvest. Six different plant tissue samples were examined for the presence of *B. bassiana*. For both application treatments, results varied between the first and second year crops due to environmental conditions. Little *B. bassiana* was isolated from plant tissue during the first year, while in the second year *B. bassiana* was recovered from corn nodes 7 and 12 and the leaf collar at the node above the primary ear, but not from the leaf collar for the primary ear. The fungus was not isolated from leaf collar surfaces, but rather from internal areas. Therefore, the fungus did not persist throughout the season on the plant surface, where it was first applied, but did persist endophytically. A significant reduction in tunneling by the European corn borer was noted between untreated corn stalks and foliar and injected corn stalks (Bing and Lewis 1991).

In a later study, Bing and Lewis (1992b) examined the ability of *B. bassiana* to control *O. nubilalis* activity at different stages in corn development. Treatments were applied to corn as a foliar application of a granular formulation of corn grits, and through injection of a conidial suspension at the whorl stage of development. Plants were infested with *O. nubilalis* at the following stages of development: V7 (whorl), V12 (late whorl) or V17 (pretassel). The later the stage of development of the plant, the less damage the European corn borer caused. Time of fungal application significantly affected amount of tunneling by European corn borer larvae. *Beauveria bassiana* colonized all stages equally. Six different plant tissue samples were examined for the presence of *B. bassiana*. The pith was more frequently colonized than the leaf collars. *Beauveria bassiana* isolated from corn maintained its pathogenicity when tested against 5th stage instars. The fungus persisted inside the plant and was present at harvest. Time of European corn borer

infestation after fungal application significantly affected the amount of tunneling by European corn borer larvae. The effectiveness of *B. bassiana* as a microbial control was regulated by environmental conditions. The authors suggested that hot, dry environmental conditions during the first year affected the fungus negatively and delayed its suppression of European corn borer larvae. *Beauveria bassiana* was found to colonize a higher percentage of plants in the second year, due to sufficient rainfall. It was concluded that *B. bassiana* does kill corn borers, but it was not determined if this action is caused by contact or consumption of the endophyte (Bing and Lewis 1992b).

In another study, a conidial suspension of *B. bassiana* was injected into corn plants at anthesis to suppress *O. nubilalis*. The fungus colonized the plants and moved primarily upward within the pith. *Beauveria bassiana* injected into corn plants significantly reduced the amount of European corn borer tunneling (Bing and Lewis 1992a).

Wagner and Lewis (2000) examined the method employed by *B. bassiana* to colonize a corn plant by means of electron microscopy. *Beauveria bassiana* spores were placed on a corn leaf substrate and found to germinate. Hyphae (less than 1%) entered the plant directly through the epidermal cuticle or the stomata to form an endophytic relationship with the plant (Wagner and Lewis 2000).

Naturally occurring endophytic isolates of *B. bassiana* have been isolated from corn, cotton and jimson weed using traditional plating techniques on selective media (Doberski and Tribe 1980). From five isolates collected, two isolates were selected to use as potential endophytic biocontrol inoculum for potato plants. These isolates were capable of endophytic colonization of potato plants (Jones 1994).

***Beauveria bassiana* as a biocontrol of plant pathogens**

The potential of *B. bassiana* to control important soilborne plant pathogens of seedlings, such as *R. solani in situ,* is largely unknown. With few exceptions, research on *B. bassiana* as a control for plant pathogens has been limited to *in vitro* studies on growth and cell lysis of plant pathogens. Antagonistic ability against *R. solani* with 22 different *B. bassiana* isolates was examined on potato dextrose agar (PDA) plates. Three isolates were inhibitory against *R. solani*, indicating there are differences among *B. bassiana* isolates and their specific plant pathogen biocontrol capacities (Lee et al. 1999). In another study, five *B. bassiana* isolates were found to have antifungal inhibitory activity against the mycelial growth of the plant parasitic fungi *Fusarium oxysporum*, *Armillaria mellea* (Vahl:Fr.) P. Kumm. and *Rosellinia necatrix* Prill. (Reisenzein and Tiefenbrunner 1997). The isolates of *B. bassiana* had different antifungal effects, but differences were small compared to nontreated controls. Mycelial growth and spore germination of the phytopathogenic fungi *Botrytis cinerea* Pers.:Fr. and *F. oxysporum* was inhibited by *B. bassiana* culture filtrate (Bark et al. 1996). Inhibition efficiency varied depending on culture medium; inhibition was greatest on PDA for *B. cinerea* and on tryptic soy agar for *F. oxysporum*. *Beauveria bassiana* was antagonistic to *Pythium ultimum* Trow and *P. debaryanum* Auct. non R. Hesse, inducing lysis of the mycelium while actively growing on or beneath the mycelia; however, *R. solani* was resistant to this isolate of *B. bassiana*, while *Septoria nodorum* (Berk.) Castell. & E.G. Germano was strongly suppressed on solid medium (Vesely and Koubova 1994).

In a greenhouse study involving treatment of onion bulbs with antagonistic fungi against *Fusarium oxysporum* f. sp. c*epae* (H.N. Hansen) W.C. Snyder & H.N. Hansen,

out of sixteen fungi, three treatments (*T. viride* isolate 144, *T. harzianum* 312 and *B. bassiana* 142), provided greater that 63% control of the pathogen (Flori and Roberti 1993). *Beauveria bassiana* isolate 11-98 applied as a conidial treatment in a 2.5% methylcellulose (MC) solution to tomato seeds prior to sowing provided protection against *R. solani* (Bishop 1999, Seth 2001).

Beauveria bassiana has the ability to become endophytic in plants. Endophytic activity may in part be responsible for its ability to act as a biocontrol of plant pathogens. Detection of *B. bassiana* in tomato tissues was accomplished with the use of polymerase chain reaction and ITS primers 1 and 4 (Leckie 2002, Ownley et al. 2005). *Beauveria bassiana* was reported to be endophytic in cocoa (*Theobroma cacao* L.*)* seedlings and was recovered from the roots, stems and leaves after two months using direct plating techniques (Posada and Vega 2005). *Beauveria bassiana* was found to colonize approximately 40% of opium poppy, (*Papaver somniferum* L.) seedlings after seed were coated with conidia and examined after six days using direct plating techniques, scanning electron microscopy and polymerase chain reaction with ITS primers 1 and 2 (Quesada-Moraga et al. 2006).

In a regional field trial on cotton, *B. bassiana* seed treatments provided protection against seedling pathogens in some soils. In the two field sites where *B. bassiana* proved successful, the soils were sandy loams and the only pathogen isolated from plant material was *R. solani* (Batson et al. 2000). Variability of the environment has long been noted as a problem for the overall efficiency of biocontrols (Ownley et al. 2003). Soilborne pathogens present the greatest threat to seedlings prior to and a few days after germination.

Research Objectives

The objectives of this study were to determine: 1) if *B. bassiana* is endophytic in cotton and can be isolated from tissue via traditional direct plating techniques; 2) if *B. bassiana* can be detected in cotton with electron microscopy and polymerase chain reaction; 3) if *B. bassiana* is capable of providing protection for cotton as a seed treatment against soilborne pathogens responsible for cotton seedling disease complex; 4) if different rates of conidia, applied to seed, alter effectiveness of *B. bassiana*; and 5) mechanisms of biocontrol used by *B. bassiana* against plant pathogens.

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Part 2. *Beauveria bassiana*: Evidence for endophytic activity in cotton following seed treatment with conidia

Abstract

Beauveria bassiana an entomopathogenic fungus is endophytic in some agronomically important plants, including tomato and corn. The primary objective of this study was to determine ability of *B. bassiana* for endophytic colonization of cotton, *Gossypium hirsutum*, following seed treatment. Cotton seedling tissues were evaluated for endophytic colonization with standard isolation techniques on culture medium, scanning electron microscopy, and polymerase chain reaction. Seed from 'Delta Pine 436' cotton was inoculated with conidia in 2% methylcellulose slurry. Seed were germinated in either a sterile or a gnotobiotic system. *Beauveria bassiana* isolate11-98C was endophytic in cotton based on standard isolation plating techniques with selective medium. Scanning electron microscopy was used to determine mode of penetration into cotton. *Beauveria bassiana* hyphae grew along cotton leaf surfaces; germination and hyphal growth were associated with areas of leaf exudates. Hyphae also germinated and grew on >50% of the root surface examined. Often, a germ tube formed from a conidium and elongated without any apparent termination of growth or penetration of the plant surface. Penetration points through epithelial cells were observed; however, not all developing hyphae on the leaf surface penetrated the cuticle. It is possible that selection sites for germination and generalized movement by *B. bassiana* toward trichomes and other cellular structures associated with exuded plant nutrients indicate the fungus is capable of utilizing specific topographic signals to locate an appropriate entry site. Three sets of primers were evaluated for detection of *B. bassiana* using polymerase chain reaction (PCR) and gel electrophoresis of PCR products. *Beauveria bassiana* was detected as a single band in positive *Beauveria* DNA controls with the use of ITS 1 and 4

primers. However, ITS 1 and 4 primers failed to detect *B. bassiana* in the presence of excess cotton DNA. These primers also amplify ITS regions in cotton. In DNA samples from *Beauveria*-colonized plants a *Beauveria* sequence could not be detected. Presumably the excess cotton DNA out-competed the fungal DNA for primer binding. MAT 1 and MAT 2 mating-type primers, also were unable to detect this isolate of *B. bassiana* in any samples. From ITS sequences detected by ITS 1 and 4 primers, additional primers were designed that did not bind to cotton DNA. Using the new ITS primers, *B. bassiana* was detected in a mixed DNA sample at a ratio of 1 part *B. bassiana* DNA to 1000 parts cotton DNA ($1:10^3$) with PCR and gel electrophoresis of PCR products. The reaction produced a single 421-bp band for *B. bassiana* and no bands for cotton. New ITS primers also detected *B. bassiana* in different tissues of cotton seedlings grown from *B. bassiana*-treated seed.

Keywords: *Beauveria bassiana*, endophytic colonization, cotton, SEM, PCR

Introduction

Beauveria bassiana (Bals.-Criv.) Vuill. was first identified in 1834, by an Italian scientist, Agostino Bassi, as the agent of silkworm (*Bombyx mori* L.) disease, commonly known as muscardine disease (Porter, 1973). In 2001, the teleomorph stage was associated with the Ascomycota, Pyrenomycetes, Hypocreales, Clavicipitaceae, and named *Cordyceps bassiana* (Yokoyama et al. 2004). *Beauveria* is closely related to the mutualistic *Neotyphodium* spp., known endophytes of grasses that can confer protection and act as a deterrent to feeding by insects (Ahmad et al. 1985; Johnson et al. 1985).

Beauveria bassiana is pathogenic on several insect species worldwide. The use of

B. bassiana as a biocontrol for insect pests has been the subject of several papers and reviews (McCoy et al. 1985, Roy et al. 2005). *Beauveria bassiana* applied to whorl-stage corn by foliar application, colonized and persisted in corn plants to provide season-long suppression of European corn borer, *Ostrinia nubilalis* Hübner (Bing and Lewis 1991). Naturally occurring isolates of *B. bassiana* were isolated sporadically from the stems of corn, cotton and jimson weed by Jones (1994) and a single selected isolate was able to endophytically colonize potatoes (Jones 1994). *Beauveria bassiana* has the ability to colonize plants endophytically. Endophytic activity may be responsible in part for the fungus' ability to act as a biocontrol against plant pathogens. *Beauveria bassiana*, applied as conidia in a 2.5% methylcellulose (MC) solution to tomato seeds prior to sowing, provided protection against *Rhizoctonia solani* Kühn, a ubiquitous soilborne fungus that causes disease in a wide range of cultivated plants (Ownley et al. 2005).

Objectives of this study were: 1) to determine extent of *B. bassiana* isolate 11-98 colonization of cotton by isolation from leaves, stems, and roots of cotton seedlings using traditional plating techniques, following seed treatment with the fungus; 2) to monitor *B. bassiana* penetration and invasion using scanning electron microscopy (SEM); and 3) to develop PCR protocols for detection of *B. bassiana* in cotton tissues.

Materials and Methods

Gnotobiotic assay for selection of cotton-adapted *B. bassiana* 11-98

Beauveria bassiana 11-98 (Bb 11-98) was isolated originally from an infected click beetle (Coleoptera: Elateridae) from Scott County, TN (Leckie 2002). The isolate was grown on Sabouraud dextrose agar (SDA) for approximately 6 weeks, and conidia

were harvested by brushing the surface of the plates with a stenciling brush. Conidia were stored at 4°C in a desiccator until needed.

Delinted black cotton seed of 'Delta Pine (DP) 436 RR' was obtained from Dr. Craig Canaday, The University of Tennessee, West Tennessee Research and Education Center, Jackson. Seeds were surface-sterilized with a 30% Clorox solution (30 ml Clorox and 70 ml deionized H_2O) and soaked for 5 min, stirring occasionally. Seeds were rinsed five times, allowed to soak for 1 min during each rinse in deionized water, then air-dried under a chemical fume hood for 3 h. Seed (2 g) were coated with *B. bassiana* spores (1 g Bb 11-98) in 2 ml 2% MC solution to which 25 µl Tween-20 had been added. Modifications to the formula were made to achieve desired conidial rates on seed.

In the gnotobiotic assay, seed of 'DP 436 RR' were coated with Bb 11-98 to achieve a final CFU of 1×10^9 CFU per seed. Treated seed were air-dried and stored at 4°C until use. Seed coated with Bb 11-98 were aseptically placed into 40 (30-mm^2) test tubes with 20 cm^3 of twice-autoclaved vermiculite and 8 to 10 ml sterile deionized water (Fig. 2-1). Seed were placed beneath the vermiculite surface and tubes were incubated in a growth chamber with a 12 hour light/dark cycle at 24 to 25°C for 2 weeks, after which germination rates were recorded. Six 21-day old plants were used for direct plating onto *B. bassiana* selective culture medium (Doberski and Tribe 1980) and remaining plants were harvested at 14 days and stored at -80°C for PCR. The gnotobiotic assay was repeated. A cotton adapted isolate (Bb 11-98C) collected after the gnotobiotic assay was used in subsequent experiments.

At 21 days, five intact seedlings were surface-sterilized by immersion for 1 min in 95% ethanol, then transferred to a 20% Clorox solution for 3 min, and placed in 95%

Fig. 2-1. Cotton seedlings in gnotobiotic assay.

ethanol for 1 min (method adapted from Jones 1994). Afterwards, the plant was rinsed in sterile deionized water and allowed to dry under a laminar flow hood. Surface-sterilized seedlings were aseptically cut into 2 to 3-mm pieces for root and stem sections; leaves were cut into 3-to 7-mm pieces before being placed on *Beauveria* selective medium (Doberski and Tribe 1980) for 6 weeks. Each plate contained five to eight pieces of plant material. Plates were incubated at 22 to 24°C for 6 weeks. A single non-surface sterilized seedling was plated on the selective medium to determine epiphytic growth of *B. bassiana*. In addition, to determine presence of fungal seedborne pathogens 20 to 25 surface-sterilized seeds were randomly selected and placed directly onto potato dextrose agar (PDA). Cultures were sealed and incubated at 22 to 24°C for 14 to 18 days.

Scanning electron microscopy

Conidia from Bb 11-98C cultures were coated onto 'DP 436 RR' seed at 1×10^{10} CFU/seed and air-dried as previously described. Seed were stored at 4°C until needed.

Seed were germinated on moistened filter paper in 150 × 15-mm Petri plates were sealed and incubated at 25°C for 7 days with continuous light. Approximately 3 to 4 days after germination, two seedlings were selected for SEM.

A modified fixation procedure, adapted from Wagner and Lewis (2000), was used for SEM. Cotyledon and root sections were cut into 5-to 7-mm pieces and fixed in 3% glutaraldehyde in phosphate buffer (0.1 M; pH 7.2) for 2 h at 20 to 22°C; plant material was then transferred to fresh fixative solution for approximately 24 h at 4°C. Specimens were rinsed in three changes of buffer, 10 ml each, and then postfixed in 1% OsO_4 for 2 h. Material was washed twice in distilled water for 30 min, and dehydrated in a graded ethanol series to 100% ethanol. Leaves were critical point dried in a DCP-1 critical drying unit (Ladd Research Industries, Inc., Willeston, VT) with CO_2. Specimens were coated with gold in a SPI-Module sputter coating unit. Photographs were taken on a Leo 1525 Field Emission Secondary Electron Microscope (FE-SEM) (Leo Gemini with Oxford INCA ED software, Pretoria, South Africa) operating at 5-kV accelerating voltage.

Isolation of plant and fungal DNA

Plant and fungal material was lyophilized (Virtis Company Inc., Gardner, NY) for 24 h in Eppendorf tubes with three to four, 2.5-mm glass beads (Biospec Products Inc., Bartlesville, OK). Dried material was pulverized with a Mixer mill (Retsch Inc, Haan, Germany); for 1 min, rotated and repeated for 1 min. Plant and fungal DNA were isolated using a PCR purification kit (Qiagen, Valencia, CA). DNA concentrations were determined with a TD 360 Mini-Fluorometer (Turner Designs Instruments, Sunnyvale,

CA) at 360 nm. DNA samples were stored at 4°C until needed.

Polymerase chain reaction

All primers were obtained from Sigma-Genosys (The Woodlands, TX) (Table 1).

MAT 1 and MAT 2 mating type primers

The PCR reaction mixture with the mating type primers (Yokoyama et al. 2004) contained 2.5 µl of 10X PCR reaction buffer with $MgCl_2$ (Promega., Madison, WI); 0.5 µl of a 2 mM mixture of dCTP, dGTP, dTTP and dATP (Promega); 1.25 µl of 20 µM MAT (forward) primer; 1.25 µl of 20 µM MAT (reverse) primer; 9.5 µl of deionized water; 0.5 µl AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA) and 10 µl of DNA sample (100 ng/µl). Tubes were placed into a Mastercycler gradient thermalcycler (Eppendorf AG, Hamburg, Germany) for amplification. Reaction parameters were as follows: 95°C for 9 min; 95°C for 45 s; 65°C with MAT 1-1 for 45 s, 68°C with MAT 1-2 for 45 s, 54°C with MAT 2-1 for 45s and 59°C with MAT 2-2 for 45 s; and 72°C for 30 s, for 35 cycles. The reaction mixture was held at 4°C until tubes were removed. Products were removed from tubes and separated on 2% (w/v) agarose-TAE gel (Tris-acetate-EDTA electrophoresis buffer) at approximately 100 volts. Agarose gels were stained with ethidium bromide solution (4 µl/400 ml buffer; Sigma Chemical Co., St. Louis, MO) and visualized with a Fisher Biotech 312-nm transilluminator FBTI 816 (Fisher Scientific, Pittsburgh, PA). Positive controls of DNA from *B. bassiana* isolate 11-98 and cotton were included in each gel. A negative control containing all reaction

Table 2-1. Primer sequences

Primer[1]	Sequence	Orientation	Reference
MAT1-F1	5' - G(A/G)GC(A/T)AA(A/G)CG(A/G)CCATT(G/)A(C/T)GC	Forward	Yokoyama et al. 2004
MAT1-R1	5' - TT(G/T)CCCATCTC(A/G)TC9A/G)CGGA(C/T(A/G)AA(A/G)GA	Reverse	Yokoyama et al. 2004
MAT1-F2	5'- CCAAGCCGGTATCAGTGAATGC	Forward	Yokoyama et al. 2004
MAT1-R2	5' – CGACCTGTTGTCGAACAAAGGT	Reverse	Yokoyama et al. 2004
MAT2-F1	5' – GC(A/G)TATATTCT(A/G)TACCGCAG	Forward	Yokoyama et al. 2004
MAT2-R1	5' – CGAGGTTGATA(T/C)TGATA(T/C)TG	Reverse	Yokoyama et al. 2004
MAT2-F2	5' – ACGCATATATT(T/C)TGTACCG(T/C)AA	Forward	Yokoyama et al. 2004
MAT2-R2	5' – GAAGGCTTTCG(AT/T)GGT(T/C)TGTAC	Reverse	Yokoyama et al. 2004
ITS 1	5'- TCCGTAGGTGAACCTGCGG	Forward	White et al., 1990
ITS 4	5' – TCCTCCGCTTATTGATATGC	Reverse	White et al., 1990
Modified ITS	5' – GAACCTACCTATYGTTGCTTC	Forward	This study
Modified ITS	5' – ATYCGAGGTCAACGTTCAG	Reverse	This study

[1] All primers were obtained from Sigma-Genosys, The Woodlands, CA.

components except the DNA sample was included to ensure no DNA contamination of PCR reaction constituents.

ITS 1 and ITS 4 primers

The PCR reaction mixture with ITS 1 and ITS 4 primers contained 2.5 µl of 10X PCR reaction buffer (Takara Bio. Otsu, Shiga, Japan); 1.0 µl of a 2 mM mixture of dCTP, dGTP, dTTP and dATP (Takara); 2.5 µl of 3 µM ITS1 primer; 2.5 µl of 3 µM ITS4 primer; 17.75 µl of deionized water; 0.25 µl Taq DNA polymerase (Takara); and 1 µl of DNA sample (100 ng/µl). Mixture was placed in 25-µl Eppendorf tubes, and tubes placed in Mastercycler gradient thermalcycler for amplification. Reaction parameters were as follows; 95°C for 9 min, followed by 35 cycles at 94°C for 1 min, 42°C for 1 min, 72°C for 1 min, for 40 cycles, followed by a final 3-min period at 72°C. The reaction mixture was held at 4°C until the tubes were removed. Products were removed from the tubes and separated on a 1% (w/v) agarose-TBE gel (Tris-Boric-EDTA Electrophoresis Grade, Fisher Scientific) at approximately 100 volts. A 50-bp + DNA step ladder (Promega) was included to determine size of PCR products. Agarose gels were stained with ethidium bromide and visualized as described earlier.

Positive controls of DNA from Bb 11-98 and cotton were included in each gel. A negative control with all reaction components except the DNA sample was included in each gel to ensure no DNA contamination of the PCR reaction constituents. Cotton and *B. bassiana* bands were excised from gels, and processed with a QIAquick Gel Extraction Kit (Qiagen) using the manufacturer's instructions. Extracted PCR products were

sequenced (The University of Tennessee Molecular Biology Resource Facility) and subjected to a GenBank Blast search (Altschul et al. 1997).

Beauveria bassiana new ITS primers

New ITS primers specific for *B. bassiana* DNA were designed by Dr. J.K. Moulton, The University of Tennessee, Knoxville. The reaction mixture consisted of 2.5 µl of 10X PCR reaction buffer (Takara); 1.0 µl of a 2 mM mixture of dCTP, dGTP, dTTP and dATP (Takara); 2.5 µl of 20 µM forward primer; 2.5 µl of 20 µM reverse primer; 16.75 µl deionized water; 0.25 µl Taq polymerase (Takara); and 1.0 µl (100 ng/µl) template DNA. Contents were placed in a 25-µl Eppendorf tube and placed in the thermalcycler for sequence amplification. Two gradient series were conducted to determine optimal annealing temperature for the primers. The optimal annealing temperature (59°C) for the new ITS primers was used in subsequent experiments. Positive controls of DNA from Bb 11-98, and cotton were included in each gel. A negative control with all reaction components except the DNA sample was included in each gel to ensure no DNA contamination of the PCR constituents.

Reaction parameters for PCR were as follows; 95°C for 2 min, 94°C for 1 min, 59°C for 1 min, 72°C for 1 min, for 40 cycles, followed by 72°C for 3 min. Reaction mixes were held at 4°C until used for agarose electrophoresis.

Products were removed from the tubes and separated on a 1% (w/v) agarose-TBE gel at 100 volts until bands had separated properly. A 50 bp+ DNA step ladder (Promega) was included to determine the size of PCR products. Agarose gels were stained with ethidium bromide and visualized with a transilluminator as described above.

The original Bb 11-98 isolate, Bb 11-98C from cotton seedlings and a putative violet *B. bassiana* isolate from seedlings treated with 11-98 were used as DNA samples. Samples were processed with a QIAquick Gel Extraction Kit (Qiagen). PCR fragments were sequenced with the Applied Biosystems (Foster City, CA) Big Dye version 3.1 cycle sequencing kit and analyzed with an MJ Research BaseStation-100 automated sequencer (BioRad Laboratories, Hercules, CA).

To determine the smallest relative concentration of DNA from *B. bassiana* that could be detected in a mixture of plant and fungal DNA; a dilution series was established with 1:1; 1:10; 1:100; 1:1000 ratios of DNA from Bb 11-98 to DNA from cotton 'DP 436 RR.'

The PCR reaction mixture for the dilution series was 2.5 µl of 10X PCR reaction buffer (Takara); 1.0 µl of a 2 mM mixture of dCTP, dGTP, dTTP and dATP (Takara); 1.25 µl of 20 µM forward primer; 1.25 µl of 20 µM reverse primer; 17.75 µl of deionized water; 0.25 µl Taq polymerase (Takara); and 1.0 µl (100 ng/µl) template DNA. Tubes were placed into the thermalcycler and the amplification procedure was conducted. Reaction parameters were as follows; 95°C for 2 min, 94°C for 1 min, approximately 59°C for 1 min for 40 cycles, followed by 72°C.

Products were removed from tubes and separated on a 1% (w/v) agarose-TBE gel. Electrophoresis was conducted at approximately 100 volts until bands had separated properly. As previously described, each gel included a 50 bp+ DNA ladder, a positive Bb 11-98 DNA control, a positive cotton DNA control, and a negative no DNA control. Reaction mixes were held at 4°C until electrophoresis. Agarose gels were stained with

ethidium bromide and visualized with a transilluminator. Bands were excised from the gels and DNA was extracted for sequencing.

Results

Gnotobiotic assay

Beauveria bassiana 11-98 was isolated from cotton seedling tissues of 'DP 436 RR.' The cotton-adapted isolate was named Bb 11-98C. From five seedlings surface sterilized prior to plating, approximately 78% of the plates had fungal isolates. A single non-surface sterilized seedling also had similar fungal isolates in culture. The majority of fungal isolates observed in culture were violet hue in color rather than the typical beige observed in the original 11-98 isolate (Fig. 2-2). From these endophytic isolates, two fungal isolates had the color and morphology typical of Bb 11-98 in culture. One of these was selected and designated isolate Bb11-98C, the assay was repeated with five additional seedlings and beige *B. bassiana* isolates were re-isolated from all parts of cotton plants (Fig. 2-3). Fungal growth from surface-sterilized cotton tissues took approximately four to six weeks for development in culture (Fig. 2-2 and 2-3).

In the first gnotobiotic assay, 79% cotton seeds germinated. In the second assay, 69% of seeds germinated. To test for potential endophytes present, seed were placed directly on PDA plates and evaluated for fungal contaminants after 10 days. Of 23 seeds placed on PDA, 100% germinated and less than 9% had fungal contaminants. The contaminants morphologically resembled *Fusarium* sp. and *Penicillium* sp. No *Beauveria* was isolated from nontreated seed.

A. B.

Fig. 2-2. Violet colored endophytic *Beauveria bassiana*. A. Several violet colonies on *B. bassiana* selective medium were recovered using plating techniques with gnotobiotically grown cotton seedlings. B. Colony of violet fungus.

A. B. C.

Fig. 2-3. Endophytic *Beauveria bassiana* 11-98C was re-isolated from 'DP 436 RR.' *Beauveria bassiana* 11-98C was isolated from the leaves (A) stems (B) and roots (C) of surface-sterilized seedlings grown in a gnotobiotic assay.

The cotton-adapted strain, Bb 11-98C, was grown on SDA for 6 weeks; conidia were harvested and stored at 4°C until needed. A morphological examination of the predominant violet colored culture suggested that it was *B. bassiana*; however, this fungus did not resemble the typical highly sporulating Bb 11-98 observed in culture. The violet colored colonies had more aerial mycelia than the Bb 11-98 isolate and did not readily sporulate. No other fungal isolates were found.

The cotton-adapted strain, Bb 11-98C, was grown on SDA for 6 weeks; conidia were harvested and stored at 4°C until needed. A morphological examination of the predominant violet colored culture suggested that it was *B. bassiana*; however, this fungus did not resemble the typical highly sporulating Bb 11-98 observed in culture. The violet colored colonies had more aerial mycelia than the Bb 11-98 isolate and did not readily sporulate. No other fungal isolates were found.

Scanning electron microscopy

Conidia of Bb 11-98C were observed that had germinated and colonized the outer surfaces of cotton seedlings grown from seed treated with Bb 11-98C conidia, including cotton cotyledons (Figs. 2-4, 2-5, 2-6, 2-7 and 2-8) and on the radicals (Fig. 2-9). Scanning electron micrographs of conidia revealed either ungerminated or in varying stages of germination and hyphal extension. Germination of dry conidia occurred after re-hydration on surface of cotton seedling. Early germination and formation of a germ tube from a single conidium was observed (Fig. 2-4). Germ tubes gradually elongated into hyphae and spread in seemingly random directions across epidermal cells of the cotyledon (Fig. 2-6). Germinating conidia and hyphae were closely associated with

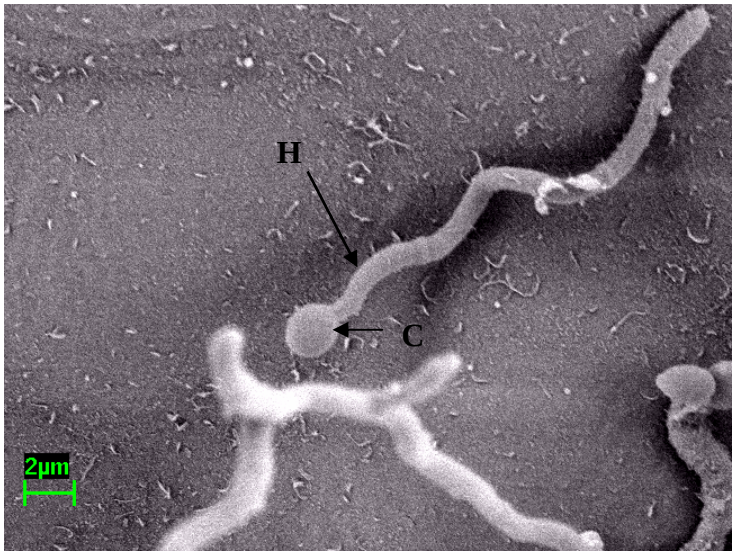

Fig. 2-4. Conidium of *Beauveria bassiana* 11-98C germinating on cotton 'DP 436 RR' cotyledon surface. H = hyphae, C = conidium. Bar = 2 *u*m.

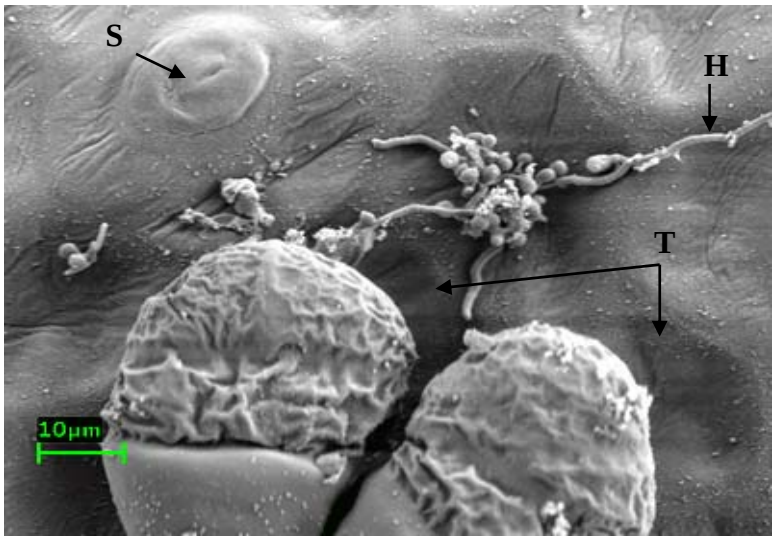

Fig. 2-5. Conidia of *Beauveria bassiana* 11-98C germinating and growing towards two trichomes. H = hyphae, T = trichome, S =stomate. Bar = 10 *u*m.

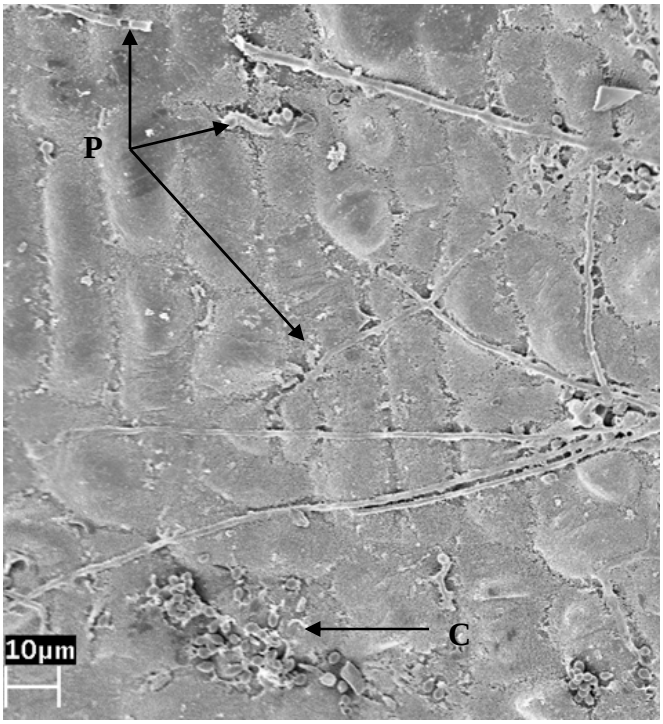

Fig. 2-6**.** Mycelia of *Beauveria bassiana* 11-98C spreading out in a seemingly random pattern, penetrating, and exiting epithelial cells. P = penetrating hyphae, C = conidia that have not germinated. Bar = 10 *u*m.

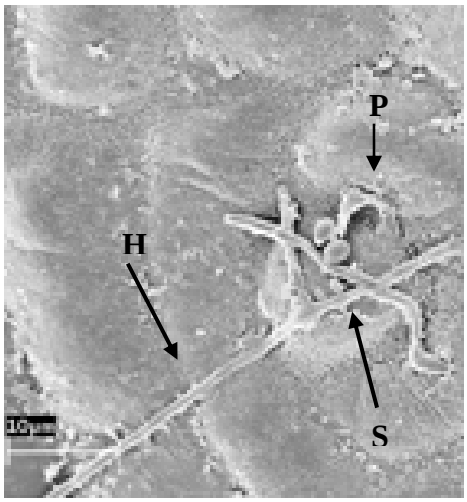

Fig. 2-7. *Beauveria bassiana* 11-98C conidia germinating outside of stomate. H = hyphae, P = penetration point S = stomate. Bar = 10 *u*m.

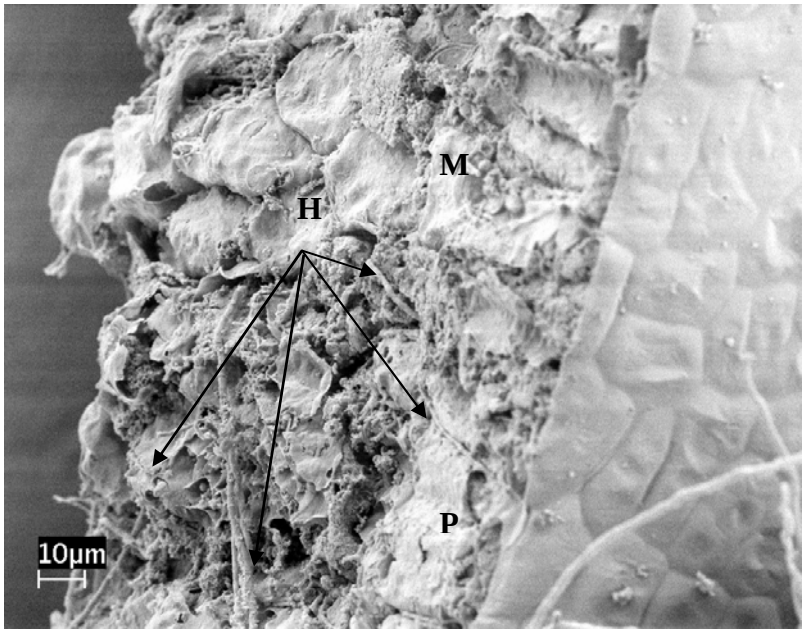

Fig. 2-8. Cotton leaf cross section showing the palisade parenchyma and spongy mesophyll. Linear structures (indicated by arrows) represent hyphae of *Beauveria bassiana* 11-98C growing throughout. H = hyphae, P = palisade leaf layer, M = mesophyll leaf layer. Bar = 10 µm.

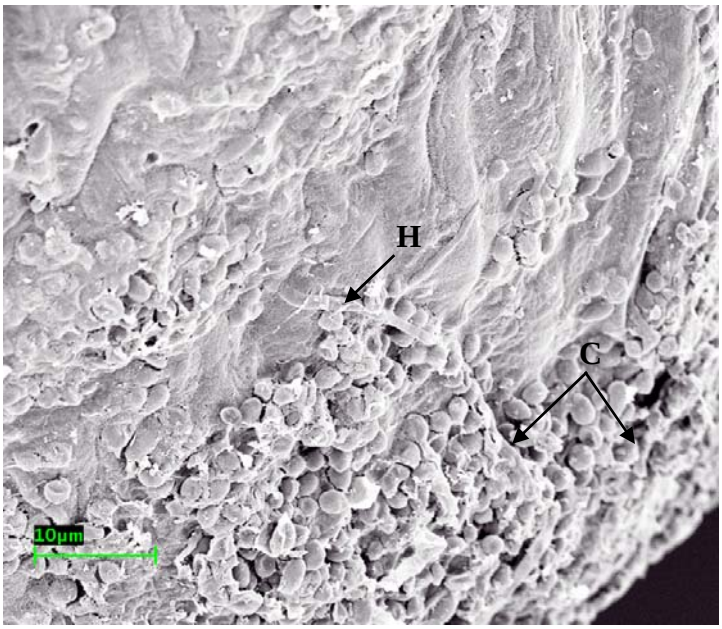

Fig. 2-9. Conidia and hyphae of *Beauveria bassiana* 11-98C pushed forward on root tip of cotton radicle three to four days after germination from conidia coated seed. C = conidia, H = hyphae. Bar = 10 µm.

trichomes on the cotyledon surface (Fig. 2-5).

Mycelia were strongly associated with tips of the cotyledons where thick mats developed and were visible to the naked eye. Several hyphae were associated with stomatal openings but penetration occurred through the epidermal cells (Fig. 2-7). In a cotyledon cross section, Bb 11-98C hyphae were observed growing through the internal leaf tissues, being associated with all layers of the cotyledon (Fig. 2-8). In general, more than 50% of the cotton radicle surface was covered with conidia (Fig. 2-9).

Polymerase Chain Reaction

Attempts to use mating type primers, MAT 1 and MAT 2 (Yokoyama et al. 2004), to detect presence of *B. bassiana* DNA in association with cotton DNA were not successful. Internal transcribed spacer region, ITS 1 and ITS 4 primers were used for detection of *B. bassiana.* When pure DNA samples were used, cotton DNA had a band approximately 800-bp in size and *B. bassiana* 11-98 had a 550-bp PCR product. Both bands were sequenced and a GenBank Blast search confirmed that 800 and 550-bp PCR products were homologous with ITS regions of cotton and *B. bassiana*, respectively. However in mixed DNA samples, ITS 1 and ITS 4 primers had a higher affinity for cotton DNA than for *B. bassiana* DNA. The band for *B. bassiana* was not visible in mixed DNA samples with a high concentration of cotton DNA (Fig. 2-10).
Because the objective was to detect the presence of *B. bassiana* in plant tissue, a more highly selective primer pair for *B. bassiana* was needed. Since *B. bassiana* was successfully detected using ITS 1 and ITS 4 primers, new ITS primers were designed to detect the ITS region within *B. bassiana* but not the ITS region in cotton.

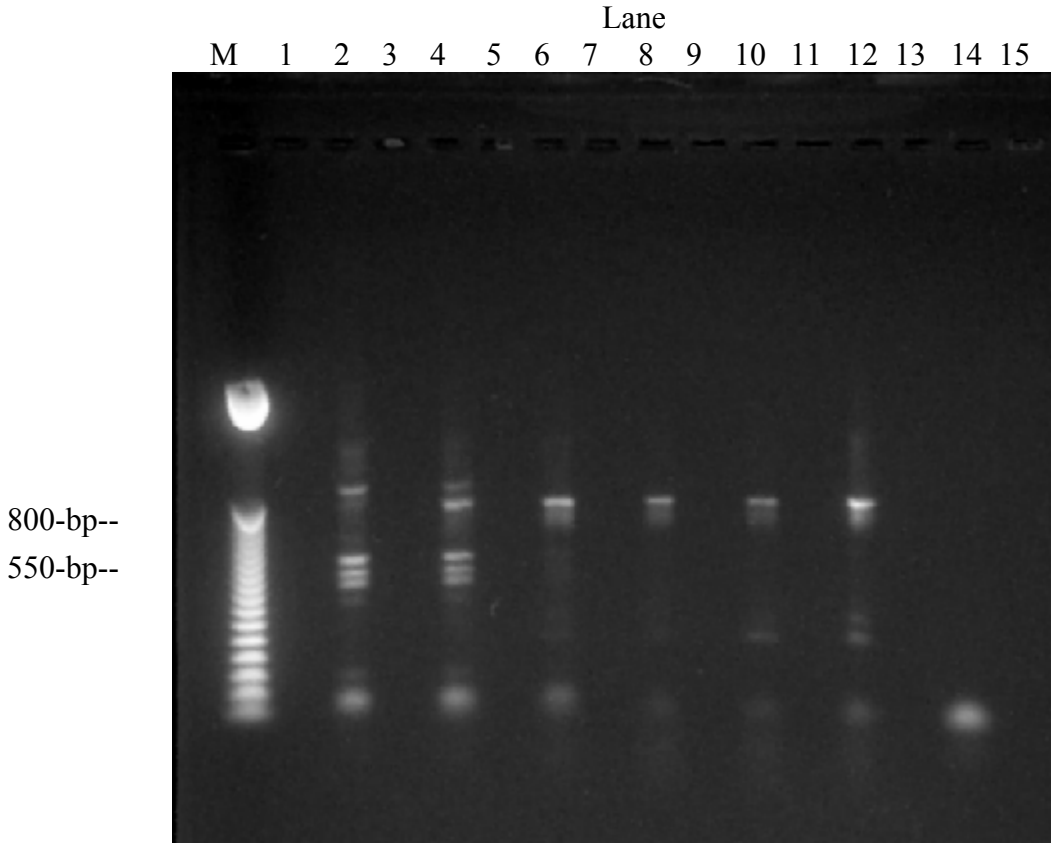

Fig. 2-10. Dilution series with *Beauveria bassiana* isolate 11-98C and cotton 'DP 436 RR' DNA using ITS 1 and ITS 4 primers. Electrophoresis of PCR products produced a strong band at approximately 800-bp in all lanes where cotton was present. A 550-bp band was present in two lanes (3 and 5) where *B. bassiana* was present. However, detection of *B. bassiana* in mixed DNA samples at dilution rates 1:10 and below was not possible due to primer affinity for plant DNA. PCR products were separated by electrophoresis in a 1% agarose-TBE gel. The 50-bp ladder (Promega, Madison, WI) (M) was used to estimate the size of PCR products. Lanes 1, 3, 5, 7, 9, 11, 13, 15: Blank; Lane 2: Bb 11-98C; Lane 4: Bb 11-98C : cotton (1:1); Lane 6: Bb 11-98C : cotton (1:10); Lane 8: Bb 11-98C : cotton (1:100); Lane 10: Bb 11-98C : cotton (1:1000); Lane 12: cotton 'DP 436 RR' DNA; Lane 14: no DNA control.

Using the new ITS primers, repeated bands for the original *B. bassiana* 11-98 (Fig. 2-11) and 11-98C (Fig. 2-12) and the violet isolate (Fig. 2-13) were sequenced, and were a 99% homologous match to *Cordyceps bassiana*, the teleomorph of *B. bassiana* (Fig. 2-14). Using new ITS primers on a dilution series of DNA from Bb 11-98C and a GenBank Blast search was conducted. The 421-bp PCR product from Bb 11-98, Bb 11-98C and the violet hued putative *B. bassiana* isolate were identical. The sequence was a cotton, *B. bassiana* was detectable in ratios as low as 1:1000 [*B. bassiana* DNA : cotton DNA template (Fig. 2-15)]. No bands were present in the lane with positive cotton DNA control. *Beauveria bassiana* also was detected using the new ITS primers, from whole plant parts in two plants, that had been grown in the gnotobiotic assay from seed treated with Bb 11-98 (Fig. 2-16).

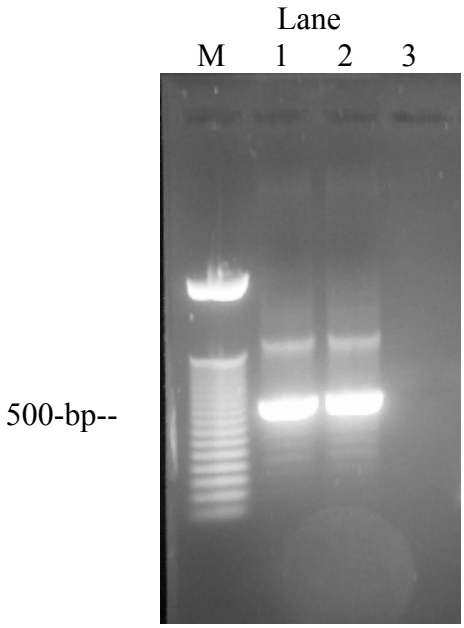

Fig. 2-11. DNA from original fungal isolate *Beauveria bassiana* 11-98 was isolated and subjected to PCR using new ITS primers. Primers were designed by Dr. J. K. Moulton (University of Tennessee. Electrophoresis of the PCR products produced a 421-bp band. PCR products were separated by electrophoresis in a 1% agarose-TBE gel. The 50-bp ladder (Promega, Madison, WI) (M) was used to estimate the size of PCR products. Lanes 1, 2: Bb 11-98 (repeated); Lane 3: Blank.

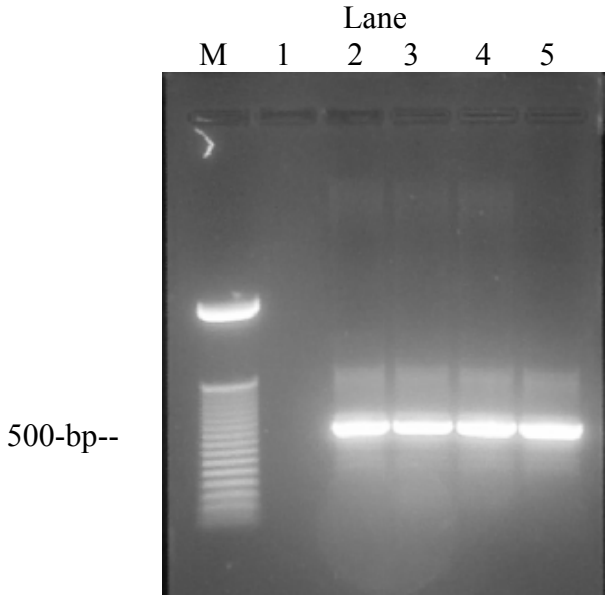

Fig. 2-12. DNA from *Beauveria bassiana* 11-98C was isolated and subjected to PCR using new ITS primers. Primers were designed by Dr. J. K. Moulton (University of Tennessee). Electrophoresis of the PCR products produced a 421-bp band. PCR products were separated by electrophoresis in a 1% agarose-TBE gel. The 50-bp ladder (Promega, Madison, WI) (M) was used to estimate the size of PCR products. Lane 1: Blank; Lanes 2, 3, 4, 5: *B. bassiana* 11-98C (repeated).

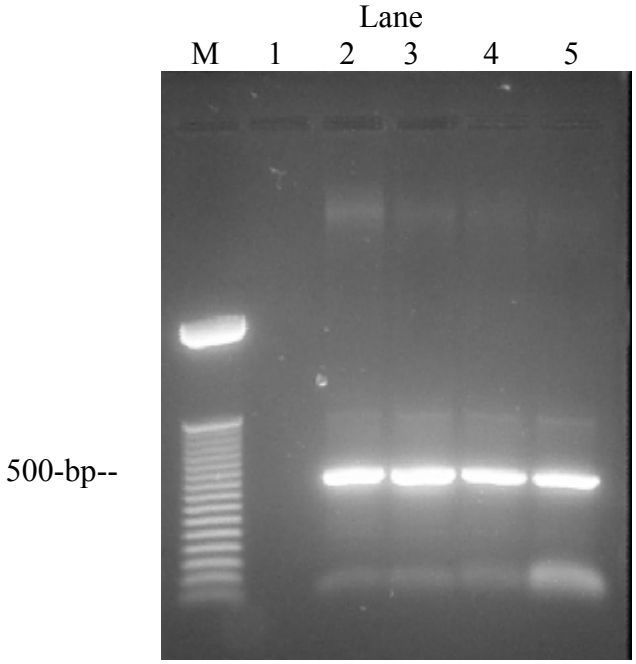

Fig. 2-13. DNA from the violet-colored putative *Beauveria bassiana* 11-98 isolate. The fungus was subjected to PCR using new ITS primers. Primers were designed by Dr. J. K. Moulton (University of Tennessee). Electrophoresis of the PCR products produced a 421-bp band. PCR products were separated by electrophoresis in a 1% agarose-TBE gel. The 50-bp ladder (Promega, Madison, WI) (M) was used to estimate the size of PCR products. Lane 1: Blank; Lanes 2, 3, 4, 5: violet colored *B. bassiana* 11-98 (repeated).

```
                    Bp
Gen Bank            1       TCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTTCAACTCCCTAA
B. bassiana 11-98           ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~

Gen Bank            51      CCCTTCTGTGAACCTACCTATCGTTGCTTCGGCGGACTCGCCCCAGCCCG
B. bassiana 11-98           ~ ~ ~ ~ ~ ~ ~ ~ CCTATTGTTGCTTCGGCGGACTCGCCCCAGCCCG

Gen Bank            101     GACGCGGACTGGACCAGCGGCCCGCCGGGGACCTCAAACTCTTGTATTCC
B. bassiana 11-98           GACGCGGACTGGACCAGCGGCCCGCCGGGGACCTCAAACTCTTGTATTCC

Gen Bank            151     AGCATCTTCTGAATACGCCGCAAGGCAAAACAAATGAATCAAAACTTTCA
B. bassiana 11-98           AGCATCTTCTGAATACGCCGCAAGGCAAAACAAATGAATCAAAACTTTCA

Gen Bank            201     ACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAACGCGA
B. bassiana 11-98           ACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGAAACGCGA

Gen Bank            251     TAAGTAATGTGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCA
B. bassiana 11-98           TAAGTAATGTGAATTGCAGAATCCAGTGAATCATCGAATCTTTGAACGCA

Gen Bank            301     CATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTC
B. bassiana 11-98           CATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTC

Gen Bank            351     AACCCTCGACCTCCCCTTGGGGAGGTCGGCGTTGGGGACCGGCAGCACAC
B. bassiana 11-98           AACCCTCGACCTCCCCTTGGGGGGGTCGGCGTTGGGGACCGGCAGCACAC

Gen Bank            401     CGCCGGCCCTGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGCAG
B. bassiana 11-98           CGCCGGCCCTGAAATGGAGTGGCGGCCCGTCCGCGGCGACCTCTGCGCAG

Gen Bank            451     TAATACAGCTCGCACCGGAACCCCGACGCGGCCACGCCGTAAAACACCCA
B. bassiana 11-98           TAATACAGCTCGCACCGGAACCCCGACGCGGCCCCGC~ ~ ~ ~ ~ ~ ~

Gen Bank            501     ACTTCTGAACGTTGACCTCGAATCAGGTAGGACTACCCGCTGAACTTAAG
B. bassiana 11-98           ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~

Gen Bank            551     CATATCAATAAGCGGAGGA
B. bassiana 11-98           ~ ~ ~ ~ ~ ~ ~ ~ ~ ~
```

Fig. 2-14. Sequence and comparison of PCR products. Products (detected with new ITS primers) from *Beauveria bassiana* isolate 11-98 with published ITS region from *Cordyceps bassiana* strain EABb 04/01-Tip (Quesada-Moraga et al. 2006). Differing nucleotides in sequences are shaded.

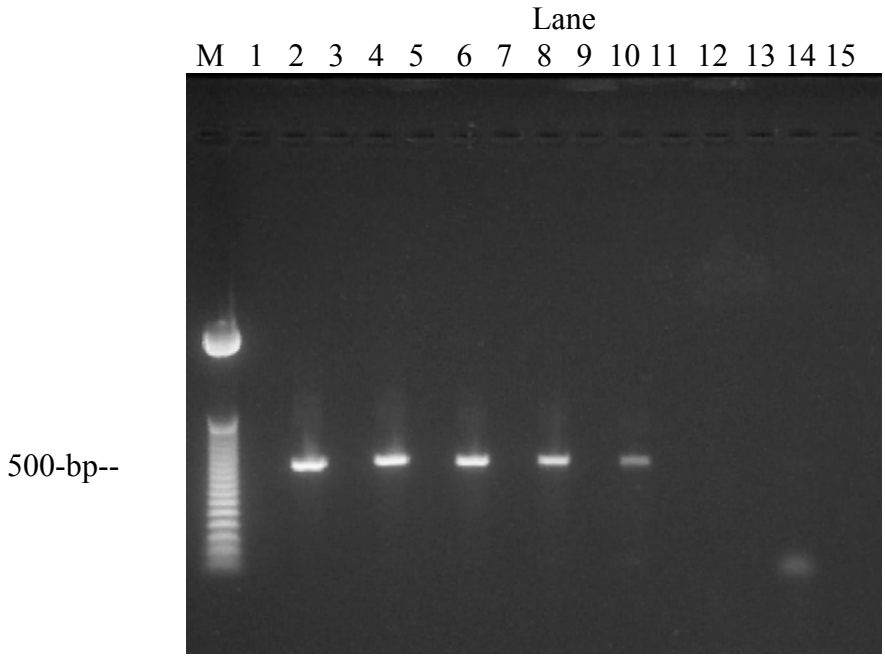

Fig. 2-15. Dilution series with *Beauveria bassiana* 11-98C and cotton 'DP 436 RR' DNA using new ITS primers designed by Dr. J. K. Moulton (University of Tennessee). Electrophoresis of the PCR products in a 1% agarose-TBE gel produced a 421-bp band in lanes where *B. bassiana* was present (Lanes 2, 4, 6, 8, 10) but not in the cotton (Lane 12) or no DNA control lane (Lane 14). The 50-bp ladder (Promega, Madison, WI) (M) was used to estimate the size of the PCR products. Lanes 1, 3, 5, 7, 9, 11, 13: Blank; Lane 2: *B. bassiana* 11-98C; Lane 4: 1:1 ratio of DNA from Bb 11-98C to cotton; Lane 6: 1:10 ratio of DNA from Bb 11-98C to cotton; Lane 8: 1:100 ratio of DNA from Bb 11-98C to cotton; Lane 10: 1:1000 ratio of DNA from Bb 11-98C to cotton; Lane 12: cotton 'DP 436 RR' DNA; Lane 14: no DNA control.

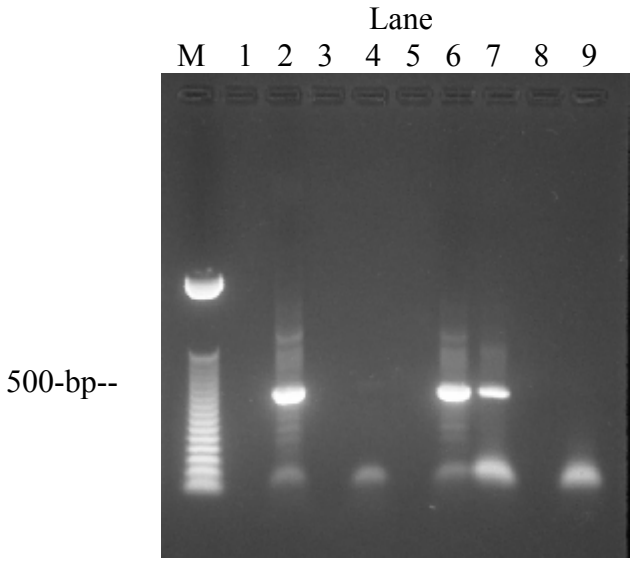

Fig. 2-16. Detection of *Beauveria bassiana* 11-98 DNA extracted from cotton 'DP 436 RR' DNA from plants grown from Bb 11-98 treated seed using new ITS primers designed by Dr. J. K. Moulton (University of Tennessee). Electrophoresis of the PCR products in a 1% agarose-TBE gel produced a 421-bp band in lanes where *B. bassiana* was present (Lanes 2, 6, 7) but not in the cotton (Lane 4) or no DNA control lane (Lane 9). The 50-bp ladder (Promega, Madison, WI) (M) was used to estimate the size of the PCR products. Lanes 1, 3, 5, 8: Blank; Lane 2: *B. bassiana* 11-98C; Lane 4: cotton 'DP 436 RR' DNA; Lane 6: Bb 11-98C in cotton whole plant material; Lane 7: Bb 11-98C in cotton whole plant material; Lane 9: no DNA control.

Discussion

Beauveria bassiana was successfully reisolated from leaf, stem and root tissue of cotton seedlings following conidial seed treatments in a sterile or gnotobiotic system. Ability of *B. bassiana* to become endophytic after foliar application has been observed with corn (Bing and Lewis 1991), potato plants (Jones 1994), tomato (Leckie 2002) and cocoa seedlings (Posada and Vega 2005).

Results of the SEM work with cotton seedlings are similar to results by Wagner and Lewis (2000) for corn. However, *B. bassiana* appeared to be selectively associated with areas of exudates on the cotton cotyledons. Following seed treatment, conidia and hyphae of Bb 11-98C were observed on all parts of the seedling, including the emerging root radical. *Beauveria bassiana* 11-98C spores also germinated and hyphal growth extended along the cotton cotyledon surface. Hyphae appeared to grow in a selective process along the cotyledon surface toward trichomes. Because no appressoria were observed, hyphae are believed to penetrate the epidermal layers of the cotton cotyledon with mechanical pressure. Growth within the cotton cotyledon was observed in a cross-section of the cotyledon. A large percentage of conidia did not germinate on the radicle; however, some hyphae were observed growing along the radicle surface. The ungerminated spores may provide the emerging radicle and developing roots with protection within a soil environment as conidia would be carried deeper into the soil profile as the radicle grows through the soil.

MAT 1 and MAT 2, mating type primers, were suitable for detection of *B. bassiana* in any DNA samples. Use of these primers may have been unsuccessful because Bb 11-98 may be a different mating type than those tested by Yokoyama et al. (2004), or

have been due to the fact that these primers contain several degenerate base pairs, and larger amounts of primer may have been needed for the PCR reactions to detect *Beauveria*.

Detection of fungi in mixed samples of plant and fungal DNA was problematic using ITS primers because these primers bind to both fungal and plant ITS sequences (Martin and Rygiewicz 2005). In studies on detection of Bb 11-98 in tomato, ITS 1 and ITS 4 primers were used (Leckie 2002, Ownley et al. 2005). In work with tomato colonized by Bb 11-98, ITS 1 and ITS 4 primers detected tomato DNA and *B. bassiana* DNA in mixed DNA samples and PCR products were 100-bp different in size. *Beauveria bassiana* 11-98 produced a single 550-bp product and tomato had a larger single-650 bp PCR product. Similarly Quesada-Moraga et al. (2006) using ITS 1-5.8S-ITS 2 primers detected a single PCR product, about 570-bp for *B. bassiana* strain EAB b 04/01-Tip and a larger single PCR product of about 750-bp for opium poppy (*Papaver somniferum* L.) in mixed DNA samples.

Using ITS 1 and ITS 4 primers with cotton and Bb 11-98 DNA, gel electrophoresis of PCR products revealed two consistent bands appearing in mixed samples of cotton and *Beauveria* at a ratio of 1:1: an 800-bp band for cotton and a smaller 550-bp band for *B. bassiana*. However, additional bands were also present. Using a higher ratio of cotton DNA, the ITS primers had greater affinity for cotton DNA and the 550-bp band for *Beauveria* was absent. The new ITS primers were selective for *B. bassiana* DNA in mixed samples of fungal and plant DNA. Using a spiked dilution series of cotton and fungal DNA, *B. bassiana* was detected at a ratio of 1:1000 (fungal to plant DNA) with new ITS primers. Using the new ITS primers, *B. bassiana* DNA was detected

from cotton tissue of seedlings grown from *Beauveria*-treated seed in a gnotobiotic assay. Since it was possible to detect *B. bassiana* in very small quantities using the new ITS primers, it may be possible to use them for detection of endophytic *Beauveria* regardless of plant type or material.

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Part 3. *Beauveria bassiana*: Effect of conidial rate and soil type on biocontrol of *Pythium*, *Rhizoctonia*, and *Thielaviopsis*

Abstract

Beauveria bassiana 11-98, an entomopathogenic fungus, is endophytic in tomato and is an effective biocontrol against *Rhizoctonia solani*. Biocontrol of cotton seedling disease complex by *B. bassiana* has been reported in selected soils in field experiments. In this study, efficacy of *B. bassiana* to control soilborne cotton pathogens, *Rhizoctonia solani*, *Pythium myriotylum*, and *Thielaviopsis basicola* was determined in different soils using different rates of *B. bassiana* conidia. 'Delta Pine 436 RR' black cotton seed were coated with different rates (1×10^5 to 10^{11} CFU) of *B. bassiana* 11-98C conidia using a 2% methylcellulose solution. Growth chamber trials were conducted using factorial experimental design in randomized complete blocks with pathogen infested versus uninfested soil, four seed treatments, and 7 to 14 replicates with two seeds per replicate. Final plant stand, height, weight and disease severity were determined 21 to 28 days after exposure. Conidial rate of *B. bassiana* impacted seedling survival in germination assays *in planta*. In growth chamber assays, however, little reduction in seed viability was noted regardless of spore rate applied as a seed coat treatment. *Beauveria bassiana* provided significant control of *R. solani* in three trials and marginal control in another. *Beauveria bassiana*-treated seed performed better in soil infested with *P. myriotylum* 7 days after sowing and in one trial when *P. myriotylum* was introduced to soil the same day as seed sowing. Two soil trials with *T. basicola* were conducted but results were inconclusive.
Keywords: *Beauveria bassiana*, biocontrol, cotton seedling disease, *Pythium myriotylum*, *Rhizoctonia solani, Thielaviopsis basicola*

Introduction

Gossypium hirsutum L. has become the dominant species in commercial cotton production. With introduction of transgenic cultivars that have engineered traits such as the toxin against lepidopteran insects produced by *Bacillus thuringiensis* Berlinger (Bt) (Clark and Russell 2000a), the gene for resistance to glyphosate in Roundup Ready (RR) (Clark and Russell 2000b), and bromoxynil (BXN), a common herbicide (Hagedorn 2005), cotton growers have been provided with additional management tools.

Even with production benefits offered by transgenic cultivars, cotton growers still face multiple challenges to yields. One of the greatest challenges to crop success is the seedling disease complex, caused by a complex of soil fungi. Symptoms of pathogenicity include: seed decay before germination, seedling decay before emergence, girdling of emerged seedlings at or near the soil surface, and rotting of root tips (Blasingame 1993). Fungi within the soil pathogen complex are *Rhizoctonia solani* (Kühn), *Fusarium* spp., *Pythium* spp., and *Thielaviopsis basicola* (Berk. & Broome) Ferraris. Any one of these fungi or several occurring in combination can produce symptoms characteristic of the disease complex and present the greatest threat to cotton during the first 30 days after planting. These fungi are soilborne pathogens capable of maintaining high inoculum loads in the absence of host plants for multiple years.

Rhizoctonia solani causes serious plant losses primarily attacking roots and lower stems of plants (Zaki et al. 1998). *Rhizoctonia* mainly exists as sterile mycelium and sometimes, as small sclerotia that show no internal tissue differentiation. The fungus is present in most soils and once established in a field remains indefinitely (Watkins 1981).

In older plants, *Pythium myriotylum* Drechsler is typically restricted to root tips

and young cells. However, in young plants the pathogen is capable of causing pre- and post-emergence death known as damping off. Primary methods of infection are by direct penetration of mycelia and through release of flagellate zoospores which are capable of movement through soils, where they invade seeds or roots either through direct penetration or by entering natural cracks in root tissues (Kucharek and Mitchell 2000).

Thielaviopsis basicola is a soilborne plant pathogen that causes black root rot, mainly recognized as cortical rot of seedling roots. The seedling disease is widespread in the southwestern United States (Olsen and Silvertooth 2001). The pathogen reproduces asexually and persists in soils for long periods as chlamydospores. Two kinds of spores are formed: pigmented, barrel-shaped chlamydospores and hyaline endoconidia. Disease is favored by wet, cool, alkaline soil in conjunction with any condition that weakens plants (Wheeler et al. 1999).

Beauveria bassiana (Bals.-Criv.) Vuill. is a well known ubiquitous entomopathogenic fungus with an extensive host range (Boucias and Pendland 1988). The fungus, commonly known as muscardine disease was first identified in 1834 by an Italian scientist, Agostino Bassi, as the agent of silkworm (*Bombyx mori* L.) disease, (Porter 1973). In 2001, the sexual stage of *B. bassiana* was classified in Ascomycota, Clavicipitaceae, genus *Cordyceps bassiana* (Yokoyama et al. 2004).

With few exceptions, research on *Beauveria bassiana* as a control for plant pathogens has been limited to *in vitro* studies on growth and cell lysis of plant pathogens (Vesely and Koubova 1994, Reisenzein and Tiefenbrunner 1997, Lee et al. 1999 and Nagaoka 2004). *Beauveria bassiana* applied as a conidial seed treatment in a 2.5% methylcellulose (MC) solution to tomato seeds prior to sowing provided protection

against *R. solani* (Bishop 1999, Ownley et al. 2005). In a regional field trial on the biocontrol of cotton seedling diseases conducted across the Southeast at 11 sites, *B. bassiana* provided protection against soilborne pathogens on cotton in some soils. In two field sites where *B. bassiana* proved successful as a biocontrol for cotton seedling disease, soils were sandy loams and *R. solani* was the only pathogen isolated from plant material (Batson et al. 2000). Environment variability has long been recognized as a problem for the overall efficiency of biocontrols (Duffy et al. 1997, Ownley et al. 2003).

Objectives of this study were: 1) to evaluate biocontrol efficacy of *B. bassiana* 11-98C (Bb 11-98C) against soilborne pathogens: *Rhizoctonia solani*, *P. myriotylum* and *T. basicola*; 2) to determine effect on biocontrol of different rates of Bb 11-98C conidia as a seed treatment; 3) to evaluate biocontrol efficacy of Bb 11-98C in different soils; 4) to evaluate impact of Bb 11-98C treatment on germination of cotton seed and; 5) to determine the relationship between germination rates of cotton and application rates of Bb 11-98C conidia.

Materials and Methods

Experimental design for disease assays

Designs for all disease assays were factorial experiments in randomized complete block s(RCB). Each assay had three to five seed treatments, seven to 14 replicates, with two seeds per replicate. Seed treatments ranged from 1×10^5 to 1×10^{11} CFU/seed of *B. bassiana* 11-98C, in disease assays with *R. solani* in potting mix (Premier Horticulture Quakertown, PA) and Staser, a sandy loam soil from Hawkins County, TN. For *P.*

myriotylum assays, seed treatment rates of *B. bassiana* 11-98 and BotaniGard at 1×10^7 CFU/seed were examined for biocontrol efficacy in Collins, a silt loam from Gibson County, TN and potting mix. Seed treatments were 1×10^9 to 1×10^{11} CFU/seed of *B. bassiana* 11-98C, in disease assays with *T. basicola* in Staser soil. Effectiveness of seed treatments was determined by measuring root, shoot and plant weight (g), plant height (mm), survival rate (%) and disease severity rating (1-6) for plant hypocotyls and roots, three to four weeks after sowing. Seedlings were rated for disease symptoms with the following hypocotyl disease index, 1 = no symptoms, 2 = a few pinpoint lesions or diffuse discolored areas, 3 = distinct necrotic lesions, 4 = girdling lesions, 5 = dead seedling attributed to post-emergence damping off, and 6 = dead seedling due to preemergence damping off. For the root disease index, ratings were 1 = no symptoms, 2 = 1 - 10% of root system discolored, 3 = 11 - 25% of root system discolored, 4 = 26 - 50% of root system discolored, 5 = > 50% of root system discolored or post-emergence death, and 6 = pre-emergence death. All data were analyzed using PROC MIXED of PC-SAS, Ver. 9.1.3. Significant main effects were further analyzed with F-protected LSD ($P = 0.05$ and $P = 0.10$).

Growth chamber assay conditions

Growth chamber conditions were 25 to 26°C, with 50% RH and a 12 h photoperiod. For assays, Ray Leach conetainers (Stuewe and Sons, Corvallis, OR) were placed in racks and plugged with three to four cotton balls to prevent rapid loss of water and soil. Immediately after sowing seeds, tops of conetainers were covered and sealed with clear plastic to retain moisture until germination began. Plastic was removed once

seedlings emerged from soil. Germination typically began by day 3 and ended by day 7. If germination did not occur during this time period, seed typically failed to germinate. Delinted untreated cotton seed of 'Delta Pine (DP) 436 RR' transgenic cultivar with the genes for glyphosate resistance was obtained from Dr. Craig Canaday, The University of Tennessee, West Tennessee Research and Education Center, Jackson.

Collection and storage of *B. bassiana* conidia

Beauveria bassiana 11-98 (Bb 11-98) was isolated originally from an infected click beetle (Coleoptera: Elateridae) from Scott County, TN (Leckie 2002). *Beauveria bassiana* 11-98 was used as a seed treatment in a gnotobiotic assay with 'DP 436 RR' and re-isolated from cotton tissue by means of traditional plating techniques (Griffin et al. 2005). Endophytic cotton adapted Bb 11-98C was used along with Bb 11-98 in subsequent assays. Both isolates were grown on Sabouraud dextrose agar (SDA) for approximately 6 weeks, and then harvested by brushing the surface of the plates with a stenciling brush. Conidia were stored at 4°C in a desiccator until needed. Fungal cultures, started from conidia, were grown on SDA at 22 to 24°C. Approximately every 6 weeks dry conidia were transferred to fresh SDA for production of spores.

***Beauveria bassiana* isolates used with assays**

Seed were coated with conidia and stored at 4° C until needed. Conidial rates for coated seed were determined through a dilution series plated onto SDA. Assays with *P. myriotylum* were conducted using the original isolate Bb 11-98 and BotaniGard (Emerald BioAgriculture Corp. Butte, MT) isolate GHA. Later assays with *R. solani* and *T.*

basicola were conducted using only Bb 11-98C.

Seed coating technique and spore rate determination of Bb 11-98, Bb 11-98C and BotaniGard

Beauveria bassiana conidia, 2% methylcellulose solution (1 ml/g of seed) and Tween 20 (25 µl/g of seed) were evenly mixed together before the addition of cotton seed. The mixture was stirred every 15 min in a biosafety cabinet until seeds were dry.

Pythium experiments were conducted with the original Bb 11-98 isolate and BotaniGard at 1×10^7 CFU/seed and 10 to 14 replicates with 3 to 4 treatments for each replicate. Seed germination assay, and disease assays with *Rhizoctonia* and *Thielaviopsis* were made with multiple conidial rates of Bb 11-98C, which were estimated based on dry mass of bulk conidia. Experimental rates ranged from 1×10^5 to 1×10^{11} CFU per cotton seed. All rates were confirmed by dilution plating of coated seeds on SDA.

Seed germination assay

Seed coated with Bb 11-98C were stored at 4°C until needed. To determine germination rates, seeds were placed on 15-cm diameter sterile Whatman filter paper, which was inserted into a 150 × 15 mm Petri dish. Approximately 6 to 7 ml sterile deionized water was added to Petri dishes, which were then sealed with Parafilm to maintain moisture. Assays were placed in an incubator at 25°C with continuous light. Three replicates of Petri dishes each contained seven cotton seeds, for a total of 21 seeds for each rate of conidia and the assay was repeated.

Disease assays- Soil types

For disease assays, a Collins silt loam, a Staser sandy loam, and a potting mix were used. Collins silt loam soil was collected from a no-till field in which cotton had been grown for several years in Gibson County at the Milan Research and Education Center, University of Tennessee, Milan, TN. Soil was sieved through a 1-cm screen to remove roots and debris. The Staser sandy loam soil was collected from a field adjacent to the Holston River from Hawkins County TN. Promix potting soil was obtained from Premier Horticulture, Quakertown, PA.

***Rhizoctonia solani* inoculum**

Rhizoctonia solani RS3 (AG-4) was obtained from Dr. Mike Benson, North Carolina State University, Raleigh, NC. The fungus was cultured on cornmeal: sand medium, which contained twice-autoclaved white quartz sand (100 g), cornmeal (3 g) and deionized water (7 ml). Ten 3-mm^2 plugs of 7-day old hyphal tips of *R. solani* were added to flasks containing medium. Inoculum was shaken thoroughly every 48 to 72 h and allowed to grow for 2 weeks. At the time of assays, inoculum was mixed into soil at 1% (w/w).

***Pythium myriotylum* inoculum**

A *P. myriotylum* isolate was obtained from Dr. Darrell Hensley, The University of Tennessee, Knoxville, TN. Inoculum was prepared to stimulate production of zoospores using a modification of the procedure from Mitchell and Rayside (1986). Two types of assays, differing in when the pathogen was applied to the soil, were examined using this

inoculum. In pre-emergence disease assays, *P. myriotylum* inoculum was applied immediately after the seed were planted. In post-emergence disease assays, *P. myriotylum* inoculum was applied approximately 7 days after cotton seed were sown.

Thielaviopsis basicola inoculum

Thielaviopsis basicola was grown for three weeks on 10 to 12% carrot agar formulated using 100 to 120 ml carrot juice per liter with 2% agar. Propagules of *T. basicola* were collected by pouring 10 ml of sterile deionized water onto the culture surface, then lightly scraping propagules free. Inoculum was a natural mixture of endoconidia and chlamydospores. This suspension was mixed with 100 ml of deionized water and added to Staser soil, which had a slightly alkaline pH.

Propagules per gram of soil were determined from dilutions of six counts per trial and averaged with a hemacytometer (American Optical, Buffalo, NY; Becker et al. 1996). Thorough mixing was enhanced by placing soil in a large plastic bag with inoculum, then placing it on a shaker at 50 to 70 rpm for 30 to 45 min, with manual adjustments to ensure equal distribution of the inoculum in soil. In a preliminary pathogenicity test, approximately 20,000 propagules (as determined with a hemacytometer) per gram of dry soil was needed for disease ($P = 0.0038$) at EC50.

Results

Germination Assay

For the germination assay (Fig. 3-1), rates of conidial seed treatments were 7.5×10^5, 4.0×10^7, 2.33×10^9, and 4.57×10^{11} CFU of Bb 11-98C per seed. The effect of Bb

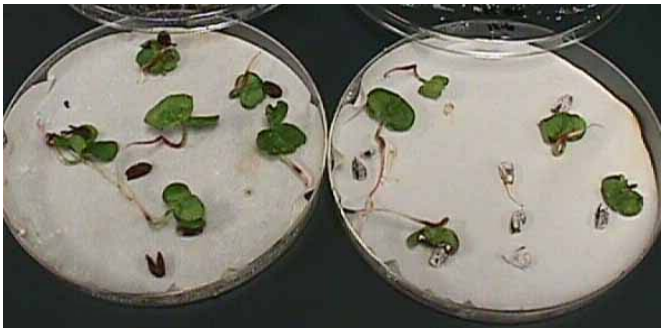

Fig. 3-1. Germination assay. Untreated seed (left) and Bb 11-98C coated seed (right) at a rate of 1×10^7 CFU/seed.

11-98C conidial seed treatment rate was significant for all variables in the germination assay. The assay was repeated with consistent results. There was a significant effect of *B. bassiana* conidial rates on seedling shoot height [Trial 1, (F = 2.87; df 4,98; P = 0.0230); Trial 2; (F = 5.81; df = 4,98; P = 0.0003)] and radicle length [Trial 1, (F = 2.7; df = 4,98; P = 0.0264) Trial 2, (F = 3.09; df = 4,98; P = 0.0192)]. Shoot height and radicle length were reduced in the soilless system when conidial rates exceeded 1×10^9 CFU/seed (Fig. 3-2). Rate of Bb 11-98C per seed was significant for percent seedling survival [Trial 1, (F = 4.59; df = 4,98; P = 0.0024); Trial 2, (F = 4.61; df = 4,98; P = 0.0019)]. At conidial rates higher than 1×10^9 CFU/seed, survival was reduced. No significant differences from control seed were noted with lower rates (Fig. 3-2).

Rhizoctonia solani **disease assays**

Four trials were conducted using potting mix, to determine the efficacy of Bb 11-98C as a biocontrol against *R. solani*. In three of the four trials, there was a significant interaction between seed treatment and pathogen presence. In one trial, only the main

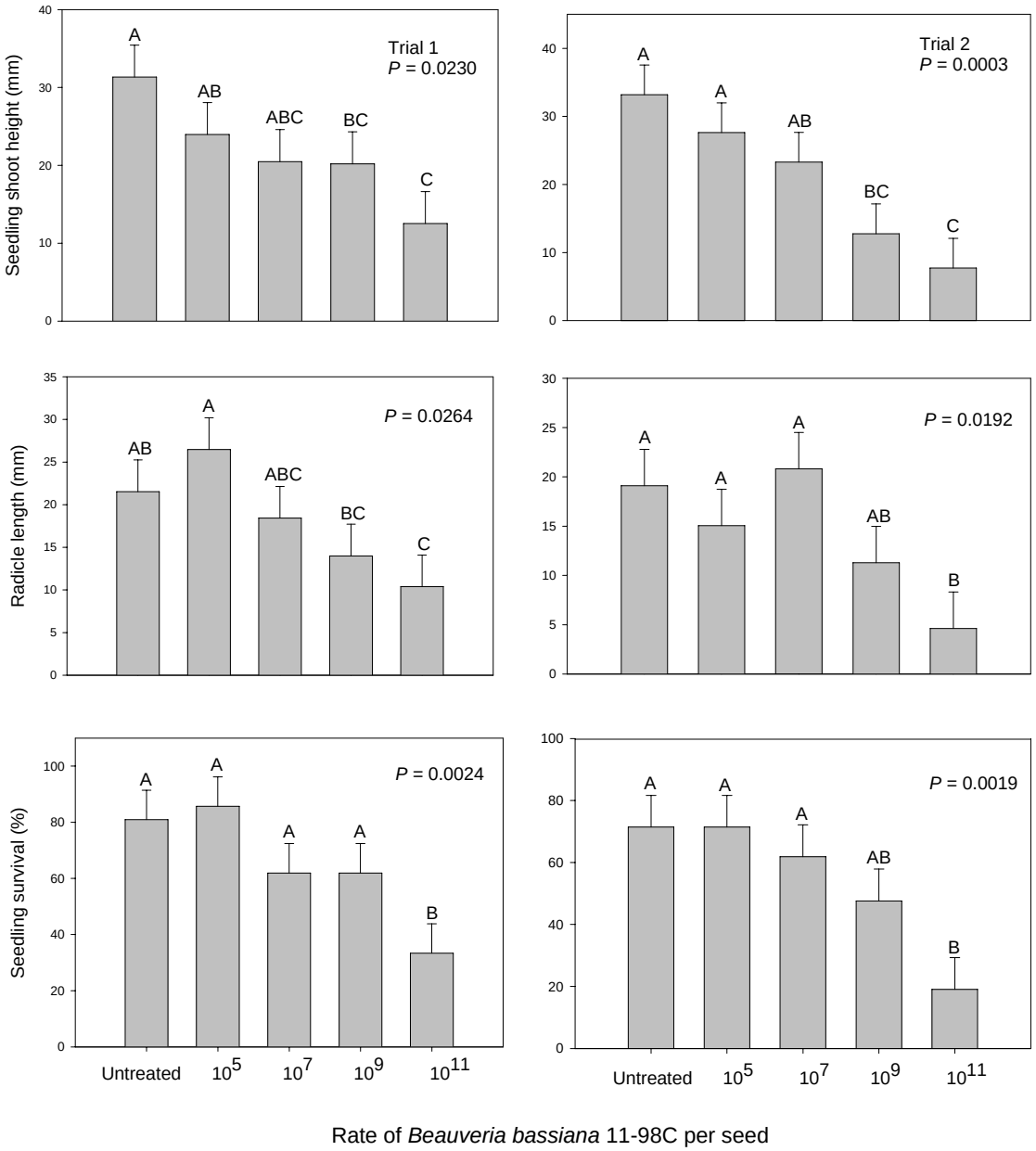

Fig. 3-2. Effect of seed treatment rate of *Beauveria bassiana* 11-98C conidia on seedling shoot height, radicle length and seedling survival in a soilless germination assay. Effects were significant for shoot height in Trial 1 ($P = 0.0230$) and Trial 2 ($P = 0.0003$), for radicle length in Trial 1 ($P = 0.0264$) and Trial 2 (P = 0.0192) and for seedling survival in Trial 1 ($P = 0.0024$) and Trial 2 ($P = 0.0019$). For each trial, bars with the same letter are not different according to an F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

effect of seed treatment was significant. These assays indicated a significant effect in conidial rate application of Bb 11-98C, which resulted in differences in levels of protection provided. The first trial included three conidial rates (1×10^5, 1×10^9 and 1×10^{11}) of Bb 11-98C, and an untreated control. Interaction of seed treatment and pathogen was significant for plant height ($F = 2.37$; df = 3,113; $P = 0.0742$), and hypocotyl disease index ($F = 3.47$; df = 3,113; $P = 0.0185$) in 3-week old seedlings. Bb 11-98C at 1×10^9 CFU/seed provided significant protection of cotton against *R. solani*. In noninfested soil, there were no differences in seedling height (Fig. 3-3). However, in infested soil seedlings treated with Bb 11-98C at 1×10^9 had significantly greater height than the untreated control (Fig. 3-3). Hypocotyl disease index in infested soil was lowest for plants treated with Bb 11-98C at 1×10^9 and did not differ from plants in noninfested soil (Fig. 3-4). The effect of seed treatment was significant for seedling weight ($F = 3.22$; df = 3,113; $P = 0.0256$), root weight ($F = 2.16$; df = 3,113; $P = 0.0973$), and shoot weight ($F = 5.32$; df = 3,113; $P = 0.0099$). Seedling weight was greatest with 1×10^9 CFU Bb 11-98C seed treatment and not different than the untreated control (Table 3-1). Similarly, root weight was largest for seedlings treated with 1×10^9 CFU Bb 11-98C but not different from the untreated control. However, shoot weight with 1×10^9 CFU Bb 11-98C was greater than all other treatments (Table 3-1).

A second trial was conducted to confirm that a difference in spore rates would affect the level of protection provided by *B. bassiana* as a biocontrol against *R. solani* in potting mix. Three conidial rates (1×10^5, 1×10^7, and 1×10^9 of *B. bassiana* isolate 11-98C) and an untreated control were examined for biocontrol effectiveness. There was a

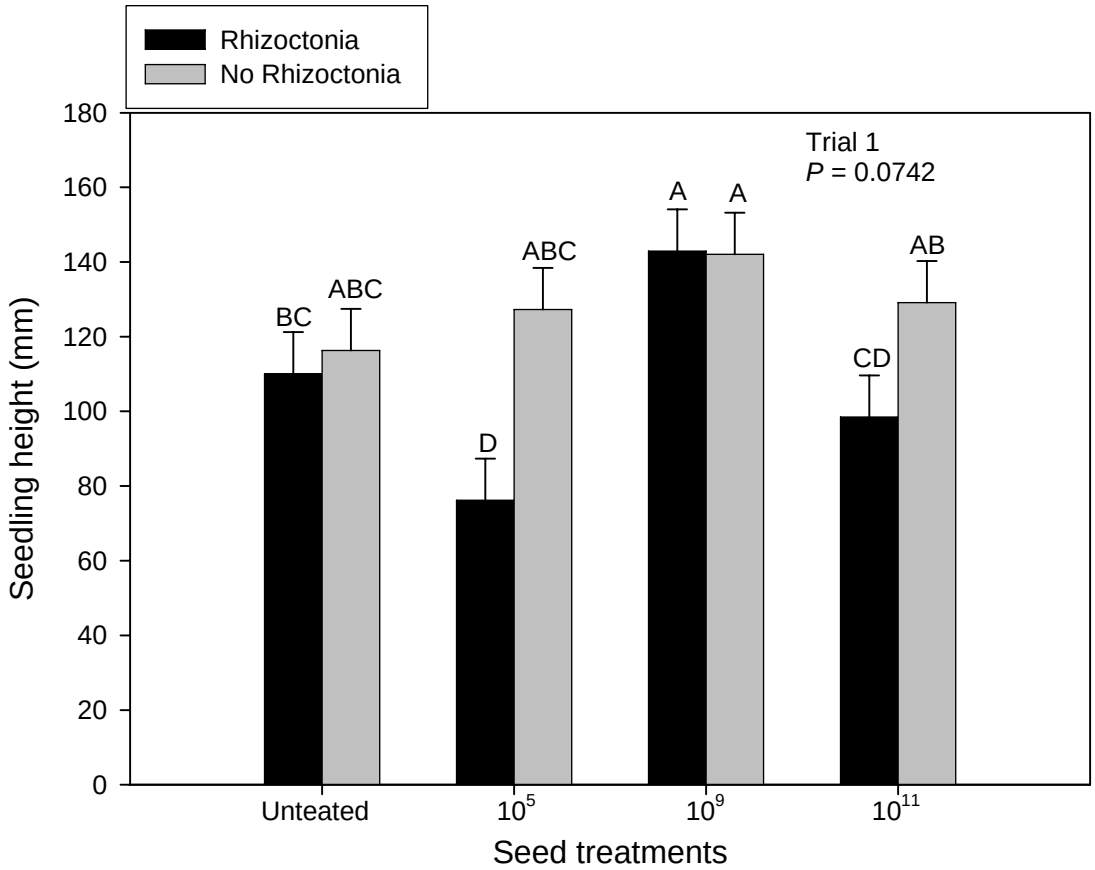

Fig. 3-3. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia and pathogen in potting mix amended with 1% (w/w) *Rhizoctonia solani* on cotton seedling height, Trial 1. Seed treatments were untreated, 1×10^5, 1×10^9, and 1×10^{11} Bb 11-98C CFU/seed. Interaction of seed treatment and pathogen was significant at $P = 0.0742$. Bars with the same letter are not different according to F-LSD at $P = 0.10$. Each bar represents least square means ± SE.

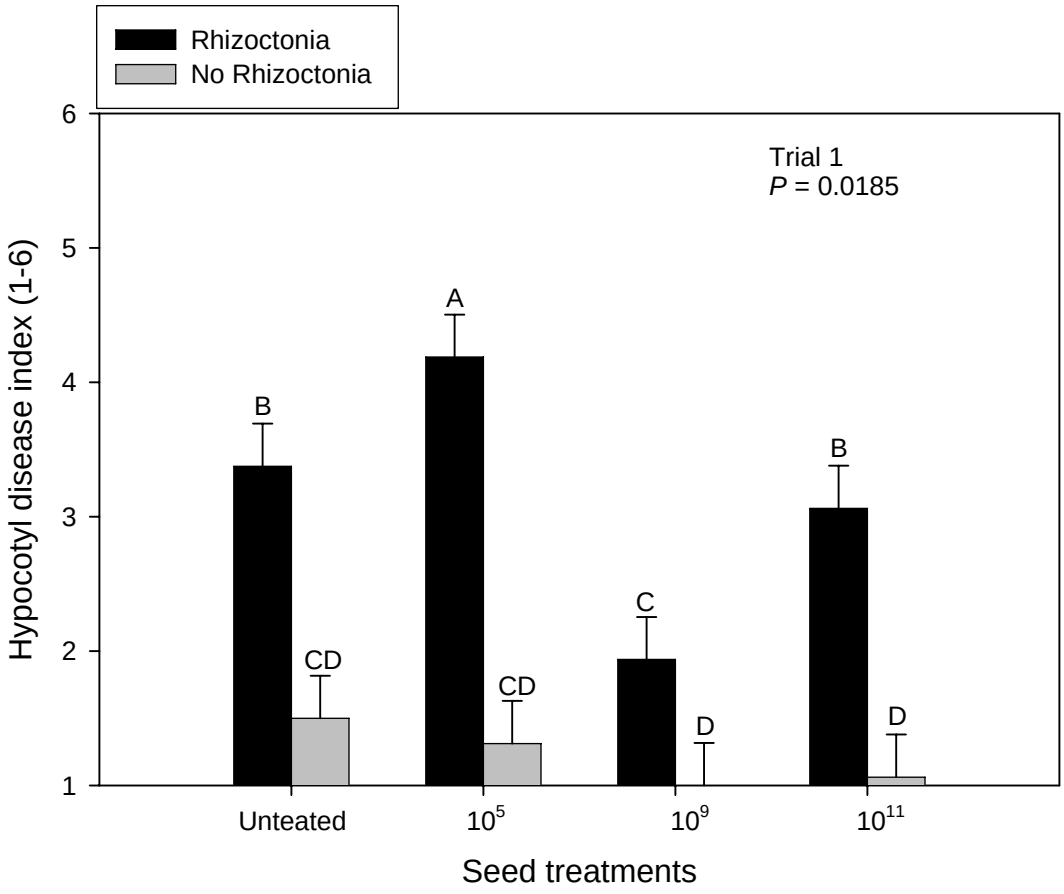

Fig. 3-4. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia and pathogen in potting mix amended with 1% (w/w) *Rhizoctonia solani* on cotton seedling hypocotyl disease index, Trial 1. Seed treatments were untreated, 1×10^5, 1×10^9, and 1×10^{11} Bb 11-98C CFU/seed. Interaction of seed treatment and pathogen was significant for hypocotyl disease index at $P = 0.0185$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

Table 3-1. Seed treatment differences with *Beauveria bassiana* in potting mix infested with 1% *Rhizoctonia solani*, Trial 1. Effect of different seed treatment rates of *Beauveria bassiana* (Bb) 11-98C conidia on seedling weight ($P = 0.0256$), shoot weight ($P = 0.0099$), and root weight ($P = 0.0973$). For each variable, least square means ± SE with the same letters are not different according to F-LSD at $P = 0.05$ or 0.10

Seed treatment (CFU/seed)	Seedling weight (g)	Shoot weight (g)	Root weight (g)
Untreated	1.8384 ± 0.1557 ab	1.0125 ± 0.08797 b	0.8259 ± 0.08235 ab
Bb 11-98C 1×10^5	1.6125 ± 0.1557 b	0.9094 ± 0.08797 b	0.7031 ± 0.08235 b
Bb 11-98C 1×10^9	2.244 ± 0.1557 a	1.2512 ± 0.08797 a	0.9931 ± 0.08235 a
Bb 11-98C 1×10^{11}	1.7525 ± 0.1557 b	0.9601 ± 0.08797 b	0.7960 ± 0.08235 ab
F-LSD *P*-value	0.05	0.05	0.10

significant seed treatment and pathogen interaction effect for plant height (F = 2.9; df = 3,113; P = 0.0379), survival rate (F = 3.09; df = 3,113; P = 0.0371), and root disease index (F = 3.87; df = 3,113; P = 0.0113). Similar to Trial 1, the 1×10^9 CFU/seed Bb 11-98C treatment produced the tallest seedlings in the presence of *R. solani* and the height of plants was significantly greater than untreated controls (Fig. 3-5). Seedling survival was greatest with 1×10^9 CFU/seed Bb 11-98C (Fig. 3-6) and root disease index was significantly lower in infested soil with 1×10^7 and 1×10^9 CFU/seed Bb 11-98C (Fig. 3-7).

In the third trial, three conidial rates (1×10^5, 1×10^7 and 1×10^9) of Bb 11-98C, an untreated control and a MC control were included as seed treatments. Pathogen effect was significant for seedling weight (F= 6.20; df = 1,181; P = 0.0137), shoot weight (F = 11.52; df = 1,181; P = 0.0008), seedling height (F = 9.6; df = 1,181; P = 0.0023), and hypocotyl disease index (F= 71.48; df = 1,181; P = 0.0001). In infested soil seedling weight, shoot weight and seedling height were lower and hypocotyl disease index was higher than in noninfested soil (Table 3-2).

The main effect of seed treatment was significant for several variables including: seedling weight (F = 4.48; df = 4,181; P = 0.0018), root weight (F =2.88; df = 4,181; P = 0.0242), shoot weight (F = 4.75; df = 4,181; P = 0.0011), seedling height (F = 3.12; df = 4,181; P = 0.0164), and hypocotyl disease index (F= 5.41; df = 4,181; P = 0.0004). For all plant growth variables, seed treatments with Bb 11-98C produced larger plants than the MC control, but were not different from the untreated control (Table 3-3). Similarly, hypocotyl disease indices were lower with the Bb 11-98C seed treatments than the MC control, but not different from the untreated control (Table 3-3).

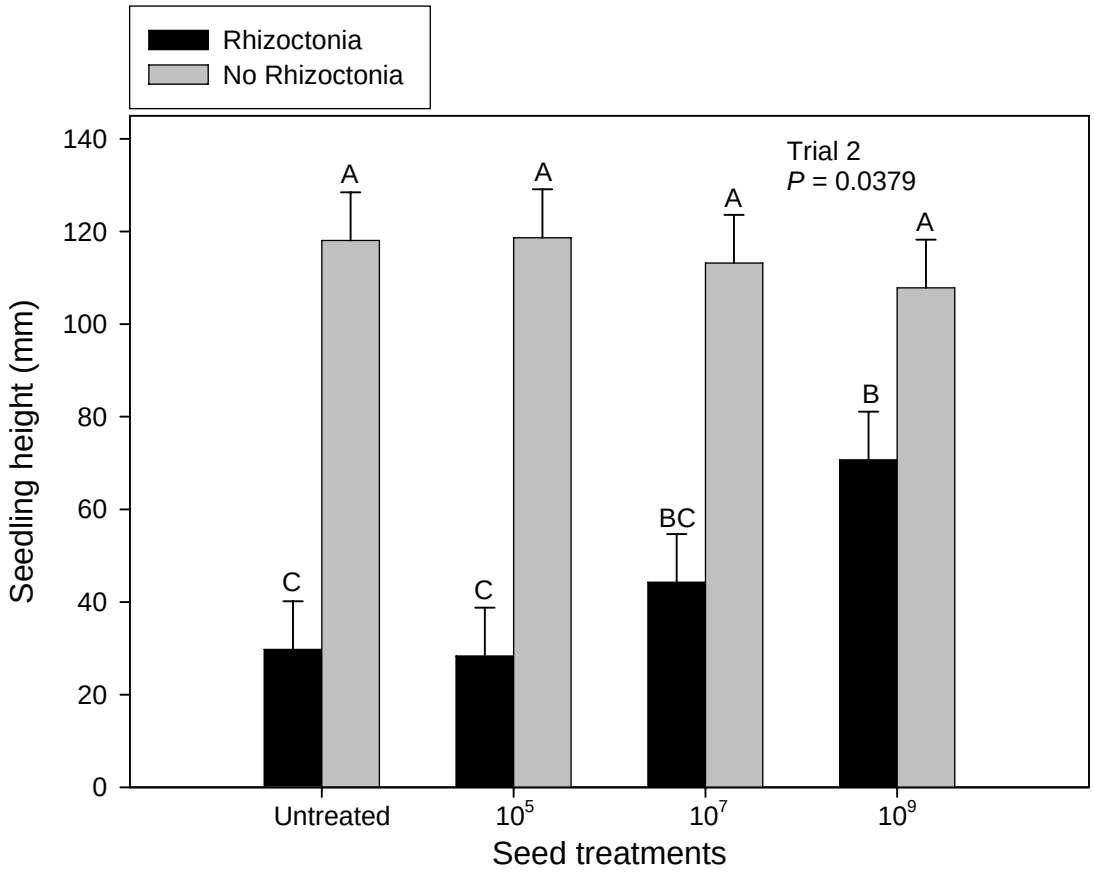

Fig. 3-5. Effect of different seed treatment rates of *Beauveria bassiana* (Bb) 11-98C conidia on cotton seedling height in potting mix amended with 1% *Rhizoctonia solani,* Trial 2. Seed treatments were untreated, 1×10^5, 1×10^7, and 1×10^9 Bb 11-98C CFU/seed. Effect of interaction of seed treatment and pathogen was significant at P = 0.0379. Bars with the same letter are not different according to F-LSD at P = 0.05. Each bar represents least square means ± SE.

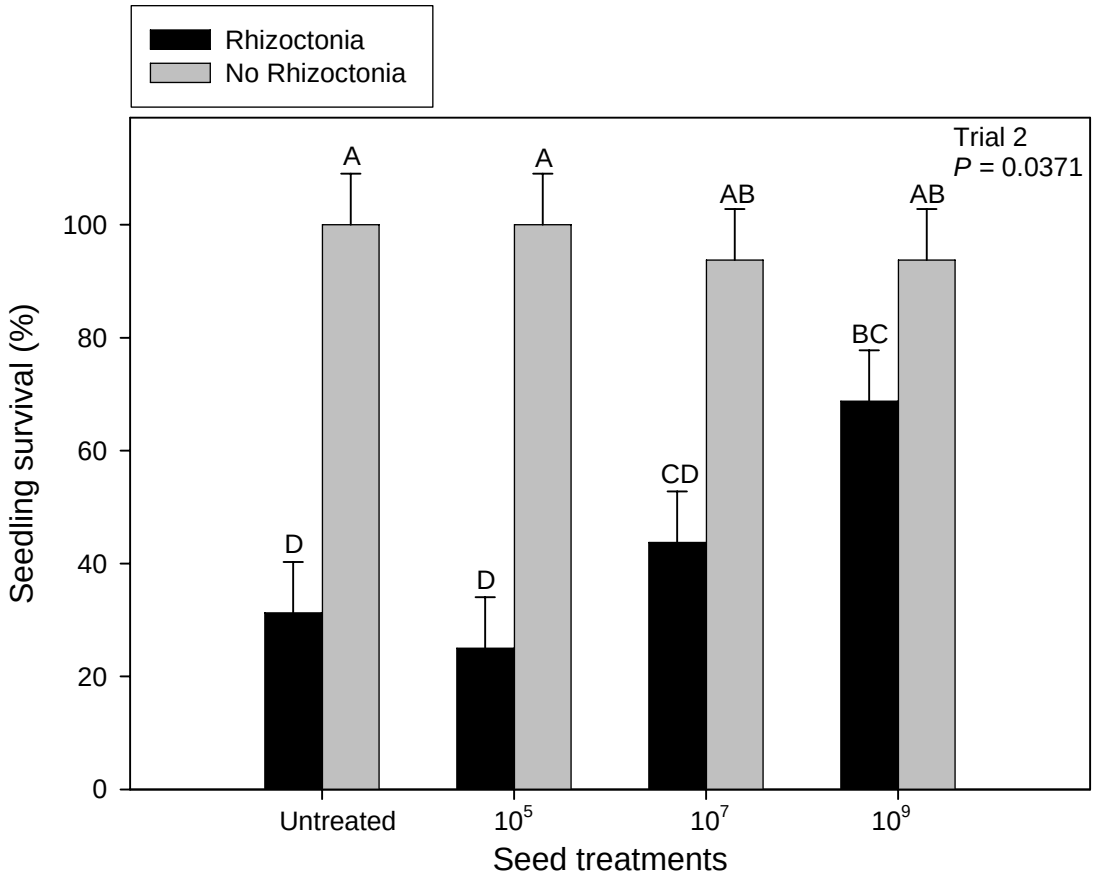

Fig. 3-6. Effect of different seed treatment rates of *Beauveria bassiana* (Bb) 11-98C conidia on percentage cotton seedling survival in potting mix amended with 1% *Rhizoctonia solani*, Trial 2. Seed treatments were untreated, 1×10^5, 1×10^7, and 1×10^9 Bb 11-98C CFU/seed. Effect of interaction of seed treatment and pathogen was significant at $P = 0.0371$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

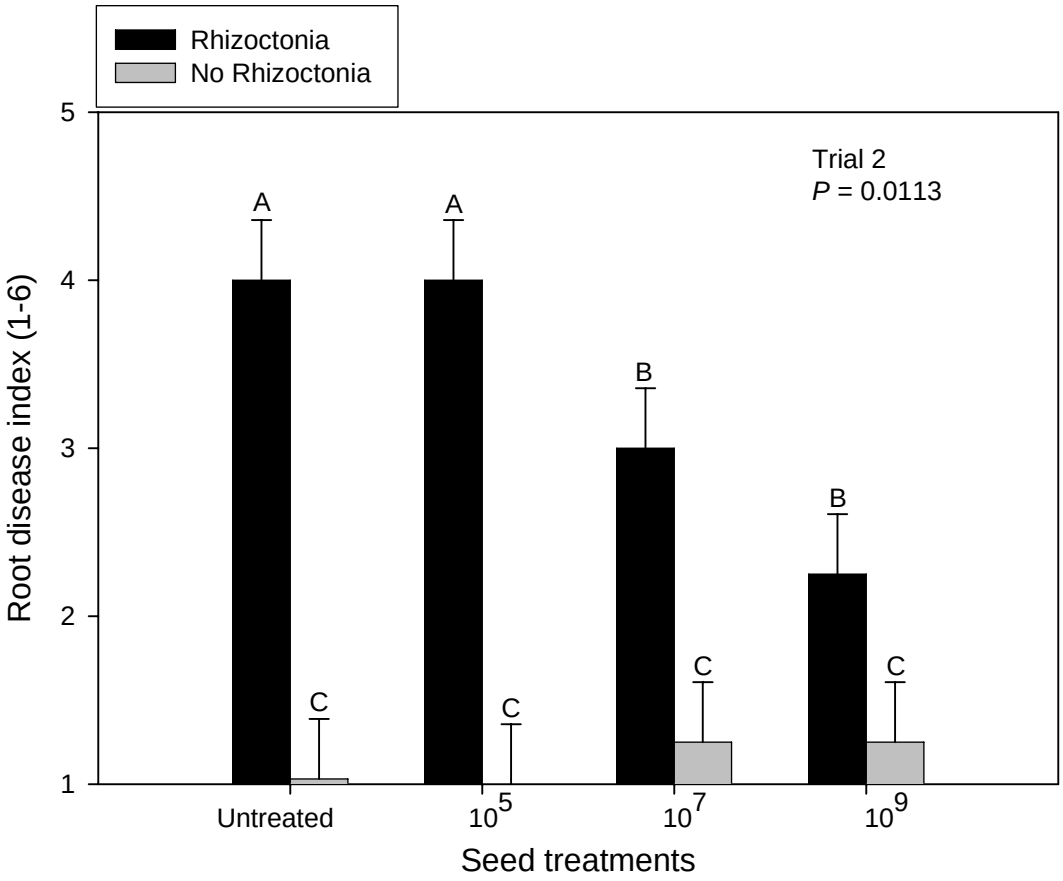

Fig. 3-7. Effect of different seed treatment rates of *Beauveria bassiana* (Bb) 11-98C conidia on cotton seedling root disease index in potting mix amended with 1% *Rhizoctonia solani*, Trial 2. Seed treatments were untreated, 1×10^5, 1×10^7, and 1×10^9 Bb 11-98C CFU/seed. Effect of interaction of seed treatment and pathogen was significant at $P = 0.0113$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

Table 3-2 Effect of pathogen on cotton seedling variables, Trial 3. The measured variables were seedling weight (P = 0.0137), shoot weight (P = 0.0008), seedling height (P = 0.0023), and hypocotyl disease index (P = 0.0001) of cotton seedlings in potting mix infested with 1% *Rhizoctonia solani*. For each variable, least square means ± SE with the same letters are not different according to F-LSD at P = 0.05

Pathogen	Seedling weight (g)	Shoot weight (g)	Hypocotyl disease index (1-6)	Seedling height (mm)
Rhizoctonia solani	1.4860 ± 0.08826 a	0.7955 ± 0.05039 b	3.49 ± 0.1673 a	107.18 ±5.5832 b
No pathogen	1.7968 ± 0.08826 b	1.0224 ± 0.05039 a	1.49 ± 0.1673 b	131.65 ± 5.5832 a

Table 3-3. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C CFU/seed on cotton seedling variables, Trial 3. Measured variables were seedling weight ($P = 0.0018$), shoot weight ($P = 0.0011$), root weight ($P = 0.0242$) and seedling height ($P = 0.0164$) and hypocotyl disease index ($P = 0.0004$) of cotton seedlings in potting soil infested with 1% *Rhizoctonia solani*. For each variable, least square means ± SE with the same letters are not different according to F-LSD at $P = 0.05$

Seed treatments	Seedling weight (g)	Shoot weight (g)	Root weight (g)	Seedling height (mm)	Hypocotyl disease (1-6)
Untreated	1.6058 ± 0.1396 a	0.9165 ± 0.07676 a	0.6892 ± 0.08058 ab	117.53 ± 8.8278 ab	2.6 ± 0.2645 b
MC	1.1543 ± 0.1396 b	0.6420 ± 0.07676 b	0.5123 ± 0.08058 b	95.0 ± 8.8278 b	3.5 ± 0.2645 a
Bb 11-98C 1×10^5	1.9275 ± 0.1396 a	1.0823 ± 0.07676 a	0.8453 ± 0.08058 a	137.73 ± 8.8278 a	2.275 ± 0.2645 b
Bb 11-98C 1×10^7	1.7478 ± 0.1396 a	0.9275 ± 0.07676 a	0.8203 ± 0.08058 a	125.13 ± 8.8278 a	2.150 ± 0.2645 b
Bb 11-98C 1×10^9	1.7718 ± 0.1396 a	0.9765 ± 0.07676 a	0.7953 ± 0.08058 a	121.70 ± 8.8278 a	1.9250 ± 0.2645 b

In the fourth trial, seed treatments were an untreated control, a MC control and 1 × 10^7 CFU/seed Bb 11-98C. Variables for which there was a significant seed treatment and pathogen interaction were: seedling weight (F = 3.42; df = 2,72; P = 0.0381), shoot weight (F = 5.06; df = 2,72; P = 0.0088), seedling height (F = 3.19; df = 2,72; P = 0.0471), hypocotyl disease index (F = 5.80; df = 2,72; P = 0.0046) and root disease index (F = 3.0; df = 2,72; P = 0.0558). In *R. solani*-infested soil, 1 × 10^7 CFU Bb 11-98C per seed resulted in 3-week old seedlings that were significantly larger in seedling weight (Fig. 3-8), shoot weight (Fig. 3-9), seedling height (Fig. 3-10), and had a lower hypocotyl disease index (Fig. 3-11) and root disease index (Fig. 3-12) than untreated seed.

Rhizoctonia in Staser sandy loam soil

Two disease assays were conducted in Staser sandy loam soil amended with 1% *R. solani* inoculum. The interaction of seed treatment and pathogen was not significant for any variable in either trial. However in both trials, there were significant main effects for pathogen and seed treatment. In trial 1, the effect of pathogen was significant for seedling weight (F = 4.29; df = 1,181; P = 0.0398), shoot weight (F = 4.47; df = 1,181; P = 0.0359), root weight (F= 3.23; df = 1,181; P = 0.0311), seedling height (F = 11.08; df = 1,181; P = 0.0011), hypocotyl disease index (F = 22.88; df = 1,181; P = 0.0001) and root disease index (F = 17.05; df = 1,181; P = 0.0001). In infested soil, seedling weight variables and seedling height were lower (Table 3-4). Hypocotyl and root disease indices were lower in noninfested soil (Table 3-4).

There were significant seed treatment effects for seedling weight (F = 2.31; df = 4,181; P = 0.0595), root weight (F = 3.44; df = 4,181; P = 0.0098), survival rate (F =

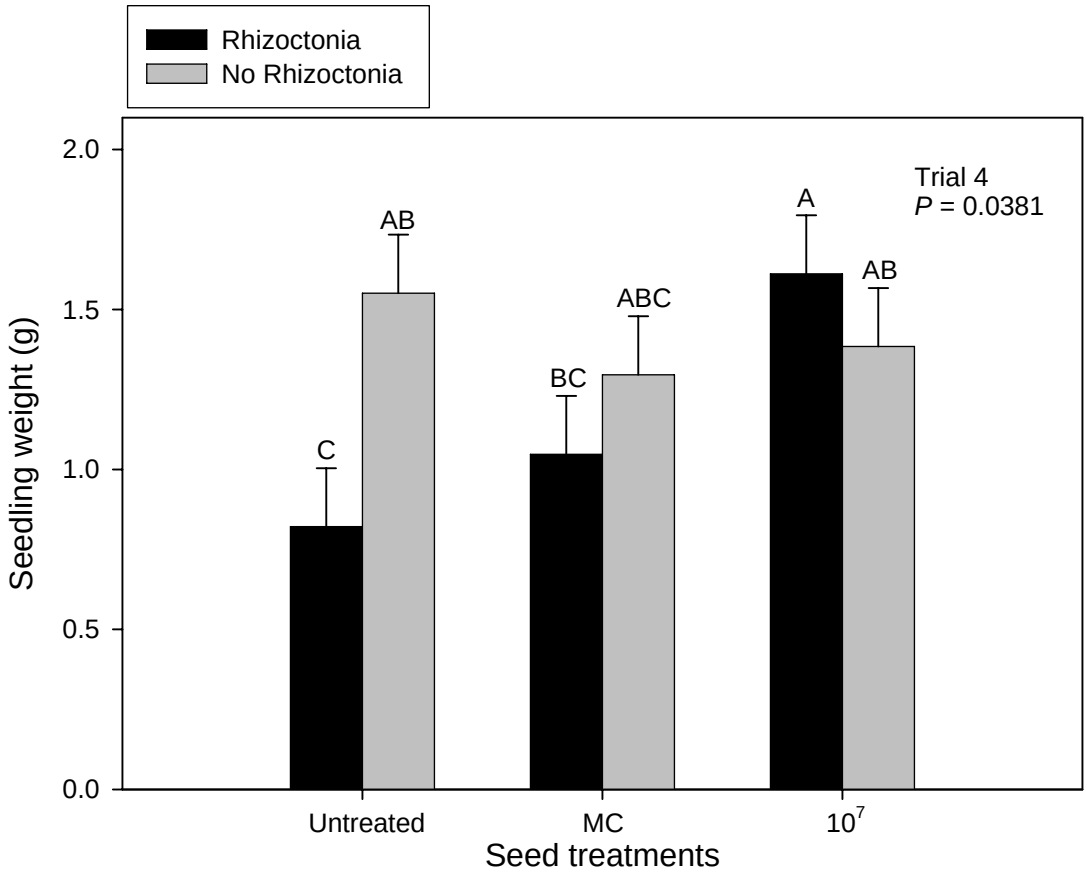

Fig. 3-8. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C on seedling weight in potting mix amended with 1% *Rhizoctonia solani*, Trial 4. Seed treatments were untreated, methylcellulose (MC), and 1×10^7 Bb 11-98C CFU/seed. Interaction of seed treatment and pathogen was significant at $P = 0.0381$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

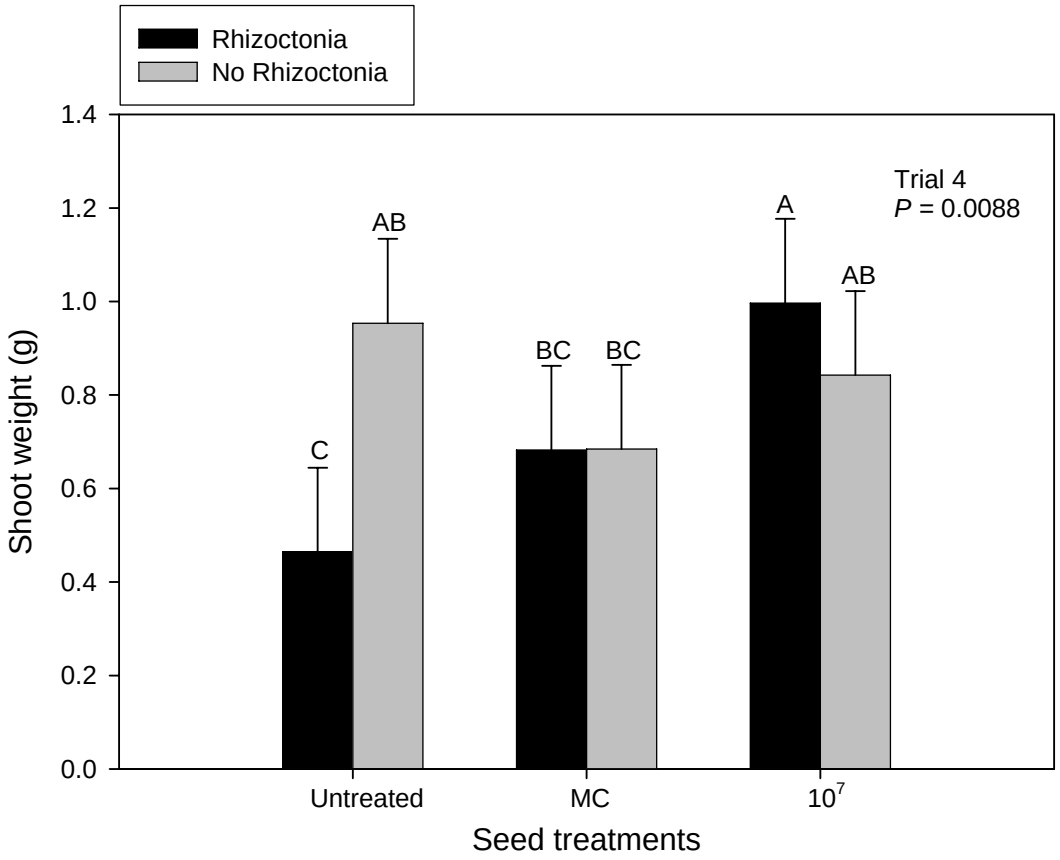

Fig. 3-9. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C on shoot weight in potting mix amended with 1% *Rhizoctonia solani,* Trial 4. Seed treatments were untreated, methylcellulose (MC), and 1×10^7 Bb 11-98C CFU/seed. Interaction of seed treatment and pathogen was significant at $P = 0.0088$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

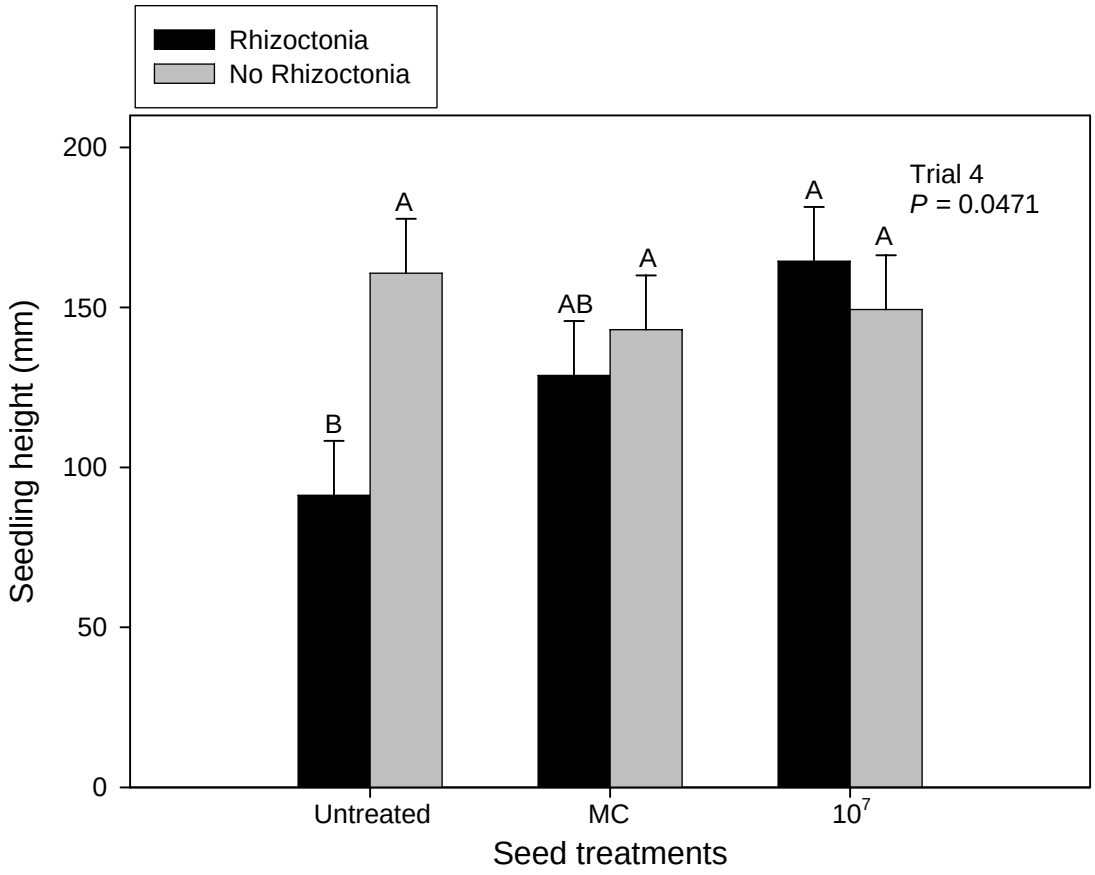

Fig. 3-10. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C on seedling height in potting mix amended with 1% *Rhizoctonia solani,* Trial 4. Seed treatments were untreated, methylcellulose (MC), and 1×10^7 Bb 11-98C CFU/seed. Interaction of seed treatment and pathogen was significant at $P = 0.0471$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

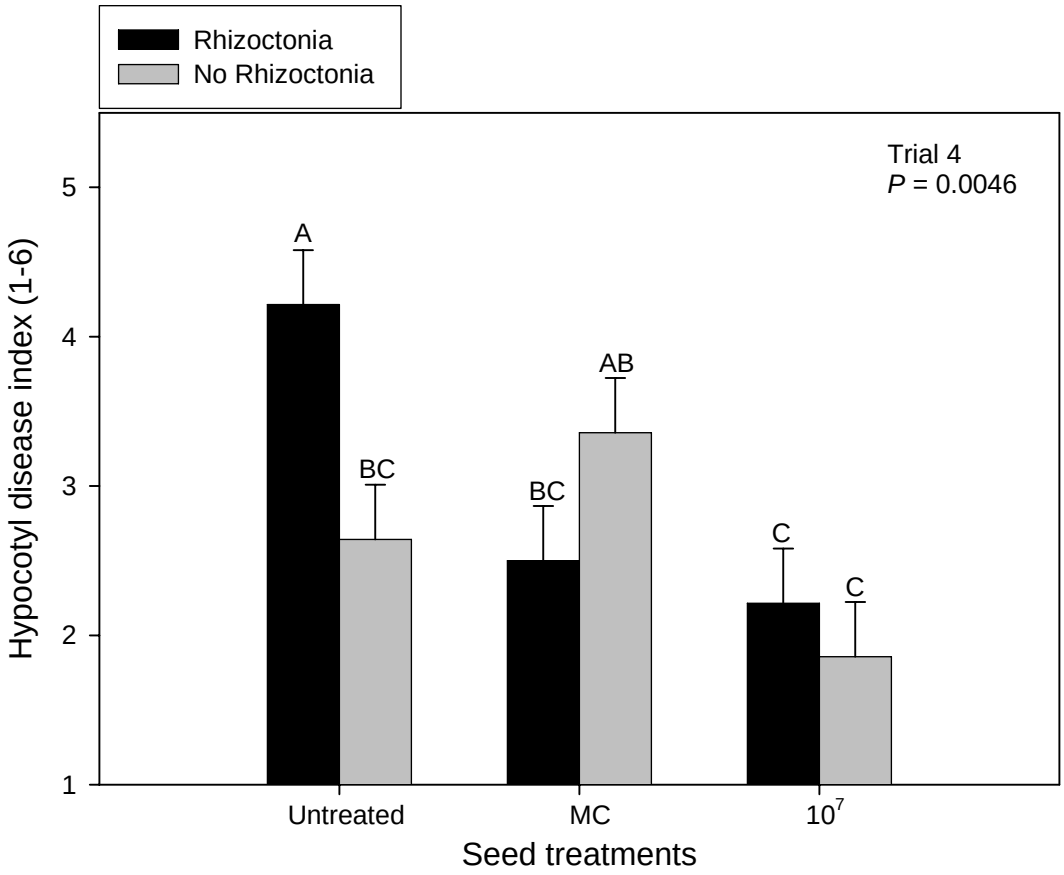

Fig. 3-11. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C on hypocotyl disease index in potting mix amended with 1% *Rhizoctonia solani*, Trial 4. Seed treatments were untreated, methylcellulose (MC), and 1×10^7 Bb 11-98C CFU/seed. Interaction of seed treatment and pathogen was significant at $P = 0.0046$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

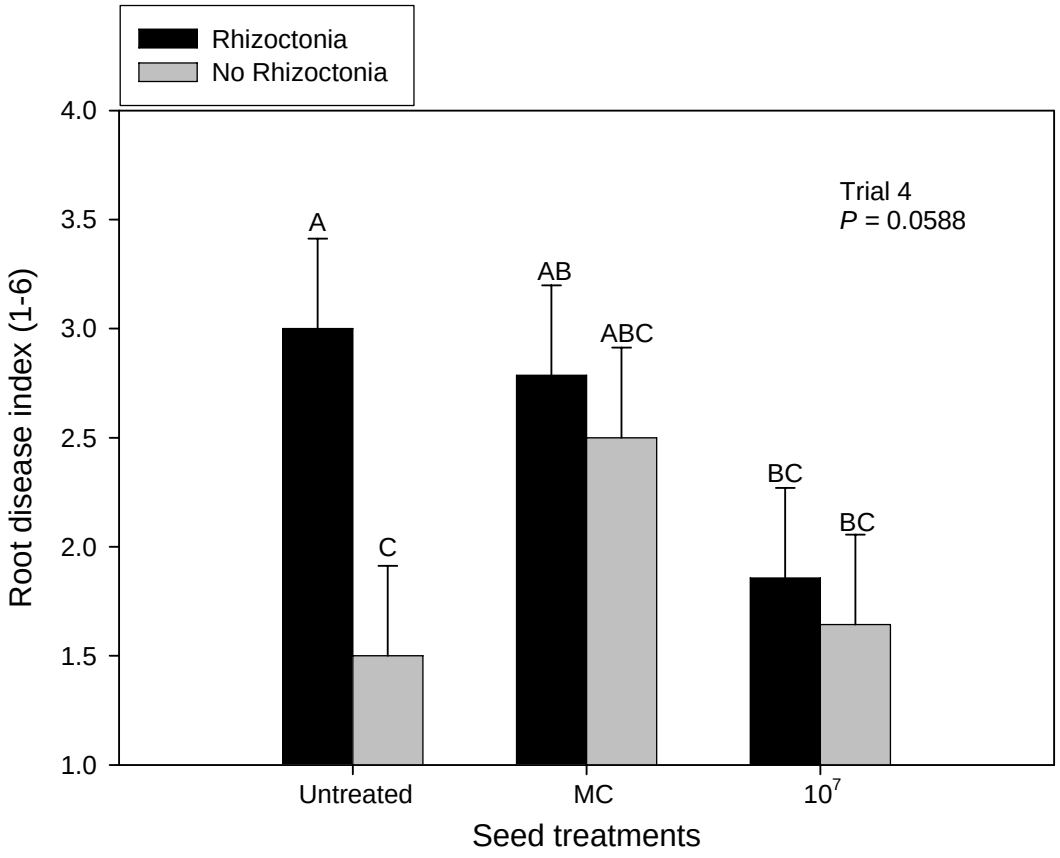

Fig. 3-12. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C on root disease index in potting mix amended with 1% *Rhizoctonia solani*, Trial 4. Seed treatments were untreated, methylcellulose (MC), and 1×10^7 Bb 11-98C CFU/seed. Interaction of seed treatment and pathogen was significant at $P = 0.0588$. Bars with the same letter are not different according to F-LSD at $P = 0.10$. Each bar represents least square means ± SE.

Table 3-4. Effect of pathogen on cotton seedling variables, Staser Trial 1. The measured variables were seedling weight (P = 0.0398), shoot weight (P = 0.0359), root weight (P = 0.0311), seedling height (P = 0.0011), hypocotyl disease index (P = 0.0001) and root disease index (P = 0.0001) of cotton seedlings in Staser sandy loam infested with 1% *Rhizoctonia solani*. For each variable, least square means ± SE with the same letters are not different according to F-LSD at P = 0.05.

Pathogen	Seedling weight (g)	Shoot weight (g)	Root weight (g)	Seedling height (mm)	Hypocotyl disease index (1-6)	Root disease index (1-6)
Rhizoctonia solani	0.9520 ± 0.07892 a	0.5938 ± 0.05442 a	0.3582 ± 0.03011 a	63.61 ± 4.5564 a	3.51 ± 0.1685 a	3.0350 ± 0.1944 a
No pathogen	1.1731 ± 0.07892 b	0.7384 ± 0.05442 b	0.4347 ± 0.03011 b	85.06 ± 4.5564 b	32.37 ± 0.1685 b	1.9 ± 0.1944 b

2.49; df = 4,181; P = 0.0449), hypocotyl disease index (F = 3.10; df = 4,181; P = 0.0169) and root disease index (F = 2.93; df = 4,181; P = 0.0221). For seedling weight, 1×10^5 and 1×10^7 Bb 11-98C were greater than the MC control, but not different from untreated seedlings (Fig. 3-13). Root weight was greater for all Bb 11-98C treatments than the MC control, but not different from the untreated control (Fig. 3-14). Percent seedling survival was greater with 1×10^5 and 1×10^9 Bb 11-98C than the MC control (Fig. 3-15). Hypocotyl disease index was lower with 1×10^5 and 1×10^9 Bb 11-98C than the MC control (Fig. 3-16), and root disease index was lower for all Bb 11-98C seed treatments than the MC control (Fig 3-17).

In a second trial, two conidial rates 1×10^7 and 1×10^9 CFU of Bb11-98C, a MC control and an untreated control were examined for biocontrol effectiveness. There was no significant interaction of seed treatment and pathogen for any measured variables in trial 2. The effect of pathogen was significant for shoot weight (F = 17.98; df = 1,98; P = 0.0001), root weight (F = 22.32; df = 1,98; P = 0.0001), seedling height (F = 29.45; df = 1,98; P = 0.0001), percent survival (F= 28.91; df= 1,98; P = 0.0001), hypocotyl disease index (F= 33.88; df = 1,98; P = 0.0001), and root disease index (F = 31.94; df = 1,98; P = 0.0001). In pathogen infested soil, shoot and root weights and seedling height were lower and hypocotyl and root disease indices were higher than in noninfested soil (Table 3-5).

Seed treatment effects were significant for seedling height (F = 2.24; df = 3,98; P = 0.0889), and survival rate (F = 3.01; df = 3,98; P =0.0340). Seeds treated with 1×10^7 and 1×10^9 CFU produced seedlings with greater height (Fig. 3-18) and a higher survival rate (Fig. 3-19) than the MC control.

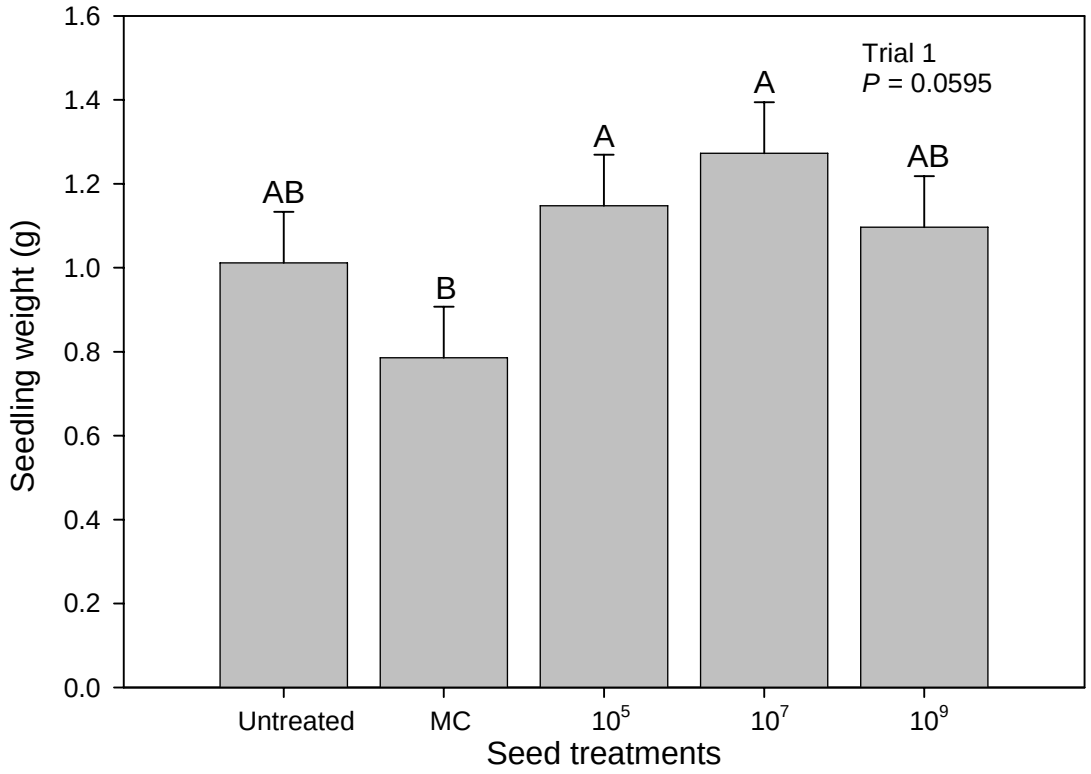

Fig. 3-13. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia on seedling weight in a Staser sandy loam soil amended with 1% *Rhizoctonia solani,* Trial 1. Seed treatments were untreated, methylcellulose (MC), 1×10^5, 1×10^7, and 1×10^9 Bb 11-98C CFU/seed. The effect of seed treatment was significant at $P = 0.0595$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

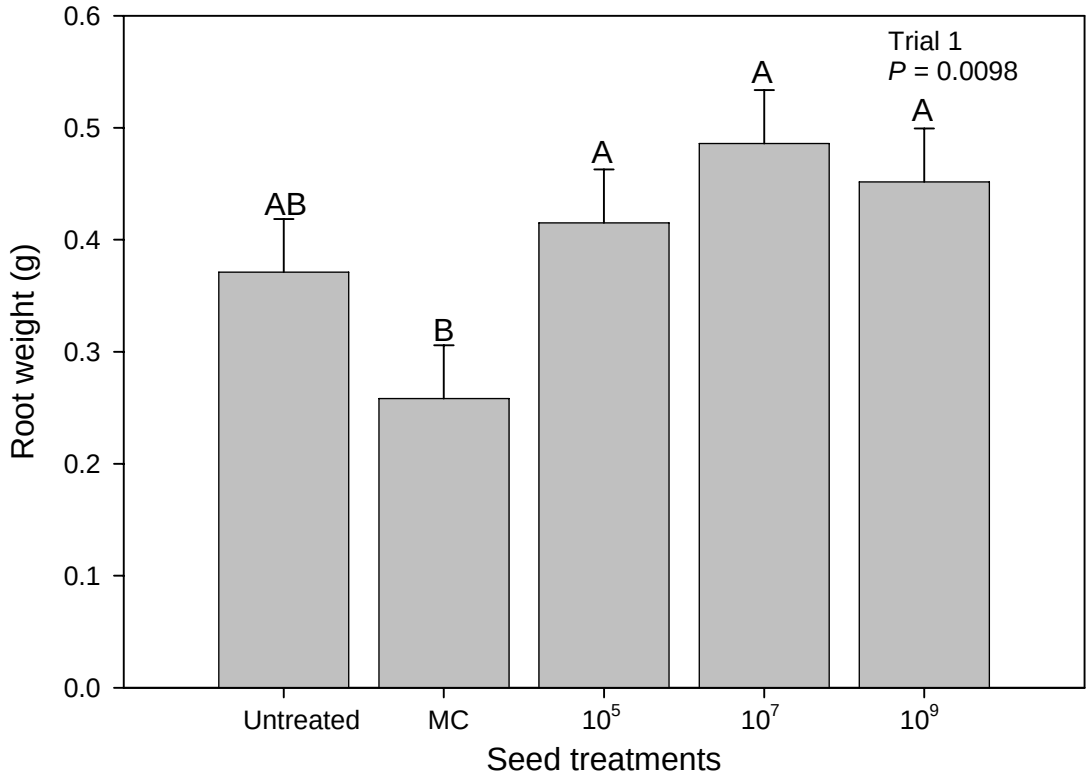

Fig. 3-14. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia on seedling root weight in Staser sandy loam soil amended with 1% *Rhizoctonia solani,* Trial 1. Seed treatments were untreated, methylcellulose (MC), 1×10^5, 1×10^7, and 1×10^9 Bb 11-98C CFU/seed. The effect of seed treatment was significant at $P = 0.0098$. Bars with the same letter are not different according to an F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

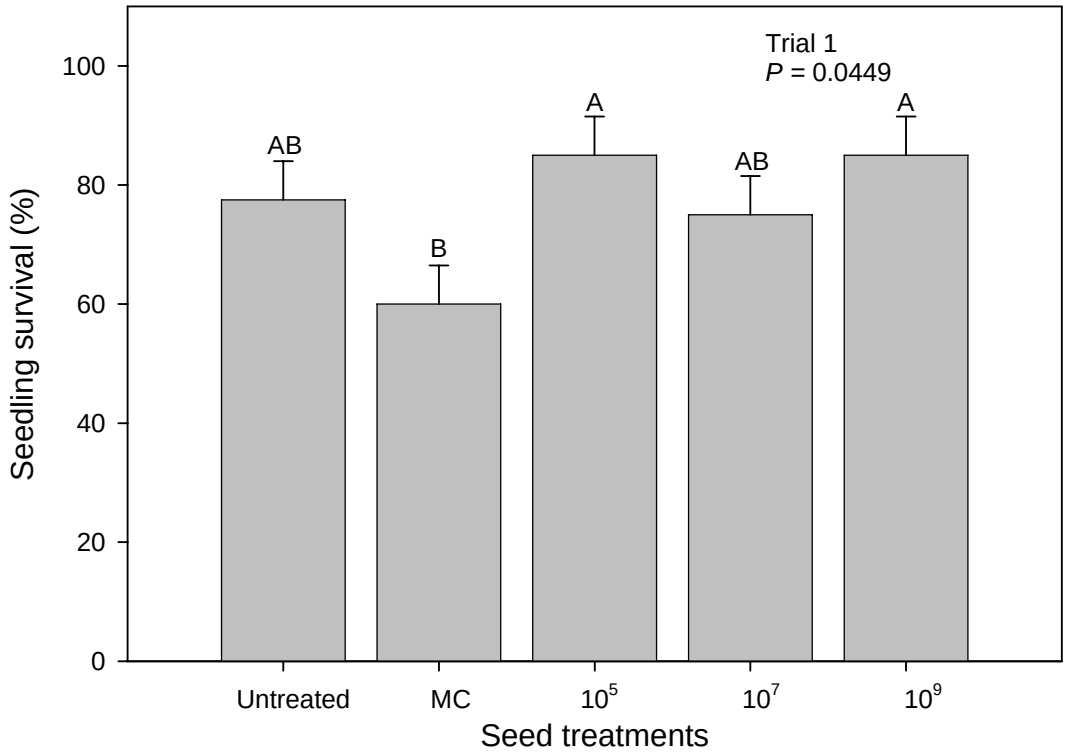

Fig. 3-15. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia on percent seedling survival in Staser sandy loam soil amended with 1% *Rhizoctonia solani*, Trial 1. Seed treatments were untreated, methylcellulose (MC), 1×10^5, 1×10^7, and 1×10^9 Bb 11-98C CFU/seed. The effect of seed treatment was significant at $P = 0.0449$. Bars with the same letter are not different according to an F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

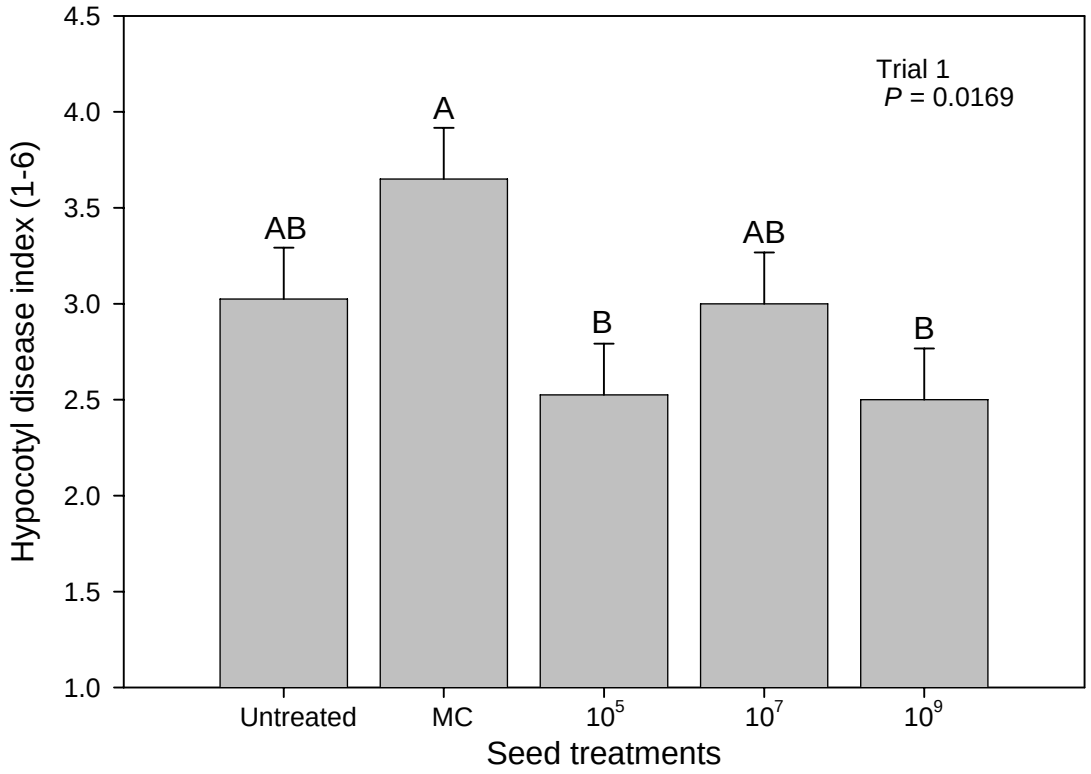

Fig. 3-16. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia on hypocotyl disease index in Staser sandy loam soil amended with 1% *Rhizoctonia solani,* Trial 1. Seed treatments were untreated, methylcellulose (MC), 1×10^5, 1×10^7, and 1×10^9 Bb 11-98C CFU/seed. The effect of seed treatment was significant at $P = 0.0169$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

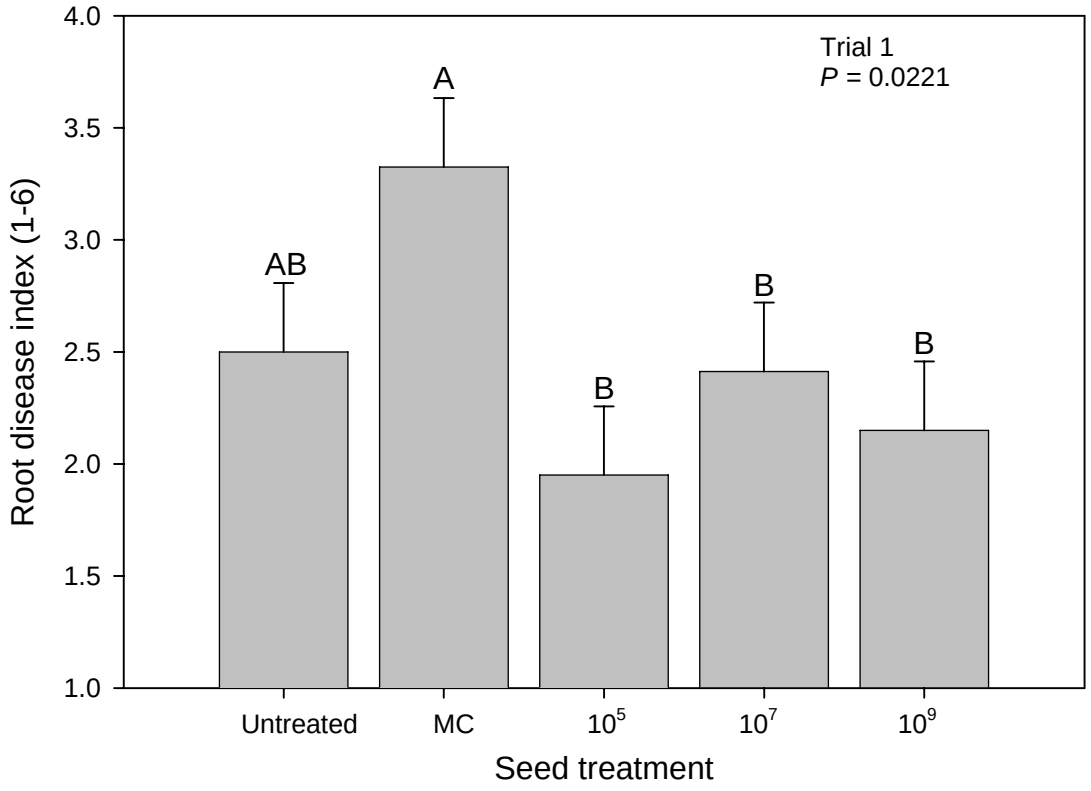

Fig. 3-17. Effect of seed treatment rate of *Beauveria bassiana* (Bb) 11-98C conidia on root disease index in Staser sandy loam soil amended with 1% *Rhizoctonia solani,* Trial 1. Seed treatments were untreated, methylcellulose (MC), 1×10^5, 1×10^7, and 1×10^9 Bb 11-98C CFU/seed. The effect of seed treatment was significant at $P = 0.0221$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

Table 3-5. Effect of pathogen on cotton seedling variables, Staser Trial 2. The measured variables were shoot weight (P = 0.0001), root weight (P = 0.0001), seedling height (P = 0.0001), hypocotyl disease index (P = 0.0001) and root disease index (P = 0.0001) of cotton seedlings in Staser sandy loam infested with 1% *Rhizoctonia solani*. For each variable, least square means ± SE with the same letters are not different according to F-LSD at P = 0.05

Pathogen	Shoot weight (g)	Root weight (g)	Seedling height (mm)	Hypocotyl disease index (1-6)	Root disease index (1-6)
Rhizoctonia solani	0.1514 ± 0.04643 b	0.1246 ± 0.04648 b	22.5893 ± 6.2916 b	5.9643 ± 0.2365 a	5.7679 ± 0.2748 a
No pathogen	0.4298 ± 0.04643 a	0.4352 ± 0.04648 a	70.8750 ± 6.2916 a	4.0179 ± 0.2365 b	3.5714 ± 0.2748 b

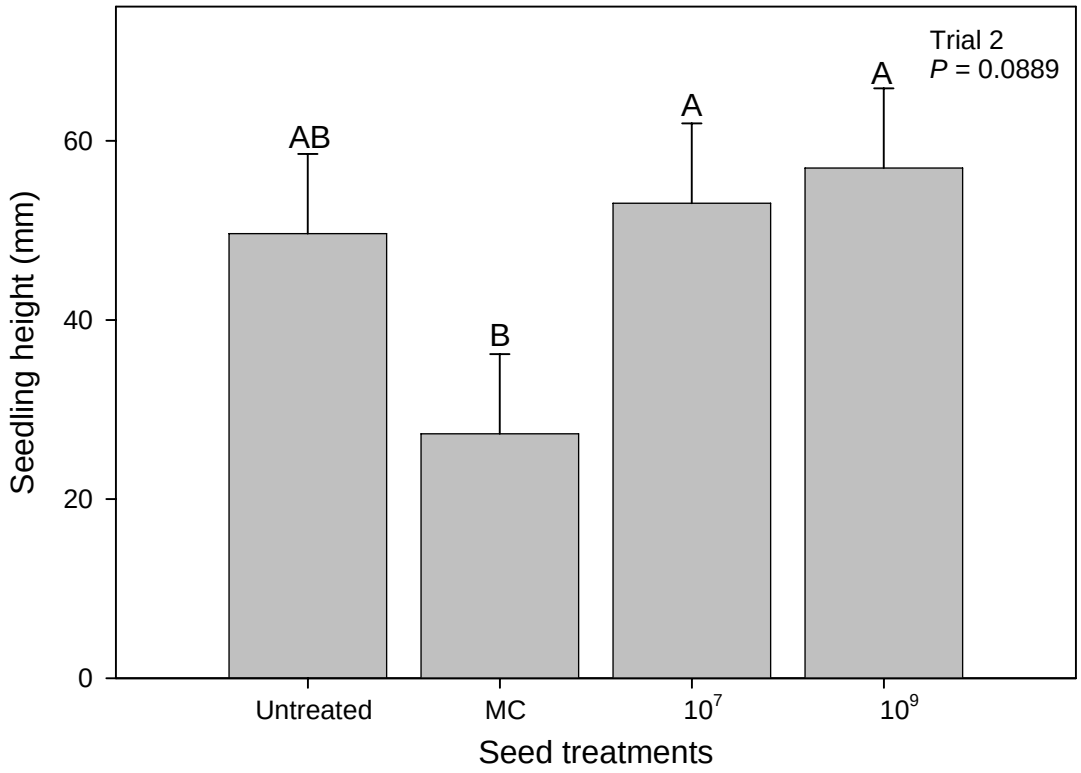

Fig. 3-18. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C conidia on seedling height in Staser sandy loam soil amended with 1% *Rhizoctonia solani,* Trial 2. Seed treatments were untreated, methylcellulose (MC), 1×10^7, and 1×10^9 Bb 11-98C CFU/seed. The effect of seed treatment was significant at $P = 0.0889$. Bars with the same letter are not different according to an F-LSD at $P = 0.10$. Each bar represents least square means ± SE.

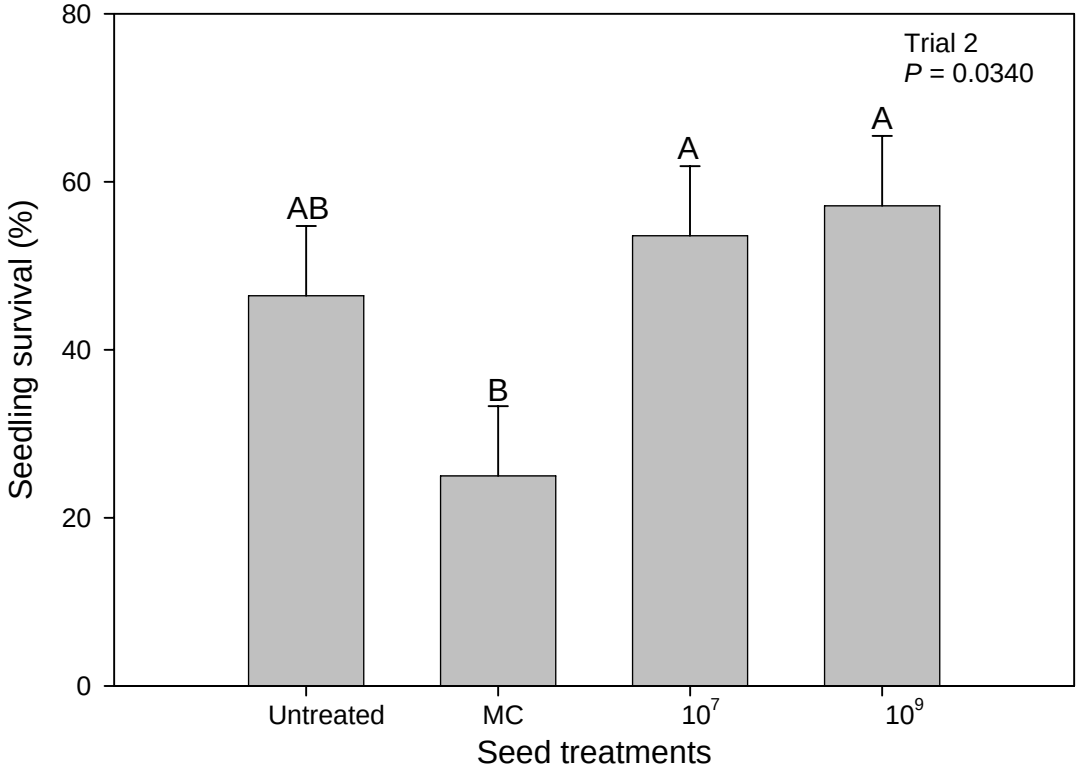

Fig. 3-19. Effect of seed treatment with *Beauveria bassiana* (Bb) 11-98C conidia on percent seedling survival in Staser sandy loam soil amended with 1% *Rhizoctonia solani*, Trial 2. Seed treatments were untreated, methylcellulose (MC), 1×10^7, and 1×10^9 Bb 11-98C CFU/seed. The effect of seed treatment was significant at $P = 0.0340$. Bars with the same letter are not different according to an F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

***Pythium myriotylum* assays**

A total of four disease assays were conducted with *P. myriotylum*; treatments were Bb 11-98 and BotaniGard (*B. bassiana* isolate GHA), at 1×10^7 CFU/seed, a MC control, and an untreated control. Two post-emergence assays were conducted with *P. myriotylum* added after seedling emergence in Collins silt loam soil. Two additional assays were conducted with *P. myriotylum* added at seeding (pre-emergence application). The first trial was in Collins soil and the second in potting soil.

In the first post-emergence trial, there were no significant effects. In the second trial, disease pressure was low and the effect of pathogen was not significant for any of the measured variables. There were significant seed treatment effects for seedling weight ($F = 4.4$; df = 3,173; $P = 0.0052$), root weight ($F = 2.5$; df = 3,173; $P = 0.0609$), survival rate ($F = 7.96$; df = 3,173; $P = 0.0001$), seedling height ($F = 7.25$; df = 3,173; $P = 0.0001$) and hypocotyl disease index ($F = 5.97$; df = 3,117; $P = 0.0007$). The 1×10^7 rate of Bb 11-98 and BotaniGard resulted in greater seedling weight (Fig. 3-20), root weight (Fig. 3-21), percent survival (Fig. 3-22), and seedling height (Fig. 3-23) than the untreated control, but were not different than the MC control. The hypocotyl disease index was significantly lower for *Beauveria* treated seed than for untreated, but was not different from the MC control (Fig. 3-24).

In the first pre-emergence trial, there were no significant effects for any measured variables. In the second trial the interaction of seed treatment and pathogen was not significant. The effect of pathogen was significant for seedling weight ($F = 6.6$; df = 1,173; $P = 0.0011$), shoot weight ($F = 4.15$; df = 1,173; $P = 0.0432$), root weight ($F = 10.04$; df = 1,173; $P = 0.0018$); seedling height ($F = 8.83$; df = 1,173; $P = 0.034$)

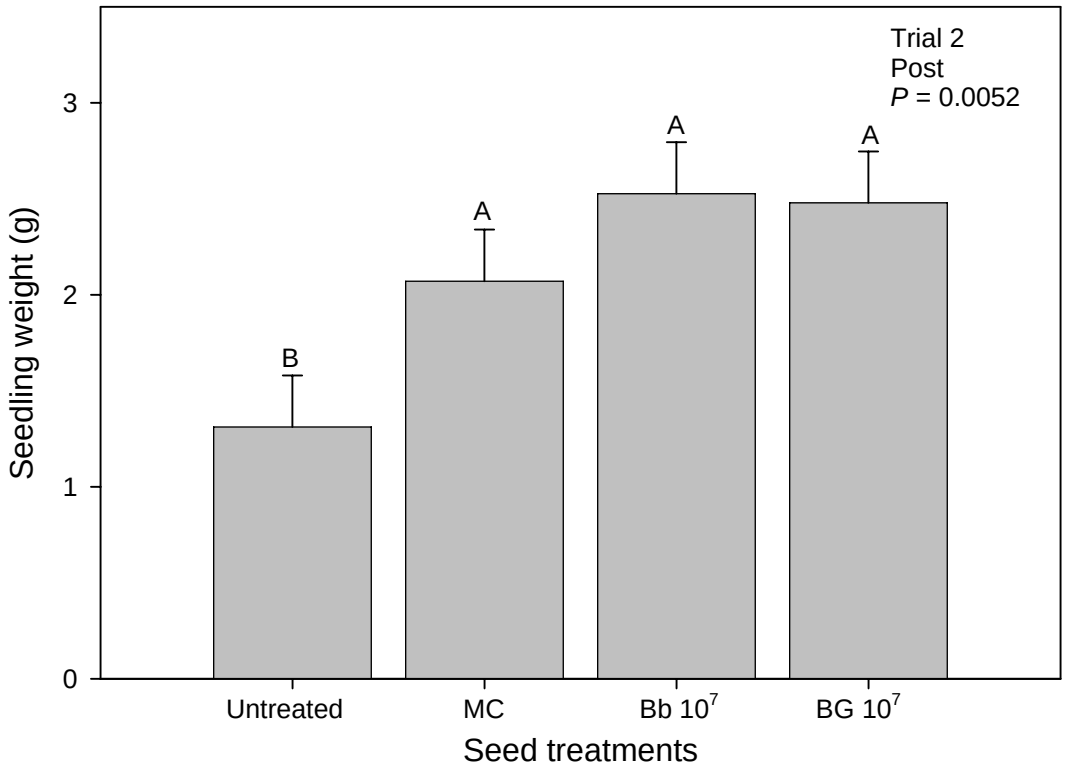

Fig. 3-20. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on seedling weight in Collins silt loam amended with *Pythium myriotylum* (post-emergence), Trial 2. Inoculum of *P. myriotylum* was added eight days after seed sowing. Seed treatments were untreated, methylcellulose (MC), 1×10^7 Bb 11-98 CFU/seed and 1×10^7 BotaniGard (BG) isolate GHA CFU/seed. The effect of seed treatment was significant at $P = 0.0052$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

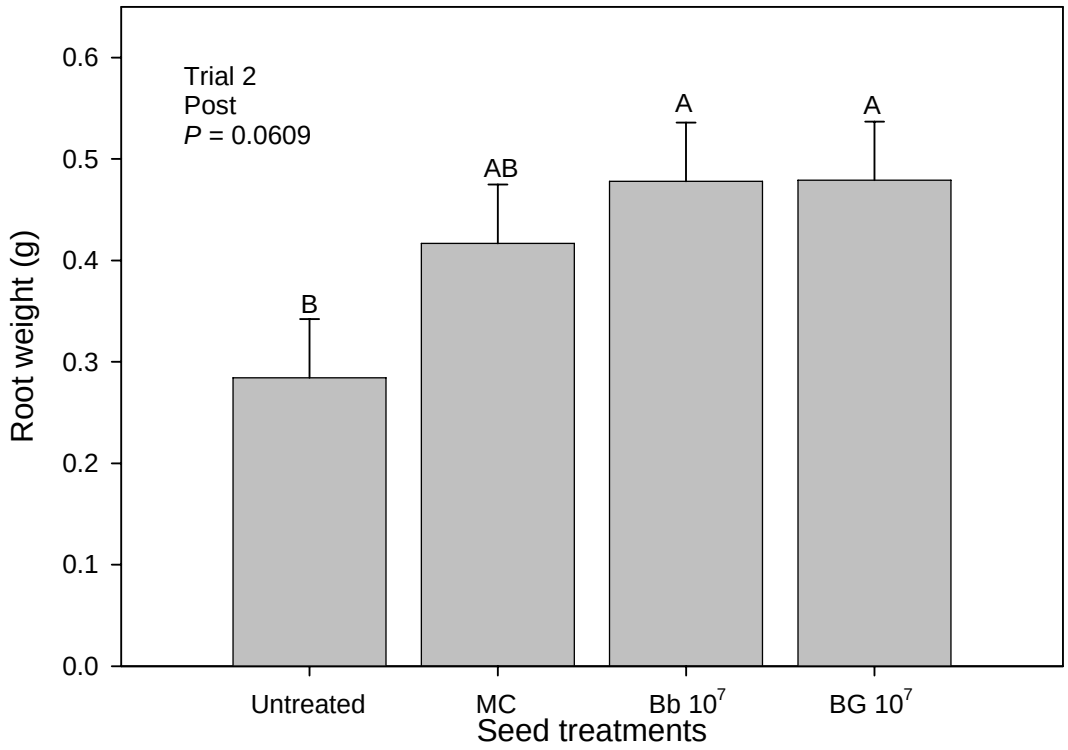

Fig. 3-21. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on root weight in Collins silt loam amended with *Pythium myriotylum* (post-emergence), Trial 2. Inoculum of *P. myriotylum* was added eight days after seed sowing. Seed treatments were untreated, methylcellulose (MC), 1×10^7 Bb 11-98 CFU/seed and 1×10^7 BotaniGard (BG) isolate GHA CFU/seed. The effect of seed treatment was significant at $P = 0.0609$. Bars with the same letter are not different according to F-LSD at $P = 0.10$. Each bar represents least square means ± SE.

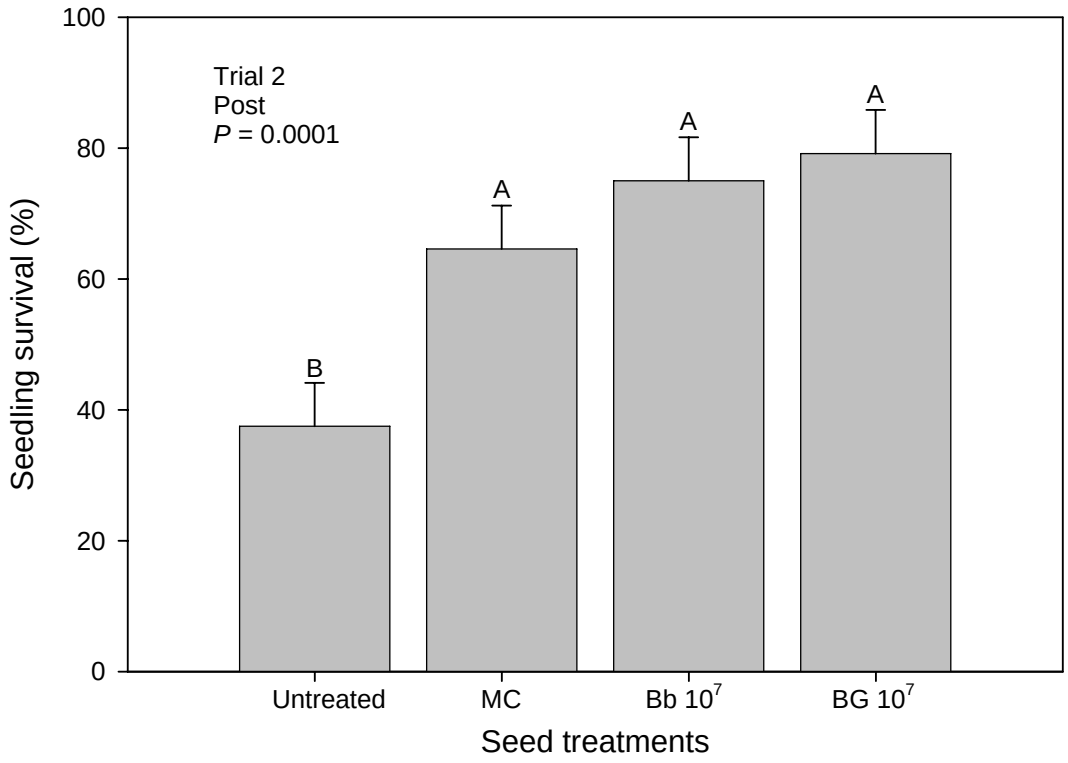

Fig. 3-22. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on percent seedling survival in Collins silt loam amended with *Pythium myriotylum* (post-emergence), Trial 2. Inoculum of *P. myriotylum* was added eight days after seed sowing. Seed treatments were untreated, methylcellulose (MC), 1×10^7 Bb 11-98 CFU/seed and 1×10^7 BotaniGard (BG) isolate GHA CFU/seed. The effect of seed treatment was significant at $P = 0.0001$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

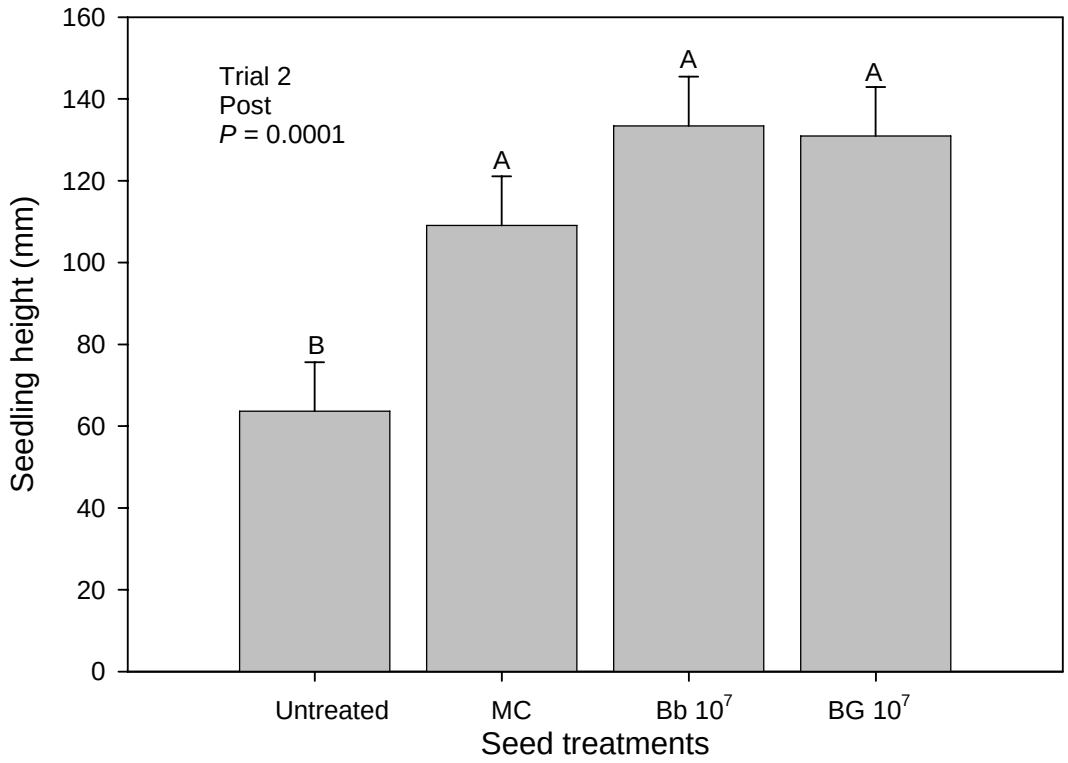

Fig. 3-23. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on seedling height in Collins silt loam amended with *Pythium myriotylum* (post-emergence), Trial 2. Inoculum of *P. myriotylum* was added eight days after seed sowing. Seed treatments were untreated, methylcellulose (MC), 1×10^7 Bb 11-98 CFU/seed and 1×10^7 BotaniGard (BG) isolate GHA CFU/seed. The effect of seed treatment was significant at $P = 0.0001$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

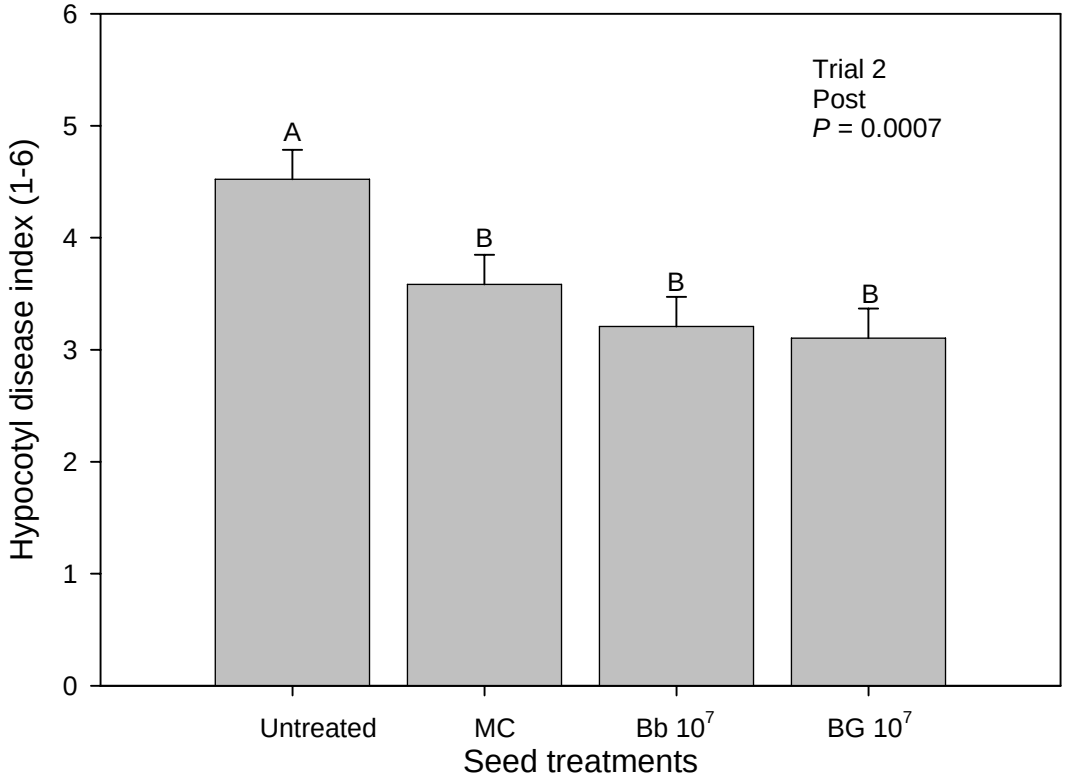

Fig. 3-24. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on hypocotyl disease index in Collins silt loam amended with *Pythium myriotylum* (post-emergence), Trial 2. Inoculum of *P. myriotylum* was added eight days after seed sowing. Seed treatments were untreated, methylcellulose (MC), 1×10^7 Bb 11-98 CFU/seed and 1×10^7 BotaniGard (BG) isolate GHA CFU/seed. The effect of seed treatment was significant at $P = 0.0007$. Bars with the same letter are not different according to F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

hypocotyl disease index (F = 7.91; df = 1,173; P = 0.0055), and root disease (F = 8.05; df = 1,173; P = 0.0051). Seedling weight, shoot weight, root weight and seedling height were lower in *Pythium* infested soil while hypocotyl and root disease indices were higher (Table 3-6).

There was a significant seed treatment effect for hypocotyl (F = 3.46; df = 3,173; P = 0.0177) and root disease (F = 2.14; df = 3,173; P = 0.0967) indices. Both isolates of *B. bassiana* resulted in lower hypocotyl (Fig. 3-25) and root (Fig. 3-26) disease indices than the MC control.

***Thielaviopsis basicola* assays**

Two trials were conducted in Staser sandy loam soil; in both assays, there was no significant interaction of seed treatment (untreated control, MC control, and 1×10^{11} CFU/seed of Bb 11-98C or BotaniGard isolate GHA) and pathogen. In trial 1, the effect of pathogen was significant for seedling weight (F = 3.35; df =1,83; P = 0.0710), root weight (F = 2,91; df = 1, 83; P = 0.0919), seedling height (F = 6.55; df = 1,83; P = 0.0123), hypocotyl disease index (F = 21.01; df = 1,83; P = 0.0001), and root disease index (F = 2.96; df = 1, 83; P = 0.0890). In *Thielaviopsis* infested soil, seedling weight, root weight and seedling height were lower, while hypocotyl and root disease indices were higher than in noninfested soil (Table 3-7). The effect of seed treatment was not significant in Trial 1.

In the second trial, seed treatments were Bb 11-98C (1×10^9 and 1×10^{11} CFU/seed) and BotaniGard GHA (1×10^{11} CFU/seed), a MC control and an untreated control. Neither the interaction nor the main effects were significant.

Table 3-6. Effect of pathogen on cotton seedling variables in potting mix with *Pythium* pre-emergence application, Trial 2. The measured variables were seedling weight ($P = 0.0110$), shoot weight ($P = 0.0432$), root weight ($P = 0.0018$), seedling height ($P = 0.0034$), hypocotyl disease index ($P = 0.0055$) and root disease index ($P = 0.0051$). For each variable, least square means ± SE with the same letters are not different according to F-LSD at $P = 0.05$

Pathogen	Seedling weight (g)	Shoot weight (g)	Root weight (g)	Seedling height (mm)	Hypocotyl disease index (1-6)	Root disease index (1-6)
Pythium myriotylum	2.57 ± 0.1530 b	1.7598 ± 0.1027 b	0.8102 ± 0.05804 b	108.16 ± 5.1852 a	2.3438 ± 0.1549 a	2.1146 ± 0.1599 a
No pathogen	3.126 ± 0.1530 a	2.0557 ± 0.1027 a	01.0703 ± 0.05804 a	129.95 ± 5.1852 b	1.75 ± 0.1549 b	1.4792 ± 0.1599 b

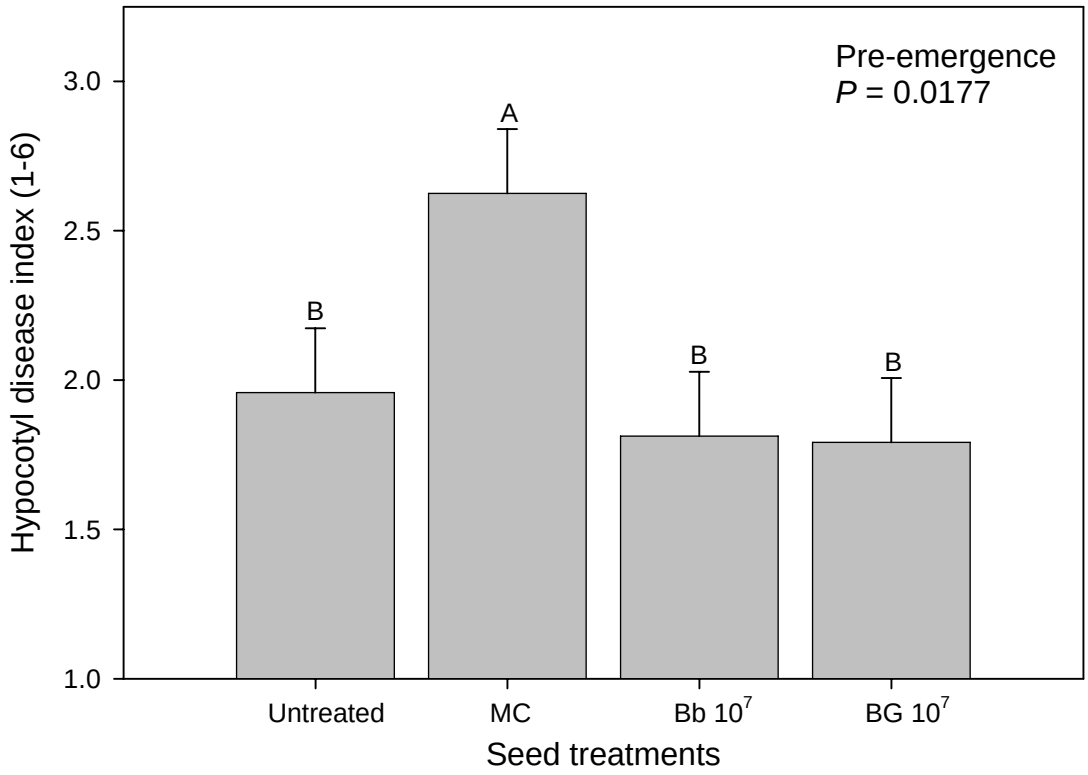

Fig. 3-25. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on hypocotyl disease index in potting mix infested with *Pythium myriotylum* added immediately after seed sowing (pre-emergence). Seed treatments were untreated, methylcellulose (MC), 1×10^7 Bb 11-98 CFU/seed and 1×10^7 BotaniGard (BG) isolate GHA CFU/seed. The effect of seed treatment was significant at $P = 0.0177$. Bars with the same letter are not different according to an F-LSD at $P = 0.05$. Each bar represents least square means ± SE.

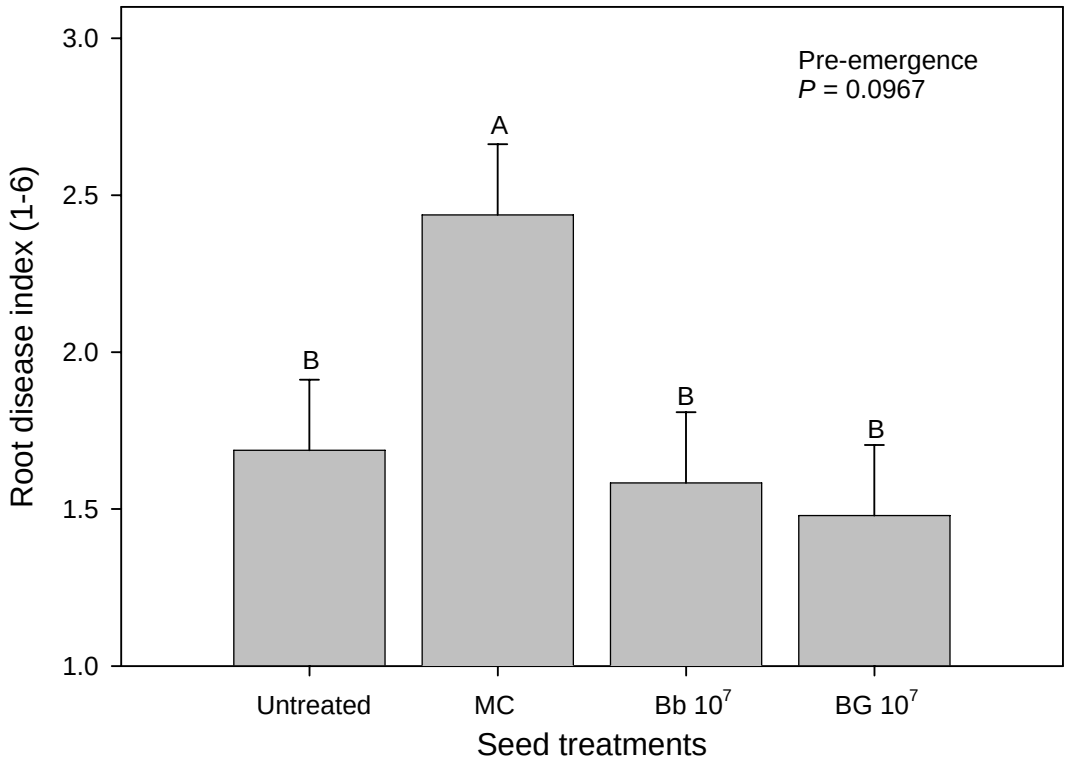

Fig. 3-26. Effect of seed treatment with *Beauveria bassiana* (Bb) conidia on root disease index in potting mix infested with *Pythium myriotylum* added immediately after seed sowing (pre-emergence). Seed treatments were untreated, methylcellulose (MC), 1×10^7 Bb 11-98 CFU/seed and 1×10^7 BotaniGard (BG) isolate GHA CFU/seed. The effect of seed treatment was significant at $P = 0.0967$. Bars with the same letter are not different according to an F-LSD at $P = 0.10$. Each bar represents least square means ± SE.

Table 3-7. Effect of pathogen on cotton seedling variables in Staser sandy loam soil with *Thielaviopsis basicola*, Trial 1. The measured variables were seedling weight ($P = 0.0710$), root weight ($P = 0.0919$), seedling height ($P = 0.0123$), hypocotyl disease index ($P = 0.0001$) and root disease index ($P = 0.0890$). For each variable, least square means ± SE with the same letters are not different according to F-LSD at $P = 0.05$ or 0.10

Pathogen	Seedling weight (g)	Root weight (g)	Seedling height (mm)	Hypocotyl disease index (1-6)	Root disease index (1-6)
Thielaviopsis basicola	1.4244 ± 0.1768 b	0.4213 ± 0.06056 b	69.528 ± 8.5682 b	4.333 ± 0.2231 a	3.5 ± 0.2817 a
No pathogen	1.8767 ± 0.1768 a	0.5673 ± 0.06056 a	100.53 ± 8.5682 a	2.9583 ± 0.2231 b	2.8750 ± 0.2817 b
F-LSD P-Value	0.10	0.10	0.05	0.05	0.10

Discussion

Microbial control of plant pathogens is an important component of efforts to reduce our reliance on chemical pesticides and increase sustainability of U.S. agriculture. Plant pathogens can become tolerant or resistant to individual or combinations of pesticides. Development of fungicide resistance forces farmers to use higher amounts of chemical pesticides to produce a crop. Soilborne fungi can persist in the soil, in some cases, for years. This persistence makes them especially difficult to combat since their lifecycles are adapted to survive long periods of time in the form of survival propagules. Public concern over potential health risks caused by chemical pesticides has helped energize interest in pest management alternatives such as biological control agents, which involve the use of a living organism to control another. *Beauveria bassiana* 11-98 has potential as a biocontrol of plant pathogens; however, to enhance its efficacy, information about application rates and a greater understanding of its function in different soil environments must be obtained.

Some biocontrol organisms are capable of enhancing growth in some plants. For example, *Trichoderma harzianum* Rifai applied as a peat-bran preparation and incorporated into a propagative mixture in a production nursery, significantly enhanced seedling height and dry plant weight in cucumber and pepper (Inbar et al. 1994). In another study, *T. harzianum* (T-203) propagules added to soil gave a 30% increase in cucmber seedling emergence up to 8 days after sowing. After four weeks the plants exhibited a 95% increase in root area and a 75% cumulative increase in root length. A significant increase in dry weight, shoot length and leaf area also was observed (Yedidia et al. 2001).

Based on results of the germination assays, certain rates of Bb 11-98C affected seedling shoot and root length and survival rate. In some cases these parameters were enhanced in the presence of Bb 11-98C. Application of Bb 11-98C at 1×10^5 CFU/seed enhanced radicle length (Trial 1) and survival rates (Trial 1 and 2); thus, at low rates *B. bassiana* may enhance growth in the absence of pathogens.

Conversely, high rates of Bb 11-98C may be detrimental to plant growth. The rate of 1×10^{11} CFU/seed appeared to be the conidial rate limit for 'DP 436 RR'. Percentage seed survival was reduced at the highest rate (1×10^{11} CFU/seed) of Bb 11-98C. Decreased survival at the 1×10^{11} rate could be due to production of secondary metabolites by Bb 11-98C. Apparent toxicity was reduced in soil.

Biological control agents are capable of producing phytotoxic effects if population rates are not optimized. In a study using *Pseudomonas fluorescens* Migula strain 2-79, a well known biocontrol agent used against take-all of wheat (*Gaeumannomyces graminis* var. *tritici* Walker), the biological control agent was found to have a negative effect on seedling height. Decline in plant growth coincided with deterioration of the biocontrol agent in storage when encapsulated on wheat seeds. Significant germination loss also was observed due to phytotoxic metabolites (Slininger et al. 1996).

In the current study, application of *B. bassiana* on the seed coat surface within a potting mix or soil substrate did not decrease germination, growth, or survival rates of seedlings regardless of spore rate. It is undetermined at this time if the production of mycotoxins inhibited seedling growth in the germination assay, or if seed nutrients may have been depleted due to the mass of germinating spores and growing hyphae.

Some biocontrols, particularly pseudomonads, have a required threshold population density of bacteria before they affect significant disease suppression. Relatively small decreases in population size can dramatically reduce protection (Johnson 1994). In a two-year study by Dorner et al. (1998) on the effect of inoculum rate of biological control agents on preharvest aflatoxin contamination of peanuts, the biocontrol agents were nontoxigenic color mutants of *Aspergillus flavus* Link:Fr. and *A. parasiticus* Speare. *Aspergillus* mutants were grown on rice for soil inoculum and added to three replicate plots at 0, 2, 10 and 50 g/m of row. Regression analysis indicated a trend toward lower aflatoxin concentration with increasing rates of inoculum and this relationship increased in the second year of treatment.

In the present study, Bb 11-98C, at a rate of 1×10^5 CFU/seed was too low to provide significant protection against *R. solani* in potting mix. As the rates of Bb 11-98C applied to seed increased, disease suppression was greater. In general, the rate of 1×10^9 CFU/seed Bb 11-98C gave optimal control in the organic potting mix. In the Staser sandy loam soil, 1×10^7 CFU/seed was the best treatment, suggesting that soil type may play a role in efficacy of Bb 11-98C against *R. solani*.

Wide-scale commercial application of biocontrols has been slowed, in part, due to inconsistent performance in field locations. Effects of various minerals, growth factors, carbon and nitrogen source, pH and temperature on the potential biocontrol agents have each been proposed as potential factors influencing success of biological control activity in a particular location (Ownley et al. 2003). Understanding which abiotic and biotic soil factors have the most influence on the biocontrol activity of *B. bassiana,* and how these factors interact, may provide ways to enhance biocontrol efficacy through regulation of

biocontrol mechanisms.

It is possible that the population density of *B. bassiana* per seed needed for effective biocontrol correlates to nutrient availability in the soil. The biocontrol activity of *Beauveria bassiana* against *R. solani* was more effective in potting mix that has higher organic matter than the sandy loam soil, which is low in organic matter.

Soil mineral components are known to have an effect on biocontrol efficacy and it is possible to create a more favorable environment for biological control to occur with managed mineral fertilization regimes. Duffy and Défago (1997) found in soilless tomato culture that mineral amendments of copper and zinc significantly improved the biocontrol activity of *Pseudomonas fluorescens* strain CHA0 against *Fusarium oxysporum* f. sp. *radicis-lycopersici* Jarvis & Shoemaker. The trace mineral zinc was positively correlated with biocontrol activity of *Pseudomonas fluorescens* 2-79 and enhanced the production of a phenazine antibiotic essential for disease suppression of take-all of wheat (Duffy et al. 1997).

In the *P. myriotylum* disease assays with post-emergence application of *Pythium*, disease was low overall and no disease suppression by *B. bassiana* was observed in the first trial. However, in the second trial, disease pressure was greater and both *Beauveria* isolates (11-98 and GHA) provided disease protection compared to the untreated controls.

Two pre-emergence assays where *Pythium* was added prior to seedling emergence were conducted. In one trial, no results were significant, while the second resulted in *Beauveria* 11-98 and BotaniGard isolates having less disease than the MC treatment for both hypocotyl and root disease indices, but effects were not different from observations of untreated seedlings. Protection against *P. myriotylum* in a pre-emergence environment

was successful using *Beauveria* isolates Bb 11-98 and BotaniGard with tomato (Clark 2006).

In this study, comparison of pre-emergence and post-emergence assays indicates that moisture levels were potentially a limiting for the efficacy of *B. bassiana* on cotton especially in the pre-emergence assays. This was most likely due to soil flooding with the application of *Pythium* zoospores immediately after seed were sown. In post-emergence assays, there was an 8-day period during which spores of *B. bassiana* could colonize the cotton seedling; seedlings treated with Bb 11-98 and BotaniGard had greater seedling shoot and root weight, and seedling height than untreated seedlings; however, disease pressure in the assay was low.

The effectiveness of *B. bassiana* 11-98 and BotaniGard isolate GHA in providing protection for seedlings against *T. basicola* was not demonstrated in the two trials. In the first trial, only one rate of isolate Bb 11-98 and BotaniGard were examined. Because the effect of pathogen was significant, it is likely that 1×10^{11} was not an effective spore rate. The second trial proved inconclusive even though different rates, 1×10^{9} and 1×10^{11} of Bb 11-98C, and 1×10^{11} of BotaniGard, were examined. Pathogen effect was significant in the first assay; however, there were no other significant effects. In the second assay no significant effects were found. For the first assay, it is possible that the rates of *Beauveria* were too high, because in the assay with *R. solani* in potting mix, the highest rate of 1×10^{11} CFU/seed did not provide protection compared to the lower 1×10^{9} CFU/seed rate. In the sandy loam soil, the lower rate of 1×10^{7} was more effective against *R. solani*.

In conclusion, rate of conidia applied to cotton seeds does influence the biological control efficacy of Bb 11-98C. Biocontrol efficacy of *B. bassiana* varies in field soil and

this is likely due partly to abiotic factors, as the effective rate of conidia on seed varied with soil type. *Beauveria bassiana* also differed in its ability to control the three different soilborne pathogens examined in this study. Efficacy was greater against *R. solani* than *P. myriotylum*, and results with *T. basicola* were inconclusive.

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Part 4. Mechanisms of action, including induced systemic resistance, employed by *Beauveria bassiana* as a biocontrol against cotton pathogens

Abstract

Beauveria bassiana (Bals.-Criv.) Vuill., an entomopathogenic fungus, can protect against soilborne plant pathogens when applied as a seed treatment. However, the mechanisms that *B. bassiana* utilizes have not been elucidated. In this study, *B. bassiana* was evaluated for its ability to induce systemic resistance (ISR) in cotton against *Xanthomonas axonopodis* pv. *malvacearum* (Smith 1901) Vauterin, Hoste, Kersters & Swings 1995. Treatments were three rates of *B. bassiana* 11-98C conidia (1×10^5, 1×10^7 and 1×10^9) and three controls [untreated, 2,6-dichloro-isonicotinic acid (INA), and untreated without *Xanthomonas* challenge]. Treatments were replicated six to eight times with three to four seedlings per replication. Cotton seeds were placed in growth pouches in a soilless system and incubated at 28°C with continuous light for five days. Conidial treatments were added to 0.2 ml sterile 1/3 Hoagland's solution and applied to roots 5 cm below radicle emergence. Likewise, INA was applied at 0.2 ml per root. After 13 days, one primary leaf of each plant was pricked with a needle and challenge-inoculated with *X. axonopodis* (1×10^9 to 1×10^{10} CFU/ml). After which, leaves were rated for bacterial leaf blight daily for 1 week. Treatment with *B. bassiana* 1×10^7 conidia resulted in lower disease ratings than the untreated control and was as effective as INA. In host-based medium, *B. bassiana* was tested for ability to produce antimicrobials that would inhibit growth of three soilborne pathogens of cotton. Although no zone of inhibition was noted for any tested pathogens, *B. bassiana* appeared to out-compete, or parasitize *Thielaviopsis basicola* (Berk. & Broome). Colony diameter of *T. basicola* was reduced after two weeks in culture. Furthermore, after two weeks *B. bassiana* continued to grow, and colony diameter expanded within all pathogen assay plates. Parasitism assays were

performed to determine if *B. bassiana* could parasitize *Rhizoctonia solani* (Kühn), *Pythium myriotylum* Dreschler or *T. basicola*. Coiling by *B. bassiana* was observed on hyphae of *P. myriotylum*; however, no coiling was observed on *R. solani*. *Thielaviopsis basicola* was unable to produce mycelia on the glass cover slips in this assay. An assay was conducted to determine if Bb 11-98C could grow on chitin- and cellulose-based media. *Beauveria bassiana* 11-98C was able to hydrolyze and use the chitin-based medium as a food source however; *B. bassiana* was not able to hydrolyze the cellulose-based medium.

Keywords: *Beauveria bassiana*, biocontrol, cellulose, chitinase, induced systemic response, ISR

Introduction

In order to better understand the efficacy of *B. bassiana* as a biocontrol agent it is important to understand mechanisms by which it protects itself and its symbiont. Mechanisms through which biocontrol agents can antagonize soilborne pathogens are generally classified as antibiosis, competition for niches and nutrients (niche exclusion), parasitism/predation, and induction of a plant defense response (Chin-A-Woeng et al. 2003).

Antibiosis is defined as antagonism mediated by specific or nonspecific metabolites of microbial origin by lytic agents, enzymes, volatile compounds or other toxic substances, such as antibiotics (Fravel 1988). Antibiotics encompass a chemically heterogeneous group of organic low-molecular weight compounds produced by

icroorganisms. At low concentrations antibiotics are deleterious to growth or metabolic activities of other organisms (Raaijmakers et al. 2000). Most antibiotics are produced by soil inhabiting microorganisms. In the past decade, numerous antibiotics have been isolated from various biocontrol strains representing different bacterial genera, such as pseudomonads, bacilli and *Streptomyces* spp.; and *Trichoderma* spp., a group of fungal biocontrol agents, which are widely distributed in soil and decaying vegetation. Each produces antimicrobial compounds and suppresses disease by diverse mechanisms (Handelsman and Stabb 1996).

Trichoderma virens (J.H. Mill., Giddens & A.A. Foster) Arx (formerly *Gliocladium virens* GL-21) can protect plants against damping-off and root rot pathogens (Paulitz and Bélanger 2001). Its spectrum of control includes the soilborne pathogens *R. solani* and *Pythium* spp. *Trichoderma virens* typically produces gliotoxin, a structurally complex antibiotic. Mutants of *T. virens* that do not produce gliotoxin are reduced in their ability to control *Pythium* damping-off. Mutants with increased or decreased antibiotic production have a corresponding effect on biocontrol (Handelsman and Stabb 1996).

Beauveria bassiana may act similarly to *Trichoderma* spp. in its biocontrol activity, and may be in part, securing its substrate through antibiosis. *Beauveria bassiana* is known to produce beauvericin, bassinolide, oosporein, beauvolide, beauvirolide, bassinin and tellinin, but not every isolate produces all the toxins listed.
Although production by *B. bassiana* of mycotoxins *in vitro* is well studied, little information is available on production of mycotoxins by *B. bassiana in planta.* As an entomopathogen, the production of toxins is thought to act as virulence factors that aid in the colonization and utilization of a food substrate until propagules are produced.

Beauvericin is an ionophore capable of inserting into the lipid of the plasma membrane causing abnormal ion transport and disrupting cells and organelles such as mitochondria (Plattner and Nelson 1994, Žižka and Weiser 1993). Bassinolide is toxic to lepidopteran species (Kanaoka et al. 1978). Oosporein, a red pigmented dibenzoquinone, has antibiotic activity against several bacteria, particularly Gram-positive species (Brewer et al. 1984, El Basyouni and Vining 1966, Taniguchi et al. 1984, Vining et al. 1962, and Wainwright et al. 1986). Oosporein may help *B. bassiana* compete against the natural bacterial microflora located within the gut of the insect. Production is generally observed after insect infection, when *B. bassiana* is within its proliferation phase; the insect is noted to turn pink or reddish in color (Stasser et al. 2000). Beauvolide and beauvirolide are structurally similar to beauvericin, but their toxic effects have not been fully demonstrated against target insects (Kuzma et al. 2001). Little is known about bassinin and tellinin (El Basyouni et al. 1968).

One mechanism of action by fungal antagonists is mycoparasitism or hyperparasitism: the utilization of other fungi or fungal pathogens as a food base. Parasitism requires specific interactions between parasite and fungal host, including ability to detect chemical gradients and mycelial surface features. Digestion of host cell walls is accomplished by a battery of excreted lytic enzymes, including proteases, chitinases and glucanases. These enzymes often have antifungal activity individually and are synergistic in mixtures or with antibiotics (Handelsman and Stabb 1996, Spadaro and Gullino 2005).

Many fungi are capable of producing lytic enzymes. Some of these enzymes are involved in the breakdown of fungal cells by degradation of cell wall constituents such as

glucans and chitins, resulting in the destruction of pathogen structures or propagules. The degradation products released can be used by the biocontrol agent as a food source (Chin-A-Woeng et al. 2003).

Several *Trichoderma* spp. are known to parasitize fungal plant pathogens. Hyphal branches grow toward the target host hyphae, coil around and attach to them with appressorium-like bodies, then penetrate the hyphae of the host fungus. *Trichoderma harzianum* Rifai produces at least three distinct chitinases as well as proteolytic and glucanolytic enzymes (Handelsman and Stabb 1996). The fungus *Trichoderma harzianum* Rifai strain KRL-AG2 (T-22) is effective in controlling soilborne pathogens such as *Pythium* spp., *Rhizoctonia solani, Verticillium* spp., *Sclerotium* spp., and others by parasitism and competition (Haggag 2002).

As an entomopathogen, the production of chitinases by *B. bassiana* would be necessary for it to break down the insect's cuticle before colonization. For *B. bassiana* to successfully utilize fungi as a food source it would be necessary for it to produce the necessary lytic enzymes capable of breaking down the components of the pathogen. Conidial swelling and germ tube formation in *B. bassiana* requires only a carbon source, but nitrogen is necessary to support hyphal development after these initial events (Boucias and Pendland 1998).

Systemic resistance induced by nonpathogenic microorganisms is termed induced systemic resistance (ISR), which is a state of enhanced defensive capacity developed by a plant when appropriately stimulated. Jasmonic acid and ethylene activate the ISR signaling pathway (Han et al. 2000). A nonpathogenic microorganism can induce a systemic effect against a pathogen while remaining spatially separated on the plant.

Plants infected by pathogens can develop resistance against later attacks on previously infected parts and even on plant tissues distant from the primary infection (Van Loon et al. 1998).

Systemic acquired resistance (SAR) differs from ISR in that salicylic acid production and pathogenesis-related (PR) proteins are produced in the plant. Systemic acquired resistance, can be induced by several necrotizing pathogens in a wide range of plants, and by chemicals such as phosphates, 2,6-dichloro-isonicotinic acid (INA) and benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) (Bigirimana et al. 1997). In contrast, ISR induced by nonpathogenic rhizosphere organisms does not involve the SA signaling pathway or the induction of PR proteins (Han et al. 2000).

Although *B. bassiana* has not been previously studied for its ability to induce ISR response in plant pathogen systems, other nonpathogenic fungi such as *T. harzianum* have been observed to induce this resistance (Bigirimana et al. 1997). *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg. [stat. anam.] (formerly *T. harzianum* 203) can penetrate the roots of cucumber seedlings and colonize the epidermis and outer root cortex. These interactions also induce host plant resistance to pathogens even in upper plant potions. *Trichoderma* root inoculation has been shown to be effective against different types of pathogens in a wide variety of plants (Shoresh et al. 2004).

Beauveria bassiana may also offer protection against fungal pathogens via other mechanisms as *B. bassiana* produces a host of antibiotics and has the ability to hydrolyze chitin. Evidence for *B. bassiana* as a dual-purpose biocontrol organism, with activity against insect pests and plant pathogens, is currently being examined. *Beauveria bassiana* applied to whorl-stage corn by foliar application colonized and persisted in corn plants to

provide season-long suppression of *Ostrinia nubilalis* Hübner (Bing and Lewis 1991). Application of *B. bassiana* to tomato seeds prior to sowing provided protection against *R. solani*, a soilborne fungus that causes disease in a wide range of cultivated plants (Ownley et al. 2005).

Objectives of this study were 1) to determine if *B. bassiana* is capable of causing an induced systemic response in cotton seedlings against *X. axonopodis* pv. *malvacearum*; 2) to determine if *B. bassiana* is capable of creating a response in cotton plants comparable to that induced by a SAR inducing chemical; 3) to determine if effect of spore rates applied to cotton roots will vary ISR response in cotton; 4) to determine if *B. bassiana* is capable of inhibiting pathogens via production of antibiotics on host based medium; 5) to determine if *B. bassiana* is capable of mycoparasitic activity against soilborne pathogens and; 6) to determine indirectly if *B. bassiana* is capable of mycoparasitic activity by hydrolysis of chitin- and cellulose-based media.

Methods and Materials

Production of *Beauveria bassiana*

Beauveria bassiana (Bb) 11-98 was isolated originally from an infected click beetle (Coleoptera: Elateridae). Following application to cotton seed, this organism was re-isolated from cotton 'Delta-Pine (DP) 436 RR' tissue by plating samples of surface-sterilized plant tissue onto a selective culture medium (Doberski and Tribe 1980); the recovered isolate (Bb 11-98C) was utilized in subsequent assays.

Cultures of *B. bassiana* were produced on Sabouraud dextrose agar (SDA) over a 4 to 6-week period at ambient room temperature (22 to 25°C) in low light. When cultures

had sporulated heavily, conidia were collected and stored at 4ºC in a desiccator.

Production of pathogen inocula for antibiosis assay

Rhizoctonia solani and *P. myriotylum* were grown on potato dextrose agar (PDA) for 3 or 4 days. *Thielaviopsis basicola* was cultured on 10% carrot juice agar (100 ml carrot juice and 20 g agar in 900 ml deionized water) and allowed to grow for 10 to 12 days at ambient room temperature (22 to 25°C) in low light.

Production of *X. aonopodis* pv. *malvacearum* for ISR assay

Xanthomonas axonopodis pv. *malvacearum* (ATCC 9924) which was originally isolated from cotton was obtained from the American Type Culture Collection, (Manassas, VA). To produce inoculum, cultures were grown for three days on nutrient broth agar. Rate of *Xanthomonas* used in the ISR assay was 1×10^9 CFU/ml for trial 1 and 1×10^{10} CFU/ml for trials 2 and 3.

Cotton cultivar

Delinted black cotton seed 'Delta Pine (DP) 436 RR', (obtained from Dr. Craig Canaday, The University of Tennessee, West Tennessee Research and Education Center, Jackson) was used in the ISR assay.

ISR assay

Cotton seed were surface-sterilized with 30% Clorox solution for 5 min. Seed were rinsed five times with sterile deionized water and air-dried in a laminar flow hood overnight. Dried seed were stored at 4ºC until use.

Seed were placed aseptically into sterile autoclaved growth pouches (Mega International, St. Paul, MN) containing paper wicks with four seed per pouch. Approximately 10 ml of sterile deionized water was added to each pouch. Germination typically occurred within 72 h.

Assays were conducted in a growth chamber and maintained at a constant 28ºC. Relative humidity was approximately 50%, prior to inoculation with *Xanthomonas*. Lights were 1,530 lumens on top and 5,100 lumens on bottom. The light inequality was rectified by alternating assay location every 24 h.

If contamination was observed in growth pouches, the pouch was discarded immediately. A minimal number, 4 to 6 pouches per assay, were discarded. Pouches also were removed if all plants in a pouch had not developed primary leaves by day 18. This was done to increase overall light exposure for the remaining seedlings. Plants were left uncovered until day 18, when inoculation of primary cotton leaves occurred.

A supplementation of sterile 1/6 Hoagland's solution was applied on day 8 of the assay then alternated every other day with sterile deionized water for about one week. Liquids were added aseptically to the corner of the paper wick in the growth pouch.

Root treatments-ISR assay

Treatments for the ISR assay included two controls of untreated seed, a chemical control with 2, 6-dichloro-isonicotinic acid (INA) and three conidial rates of *B. bassiana*. The two untreated seed controls received 200-µl 1/3 Hoagland's solution applied directly along the root surface.

Root drench treatments with Bb 11-98C spores were prepared by adding dry conidia to test tubes with 20 ml of sterile 1/3 Hoagland's solution and approximately 25 µl of Tween 20. Solution was mixed by vortex and tubes were intermittently placed on a rotary shaker (150-160 rpm) 2 to 3 h. Population density of Bb 11-98C was determined by dilution plating on SDA.

Conidial treatments were added to 0.2-ml sterile 1/3 Hoagland's solution and applied to roots 5 cm below radical emergence. For *B. bassiana* treatments, three rates of conidia (1×10^5, 10^7 and 10^9) of isolate Bb 11-98C were applied as 0.2-µl root drench treatments.

A 200-µl solution of 2,6-dichloroisonicotinic acid (INA) at 50 µg/ml in sterile deionized water was added, in two100-µl aliquots, to each individual root to ensure thorough coverage.

Thirteen days after the cotton seedlings received the root drench treatments, (18 days after seeding the pouch) a single primary leaf was challenged with *X. axonopodis* pv. *malvacearum*. All treatments except one control treatment were inoculated. Upper surface of one true leaf per cotton plant was pricked three to four times with a 26-gauge sterile disposable needle while a rubber stopper was held against the lower leaf surface. *Xanthomonas* was applied to the upper leaf surface with a cotton swab. Leaves selected

for inoculation were approximately 1.5- 2 cm wide by 2-3 cm long. None of the plants had more than one leaf large enough for inoculation on day 18.

After inoculation, growth pouches with seedlings were placed back into containers, placed in a large plastic box, and returned to the growth chamber. The outer plastic box was covered with clear plastic to increase relative humidity (RH) within assays containers. Humidity was measured daily and maintained at 85 to 90% RH for the next six days.

Measurement of disease severity index began on day 19 and was completed on day 24 after seeding. Disease severity index was recorded 1, 2, 3, 4, and 6 days after *Xanthomonas* inoculation. Disease severity index was 1 = symptomless, 2 = fewer than three lesions per leaf, 3 = more than three lesions per leaf, less than 25% leaf area affected by disease, 4 = lesions covering 25 to 50% of leaf surface area, 5 = lesions covering more than 50% of leaf surface area, 6 = one or more leaves dead. Symptoms developed within 24 h.

Statistical analysis and experimental design of the ISR assay

Treatments were replicated (5 to 12) with 3 to 4 seedlings per replicate, and the experiment was repeated to yield three complete trials. Disease severity data was analyzed with PROC MIXED of PC-SAS version 9.1. Least significant differences were determined according to Fisher's protected LSD ($P = 0.05$).

Antibiosis Assay

To prepare host-based medium, 'DP 436 RR' cotton plants were grown for 28 days in Promix Premier potting soil in a growth chamber at 25 to 26°C. Seedlings were harvested by cutting the plant at the crown. Soft stems and cotyledons were comminuted in a Waring blender, and then 2 L deionized water was added. Mixture was heated and allowed to simmer for 30 to 45 min. Using cheese cloth, supernatant was collected. Cotton agar was prepared by adding 2% agar to 200 ml of harvested cotton supernatant, and autoclaving for 30 min.

Beauveria bassiana 11-98C was grown for seven days on SDA, and then 10-mm^2 plugs of *B. bassiana* mycelia were placed on opposing sides of the cotton agar plates. For controls, two 10-mm^2 plugs of uninoculated SDA were placed on opposing sides of the plates. *Beauveria bassiana* was allowed to grow for 7 days on the cotton agar (Fig. 4-1).

After seven days, a single 3-mm diameter plug of three cotton pathogens, *P. myriotylum*, *R. solani* (which had been grown for 5 days on PDA) or *T. basicola* (which had grown for two weeks on 12% carrot agar) were placed in the center of Petri dishes, equidistant from the *B. bassiana* and SDA control plugs. There were seven replicate plates for each pathogen. Petri dishes were left without Parafilm throughout the assay to help reduce moisture within plates.

Parasitism assay

Dilute PDA (1/5) was prepared and actively growing hyphal tips of 10-mm^2 plugs were taken from the *R. solani*, *P. myriotylum* and *T. basicola* cultures. In the center of the dilute PDA plates, flame-sterilized, cooled microscope slides (25 x 75 mm) were placed

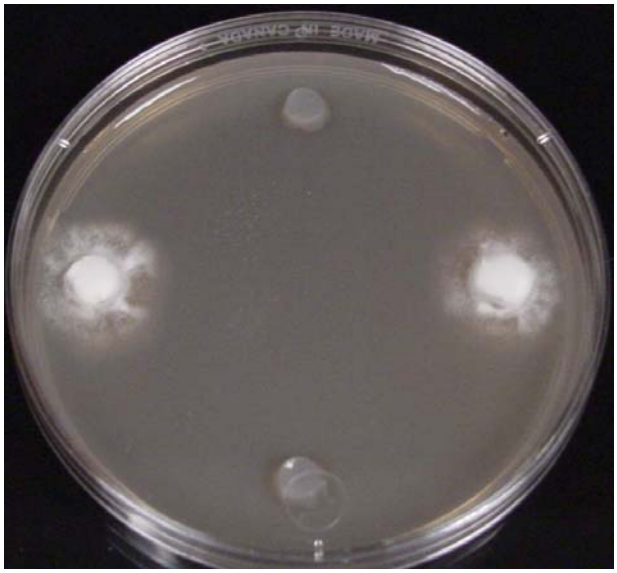


Fig. 4-1. *Beauveria bassiana* after growing on cotton agar for approximately 7 days.

on the agar. Plugs were aseptically placed using forceps onto the microscope slides. For controls, a single plug of each soilborne pathogen (*R. solani*, *P. myriotylum* and *T. basicola*) and *B. bassiana* was placed individually in the center of the microscope slide and a cover slip (24 mm x 50 mm) was lightly but securely placed on top of the plug. Controls were repeated three times for each individual fungus. For the parasitism challenge, Petri dishes contained a single soilborne pathogen and *B. bassiana*; the pathogen plug and the *B. bassiana* plug were placed 20 mm from the edge of the slide in opposite directions. The area between the two plugs was approximately 11 to 15 mm (Ownley and Windham 2004). After 3 days plates, were examined daily for hyphal growth (of *B. bassiana* or pathogen) along the glass cover slides or the cover slips toward the opposing fungus. Six replicates of *B. bassiana* and each soilborne pathogen (*R. solani*, *P. myriotylum* and *T. basicola*) were assayed. Digital photomicrographs of hyphal

growth along cover slips where taken at 100 × magnification.

Chitin and cellulose utilization assay

To determine if Bb 11-98C can hydrolyze chitin or cellulose, it was grown on media containing these polymers as the sole carbon source. A chitin-based medium was prepared, (10 g ball-milled chitin, 1 g $MgSO_4 \cdot 7H_2O$, 1 g K_2HPO_4 and 15 g agar in 1L deionized water), and 8 to 10 ml of this medium was poured directly onto a plate already containing 8 to 10 ml of solidified 2% water agar. Cellulose-based medium (12 g ball-milled cellulose, 0.5 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$ and 15 g agar in 1L deionized water) was prepared. Conidia of Bb 11-98C were suspended in a phosphate buffer solution, and 0.1 ml was pipetted onto the center of seven plates each, of the chitin-based medium and cellulose-based medium. Cultures were incubated for 14 to 28 days and examined. Substrate specific stains were used. For chitin hydrolysis, methyl blue dye (Sigma-Aldrich Co., St. Louis, MO) was mixed with deionized water at 0.01g/ml and filtered in a 250-ml filter system vacuum (Corning Inc., Corning, NY). Approximately 8 to 10 ml was applied to uninoculated control and treated plates on which Bb 11-98C had been grown earlier. To avoid a false reading caused by presence of *B. bassiana* culture growing in the medium, 8 to 10 ml of sterile deionized water was poured onto the culture and a brush was used to gently scrape the medium surface. The procedure was repeated twice to ensure removal of *B. bassiana*. Methyl blue dye was added for 25 to 30 min then removed with a pipette. The medium was rinsed twice with sterile deionized water. For the cellulose medium, a sterile Congo Red (Fisher Scientific, Fairlawn, NJ) solution was applied (0.1 g dye/100 ml deionized water) for 15 min to

uninoculated control and treated plates. Exposure was followed by a 5 min soak with sterile NaCl solution (5.84 g/100 ml deionized water). Petri dishes were left without Parafilm throughout the assay to reduce moisture within the plates.

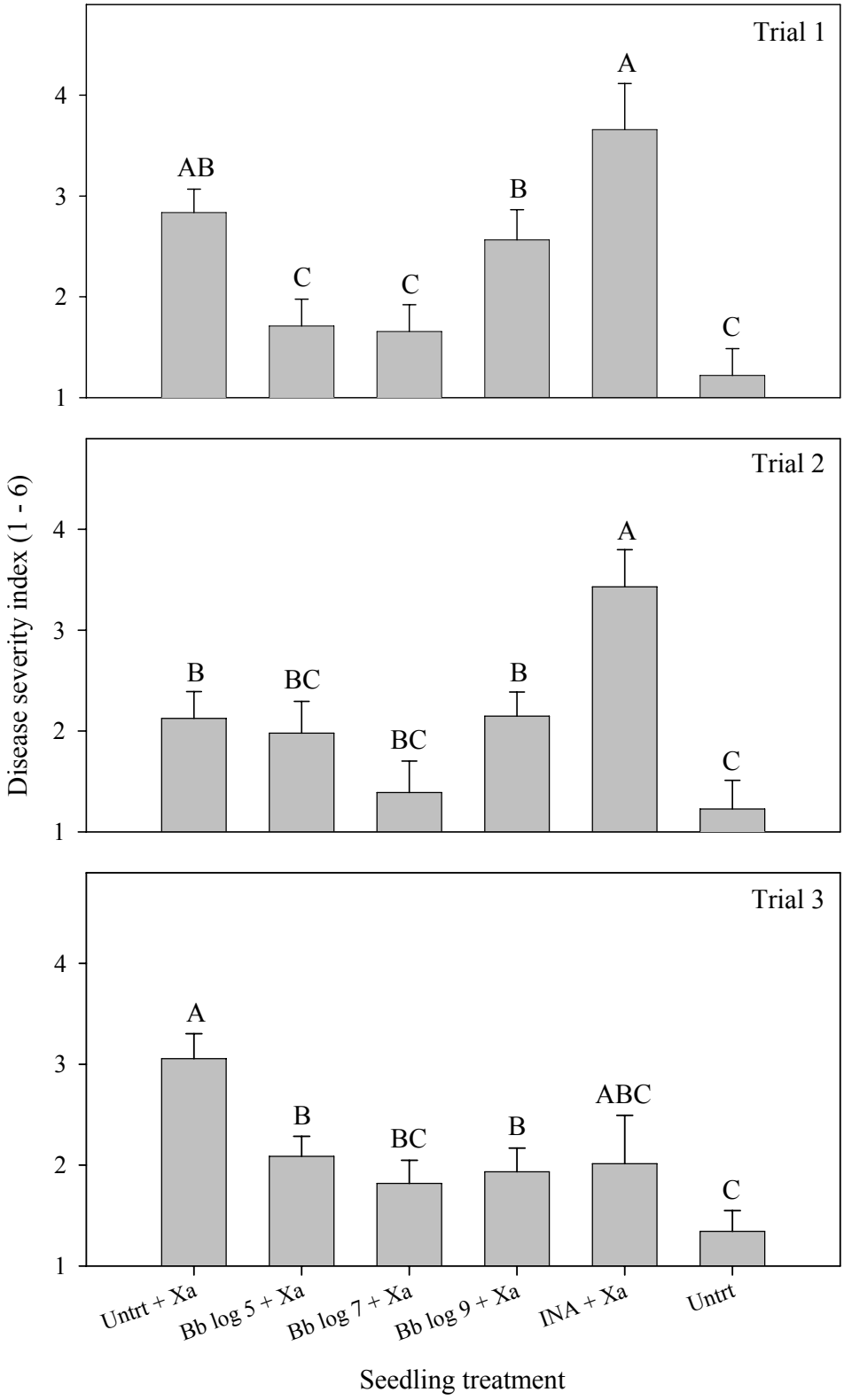
Results

ISR Assay

Effects of induced systemic resistance reaction by cotton seedlings, caused by *B. bassiana* 11-98C applied as a root drench, were observed two weeks after initial challenge by bacterial blight pathogen, *X. axonopodis* pv. *malvacearum*. Symptoms of *Xanthomonas* infection were noticeable on leaves within 48 h. Seedling treatment was significant for disease severity in all three assays. Six days after pathogen challenge, the effect of seedling treatment was significant ($F = 8.34$; df = 5,87; $P = 0.0001$) for Trial 1, ($F = 5.61$; df = 5,58; $P = 0.0003$) for Trial 2, and ($F = 6.66$; df = 5,70; $P = 0.0001$) for Trial 3.

For trial 1, among treatments challenged with *Xanthomonas*, cotton seedlings treated with *B. bassiana* conidia at 1×10^5 and 1×10^7 CFU/ml had lower disease severity than all other treatments (Fig. 4-2). The 1×10^5 and 1×10^7 CFU/ml Bb 11-98C treatments had disease severity indices as low as the untreated control that was not challenged with *Xanthomonas*. For Trial 2, cotton seedlings treated with *B. bassiana* conidia at 1×10^5 and 1×10^7 CFU/ml were intermediate in disease between the untreated control inoculated with *Xanthomonas* and the untreated control without pathogen challenge. In Trial 3, all Bb 11-98C treatments had lower disease than the

Fig. 4-2. Evidence of induced systemic resistance in cotton caused by *Beauveria bassiana* (Bb) 11-98C applied as a root drench 13 days prior to pathogen challenge. Disease severity index (1-6) was measured at six days after *Xanthomonas axonopodis* pv. *malvacearum* (Xa) inoculation of cotton leaves. *Beauveria bassiana* 11-98C was applied to cotton root systems at three rates; (log 5 = 1×10^5, log 7 = 1×10^7 and log 9 = 1×10^9 CFU/ml). Controls were an untreated control with *Xanthomonas* challenge (Untrt + Xa), an untreated control with no pathogen challenge (Untrt), and an SAR chemical control, 2, 6-dichloro-isonicotinic acid (INA) with pathogen challenge (INA + Xa). Effects of seedling treatment were significant for Trial 1 ($P = 0.0001$), 2 ($P = 0.0003$) and 3 ($P = 0.0001$). For each trial, bars with the same letter are not different according to an F-protected LSD ($P = 0.05$). Each bar represents least square means ± SE.


Trial 1
Trial 2
Trial 3
Disease severity index (1 - 6)
1
2
3
4
AB
C
C
B
A
C
B
BC
BC
B
A
C
A
B
BC
B
ABC
C
Untrt + Xa
Bb log 5 + Xa
Bb log 7 + Xa
Bb log 9 + Xa
INA + Xa
Untrt
Seedling treatment

untreated control inoculated with *Xanthomonas*. The rate of 1×10^7 CFU/ml Bb 11-98C had the least disease and was not different then the untreated control without *Xanthomonas*. In all three trials, 1×10^7 CFU/ml Bb 11-98C had disease severity indices as low as the untreated control without *Xanthomonas*.

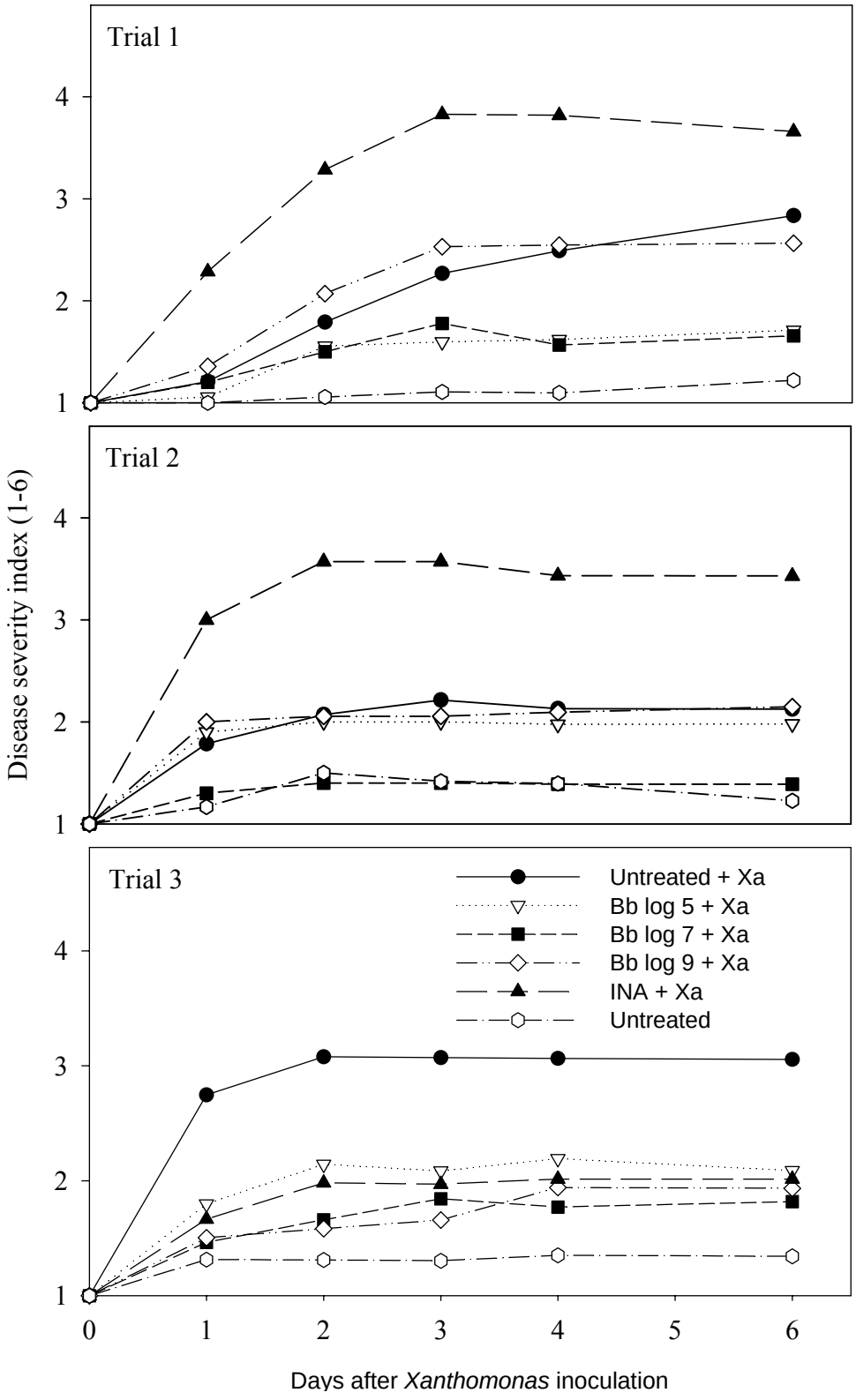
Over the six days of the assay, the disease severity indices were consistently lower for Bb 11-98C treatments based on disease progress curves (Fig. 4-3). In contrast, INA treatment was inconsistent between trials with greater variability between replicates.

Antibiosis assay

Beauveria bassiana, P. myriotylum, *R. solani* and *T. basicola* grew well on the cotton agar. After seven days the colony diameter of *B. bassiana* grew approximately 1 to 1.25 cm from the edge of the initial plug. Initially all three pathogens exhibited faster growth in culture than *B. bassiana* (Figs. 4-4, 4-5, and 4-6), with the most rapid growth by *P. myriotylum* and *R. solani*.

Thielaviopsis basicola was the slowest growing pathogen. By day 5 of the antibiosis assay, *T. basicola* exhibited an unusual colony growth pattern that indicated it was hindered by *B. bassiana* but not inhibited by antibiosis activity, since no zone of inhibition was observed. By 14 days, *T. basicola* was almost completely eliminated in some plates. *Thielaviopsis basicola* was either out-competed by *B. bassiana* or another mechanism of action was employed. Growth of *T. basicola* was much slower (Fig. 4-4 A-C) than the other pathogens, and growth on cotton agar was greatly restricted (Fig. 4-4 B-C) by *B. bassiana* 11-98C. After three weeks, growth of *T. basicola* appeared to have halted.

Fig. 4-3. Effect of *Beauveria bassiana* (Bb) 11-98C applied as a root drench to cotton seedlings 13 days prior to pathogen challenge on disease progress caused by *Xanthomonas axonopodis* pv. *malvacearum.* Disease severity index (1-6) was measured at 1, 2, 3, 4 and 6 days after pathogen challenge inoculation on cotton leaves. *Beauveria bassiana* (Bb) 11-98C was applied to cotton root systems at three rates; (log 5 = 1×10^5, log 7 = 1×10^7 and log 9 = 1×10^9 CFU/ml). Controls were an untreated control with *Xanthomonas* challenge (Untreated + Xa), an untreated control with no pathogen challenge (Untreated), and an SAR chemical control, 2, 6-dichloro-isonicotinic acid (INA) with pathogen challenge (INA + Xa). Symbols represent the mean of 5 to 12 replicates.


Trial 1
Trial 2
Trial 3
Disease severity index (1-6)
Days after *Xanthomonas* inoculation
Untreated + Xa
Bb log 5 + Xa
Bb log 7 + Xa
Bb log 9 + Xa
INA + Xa
Untreated
1
2
3
4
0
1
2
3
4
5
6

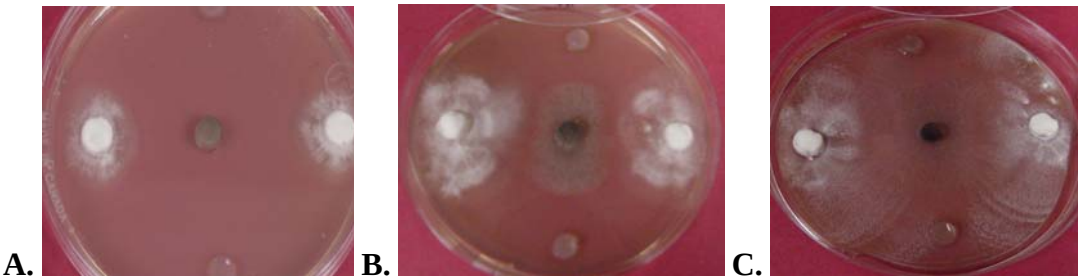


Fig. 4-4. Antibiosis assay with *Beauveria bassiana* (Bb) 11-98C and *Thielaviopsis basicola*. A. Day 1: inoculation of a 3-mm-diameter plug of *T. basicola*. B. Day 10: Bb 11-98C limited growth of *T. basicola* and out-competed the pathogen for new space on cotton-based medium. C. Day 28: Bb 11-98C maintained its space and contained the spread of *T. basicola* into the medium. Bb 11-98C also sporulated.

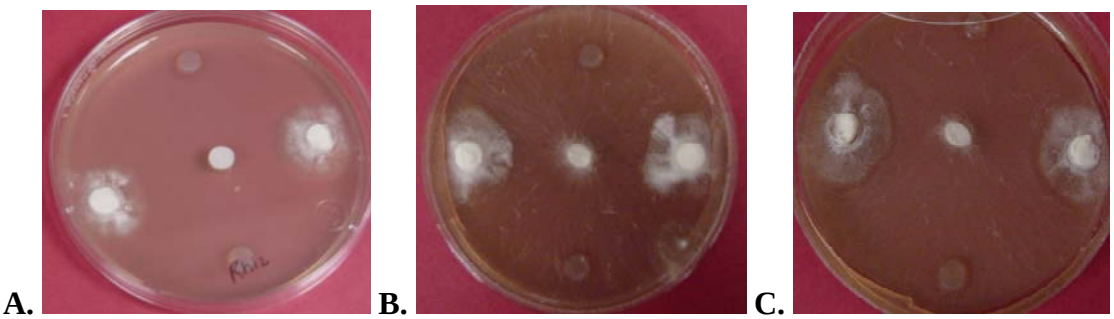


Fig. 4-5. Antibiosis assay with *Beauveria bassiana* (Bb) 11-98C and *Rhizoctonia solani*. A. Day 1 after a 3-mm-diameter plug of *R. solani* was introduced to cotton based medium. B. Day 4: Bb 11-98C was out- competed for new space on the cotton based medium surface. C. Day 21: Bb 11-98C maintained its initial colony diameter.

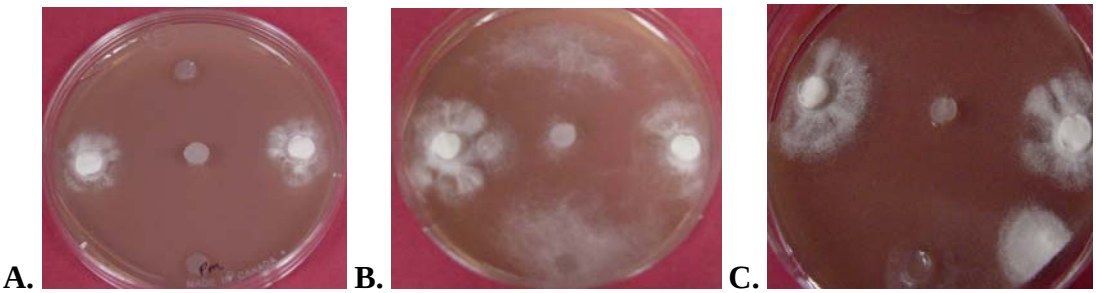


Fig. 4-6. Antibiosis assay with *Beauveria bassiana* (Bb) 11-98C and *Pythium myriotylum*. A. Day 1 after a 3-mm-diameter plug of *P. myriotylum* was introduced to cotton-based medium. B. Day 3: Bb 11-98C was out- competed for new space on the cotton-based medium surface. C. Day 21: Bb 11-98C maintained its initial colony diameter.

Rhizoctonia solani took approximately four days to grow to the edge of the plate and past SDA control plugs (Fig. 4-5 B). *Beauveria bassiana* colonies continued to maintain their initial colony diameter even though by day 5 aerial mycelia of *R. solani* could be seen growing around the *B. bassiana* colonies. By day 7, using a dissecting microscope (Steromaster, Fisher Scientific, Pittsburgh, PA) hyphae of *R. solani* were observed extending into the *B. bassiana* colony. Very little activity was observed by *B. bassiana* against *R. solani* after two weeks; however *B. bassiana* continued to maintain its initial colony diameter and was not out-competed: hyphae continued to extend outward into the *R. solani* colony. Three weeks after initial inoculation with *R. solani*, *B. bassiana* continued to maintain its initial colony diameter (Fig. 4-5 C).

Within two to three days, *P. myriotylum* grew to the edge of agar plates past the location of SDA control plugs (Fig. 4-6 B). *Beauveria bassiana* was out-competed for new space on the cotton-based medium surface; however *B. bassiana* continued to maintain its original colony boundaries. After 12 days, *B. bassiana* had begun to grow into the *P. myriotylum* colony. Three weeks after the *P. myriotylum* plug was introduced, *B. bassiana* continued to maintain its initial colony diameter. *Beauveria bassiana* 11-98C sporulated and produced hyphae on areas occupied by *P. myriotylum* (Fig. 4-6 C).

Parasitism assay

Within 15 days of initiation of the parasitism assay with *B. bassiana* and *P. myriotylum,* Bb 11-98C was observed coiling around *P. myriotylum* hyphae (Fig. 4-7). No coiling was observed around *R. solani* hyphae. No hyphae were observed around *T. basicola*, which produced only endoconidia (Fig. 4-8). Both, *P. myriotylum* and *R. solani*

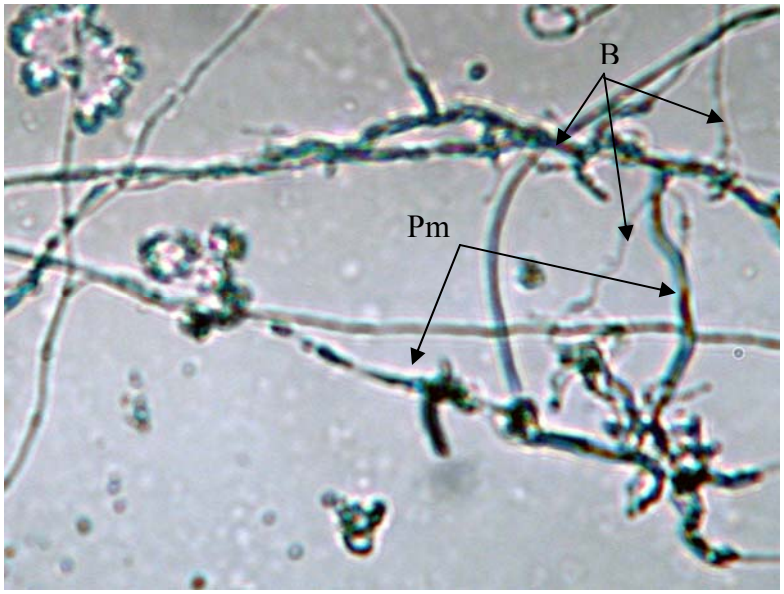


Fig. 4-7. Small hyphae of *Beauveria bassiana* isolate 11-98C (B) coiling around larger hyphae of *Pythium myriotylum* (Pm). Magnification = 100 ×.

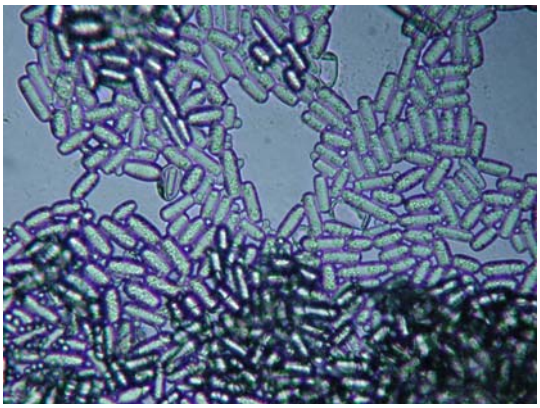


Fig. 4-8. Endoconidia of *Thielaviopsis basicola*. Endoconidia produced by *T. basicola* plug after approximately two weeks on a glass cover slip.

grew out from the initial agar plug into dilute PDA within five days. Growth of hyphae was slower on the underside of the glass cover slip. Hyphal contact between *B. bassiana* and *P. myriotylum* or *R. solani* was observed after approximately 9 to 11 days.

In the assay with *P. myriotylum* and *B. bassiana*, *P. myriotylum* grew at a faster rate than *B. bassiana*; however *B. bassiana* continued to grow along the cover slip even when in contact with *P. myriotylum*. Based on digital photomicrographs of *P. myriotylum* and *B. bassiana* hyphae, *B. bassiana* hyphae coiled around the larger *P. myriotylum* hyphae (Fig. 4-7).

In assays with *R. solani* and *B. bassiana*, similar to the *Pythium* trials, *B. bassiana* grew more slowly. By day 18, hyphae of *B. bassiana* was out-competed by *R. solani*, which covered the underside of the glass cover slip surface. In the area where the two hyphae did come into contact, no coiling was observed.

No coiling was observed by *B. bassiana* around *T. basicola* since *T. basicola* did not produce any hyphae which would extend along the glass cover slip surface for observation. *Thielaviopsis basicola* did produce copious amounts of endoconidia (Fig. 4-8) along the under surface of the glass cover slip and chlamydospores also were observed. Appearance of *T. basicola* was similar in controls without *B. bassiana*.

Chitin hydrolysis assay

Visual determination of chitin hydrolysis was apparent, even without the use of the methyl blue dye; however, the stain produced more visible results. Plates inoculated with *B. bassiana* conidia produced a clear zone around growing the *B. bassiana* colony in the chitin-based medium. Clearing was apparent after nine days and continued to expand

over time (Fig. 4-9 A). When methyl blue dye was applied to chitin-based agar, the outline where *B. bassiana* had not extended hyphae and hydrolyzed chitin was apparent (Fig. 4-9 B). Chitin-based medium control plates did not show any clearing during the same time period (Fig. 4-9 C). Chitin based medium control plates treated with methyl blue did not produce any differential outline, but the dye colored all parts of the plate equally (Fig. 4-9 D). This indirect assay demonstrated that *B. bassiana* isolate 11-98C has the capacity to utilize chitin (Fig. 4-9).

Cellulose hydrolysis assay

No differences were observed between cellulose medium inoculated with *B. bassiana* and uninoculated control medium. *Beauveria bassiana* sporulated on medium, but mycelia growth was limited and the cellulose-based medium was not utilized after treatment with Congo Red dye, which was used to aid in visual detection.

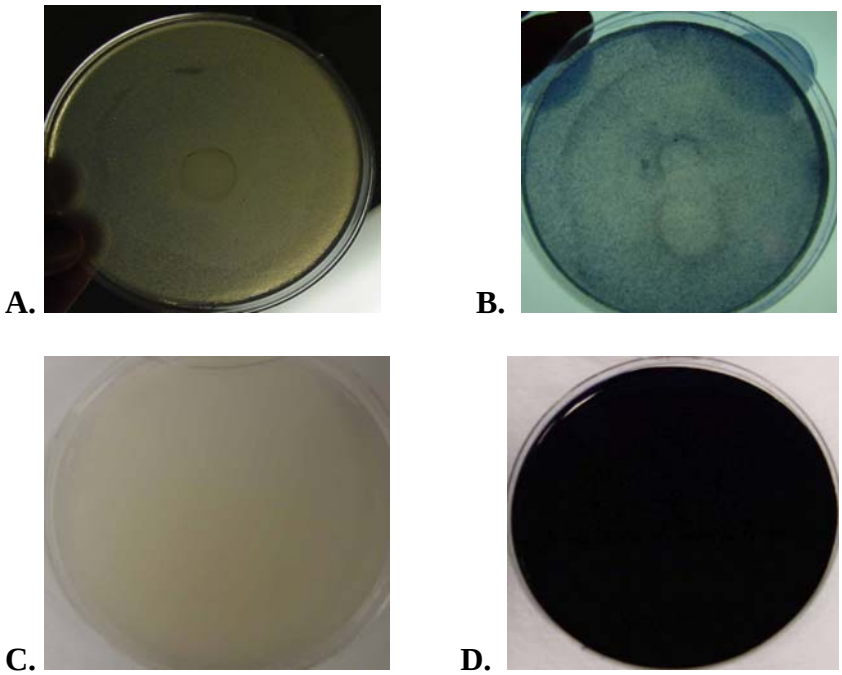


Fig. 4-9. Chitin hydrolysis assay. A. Chitin-based medium on which *Beauveria bassiana* isolate 11-98C spores in a phosphate buffer solution were added directly to plates and allowed to grow for two weeks at 22°C. B. Chitin-based medium plate, to which conidial spores had been added and allowed to grow for 18 days, stained with methyl blue for 15 to 20 min. C. Uninoculated chitin-based medium control on which no *B. bassiana* spores were added. D. Same plate as C, to which approximately 8 ml methyl blue stain was added and allowed to stand for 15 to 20 min.

Discussion

Techniques and treatments that stimulate plant host defense responses can be powerful tools for growers since neither synthetic elicitors nor biocontrols in general exhibit any direct antimicrobial activity. Unlike traditional pesticides biocontrols provide a way to control disease without asserting direct selective pressure on the pathogen population. This is important because even transgenic cotton cultivars exert direct selective pressure on the organisms they are attempting to control (Vallad and Goodman, 2004).

In the ISR assays, seedlings treated with *B. bassiana* are less susceptible to *X. axonopodis* pv. *malvacearum* than untreated plants inoculated with the pathogen. The SAR inducer, INA, slowed cotton development. Seedlings that received this treatment were healthy, but a large number of the seedlings had delayed development of their primary leaves.

A consistent theme in several field experiments using BTH or INA as SAR inducers was the reduction of crop yield (Louws et al. 2001, Vallad and Goodman 2004). Often these reductions were statistically insignificant. Tomato seedlings treated with BTH were smaller than nontreated plants in greenhouse experiments (Vallad and Goodman 2004). Growth of pepper plants was also greatly influenced by BTH (Romero et al. 2001). Decreased effects on plant growth were apparent across several pepper cultivars when compared with plants treated with copper hydroxide, but only when plants were infected with *Xanthomonas campestris* pv. *vesicatoria* Doidge Dye (causal agent of bacterial spot). In experiments that excluded *X. campestris* pv. *vesicatoria*, there were no significant yield differences between BTH-treated and copper hydroxide-treated plants

(Romero et al. 2001). Alternaria leaf spot caused by *Alternaria macrospora* Zimm., bacterial blight (caused by *Xanthomonas campestris* pv. *malvacearum*) and Verticillium wilt (caused by *Verticillium dahliae* Kleb.) of cotton were controlled in a series of field experiments with the use of BTH and INA (Colson-Hanks and Deverall 2000). Single applications of either BTH or INA reduced symptoms of both Alternaria leaf spot and bacterial blight. Multiple applications of BTH or a single application of INA reduced severity of Verticillium wilt of cotton (Colson-Hanks et al. 2000). Plants challenged with limited insect damage benefit from application of SAR-inducing chemicals. However in their absence, induced plants have been found to be less reproductively fit in terms of flower development and pollen production (Agrawal 1999). Heil et al. (2000) observed effects on vegetative growth and seed production of wheat plants induced with BTH in the absence of disease pressure, regardless of growing conditions or fertilization regiment. However, they found that BTH most impeded growth during production of lateral shoots and under nitrogen-limiting conditions, demonstrating the importance of plant growth stage and nutritional status when assessing physiological costs of induced resistance (Heil et al. 2000).

Many plant disease biocontrol agents are not broad spectrum but instead are more specific in nature, capable of providing protection for a limited number of hosts against a limited number of pathogens. Biocontrol with induced systemic resistance in *Trichoderma harzianum* T39 against the necrotrophic fungus *Botrytis cinerea* Pers.:Fr. has been demonstrated. Resistance was conferred by *T. harzianum* T39, as a soil treatment applied 7 days before challenge inoculation with a foliar application of *B. cinerea. Trichoderma harzianum* T39 reduced stem infections in pepper and reduced leaf

infections in tomato, lettuce, pepper, tobacco and bean (DeMeyer et al. 1998). It is not known whether ISR mediated by *B. bassiana* functions as a broad-spectrum control agent since it has not been tested with other plant species; however, it is possible that seed treatment will provide some protection in tomato because it has provided protection against damping off in tomato caused by *R. solani* (Bishop 1999, Ownley et al 2005, Seth 2001) and *P. myriotylum* (Clark 2006).

The ISR response in this assay was extended compared to work by Han et al. (2000), in which the response was only 7 to 8 days between ISR induction and pathogen inoculation. In this study, response was extended to 13 days as this amount of time was needed for cotton seedlings to produce primary leaves. This resulted in a large portion of INA plants being excluded from data collection because primary leaves had not yet formed or were too small to use. It is probable that INA concentration was too high for cotton seedlings. A reduced rate may help by not delaying plant development or another chemical SAR inducer might prove more useful. In all trials, Bb 11-98C at conidial treatment rate of 10^7 had less disease than untreated control plants inoculated with *Xanthomonas* and the INA-treated plants.

Inhibitory effects of *B. bassiana* against *R. solani* were examined in an *in vitro* study with 22 different *B. bassiana* isolates, on PDA. Three isolates were inhibitory against *R. solani*, indicating there are differences among *B. bassiana* isolates in their ability to inhibit growth of plant pathogens *in vitro* (Lee et al. 1999). In another study, *B. bassiana* isolates had inhibitory activity against mycelial growth of the plant parasitic fungi *Fusarium oxysporum* Schltdl.:Fr., *Armillaria mellea* (Vahl:Fr.) P. Kumm. and *Rosellinia necatrix* Prill. (Reisenzein and Tiefenbrunner 1997). Mycelial growth and

spore germination of the phytopathogenic fungi *Botrytis cinerea* and *F. oxysporum* were inhibited by the culture filtrate of *B. bassiana* (Bark et al. 1996); inhibition efficiency varied depending on culture medium, which was the highest on PDA for *B. cinerea* and on tryptic soy agar for *F. oxysporum*.

Although *B. bassiana* produces numerous toxins, in the present antibiosis assay, evidence of *in vitro* antibiosis was not apparent on cotton-based medium. The range of toxins produced by *B. bassiana* 11-98C has not been determined. It may be that Bb 11-98C simply does not produce a toxin that inhibits growth of *R. solani*, *P. myriotylum* and *T. basicola*. Many of the *Beauveria*-related toxins have only recently been elucidated, or are difficult to isolate and few standards are available. It is known that Bb 11-98 does produce beauvericin and that beauvericin production occurs during colonization of tomato plants (Powell, 2005). *Beauveria bassiana* 11-98 and 11-98C also produce oosporein, as evident by the deep red color of liquid culture after a few days (Ownley, personal communication). Another possibility is that, because Bb 11-98C has been host adapted by passage through cotton, selection may have occurred against production of certain toxins. A possible loss of toxins may make it easier for *B. bassiana* 11-98C to colonize a cotton host.

Possible parasitism of *Pythium* species by *Beauveria bassiana* was observed by Vesely and Koubova (1994) when *B. bassiana* was antagonistic to *Pythium ultimum* Trow and *P. debaryanum* Auct. non R. Hesse. *Beauveria bassiana* induced lysis of the mycelium while actively growing on or beneath the mycelia. In the present study, hyphae of Bb 11-98C coiled around the larger hyphae of *P. myriotylum*. This coiling behavior was not observed with *R. solani* and was not applicable to *T. basicola* as this fungus did

not produce mycelia on the glass surface as a substrate on which to produce mycelia. It is possible that *B. bassiana* is capable of coiling behavior with *R. solani* and *T. basicola*, however this assay was limited in visualization and did not provide a suitable surface for *T. basicola* to develop.

Utilization of culture substrates is an indirect though valid method to determine if *B. bassiana* is capable of utilizing soilborne pathogens as a potential food source. In the hydrolysis assay with chitin-based medium, Bb 11-98C hydrolyzed the chitin. Although utilization of chitin is not a novel concept for *B. bassiana*, it is important to note that Bb 11-98C was host-adapted to cotton and this assay confirms that the isolate has maintained this capability. For the cellulose-based medium, *B. bassiana* 11-98C did not hydrolyze the medium after three weeks of incubation. Mycelial production, if present, was very sparse and difficult to detect. No clearing of the cellulose medium was noted. These results seem to contradict the parasitism assay where Bb11-98C coiled around *P. myriotylum* hyphae. *Beauveria bassiana* 11-98C may not produce all the enzymes needed to hydrolyze a solid cellulose-based medium. Cellulose is composed of long chains of glucose residues linked by β-1,4-glycosidic bonds and several enzymes are involved in hydrolysis. Enzymes such as exocellobiohydrolase, which is capable of exo-type activity, cellulase, endoglucanase or carboxymethylcellulase, which randomly split β-1,4-glycosidic bonds to form cellobiose, cellotriose, or cellotetraose can be used by organisms to hydrolyze cellulose. If Bb 11-98C was missing an enzyme required for some step of cellulose hydrolysis, there would be no clearing of the cellulose-based medium.

Beauveria bassiana 11-98C is capable of inducing an ISR response in cotton as

demonstrated with the *X. axonopodis* challenge, it also may be able to out-compete or parasitize *T. basicola*, as demonstrated in the antibiosis assay, even though parasitism was not conclusive due to the failure of *T. basicola* to grow along the glass surface in the parasitism assay. *Beauveria bassiana* 11-98C was able to utilize the chitin-based medium which would suggest that it could function as a hyperparasite against *T. basicola* and *R. solani*. Furthermore, *B. bassiana* 11-98C did exhibit coiling around *P. myriotylum*, but was unable to utilize the cellulose-based medium possibly due to the lack of all necessary enzymes needed for hydrolysis of this medium.

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Part 5. Potential of *Beauveria bassiana* as a biocontrol for plant pathogens

Research Possibilities

Although the research in this document supports the use of *Beauveria bassiana* as a biocontrol agent against plant pathogens, many questions remain unanswered. Many avenues have yet to be explored, and opportunities to test *B. bassiana* in other systems still wait.

Antibiosis

The lack of a clear zone of inhibition in the antibiosis assays on host-based medium with three soilborne plant pathogens should not be taken as conclusive evidence that Bb 11-98C does not produce antifungal compounds in the plant. Mobility of metabolites may have been reduced in the solid medium and therefore did not inhibit pathogen development. Sterile culture filtrates from liquid culture added to solid media should be evaluated to determine if pathogen growth is inhibited. This would address questions about the ability of metabolites to diffuse through the media.

The violet isolate produced on the host-based medium may prove to be an important experimental tool in elucidating antibiosis. The violet isolate, which was changed morphologically after colonizing cotton, may have also changed physiologically in response to the host plant environment that would enable *B. bassiana* to inhibit the pathogen.

Beauveria bassiana does not produce all of its secondary metabolites constitutively, for example, oosporein is typically produced after colonization of an insect has occurred. It is possible that *B. bassiana* responds to environmental triggers before producing some secondary metabolites. A reddish tinge was observed around two Bb 11-

98C plugs late in the antibiosis assay with *Thielaviopsis basicola*. Pigment production by Bb 11-98C may have been stimulated by cotton components within the agar or by mycelial contact with *T. basicola*.

Regardless HPLC assays to determine if mycotoxins are produced within the host plants is important, particularly with plant parts intended for consumption. Beauvericin was determined to be present in the leaves of tomato (Powell 2005), but no determination was made for the fruits in part due to the high number of interfering compounds. Isolation of beauvericin from cotton may prove more difficult as only seeds and seed oils are utilized in food production. Growing cotton plants and determining if *B. bassiana* or any secondary compounds could be extracted from ground seed would be a time consuming process but a conclusive way to determine if *B. bassiana* produces these compounds during the plant growth cycle. A greenhouse project where plants are systematically sacrificed throughout the lifecycle should yield this information.

Parasitism and competition assays using *Beauveria bassiana* against plant pathogens

A possible explanation of the microscopic evaluations of the coiling action by Bb11-98C around the *P. myriotylum* is that Bb 11-98C parasitizes *Pythium*. Using a similar methodology to the assay with cellulose medium, information about the ability of *B. bassiana* to hydrolyze a solid based medium in which β-1-3 and 1-6 glucans are present could be generated. It is possible that *B. bassiana* has a sequence to enzymatic production and hydrolysis of the cell walls of *Pythium* spp. Evidence to support that there can be sequence to enzyme production was shown with *Trichoderma harzianum*. *Trichoderma* was found to produce first glucanases, then cellulases (Benhamou and Chet

1997). To simplistically determine if there is similar sequential production of enzymes produced by Bb 11-98C, a series of culture media would be prepared. Cultures would be grown on media augmented with ß-1-3 glucans, β-1-6 glucans or cellulose. Hydrolytic activity of Bb 11-98C in these cultures would be monitored. After Bb 11-98C is grown on media containing the glucans, a section of the agar containing growing hyphae would be transferred to a plate containing a new compound, the other glucans or cellulose. Using this method, it may be possible to determine a sequence for enzyme production. In addition, SEM work to examine hyphal contact between *B. bassiana* and *Pythium* or other plant pathogens is needed.

Storage and Production

If Bb 11-98C is to be a viable commercial biological control agent for plant pathogens, then the viability of conidia coated on seed must be determined over time. Testing could be done with simple dilution plating techniques. Based on results from the germination assay, one could conclude that *B. bassiana* may be capable of producing secondary compounds which could reduce seed germination. It is possible that metabolite production, even at low levels, could negatively affect the viability of seeds. A determination of the viability of seed coated with conidia (as well as different conidial rates) is needed. Simple seed viability trials over time would answer these questions. Both questions need to be considered as seed coating with *B. bassiana* is essential to control of soilborne pathogens.

Furthermore, quality control of conidial coating technique needs to be examined. A more efficient, consistent method of seed coating should be developed since the

method currently used is labor intensive and time consuming. Quesada-Moraga et al. (2006) sprayed conidia in a rotating drum; this application has the potential to achieve more even distribution of conidia.

Endophytic action and dual-control possibility

The capability of *B bassiana* to act as an endophyte within plants can be systematically examined now that a protocol has been established. *Beauveria bassiana* is known to colonize tomato (Leckie 2002), corn (Bing and Lewis 1991), snap bean (Ownley, personal communication), jimson weed (Jones 1994), cocoa (Posada and Vega 2005), opium poppy (Quesada-Moraga et al. 2006), and cotton (Griffin 2006); thus, it is likely to be capable of colonizing many other agronomically important crop plants. Bb 11-98 was isolated using traditional plating techniques from leaf tips of tomato plants of varying ages (Ownley, personal communication). Also, Posada and Vega (2005) isolated *B. bassiana* from all parts of 2-month-old cocoa plants using the same technique. Real-time PCR would give information on the location of accumulation of *B. bassiana* within selected host plants.

The route *B. bassiana* travels as it colonizes a plant is also currently unknown and may not be the same for every plant species that *Beauveria* is able to colonize. Transport of *B. bassiana* may occur through xylem vessels of the plant (Wagner and Lewis 2000). Additional light microscopy and transmission electron microscopy may help to gain more information to answer these questions.

The ability of *B. bassian*a to act as a dual biocontrol has potential in crops on which products like BotaniGard (Emerald BioAgriculture Corp. Butte, MT) are already

in use. Bing and Lewis (1991) showed endophytic growth by *B. bassiana* was possible using foliar spray application. However a colonized seed coat will allow for endophytic growth during seeding and plant development and has the advantage that the plant is colonized prior to an attack. This is particularly important since it was established in this study that Bb 11-98C can induce systemic resistance in cotton seedlings. Cooperation would involve sample testing for colonization in plants and collection of insect pest to check for entomopathogenic activity by *B. bassiana* over time. Information gathered from a study such as this would be especially useful if *B. bassiana* efficiency could be tested in soils which harbor plant pathogens. This system may not be viable for cotton as seed produced for growers is almost always treated with pesticides.

Soils and nutrients

Disease assays with cotton demonstrate that differences in soil characteristics can affect efficacy of *B. bassiana* as a biocontrol. Nutritional content varies among soils and often within a field. Comparison of *B. bassiana* in different soils should be conducted. In addition, analysis of the impact of micronutrients on the ability of *B. bassiana* to grow (and potentially colonize crop plants) could be correlated with its ability to provide protection against different pathogens. Fungal growth capacity on (host-based) media in which varying amounts of micronutrient supplement(s) are added should be determined. In addition to radial growth measurements and colony morphology, production of compounds by the fungus in response to the micronutrients should be examined. In order to use this information, secondary metabolite production against test pathogens would be necessary. This would not necessarily relate to biocontrol efficiency, but it would give a

working database of information from which to make comparisons later.

Induced systemic response assays

Continued examination of the ability of *B. bassiana* to induce systemic resistance in other plants is needed. Other plant pathogens should also be evaluated. It has been speculated that the ISR response in plants is most effective against bacterial pathogens however, examination of foliar fungal pathogens is needed. Other than pathogen protection, physiological changes in the plant in response to endophytic growth of *B. bassiana* are unknown. In cotton, for example production of known defense chemicals such as terpenoids, gossypol and hemigossypol, in response to *B. bassiana* colonization should be monitored.

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Vita

Mary Ruth Griffin was born on August 24, 1968, and is the daughter of Robert and Patricia Brewer. She grew up in Rogersville, Tennessee, and attended Cherokee Comprehensive High School, graduating in 1986. While attending high school, Mary Ruth earned extra money working as a reporter for her hometown newspaper, "The Rogersville Review". She later became a student editor for The University of Tennessee's newspaper, "The Daily Beacon". As an undergraduate she was a student member of The Society of Professional Journalists and received a reward of accommodation from the University of Tennessee's athletics facility for many feature articles she wrote. While taking a year off from school she discovered a talent for teaching after worked as a substitute teacher.

After returning to college, Mary Ruth worked in several botanical labs and greenhouses as part of the university's work study program. In 1992, Mary Ruth completed her undergraduate degree at the University of Tennessee, with a degree in Liberal Arts with a major in Botany and a minor in Secondary Education. Upon graduation, Mary Ruth worked as a science teacher in her hometown at Cherokee High School. She then worked at Harrison Chilhowee's King's Academy in Seymour, TN for two years. While there she completed one year of theological studies through the school's adult ministry school as well as a course on teaching English as a second language.

Mary Ruth went on to take a science teaching position at Sevier County High School in Sevierville, Tennessee. Her teaching experiences included Physical Science, Ecology, Earth Science, all levels of Biology and Foundational Mathematics. While at Sevier County High School she served as the dance team sponsor, worked on several committees including SACS and helped facilitate recognition of SCHS as Tennessee's only high school having arboretum status. During this time she completed her Master of Arts degree in Secondary Education from UT in 1998.

Mary Ruth also attended the University of Tennessee for four years as she completed a Ph.D. in Plant Soils and Insects with a concentration in Plant Pathology. Her major professor is Dr. Bonnie Ownley. Mary Ruth currently resides in Dandridge, Tennessee along with her nine year old son Daniel Austin.