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Evaluations of biological control agents for the management of soybean cyst nematode (*Heterodera glycines*) in soybean (*Glycine max* L. Merr.)

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EVALUATIONS OF BIOLOGICAL CONTROL AGENTS FOR THE MANAGEMENT OF SOYBEAN CYST NEMATODE (*Heterodera glycines*) IN SOYBEAN (*Glycine max* L. Merr.)

by

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EVALUATIONS OF BIOLOGICAL CONTROL AGENTS FOR THE MANAGEMENT OF SOYBEAN CYST NEMATODE (*Heterodera glycines*) IN SOYBEAN (*Glycine max* L. Merr.)

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The soybean cyst nematode (SCN, *Heterodera glycines*) is the most yield limiting pathogen of soybeans (*Glycine max* L. Merr.). Current management strategies of crop rotation and using resistant varieties are not completely effective and alternative management strategies are needed. Commercial seed treatments with biological agents are available to protect against yield loss from SCN, but have not been evaluated in Nebraska. Field studies were conducted in eight Nebraska locations (six infested with SCN and two non-infested) during 2014 and 2015 to evaluate seed treatment effects on soybean establishment, SCN population density, and yield. The seed treatments were CruiserMaxx® Advanced, Clariva®Complete Beans containing Clariva®pn (*Pasteuria nishizawae*), and Poncho®/ VOTiVO® containing *Bacillus firmus* I-1582; all treatments contained the same fungicides and an insecticide with the same mode of action. Average yields in the SCN infested fields ranged from 45 to 72 bu/A and initial SCN population densities ranged from 200 to 4,300 eggs/100 cc's of soil. No statistical differences were found among the three treatments in either yield or SCN reproduction

at any individual location or when the SCN infested locations were combined in either growing season. The use of cover crops (cereal rye, *Secale cereale*), and other bacteria have inconsistently reduced SCN populations in previous studies. The use a cover crop as a means to establish a biocontrol agent has not been investigated. Greenhouse experiments were conducted to evaluate the ability of the bacterium *Lysobacter enzymogenes* C3 to colonize the rhizospheres of cereal rye and soybean from populations applied to seed. The bacterium was found to colonize cereal rye roots to higher population levels than soybean over 4 week periods. C3 root populations on cereal rye increased by a thousand fold from seed populations. Based on these studies the potential for biocontrol for SCN exists, but more research is needed to determine optimum conditions for biocontrol agents to be effective tools in sustainable soybean production.

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CHAPTER I

THE SOYBEAN CYST NEMATODE AND INTEGRATING BIOLOGICAL CONTROL IN MANAGEMENT STRATEGIES

1.1 Soybean Production and Major Diseases Affecting Yield

Soybean (*Glycine max* L. Merr.) was cultivated over 5,000 years ago in China and since then has become an important agricultural crop in many countries (Singh and Shivakumar, 2010). However, only within the last 200 years have the importance and demand of soybean production increased. Soybeans have the highest protein and vegetable oil output of crops, giving it high commercial value (LiJuan, Ruzhen, Singh, 2010; Singh and Shivakumar, 2010). Due to this, soybeans are mainly cultivated for oil extraction and protein, where oilseed production in the United States is mainly accounted for by soybeans (Ash et al., 2006; Singh and Shivakumar, 2010). Over the past ten years the production rates of soybeans have steadily increased and the United States is the number one producer globally, only trailing corn in crop production (Hartman et al., 2011; Sadras et al., 2014). In 2015, there were 82,650,000 acres of soybeans planted yielding 3,929,885,000 bushels (USDA ERS, 2016). More than 80% of these acres are produced in the North Central region of the United States, even though soybeans can be grown in a variety of temperate climates (Ash et al., 2006; Cooper et al., 2008). Nebraska ranks fifth in production of soybeans and has the highest average yield per acre (USDA & NASS, 2016).

A variety of different diseases that can reduce soybean production. Between the years of 1996-2007 a survey was conducted in the states that produce soybeans on the effects of disease on soybean yield (Wrather and Koenning, 2009). During those years

the soybean cyst nematode (*Heterodera glycines*; SCN) suppressed yield each year more than any other disease. On average, SCN causes a loss of 120 million bushels a year, which would amount to a loss of $720 million a year from the disease (Wrather and Mitchum, 2010). Phytophthora root and stem rot (*Phytophthora sojae*), seedling diseases (*Rhizoctionia solani*, *Pythium spp.*, *Fusarium spp.*) and Sudden Death Syndrome (*Fusarium virguliforme*) all suppressed yield significantly and were ranked from 2nd to 5th depending upon the year and region (Wrather and Koenning, 2009). Unlike the other top diseases on soybean, damage caused by SCN is not restricted to any particular region and is a problem throughout soybean producing areas (Niblack, 2005).

1.2 Soybean Cyst Nematode

1.2.1 Origin and Distribution

SCN has been observed since 1881 in Japan, but was officially reported in 1915 and was often mistaken for *Heterodera schachtii* or other *Heterodera* species (Hori, 1916). Korea (1936), Manchuria (1938), once an independent state and now located in China, and the United States (1954) all reported SCN shortly thereafter (Yokoo, 1936; Nakata and Asuyana, 1938; Winstead, Skotland, and Sasser, 1955). SCN was first found in North Carolina in the United States, where it is thought to have been imported from Japan through bulbs and soil samples (Spears, 1955; Noel, 1986). The pathogen rapidly spread across the country through farm machinery, flood waters, contaminated seed, wind, and birds (Riggs, 1977). SCN was first discovered in Nebraska in 1986, and can now be identified in 58 counties throughout the state (Powers et al., 1989; Wilson and Giesler, 2016). Once discovered in a field, the distribution of SCN is not uniform and will

be random or aggregated in certain topography elements (Wrather and Mitchum, 2010). Reproduction will depend upon the soil type, presence of crop host, and presence of natural enemies of SCN (Wrather and Mitchum, 2010).

1.2.2 Life Cycle

Understanding the life cycle and how the SCN can reproduce on other plant hosts is vital in how the disease is managed. The optimal soil temperature for SCN reproduction is 25°C and takes 28 days to complete (Wrather, Anand, and Dropkin, 1984; Chen et al., 2011). When the environment is not at optimal conditions the length in the SCN life cycle can be extended to 40 days allowing for the possibility of several lifecycles to complete during a field season (Wrather, Anand, and Dropkin, 1984). This obligatory parasite begins its life cycle when second-stage juvenile nematodes (J2) hatch from eggs in the soil from external signals, plant exudates, and from internal signals (Niblack, 2005; Niblack et al., 2006). What causes SCN eggs to become dormant are more understood than the hatching mechanisms, as soil temperature, insufficient host, or both, will cause dormancy and no hatching will occur until favorable conditions resume (Niblack et al., 2006). Once hatched, the J2 locates the host root and pierces the cell wall using its stylet and enzymatic degradation (Niblack, 2005). Once inside the root, the J2 creates a feeding site called a syncytium to obtain nutrients, where the developing nematodes are immobile at this stage and swell into a sausage like state (Figure 1.1 B) (Niblack, 2005; Chen et al., 2011). Three molts of the nematode occur inside the host plant, where at the end of the last molt sex is determined. The male nematodes leave the root, regaining their vermiform shape. Females continue to swell,

until they eventually break through the root system. Males search for the females that have broken through the root to mate and anywhere from 40 to 600 eggs could be produced inside the female body (Niblack, 2005). On average 200 eggs are developed inside the female and some of the eggs are deposited in a gelatinous matrix on the outside of the body (Figure 1.1 C). The female body then dies, changing in color from a yellow to dark brown (Figure 1.1 D). This structure is now called a cyst and is the female nematodes body forming a tough protective layer to protect the eggs inside. Eggs can remain viable inside a cyst for many years and hatch when the conditions are again optimal. SCN can be moved throughout the soil by equipment, floods, wind, or animals, but generally only moves a few centimeters on its own throughout the year creating patches in a field.

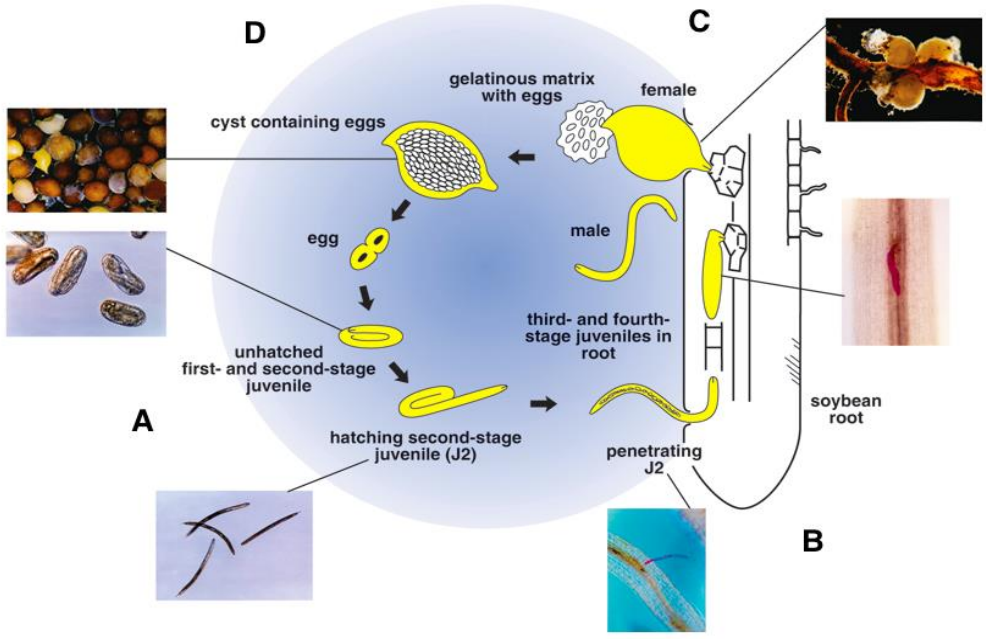

Figure 1.1. Life cycle of soybean cyst nematode (SCN) on a root system where the entire life cycle takes around 24-28 days at 25°C and can have multiple cycles in a growing season (Chen et al. 2011; drawn by Dirk Charlson, Iowa State University).

1.2.3 Field Symptoms

When SCN is present at high population densities in a field, symptoms begin to be exhibited by the host plant. However, lower soybean yield may be the only above ground symptom, where 25-30% yield loss has been observed (Wilson and Giesler, 2016; Wrather and Mitchum, 2010). Along with decreasing yield, plants may display stunting and chlorosis; however, there are many crop production problems that can result in these same symptoms. Having the potential of numerous soybean health problems presenting these symptoms, it is key to look for the presence of cysts on potentially damaged plants or to collect a soil sample for SCN analysis. Cysts can visually be detected on the root systems of soybeans a month after infection, but they are difficult to observe when populations are low. Proper field soil sampling will ensure that SCN population densities are correctly assessed. Once population densities of SCN in a field are known then proper management practices can be implemented. Population densities are based on SCN egg counts per 100cc's of soil where 0 eggs are non-detectable, up to 500 eggs is very low, 500-2,000 is low to moderate, 2,000-5,000 is moderate to high, and greater than 5,000 is a high population density of SCN (The soybean cyst nematode problem, n.d.). SCN population densities can range from 0 to 136,000 eggs per 100cc's of soil in Nebraska soybean fields, but initial SCN population densities average between 1,700 to 2,100 eggs per 100cc's of soil (Giesler and Wilson, 2011).

1.3 SCN Management Strategies

When implementing management strategies, it is important to know that SCN populations are suppressed and not eradicated through any practice. The cyst structure allows SCN to persist in the soil environment, even under adverse conditions (Wrather and Mitchum, 2010). If a field is not infested with SCN, it is important to avoid the introduction of the pathogen. The nematode moves any way that the soil can move, through machinery or on infected plant material (Riggs, 1977). Since eradication is not possible, being diligent to keep a field SCN free to prevent introduction is key. Once a field is identified to have SCN there are practices to manage the density of the nematode. These practices include the use of resistant cultivars, crop rotation to a non-host, chemicals, tillage, and control of alternative hosts. Even though these are the common management practices to suppress SCN, they are not always effective.

1.3.1 Host Resistance

Resistant cultivars are the most effective practice to manage SCN (Chen et al., 2001a). There have been seven sources of resistance to SCN identified, but there are only three that are commercially available including PI 88788, PI 437654 (Hartwig or CystX), and PI 548402 (Peking). Out of these three, around 95% of the SCN resistant varieties come from PI 88788 resistant source (Chen et al., 2011). Peking resistance was found to be inherited through four genes, *rhg1*, *rhg2*, *rhg3*, and *Rgh4*, where *Rhg4* is dominantly inherited compared to recessive inheritance of the other three genes (Caldwell et al., 1960; Matson and Williams, 1965). PI 88788 has an additional resistance gene of *Rhg5*, which is also dominantly inherited (Rao-Arelli, 1994). The greatest

resistance of SCN is held on the *rhg1* and *Rhg4* regions (Concibido et al., 2004). In cultivars with Peking resistance, the host response upon SCN infection is necrosis and cell wall thickening of the syncytium (Endo, 1965; Riggs et al., 1973). The disintegration of the syncytium cells is the host response for cultivars with PI 88788 upon infection with SCN (Kim et al., 1987).

1.3.2 Crop Rotation

Rotating to a non-host crop where SCN cannot reproduce helps reduce population densities (Niblack, 2005), and can greatly reduce population densities of SCN after one year of rotation, generally to non-damaging levels (Koenning et al., 1993; Perez-Hernandez, 2013). However, multiple years of rotation might be necessary if SCN population densities are high. The number of years in rotation, geographical location, and nematode density all impact the effectiveness of a crop rotation (Miller et al., 2006). From greenhouse studies, SCN population densities were lower on monocots (corn, wheat, barley, and oats) compared to leguminous plants (Warnke et al., 2006), and a rotation to corn is a common practice in many soybean growing areas (Niblack, 2005; Giesler and Wilson, 2011). Rotation to corn annually reduces SCN population densities, where Nebraska field studies have shown SCN population decreases ranging from 0 to 94% (Perez-Hernandez, 2013).

1.3.3 Chemical Control

Historically, nematicides had been successful in the management of parasitic nematodes, however, they are also toxic to the environment and can cause adverse health effects on humans if they build up as residues in the soil and infiltrate the ground

water (Abawi and Widmer, 2000). Many products have been banned by the Environmental Protection Agency, creating a need to replace these products (Frye, 2009). Nematicides are available for use in soybean fields infested with SCN, however, they do not provide protection over the entire season (Hooks et al., 2011), and are generally not a cost effective practice (Koenning et al., 1993). Fluopyram (ILeVO®, Bayer CropScience) is a fungicide that is available as a seed treatment that also has nematicidal activity (Zaworski, 2014). Based on greenhouse studies, treatments with fluopyram had a reduction in SCN populations compared to seed without the fungicide (Zaworski, 2014; Broderick et al., 2015). Aldicarb (Temik), is a nematicide that is not marketed for SCN control unless population densities are high due variability of chemical activity with soil texture and environmental conditions (Frye, 2009).

1.3.4 Effects of Soil Texture and Tillage

Tillage practices on plant-parasitic nematodes, including SCN, have yielded inconclusive results (Chen et al., 2001b). In Midwestern regions (Minnesota, Illinois, and Iowa) SCN reproduction was higher in no-till fields, when only tillage practices were taken into consideration (Thomas, 1978; Chen et al., 2001b; Noel and Wax, 2003). The amount of crop residue, environmental conditions, soil type, and initial SCN population density are all additional factors that could affect tillage interactions with SCN population densities (Chen et al., 2001b). No-till practices have been show to slow the spread of SCN (Chen et al., 2001b). Soils across the US with a high clay content were found to be negatively correlated with SCN population densities in no-tilled fields (Workneh et al., 1999).

SCN and microbe survival are affected by soil moisture, which is related to soil texture (Alston and Schmitt, 1988). Through tillage practices, organic matter is distributed throughout the soil profile and left concentrated on-top in no-till, causing microbial biomass to be high on the surface of no-till soils (Arshad et al., 1990; Angers et al., 1993; Kandeler and Böhm, 1996). Tillage has also been shown to reduce the aggregate stability, which are necessary as a microhabitat for microorganisms (Lienhard et al., 2013). These microhabitats are places where opportunistic parasitic bacteria could survive (Tian et al., 2007).

1.3.5 Controlling Alternative Hosts

Controlling weed populations in infested fields is another important management practice to keep population densities of SCN from increasing. Alternative hosts of SCN, especially winter annual weeds identified under greenhouse conditions, include Purple deadnettle (*Lamium purpureum* L.), henbit (*L. amplexicaule* L.), field pennycress (*Thlaspi avense* L.), and shepherd's purse (*Capsella bursa-pastoris* L. Medik) (Venkatesh et al., 2000; Creech et al., 2007). Henbit and field pennycress are of the main concerns in Nebraska, as SCN has been observed to complete its lifecycle under field conditions on these species (Giesler and Wilson, 2011). Improper management of winter annuals could allow for the increase of SCN population densities, however, it depends upon the density of weeds in a field and environmental conditions (Creech et al., 2008). Winter annual weeds can be controlled through herbicides and winter cover crops (Creech and Johnson, 2006).

1.3.6 Challenges

One of the main issues with current management strategies is that not all of the resistance sources are widely available. The common use of PI 88788 in the Midwestern regions allows for a shift in SCN population virulence reducing the effectiveness of the resistant varieties with virulence ranges from 60-78% on PI 88788 in Missouri, Illinois, and Minnesota (Mitchum et al., 2007; Niblack et al., 2008; Zheng and Chen, 2011). Rotation out of soybean and to a non-host crop may become economically unfeasible during the longer necessary rotations if the non-host crop has a lower value or is restricted by a government program (Koenning et al., 1993). Soil or seed treatments with a nematicide may not be economically feasible because they are dependent on multiple variables including, soil texture, environment, and initial SCN population densities (Frye, 2009). Sustainable management strategies to affect SCN are being developed to address current challenges for SCN management soybean production.

1.4 Biological Control for SCN

1.4.1 Soil Inhabitants

There are numerous genera of microorganisms that reside in the soil as natural enemies of nematodes (Tian et al., 2007). Two of the more extensively studied genera of bacteria are *Bacillus* and *Pasteruia* (Emmert and Handelsman, 1999; Meyer, 2003). Strains of these species have been used commercially as biological control agents against nematodes and each has been shown to reduce SCN population density (Noel and Stanger, 1994; Siddiqui and Mahmood, 1999).

1.4.2 Bacillus

Microorganisms in the genus *Bacillus* are free living bacteria that exist as saprotrophs in the soil in association with plants (Emmert and Handelsman, 1999). *Bacillus* spp. produce resistant spores called endospores that allow for increased bacterial survival as well as formulation into stable products (Emmert and Handelsman, 1999). Species in the genus *Bacillus* are known as a rhizobacteria because they are able to colonize the root systems of plants (Siddiqui and Mahmood, 1999). On a colonized plant, they can improve plant growth and suppress diseases through the production of metabolic-by-products, enzymes, toxins, or induced resistance (Emmert and Handelsman, 1999; Tian et al., 2007). *Bacillus firmus* is a species that has been shown to be antagonistic against plant parasitic nematodes, specifically *Meloidogyne* spp. that are also sedentary endoparasites like SCN (Wilson and Jackson, 2013). *M. incongnita* populations are reduced through *B. firmus* endospores colonizing eggs, and producing secondary metabolites that decrease egg hatching and paralyze juveniles (Mendoza et al., 2008). This was also made into a biological control seed treatment through Bayer Crop Science®, producing Poncho/VOTiVO™, in which an insecticide (Clothianidin) and the spores of *B. firmus* (I-1582) coat the seed (Bayer CropScience, 2016). This bacterium is a rhizobacteria and colonizes the roots of the host plant to prevent nematode attraction to the root by consuming root exudates, competing with the nematode for nutrients and space (Siddiqui and Mahmood, 1999; Bayer CropScience, 2016). *B. firmus* continues to grow along with the root system providing a living protection against SCN,

where the nematode will seek out an alternate food source or die from starvation (Bayer CropScience, 2016).

1.4.3 Pasteruia

All *Pasteuria* spp. are obligate parasites affecting a range of nematode species. *Pasteuria nishizawae* (Pn), is the only species of *Pasteruia* observed to parasitize SCN (Siddiqui and Mahmood, 1999; Noel et al., 2005). When juveniles of SCN encounter endospores of Pn in the soil, the endospores attach to the nematode cuticle (Atibalentja et al., 2004; Noel et al., 2005). Once the juvenile enters the root of soybean, the endospore germinates to create a germ tube that penetrates the cuticle of the nematode (Figure 1.2 A). Microcolonies then develop inside the body of the nematode and eventually spread throughout the entire body (Figure 1.2 B). The microcolonies of Pn have only been observed in the bodies of juvenile or adult females and in cyst, but never in juvenile or adult males (Noel et al., 2005). Then once the parasitized juvenile female or cyst decomposes the mature endospores that are contained inside of parasitized female are released back into the soil. Endospore attachment has been observed on other *Heterodera spp.* but, the life cycle of the bacterium cannot be completed on other nematode species. Being an obligate parasite, it was previously difficult to produce the endospores that infect the nematodes without first rearing them on their host (Sayer et al., 1991). In the last decade, cultures were able to be produced from a single species without a living nematode host (Atibalentja et al., 2004; Gerber et al., 2006). These advancements allowed for the production of a biological seed treatment Clariva™pn (Pn-1) (Syngenta® Crop Protection) (Hewlett et al., 2013).

Clariva™ Complete Beans contain seed treatment applications of insecticide, fungicide, and the Pn-1 endospores which are classified as a biological nematicide (Callanan and Alderfer, 2014).

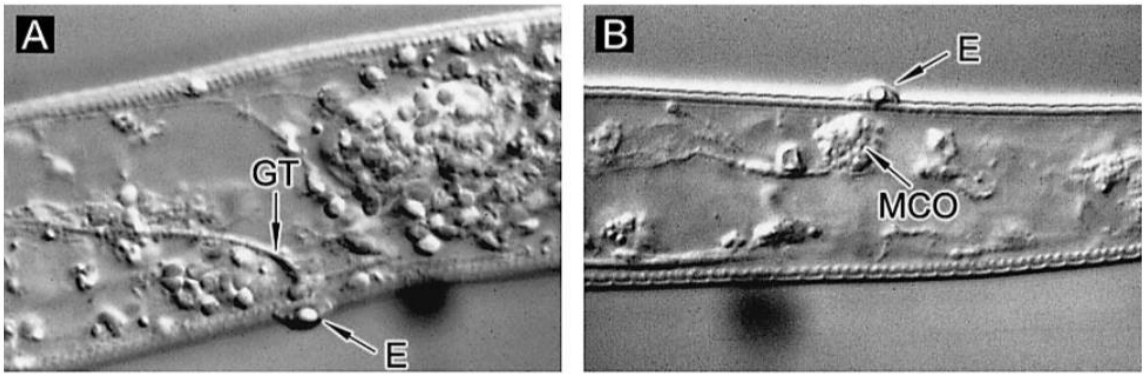

Figure 1.2. Life cycle of *Pasteuria* spp. NA isolate from North America that parasitizes SCN. A) A *Pasteuria* endospore (E) attached to juvenile nematode (J2) cuticle and a germ tube (GT) is generated to penetrate the cuticle once the J2 enters the root system. B) Underneath the endospore, primary vegetative microcolonies (MCO) are formed inside J2, J3, and immature females where they spread throughout the entire nematode body and the parasitized females ultimately release new endospores (Atibalentja et al., 2004).

1.4.4 Lysobacter

Another genus of soil and root inhabiting bacteria that has strains with promise as potential biological control agents of SCN is *Lysobacter*. This is another bacterial group that is shown to be a component of suppressive soils, but little is known about its population dynamics in soil (Postma et al., 2011). No spore structures are produced to allow the bacterium survive harsh environmental conditions, but they have a range of micropredatory activity (Christensen and Cook, 1978). *Lysobacter* species can be found in diverse habitats, but are mainly isolated in soil and water environments (Hayward et al., 2010). *Lysobacter antibiotcus* was shown to be effective in controlling root knot nematodes on tomato in field experiments (Zhou et al., 2016). *Lysobacter enzymogenes*

strain C3, first identified from the foliage of Kentucky bluegrass in Nebraska (*Poa pratensis* L.), has been extensively studied in a wide array of biocontrol activity (Giesler and Yuen, 1998). Being able to produce extracellular enzymes, antibiotics, and colonize host systems have allowed C3 to help control plant pathogens. Whereas *Lysobacter spp.* have been studied for use as biocontrol agents and have had issues with large scale success (Hayward et al., 2010). The mechanisms of how C3 effects nematode populations are still in the beginning stages and not well understood (Hayward et al., 2010). In laboratory experiments, C3 was previously shown to be nematistatic and inhibit SCN reproduction of *H. schachtii* (Chen et al., 2006). One possible mechanism is that C3 produces an extracellular enzyme, chitinase, and the egg cases of cyst nematodes have a layer of chitin allowing the bacteria to degrade them. Another mechanism is the production of an antifungal secondary metabolite HSAF (dihydromaltophilin) that was found to be toxic to nematodes (Yuen et al., 2006). Field efficacy against SCN has not been verified with C3 because practical methods to introduce the bacterium into the soil profile have not been developed. Since this bacterium cannot produce spores, this limits the introduction method into the soil profile directly to the soil or to a seed due to the poor survival rates.

1.4.5 Cover Crops

Biological control is the suppression of an organism through the use of another organism, making some non-host crops have the potential to be biological controls (Gardener and Fravel, 2002). There are some non-host crops that can reduce nematode population densities through releasing toxins that are detrimental to the nematodes,

but the mechanisms are not fully understood (Miller et al., 2006). One mechanism that is thought to allow for the reduction of a variety of soil diseases is the development of a suppressive soil (Eastburn, 2013). Grass cover crops, such as cereal rye (*Secale cereale*) and annual rye grass (*Lolium multiflorum*), can result in a reduction in population density of SCN from the nematode not having a suitable host (Hoorman, 2011). Annual ryegrass can be difficult to control if not managed properly and become a competitor to annual crops (Ackley, 2013). Cereal rye in comparison is the best cool season cover crop, where rather than becoming the competitor it naturally suppresses weeds allelopathically, it can decrease multiple soybean diseases including SCN, just not consistently in field studies (Eastburn, 2013; Rye, 2012). Among the other benefits of cereal rye as a cover crop are reduced soil erosion, the enhancement in soil tilth, and the ability to recycle nutrients (Zasada et al., 2005). Addition of organic matter from the cover crops allows for the enhancement of microorganism diversity that are naturally present in the soil to help prevent disease (Garbeva et al., 2004). Microbial communities have been found to be more diverse after a rotation of different crops (grassland versus arable) with more suppressive microbes in grassland soils (van Elsas, Garbeva, and Salles, 2002; Garbeva et al., 2004). There is preliminary evidence that C3 favors grass root systems compared to non-grass (Yin, 2010). This provides a potential solution where C3 could establish on grass root systems in between soybean growing seasons.

1.5 Field Trials with Commercial Biological Controls

Field trials of Poncho/VOTiVO™ and Clariva™ have taken place in Wisconsin, Michigan, Iowa and Minnesota to determine the efficacy of the seed treatments in

different environments. In each study the biological control agents were compared to a standard fungicide and insecticide seed treatment. In Minnesota the field trials with Poncho/VOTiVO™ had no improvement in yield (Koch and Rich, 2016) and neither did the trials with Clariva™ along with no differences in SCN reproduction between seed treatments (Potter et al., 2015). Yield was also not improved by Clariva™ in Michigan making this not cost effective from the additional seed treatment cost (Staton and Seamon, 2015). Poncho/VOTiVO™ consistently increased plant stand in Wisconsin, however, yield was not increased compared to a base fungicide and insecticide treatment (Gaspar et al., 2014). Iowa was the only state were the results showed significant yield differences ranging -2.2 to +4.6 bushels/A in eight of twenty-four field experiments and decrease in SCN reproduction with the use of Clariva™; however, decreases in SCN population densities was not related to the locations with yield increases (Tylka et al., 2015). However, in Syngenta® field studies, Clariva™ Complete Beans increased soybean yields an average of 4.1% compared to seed treatments with only an insecticide and fungicide (Callanan and Alderfer, 2014). Having variable and inconsistent results that are based upon unpredictable factors was common in all the studies. The main issue with recommending the alternative strategies, such as the use of biological control agents and cover crops to specifically reduce nematode populations, is that they have been inconsistent in field studies.

1.6 Research Objectives

One objective of this study was to evaluate the efficacy of commercial seed treatments without biological control agents (Crusier Maxx Advanced) and with

biological control agents, Poncho/VOTiVO with *Bacillus firmus* (I-1582) and Clariva Complete with *Pasteuria nishizawae* Pn-1 for their effects on soybean yields and SCN population density under Nebraska conditions. Field scale evaluations of experimental biocontrol agents such as *L. enzymogenes* C3 have been hampered by the lack of effective methodology to deliver the agents in row crop settings. Based on this, the second objective was to compare the population dynamics of C3 in the rhizosphere of cereal rye and soybean to determine if either plant would serve as a better delivery source of C3 into the soil profile as a beginning process to develop a combined biological control strategy with a cover crop.

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CHAPTER II

EVALUATION OF THE EFFECTS OF BIOLOGICAL SEED TREATMENTS ON YIELD AND SOYBEAN CYST NEMATODE (*HETERODERA GLYCINES*) POPULATIONS

2.1 Introduction

Global production of soybean (*Glycine max* L. Merr.) has increased in the past 40 years and is the worlds' most important oilseed crop (Hartman et al., 2011). The United States is the number one producer globally and Nebraska is ranked fifth in production of soybeans in the US (Sadras et al., 2014; USDA & NASS, 2016). Disease is the main cause of yield reduction and soybean cyst nematode (SCN, *Heterodera glycines*) causes the highest yield loss across all soybean producing areas in the United States (Wrather and Koenning, 2009). Yield loss due to SCN is over $720 million each year in the United States (Wrather and Mitchum, 2010) and over $45 million a year in Nebraska (Wilson and Giesler, 2014). Yield loss is the main indicator that SCN may be present, as there can be a 10-20% yield loss with no visible host symptoms (Davis and Tylka, 2000).

Population densities of SCN in a field can vary from non-detectable to greater than 5,000 eggs/100 cc soil depending on the soil type, presence of crop host, and natural enemies of SCN (Wrather and Mitchum, 2010). In Nebraska, SCN population densities have been found to be as high as 136,00 eggs/100cc's of soil with most fields being at manageable levels between 1,700 to 2,100 eggs per 100cc's of soil (Giesler and Wilson, 2011). The best way to determine if a field is infested with SCN is to collect soil samples and once a field becomes infested the nematode cannot be eradicated. This is

due to the cyst structures that are formed that allow the eggs inside to remain viable in the soil for many years' even under harsh environmental conditions (Wrather, Anand, and Dropkin, 1984).

Current management practices include the use of resistant varieties, crop rotation, and sanitation. The use of resistant varieties is the most effective management practice to control SCN (Chen et al., 2001a). There are seven sources of resistance, but only three are commonly used which are PI 88788, PI 437654 (Hartwig or CystX), and Peking (Chen et al., 2011). Over 95% of resistant varieties contain PI88788 leaving a limited selection when it comes to rotating resistant sources (Chen et al., 2011). Rotation of these resistance sources is recommended to help manage shifts in SCN virulence (Niblack et al., 2008). Increases in virulence on PI 88788 has been found to range from 60-78% in the Midwestern states, reducing the effectiveness of the resistant varieties (Mitchum et al., 2007; Niblack et al., 2008; Zheng and Chen, 2011). Crop rotation to a non-host, usually corn, can greatly reduce population levels after a one-year rotation (Perez-Hernandez, 2013). Multiple years out of soybean may be necessary if SCN population levels are high, however, a one-year rotation typically reduces the populations to non-damaging levels (Miller et al., 2006; Koenning et al., 1993). If a field is not already infested with SCN it is important not to introduce the pathogen by washing equipment and planting fields known to have SCN last as the pathogen cannot be eradicated once present in a field. Due to the challenges with current management strategies more sustainable methods are being explored to manage SCN.

Biological control is a practice where a disease is suppressed through the use of microbial antagonists (Pal and Gardener, 2006). Bacteria are natural enemies of nematodes in the soil and two common soil inhabitants' that have potential to control nematode populations are *Bacillus* and *Pasteruia* spp. (Emmert and Handelsman, 1999; Siddiqui and Mahmood, 1999; Meyer, 2003). The option of utilizing biological control agents as seed treatments to manage SCN has become commercially available in recent years. One product is Poncho/VOTiVO™ from Bayer Crop Science® that contains the spores of *Bacillus firmus* (I-1582) along with an insecticide, Clothianidin (Bayer Crop Science®). This bacterium is a rhizobacteria and colonizes the roots of the host plant to prevent nematode attraction to the root by consuming root exudates, competing with the nematode for nutrients and space (Siddiqui and Mahmood, 1999; Bayer CropScience, 2016). Clariva™pn, a product from Syngenta®, contains endospores of *Pasteruia nishizawae* that parasitizes juvenile SCN in the soil by the endospores attaching and penetrating the cuticle of the nematode (Noel et al., 2005). Then the bacteria develops inside the entire body, degrading the nematode to release more endospores back into the soil (Noel et al., 2005).

Even though there is a reported increase in yield with the use of commercial biological seed treatments, results have not been consistent as biological control agents are highly sensitive to environmental conditions (Paulitz and Bélanger, 2001). Syngenta® has observed yield increases of 4.1% when using Clariva™Complete seed treatments, which contains a biological control, insecticide, and fungicide, compared to usual seed treatments of only insecticide and fungicides (Callanan and Alderfer, 2014).

Field studies in Minnesota and Michigan have indicated that both products, Poncho/VOTiVO™ and Clariva™, do not provide a yield increase compared to a fungicide and insecticide base seed treatment or reduce nematode populations compared to using a resistant variety source (Potter et al., 2015; Staton and Seamon, 2015; Koch and Rich, 2016). Iowa in comparison, had five out of twenty-four locations yield statistically greater than the base fungicide and insecticide seed treatment with a high of 91.5 bushels/A in a strip plot study; however, SCN population density reduction was not associated with the locations with greater yield (Tylka et al., 2015).

Field evaluations of Poncho/VOTiVO™ (*B. firmus* I-1582) and Clariva™ Complete (*P. nishizawae*) seed treatments have not been done in Nebraska. Therefore, the objective of this study was to determine the effects of commercially available biological seed treatments in Nebraska on soybean plant population, SCN population density, and yield.

2.2 Materials and Methods

2.2.1 Experimental locations

Over the course of two years (2014 and 2015), eight field experiments were conducted in Nebraska. Each year there were three different field locations that were naturally infested with SCN and one location that was non-infested, and all field sites were different. In 2014, the three infested locations were near Battle Creek, Columbus, and Plattsmouth, NE. In the 2015 growing season, the infested locations were near West Point, Columbus, and Plattsmouth, NE. The non-infested location was near Mead, NE in both years.

All sites were irrigated fields following corn the previous year under no-tillage management conditions. All sites were planted at 140,000 seeds per acre with the soybean variety NK S28-A2. This variety is a Syngenta product and is from the brand Northrup King (NK®) seed and is resistant to SCN using the PI 88788 resistance source (Syngenta, 2015). The planting and harvest dates for all locations, along with rainfall in August and the entire season are presented in Table 2.1. Herbicide programs for the fields are presented in table 2.2.

2.2.2 Treatments and experimental design

The same sets of treatments were tested in all eight experiments (Table 2.3). Seed was treated by Syngenta® with the active ingredients following the label rates (Table 2.3). Poncho/VOTiVO does not typically include a fungicide component, however, to make treatments comparable, fungicide components with the same composition were added making only the biological control agents different. Insecticide components (thiomethoxam and clothianidin), are different active ingredients, but contain the same mode of action. Each experiment was a randomized complete block design that contained eight replications per treatment. The individual plots in all experiments except West point (2015) consisted of four rows that were 3.0 m wide by 5.1 m long with 0.7 m spacing between the rows. At West Point (2015) the individual plots for the entire location were four rows that were 3.0 m wide by 6.7 m long with 0.7 m spacing between the rows.

2.2.3 Plant population assessments

Plant populations were determined by counting the number of plants in two 3.0 m sections of each plot in the center two rows that were marked with flags at the first assessment. Three assessments were done during the season: 12-26 days after planting (DAP), 21-39 DAP, and at maturity (131-149 DAP). All assessments for plant population were performed in the same 3.0 m sections each time. Plant counts were then extrapolated to the plant populations per acre, by adding the counts from each 3.048 m section together and multiplying the result by 43,560 ft^2/acre. The outcome was then divided by 50 to give the expected plant population per acre from the two 3.0 m sections.

2.2.4 SCN population density assessments

Initial SCN populations (P_i) were determined shortly after emergence of soybeans. Final SCN populations (P_f) were determined after harvest. Using a soil probe, 12 soil cores 15-20 cm in depth from each plot were collected from the center two rows. The soil was transferred back to be processed in the laboratory and stored at 4°C. The soil from each plot was crushed and mixed thoroughly, then by volumetric displacement, 100 cc of crushed soil was added to water. The soil and water mixture was allowed to sit for at least 20 minutes when the water was poured through a 25 mesh sieve over a 60 mesh sieve, trying to expel the soil contents. More water was added to disturb the settled soil and then the water was passed again through the sieves. The decanting of the water off the settled soil through the sieves was done a total of four times for each sample. Contents of the 60 mesh sieve were then rinsed

onto 120 mesh sieve nested over a 500 mesh sieve. A rubber stopper was used to grind any material collected on the 120 mesh sieve to release SCN eggs. Material was ground until only coarse sand particles and organic matter remained and was then gently washed onto the 500 mesh sieve. Contents on the 500 mesh sieve were rinsed into a beaker using less than 20 milliliters (mL) of water. The eggs were then stained using acid fusion, by adding one mL of stain to the sample and boiling the sample for 30 seconds and bringing the final volume up to 20 mL. SCN eggs were counted under a dissecting microscope in a tray by taking 1 mL out of the stock solution and adding another 1 mL of distilled water to fill the bottom of the tray. Once the eggs were counted the SCN population in the plot was determined by taking the number of eggs counted multiplied by 2 (how many mL fills the tray) and then multiplying by 20 mL (original volume from processed soil). The Reproduction Factor (Rf) can be calculated at each site by comparing the average SCN populations. Dividing the P_f SCN population by the P_i SCN population the Rf can be calculated. If the Rf value is above 1.0 then the SCN population increased, below 1.0 the SCN population decreased, or at 1.0 then there was no change in the SCN population.

2.2.5 Yield Assessment

At harvest each of the plots were cut to 4.5 m in length and the two center rows were harvested. An Almaco plot combine was used to determined yield and grain properties. Yield is reported as bushel per acre (bu/A) with weights converted for 13.0% moisture.

2.2.6 Statistical Analysis

Initially a combined analysis was performed on plant population assessments (plant/A), Rf, and yield (bu/A) from all locations in 2014, 2015, and then from both years combined with PROC GLIMMIX using Statistical Analysis System at significance level of $\alpha \leq 0.05$ (SAS; SAS Institute, Cary NC). A combined analysis of the infested or non-infested locations in 2014, 2015, and both years combined was performed on the plant populations (plant/A), Rf (infested sites) and yield (bu/A) with PROC GLIMMIX in SAS at significance level of $\alpha \leq 0.05$. The data from each year was then analyzed separately by location for plant populations, Rf, and yield (bu/A) at all locations with PROC GLIMMIX using SAS at significance level of $\alpha \leq 0.05$. Rf data was log transformed prior to analysis using $\log_{10}((P_f + 1)/ (P_i + 1))$, however all data presented in graphs is in P_f/P_i (Chen et al., 2001b). Graphs of each outcome were produced using SigmaPlot (Systat Software, San Jose, CA).

2.3 Results

Plant Populations

The non-infested location (Mead, 2014) was the only site where treatment was significant in plant population assessments for harvest time points (Table 2.5). CruiserMaxx Advanced was significantly lower in plant population (0.0294) than the biologically treated seed at harvest, with an average plant population of 84,942 (Table 2.5). Columbus was the only location, however, to have plant populations that were significantly different at 21-39 days after planting (Table 2.5). Clariva Complete had an

average population of 140,263 that was significantly higher than CruiserMaxx Advanced or Poncho/VOTiVO with a Pr > F of 0.0007 (Table 2.5).

In 2014, treatment was not significant for plant populations (0.9405) and was not significant for any individual time collection points; however, location and time was significant (<0.0001) (Table 2.11). In 2015 treatment was not significant overall (0.7202), but, the harvest collection time point overall was significantly different at $\alpha \leq 0.10$ (0.0907), with Clariva Complete plant stands averaged over all locations being higher than Poncho/VOTiVO or CruiserMaxx Advanced (Table 2.11). In both 2014 and 2015, when all locations were analyzed results were comparable to infested locations being analyzed separately (Table 2.11). When only the infested locations were analyzed for combined years plant population was not significant (0.1352) (Table 2.8). In a combined analysis of the non-infested locations, treatment was not significant (0.5139) (Table 2.7). When the plant population assessment data from all locations and years was combined there was no treatment effect from any of the three individual time collection points, but year, location, year*location, and year*time were significant at Pr > F < 0.001 (Table 2.9). However, treatment was significant (0.0128) for plant population assessment when all collection times and years are combined (Table 2.9).

SCN Population Density

Initial population densities (P_i) of SCN infested sites ranged from 200-5,105 (eggs/100cc of soil) and final population densities (P_f) ranged from 335-2,630 (eggs/100cc of soil), where only the P_f treatments at Battle Creek were significant (0.0358) in population density (Table 2.6). Individual locations varied in the reproduction

factor (Rf) from 2014 to 2015 (Figure 2.1), where a Rf greater than 1.0 means that SCN populations increased, or below 1.0 SCN population densities decreased. In 2015 all locations had populations and treatments that were able to reproduce (Figure 2.1). Individual locations showed no treatment effect on logRf (Table 2.6) and there was no overall treatment effect in either 2014 (0.3795) or 2015 (0.7312) (Table 2.11). In 2014 location was significant (0.0007), however was not in 2015 (0.2551) (Table 2.11). When all locations from both years were combined, treatment (0.9364) was not significant, however, both location (0.0332) and year (0.0746) were found to be significant. There was no location by treatment interaction (0.7209) identified in the combined analysis for the SCN infested sites (Table 2.8).

Yield

Average yields were higher in 2015 than 2014, where the average yield ranges from 45.4-77.0 bu/A in all locations, and 45.4-71.9 bu/A in only infested locations (Figure 2.2). In 2014 the yield ranges were 45.4- 77.0 bu/A overall locations or 45.4 – 56.3 bu/A over infested locations (Table 2.5). In 2015 yield ranges were from 52.6-71.9 bu/A (Table 2.5). Treatment was not significant at any of the individual locations (Table 2.5). Overall the seed treatments had no effect on yield in location from either 2014 (0.6719) or 2015 (0.2917) that were infested with SCN (Table 2.11). Location was not significantly different in 2014 (0.2217), but was in 2015 (<0.0001) (Table 2.11). When the 2014 and 2015 were combined for infested locations treatment was not significant (0.8075), location and year were significant with a Pr $>$ F of <0.0001, as well as the

location*year with a Pr > F of 0.0058 (Table 2.8). Treatment was not significant (0.8811) when the non-infested locations were combined from 2014 and 2015 (Table 2.7).

2.4 Discussion

There were no treatment effects on yield in 2014, 2015, or overall when year and locations were combined. This could have been due to the environmental conditions that were observed over the two years for this study, as there was little water stress especially during maturity in August (Table 2.1). Water stress can accelerate leaf senescence and reduce the end yield by reducing the seed size and seed number per plant (Brevedan and Egli, 2003). Drought and water stress can intensify the symptoms of SCN that are muted with sufficient or excess rainfall, mainly yield loss (Tylka, 2012). Yield was higher in 2015 and the environmental factors were similar between the two growing seasons. The initial SCN population densities were higher in 2014 and could have affected overall productivity and resulted in overall lower yields. However, in the non-infested location in 2015 the yields were lower than in 2014, where a non-infested location would typically suggest how a field would yield without SCN (Table 2.5, Figure 2.2). In similar studies that compared the biological control seed treatments with just the fungicide/insecticide seed treatments, the biological seed treatments yielded more in Minnesota field studies (Potter et al., 2015), there was a yield loss of 0.8-4.0 bushles/A in Michigan (Koch and Rich, 2016), and there was an average yield increase of 0.2 bushels/acre in Iowa field studies (Tylka et al., 2015). In Iowa, there were eight locations out of 24 that significantly yielded higher between Clariva and the base fungicide/insecticide treated seed, however, the locations that

yielded higher did not necessarily have the reduction in SCN populations as mentioned above (Tylka et al., 2015).

Results from other field studies displayed variable SCN population reduction similar to the trials reported here. In Minnesota only a single site showed a reduction in SCN populations by Clariva (Potter et al., 2015), Michigan had an increase in SCN populations at all locations Koch and Rich, 2016), and Iowa field trials showed that Clariva treated seed reduced SCN population densities in both small plot and strip trials (Tylka et al., 2015). The SCN population densities varied in the studies reported here depending upon the year because the locations changed, showing the differences in HG types (Table 2.4). These variabilities in HG type can help explain the increase in SCN populations during 2015. Plattsmouth (2015) was found to be HG type 2.5.7, along with the Columbus (2014 and 2015) location, meaning that the nematode population in the field was able to reproduce on the cultivar planted (Figure 2.1). Many of the nematodes were only able to reproduce between 12-15% on PI 88788. There have been sources that show linkage between 2.5.7 virulence, and the ability of the nematodes to reproduce on PI 5484316 was between 11-29%, which is close to being susceptible and could have added to the virulence and increase in SCN populations (Colgrove and Niblack, 2008; Broderick, 2016). The other HG types observed besides 2.5.7, contradict findings as reproduction of SCN would not be expected from any of those locations (Table 2.4). There could still be reproduction on PI 88788, just at lower level than the 10% required to meet the requirements for the HG test.

The soil types were different between locations, however, since the 'texture by feel' technique was used to determine soil type and exact percentages cannot be determined it is hard to speculate how the soil type played a role. However, in almost all of these analyses presented, location was significantly different. This could be due to a number of reasons, environmental conditions, overall plant health, or the differences in soil type. While initial SCN population density varied among locations, SCN populations tend to be lower in no-tilled soils with a high clay content (Workneh et al., 1999). Soil type could be linked to the efficacy of the biological seed treatments used in these trials, but it has not been examined.

No consistent effects were observed on plant populations, only two individual time points at two locations were significant (Table 2.5). Overall plant population were relevant to general soybean production with the exception of Battle Creek, which could have stemmed from a hail event in 2014 on 2 June. There were also portions of the field that were located near the entrance and allowed for standing water early due to precipitation events. Both of these, could factor into the lower stand counts for Battle Creek. Similar trials in Minnesota also found that there were no differences among treatments and plant population assessments (Potter et al., 2015). As soybean is able to compensate for reduced plant populations, observed effects of plant population density did not relate to changes in yield (Carpenter and Board, 1997). This compensatory effect, along with favorable conditions in both years could be why no treatment effects were observed on yield.

In 2015 the seed treatment costs for Clariva Complete and Crusier Maxx Advanced were $26.80 and $16.10, respectively, (Staton and Seamon, 2015). A base application of standard fungicide/insecticide seed treatment of Cruiser Maxx costs $16.10, where the additional application of biological seed treatments brought total costs to $22.90 (Poncho/VOTiVO) and $26.80 (Clariva Complete) (Battel et al., 2014; Staton and Seamon, 2015). Having the addition of the biological seed treatment, a 0.7 and 0.9 yield increase would need to be observed with Poncho/VOTiVO and Clariva Complete, respectively, to have the additional seed treatment costs be economically effective at soybean prices of $9.15/bushel. Based upon these results where plant populations, SCN population density, and yield all showed no treatment effect from the biological control seed treatments, current management recommendations would be to test the field for the presence of SCN. If SCN eggs are detected at manageable ranges, planting resistant cultivars and rotation to a non-host crop would be the most economical and sustainable management practice.

From the variabilities of response to seed treatments with biological agents between years and geographical area, more research needs to be expanded on the use of biological control seed treatments and SCN management. This expansion could include more experiments on Clariva Complete and Ponco/VOTiVO, other bacterial organisms that are nematicidal or nematistatic, and other mechanisms of dispersion into the soil profile for applied agents. More research is also needed when soybean production occurs under stressful environmental conditions, especially during the seed filling stages when factors affecting roots are more pronounced. With more research on

seed treatments with biological control agents and other biocontrols it may be possible to develop a profitable SCN management practice for growers that mitigates the impact of SCN in a way that is sustainable for the environment.

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2.6 Figures

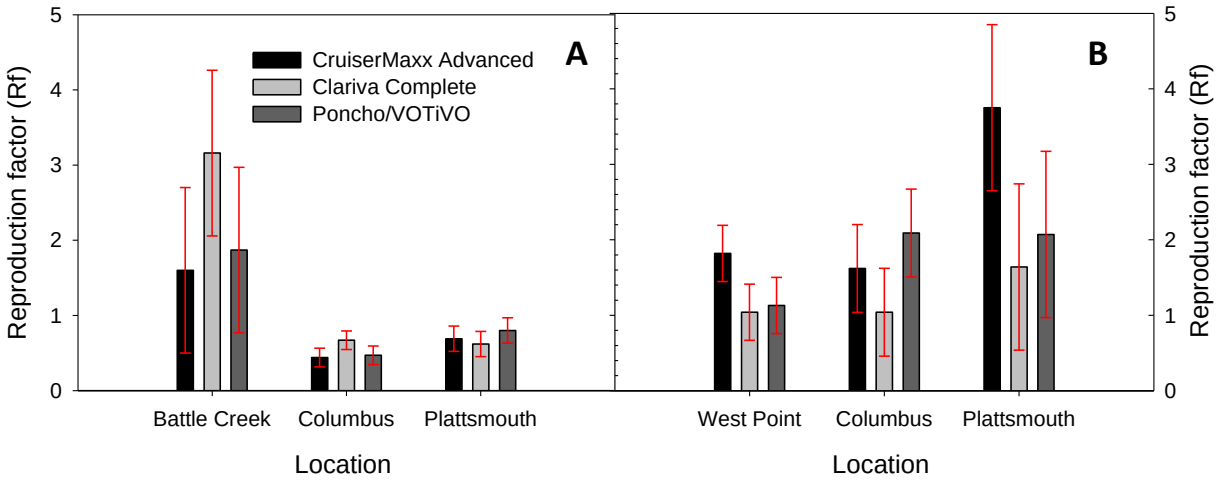

Figure 2.1. Comparison of seed treatment effects on reproduction factors (Rf) from 2014 (A) and 2015 (B) from naturally infested locations with SCN. Locations were different between the two years, but had similar soil types. An Rf greater than 1.0 means that SCN population increased and below 1.0 represents a decrease in population density. There was not a treatment effect observed at $\alpha \leq 0.05$; red error bars are the standard error of the means.

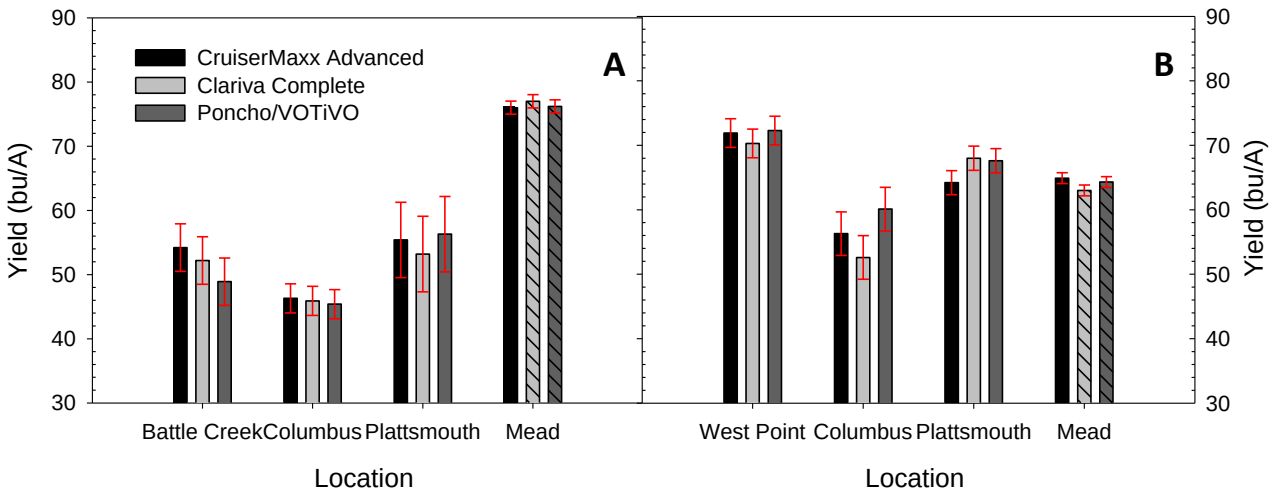

Figure 2.2. Comparison of yield (bu/acre) during the growing seasons of 2014 (A) and 2015(B). Locations were different between the two years, where Mead is the non-infested location each year designated by diagonal hash marks in the bars. Treatment was not found to be a significant factor at $\alpha \leq 0.05$; however, year and location did have an effect on yield. Red error bars are the standard error of the means.

2.7 Tables

Table 2.1. Planting date, harvest date, and rainfall during August and throughout the entire growing season for eight Nebraska field trial locations established to evaluate the effects of SCN seed treatments.

Location (Year)	Planting Date	Harvest Date	August Rainfall (cm.)	Season Rainfall (May-Oct) (cm.)
Battle Creek (2014)	23 May 2014	27 Oct 2014	13.49	55.63
Columbus (2014)	30 May 2014	29 Oct 2014	20.32	69.85
Plattsmouth (2014)	21 May 2014	21 Oct 2014	25.07	71.63
Mead (2014)	21 May 2014	22 Oct 2014	20.39	59.18
West Point (2015)	18 May 2015	13 Oct 2015	13.03	51.56
Columbus (2015)	22 May 2015	20 Oct 2015	11.99	59.18
Plattsmouth (2015)	8 Jun 2015	26 Oct 2015	23.19	74.17
Mead (2015)	1 Jun 2015	13 Oct 2015	18.54	47.24

Table 2.2. Herbicide programs for the field locations established to evaluate the effects of SCN seed treatments during the 2014 and 2015 growing seasons.

Location (Year)	**Herbicide**	
	Pre-emergent	*Post-emergent*
Battle Creek (2014)	Valor SX (2 oz/A)	Roundup 22oz/A (6/27/14) Roundup 22oz/A (7/28/14)
Columbus (2014)	No pre-emergent applied	Roundup 22oz/A (6/27/14) Roundup 22oz/A (7/30/14)
Plattsmouth (2014)	Authority (5 oz/A) Prowl 33 (3 pt/A) Salvo (1 pt/A)	Roundup 22 oz/A (8/18/14)
Mead (2014)	Valor SX (2 oz/A)	No post application applied
West Point (2015)	Valor SX (2 floz/A)	Roundup 32 oz/A (6/8/15) Roundup 32 oz/A (7/8/15)
Columbus (2015)	Authority (5oz/A)	Roundup 32 oz/A (6/11/15) Roundup 32 oz/A (6/25/15) Roundup 32 oz/A (7/9/15) Roundup 32 oz/A (7/27/15)
Plattsmouth (2015)	Authority (5oz/A)	No post application applied
Mead (2015)	Valor SX (2 oz/A)	No post application applied

Table 2.3. Seed treatment components and respective rates[z] used in field trials to evaluate the effects of biological agents on SCN management.

Component	**Seed Treatments Active Ingredient (trade name)**		
	Cruiser Maxx Advanced	*Clariva Complete*	*Poncho/VOTiVO*
Insecticide	Thiomethoxam (Cruiser)	Thiomethoxam (Cruiser)	Poncho/VOTiVO (Clothianidin[y])
Fungicide[x]	Mefenoxam (Apron XL) Fludioxonil (Maxim 4FS) Sedaxane (Vibrance)	Mefenoxam (Apron XL) Fludioxonil (Maxim 4FS) Sedaxane (Vibrance)	Mefenoxam (Apron XL) Fludioxonil (Maxim 4FS) Sedaxane (Vibrance)
Microbial Agent	None	Clariva pn (*Pasteruia nishizawae* – Pn1)	Poncho/VOTiVO (*Bacillus firmus* I-1582)

[z]All treatments applied by Syngenta Crop Protection; Thiomethoxam – 0.0113 mg ai/ seed; Clothianidin – 0.13 mg ai/ seed; Mefenoxam – 0.0038; mg ai / seed; Fludioxonil – 0.075 mg ai/seed; Sedaxane – 0.0038 mg ai/seed; Clarivia pn – 59.13 mL/45.36 kg seed; *Bacillus firmus* I-1582 – 0.13 mg/ai seed

[y]Clothianidin and Thiomethoxam are the same mode of action

[x]The fungicides in the bolded column would not normally be treated with Poncho/VOTiVO, and would normally be treated with Bayer Crop Science branded fungicides.

Table 2.4. Soil and HG types[z] of field locations infested with SCN for experiments conducted in 2014 and 2015.

Location (Year)	HG Type[y]	Soil Type[x]
Battle Creek (2014)	1.3.6.7	Loamy, Sand
Columbus (2014)	2.5.7	Loamy, Sand
Plattsmouth (2014)	0[w]	Silty, Clay Sand
West Point (2015)	7	Sandy, Loam
Columbus (2015)	2.5.7	Loamy Loam
Plattsmouth (2015)	2.5.7	Silt Sand

[z]HG (*Heterodera glycines*) Type is a test to determine the ability of SCN field populations to reproduce on the seven sources of resistance compared to a susceptible indicator line. The sources of resistance and their HG types are PI 548402 (1), PI 88788 (2), PI 90763 (3), PI 437654 (4), PI 209332 (5), PI 89772 (6), and PI 5484316 (7).

[y]Any type with a 2 in the designation could reproduce on PI 88788 which was the resistance source used. If a number is not listed in the HG type, that source of resistance held SCN reproduction to 10% or less than on standard susceptible variety. None of the resistance sources were found to be greater than 30% reproduction, making them non-virulent populations.

[x]Soil type was determined through the texture by feel technique by Ward laboratories in Kearney, Nebraska.

[w]SCN populations could not reproduce on any resistant sources.

Table 2.5. Average plant population and yield of each seed treatment in trials from 2014 and 2015 in eight different locations from Nebraska.

Location *(Year)*	**Treatment**[z]	**Plant Population/A**[y]			**Yield (bu/A)**
		12-26 DAP[x]	*21-39 DAP*	*Harvest*	
Battle Creek (2014)	1. CruiserMaxx Advanced	45,193	50,421	42,144	54.2
	2. Clariva Complete	44,322	45,520	41,709	52.2
	3. Poncho/VOTiVO + equivalent fungicides (ef)	48,569	45,738	38,224	48.9
	PR>F	0.7514	0.7179	0.6743	0.1862
Columbus (2014)	1. CruiserMaxx Advanced	87,447	86,249	65,231	46.3
	2. Clariva Complete	88,753	86,576	67,627	45.9
	3. Poncho/VOTiVO + ef	89,189	90,278	65,667	45.4
	PR>F	0.8603	0.5635	0.2260	0.9567
Plattsmouth (2014)	1. CruiserMaxx Advanced	99,970	105,415	85,813	55.4
	2. Clariva Complete	104,108	109,227	82,002	53.2
	3. Ponch/ VOTiVO + ef	98,881	102,257	78,626	56.3
	PR>F	0.2569	0.1360	0.2637	0.8254
Mead[v] (2014)	1. CruiserMaxx Advanced	89,189	101,168	**84,942***	76.0
	2. Clariva Complete	95,505	107,811	90,605	77.0
	3. Ponch/ VOTiVO + ef	93,218	106,287	90,496	76.2
	PR>F	0.4474	0.1440	**0.0294**[u]	0.7719
West Point (2015)	1. CruiserMaxx Advanced	59,024	122,730	97,574	71.9
	2. Clariva Complete	60,439	118,266	95,288	70.3
	3. Poncho/VOTiVO + ef	57,935	115,543	87,991	72.3
	PR>F	0.9552	0.6020	0.3727	0.7988
Columbus (2015)	1. CruiserMaxx Advanced	127,522	133,511	124,473	56.3
	2. Clariva Complete	131,987	**140,263***	127,958	52.6
	3. Poncho/VOTiVO + ef	126,106	133,184	129,047	60.1
	PR>F	0.1769	**0.0007**[u]	0.4611	0.2504
Plattsmouth (2015)	1. CruiserMaxx Advanced	135,363	138,085	114,563	64.2
	2. Clariva Complete	136,670	139,174	123,819	68.0
	3. Poncho/VOTiVO + ef	137,214	144,075	110,534	67.6
	PR>F	0.8943	0.2482	0.0649	0.1871
Mead[v] (2015)	1. CruiserMaxx Advanced	135,798	136,234	125,780	64.9
	2. Clariva Complete	136,887	139,065	124,691	63.0
	3. Ponch/ VOTiVO + ef	128,175	133,402	114,563	64.3
	PR>F	0.0623	0.3362	0.1765	0.2431

[z]All treatments applied by Syngenta Crop Protection; Thiomethoxam – 0.0113 mg ai/ seed; Clothianidin – 0.13 mg ai/ seed; Mefenoxam – 0.0038; mg ai / seed; Fludioxonil – 0.075 mg ai/seed; Sedaxane – 0.0038 mg ai/seed; Clarivia pn – 59.13 mL/45.36 kg seed; *Bacillus firmus* I-1582 – 0.13 mg/ai seed

[y]Plant population determined by counting the number of plants in two 3.048 m sections of row of each plot

[x]Days after planting

[v]SCN non-infested location

[u]Treatment is statistically different at P= 0.05, with an asterisk (*) indicating which treatment that is significantly different from others

Table 2.6. SCN population density of each seed treatment in trials from 2014 and 2015 at six locations naturally infested with SCN in Nebraska.

Location *(Year)*	**Treatment**[z]	**SCN population (eggs/100 cc of soil)**		
		Initial (P_i[y])	*Final (P_f[x])*	*Rf*[w]
Battle Creek (2014)	1. CruiserMaxx Advanced	440	435	1.60
	2. Clariva Complete	1,120	910	3.16
	3. Poncho/VOTiVO + equivalent fungicides (ef)	1,063	1,107	1.87
	PR>F	0.3149	0.0358	0.3424
Columbus (2014)	1. CruiserMaxx Advanced	2,015	850	0.44
	2. Clariva Complete	2,610	1,290	0.67
	3. Poncho/VOTiVO + ef	3,105	1,155	0.47
	PR>F	0.3321	0.3566	0.3017
Plattsmouth (2014)	1. CruiserMaxx Advanced	3,335	2,205	0.69
	2. Clariva Complete	5,105	2,340	0.62
	3. Ponch/ VOTiVO + ef	3,860	2,630	0.80
	PR>F	0.2820	0.8009	0.7327
West Point (2015)	1. CruiserMaxx Advanced	270	420	1.82
	2. Clariva Complete	465	385	1.04
	3. Poncho/VOTiVO + ef	270	305	1.13
	PR>F	0.2319	0.6393	0.9816
Columbus (2015)	1. CruiserMaxx Advanced	1,675	2,080	1.62
	2. Clariva Complete	2,225	1,570	1.04
	3. Poncho/VOTiVO + ef	2,690	1,570	2.09
	PR>F	0.3966	0.2276	0.1087
Plattsmouth (2015)	1. CruiserMaxx Advanced	245	665	3.75
	2. Clariva Complete	335	515	1.64
	3. Poncho/VOTiVO + ef	200	335	2.07
	PR>F	0.6194	0.4776	0.9160

[z]All treatments applied by Syngenta Crop Protection; Thiomethoxam – 0.0113 mg ai/ seed; Clothianidin – 0.13 mg ai/ seed; Mefenoxam – 0.0038; mg ai / seed; Fludioxonil – 0.075 mg ai/seed; Sedaxane – 0.0038 mg ai/seed; Clarivia pn – 59.13 mL/45.36 kg seed; *Bacillus firmus* I-1582 – 0.13 mg/ai seed

[y]The initial SCN population (P_i) was collected during the spring after planting.

[x]The final SCN population (P_f) was collected during the fall after harvest.

[w]Reproduction factor (Rf) was calculated for each plot and the average among replicate plots is reported. The $\log_{10}((P_f + 1)/ (P_i + 1))$ was used for statistical analysis, but is not reported. Rf values below and above 1.0 indicate the SCN population decreased or increased, respectively, during the season.

Table 2.7. Combined analysis of seed treatment in trials from 2014 and 2015 in non-infested locations from Nebraska GLIMMIX procedure with a confidence level of $\alpha \leq 0.05$.

		Response variable[z]	
Effect	**DF**	*Plant population[y]*	*Yield*
Year	1	**<0.0001*[x]**	**<0.0001***
Treatment[v]	2	0.5139	0.8811
Year*Treatment	2	**0.0024***	0.2818
Time	2	0.0811	NA
Time*treatment	4	0.9220	NA
Year*time	2	**0.0369***	NA
Year*treatment*time	4	0.8917	NA
Treatment 12-26 DAP[t]	2	0.4789	NA
Treatment 21-39 DAP	2	0.3815	NA
Treatment Harvest	2	0.4276	NA

[z]Combined data from all locations in 2014 and 2015 for each response variable

[y] Plant population determined by counting the number of plants in two 3.0 m sections of row of each plot

[x]Treatment is statistically different at P= 0.05, with an asterisk (*) indicating which effect that is significantly different

[v]This is the treatment effect of combined years and combined collection time points

[t]Days after planting

Table 2.8. Combined analysis from 2014 and 2015 at six locations naturally infested with SCN in Nebraska GLIMMIX procedure with a confidence level of $\alpha \leq 0.05$.

		Response variable[z]		
Effect	**DF**	*Plant population[y]*	*SCN population[x] (log Rf)*	*Yield*
Year	1	**<0.0001[w]***	**0.0746**	**<0.0001***
Location	2	**<0.0001***	**0.0332***	**<0.0001***
Treatment[v]	2	0.1352	0.9364	0.8075
Year*Location	2	**<0.0001***	**0.0004***	**0.0058***
Year*Treatment	2	0.6967	0.2871	0.6686
Location*Treatment	2	0.3894	0.7209	0.9728
Year*Location*Treatment	4	0.9971	0.7747	0.6040
Time	2	0.6500	NA	NA
Time*treatment	4	0.7941	NA	NA
Year*time	2	**<0.0001***	NA	NA
Year*treatment*time	4	0.9860	NA	NA
Year*treatment*time*location	8	0.4996	NA	NA
Treatment 12-26 DAP[t]	2	0.8861	NA	NA
Treatment 21-39 DAP	2	0.9426	NA	NA
Treatment Harvest	2	0.4178	NA	NA

[z]Combined data from all locations in 2014 and 2015 for each response variable

[y] Plant population determined by counting the number of plants in two 3.048 m sections of row of each plot

[x]Reproduction factor (Rf), $\log_{10}((P_f + 1)/ (P_i + 1))$ was used for statistical analysis, but is not reported. Rf values below and above 1.0 indicate the SCN population decreased or increased, respectively, during the season.

[w]Treatment is statistically different at P= 0.05, with an asterisk (*) indicating which effect that is significantly different

[v]This is the treatment effect of combined years and combined collection time points

[t]Days after planting

Table 2.9. Combined analysis of seed treatment in trials from 2014 and 2015 in eight different locations from Nebraska GLIMMIX procedure with a confidence level of $\alpha \leq 0.05$.

		Response variable[z]	
Effect	**DF**	*Plant population[y]*	*Yield*
Year	1	**<0.0001[x]***	**<0.0001***
Location	3	**<0.0001***	**<0.0001***
Treatment[w]	2	**0.0128***	0.8075
Year*Location	3	**<0.0001***	**<0.0001***
Year*Treatment	2	0.3237	0.6714
Location*Treatment	6	0.3894	0.9812
Year*Location*Treatment	6	0.9971	0.7905
Time	2	0.5399	NA
Time*treatment	4	0.6748	NA
Year*time	2	**<0.0001***	NA
Year*treatment*time	4	0.9721	NA
Year*treatment*time*location	12	0.4815	NA
Treatment 12-26 DAP[v]	2	0.7154	NA
Treatment 21-39 DAP	2	0.8165	NA
Treatment Harvest	2	0.2676	NA

[z]Combined data from all locations in 2014 and 2015 for each response variable

[y] Plant population determined by counting the number of plants in two 3.048 m sections of row of each plot

[x]Treatment is statistically different at P= 0.05, with an asterisk (*) indicating which effect that is significantly different

[w]This is the treatment effect of combined years and combined collection time points

[v]Days after planting

Table 2.10. Individual analysis of seed treatment in trials of years 2014 and 2015 in eight different locations from Nebraska GLIMMIX procedure with a confidence level of $\alpha \leq 0.05$.

			Response variable[z]	
Year	**Effect**	**DF**	*Plant population[y]*	*Yield*
2014	Location	3	**<0.0001[x]***	**<0.0001***
	Treatment[w]	2	0.8765	0.7340
	Location*Treatment	6	0.1444	0.8006
	Time	2	**<0.0001***	NA
	Time*treatment	4	0.9970	NA
	Treatment 12-26 DAP[t]	2	0.7058	NA
	Treatment 21-39 DAP	2	0.9177	NA
	Treatment Harvest	2	0.7460	NA
2015	Location	3	**<0.0001***	**<0.0001***
	Treatment[w]	2	0.4120	0.1728
	Location*Treatment	6	0.3054	0.3495
	Time	2	**<0.0001***	NA
	Time*treatment	4	0.9818	NA
	Treatment 12-26 DAP[v]	2	0.6994	NA
	Treatment 21-39 DAP	2	0.3316	NA
	Treatment Harvest	2	**0.0107***	NA

[z]Combined data from all locations in 2014 and 2015 for each response variable
[y] Plant population determined by counting the number of plants in two 3.048 m sections of row of each plot
[x]Treatment is statistically different at P= 0.05, with an asterisk (*) indicating which effect that is significantly different
[w]This is the treatment effect of combined years and combined collection time points
[v]Days after planting

Table 2.11. Individual analysis of years 2014 and 2015 at six locations naturally infested with SCN in Nebraska GLIMMIX procedure with a confidence level of $\alpha \leq 0.05$.

			Response variable[z]		
Year	**Effect**	**DF**	*Plant population[y]*	*SCN population[x] (log Rf)*	*Yield*
2014	Location	2	**<0.0001[w]***	**0.0007***	0.2217
	Treatment[v]	2	0.9405	0.3795	0.6719
	Location*Treatment	4	0.1444	0.4560	0.7313
	Time	2	**<0.0001***	NA	NA
	Time*treatment	4	0.9228	NA	NA
	Treatment 12-26 DAP[t]	2	0.9266	NA	NA
	Treatment 21-39 DAP	2	0.9553	NA	NA
	Treatment Harvest	2	0.4820	NA	NA
2015	Location	2	**<0.0001***	0.2551	**<0.0001***
	Treatment[v]	2	0.7202	0.7312	0.2971
	Location*Treatment	4	0.3054	0.7624	0.3575
	Time	2	**<0.0001***	NA	NA
	Time*treatment	4	0.9921	NA	NA
	Treatment 12-26 DAP[t]	2	0.8937	NA	NA
	Treatment 21-39 DAP	2	0.7524	NA	NA
	Treatment Harvest	2	**0.0907**	NA	NA

[z]Combined data from all locations in 2014 and 2015 for each response variable

[y] Plant population determined by counting the number of plants in two 3.048 m sections of row of each plot

[x]Reproduction factor (Rf), $\log_{10}((P_f + 1)/ (P_i + 1))$ was used for statistical analysis, but is not reported. Rf values below and above 1.0 indicate the SCN population decreased or increased, respectively, during the season.

[w]Treatment is statistically different at P= 0.05, with an asterisk (*) indicating which effect that is significantly different

[v]This is the treatment effect of combined years and combined collection time points

[t]Days after planting

CHAPTER III

POPULATION DYNAMICS OF THE BIOCONTROL AGENT *LYSOBACTER ENZYMOGENES* IN THE RHIZOSPHERES OF SOYBEAN (*GLYCINE MAX* L. MERR.) AND CEREAL RYE (*SECALE CEREALE*)

3.1 Introduction

Variability in the effectiveness of host resistance and crop rotation as management strategies for soybean cyst nematode (*Heterodera glycines*, SCN) has generated the need for additional tools to be developed. Currently, there are two separate biological control approaches being developed against SCN. One approach is to establish populations of bacterial antagonists of SCN in the soil or roots. Clariva™ Complete Beans containing *Pasteruia nishizawa* and Poncho/VOTiVO™ containing *Bacillus firmus* are commercially available nematicidal seed treatments having different modes of action in protecting the root system from SCN (Callanan and Alderfer, 2014; Bayer CropScience, 2016). Field evaluations conducted in various locations in Nebraska, however, have not shown these systems to be effective (Musil, unpublished data), other states in the Midwestern region have performed similar field studies with inconsistent results across regions (Tylka et al, 2015; Potter et al., 2015; Staton and Seamon, 2015). Fortunately, there are many of genera of bacteria besides *Bacillus* or *Pasteruia* that have exhibited antagonistic or competitive activity against plant parasitic nematodes and could potentially be developed as a biocontrol for SCN (Siddiqui and Mahmood, 1999; Tian et al., 2007). Among them are species of *Lysobacter* that can produce a wide array of extracellular enzymes, antibiotics, and can colonize the host systems (Christensen and

Cook, 1978; Hayward et al., 2010). A particular species, *Lysobacter enzymogenes* strain C3, has been studied as a biological control agent against fungi with varied success when evaluated in field trials (Giesler and Yuen, 1998). In laboratory experiments C3 was able to inhibit SCN reproduction through nematicidal effects of chitinase, however, its ability to reduce nematode populations has not been field tested (Chen et al., 2006). Based off distribution soil sampling, C3 was found to prefer grass species over non-grass species (Yin, 2010).

Grass species and other cover crops, including cereal rye, do not only enhance microorganism diversity, but can help prevent soilborne diseases by inducing a suppressive soil (Garbeva et al., 2004; Eastburn, 2013). Nematodes, including SCN, have been suppressed by growing annual ryegrass (*Lolium multiflorum*) and cereal rye as a cover crop, however annual ryegrass can become a competitor to annual crops if not properly maintained (Hoorman, 2011; Ackely, 2013). Cereal rye is easier to manage, however the field trials of SCN suppression are not as consistent (Rye, 2012; Eastburn, 2013).

In developing new strategies for management both cereal rye as a cover crop and the introduction of C3 into the soil profile are both potential options on their own for reducing of SCN populations in the soil. The use of both a cover crop along with a biological control agent for a management practice, however, has not been explored. As a first step in studying the combined strategies, the potential of C3 to colonize the rhizosphere of cereal rye and soybean needed to be determined. Therefore, the objectives of this research were to: 1) determine if plant species would affect

population dynamics of C3 in the soil, 2) determine if plant species would affect population density of C3 population levels in the root system and distributed over plant parts.

3.2 Materials and Methods

3.2.1 General microbiological methods

Strain C3R5, rifampicin-resistant spontaneous mutant of *Lysobacter enzymogenes* C3, was used in all experiments. The strain was stored at -80°C in storage broth. To create cell suspensions of C3R5, the bacteria was transferred from cold storage to 10% tryptic soy agar (TSA). The plates were then incubated for three days at 28°C. A single colony was suspended in 1 mL of sterile phosphate buffer, pH 7.1 (PB). The suspension was then spread onto TSA (250 µL per plate) and incubated at 28°C for two days. Bacterial cells were then scraped off the plates with a sterile plastic scraper and suspended in PB. Using a spectrophotometer to measure absorbance of the cell suspension at 595 nm, the concentration of the cell suspension was diluted to between 10^8-10^9 colony forming units (CFU)/mL.

3.2.2 General plant growth methods

Cereal rye (Olsen Livestock & Seed) and soybean AG-4703(resistant to SCN, Asgrow) were used in all experiments. In some experiments, soybean 'Williams-82', susceptible to SCN, also was used. Seeds were surface disinfested by soaking in a 3% bleach solution for five minutes and rinsed three times in sterile distilled water (SDW). The seeds were allowed to dry and refrigerated until use. Sanitized seeds were planted into a pasteurized sandy soil and, in some experiments, a pasteurized loam soil held in

various types of containers described below. The soil was moistened to field capacity prior to planting of seed. Soybean seeds were planted 2.5 cm deep, while cereal rye seeds were planted 1.3 cm deep. The planted units were kept on a bench in greenhouse where air temperatures generally ranged from 23-28°C.

3.2.3 General C3 population assay procedures

To enumerate C3 on C3-treated seed, a single seed was placed into a microcentrifuge tube and 1 mL of PB was added. The seed was soaked for 30 minutes and vortexed for 15 seconds, after which the liquid was used for C3 population assay. Soil and root samples were collected from greenhouse experiments by emptying the contents of containers containing plants and soil into a plastic tote and separating the plants from soil. Then, samples of soil (6-10 ml) were collected from the vicinity of roots using an alcohol-sterilized scoopula. Each soil sample was weighed and placed in conical tubes, and 5 mL of PB was added to suspend the sample. Each tube was then vortexed for 1 minute and then the contents were assayed for C3 populations. Dry weights of soil were determined after soil was allowed to dry in a 60°C oven for 48 hours. The root system of the plant removed each container was gently tapped to remove excess soil. The shoots were cut off and the roots were placed into pre-weighed mesh sample bags (AgDia) into which 5-10 mL of PB was added depending upon root size. Roots were then ground with a grinding device (AgDia) attached to a drill press to uniformly extract bacteria into the PB. The liquid was then assayed for C3 populations. Dry weights of roots were determined after the roots were allowed to dry in an oven for 48 hours at 60°C.

C3 population levels in liquid extracts seed, soil and roots were determined using a technique referred to as the 8-spot method, essentially a most-probable numbers method (Harris and Sommers, 1967) modified by Yuen et al., 1991. Starting with the sample extracts, a series of seven 10-fold dilutions (50 µL into 450 µL) were made in PB. A repeating pipetter was used to take up 50 µL of each dilution and dispense the dilution as eight 10-µL drops onto a quadrant on plates of C3R5-selective agar media. The C3R5-selective medium consisted of 10% TSA and 5 g/L baker's yeast (an indicator for lytic activity typical of *Lysobacter*); the antibacterial drugs rifampicin (200 mg/L), kanamycin (100 g/L) and penicillin (100/L); and the fungicides cyclohexamide (200 mg/L); Benlate (12.5 g a.i./L) and amphotericin (4 g/L). The cultures were incubated at 28°C for 3-5 days. Spots where 10-µL drops were applied were examined for bacterial growth surrounded by clearing zones resulting from the lysis of yeast cells. For each dilution series, the total number of spots with growth indicative of C3R5 was counted and used to calculate cell density (CFU/mL) in the original sample (seed, soil or root) extract (Harris and Sommers, 1967; Yuen et al., 1991). C3 soil populations were expressed as CFU/dry weight soil. C3 populations on seed and roots were expressed as CFU/dry weight seed-root, and as CFU/seed or root system. Prior to statistical analysis, the population data were converted to $\log_{10}$ units based on the assumption that C3 populations in the rhizosphere follow a lognormal distribution (Loper et al., 1984).

3.2.4 Plant species effect on C3 populations in soil

An experiment (Experiment 1) was conducted to determine if the presence of soybean or cereal rye roots have any effects on populations of C3 present in the soil.

There were two trials of the experiment, the trials differing primarily as to the type of container used in growing the plants and the number of plants per container. In the first trial, three seeds each of cereal rye and soybean cvs AG-4703 and William-82 were planted equal distances apart in pasteurized sandy soil contained in plastic tri-corner beakers (800 mL with no holes). The tri-corner beakers were placed into a water bath kept at 28°C. Non-planted beakers of the soil were used as the no-plant control. Seven days after planting, when plants had emerged, 2 x 10^7 CFU of C3R5 was added to the soil in each pot by drenching 50 µL of a 4 x 10^9 CFU/mL cell suspension to the center of each tri-corner beakers, followed by 4.95 mL of SDW. Planted and non-planted pots of soil were drenched with 5 ml SDW as the no-bacteria controls. Soil and root samples were collected 4 hours (= day 0) after addition of C3 to the soil, and then every 7 days up to 28 days. There were three replicates of soil and root samples collected per treatment (i.e. plant-bacteria combination) at each sampling date.

In the second trial of Experiment 1, individual conetainers (164 mL, Ray Leach "Cone-tainers") filled with sandy soil were planted with only one seed of cereal rye or the two soybean cultivars. Soil with no seed was the no-plant control. There were six replications for each plant/cultivar that were arranged in a completely randomized design on the bench top. Seven days after planting, 3.5 x 10^8 CFU of C3R5 was added to each conetainer of soil by drenching with 50 µL of a cell suspension of C3R5 at 7 x 10^9 CFU/mL and then with 2.95 mL of SDW. Planted and non-planted conetainers of soil were treated with 3 mL of PB to produce no-C3 controls. All subsequent procedures were the same as those for the first trial of the experiment.

3.2.5 Plant species effects on C3 root colonization

Two experiments were conducted to compare cereal rye and soybean as host plants for root colonization by C3 starting from treated seed. Each experiment was conducted twice. Two different methods were used between the experiments to apply C3 to seed because seed treatment methods for C3 have not been evaluated prior to this study. In one experiment, bacteria were applied as a dried powder formulation. In the other, bacteria were applied as a liquid seed treatment.

A modification of the method described by (Kloepper and Schroth, 1981) was used to create a powder seed treatment formulation (Experiment 2). C3 was cultured on 100% sucrose agar plates for 2 days and then cells were scrapped off using less than 20 mL of PB. The concentration of the cell suspension was checked using a spectrophotometer to make sure that it was was higher than 10^9 CFU/mL. 20% xanthum gum (5.0 g) and 15 mL of the cell suspension were mixed in a sterile petri dish. This mixture set for five minutes and then was placed into a plastic bag along 40.0 g sterile talc. The bag was filled with air and shaken until small pellets were formed. A dowel was used to flatten the material in the bag to an even thickness and then cut open under aseptic conditions. The opened bag was placed onto a sterile tray and covered with aluminum foil, which was then placed in an incubator at 12°C to dry for three days. Once the mixture was dry, it was ground into fine powder using a mortar and pestle. The same procedure was followed to make the control (no bacteria) formulation except that 15 mL of PB was mixed with xanthan gum instead of the cell suspension. C3 populations in the powder was enumerated by adding 100 mg of the powder to 1 mL of PB, allowing

the mixture to sit for 30 minutes, and vortexing for 15 seconds before making serial dilutions.

Soybean (AG-4703) and cereal rye seeds were coated with the C3 or control powder formulation by adding together 40 seeds, 2.5 mL 1% methyl cellulose and 5.0 g powder formulation in a plastic bag. The plastic bag was filled with filtered air and gently shaken to coat all the seeds evenly. Coated seed were immediately planted.

The liquid seed treatment consisted of a culture of C3 in 125 mL of 10% tryptic soy broth (TSB) (Experiment 3). Broth cultures were placed on an incubated shaker at 28°C and 180 rpm for six hours when the culture reached 10^8 cells/mL, as determined by absorbance measured it was removed from the shaker. Using sterile cheese cloth Surface sanitized soybean and cereal rye seed held in sterile cheese cloth were dipped into the liquid cell culture until all the seeds were coated. Seeds were immersed in sterile 10% TSB as the no-bacteria control treatment Seeds were allowed to dry before planting.

In both experiments, seed treated with C3 or the no-bacteria controls were planted in individual conetainers (164 mL) in a pasteurized loam soil that was previously watered. The containers of soil were set up in a completely randomized design on the greenhouse bench top, where the temperature ranged from 23-28°C. Plants were watered daily with 5 mL of distilled water. Treated seed was assayed for C3 populations at planting (= day 0), and root samples were collected for C3 population assay at days 7, 14, 21, and 28 days after planting. Plant shoots also were collected for dry weight measurement after drying for 48 hours at 60°C to determine if seed treatment with C3

has any effects on plant growth. There were four replicate samples per treatment at each sampling date.

3.2.6 Spatial distribution of C3 in root systems of soybean and cereal rye

An experiment was conducted twice to determine whether or not the colonization of C3 on soybean and cereal rye roots was specific to particular regions of the root system. An additional factor in this experiment was a comparison of C3 root colonization in sandy soil and loam soil. Using the liquid seed treatment method described above, soybean and cereal rye seeds were treated with a broth culture of C3R5 or with TSB (no-bacteria control). A single treated seed was planted in a conetainer (164 mL) filled with either pasteurized sandy soil or pasteurized loam soil. The planted containers were maintained on a greenhouse bench as described above. Seven days after planting, three replicate plants per treatment (bacteria-plant-soil combination) was destructively sampled. After the shoots were cut off at the soil line, the roots from each plant were collected and sectioned into zones (3 zones for cereal rye, 4 zones for soybean (Figure 3.1). On the cereal rye roots, they were cut using a sterile razor blade at 2.5 cm and 5.0 cm down the root leaving three zones (0-2.5 cm, 2.5 -5.0 cm, and 7.6 cm-end of root). On the soybean roots, the lateral roots were cut off at 2.5 cm (0-2.5 cm) and at 7.6 cm (2.5-7.6 cm), the tap root was from 7.6 cm to the end of the root, and then the central root from where the lateral roots were cut off (2.5-7.6 cm), leaving four zones. Each “zone” sample was ground and extracted separately for C3 population determination as described above.

3.2.7 Statistical Analysis

All experiments, or trials of an experiment, were analyzed separately using PROC GLIMMIX in SAS at a significance level of $\alpha \leq 0.05$ (SAS Institute, Cary NC).

3.3 Results and Discussion

3.3.1 Plant species effect on C3 populations in soil

Soil and root populations of C3 in the presence of cereal rye and two soybean cultivars were examined Experiment 1 (soil drench experiment) testing the hypotheses that the presence of plant roots would increase population densities of C3 that was applied to soil, and that these effects would vary between plant species. These hypotheses, however, were not supported by the results. In both trials of Experiment 1, there was no overall 'environment' (roots or soil associated with soybean 'Williams 82', soybean 'AG-4703, cereal rye, and non-planted soil) effect in the analysis of fixed effects (Table 3.1). This meant that there were no significant differences in C3 soil populations regardless of the presence or absence of a root system. In addition, there were no significant differences between soil and root populations of C3 for a given plant.

In the first trial of this experiment, there was no significant 'environment by time' effect found in the analysis of fixed effects. The 'environment by time' interaction was significant in the second trial. This was related to differences in C3 population among various soils at Day 0 and Week 1 and Week 3, but the differences were not consistent across the three sampling dates (Figure 3.3 A).

The primary hypothesis in this experiment, that soil populations of C3 associated with one or all plant types tested would be higher than C3 populations in soil with no

plants, was based on theory that roots would exude nutrients that would support multiplication of C3 already present in the soil. Soil and root populations associated with a particular plant also were compared in this experiment with the hypothesis that C3 populations on or in the roots would be higher than C3 populations in the bulk soil because of the greater availability of nutrients at the root surface. The apparent absence of a root effect on soil populations and the lack of a population increase at the roots could have been due to experimental variability associated with the soil sampling and population assay procedures. Another explanation may be related to the root systems sampled in this experiment being comprised primarily of mature root regions rather than root tips. Because mature root tissues exude much less than root tips, the roots may not have exerted a strong enough effect (i.e. did not exude sufficient nutrients) to exert a detectable effect on C3 populations.

There was a significant 'time' effect in both trials of this experiment (Table 3.1), indicating that C3 populations in general changed over the course of the experiment. In the first trial, there was a general decrease in C3 population densities from time 0 to week 2, followed by an increase through week 4 (Fig. 3.2). The increase in soil populations observed in the first trial suggests that there was sufficient nutrients in the soil used in this experiment to support C3 population growth. In the second trial, C3 population densities gradually declined steadily over the 4 week experiment period (Figure 3.3). The decline in C3 soil populations occurring in both trials would suggest that the soil conditions in general were not conducive to C3 multiplication.

3.3.2 Plant species effects on C3 root colonization

The ability of C3 to colonize roots from populations applied to seed was investigated in a pair of experiments, one (Experiment 2) in which the bacterium was applied in dry powder and the second (Experiment 3) in which the bacterium was applied as a liquid (broth culture) treatment. The hypotheses tested in both experiments were 1) that C3 can colonize roots from seed and 2) that C3 will colonize the root differently based upon plant species. In the first trial of Experiment 3 (dry powder seed treatment), there was a significant 'time' effect for C3 population densities and total C3 populations per root system (Table 3.2), meaning these parameters changed significantly over time. In the second trial, the 'time' factor was not significant for either parameter. The general temporal trends were similar, however, between the two repetitions; C3 population densities (numbers per g root), in general, declined gradually over the 4 week experiment period (Figure 3.4 A&B) while total C3 populations per root system remained level (Figure 3.4 C&D). While these trends suggest that C3 did not multiply very rapidly in this experiment, the finding that total populations per root system were level over an extended time does suggests that survival and multiplication by C3 the root systems, i.e. colonization, did occur at a rate that balanced C3 cell death.

There was no significant 'plant' effect for either population parameter in both trials of this experiment (Table 3.2), indicating root populations of C3 were similar between cereal rye and soybean. In the first trial, there was significant 'plant' by time interaction for both population parameters (Table 3.2). C3 population density on

soybean was significantly higher than on cereal rye only on the 4th week sampling date, whereas total C3 population per root system was higher on cereal rye compared to soybean on the 1st and 2nd weekly sampling dates. Results in Experiment 3, in which seeds were treated with broth cultures of C3, provided more definitive evidence that C3 can colonize roots starting from seed populations. The 'time' effect was significant for both population parameters in trial 1 (Table 3.3). C3 populations trended upwards indicating that the bacterium multiplied on roots over the 4 weeks (Figure 3.5 A & C). In second repetition, there was no significant 'time' effect (Table 3.2); C3 populations averaged both plants remained unchanged through the course of the experiment. In both trials of this experiment, the 'plant' effect was significant at the 90% confidence level for both population parameters (Table 3.3). Averaged across sampling dates, cereal rye root populations of C3 were higher than corresponding populations on soybean roots.

The 'plant by time' interaction was significant for both population parameters in trial 1 (Table 3.3). For both parameters, C3 populations on cereal rye were higher than on soybean on week 1 and week 2 sampling dates (Figure 3.5 A & C).

From the collective results from these two experiments, it can be concluded that C3 can colonize the root system of cereal rye higher to higher numbers than the root system of soybean. This plant species effect was particularly evident during the first week of these experiments despite soybean exhibiting more rapid root growth than cereal rye (Appendix Figures 1A and 2A). One possible explanation for higher C3 populations being collected on roots of cereal rye over soybean is that there was more

soil attached to the roots of cereal rye (Figure 3.1) and that populations of C3 associated with cereal rye root was actually in the attached soil. The apparent preference for cereal rye in this study is in line with the study by Yin (2010), that *L. enzymogenes* preferred grasses over non-grasses; which suggests that C3 is indeed more adopted to colonize cereal rye over soybean.

3.3.3 Growth effects of strain C3

Root and shoots from C3-treated and control plants grown in Experiments 2 and 3 were collected and weighed to determine whether or not seed treatment with C3 would influence root and/or shoot growth. Biomass measurements (fresh and dry root weights; fresh and dry shoots weights) from soybean and cereal rye were analyzed separately. Mean biomass values at each sampling date are presented in Supplemental Figures 1 and 2 in the Appendix. Results from Proc GLIMMIX analysis of all the data are presented in Supplemental Table 1 in the Appendix. Among the two plants, two experiments and two trials per experiment, there were eight instances in which a significant treatment effect was found in the Proc GLIMMIX analysis. The results associated with those eight cases are presented in Table 3.7.

In soybean, there were significant treatment effects for root and shoot variables in both trials of Experiment 2, which involved a dry powder seed treatment (Table 3.7). The weight of C3 plant parts, averaged across 4 sampling dates, were higher than that of the corresponding no-bacteria control. No significant treatment effect, however, was found in soybean, in the two trials of Experiment 3 in which C3 was applied to seed as a broth culture.

In cereal rye, significant treatment effects were found for root weights in one trial of each of the two experiments. How C3 seed treatment affected root biomass, however, appeared to be dependent on the application method. Seed treatment with C3 increased root mass when the bacterium was applied in dry powder form, but it decreased root mass compared the control when a broth culture of the bacterium was used (Table 3.7).

The collective data from these experiments indicate that strain C3 has the potential to benefit plant growth, but that effect is not consistent. Furthermore, it possible that C3 might have a deleterious effect under certain conditions. A possible explanation for the positive effects stemming from C3 seed treatment might be the inhibition of deleterious rhizosphere bacteria and fungi that reduce plant growth while not inducing symptoms. Strain C3 was demonstrated to be a potent antagonist of fungi and the species *L. enzymogenes* is recognized to lyze bacteria via secondary metabolites (Kobayashi and Yuen, 2007). Inhibition of deleterious rhizosphere microbes by C3 as a mechanism for the growth stimulation effect would be consistent with that mechanism being commonly associated with plant growth promoting rhizobacteria, or PGPR (Lugtenberg and Kamilova, 2009).

The negative effect of C3 of cereal rye following broth culture seed treatment might be related to “root-growth inhibitor” (RGI), a yet unidentified factor produced by strain C3 that can prevent inhibit radicle emergence and slow root elongation (Li and Yuen, 2003). The effects of RGI were most apparent when C3 was applied in high numbers (>10^8 CFU per seed), and grasses, as well as soybean, were particularly

sensitive (Li and Yuen, 2003). It is conceivable that RGI was produced by the bacterium while growing in broth culture and sufficient amounts of the factor may have been applied to seed via the broth to affect root growth. Because C3 cells applied in the dry powder were grown on an agar medium, the RGI might not have been present in the cell that were used to produce the dry powder formulation. Why soybean did not suffer the same effects from C3 broth culture seed treatment in the same experiment cannot be explained.

3.3.4 Spatial distribution of C3 in root systems of soybean and cereal rye

C3 population densities in various regimes of cereal rye and soybean root sections were determined to test the hypothesis that C3 colonization is specific to particular root regions. The experiment was conducted in sand and loam soils to determine if C3 colonization is affected by soil type. In soybean and cereal rye, in both soils, the highest population densities of C3 were found at the top and decreased with increasing depth down the soil profile. This suggests that C3 is not a root tip colonizer and is more adapted to colonize mature root regions instead.

This trend was particularly apparent in cereal rye (Table 3.4) and consistent across the two trials of the experiment. The only exception to this trend was the absence of C3 from the top portion of the central root (Zone 2) of soybean grown in sand soil in repetition 1 (Table 3.5). The aberrant result might have been due to an error in the assay of that set of samples, because it is inconsistent with all other results from both soils and both trials.

The above-ground shoot parts from the cereal rye and soybean plants in this experiment also were analyzed for C3 to determine if the bacterium can colonize shoots from populations originating from seed. C3 was found to be present in both trials of soybean shoots. The bacterium was detected in rye shoots in the first trial, but not in the second (Table 3.6). The difference in C3 between soybean and cereal rye shoots was not significant (P=0.3155). The detection of C3 in the shoots starting from populations applied to just a seed is consistent with previous findings; although *L. enzymogenese* was originally described as a soil-inhabiting species (Christensen and Cook, 1978), C3 and other strains of the species can colonize leaves endophytically as well as epiphytically (Li, 2014).

3.4 Conclusions

While all of the experiments in this study were conducted under greenhouse conditions and relatively artificial soil environment, the results suggest a number of directions for further research that potentially may lead to practical use of strain C3, or similar bacteria, to address SCN and other soilborne pathogens of soybean.

First, it was demonstrated in this study that C3 can colonize soybean roots from populations applied to seed, and thus seed treatment appears to be a practical approach to deliver the strain to the roots of this crop. Further research is needed to determine the population densities and distributions that C3 can establish in soybean roots under field conditions. In addition, the population densities of C3 needed to influence the activity of SCN and other soilborne pathogens need to be determined.

Second, the finding that C3, starting from numbers as low as 10^3 CFU/seed, has the potential to multiply to 10^7 CFU per root system (Figure 3.5) offers a potential method to deliver the biocontrol agent to the soil profile in agricultural fields. By planting cereal rye seed treated with the bacterium, C3 can be introduced into the soil and distributed uniformly across a field. Cereal rye is typically planted at high seeding densities (50-200 lbs/acre) that can result in more than 2 million plants per acre. Assuming that the root system of each C3-treated plant ultimately yields 10^7 CFU of the bacterium, this translates into more than 10^{13} CFUs being added uniformly to the soil profile across an acre of land. Whether or not C3 can multiple to the same populations under field condition needs to be investigated. Additionally, the degree to which populations of C3 propagated in situ in cereal rye root systems can persist in the soil needs to be determined.

Third, better seed treatment methods need to be developed for C3 that optimize survival and multiplication of the bacterium while preventing negative effects. Two seed treatment methods were used in for experiments in study because no seed treatment method had been identified specifically for C3 that was reliable and efficient. The powder method, which is more typical of methods used to treat seed for field planting in the field, is considerably more labor and resource intensive than soaking seeds in a broth culture. The latter, on the other hand, would not be practical or compatible with conventional planting systems. The broth seed treatment appeared to support better multiplication of C3 than the powder method, perhaps due to the nutrients from the broth supporting a flush of bacterial growth. That method, however, also was associated

with growth depression in cereal rye, presumably caused by the action of a root-growth inhibiting factor excreted by C3 into the broth. But because the two methods were employed in separate experiments, it cannot be definitively concluded that the observed effects were due to the seed treatment methods and not to environmental conditions or other factors occurring within the different experiments. Future experiments comparing the two methods directly need to be conducted.

Fourth, the presence of plant roots in soils containing C3 did not enhance C3 populations in the soil or on roots. This result indicated that there is little potential for using cereal rye as means to stimulate indigenous populations of *L. enzymogenes* already in the soil. Perhaps, the planting of cereal rye might have an effect over the long-term if cereal rye was planted repeated, but even if that were to occur, the non-uniform distribution of indigenous populations would likely not be affected.

Lastly, it was shown that C3 can stimulate root growth. Although growth stimulation by C3 was observed previously on occasion (G. Yuen, personal communication), it had not been verified through repeated experiments. Further investigation should be made into the mechanisms behind the growth stimulation effect and the conditions under which the effect is expressed. That information might contribute to C3 ultimately being used for plant growth promotion, as well as biological control of pathogens.

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3.6 Figures

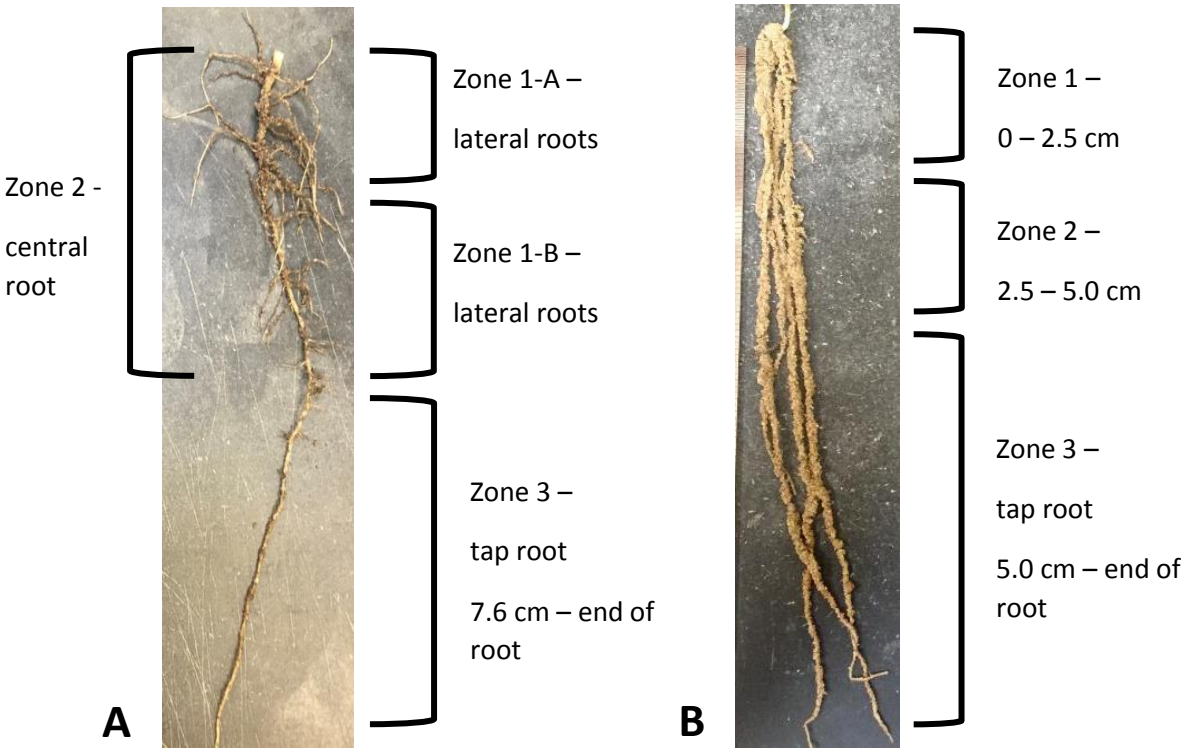

Figure 3.1. Root zones designated in 1-week old plants of soybean (A) and cereal rye (B).

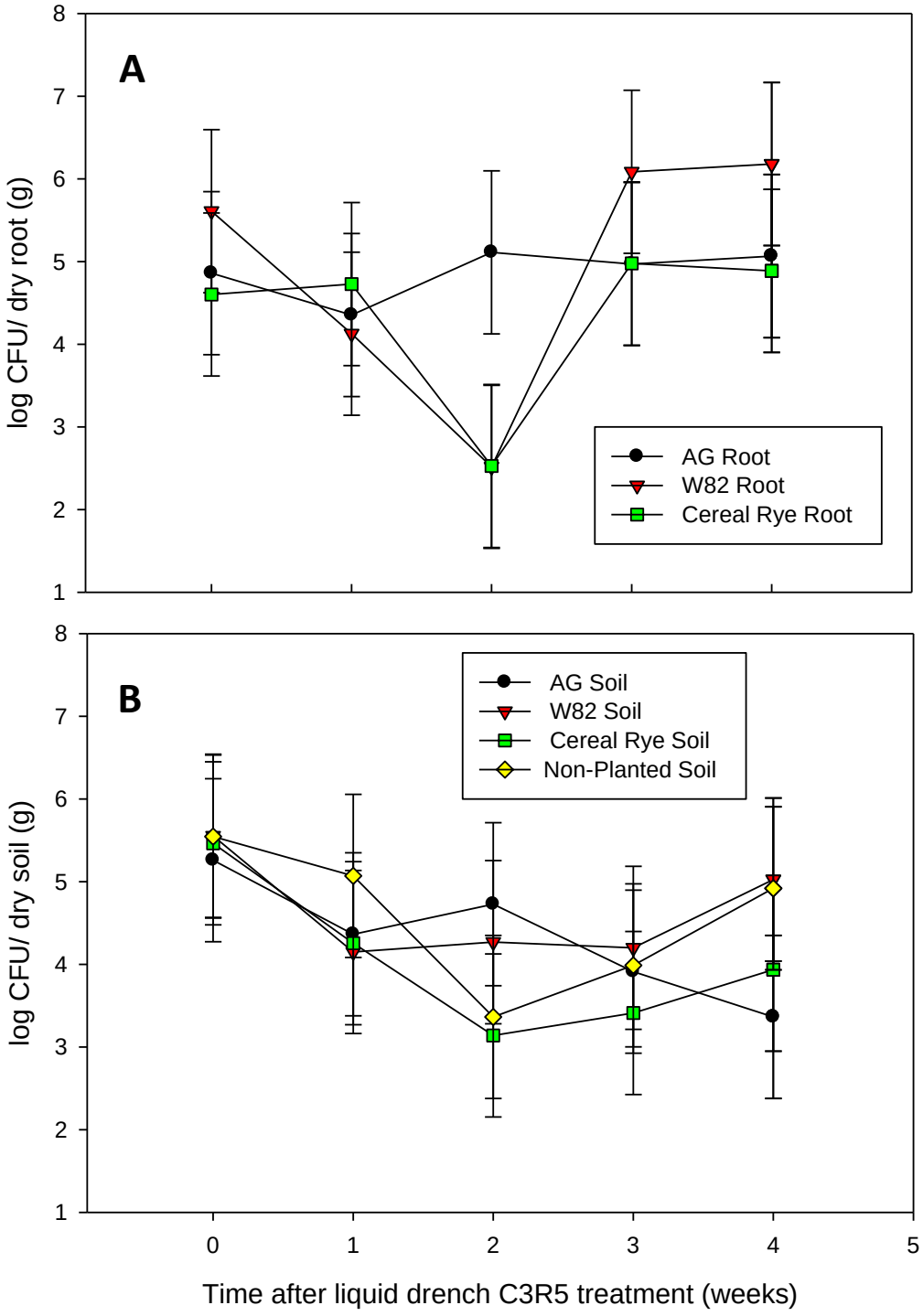

Figure 3.2. Population density of *Lysobacter enzymogenes* strain C3 in associated soil and roots of soybean 'AG-4703' (AG), soybean 'Williams 82', and cereal rye, and in non-planted soil in Trial 1 of Experiment 1. C3 was applied to soil after plant emergence as a drench with a cell suspension. C3 root populations (A) and C3 root soil populations (B) are presented separate figures for the sake of clarity. Error bars are the standard error of means.

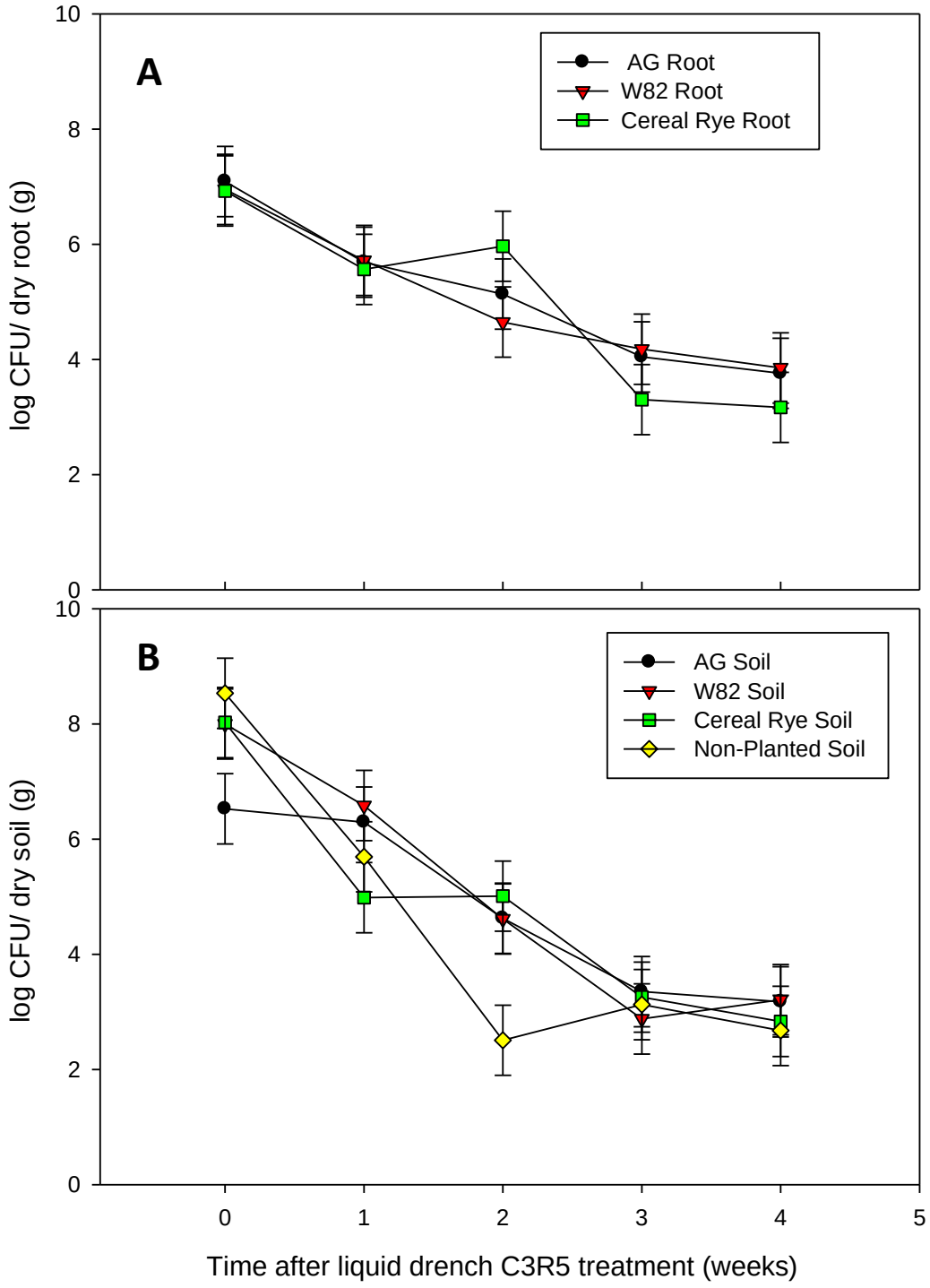

Figure 3.3. Population density of *Lysobacter enzymogenes* strain C3 in associated soil and roots of soybean 'AG-4703' (AG), soybean 'Williams 82', and cereal rye, and in non-planted soil in Trial 2 of Experiment 1. C3 was applied to soil after plant emergence as a drench with a cell suspension. C3 root populations (A) and C3 root soil populations (B) are presented separate figures for the sake of clarity. Error bars are the standard error of means.

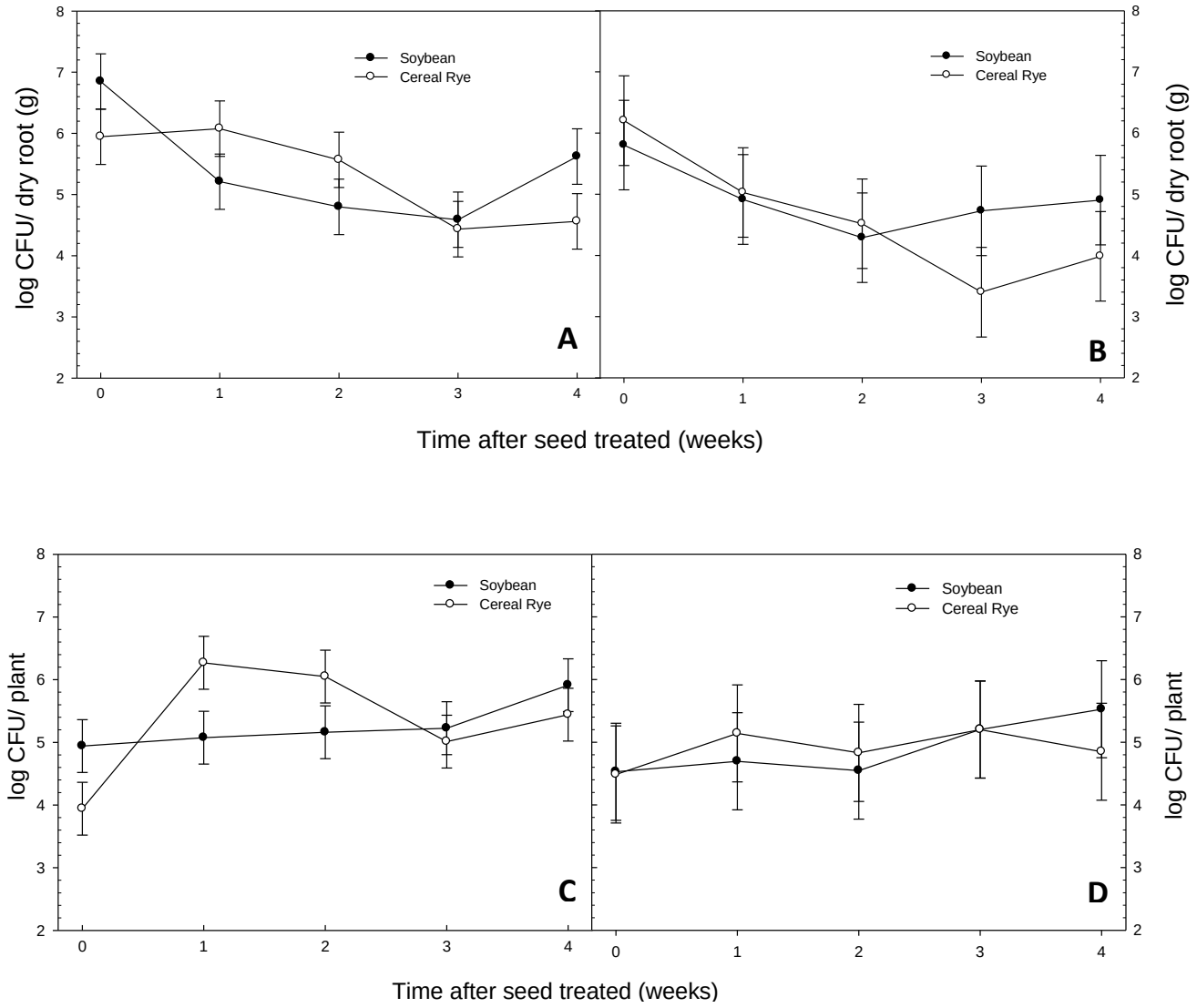

Figure 3.4. Populations of *Lysobacter enzymogenes* strain C3 in the rhizospheres of soybean (AG-4703) and cereal rye in Experiment 2 (C3 applied through a powdered seed treatment of the bacterium). Data presented in top panels (A & B) are C3 populations expressed on a density basis (i.e. members per g root); data presented in bottom panels (C & D) are C3 populations expressed in a total number basis (i.e. numbers per root system). Data in A & C are from trial 1; data in B & D from trial 2. Error bars are the standard error of means.

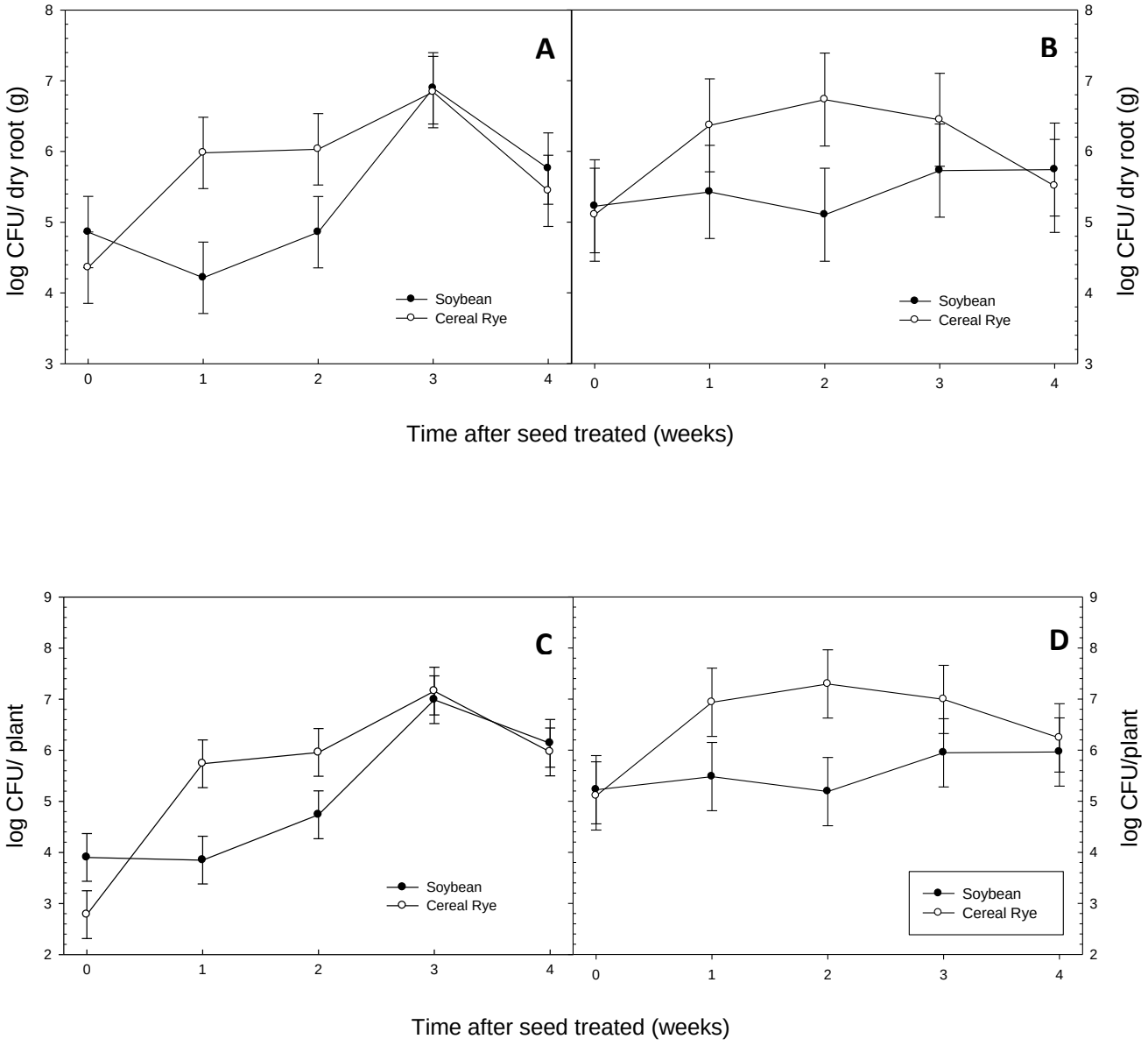

Figure 3.5. Populations of *Lysobacter enzymogenes* strain C3 in the rhizospheres of soybean (AG-4703) and cereal rye in Experiment 3 (C3 applied through a liquid seed treatment of the bacterium). Data presented in top panels (A & B) are C3 populations expressed on a density basis (i.e. members per g root); data presented in bottom panels (C & D) are C3 populations expressed in a total number basis (i.e. numbers per root system). Data in A & C are from trial 1; data in B & D from trial 2. Error bars are the standard error of means.

3.7 Tables

Table 3.1. Type III fixed effects from PROC GLIMMIX (SAS) analysis of *Lysobacter enzymogenes* C3 populations data from Experiment 1 (soil drench experiment). Where‘t’ is equal to time (week) over a four-week time period; ‘environment’ is root or soil C3 populations associated with soybean Williams 82, soybean AG-4703, cereal rye, or no plant. Data from each trial was analyzed separately.

Type III Tests of Fixed Effects				
Trial	*Response variable*	*DF*	*F Value*	*Pr > F*
1 N = 70	t	4	4.93	**0.0015**
	environment	6	1.02	0.4172
	t*environment	24	1.29	0.2035
2 N = 175	t	4	114.44	**<.0001**
	environment	6	1.3	0.2592
	t*environment	24	2.84	**<.0001**

Table 3.2. Type III fixed effects from PROC GLIMMIX (SAS) analysis of *Lysobacter enzymogenes* C3 population data from two trials of Experiment 2 (powder seed treatment experiment). Where 't' is equal to time (week) over a four-week time period and 'plant' is equal to soybean or cereal rye, and 'n' is equal to 30 for both plant species. C3 populations were analyzed on a per g root and per plant (root system) basis. The two types of data were analyzed separately for each trial.

Type III Tests of Fixed Effects					
Data type	*Trial*	*Response Variable*	*DF*	*F Value*	*Pr > F*
log CFU/root (g)	1	t	4	9.63	**<.0001**
		Plant	1	0.22	0.6388
		t*Plant	4	3.97	**0.0106**
log CFU/root (g)	2	t	4	4.3	**0.0072**
		Plant	1	0.85	0.3646
		t*Plant	4	1.11	0.3686
log CFU/ plant	1	t	4	6.94	**0.0004**
		Plant	1	0.04	0.8450
		t*Plant	4	5.25	**0.0025**
log CFU/ plant	2	t	4	0.53	0.7122
		Plant	1	0.56	0.4591
		t*Plant	4	0.88	0.4888

Table 3.3. Type III fixed effects from PROC GLIMMIX (SAS) analysis of *Lysobacter enzymogenes* C3 population data from two trials of Experiment 3 (liquid seed treatment experiment). Where 't' is equal to time (week) over a four-week time period and 'plant' is equal to soybean or cereal rye, and 'n' is equal to 30 for both plant species. C3 populations were analyzed on a per g root and per plant (root system) basis. The two types of data were analyzed separately for each trial.

Type III Tests of Fixed Effects					
Data type	*Trial*	*Response Variable*	*Num DF*	*F Value*	*Pr > F*
log CFU/root (g)	1	t	4	11.14	**<.0001**
		Plant	1	3.36	0.0767
		t*Plant	4	3.92	**0.0112**
log CFU/root (g)	2	t	4	1.21	0.3284
		Plant	1	3.99	0.0550
		t*Plant	4	1.39	0.2622
log CFU/ plant	1	t	4	35.71	**<.0001**
		Plant	1	3.62	0.0666
		t*Plant	4	6.37	**0.0008**
log CFU/ plant	2	t	4	2.3	0.0820
		Plant	1	10.16	**0.0033**
		t*Plant	4	1.79	0.1564

Table 3.4. Population density of *Lysobacter enzymogenes* C3 in different root regions of cereal rye grown in sand and loam soils. Each value is the mean from three plants assayed 7 days after a liquid seed treatment with C3. Results from each of the two trials of the experiment were analyzed and presented separately. Values with the same letter within an experiment trial are not significantly different at $\alpha \leq 0.05$.

	C3 population density (log CFU/g dry root)			
	Trial 1		Trial 2	
	Sand	*Loam*	*Sand*	*Loam*
Zone 1 (top 0 -2.5 cm of roots)	6.0947 A	6.6442 A	6.6031 A	5.131 B
Zone 2 (2.5 – 5.0 cm below root surface)	4.2319 BC	4.5311 B	2.9357 C	0.00 D
Zone 3 (5.0 cm to end of root)	3.1547 C	4.3375 BC	0.00 D	0.00 D
Pr>F		0.0006		<0.0001

Table 3.5. Population density of *Lysobacter enzymogenes* C3 in different root regions of soybean grown in sand and loam soils. Each value is the mean from three plants assayed 7 days after a liquid seed treatment with C3. Results from each of the two trials of the experiment were analyzed and presented separately. Values with the same letter within an experiment trial are not significantly different at $\alpha \leq 0.05$.

	C3 population density (log CFU/g dry root)			
	Trial 1		Trial 2	
	Sand	*Loam*	*Sand*	*Loam*
Zone 1-A (0-2.5 cm of lateral roots)	4.900 A	4.024 AB	2.6681 AB	2.0855 BC
Zone 1-B (2.5-7.6 cm of lateral roots)	1.430 BC	0.00 C	0.00 D	0.8755 CD
Zone 2 (0-7.6 cm of central roots)	0.00 C	5.213 A	4.1045 AB	4.7099 A
Zone 3 (7.6 cm to end of root)	5.349 A	2.851 AB	0.00 D	0.00 D
Pr>F	0.0011		0.0008	

Table 3.6. Population densities of *Lysobacter enzymogenes* C3 in the shoots of soybean and cereal rye plants treated with C3 by seed application. Data are means of determined from six plants grown in sand and loam soils assayed 7 days after a liquid seed treatment with C3.

	Shoot Population of C3 (log CFU/g dry shoot) ± SE	
	Soybean	Cereal rye
Experiment 1	4.541 ± 1.2861	2.39 ± 1.6913
Experiment 2	3.9916 ± 1.8988	0.00

Table. 3.7. Comparison of seed treatment with *Lysobacter enzymogenes* strain C3 with no-bacteria control for root and shoot biomass parameters in soybean and cereal rye. In Experiment 2, C3 was applied to seed in powder form while control seed were treated with powder carrier minus bacteria. In Experiment 3, C3 was applied to seed as a broth culture while control seed were treated with sterile tryptic soy broth. Data represent means of four samples collected 1, 2, 3, and 4 weeks after planting.

Plant	Experiment	Trial	Variable	Weight (g) in C3 treatment	Weight (g) in Control	Pr > F
Soybean	2	1	Fresh root wt	3.5	3.1	0.0432
Soybean	2	1	Fresh shoot wt	1.0	0.9	0.0289
Soybean	2	2	Fresh root wt	3.3	2.5	<.0001
Soybean	2	2	Dry root wt	2.4	1.8	0.0002
Cereal rye	2	2	Fresh root wt	4.6	3.9	0.0496
Cereal rye	2	2	Dry root wt	3.9	3.4	0.0374
Cereal rye	3	1	Fresh root wt	0.5	0.6	0.0379
Cereal rye	3	1	Dry root wt	0.07	0.09	0.0151

APPENDIX

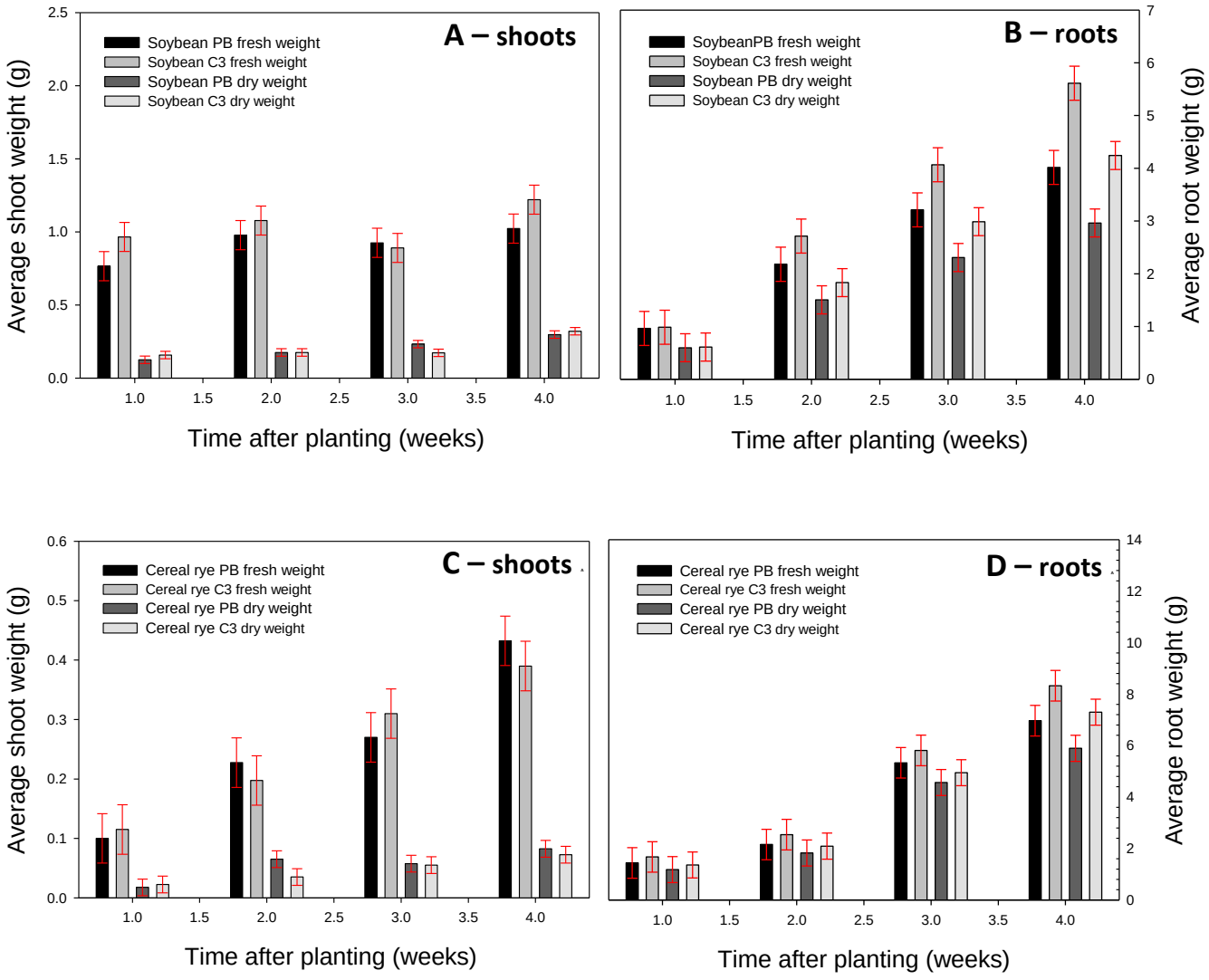

Figure 1A. Comparison of overall growth of soybean (AG-4703) shoots (A), roots (B) and cereal rye shoots (C), roots (D) with a seed treatment of powdered *Lysobacter enzymogenes* strain C3R5 or phosphate buffer (PB). Data represent means of four samples collected 1, 2, 3, and 4 weeks after planting and are a representation of two replicates. Red error bars are the standard error of means.

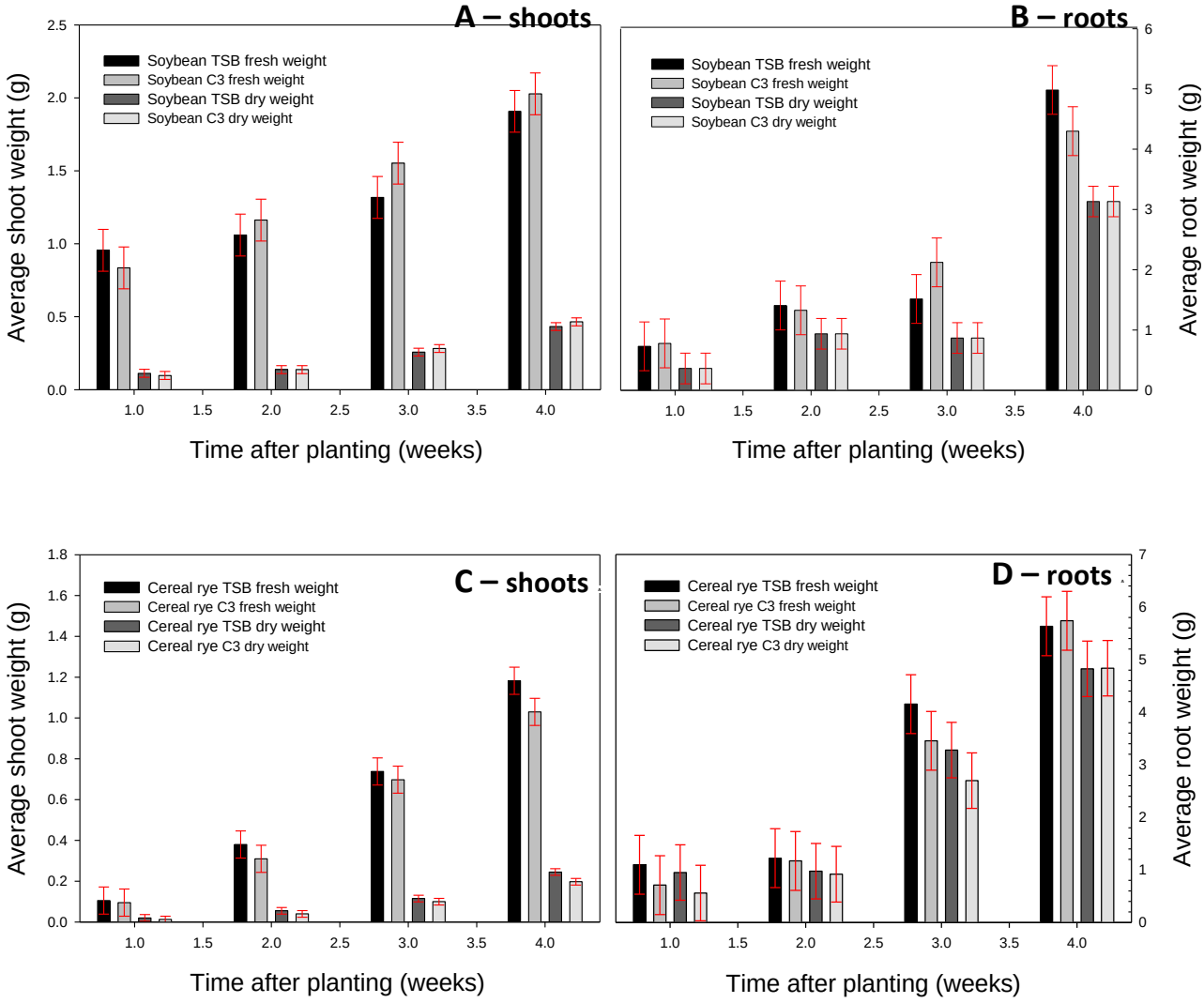

Figure 2A. Comparison of overall growth of soybean (AG-4703) shoots (A), roots (B) and cereal rye shoots (C), roots (D) with a seed treatment of a liquid *Lysobacter enzymogenes* strain C3R5 or tryptic soy broth (TSB). Data represent means of four samples collected 1, 2, 3, and 4 weeks after planting and are a representation of two replicates. Red error bars are the standard error of means.