ABSTRACT

HANSEL, JEANA CLAIRE. Evaluation of an Isolate of *Bacillus subtilis* for Biocontrol of Phytophthora Blight of Pepper. (Under the direction of Dr. Jean B. Ristaino).

Bacillus subtilis is a gram-positive, endospore-forming rhizobacterium that has been used for biological control of plant pathogens. *Bacillus subtilis* has been a successful biocontrol agent (BCA) due to its use of multiple, often generalistic mechanisms of antagonism, its ability to associate with many plant species, and its ability to thrive in a wide range of environments. Biocontrol has become a topic of interest due both to a growing public desire for organic and sustainable produce, and to the increasing numbers of plant pathogens developing resistance to traditional chemical controls. One such plant pathogen is the soilborne oomycete *Phytophthora capsici*, which is the causal agent of Phytophthora blight and cucurbits. *Phytophthora capsici* has developed resistance to the primary chemical control, mefenoxam, and research into alternate control methods is necessary. This project evaluated an isolate of *B. subtilis* for biocontrol efficacy on Phytophthora blight of bell pepper in greenhouse and in field conditions in North Carolina. In the greenhouse, *B. subtilis* applied at 30 lb A^{-1} reduced disease caused by *P. capsici* compared to an untreated control. Additionally, *B. subtilis* was effective at reducing Phytophthora blight after only a single treatment application, and reduced disease when tested in four different soil types. In the field, three applications of *B. subtilis* reduced Phytophthora blight compared to an untreated control. We conclude that this isolate of *B. subtilis* is effective and consistent in managing Phytophthora blight compared to an untreated control in greenhouse and field settings and may be a good control option for organic vegetable growers and home gardeners.

Evaluation of an Isolate of *Bacillus subtilis* for Biocontrol of Phytophthora Blight of Pepper

by
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DEDICATION

This work is dedicated to my own microbiome, the 39 trillion bacterial cells in my body. I couldn't have done it without you.

BIOGRAPHY

Jeana Claire Hansel was born on May 3, 1996 in Atlanta, Georgia, to Kathryn and Joel Hansel. She developed an interest in plant pathology through a combination of her admiration of her cardiologist grandfather and an aversion to blood, eventually deciding to become a "plant doctor", as plants are, largely, without blood. She cultivated this interest by undertaking an internship under plant pathology professor Dr. Phil Brannen at the University of Georgia, where she traveled throughout the state to collect data on diseases of fruit crops. She graduated from the University of Georgia in May 2017 with a Bachelor of Science in Horticulture and a certificate in Integrated Pest Management. She moved to Raleigh in August 2017 in pursuit of a Master of Science in Plant Pathology at North Carolina State University, under the guidance of Dr. Jean Ristaino. In September 2019, she will be moving to Arlington, Virginia to begin her career as a Plant Pathologist in the Office of Pesticide Programs at the US Environmental Protection Agency.

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CHAPTER I: LITERATURE REVIEW

Background and Significance

In recent decades, plant pathology has begun to shift away from traditional disease management programs that involve primarily chemical treatments in favor of more balanced integrated pest management (IPM) programs as a combined result of increased environmental awareness and increasing pathogen resistance to standard chemical controls. IPM programs combine chemical disease management with other effective disease management practices, such as host resistance, cultural control, and biological control, often abbreviated to biocontrol. Biological control, as originally defined by the National Academy of Science in 1987, is the use of natural or modified organisms, genes, or gene products to reduce the effects of undesirable organisms (pests) and to favor desirable organisms such as crops, trees, animals, and beneficial insects and microorganisms.

The main limitation encountered in most biocontrol efforts is the lack of understanding of the influence of environmental factors on the efficacy of these treatments, often resulting in treatments that show promise *in vitro* and in greenhouse settings but fail to deliver in field settings. This project aims to broaden the scope of potential biocontrol of soilborne pathogens, using Phytophthora blight, caused by *Phytophthora capsici*, as a model. *Phytophthora capsici* has shown increasing resistance to one of the leading chemical controls, mefenoxam, over the past few decades (Parra and Ristaino 2001). As such, development of efficacious alternative management methods for Phytophthora blight is needed.

***Phytophthora*: Its overview, historical significance, and biology**

The genus *Phytophthora*, meaning plant-destroyer in Greek, is a diverse group of plant pathogenic oomycetes, with over 140 described species occupying a myriad of niches and host ranges (Erwin and Ribeiro 1996; Kroon et al. 2012). The genus was first described by Anton de Bary in 1875, when he identified and described the causal agent of late blight of potato, *P. infestans* (de Bary 1876). The genus *Phytophthora* is classified under the phylum Oomycota, which includes other genera of important plant pathogens such as *Pythium* spp. and *Peronospora* spp. The phylum Oomycota was originally placed in Kingdom Fungi but has since been reclassified under kingdom Chromista due to its members possessing biflagellate motile zoospores featuring ciliation on the anterior flagellum, cell walls made of cellulose rather than chitin as in fungi, and possession of colored plastids consistent with other members of kingdom Chromista (Cavalier-Smith 1981; Erwin and Ribeiro 1996; Cavalier-Smith 2018). Furthermore, most of the life cycle of oomycetes is spent in a diploid state, whereas fungal cells are principally haploid (Erwin and Ribeiro 1996). *Phytophthora* spp., similar to other oomycetes and many members of Kingdom Fungi, have a body (thallus) made of thin filaments called hyphae (Erwin and Ribeiro 1996).

Figure 1.1 illustrates the life cycle of *Phytophthora capsici*. As previously mentioned, *Phytophthora* spp. produce a biflagellated motile asexual infectious spore known as a zoospore. These zoospores are borne from (zoo)sporangia, which are borne on stalklike sporangiophores from the hyphae under optimal, typically aqueous, conditions (Erwin and Ribeiro 1996). The sexual spore of oomycetes such as *Phytophthora* spp. is called the oospore, which is produced from the antheridium ("male") and the oogonium ("female") under optimal conditions (Erwin and Ribeiro 1996). Depending on the species, *Phytophthora* spp. can be homothallic or heterothallic. *Phytophthora capsici* is a heterothallic *Phytophthora* species, meaning isolates are either

designated as an A1 or A2 mating type, and both mating types must be present for sexual reproduction and subsequent meiosis and production of sexual oospores to occur (Erwin and Ribeiro 1996).

Phytophthora capsici and Phytophthora blight of pepper

Phytophthora capsici is the causal agent of Phytophthora blight of pepper and cucurbits, first described by Leon Leonian on chili pepper in New Mexico (Leonian 1922), but has since been described in a multitude of states in the US and is considered to be ubiquitous (Granke et al. 2012). *P. capsici* is soilborne and spreads primarily through water and movement of infected soil (Granke et al. 2012). *P. capsici* is responsible for significant economic losses each year due both to management costs and to widespread crop loss (Hausbeck and Lamour 2004). *P. capsici* can infect every part of its plant hosts, leading to wilting, root rot, crown rot, stem rot, and less commonly leaf and fruit rot from splashing irrigation and rainwater (Granke et al. 2012; Lamour et al. 2012). Although *P. capsici* is most devastating on solanaceous and cucurbitaceous crops, *P. capsici* has been documented as pathogenic on over twenty unrelated botanical families (Reis et al. 2018).

Phytophthora capsici is a heterothallic species and requires an A1 and an A2 isolate in order to produce oospores. Isolates of both mating types are often found within *P. capsici*-infested fields in the US, leading to common occurrences of sexual reproduction (Granke et al. 2012). This can exacerbate management challenges, as the oospore is the only infectious propagule produced by *P. capsici* that can survive overwintering and long periods of time without a suitable host (Babadoost and Pavon 2013; Dunn et al. 2010). In addition to enabling longer-term survival of populations, sexual reproduction may also significantly contribute to the development of fungicide resistance in *P. capsici* populations. Fungicide resistance was found to be independent of mating type in *P. capsici* oospore isolates and can therefore theoretically be selected for through sexual

recombination (Lamour and Hausbeck 2000). Furthermore, it has been observed that geographically distant strains of *P. capsici* exhibit different levels of virulence and fungicide resistance, indicating independent co-development of fungicide resistance in sexually isolated populations (Nawaz et al. 2018). This is important from a management and quarantine perspective as this divergence and subsequent potential reconvergence of distinct gene pools through transport of infected soil or plant material could lead to the dissemination and further development of highly virulent and/or fungicide-resistant strains.

Management of *P. capsici*

Management of *Phytophthora* diseases, including Phytophthora blight, has traditionally used a chemical approach (Ristaino and Johnston 1999). As *P. capsici* is a soilborne pathogen, a highly effective and popular method for management was soil fumigation with methyl bromide prior to its prohibition in 2005 (Ezziyyani et al. 2007). The primary group of fungicides used to control *P. capsici* are the phenylamide fungicides, including metalaxyl and more commonly mefenoxam. Mefenoxam is the active enantiomer of metalaxyl and thus can be applied at lower rates than metalaxyl (Parra and Ristaino 2001). However, in the past several decades, isolates of *P. capsici* that have developed resistance to metalaxyl and mefenoxam have been increasingly reported (Café-Filho and Ristaino 2008; Parra and Ristaino 2001). Emerging mefenoxam resistance has led to increased attempts at disease management through other means, such as development of host resistance, cultural controls, and biological control methods (Granke et al. 2012; Ristaino and Johnston 1999).

Metalaxyl and mefenoxam are not the only chemicals labeled for management of Phytophthora blight. For example, the fungicides cyazofamid, dimethomorph, and mandipropamid have all shown ability to reduce disease caused by *P. capsici* in cucurbits (de Souza et al. 2016).

Cyazofamid-resistant isolates of *P. capsici* have been reported (Kousik and Keinath 2008). Despite the variety of chemicals labeled for management of Phytophthora blight and the threat of resistance development, mefenoxam remains a commonly used chemical due to its efficacy in managing susceptible isolates of *P. capsici* (Granke et al. 2012). Treatment programs for Phytophthora blight generally recommend applying fungicides in tank mixes or alternating between different modes of action throughout the growing season to avoid fungicide resistance development (Granke et al. 2012).

Many pepper cultivars have been developed that are tolerant to Phytophthora blight, but currently there are no available cultivars that are fully resistant to the pathogen (Granke et al. 2012). Furthermore, many tolerant or partially resistant cultivars of pepper are less horticulturally desirable or have lower yields than fully susceptible cultivars. Therefore, most growers would rather use a fully susceptible and more agronomically desirable cultivar combined with other management methods or even risk losing part of their harvest than to plant a lower-yielding or less valuable cultivar (Barchenger et al. 2018).

Cultural control strategies are also a viable and widely used method for management of Phytophthora blight. In fields infested with *P. capsici* oospores, host rotation for a few years is necessary for disease management, as the overwintering oospore is only viable for up to 48 months after formation (Babadoost and Pavon 2013).

Other cultural control strategies for management of *P. capsici* primarily involve strategies that focus on managing water, as *P. capsici* is a water mold and many stages of its life cycle benefit from saturated conditions. *Phytophthora capsici* forms sporangia in the presence of water, and these sporangia release swimming zoospores in cool, water-saturated conditions. Water management strategies have been used successfully to reduce Phytophthora blight disease

incidence and severity; these strategies include reducing irrigation frequency, using raised bed structures, and avoiding planting in low areas that tend to accumulate water (Ristaino et al. 1992; Ristaino and Johnston 1999). The use of straw mulch and cover crops has also been used to successfully reduce disease spread across planted rows by suppressing splash dispersal of inoculum (Ristaino et al. 1997; Ristaino and Johnston 1999).

History of & potential for biological control of Phytophthora diseases

'Suppressive' and 'conducive' soils have long been studied to examine differences in their impact on *Phytophthora* spp. propagules and disease (Baker and Cook 1974; Shea and Broadbent 1983). 'Suppressive' soils are described as "soils in which the pathogen does not establish or persist, establishes but causes little or no damage, or establishes and causes disease for a while but thereafter the disease is less important, although the pathogen may persist in the soil" (Baker and Cook 1974). In 1983, Malajczuk proposed that 'suppressive' soils were due to a microbiological agent, as 'suppressive' soils became 'conducive' to disease when a susceptible host was transplanted after sterilization by autoclaving, steam sterilization, and irradiation. Malajczuk compared suppressive versus conducive soils in a field planted with *Eucalyptus* sp. and found a higher percentage of microorganisms antagonistic to *Phytophthora cinnamomi* in the rhizosphere of suppressive soils than in conducive soils (Malajczuk 1983).

Several strains of different rhizobacterial genera and species have been shown to have antagonistic activity *in vitro* on *Phytophthora* spp. Bacterial genera reported to suppress growth of *P. capsici in vitro* include *Streptomyces* spp., *Bacillus* spp., and *Paenibacillus* spp (Chen et al. 2016).

Biological control mechanisms

Antagonism is one mechanism of biocontrol and can categorized into being either "direct" or "indirect". Direct antagonism, such as parasitism, antibiosis, or production of metabolites such as lytic enzymes, occurs when the antagonist directly targets or inhibits the growth or pathogenicity of the pathogen. Conversely, indirect antagonism refers to mechanisms of pathogen inhibition such as resource competition or induction of host resistance. In these cases, the ability of an antagonist to reduce pathogen growth or pathogenicity occurs without the antagonist directly and actively targeting the pathogen (Heydari and Pessarakli 2010).

Previous studies examining rhizobacterial antagonism of *Phytophthora* spp. have documented the use of many different mechanisms of antagonism depending on the antagonist (Pal and Gardener 2006). Rhizobacterial antagonists of *P. infestans* have been demonstrated to colonize hyphae and produce lytic enzymes, siderophores, and antibiotics (Hollywood 2004). A study conducted by Sharma *et al.* examined antagonistic *Bacillus* spp. strains on *P. capsici* and proposed cellulolytic activity to be a primary method of antagonism, as cell walls of *Phytophthora spp.*, unlike the chitinous cell walls of fungi are made of cellulose (Erwin and Ribeiro 1996). Most lytic enzymes or antibiotics have shown to have a degradative effect on various infectious propagules of *Phytophthora* spp., including sporangia, oospores, and hyphae (Malajczuk 1983).

In several prior studies of biocontrol of *Phytophthora* spp., several antagonists also functioned as plant growth promoters (PGPs) (Hollywood 2004; Sharma et al. 2015). Sharma *et al.* concluded that these PGP properties, in the case of *Bacillus* spp., were correlated with soil phosphate solubilization and increased plant indole-3-acetic acid (IAA) production. It is worth noting that IAA, while acting as a plant growth promoter, can promote disease development as

well, and thus the role of IAA produced by potential biocontrol agents warrants further examination (González-Lamothe et al. 2012).

Biological control products on the market for management of Phytophthora blight

Many rhizobacterial agents with varying modes of action have been identified to control *P. capsici*, including several formulations already available to the public. One such agent is the antagonistic fungus *Trichoderma asperellum* T34 (ASPERELLO™ T34, Biobest Group). T34 acts as a resistance-inducing endophyte that stimulates plant immune responses from within the roots (Segarra et al. 2013). T34 provided *P. capsici* control comparable to the chemical control agent etridiazole in greenhouse experiments on susceptible pepper plants (Segarra et al. 2013).

Another biocontrol agent that has been observed to control *P. capsici* is the rhizobacterium *Bacillus subtilis* QST-713 (Serenade®, Bayer CropSciences). QST-713 is a broad-spectrum antagonist that suppresses plant pathogens by producing inhibitory lipopeptides (Marrone 2002). In a greenhouse experiment on zucchini, QST-713 significantly reduced *P. capsici* disease severity by up to 69% (Gilardi et al. 2015).

A third commercially available biocontrol agent that has demonstrated control of *P. capsici* is another strain of *B. subtilis*, *B. subtilis* GB03 (Kodiak®, Gustafson). GB03 is another broad-spectrum antagonist that produces iturin-class antibiotics to inhibit plant pathogens (Brannen and Kenney 1997). GB03 applied in a bell pepper field in Florida significantly reduced *P. capsici* incidence of Phytophthora blight compared to an untreated control by up to 23% (Nemec et al. 1996).

Bacillus subtilis

Bacillus subtilis is an aerobic rod-shaped Gram-positive endospore-forming bacterium commonly found in soils and aquatic environments. The endospores produced by *B. subtilis* are resistant to stressors such as heat, cold, pH, chemicals, and radiation, and can survive in soils for years after formation and germinate when conditions are suitable for bacterial growth (Setlow 2006). The hardiness of these endospores makes *B. subtilis* and other endospore-forming rhizobacteria good candidates for commercial biological control agents, as commercial formulations composed of endospores are more shelf-stable than formulations comprised of vegetative cells (Schisler et al. 2004).

Bacillus subtilis strains often use multiple antagonistic mechanisms to suppress pathogens and/or bolster host defenses. Antagonistic mechanisms found in *B. subtilis* isolates include production of antimicrobial compounds such as degradatory enzymes, antibiotics, and lipopeptides, induction of systemic acquired resistance in plant hosts, and plant growth promotion via stimulation of plant growth promoting hormones such as IAA or gibberellic acid (Shafi et al. 2017; Sharma et al. 2015). The use of multiple antagonistic mechanisms is important for reliable management of plant diseases, as this makes it more difficult for the pathogen to overcome the antagonist, and can also allow the biocontrol agent to simultaneously control multiple pathogens if antagonistic methods are more generalized and not specific to a single pathogen (Shafi et al. 2017).

Bacillus subtilis is also ubiquitous in soils of all different types and chemistries and can associate itself with the roots of many plant species (Earl et al. 2008). This omnipresence and promiscuity of *B. subtilis* are more assets lending to its attractiveness as a commercial biocontrol.

Practical limitations of biological control

There are practical limitations of biological control of Phytophthora diseases. Growers are often hesitant to abandon long-standing and effective chemical schedules (Cook 1993). Limitations of widespread grower adoption of biologicals include low fitness and oft-observed inconsistencies of biological controls in field conditions (Erwin and Ribeiro 1996). Even when biocontrol agents applied in the field survive and suppress disease, long-term survival is typically low (Cook 1993). This subsequently makes multiple applications necessary for continued suppression, resulting in low public opinion of biological control as a realistic alternative to traditional chemical controls (Cook 1993). The reason for decreased biocontrol efficacy in field conditions compared to *in vitro* or under controlled conditions has largely been attributed to environmental factors, including abiotic differences in soil chemistry, composition, moisture, and temperature, and biotic factors such as competing microbiota and pathogen strains (Heydari and Pessarakli 2010; Hollywood 2004). These factors are often used to explain the phenomena around reduced biocontrol efficacy from greenhouse to field studies, as well as low comparative efficacy to chemical control agents. Several solutions for these problems, such as genetic engineering or formulations tailored to climate, host, and soil type have been proposed, but none have been fully adopted. It is valuable to investigate environmental conditions which impact biocontrol efficacy in order to combine cultural control methods with biological control methods for successful IPM programs.

Conclusions

Biological control of plant pathogens has become a topic of interest over the past several decades, owing both to increasing numbers of fungicide-resistant strains of plant pathogens and to the growing trend towards organic and sustainable agriculture. The soilborne oomycete plant pathogen *Phytophthora capsici*, causal agent of Phytophthora blight of pepper and cucurbits, is an ideal pathogen for biocontrol research using rhizobacterial antagonists. Prior studies have identified several rhizobacterial strains to be capable of controlling *P. capsici*, including the commercial strains *Bacillus subtilis* QST-713 (Serenade®, Bayer CropScience), and *B. subtilis* GB03 (Kodiak®, Gustafson) (Gilardi et al. 2015; Nemec et al. 1996).

Proponents of IPM and biological control envision a future where soil microbiome manipulation and maintenance is a cornerstone of plant pest management. Current practical limitations include a lack of understanding of ecological factors impacting survival of BCAs and lack of understanding of mechanisms of action in field systems. Previous studies on biological control of soilborne plant pathogens have mainly concluded that despite any potential promise shown *in vitro* or in greenhouse settings, uncontrollable ecological variables in field trials are often responsible for notoriously low success rates and low fitness of BCAs (Heydari and Pessarakli 2010; Hollywood 2004). This project aims to evaluate a strain of *Bacillus subtilis* for its potential to control *P. capsici* on bell pepper, and to experimentally adjust application methods and environmental conditions to maximize management efficacy in greenhouse and field conditions.

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FIGURES

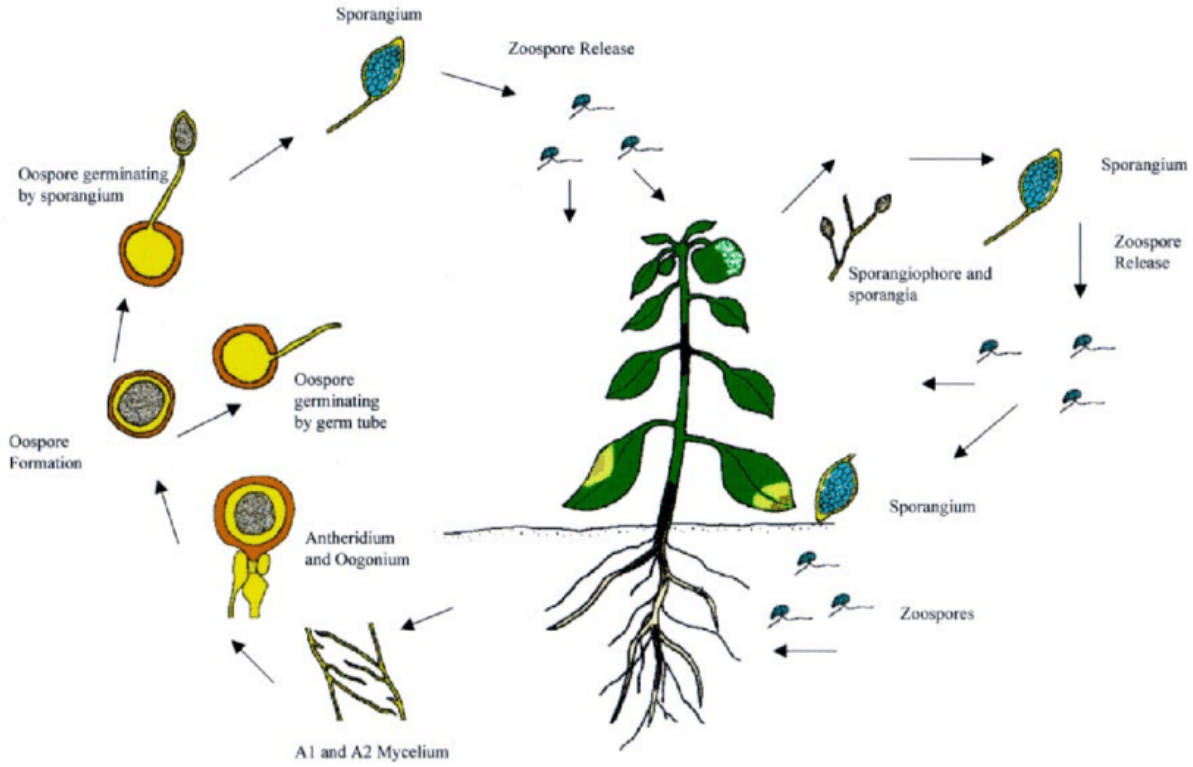

Figure 1.1: Life cycle of *Phytophthora capsici* (Ristaino & Johnston 1999).

CHAPTER II. Evaluation of an isolate of *Bacillus subtilis* for management of Phytophthora blight of pepper in greenhouse conditions

ABSTRACT

Phytophthora blight, caused by *Phytophthora capsici*, is one of the most economically significant diseases of bell pepper in the US (Granke et al. 2012). Over the past several decades, isolates of *P. capsici* exhibiting resistance to mefenoxam have been reported. This threat of resistance coupled with an increased market for organically grown crops has led to interest in disease management through biological control. *Bacillus subtilis* isolate AFS032321 was evaluated for control of Phytophthora blight of bell pepper in the greenhouse. A wettable powder formulation of the strain was applied as a soil drench at transplant prior to pathogen inoculation. *B. subtilis* significantly reduced area under the disease progress curve (AUDPC) by up to 52% compared to an untreated control. Comparisons between beginning treatment applications made weekly from plant seeding (5 treatments total) or a single treatment at transplanting 5 weeks after seeding indicated no significantly different effects of increased applications. While not significant, *B. subtilis* slightly reduced disease caused by a mefenoxam-resistant isolate of *P. capsici*, compared to both untreated and mefenoxam controls. The biocontrol efficacy of *B. subtilis* in four different soil types was also evaluated, and the results indicate that this *B. subtilis* isolate's biocontrol efficacy was not affected by soil type. While the precise mechanism(s) of action is unclear, a dual-culture test on lima bean agar suggested direct antagonism, as *B. subtilis* significantly inhibited colony growth of *P. capsici.*

INTRODUCTION

In recent decades, traditional disease management programs have shifted from primarily chemical treatments to more balanced integrated pest management (IPM) programs as a result of increased environmental awareness and increasing pathogen resistance to standard chemical controls. IPM programs combine chemical disease management with other effective disease management practices, such as host resistance, cultural control, and biological control. Biological control, as originally defined by the National Academy of Science in 1987, is the use of natural or modified organisms, genes, or gene products to reduce the effects of undesirable organisms (pests) and to favor desirable organisms such as crops, trees, animals, and beneficial insects and microorganisms.

Integrated pest management programs are especially important in pathosystems that have a high risk for fungicide resistance development. One such pathosystem is Phytophthora blight of pepper and cucurbits, caused by the oomycete plant pathogen *Phytophthora capsici*. *Phytophthora capsici* has developed resistance to one of the leading chemical controls, mefenoxam (Café-Filho and Ristaino 2008, Parra and Ristaino 2001). Development of mefenoxam resistance has been observed in geographically isolated populations of *P. capsici*, indicating that resistance is a global problem (Nawaz et al. 2018). Mefenoxam-resistant isolates of *P. capsici* have also been observed to transfer resistant phenotypes to offspring (Lamour and Hausbeck 2000). As *P. capsici* readily sexually reproduces in US fields (Granke et al. 2012), the pathogen's ability to produce mefenoxam-resistant progeny may prove problematic, as resistance can be selected for and spread across an entire field (Lamour and Hausbeck 2000). Due to fungicide resistance and a growing public interest in organically grown vegetables, efficacious alternative management methods for control of Phytophthora blight are needed.

Bacillus subtilis is a ubiquitous rhizobacterial species. Several isolates of *B. subtilis* have already been successfully commercialized into biocontrol agents (Schisler et al. 2004). *B. subtilis* has several characteristics that contribute to its desirability as a biocontrol agent, including its production of resilient endospores, multiple mechanisms of action including direct antagonism, plant growth promotion and induced resistance, and its ability to colonize root systems of many different plant species (Earl et al. 2008; Schisler et al. 2004; Setlow 2006, Shafi et al. 2017).

Soilborne disease control by isolates of *B. subtilis* has been observed in many pathosystems across different pathogen kingdoms, including clubroot on canola caused by the oomycete *Plasmodiophora brassicae* (Lahlali et al. 2013), stem rot of groundnut caused by the fungus *Sclerotium rolfsii* (Shifa et al. 2014), and bacterial wilt of mulberry caused by the bacterium *Ralstonia solanacearum* (Ji et al. 2008). These broad-spectrum antagonistic abilities of *B. subtilis* also make it an ideal biocontrol agent in cropping systems with complex disease problems (Earl et al. 2008; Shafi et al. 2017).

Several commercial formulations of *B. subtilis* are labeled for management Phytophthora blight. *Bacillus subtilis* QST-713 (Serenade®, Bayer CropSciences) significantly reduced disease severity caused by Phytophthora blight by up to 69% in a greenhouse experiment on zucchini (Gilardi et al. 2015). *B. subtilis* GB03 (Kodiak®, Gustafson) applied in a bell pepper field in Florida significantly reduced incidence of Phytophthora blight compared to an untreated control by up to 23% (Nemec et al. 1996).

Screening of potential biocontrol agents, including the *B. subtilis* isolate used in this study, typically follows a selective scaling-up process of isolation from suppressive soils, *in vitro* evaluations, controlled environment *in planta* studies, then field trials. AgBiome, Inc. (Research

Triangle, NC) isolated and performed primary screenings of this *B. subtilis* isolate for biocontrol potential and formulated it as a shelf-stable wettable powder endospore formulation for screening in multiple pathosystems, including these screenings on Phytophthora blight of pepper.

The objective of this study was to examine the efficacy of an isolate of *B. subtilis* for biocontrol of Phytophthora blight of pepper. First, this *B. subtilis* isolate was evaluated *in vitro* in a dual-culture assay for its ability to suppress culture growth of *P. capsici* (Irabor and Mmbaga 2017). We subsequently examined the rate of biocontrol application, timing and number of applications, and the effect of soil type on biocontrol of Phytophthora blight. Biocontrol efficacy of *B. subtilis* was also compared for a mefenoxam-resistant and sensitive isolate of *P. capsici*, as there is a need for alternative methods of control in fields infested with mefenoxam-resistant strains of the pathogen (Parra and Ristaino 2001).

MATERIALS AND METHODS

Planting material. A *Phytophthora capsici*-susceptible bell pepper (*Capsicum annuum*) cultivar, Red Knight, was used for all greenhouse and Phytotron experiments. Seeds were sourced from Johnny's Selected Seeds (Fairfield, ME), and were not treated with any pesticides prior to planting. Seeds were germinated on damp filter paper in sealed petri dishes incubated in the dark at 25°C for one week before planting in seedling cells. Seedlings were planted into 9.1 cm styrofoam cups or 10.2 cm pots after 5 weeks. Plants were watered twice daily. Experiments were done either in the greenhouse or in a controlled-environment incubator at the Phytotron at North Carolina State University in Raleigh, NC. During the winter months, the temperatures in the greenhouses were unsuitable for plant growth and pathogen infectivity so experiments were moved to the Phytotron. In all experiments, temperatures were 25°C (approx. in greenhouse). A soilless potting medium (Sungro® Fafard® 4P Mix: Sphagnum peat moss, bark, perlite, vermiculite, dolomitic lime) was used for experiments in the greenhouse, while in the Phytotron experiments, a soilless potting mix (Sunshine Redi-Earth Pro Growing Mix: Canadian Sphagnum peat moss, vermiculite, dolomitic lime, silicon dioxide) was mixed with 50% sand.

Pathogen zoospore inoculum preparation. *Phytophthora capsici* was cultured on V8 juice agar (800 mL diH_2O, 200 mL V-8 juice, 2 g $CaCO_3$, and 17 g agar) for 7 days at room temperature (21 ± 2°C) (Ristaino, 1990). Cultures were then divided using a sterile scalpel into pieces and flooded with a 2% sterile soil extract (20 g soil liter $^{-1}$ water) and incubated at 25°C for 48-72 hours until sporangia were visible under a dissecting microscope (10x magnification). Cultures were incubated at 4°C for two hours to stimulate zoospore cleavage from sporangia and then brought back to 25°C for one hour to induce zoospore release. The contents of the plates were filtered through two layers of cheesecloth to filter out agar pieces and zoospores were concentrated. A one

mL aliquot of the filtrate was treated with two drops of lactophenol cotton blue to encyst zoospores for counting. Zoospores were quantified with a hemocytometer and zoospore concentration was adjusted with diH_2O to 5.0 x 10^4 zoospores mL^{-1}.

***P. capsici* isolate selection.** Four isolates of *P. capsici* were tested for growth, sporulation ability, virulence, and aggressiveness on susceptible bell pepper. Isolates 415 and FF3-C1 were first tested, then isolates 399 and NCCP21 were tested. Whole-plant testing was conducted in the greenhouse, with four whole-plant replications for each treatment. Plants were inoculated with 10 mL of a 5.0 x 10^4 solution per plant. Negative controls were inoculated with 10 mL of water only. Plants were placed in pans filled with 5 cm of water for 3 hours after inoculation to encourage zoospore movement to plant roots. Each test was conducted twice, and plants were rated for disease severity every two days for ten days after inoculation. Disease severity was rated every other day on an ordinal scale of 0 to 4, where 0 = no disease; 1 = mild wilting; 2 = moderate wilting and/or crown lesion; 3 = severe wilting and stem lesion; 4 = plant death (Figure 2.1).

Stem lesions were re-cultured on a *Phytophthora*-selective medium after infection to ensure that the causal agent of symptoms was the inoculated pathogen. *P. capsici* isolate NCCP21 was selected for all subsequent experiments.

***In vitro* growth inhibition by *Bacillus subtilis* AFS032321.** To determine whether *B. subtilis* suppressed *in vitro* growth of *P. capsici*, a growth inhibition assay was conducted. *Bacillus subtilis* strain AFS032321 was provided as a wettable powder endospore formulation by AgBiome, Inc (Research Triangle Park, NC). *Bacillus subtilis* was isolated from the wettable powder formulation and maintained in pure culture by plating powder on Luria-Bertani media and subsequently transferring pure bacterial colonies.

One 5-mm agar disk from a 7-day old culture of *P. capsici* isolate NCCP21 was placed in the center of a lima-bean agar plate (Sinclair and Dhingra 1995). At the same time, four 5-mm Luria-Bertani agar disks from a culture of *B. subtilis* AFS032321 were placed equidistant from one another and 3 cm away the center *P. capsici* agar disk (Irabor and Mmbaga 2017). Negative controls consisted only of a center agar disk of *P. capsici*. Colony diameters of *P. capsici* were measured after 7 days of incubation at room temperature (23 ± 2°C). Percent inhibition of colony growth was determined compared to the negative control. EC_{50} was calculated and EC_{50} graphs were generated using the AAT Bioquest EC_{50} calculator.

Rate of *B. subtilis* needed for effective biocontrol. *Bacillus subtilis* was tested for biocontrol efficacy at rates of 40, 30, 15, and 5 lb A^{-1} to determine the best rate of application for future experiments. The wettable powder formulation was dissolved in deionized water and applied as a soil drench to 5 week-old pepper plants three days before inoculation with *P. capsici* zoospores. Plants were inoculated as described above. Plants were inoculated using *P. capsici* zoospores at a rate of 2.5 x 10^5 zoospores per plant. This experiment was performed twice with six single-plant replications of five treatments. Plants were rated every other day for eighteen days after inoculation. In all subsequent experiments, *B. subtilis* was applied at 30 lb A^{-1}.

Timing of *B. subtilis* application. *Bacillus subtilis* was applied to plants either weekly from time of seeding (n=5) or applied once at five weeks of age after transplanting to pots. The objective of this experiment was to determine if multiple applications of *B. subtilis* improved biocontrol of Phytophthora blight.

The experiment was conducted in the Phytotron in a randomized complete block two-factor factorial. The primary factor was treatment onset: either weekly from plant seeding or once at five

weeks of plant age. The secondary experimental factor was treatment type: an untreated control, *B. subtilis* at 30 lb A^{-1} applied weekly, or Ridomil Gold SE at 1 pt A^{-1}applied monthly. Plants treated with *B. subtilis* from seeding received five treatments, whereas plants treated with *B. subtilis* at transplant were only treated once. Plants treated with Ridomil Gold from seeding received two treatments in accordance with the product label. Plants were inoculated with *P. capsici* zoospores at a rate of 2.5 x 10^5 zoospores per plant. This experiment was performed twice, with six single-plant replications per treatment combination. Plants were rated every other day for disease incidence and severity for eighteen days after inoculation.

Efficacy of biocontrol of a mefenoxam-resistant isolate of *P. capsici*. *Phytophthora capsici* isolate 317, a mefenoxam-resistant isolate, was compared to the mefenoxam-sensitive *P. capsici* isolate NCCP21 to compare the efficacy of biocontrol of Phytophthora blight with *B subtilis*.

Both *P. capsici* isolates were first tested for mefenoxam sensitivity and the EC50 was determined for each isolate. Clarified V8 juice medium (150 mL clarified V8 juice, 850 mL diH_2O, 2 g $CaCO_3$, 17 g agar) was amended with mefenoxam at 0, 0.1, 1, 10, 100, and 1000 ppm and inoculated with a 5mm agar disk from a 7-day old culture of *P. capsici*. There were 3 plates per level of fungicide. Colony diameters were measured after 7 days of incubation at room temperature (23 ± 2°C). If *P. capsici* growth on 10 and 100 ppm mefenoxam-amended media was less than 40% of the nonamended control, the isolate was classified as sensitive; conversely, if colony diameter growth on 10 and 100 ppm mefenoxam-amended media was greater than 40% of nonamended media, the isolate was classified as resistant to mefenoxam (Parra and Ristaino 2001).

The efficacy of biocontrol with *B. subtilis* on Phytophthora blight caused by each of the *P. capsici* isolates was tested in the Phytotron as a randomized complete block two-factor factorial.

The first factor was *P. capsici* isolate: either mefenoxam-sensitive (NCCP21) or mefenoxam-resistant (317). The second factor was treatment type: an untreated control, the *B. subtilis* at 30 lb A^{-1}, or Ridomil Gold SE at 1 pt A^{-1}. Plants were inoculated with zoospores at a rate of 2.5 x 10^5 zoospores per plant. This experiment was performed twice, with seven single-plant replications per treatment combination. Plants were rated every other day for disease incidence and severity up until eighteen days after inoculation.

Efficacy of biocontrol in different soil types. Efficacy of biocontrol of Phytophthora blight using *B. subtilis* was compared in four different soil types. Soils tested included a clay soil, a sandy soil, the Phytotron soil mix comprised of 50% Sunshine Redi-Earth Pro Growing Mix (Canadian Sphagnum peat moss, vermiculite, dolomitic lime, silicon dioxide) and 50% cement sand; and a soilless potting medium from the greenhouse (Sungro® Fafard® 4P Mix). This experiment was conducted as a randomized complete block two-factor factorial with four levels of soil type and three levels of treatment (untreated, *B. subtilis*, or Ridomil Gold). Five-week-old peppers were transplanted into 4" pots containing one of the 4 soils and treated with either water, Ridomil Gold SG at 1 pt A^{-1}, or *B. subtilis* at 30 lb A^{-1}. Each treatment/soil combination contained 5 single-plant replications. Plants were inoculated with *P. capsici* zoospores at a rate of 2.5 x 10^5 zoospores per plant three days after treatment. This experiment was performed twice, and plants were rated every other day for disease incidence and severity up until eighteen days after inoculation.

Data analysis and computations. Area under the disease progress curve (AUDPC) was calculated via previously described methods (Shaner and Finney, 1977) for average severity measurements. AUDPC, final severity ratings, and final percent incidence were analyzed for statistical differences among treatments using PROC GLM in PC-SAS (Cary, NC). If significant treatment differences

were found ($p<0.05$), treatment means were used to calculate Fisher's Least Significant Difference test to identify significant differences among treatments.

Data was also analyzed for disease incubation period for each plant. Final binary incidence was recorded for each plant and analyzed using a chi-square analysis of fit in PROC GLIMMIX in PC-SAS. Incubation period, or the length of time between inoculation and symptom development, was recorded for plants that developed disease and analyzed using PROC GLM in PC-SAS.

EC_{50} data for mefenoxam resistance in two *P. capsici* isolates was calculated by fitting a logarithmic dose-response curve to data points corresponding to percent colony growth inhibition at different rates of mefenoxam amendment compared to a nonamended control plate. The EC50, or concentration of mefenoxam amendment required to reduce colony growth by 50% compared to a nonamended control, was calculated using the equation of the fitted dose-response curve (Van Ewijk and Hoekstra 1993).

RESULTS

***P. capsici* isolate selection.** Four isolates of *P. capsici* were tested for pathogenicity and growth characteristics. Data collected included average zoospores mL^{-1}, area under the disease progress curve (AUDPC) and average final disease severity (Table 2.1). Isolates 415 and FF3-C1 produced lower numbers of zoospores and did not cause significant disease on susceptible pepper, although isolate 415 did slightly outperform FF3-C1 in both categories. Both isolates 399 and NCCP21 produced high numbers of zoospores, and NCCP21 caused the highest disease severity when rated ten days after inoculation. NCCP21 was selected as the isolate for use in subsequent experiments since it produced abundant zoospores and was highly aggressive on susceptible pepper plants.

***In vitro P. capsici* growth inhibition by *B. subtilis*.** In the dual-culture assay, *B. subtilis* AFS032321 reduced *P. capsici* colony growth *in vitro* by an average of 59% compared to a negative control (Figure 2.2). Colony diameter of *P. capsici* after 7 days of incubation was reduced from 8.6 cm to 3.5 cm.

Rate of *B. subtilis* needed for biocontrol. The effect of the rate of *B. subtilis* application on biocontrol of Phytophthora blight was evaluated with four application rates of the biocontrol agent. Rate of *B. subtilis* application had a significant effect on both AUDPC and final disease severity, but not final disease incidence or disease incubation period (Table 2.2; Figures 2.3.1 & 2.3.2). Mean final disease severity ratings were reduced from 3.8 in the control to 2.9 and 2.1 at the 30 and 40 lb A-1 application rates, respectively (Figure 2.3.1). A significant reduction in AUDPC was observed at the 30 and 40 lb A^{-1} application rates compared to the untreated control: the AUDPCs were 20.5 and 15.5 respectively at the 30 and 40 lb A^{-1} *B. subtilis* application rates compared to 30.7 in the untreated control.

Timing of *B. subtilis* application. The effect of time and frequency of application of *B. subtilis* on biocontrol of Phytophthora blight was evaluated (Table 2.3; Figures 2.4.1 & 2.4.2). Timing and frequency of biocontrol application had no significant effect on AUDPC and final disease severity. There were no significant differences among AUDPC or final disease severity for the interaction effect of treatment and timing. However, treatment type significantly affected AUDPC and final disease severity. Mean AUDPC decreased from 31.9 in the untreated controls to 21.8 in the *B. subtilis* treatments to 1.46 in the Ridomil Gold treatments (Figure 2.4.2). Mean final disease severity ratings decreased from 3.9 in the untreated control to 2.8 in the *B. subtilis* treatment to 0.25 in the Ridomil Gold treatment (Figure 2.4.1). Neither treatment nor application timing significantly affected final disease incidence (Table 2.3). Treatment significantly affected incubation period, which was extended from 7.5 days in the untreated control plants to 8.85 days in the *B. subtilis*-treated plants and 10.67 days in the Ridomil Gold-treated plants (Figure 2.4.1).

Efficacy of biocontrol of a mefenoxam-resistant isolate of *P. capsici*. *Phytophthora capsici* isolate NCCP21 is sensitive to mefenoxam, and *P. capsici* isolate 317 is resistant to mefenoxam (Table 2.4; Figure 2.5) (Parra and Ristaino 2001). The EC_{50}, or effective concentration of mefenoxam required to reduce colony growth by 50 percent, was 0.16 mg mL^{-1} for the mefenoxam sensitive isolate and 146.2 mg mL^{-1} for the mefenoxam-resistant isolate.

In the whole-plant tests comparing biocontrol efficacy in each of the *P. capsici* isolates, significant differences were identified for AUDPC, final disease incidence, and final disease severity for the interaction effect of treatment and isolate, suggesting that treatment effects were different between isolates. Isolates were analyzed separately for significant differences between treatments (Table 2.6).

Final disease severity, incidence, and AUDPC were significantly different between treatments in the mefenoxam-sensitive *P. capsici* isolate. Final disease severity decreased from 3.5 in the untreated control to 1.9 in the *B. subtilis* treatment to 0.3 in the Ridomil Gold treatment, with significant distinctions between each treatment (Figure 2.6.1). AUDPC decreased from 28.8 in the untreated control to 12.7 in the *B. subtilis* treatment to 2.0 in the Ridomil Gold treatment, all of which were significantly distinct from one another (Figure 2.6.2). Final disease incidence decreased from 100 percent in the untreated control to 57 percent in the *B. subtilis* treatment to 7 percent in the Ridomil Gold treatment (Figure 2.6.1). No significant differences in disease were identified between treatments for the mefenoxam-resistant *P. capsici* isolate (Table 2.6).

Disease incubation period was significantly different between the two isolates (Table 2.5). Plants inoculated with the mefenoxam-resistant isolate showed symptoms after 9.74 days, where plants inoculated with the mefenoxam-sensitive isolate showed symptoms after 8 days (Figure 2.7.1).

Efficacy of biocontrol in different soil types. Soil pH and cation exchange capacity (CEC) are reported in Table 2.7. Soil type did not significantly affect final disease severity, AUDPC, final incidence, or incubation period of Phytophthora blight (Table 2.8). The interaction effect between treatment and soil type was also not significantly different for any measurements, indicating statistically similar treatment effects across different soil types. Treatment alone significantly affected both AUDPC and final disease severity. While soil type did not significantly affect disease progress or treatment efficacy, disease in the clay soil was slightly lower than the other soils (Figures 2.7.1, 2.7.2, and 2.7.3). The untreated control had the highest AUDPC at 21.5, followed by the biocontrol at 12.7, and Ridomil with 1.2. All treatments were significantly distinct from one another. Average final severity ratings for treatment were 2.9 in the untreated control, 2.0 in the

B. subtilis treatment, and 0.3 in the chemical control. The untreated control and *B. subtilis* were not significantly different, but the disease severity in the chemical control group was significantly reduced compared to the other two.

DISCUSSION

Bacillus subtilis AFS032321 significantly inhibited *Phytophthora capsici* colony growth in an *in vitro* screening assay, suggesting the production of antibiotics or other directly antagonistic compounds such as lytic enzymes impacted colony growth (Irabor and Mmbaga 2017). Previous studies on *B. subtilis* isolates antagonistic to *Phytophthora* spp. have identified antibiotics effective in controlling *Phytophthora* spp (Chung et al. 2008; Shafi et al. 2017). Whether direct antagonism is the only mechanism of action of this *B. subtilis* isolate is unknown, as antagonistic isolates of *B. subtilis* often show multiple methods of antagonism including induced systemic resistance and competition for space and nutrients (Earl et al. 2008, Shafi et al. 2017). Colony growth inhibition can indicate biocontrol efficacy *in planta*, making dual-culture assays a fast and easy step in initial biocontrol screening procedures (Pliego et al. 2011).

Bacillus subtilis effectively controlled Phytophthora blight compared to an untreated control at high rates (30 and 40 lb A^{-1}) of application. The rates at which *B. subtilis* effectively controlled Phytophthora blight are much higher than the application rates for commercial *B. subtilis* wettable powder biocontrol products labeled on pepper. The maximum application rate of Serenade Opti (*B. subtilis* QST 713) and Companion (*B. subtilis* GB03) for peppers is 1.5 lb A^{-1}, applied weekly. The lowest rate tested for this isolate of *B. subtilis* was 5 lb A^{-1}, which had no significant effect on Phytophthora blight. This isolate of *B. subtilis*, if commercialized, may not provide reasonably cost-effective management of Phytophthora blight in large fields, due to the high rates of application needed to attain adequate disease control compared to current commercial *B. subtilis* biofungicides. As such, it would likely be marketed to small-scale growers and home gardeners seeking an effective organic treatment for Phytophthora blight.

In the application timing experiment, no cumulative effect on disease reduction was observed with multiple *B. subtilis* applications. Phytophthora blight was not significantly different between plants treated once at transplanting and plants treated five times starting from plant seeding. A previous study on repeated applications of *B. subtilis* for biocontrol of yellow rust of wheat found low, if any, significant reductions of disease with increased application frequency (Reiss and Jørgensen 2017). This is a positive aspect for candidates for commercial biocontrol, as single treatment applications are more cost- and labor-effective than multiple applications, especially if the same biocontrol efficacy can be attained.

When tested for biocontrol of a mefenoxam-resistant P. capsici isolate, *B. subtilis* provided insignificant control compared to the untreated control and mefenoxam but appeared to reduce disease slightly compared to the other two treatments (Figure 2.6.2). There may be potential for *B. subtilis* AFS032321 to effectively manage mefenoxam-resistant strains of *P. capsici*. Further research into the potential for this *B. subtilis* isolate to effectively and realistically manage mefenoxam-resistant strains of *P. capsici* is warranted, as mefenoxam resistance is a prevalent threat in *P. capsici* populations in US field systems (Granke et al. 2012; Parra and Ristaino 2001).

Tests of biocontrol efficacy in different soil types revealed no significant difference in biocontrol efficacy of *B. subtilis* among the four soils tested. Disease management in different environments is a very positive trait for potential commercial biocontrol agents. The clay soil developed lower disease than the other soils, even in the untreated control (Figures 2.7.1 and 2.7.2). This may be evidence of either a competitive pre-established soil microbiome with antagonistic microbes in this clay soil and/or soil chemical or physiological effects that were inhospitable to the *P. capsici* isolate tested. Studies have also suggested that soil porosity is negatively correlated with disease

in soilborne *Phytophthora* species (Liu et al. 2008), as swimming zoospores have lower motility in soils with fewer and smaller pores (Duniway 1976), such as clay soils.

This isolate of *B. subtilis* has several qualities that make it a good candidate for commercialization. The same level of Phytophthora blight control was observed in one application and five weekly applications of *B. subtilis*. This is attractive from cost, time, and labor standpoints. Duration of biocontrol activity of this isolate of *B. subtilis* against Phytophthora blight has not been studied yet but may be useful for field scenarios. *Bacillus subtilis* treatments may effectively be combined with disease forecasting technologies so the biocontrol agent can be applied before pathogen activity or early in the season. *Bacillus subtilis* was able to control Phytophthora blight in four different soils, indicating that this isolate can germinate, grow, and control disease in different soil physical and chemical conditions. If biocontrol efficacy is maintained in many different conditions, then the range of potential customers increases accordingly, leading to greater commercial success. While biocontrol efficacy was only observed at 30 and 40 lb A^{-1} rates of application, this biocontrol agent would be marketable to small-scale organic growers, which make up about 75 percent of organic vegetable growers (Fernandez-Cornejo et al. 1998) and homeowners with small vegetable gardens.

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TABLES

Table 2.1: Evaluation of four isolates of *P. capsici* for sporulation and virulence on susceptible peppers.

***P. capsici* isolate**	**Source**	**Zoospores mL^{-1}[a]**	**Final Disease Severity**	**AUDPC[b]**
399	Pepper	1.66×10^5	1.38	4.13
415	Pepper	6.35×10^4	1.29	3.58
FF3-C1	Tomato	1.12×10^4	0.83	1.83
NCCP21	Pepper	1.05×10^5	3.38	12.13

[a] Concentrations are averaged over two extractions.
[b] AUDPC – Area under the Disease Progress curve

Table 2.2: Effect of different rates of *B. subtilis* application on final disease severity, area under the disease progress curve, final incidence, and disease incubation period of Phytophthora blight on susceptible peppers. Significant ($p<0.05$) values are followed by an asterisk (*).

	Rate of Application	
Dependent Values	F[a]	p[b]
Final Disease Severity	16.93	<0.001*
AUDPC[c]	16.77	<0.001*
Final Disease Incidence	0.00	1.000
Incubation Period	2.22	0.080

[a] ANOVA F-statistic.
[b] Level of marginal significance of F-value.
[c] AUDPC – Area under the Disease Progress curve.

Table 2.3: Effects of treatment (negative control, *B. subtilis*, or Ridomil Gold) and application timing and frequency (5 times from seeding or once at transplant) on final disease severity, area under the disease progress curve, final incidence, and disease incubation period of Phytophthora blight on susceptible peppers. Significant ($p<0.05$) values are followed by an asterisk (*).

	Treatment		**Timing**		**Treatment*Timing**	
Dependent Values	F[a]	p[b]	F	p	F	p
Final Disease Severity	165.31	<0.001*	2.93	0.091*	1.28	0.283
AUDPC[c]	130.46	<0.001*	0.89	0.350	0.42	0.657
Final Disease Incidence	0.00	0.999	0.00	0.990	0.00	1.000
Incubation Period	4.47	0.016*	0.05	0.830	0.30	0.743

[a] ANOVA F-statistic.
[b] Level of marginal significance of F-value.
[c] AUDPC – Area under the Disease Progress curve.

Table 2.4: Mean percent growth compared to a nonamended control of two isolates of *Phytophthora capsici* on mefenoxam-amended V8 juice media after seven days of growth.

***P capsici* isolate**	**Mefenoxam Concentration**					
	0ppm	0.1ppm[a]	1ppm	10ppm	100ppm	1000ppm
NCCP21	100.0	65.9	7.9	0.0	0.0	0.0
317	100.0	99.4	101.6	102.2	79.3	0.0

[a] ppm = parts per million mefenoxam; mg mL^{-1}

Table 2.5: Effects of treatment (negative control, *B. subtilis*, or Ridomil Gold) and mefenoxam sensitivity of isolate of *P. capsici* on final disease severity, area under the disease progress curve, final incidence, and disease incubation period of Phytophthora blight on susceptible peppers. Significant ($p<0.05$) values are followed by an asterisk (*).

	Treatment		**Isolate**		**Treatment*Isolate**	
Dependent Values	F[a]	p[b]	F	p	F	p
Final Disease Severity	8.27	<0.001*	1.65	0.202	12.52	<0.001*
AUDPC[c]	12.55	<0.001*	0.01	0.913	13.94	<0.001*
Final Disease Incidence	0.41	0.667	0.00	0.982	4.23	0.018*
Incubation Period	0.36	0.701	7.33	0.009*	1.49	0.235

[a] ANOVA F-statistic.
[b] Level of marginal significance of F-value.
[c] AUDPC – Area under the Disease Progress curve.

Table 2.6: Effects of treatment (negative control, *B. subtilis*, or Ridomil Gold) on final disease severity, area under the disease progress curve, final incidence, and disease incubation period of Phytophthora blight on susceptible peppers, separated by two *P. capsici* isolates. Significant ($p<0.05$) values are followed by an asterisk (*).

	NCCP21 (Mefenoxam-Sensitive)		**317 (Mefenoxam-Resistant)**	
Dependent Values	F[a]	p[b]	F	p
Final Disease Severity	22.72	<0.001*	1.03	0.365
AUDPC[c]	24.79	<0.001*	0.70	0.504
Final Disease Incidence	10.62	0.002*	0.18	0.670
Incubation Period	0.07	0.796	3.21	0.084

[a] ANOVA F-statistic.
[b] Level of marginal significance of F-value.
[c] AUDPC – Area under the Disease Progress curve.

Table 2.7: Selected soil qualities for soils used to examine biocontrol efficacy of *B. subtilis* on Phytophthora blight of pepper in different soil types.

Soil	**pH**	**CEC[a]**
Clay	7.4	24.0
Sand	5.4	3.8
50:50 Mix	6.0	50.0
Soilless Potting Medium	6.2	27.8

[a]CEC - Cation Exchange Capacity, reported as meq 100 $cm^{3\ -1}$.

Table 2.8: Effects of treatment (negative control, *B. subtilis*, or Ridomil Gold) and soil type (clay, sand, 50:50 mix, or soilless potting medium) on final disease severity, area under the disease progress curve, final incidence, and disease incubation period of Phytophthora blight on susceptible peppers. Significant ($p<0.05$) values are followed by an asterisk (*).

	Treatment		**Soil**		**Treatment*Soil**	
Dependent Values	F[a]	p[b]	F	p	F	p
Final Disease Severity	15.26	<0.001*	0.98	0.408	1.22	0.313
AUDPC[c]	14.61	<0.001*	0.33	0.806	0.97	0.456
Final Disease Incidence	0.06	0.994	0.64	0.592	0.15	0.988
Incubation Period	2.33	0.123	1.38	0.277	0.27	0.896

[a] ANOVA F-statistic.
[b] Level of marginal significance of F-value.
[c] AUDPC – Area under the Disease Progress curve.

FIGURES

Figure 2.1: Visual representation of the severity scale used to rate Phytophthora blight of pepper. 0 = healthy; 1 = wilting present; 2 = wilting & crown lesion; 3 = wilting & stem lesion; 4 = plant death.

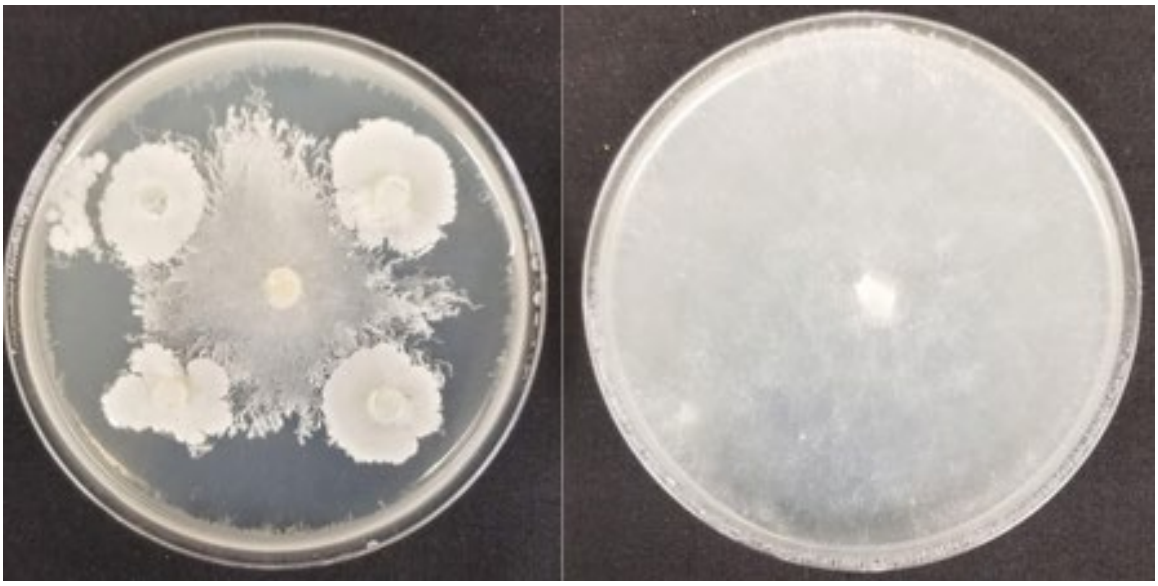

Figure 2.2: Dual-culture assay with *Bacillus subtilis* AFS032321 and *Phytophthora capsici* NCCP21 on Lima-Bean Agar. Left: experimental plate with four plugs of *B. subtilis* and one center plug of *P. capsici*; right: control plate with *P. capsici* only.

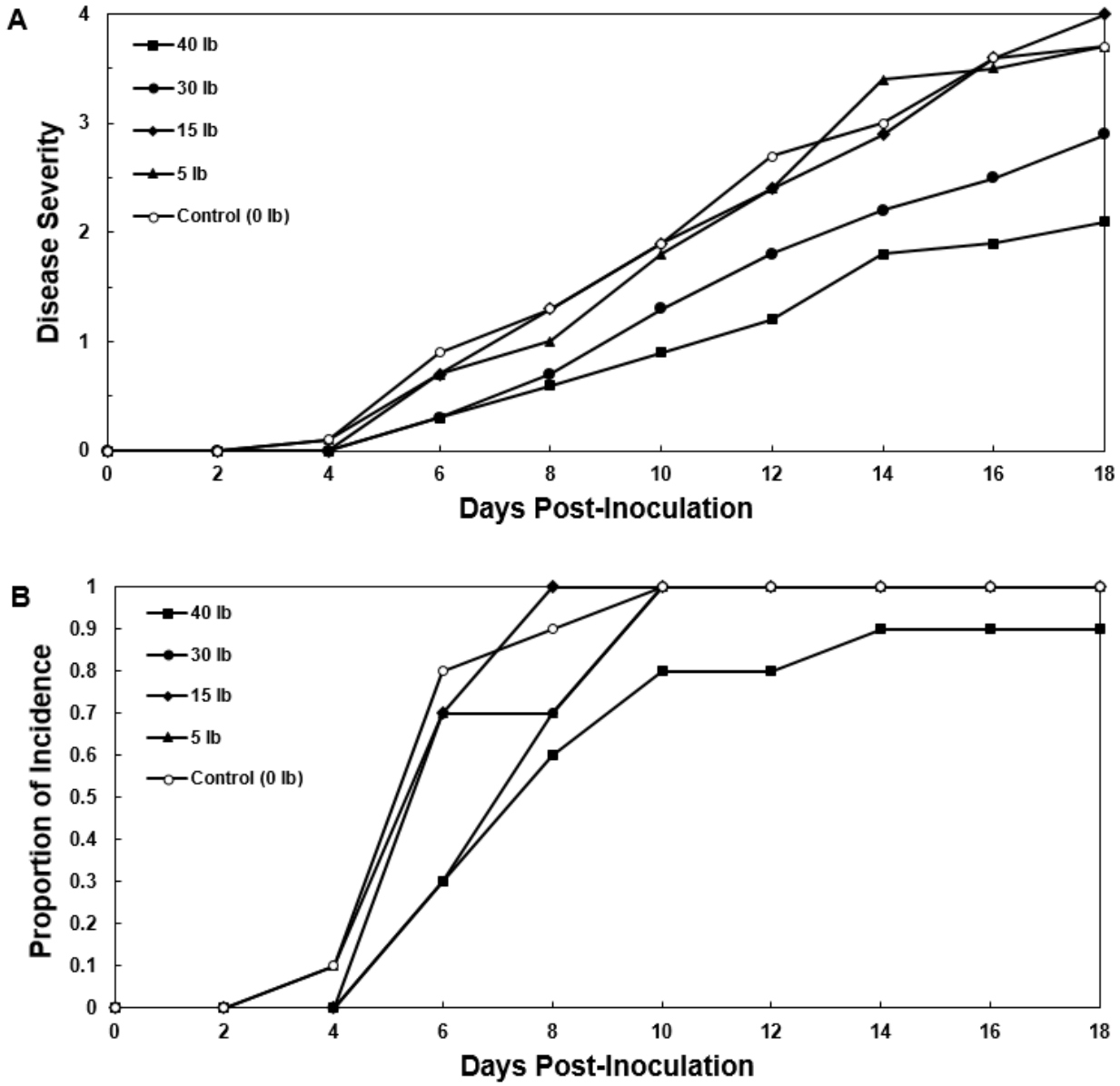

Figure 2.3.1: Disease severity of Phytophthora blight over time (A) and incidence over time (B) for four applied rates of *B. subtilis* AFS032321. Data points are means pooled from two trials, each assigned five plants per treatment (n=10).

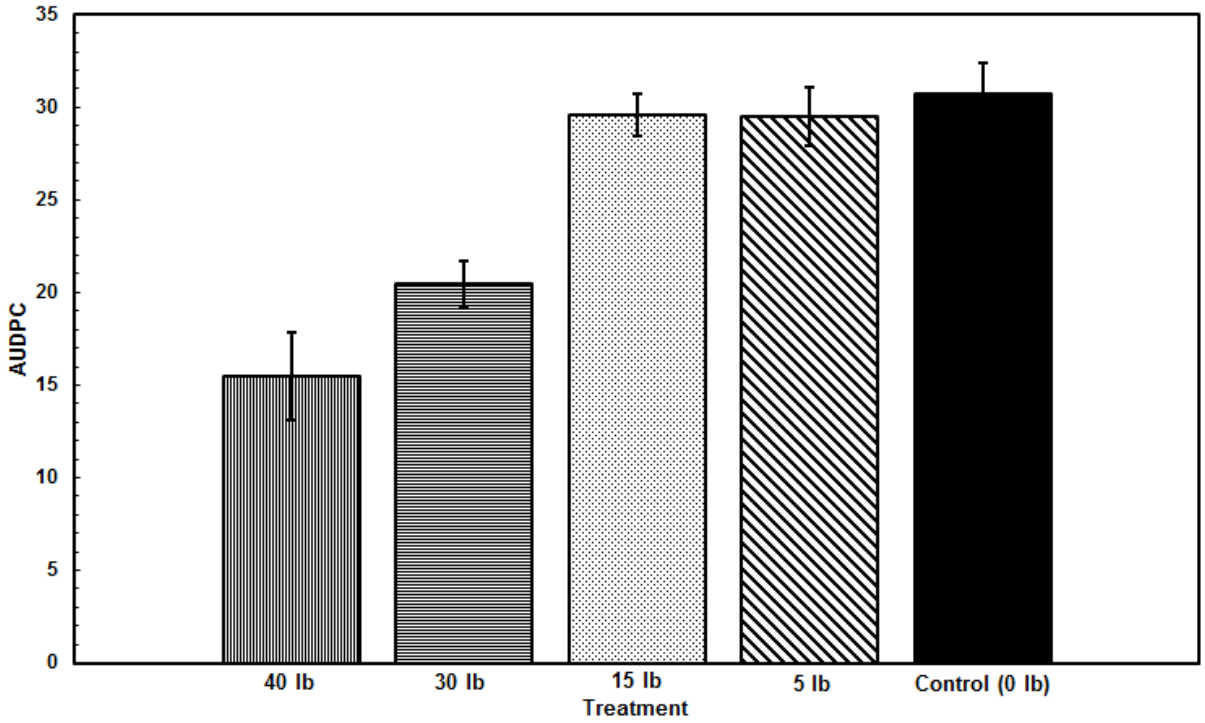

Figure 2.3.2: The area under the disease progress curve (AUDPC) for Phytophthora blight for four applied rates of *B. subtilis* AFS032321. Error bars represent standard error of the mean.

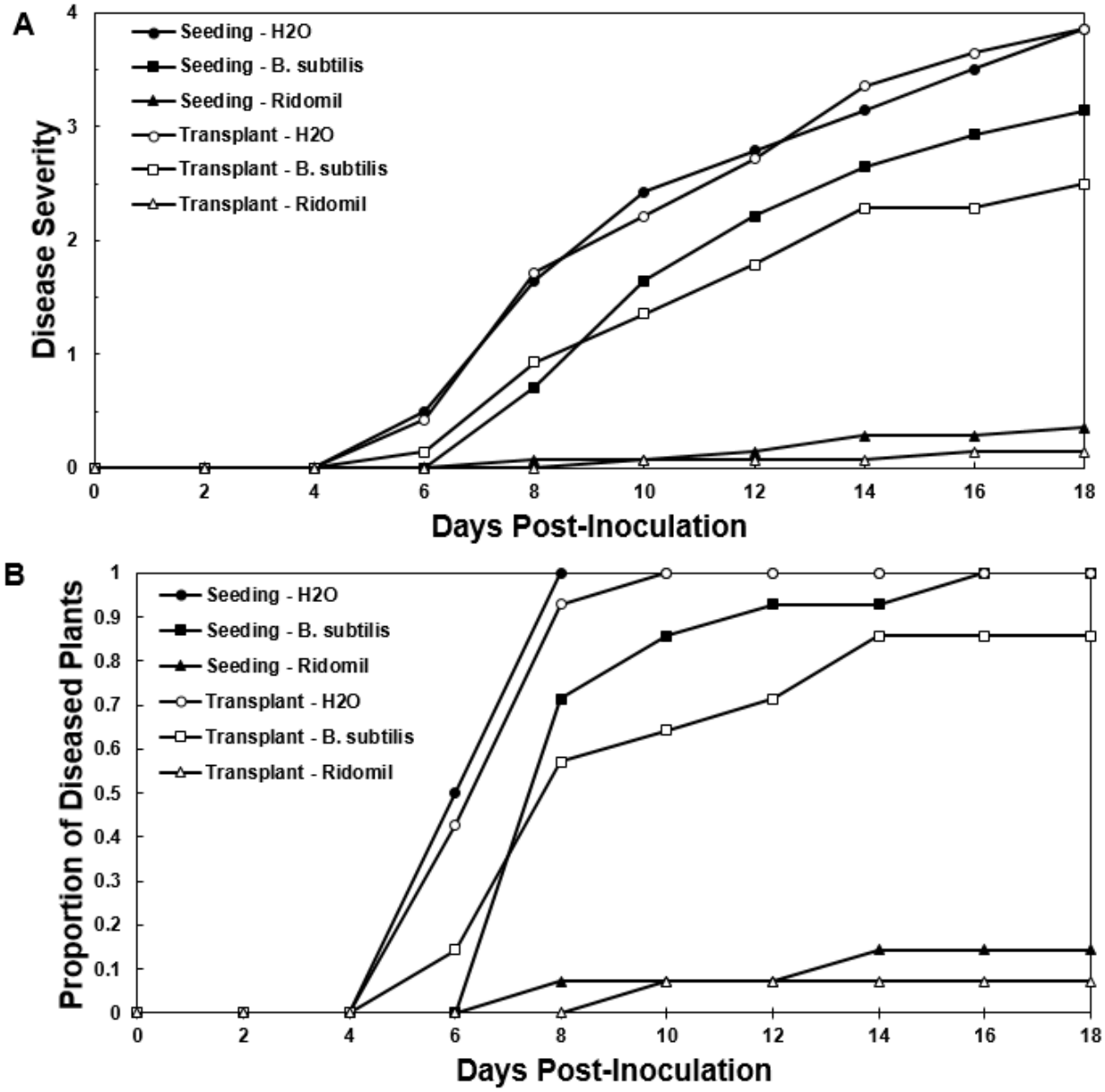

Figure 2.4.1: Disease severity (A) and incidence (B) of Phytophthora blight for three treatments (negative control, *B. subtilis*, or Ridomil) applied to plants either starting from seeding or at a 5-week transplant.

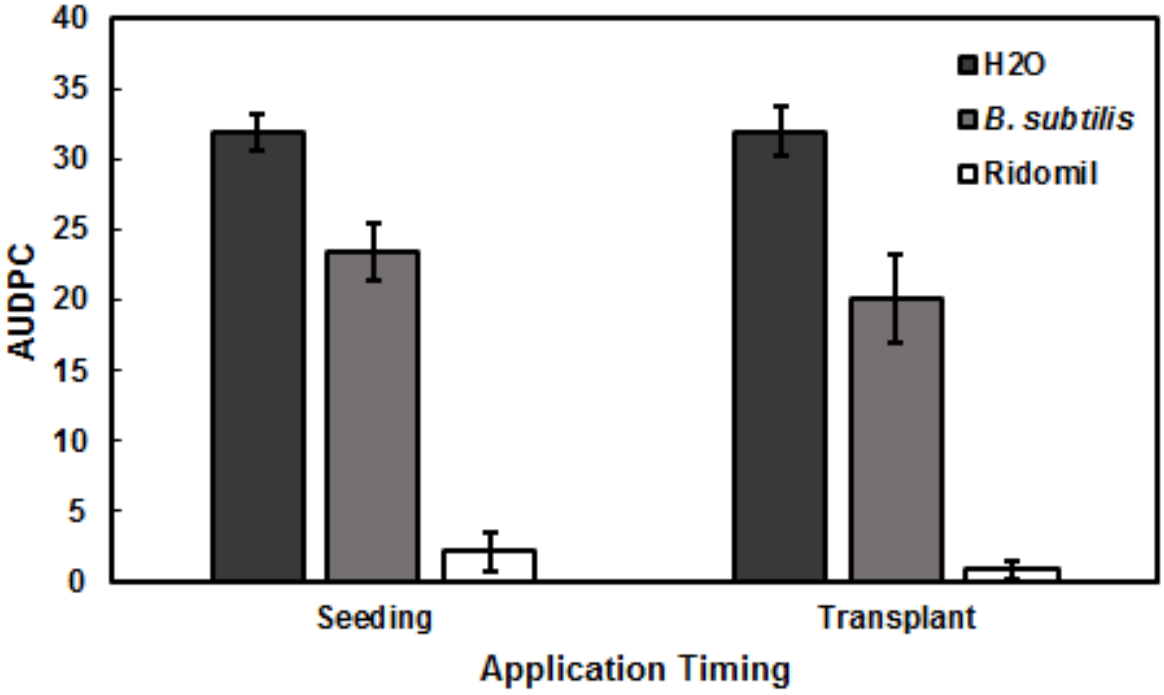

Figure 2.4.2: Area under the disease progress curve (AUDPC) of Phytophthora blight for three treatments (negative control, *B. subtilis*, or Ridomil) applied to plants weekly starting from seeding (5 applications total) or once at a 5-week transplant. Error bars represent standard error of the mean.

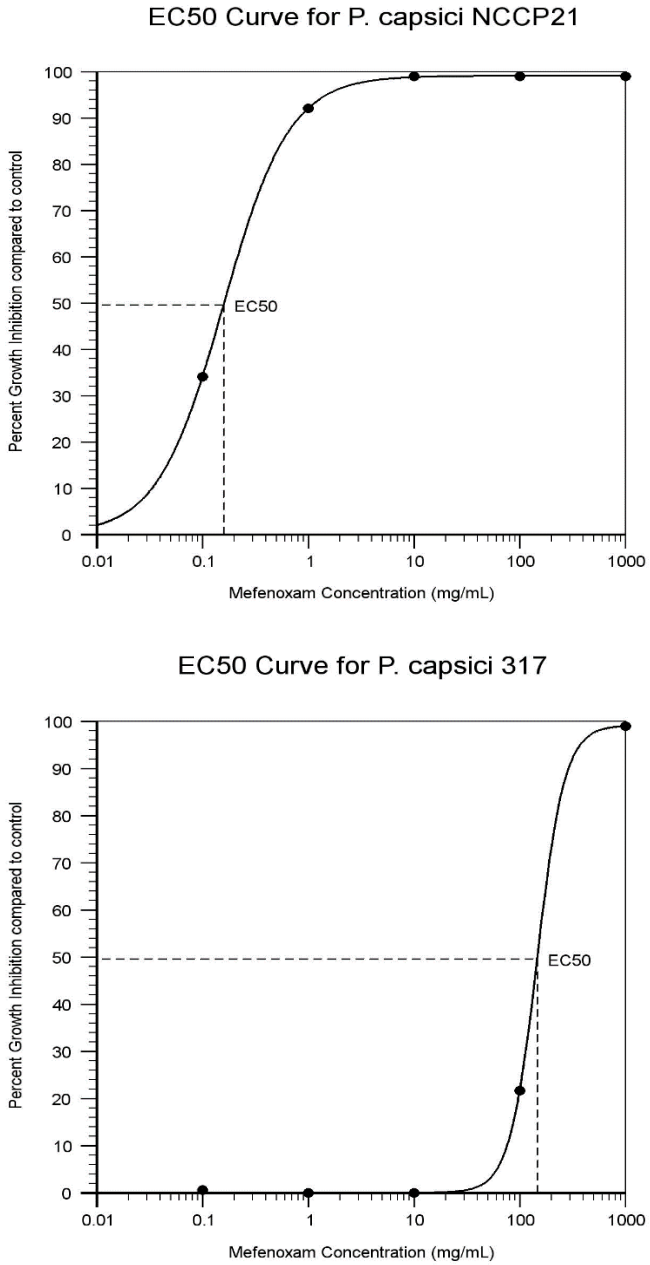

Figure 2.5: Percent growth inhibition for mefenoxam-sensitive (NCCP21) and mefenoxam-resistant (317) isolates of *P. capsici* on mefenoxam-amended media (0, 0.1, 1.0, 10, 100, 1000ppm). Graphs generated through and retrieved from AAT Bioquest EC_{50} calculator.

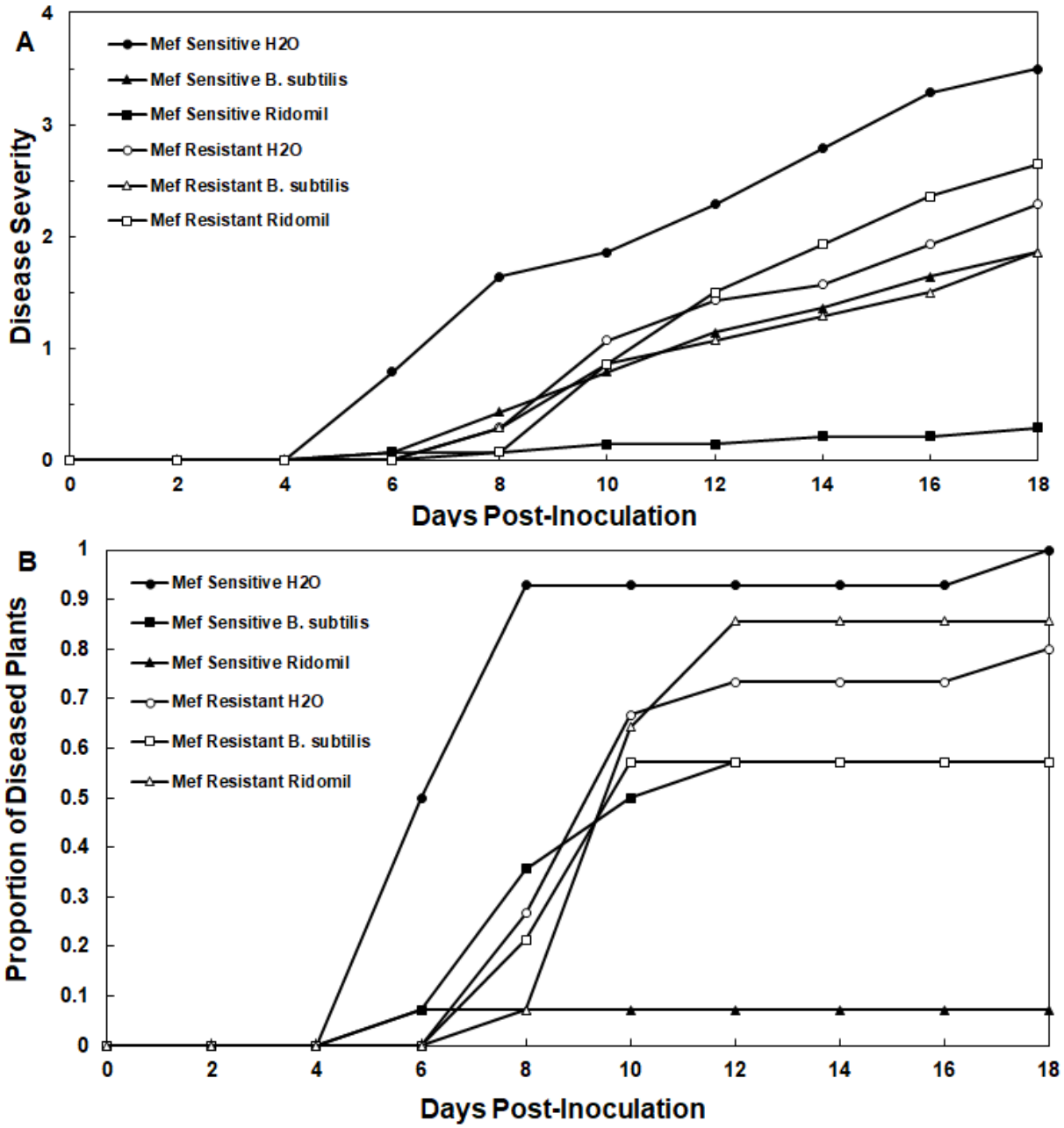

Figure 2.6.1: Disease severity (A) and incidence (B) of Phytophthora blight caused by a mefenoxam-sensitive isolate (NCCP21) and a mefenoxam-resistant isolate (317), as affected by *B. subtilis* or Ridomil Gold.

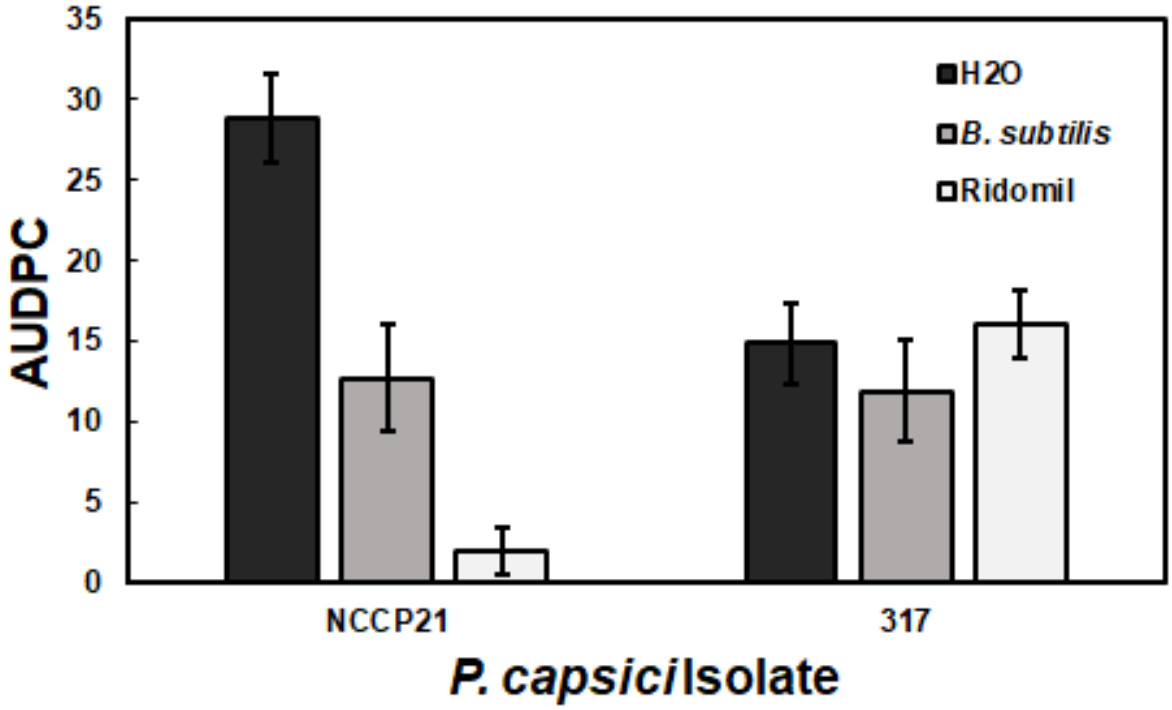

Figure 2.6.2: Area under the disease progress curve (AUDPC) measurements for Phytophthora blight caused by one of two isolates of *P. capsici*: Isolate NCCP21 is sensitive to mefenoxam (Ridomil), while isolate 317 is resistant to mefenoxam.

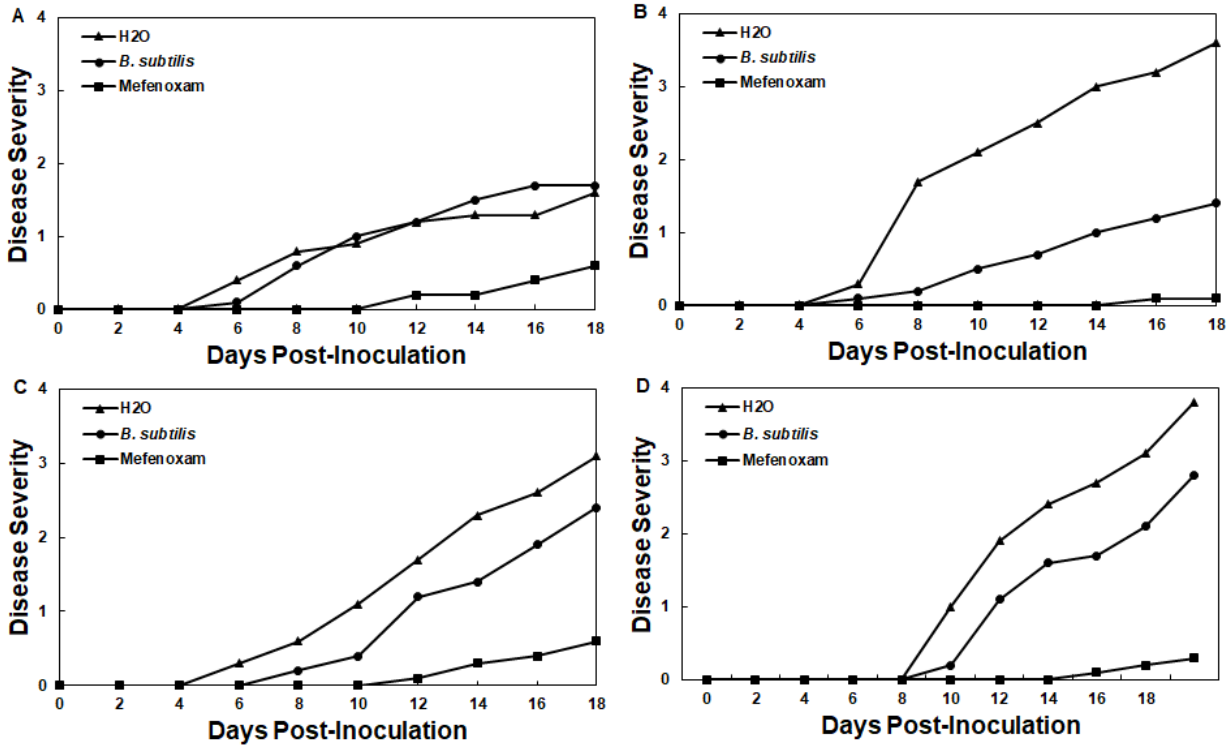

Figure 2.7.1: Disease severity over time caused by Phytophthora blight in different soil types. **A:** Clay soil; **B:** Sandy soil; **C:** 50:50 mix; **D:** soilless potting medium.

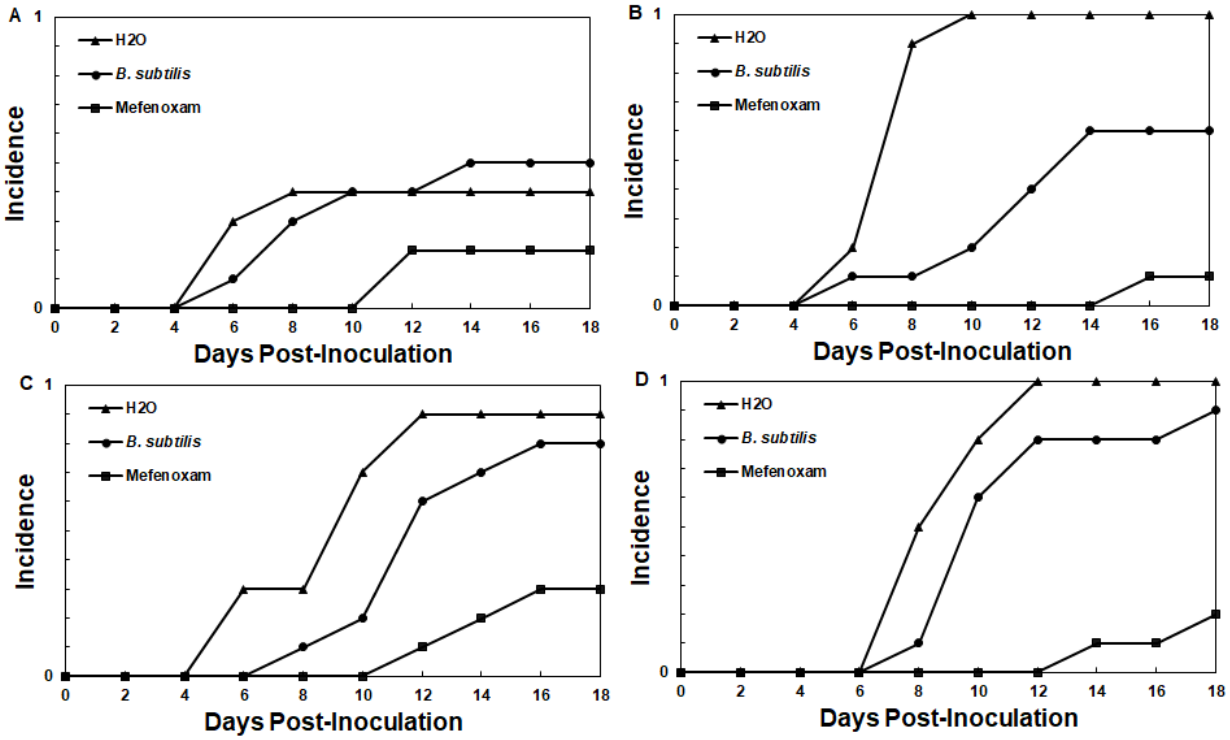

Figure 2.7.2: Disease incidence over time caused by Phytophthora blight in different soil types. **A:** Clay soil; **B:** Sandy soil; **C:** 50:50 mix; **D:** soilless potting medium.

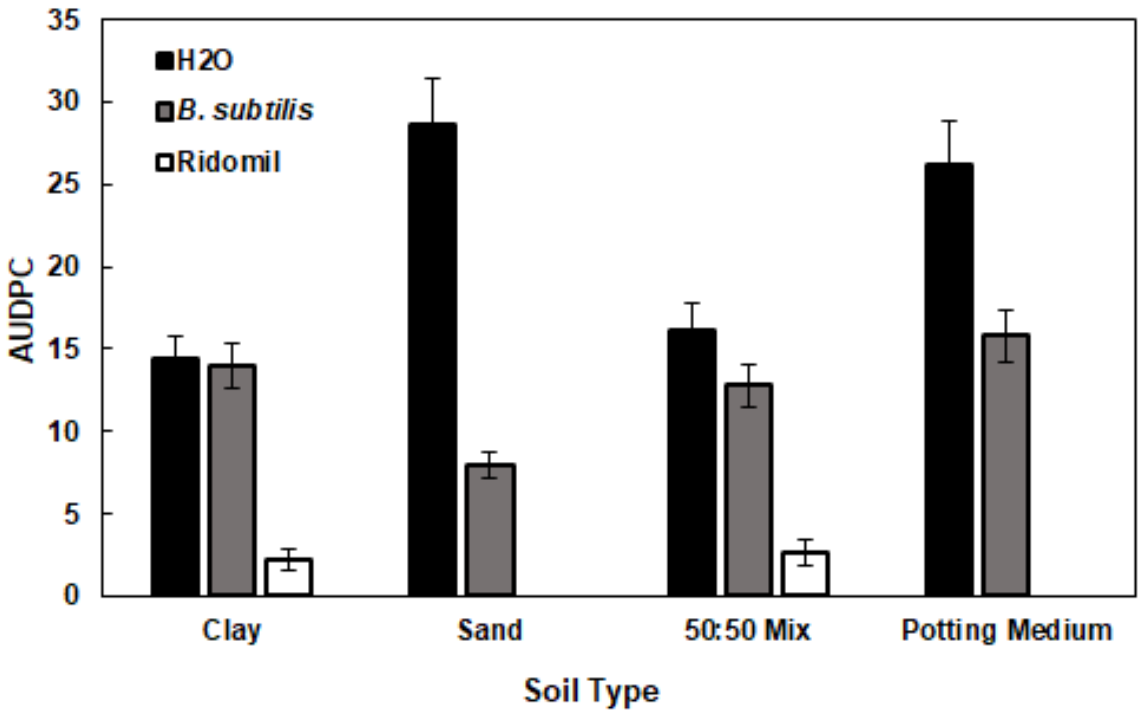

Figure 2.7.3: Area under the disease progress curve (AUDPC) of Phytophthora blight, affected by treatment (negative control, *B. subtilis*, or Ridomil Gold) and soil type. Error bars represent standard error of the mean

CHAPTER III. Evaluation of an isolate of *Bacillus subtilis* for management of Phytophthora blight of pepper under field conditions in North Carolina

ABSTRACT

Phytophthora blight, caused by *Phytophthora capsici*, is one of the most economically significant diseases of bell pepper in the US. Over the past several decades, isolates of *P. capsici* exhibiting resistance to mefenoxam have been reported. This threat of resistance coupled with an increased market for organically grown crops has led to interest in disease management through biological control. An isolate of *Bacillus subtilis* was evaluated for biocontrol potential of Phytophthora blight of pepper under field conditions in North Carolina over two growing seasons. *B. subtilis* isolate AFS032321 in a wettable powder formulation was applied as a soil drench at rates of 40, 30, 15, and 5 lb A^{-1} and compared to an untreated control and a fungicide (Ridomil Gold SE) for control of Phytophthora blight. In both growing seasons, *B. subtilis* provided control of Phytophthora blight of pepper. In 2018, disease incidence and severity of Phytophthora blight was reduced by *B. subtilis* at rates of 30 and 40 lb A^{-1} and were not significantly ($p < 0.05$) different from the chemical control. In 2019, disease incidence and severity of Phytophthora blight was significantly reduced at all rates of *B. subtilis* except for the 15 lb A^{-1} rate compared to the untreated control. *Bacillus subtilis* did not affect the spatial dynamics of disease spread within rows. The AFS032321 strain of *B. subtilis* shows promise as an alternative to fungicides for management of Phytophthora blight in the field.

INTRODUCTION

Phytophthora blight of pepper and cucurbits is a soilborne disease caused by the oomycete *Phytophthora capsici* Leonian. Phytophthora blight is responsible for significant economic losses each year due both to management costs for fungicides and to widespread crop loss (Hausbeck and Lamour 2004). *P. capsici* can infect every part of its plant hosts, leading to wilting, root rot, crown rot, stem rot, and, less commonly, leaf and fruit rot from splashing irrigation and rainwater (Granke et al. 2012; Lamour et al. 2012). Management strategies for Phytophthora blight, while primarily relying on chemical controls, also include cultural control methods, such as reduction of soil moisture and the use of tolerant plant varieties (Granke et al. 2012; Hausbeck and Lamour 2004; Ristaino and Johnston 1999).

Some strains of *P. capsici* are resistant to mefenoxam, one of the leading oomycete-targeted fungicides (Café-Filho and Ristaino 2008, Parra and Ristaino 2001). Development of mefenoxam resistance has been observed in geographically-isolated populations of *P. capsici*, in many areas of the world, indicating a global problem (Nawaz et al. 2018). Mefenoxam-resistant isolates of *P. capsici* have been observed to develop progeny that are also fungicide-resistant (Lamour and Hausbeck 2000). As *P. capsici* readily reproduces sexually in US fields (Granke et al. 2012), the pathogen's ability to survive in soils and produce fungicide resistant progeny is problematic. Fungicide resistance is under selection and the pathogen can spread rapidly across an entire field if chemical treatments fail (Lamour and Hausbeck 2000). Efficacious alternative management methods for control of Phytophthora blight are needed. Additionally, a growing public interest in organically grown vegetables warrants research into alternative strategies for managing the disease, including biocontrol.

Bacillus subtilis is a bacterial species commonly found in soils and aquatic environments and has several characteristics that make it an ideal biocontrol agent. *B. subtilis* associates with plant roots of many species (Earl et al. 2008). In addition, studies of isolates of *B. subtilis* have shown that the species can produce over two dozen antibiotics (Stein 2005), many of which are antagonistic to plant pathogens. The production of these antibiotics allows *B. subtilis* to outcompete plant pathogens in the rhizosphere, thus forming a beneficial relationship between *B. subtilis* and plants. In addition to these antibiotics, *B. subtilis* produces a resilient endospore as part of its life cycle, which makes commercial formulations of *B. subtilis* easier to produce and more shelf-stable (Schisler et al. 2004; Stein 2005). Several isolates of *B. subtilis* have already been successfully commercialized into biocontrol agents (BCAs) (Schisler et al. 2004).

Disease control of soilborne pathogens using *B. subtilis* has been demonstrated for several different kinds of plant pathogens including clubroot on canola caused by the oomycete *Plasmodiophora brassicae* (Lahlali et al. 2013), stem rot of groundnut caused by the Basidiomycete fungus *Sclerotium rolfsii* (Shifa et al. 2014), and bacterial wilt of mulberry caused by the bacterium *Ralstonia solanacearum* (Ji et al. 2008). The broad-spectrum antagonistic abilities of *B. subtilis* also makes it an ideal biocontrol agent in cropping systems with complex disease problems (Earl et al. 2008; Shafi et al. 2017).

Several commercial formulations of *B. subtilis* are currently on the market for biocontrol of Phytophthora blight, including Serenade and Kodiak, and these formulations have been demonstrated to reduce disease. *Bacillus subtilis* QST-713 (Serenade®, Bayer CropSciences) significantly reduced disease severity of Phytophthora blight caused by *P. capsici* by 69% in a greenhouse experiment on zucchini (Gilardi et al. 2015). In a bell pepper field trial in in Florida,

B. subtilis GB03 (Kodiak®, Gustafson) significantly reduced incidence of Phytophthora blight disease caused by *P. capsici* compared to an untreated control by 23% (Nemec et al. 1996).

The objective of this research was to examine the efficacy of an experimental strain of *Bacillus subtilis* (AgBiome AFS032321) under field conditions for control of Phytophthora blight of bell pepper. In other research underway (Chapter II), we found that the strain reduced disease in a series of greenhouse trials and a variety of soil types. Our specific objectives were: 1) To test four rates of application of *B. subtilis* for control of Phytophthora blight on a susceptible pepper variety; 2) To compare the efficacy of biocontrol to the efficacy of chemical control using the oomycete-targeted fungicide mefenoxam; and 3) to measure the impact of the biological compared to the chemical control on disease incidence, severity, yield and spread within an inoculated field.

MATERIALS & METHODS

Site selection and field preparation. Field trials were conducted in 2018 and 2019 at the Sandhills Research Station in Jackson Springs, NC (35°11'44"N latitude and 79°40'59"W longitude), in a plot previously used for *P. capsici* pepper trials (Parada-Rojas and Quesada-Ocampo 2019; Romero et al. 2001). The soil type in the field was a mixture of Ailey loamy sand and Candor sand (USDA Web Soil Survey). Prior to planting, the field was treated with a pre-emergent herbicide: Trevlon at 1.5 pt A^{-1} in 2018, and Dual II Magnum at 12 oz A^{-1} in 2019. The plot was also deep-tilled prior to planting in 2019 to bury sclerotia of *Sclerotium rolfsii* which was observed on carrots in a prior test in parts of the field. Weed management was done in the weeks after planting by tilling between rows and hand-picking weeds in planted rows. Plants were irrigated with 0.5 inches of water 2-3 times a week via an overhead irrigation system, the main line of which was set up down the row between the northern and southern subplots. A 17-17-17 fertilizer at 150 lb A^{-1} was applied twice during the growing season in both years, and an additional 15.5-0-0 fertilizer application was made in 2019.

Weather data was recorded from a weather station located 1 km from the field at the research station. Relevant weather data for each growing season was retrieved from the NC Climate Retrieval and Observations Network of the Southeast Database (CRONOS) and is summarized in Table 3.1.

Plant material. *Capsicum annuum* (cv. Red Knight), a bell pepper cultivar susceptible to Phytophthora blight of pepper, was seeded in 200-cell Todd flats (February 14, both years) in a soilless potting medium (Sungro® Fafard® 4P Professional Growing Mix). Plants were grown in a greenhouse at the Method Road Greenhouse Complex at NC State University. Seedlings were watered twice daily, and fertilized once per week with a water-soluble 20-20-20 fertilizer. Plants

were brought to the Sandhills Research Station in late April and left outside to harden off for one day in 2018 and three days in 2019 before transplanting into the field plot. In 2018, plants were transplanted by hand, and in 2019, plants were transplanted using a mechanical transplanter.

Plot design. The experiment was set up as a randomized complete block design with four subplots. Each treatment row contained 13 plants. Plants were spaced 0.5 meters apart on 1.1-meter wide row centers. Buffer rows were also planted on either side of treatment rows to prevent spread between treatment rows. Treatment rows were separated down rows by unplanted 1.5-meter alleys in 2018 and 3.0-meter alleys in 2019.

Treatments. There were six treatments in each year. Treatments included a pathogen only-inoculated control or pathogen plus *B. subtilis* AFS032321 at applied rates of 40, 30, 15, and 5 lb A^{-1}, or a pathogen-inoculated-chemical control (Ridomil Gold SE, 1 pt A^{-1}).The biological control treatments were applied three times during the season either at planting, two weeks after planting or 4-6 weeks after planting (2019 and 2018, respectively). The pathogen-only inoculated plots were treated with water at the same time as the biological control treatments. The chemical control was applied twice as a soil drench the day after planting and one month after transplanting, in accordance with the label. The *B. subtilis* treatment (provided by AgBiome, RTP, NC) was formulated as a wettable powder containing 10^9 CFUs per gram. The formulation was dissolved into water at an equivalent volume of 670 liters A^{-1}, and then applied at rates of 40, 30, 15, and 5 lb A^{-1} in a volume of 28 mL per plant. The biocontrol and was applied as a drench at the base of each plant.

Inoculum preparation and inoculation. Inoculum of *Phytophthora capsici* (isolate NCCP21) was prepared by seeding a sterile V8 broth-infused vermiculite culture with six agar disks of week-

old cultures of *P. capsici* grown on unclarified V8 juice agar (800 mL diH_2O, 200 mL V8 juice, 2 g $CaCO_3$, 17 g agar). Vermiculite culture was prepared using previously described methods (Ivors 2015). The V8 Juice Broth (200mL diH2O, 50mL V8 juice, 0.5g CaCO3) was added to 500 cm^3 of vermiculite in 1-quart mason jars. Jars were sealed with a vented lid plugged with a foam stopper and autoclaved in a water-filled pan on two successive days before being seeded with agar disks of *P. capsici*. Vermiculite culture was shaken weekly to break up and redistribute mycelium and used to inoculate the field after four weeks of incubation at room temperature (23 ± 2°C).

The 2018 trial was initially planned to rely on natural inoculum to induce disease. The field had a history of disease, and both mating types of *P. capsici* were applied two years prior to the test (Parada-Rojas and Quesada-Ocampo 2019). To confirm the presence and amount of inoculum, soil samples from the field plot were plated on Masago's medium, a *Phytophthora*-selective agar medium (Masago et al. 1977; Ivors 2015). However, due to low disease rates in the beginning of the 2018 season, each plot was also artificially inoculated to supplement the natural inoculum. In the 2018 trial, plants were inoculated with 10 cm^3 of vermiculite culture at the base of each plant at the first three plants in each treatment row 43 days after planting.

In the 2019 trial, three inoculations with *P. capsici* were done to induce disease in plants. Plants were initially inoculated 14 days after transplanting using previously described methods (Ristaino et al. 1992). 2 cm^3 of inoculated vermiculite culture was mixed into 206 cc of non-inoculated vermiculite culture and spread down the first meter of row and buried 10 cm, on the side of the row closest to the irrigation line. The second inoculation was performed at a higher rate 35 days after planting, in which 25 cm^3 of inoculated vermiculite culture was mixed into 183 cm^3 of non-inoculated vermiculite culture and spread down the first meter of each treatment row. A third and final inoculation was performed 42 days after planting using a method consistent with

the 2018 inoculation: 12.5 cm^3 of inoculated vermiculite culture was buried directly at the base of the first three plants in each inoculated row. Care was taken not to disturb plant roots when burying inoculum. After each inoculation, the field was irrigated for three hours to encourage infection (Ristaino et al. 1992).

Disease rating. Disease data was collected weekly after transplanting for twelve weeks. Plants were rated individually for disease incidence and severity of Phytophthora blight. Disease severity was rated on an ordinal scale of 0 to 4, where 0 = no disease; 1 = mild wilting; 2 = moderate wilting and/or crown lesion; 3 = severe wilting and/or stem lesion; 4 = plant death.

Yield. Plants were harvested twice in 2018 at ten and eleven weeks after transplanting, and once in 2019 at nine weeks after transplant. Fruit was green at harvest, and only fruits 7.6 cm in length or longer were harvested. After harvest, fruits were sorted by treatment plot into "marketable" and "cull" categories based on appearance, counted, and weighed.

Data collection & statistical analysis. Area under the disease progress curve (AUDPC) was calculated using previously described methods (Shaner and Finney 1977) for average severity measurements and percent disease incidence for each subplot. AUDPC data was analyzed for statistical differences among treatments along with final disease incidence percentage and final severity ratings using PROC GLM in PC-SAS (Cary, NC). If significant treatment differences were found ($p < 0.05$), treatment means were subjected to a Fisher's Least Significant Difference test to identify significant differences among treatments.

Disease spread down rows was evaluated using PROC LOGISTIC in PC-SAS using final incidence ratings. Each plant in a treatment row was assigned a value from 1 to 13 based on its position within the row. Uninoculated plants (positions 4 to 13) were analyzed for disease

incidence based on treatment, position, and the interaction effect of treatment and position to determine if the biocontrol or fungicide treatments reduced disease spread down rows from inoculated plants.

RESULTS

Treatment effects on Phytophthora blight incidence and severity. ANOVA was performed on data for final incidence and severity of Phytophthora blight and area under the disease progress curve (AUDPC) for disease incidence and severity and are reported in Table 3.2. In both seasons, treatment had no significant ($p < 0.05$) effect on the final disease incidence, but significantly reduced AUDPC for both incidence and severity (Table 3.2). In 2018, final disease severity was significantly different among treatments, but not in the 2019 season.

In 2018, the 30 and 40 lb A^{-1} rate of *B. subtilis* significantly reduced AUDPC for both disease incidence and severity. The chemical control reduced AUDPC for incidence and severity to a larger extent than any level of the biocontrol (Tables 3.3 & 3.4; Figures 3.1, 3.2, 3.3, 3.4). In 2018, the 40 lb A^{-1} rate of *B. subtilis* reduced final disease severity to levels similar to the chemical control (Table 3.4; Figure 3.3a). Disease severity in the pathogen only untreated control was higher than the other treatments and not different than any rate of *B. subtilis* for AUDPC incidence and severity (Tables 3.3 & 3.4).

In 2019, *B. subtilis* at the 40, 30, and 5 lb A^{-1} application rates significantly reduced incidence AUDPC compared to the untreated control (Table 3.3; Figure 3.2). *B. subtilis* applications at 40 and 5 lb A^{-1} significantly reduced severity AUDPC compared to the untreated control (Table 3.4; Figure 3.4). For all tested rates of *B. subtilis*, final disease severity was not significantly different from the chemical control, and lower than the pathogen-only untreated control (Table 3.4; Figure 3.3).

Treatment effects on spatial spread of disease. A chi-square analysis of fit was done for final incidence in each treatment row to examine differences among treatments, plant position within row, and the interaction effect of treatment and position (Table 3.5). For 2018 and 2019, spatial

variables did not have any significant effect on disease incidence, indicating final incidence was evenly distributed across treatments and plant positions. As no significant correlations between treatment and disease incidence by plant position were detected, we conclude that none of the treatments affected disease spread down rows.

Yield. Yield data was collected in each year. Fruits were sorted by marketable, cull, and total fruit weight (Table 3.6). Treatment had no significant effect on marketable or cull fruit weight but did affect total fruit weight in 2018 (Table 3.2). Total fruit weight was greater in plots treated with *B. subtilis* at the 5 lb A^{-1} application rate than the other rates of *B. subtilis* and were not significantly different from the chemical or untreated controls (Table 3.6). When comparing the two growing seasons, yield in 2019 were much lower than in 2018 due to grazing by deer in the plots. Yield data for the 2019 trial was also sorted by deer damage, as there was extensive deer grazing in the 2019 trial. Moderately and severely defoliated plants were correlated with lower marketable fruit weight and subsequent lower total harvest weight than plots with less deer damage.

DISCUSSION

In both growing seasons, *Bacillus subtilis* AFS032321 provided control of Phytophthora blight of pepper. However, when comparing the 2018 and 2019 seasons, the overall conclusions about this isolate's efficacy in reducing disease were different. In the 2018 season, *B. subtilis* only reduced disease to a level comparable to the chemical control when applied at 40 lb A^{-1}. (Tables 3.3 & 3.4). In 2019, *B. subtilis* significantly reduced disease at 40, 30, and 5 lb A^{-1} rates of application, compared to an untreated control. In both years, *B. subtilis* was not as effective as the chemical control at reducing Phytophthora blight.

Much of the research into soilborne plant disease biocontrol has been in controlled settings in the greenhouse, but several field studies on biocontrol of *P. capsici* with *B. subtilis* have been published. Results of this trial are comparable to a 1995 field study on Phytophthora blight of pepper at two field sites in Florida in which *B. subtilis* 'Kodiak' significantly reduced disease at one site, and slightly reduced disease at the other site (Nemec et al. 1996). Another study done in Mexico included several isolates of *B. subtilis*, all applied singly. In that work, biocontrol was more effective than a chemical control on a root rot disease complex on bell pepper that included *P. capsici* (Guillén-Cruz et al. 2006).

The variability in biocontrol efficacy observed between the two seasons of this trial may indicate that environmental factors impacted biocontrol efficacy over two seasons. This may be due in part to increased rainfall in the 2019 season (Table 3.1). While *B. subtilis* has been observed to perform more favorably under more aqueous conditions (Reddy and Rahe 1989), *P. capsici* also favors saturated soils (Granke et al. 2012; Ristaino and Johnston 1999). Subsequently, it is also worth noting that Phytophthora blight incidence and severity was higher pathogen-only untreated control in the 2019 season compared to the 2018 season (Tables 3.3 & 3.4), although this increased

disease may also have been due to the fact that we inoculated more than once to get disease going in 2019. Greenhouse studies on this isolate's biocontrol efficacy have shown that efficacy declines under higher *P. capsici* disease pressure, which may also indicate a source of variability.

While some *B. subtilis* isolates have been observed to promote plant growth and therefore increase yield (Shafi et al. 2017; Sharma et al. 2015), the isolate we tested had little effect on pepper yield in our field tests. However, we did not measure plant growth in this work. The only significant treatment effect on yield was a weakly significant effect on total plant yield in 2018 (Tables 3.2 and 3.6), in which *B. subtilis* at the lowest rate of application significantly increased yield compared to higher rates of application. The 2019 season was not ideal for measuring this isolate's effects on pepper yield due to extensive yield losses from deer grazing later in the season; deer had fed on foliage and flowers, leading to higher incidence of sun scalding and decreased fruit set overall (Rabinowitch et al. 1983). It might be worthwhile to further investigate this isolate's potential as a plant growth promoter under more controlled conditions, as a product that manages disease while increasing crop yield is very attractive from a commercial standpoint (Brannen and Kenney 1997; Domenech et al. 2006; Shafi et al. 2017).

Biocontrol treatments have long been criticized for observed inconsistencies and variability in efficacy, especially in field settings (Guetsky et al. 2001; Handelsman and Stabb 1996; Hollywood 2004). Interestingly, this variability has been observed to decrease significantly when multiple biocontrol agents are applied in consortium, as opposed to a single biocontrol agent (Domenech et al. 2006; Guetsky et al. 2001). Postulated reasons for improved consistency in efficacy when using multiple biocontrol agents include increased rhizospheric competency and employment of different modes of action. The future of biocontrol and integrated pest management may involve formulations of 10-15 "core strains" of biocontrol agents for each crop system that all show activity

on a variety of pathogens and exhibit growth potential in a wide array of field conditions (Hollywood 2004).

Further research on this isolate and other potential biocontrol agents should focus on improving consistency in disease management and reducing efficacy variability. This can be accomplished through applying multiple biocontrol agents simultaneously or rotating biocontrol treatments with chemical controls, as both of these methods have shown improved efficacy over applying a single biocontrol (Guetsky et al. 2001, Fravel 2005). Research in controlled environments, such as greenhouses or growth chambers, that examines various environmental factors, such as soil pH and moisture, should also be performed to investigate the ideal rhizosphere conditions for this biocontrol agent and other biocontrol agents. In our other work, greenhouse screenings on this isolate of *B. subtilis* have shown that its biocontrol efficacy is independent of soil type, which is valuable information when developing new commercial biocontrol agents. Lastly, research into methods of antagonism for biocontrol candidates are important, especially when investigating the use of multiple antagonists for biocontrol, as isolates exhibiting different methods of antagonism would ideally synergize and provide better disease management than if applied singly (Guetsky et al. 2001; Hollywood 2004).

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TABLES

Table 3.1: Climate data for each growing season for a Phytophthora blight of pepper biological control trial, conducted at the Sandhills Research Station in Jackson Springs, NC. Data retrieved from the North Carolina Climate Office.

	2018			2019		
Weather Parameter	May	June	July[a]	May	June	July[b]
Mean maximum daily air temperature (°C)	25.9	32.1	32.6	29.0	29.9	32.9
Mean daily air temperature (°C)	16.5	25.8	26.3	23.1	24.2	26.7
Mean daily soil temperature (°C)	25.2	29.8	30.7	26.3	27.8	29.8
Mean soil moisture (m^3/m^3, expressed as %)	16.1	15.8	16.2	17.3	15.5	20.2
Total rainfall (mm)	46.0	39.1	25.6	64.5	107.9	59.9

[a]July 1-July 15 (trial takedown).
[b]July 1-July 17 (trial takedown).

Table 3.2: ANOVA (F and p-values) for disease and yield data as affected by treatments (four rates of *B. subtilis* or mefenoxam) in each of two years of a Phytophthora blight of pepper in the Sandhills field trial.

		2018		2019	
	Measurement	F-value	p-value [b]	F-value	p-value
Disease	Final Disease Incidence	2.78	0.054	1.41	0.267
	Incidence AUDPC[a]	3.89	0.017*	5.90	0.002*
	Final Disease Severity	2.98	0.044*	2.01	0.125
	Severity AUDPC	3.41	0.028*	5.27	0.037*
Yield	Marketable Weight	2.57	0.069	0.23	0.945
	Cull Weight	2.52	0.073	0.98	0.459
	Total Weight	2.92	0.046*	0.48	0.789

[a]: Area under the disease progress curve.
[b] Statistically significant p-values ($p < 0.05$) are marked with an asterisk. Differences between treatment means were separated using a Fisher's Least Significant Difference test.

Table 3.3: Final disease incidence and AUDPC for Phytophthora blight as impacted by four rates of biocontrol and mefenoxam for two growing seasons.

	Final Disease Incidence (%)		AUDPC[x]	
Treatment and rate A^{-1}	2018	2019	2018	2019
Untreated Control	44.23	50.00	11.5 a[y]	19.9 a
B. subtilis (40 lb)	41.03	34.62	9.6 ab	11.6 b
B. subtilis (30 lb)	58.97	38.46	10.5 a	11.0 b
B. subtilis (15 lb)	50.00	26.93	12.0 a	13.5 ab
B. subtilis (5 lb)	42.31	34.61	13.2 a	11.0 b
Ridomil Gold (1 pt)	26.92	21.15	4.7 b	1.3 c
LSD, 5%	NSD[z]	NSD	4.9	7.3

[x] Area under the disease progress curve.
[y] If ANOVA treatment means were significant ($p<0.05$), then differences between treatment means were separated using a Fisher's Least Significant Difference test. Treatment means followed by the same letter were not significantly different.
[z]No significant difference among treatment means.

Table 3.4: Final disease severity ratings and AUDPC for Phytophthora blight as impacted by four rates of biocontrol and mefenoxam across treatments for two growing seasons.

	Final Severity		AUDPC[x]	
Treatment and Rate A^{-1}	2018	2019	2018	2019
Untreated Control	1.44 a [b]	1.62	37.9 a	63.7 a
B. subtilis (40 lb)	1.33 ab	1.25	25.6 ab	38.0 b
B. subtilis (30 lb)	1.82 a	1.25	28.7 ab	38.2 b
B. subtilis (15 lb)	1.46 a	1.02	36.3 a	47.2 ab
B. subtilis (5 lb)	1.60 a	1.21	40.8 a	34.3 b
Ridomil Gold (1 pt)	0.92 b	0.67	14.2 b	4.0 c
LSD, 5%	0.50	NSD[z]	17.1	25.3

[x] Area under the disease progress curve.
[y] If ANOVA treatment means were significant ($p<.05$), then differences between treatment means were separated using a Fisher's Least Significant Difference test. Treatment means followed by the same letter were not significantly different.
[z] No significant difference among treatment means.

Table 3.5: Chi-square values for tests of significance for Phytophthora blight disease incidence in plants among treatments (one of four rates of biocontrol, mefenoxam, or an untreated control), plant positions within rows, and the interaction effect of treatment and position in row. Data was taken from final disease ratings in each growing season.

	2018		2019	
Metric	Chi^2-value	p-value	Chi^2-value	p-value
Treatment	0.11	0.740	2.22	0.137
Position	0.01	0.922	1.14	0.286
Treatment*Position	0.58	0.446	0.22	0.642

Table 3.6: Yield of bell pepper as impacted by Phytophthora blight, four rates of *B. subtilis* or mefenoxam for two growing seasons.

	Marketable (kg) [x]		Cull (kg)		Total (kg)	
Treatment and Rate (A^{-1})	2018	2019	2018	2019	2018	2019
Untreated Control	4.7	1.0	2.3	2.8	6.9 abc [y]	3.8
B. subtilis (40 lb)	3.4	1.1	1.9	2.8	5.3 a	3.9
B. subtilis (30 lb)	4.2	1.0	1.8	2.3	6.0 ab	3.3
B. subtilis (15 lb)	3.9	1.5	1.5	3.3	5.4 a	4.8
B. subtilis (5 lb)	6.1	1.0	2.5	3.0	8.6 c	4.0
Ridomil Gold (1 pt)	6.0	1.2	1.8	2.3	7.8 bc	3.5
LSD, 5%	NSD[z]	NSD	NSD	NSD	2.3	NSD

[x]Fruits were harvested at the green stage and separated into either marketable or cull categories, dependent on appearance. The 2018 data is pooled from two harvests, while 2019 data is from a single harvest.

[y] If ANOVA treatment means were significant ($p<.05$), then differences between treatment means were separated using a Fisher's Least Significant Difference test. Treatment means followed by the same letter were not significantly different.

[z]No significant difference among treatment means.

FIGURES

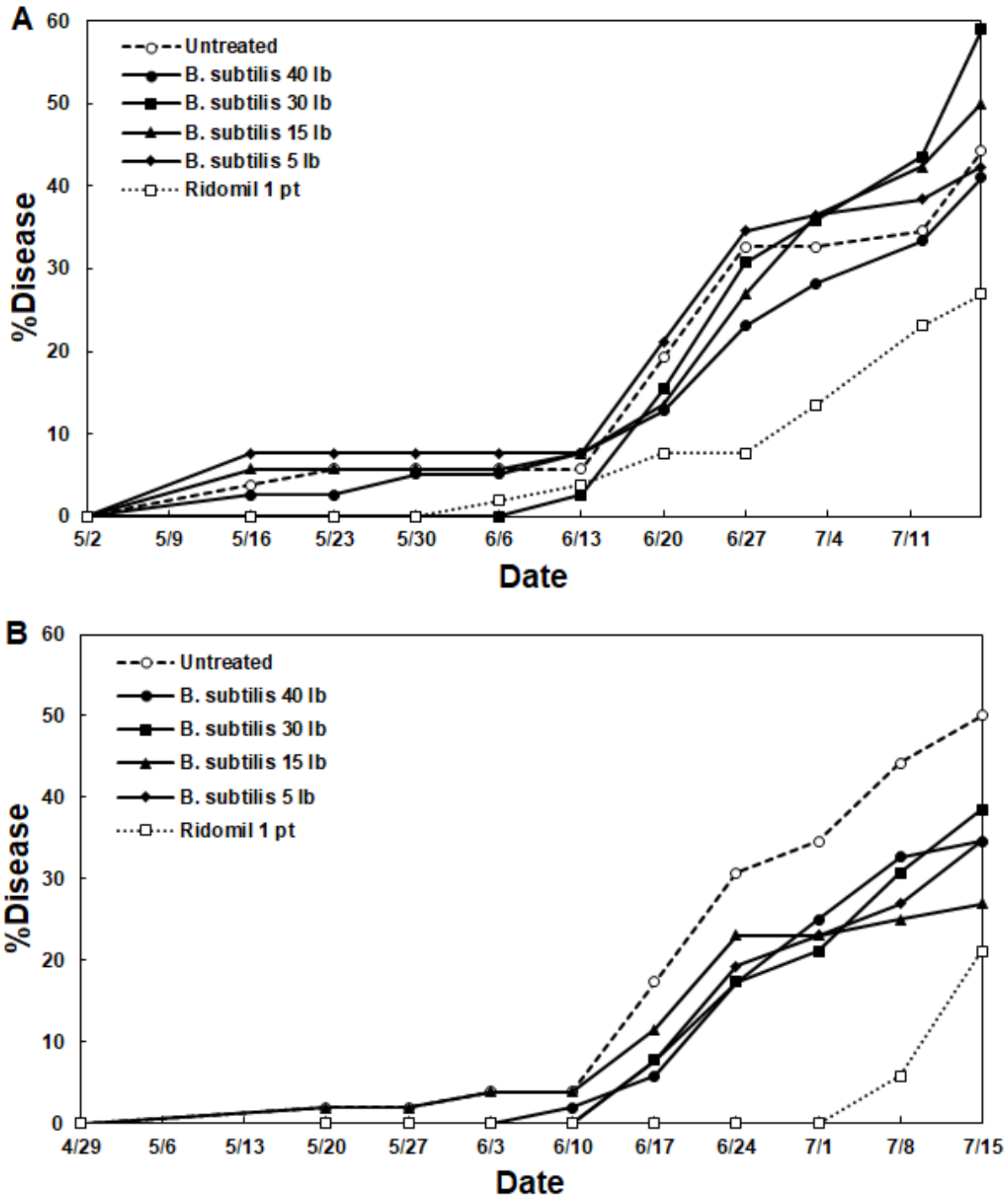

Figure 3.1: Phytophthora blight disease incidence on susceptible bell peppers in the field in 2018 (A) and 2019 (B) as affected by four rates of *B. subtilis* or mefenoxam.

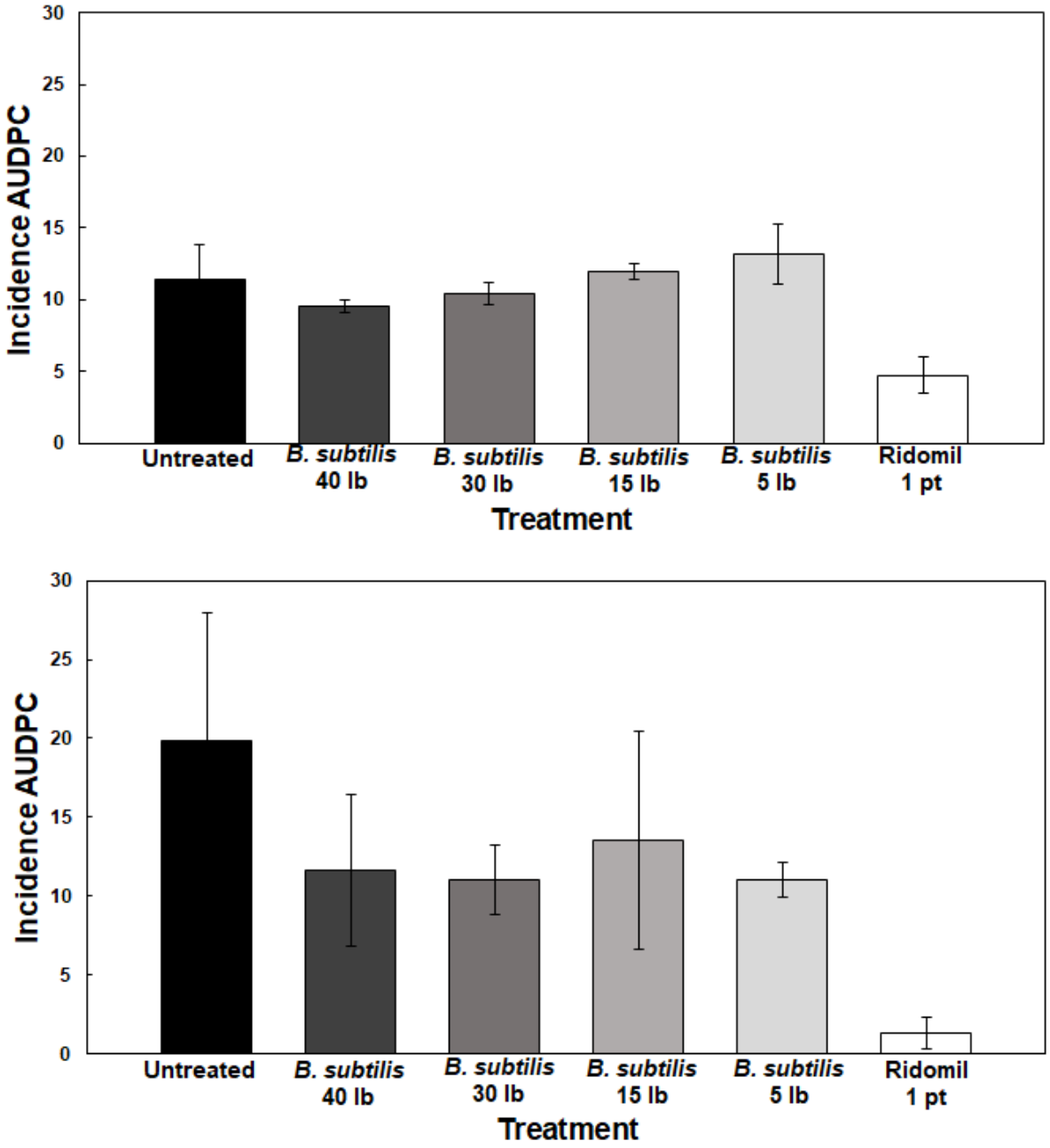

Figure 3.2: Area under the disease progress curve (AUDPC) for Phytophthora blight incidence on susceptible peppers in the field in 2018 (A) and 2019 (B) as affected by four rates of B. subtilis or mefenoxam.

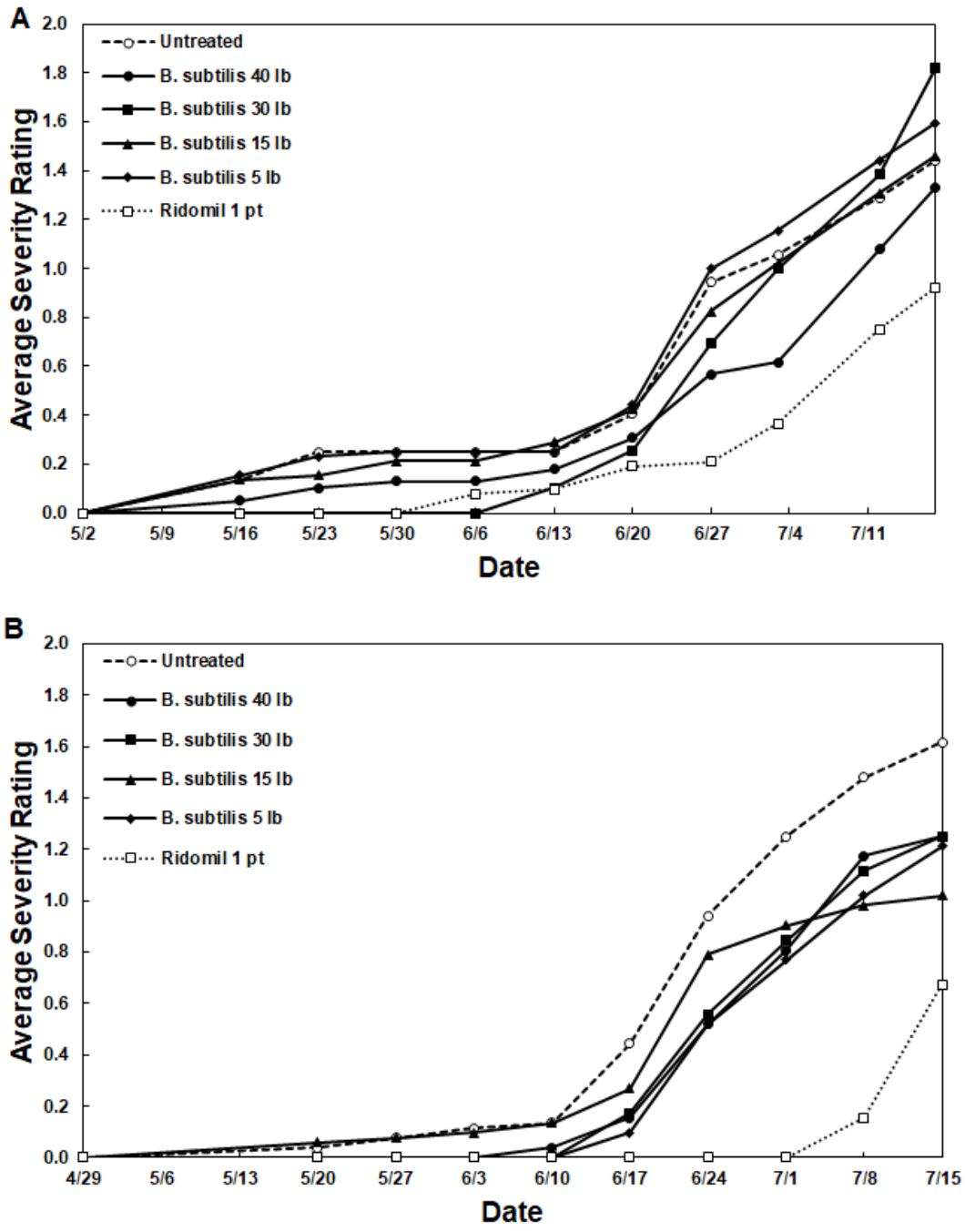

Figure 3.3: Phytophthora blight disease severity on susceptible bell peppers in the field in 2018 (A) and 2019 (B). Each data point represents the average severity rating, on a scale of 0-4 of Phytophthora blight in a treatment at that timepoint, averaged over four treatment rows, each containing thirteen plants.

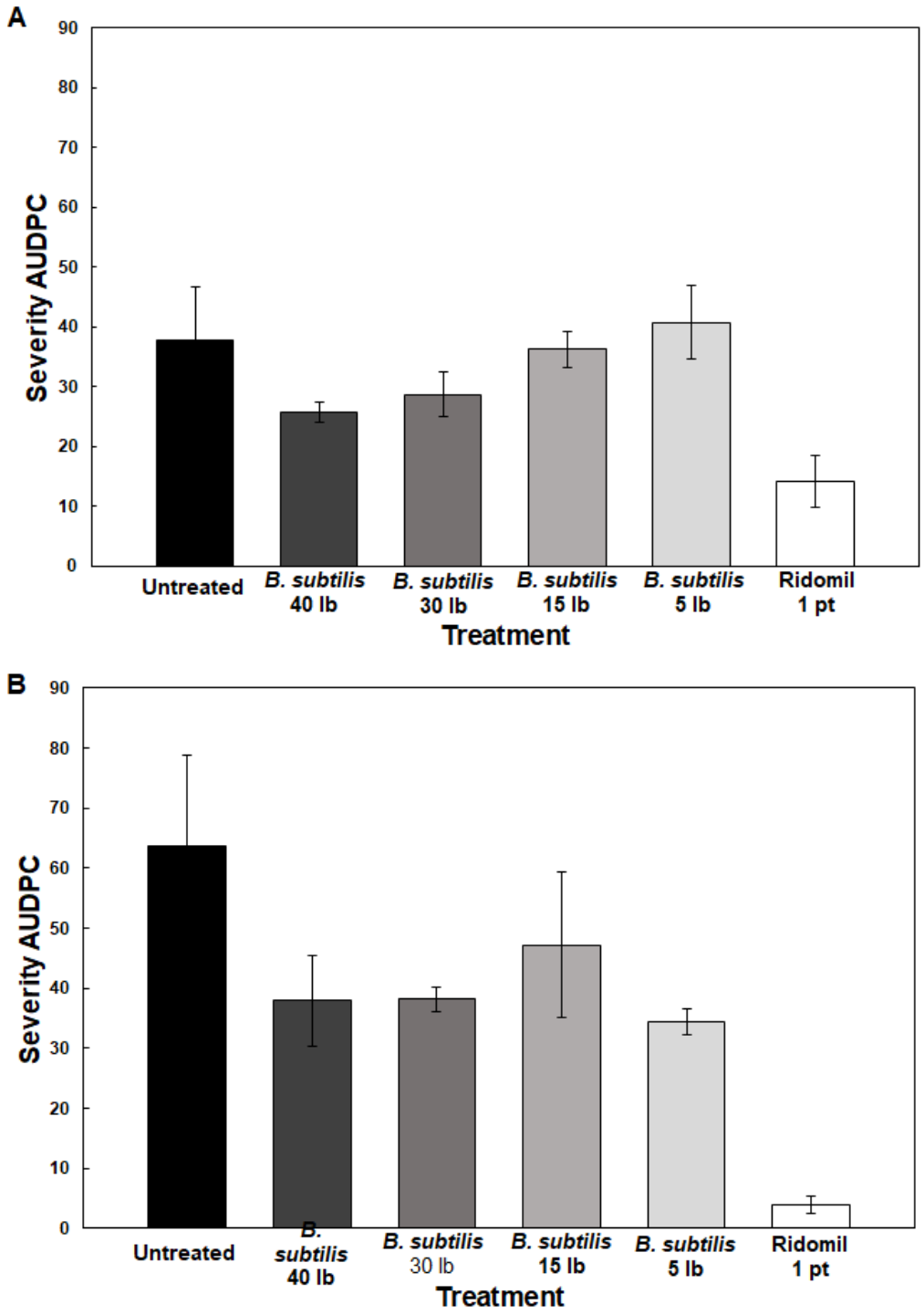

Figure 3.4: Area under the disease progress curve (AUDPC) for disease severity of Phytophthora blight in bell peppers in 2018 (A) and 2019 (B) in the field. Error bars represent standard errors of the mean.