Biocontrol potential of Bradyrhizobium japonicum against soybean sudden death syndrome

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Biocontrol potential of *Bradyrhizobium japonicum* against soybean sudden death syndrome

by

Tra Thi Thanh Huynh

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Plant Pathology

Program of Study Committee:
Xiao Bing Yang, Major Professor
Gwyn Beattie
Thomas Harrington
Andrew Lenssen
Huaiqing Wu

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2019

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DEDICATION

This dissertation is dedicated to my family for their endless love and support.
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ABSTRACT

Soybean [Glycine max (L.) Merr.] is the leading oilseed crop which accounts for 60% of the global oilseed production. Soybean sudden death syndrome (SDS) is among the most important soil-borne fungal diseases that threaten soybean production in US. Rhizobia play an important role as a nitrogen source for soybean via the fixation of atmospheric nitrogen and its subsequent conversion to useable forms. Slow-growing Bradyrhizobium japonicum (BJ) is the best known species nodulating soybean. There are currently a few reports of the use of Bradyrhizobium spp. in row crop diseases management. In literature, Bradyrhizobium isolates successfully reduced symptoms of diseases caused by Macrophomina phaseolina, Fusarium solani, and Fusarium spp. However, to the best of our knowledge, no information on the role of Bradyrhizobium japonicum in the management of SDS is established. In view of this knowledge gap, this study was designed to: (i) determine the effects of B. japonicum seed treatments and/or soil inoculant on the incidence and severity of the SDS under controlled conditions; (ii) evaluate the effects of B. japonicum seed treatments on SDS under field conditions (irrigated and non-irrigated).

To accomplish the first objective, two independent experiments were conducted twice under growth chambers and greenhouse conditions using a BJ strain from Iowa State University. Treated seeds (germinated or non-germinated) planted in clay pots or foam cups were exposed to three different growth chamber temperatures (15°C, 20°C and 25°C). Ten days later, treated seeds were shifted to a greenhouse at 25°C for the rest of study period. Treatments with BJ inoculated on the seeds planted in FV infested soil were found significantly effective in reducing SDS incidence and severity compared with untreated controls. In addition, BJ treatment of non-
germinated or germinated seeds had significant influence on SDS incidence at low temperature. No significant effects on plant biomass were detected.

To address the second objective, field trials were conducted during 2015 to 2017 cropping seasons using four BJ strains in seed treatments. At R6 (full seed set) growth stage, BJ treated plots showed lower SDS disease index compared with untreated plots in 2015 and 2016. Although no significant effects of treatments on nodule count and plant biomass, higher nodules count at V5 (fifth trifoliate) were observed in BJ treated plots compared with untreated plots. In addition, the BJ-treated plots showed yield advantage compared with untreated controls across three years.

The results from these studies showed that B. japonicum can reduce SDS. Current findings also suggest that B. japonicum can be a potential biocontrol agent, which is environmentally friendly, and as one of the alternatives to soybean growers in Iowa.
CHAPTER 1.
GENERAL INTRODUCTION

Dissertation Organization

The dissertation is arranged into four chapters. The first chapter includes an introduction and a literature review of importance, distribution, symptomatology, management approaches of soybean Sudden Death Syndrome (SDS) and using *Bradyrhizobium* spp. in managing plant diseases in general and particularly in suppressing Fusarium diseases on soybean, followed by a justification and research objectives. The second chapter is a greenhouse study on using *Bradyrhizobium japonicum* on germinated and non-germinated seeds to control SDS under controlled conditions. Chapter 3 shows the effects of *B. japonicum* seed treatments on SDS in irrigated and natural fields. Both chapters 2, 3 are written in the format of scientific journal articles. Each includes an abstract, introduction, materials and methods, results, and discussion, to be submitted to peer-reviewed scientific journals. The two manuscripts in this dissertation are the first attempts to understand the effects of treatments with *Bradyrhizobium japonicum* on soybean sudden death syndrome occurrence as well as the host-pathogen and host-symbiont interactions under different cultural practices. Chapter 4, the final chapter, provides comprehensive points in the research and conclusions drawn from the entire set of studies.
Significance of soybean production

Soybean [\textit{Glycine max} (L.) Merr.] is the main oilseed crop and accounts for 60\% of the global oilseed production (USDA 2019a). Soybean is an important protein source for human and animal feed because it has the highest protein contents (40\%) among food crops (Krishnan et al., 2005). Soybean is also used for biodiesel production, which requires lower energy inputs than using corn or sunflower oil (Pimentel and Patzek 2005). Moreover, soybean plays an important role in crop rotations by providing crop disease disruption (Curl 1963; Abbott and Murphy 2003), improving soil quality (Munkholm et al., 2013) and increasing yields of other crops, especially maize (\textit{Zea mays} L.) (Peterson and Varvel 1989; Crookston et al., 1991).

Soybean was cultivated in China around 3000 to 5000 years ago (Hymowitz 1970) and was first reported in the U.S in 1765, when it was cultivated in Georgia (Hymowitz and Harlan 1983). Since then, soybeans have been grown extensively in the United States. Currently United States is the largest soybean producer, accounting for 34\% of soybean production in the world followed by Brazil (30\%), Argentina (18\%), China (4\%) and India (3.95\%) (faostat.fao.org). In 2018, with 89.1 million acres planted in 31 states, soybean production in the US was 4.54 billion bushels with an estimated average yield of 51.6 bushels/acre. In 2018, Illinois and Iowa are the two biggest producing states in the US with 698.75 and 564.87 million bushels, respectively followed by Minnesota (389.35 million bushels), Indiana (346.32 million bushels) and Nebraska (333.35 million bushels) (USDA 2019b). Soybean is ranked the country’s second largest crop after corn in cash sales and the first in crop exports.
Soybean Sudden Death Syndrome

Causal agents of SDS and their distribution

Soybean sudden death syndrome was first noticed in Arkansas in 1971 (Roy, 1997). It was called the disease sudden death syndrome because of the fast onset of foliar symptoms (Hirrel 1983, 1987). Aoki et al. (2003) suggested that two distinct species are classified for SDS in soybeans: *Fusarium virguliforme* O'Donnell and Aoki and *F. tucummaniae* causing SDS in North America and South America, respectively. Since its first detection, SDS has been a major threat to United States soybean production (Yang and Lundeen 1997; Scherm and Yang 1999; Kurle 2003).

By 2016, the disease had been reported on 3 different continents (South America, North America and Africa) (Navi and Yang 2016a). The SDS in North America is caused by *F. virguliforme* (Aoki et al., 2003), in South Africa caused by *F. virguliforme* (Tewoldemedhin et al., 2014) and *F. brasiliense* (Tewoldemedhin et al., 2017) and in South America by a complex of *F. virguliforme, F. tucummaniae, F. brasiliense, F.cuneirostrum* (Aoki et al., 2005) and *F. crassistipitatum* (Aoki et al., 2012).

Symptoms and signs of SDS

The pathogen causing SDS can infect host tissues with a transiently symptomless stage with internal colonization (Navi and Yang 2008). The SDS pathogen infects soybean as early as seed germinated in spring and the fungus colonizes soybean roots in xylem (Navi and Yang, 2008). The first symptoms appearing on roots of seedlings infected by SDS pathogens include necrosis which is similar to other root rot pathogens. Reddish brownish discoloration occurs
from tap root to lower stem while pith is remaining white (Hartman et al. 2015). The fungus produces phytotoxins (Jin et al., 1996) which are then translocated throughout the plant via the xylem (Navi and Yang 2008). Four phytotoxins have been documented to be related to foliar symptoms but none of them was reported to be associated with SDS foliar symptoms under field conditions. The first phytotoxin identified named radicicol (or monorden) was associated with interveinal necrosis and marginal curling (Baker and Nemec 1994). A 17-KDa effector that caused chlorosis on cotyledons and detached leaves were the second phytotoxin (Jin et al. 1996). FvTox1 was the third phytotoxin; it reduced chlorophyll and induced chlorosis in soybean leaf discs and was reported as the major toxin causing foliar symptoms (Pudake et al., 2013; Brar et al. 2011). FvNIS1 is the most recent phytotoxin identified as associated with SDS foliar symptoms (Chang et al., 2016).

The diseased plants often remain symptomless until reaching the reproductive stages under field conditions. The most striking visual foliar symptoms commonly happen after flowering from the mid to late reproductive stages (Hartman et al., 2015). Symptoms are found on upper leaves as interveinal chlorotic spots producing a mottled or mosaic appearance (Scherm and Yang 1996; Roy et al., 1997). Marginal and chlorotic mottling is also observed on young leaves (Rupe et al., 1991). As the disease progresses, chlorotic spots expand and turn into interveinal necrosis while the midveins and major veins stay green. Severely affected leaves may fall off, and petioles attached to the stem (Roy et al., 1989; Rupe and Gbur 1995; Hartman et al., 2015). Flower and pod abortion results from early onset of the disease while later onset of the disease may cause complete defoliation and increase susceptibility to infection by late harvest pathogens such as *Phomopsis* and *Diaporthe* species (Roy et al., 1997; Luo et al., 2000). Under field condition, diagnosis of SDS can be confusing because some other diseases can have similar
symptoms, for example brown stem rot \((\text{Phialophora gregata})\) or red crown rot \((\text{Cylindrocladium parasiticum})\) \cite{roy1997}. Bluish masses of fungal conidia on taproot surfaces can be a helpful diagnostic sign of the disease \cite{hartman2015}.

**Fusarium virguliforme disease cycle**

While sexual stage of *Fusarium virguliforme* (FV) is not yet known, macroconidia, microconidia and chlamydospores are three types of FV asexual spores \cite{roy1989, aoki2003, aoki2005}. Macroconidia, the two to five celled spore with falcate shape, form predominantly from monophialides on conidiophores \cite{lawrence1989, aoki2003}; whereas, tiny, comma shaped microconidia are rarely produced \cite{aoki2003}. Thick-walled chlamydospores, the overwintering survival structure, are considered as the primary inoculum source of this fungus. The chlamydospore density in soil is found to be correlated to SDS severity because soybean seedlings contact the chlamydospore or conidia in the soil as they are growing \cite{scherm1998}.

Early in the spring when cool wet soil conditions favors for FV infection, chlamydospores germinate and produce hyphae that directly penetrates soybean roots. Colonization can occur as soon as seed germination \cite{gao2006, navi2008}. The fungal hyphae penetrate into the root system then grow both intercellularly and intracellularly \cite{roy1997}. The early colonization into the cortical tissues allows the fungus colonize xylem which in turn provides pathways for translocation of phytotoxins, resulting in foliar symptom expression \cite{roy1997, navi2008, gongora2011}. Reproduction happens on root surfaces, macroconidia in sporodochia are formed \cite{roy1997}. Rain-splashing, water runoff, or air can help macroconidia to be dispersed during soybean
harvest or produce chlamydospores in the presence of root exudates (Melgar et al., 1994). The fungus also can survive in cysts of the soybean cyst nematode which can withstand wide soil temperature fluctuations and resist desiccation (Rupe et al., 1993).

**Factors impacting SDS occurrence**

It is reported that wet and cool conditions in the rhizosphere can trigger the development of SDS (Scherm and Yang 1996; Scherm et al., 1998). Also, foliar symptoms are affected by soil temperature and developed differently than root disease severity (Scherm and Yang 1996). In controlled conditions, the largest foliar symptoms were observed within soil temperatures of 22°C to 24°C range tested whereas the smallest SDS disease severity were observed at the lowest temperature setting of 15°C. Root severity and temperature have negative correlation in which highest severity at lowest temperature at 15°C and lowest severity at highest temperature of 30°C. There were no correlation between root rot severity and foliar severity (Scherm and Yang 1996). Soil moisture is also a factor affecting SDS occurrence. Well-watered plants in greenhouse and field had greater foliar incidence and severity than non-irrigated plants (Roy et al., 1989; Melgar et al., 1994). SDS foliar symptoms were found to increase when soil moisture increased. This was proved by an outbreak that occurred in East Central Illinois and South and Center of Iowa in 1993, a year with a record summer flood in the US Soybean Belt (Hartman et al., 1995). In 1993 outbreak, four Iowa counties SDS were reported, and that built up a great source of inoculum in soil. This FV inoculum density in the soil is associated with disease pressure (Hershman et al., 1990; Gongora-Canul et al., 2012). Continuously, in 1998 epidemic outbreak, with great severity and prevalence Sanogo and Yang (1999) predicted that SDS could become a major production concern in Iowa (Sanogo and Yang 1999). Leandro et al (2013) found that greater total
precipitation during growing season was observed in SDS epidemic years. Particularly, the SDS outbreak in Iowa in 2010 revealed that the pathogen had been more extensively distributed when soil moisture was high in June corresponding to the onset of flowering than previously reported. In addition to high soil moisture, soil fertility were reported to be associated with SDS (Rupe et al., 1989, Scherm and Yang 1996).

*Heterodera glycines*, the soybean cyst nematode (SCN), together with SDS fungus are two major soilborne pathogens of soybean. This disease complex has been investigated for nearly three decades (Roy et al., 1989; Melgar et al., 1994; Gao et al., 2006; Westphal et al., 2014). Hirrel (1987) identified a potential interaction between SDS and SCN based on observing that SDS symptoms are more severe in fields with SCN. Plants had greater SDS incidence and severity when co-inoculated with SCN and FV than plants infected with FV alone (Melgar et al., 1994; Xing and Westphal 2006). Roy et al (1997) found that FV also can infect SCN cysts acting as survival and overwintering structure of the pathogen. Conversely, Gao et al (2006) showed that high levels of SDS may decrease the population of SCN due to root mass reduction and limiting infection sites of SCN (Gao et al., 2006).

**SDS management**

SDS is a threat to soybean yield. In 2010 outbreak, an estimation of 4.7 million metric tons yield loss in the United States was caused by SDS (Bradley and Koenning 2014). In fact, growing season in 2010 was favorable for SDS and this year demonstrates how much the impacts of SDS can have on soybeans (Leandro et al., 2013). Because of the destructive nature of SDS and its wide geographical range, finding an appropriate management approach is necessary while management options for the disease are still limited. Here, some management
options including crop rotation, resistant cultivar, tillage, planting date and seed treatments will be discussed.

**Crop rotation**

Corn-soybean rotation is typical agricultural production across the U.S Midwest to increase crop yields (Pedersen and Lauer 2004; King et al., 2016). However, because FV chlamydospore or macroconidia can remain in crop residue or soil for many years, this makes corn-soybean rotation ineffective in reducing SDS occurrence (Roy et al. 1997). In addition, study by Navi and Yang (2016b) in greenhouse and field conditions in Iowa suggested that crop residues (corn kernels and roots) amended in soil resulted in high FV population densities. On the other hand, long term rotation and diversification in cropping system had shown to reduce *F. virguliforme* density in the soil, in further to suppress SDS and protect yield (Leandro et al., 2018). However, long term crop rotations with diverse crops are not widely adopted by growers.

**Resistant cultivars**

Planting resistant soybean cultivars is a primary choice to manage SDS (Rupe et al., 1991; Leandro et al., 2013). Soybean cultivars with partial resistance to SDS currently have been identified and developed (Hartman et al. 1997; Mueller et al. 2002, 2003). DNA markers have Selecting dual resistance to SDS and Soybean Cyst Nematode for soybean cultivars have been developed based on DNA markers (Prabhu, 1999,; Luckew et al, 2013). Quantitative trait loci (QTL) were identified and used among the sources of resistance in screening and breeding programs (Shultz et al., 2007; Luckew et al., 2013). More than 80 QTL were associated with soybean resistance to SDS (Chang et al., 2018). Tan et al (2018) identified two QTL control the
field resistance to SDS and confirmed the interaction between them. Also, single nucleotide polymorphisms (SNPs) for SDS resistance have also been reported (Zhang et al., 2015; Bao et al., 2015, Swaminathan et al., 2019). So far, the complexity on information of QTL and SNPs together with interactions among FV species, SCN, soybean genotypes and environment had made genetic mapping for SDS resistance more complicated so that validity of reliable QTL and its function under field experiments is necessary (Tan et al., 2018; Chang et al., 2018).

**Tillage**

Tillage is one of the cultural practices to manage SDS; however, results are inconsistent. Study of Wrather et al (1995) showed that tilled plots had lower SDS foliar severity was than no tilled plots. Luo et al (2001) suggested that removal of root residue or accelerating residue decay may help in SDS control. Also, Vick et al (2003) found that through increasing the drainage soil capacity by deep ripping soils that have SDS history may reduce SDS in following years. The study also concluded that subsoil tillage reduced soil bulk density, changed soil porosity higher, decreased soil moisture, and lowered SDS foliar severity compared to no-till plots. However, no tilled system has been used commonly for a long time in the U.S agricultural production because of its economic and environmental benefits (Uri 2000); whereas more studies are necessary to determine if tillage have long-term benefits in SDS management.

**Planting date**

Delaying to plant soybean is another option to reduce SDS (Wrather et al., 1995). The reason for delaying planting date is early root infections by FV happening under the cool and wet conditions can reveal the greatest SDS severity later in the growing season. Studies of Gongora-Canul and Leandro (2011) showed that fungal inoculation at the time of planting brought out the
most severe foliar symptoms compared inoculation weeks later and that at warmer temperatures at planting foliar symptoms were less severe. Although there were SDS severity reductions when delayed planting were applied; however, growers are not recommended to use this practice for SDS management because delaying too long can cause a risk of reducing yield (Hartman et al., 2015). In term of yield loss, Marburger et al (2016) suggested that growers may choose cultivars with high yield potential instead of losing planting dates that maximize yield for the purpose of reducing SDS.

**Seed treatments**

Seed treatment is one of the common tool today to control SDS. From 2001 to 2011, according to CropLife Foundation (2013), the seed treatments on soybean increased from 5 to 18%. The number of active ingredients has risen, as products are considered to be farmer-friendly and environmentally responsible (Schwinn, 1994). Seed treatments are the fastest agricultural chemical sector in the world with a value of $3.957 billion in 2018 (Research and Markets, 2018). Contact and systemic are the two types of fungicides used in seed treatments. A contact fungicide can control pathogen for a limited time as it adheres only on seed surfaces (Mueller et al., 2013); on the other hand, a systemic fungicide provides more persistent protections by its translocation into the plant after being applied. Several commercial fungicides for soybean were reported to be effective against some *Fusarium* spp. (Paul et al., 2008; Yoshida et al., 2008; Ellis et al., 2011). However, until 2010 there were no registered seed treatments that were reported to effectively reduce root or foliar symptoms of SDS. Recently, some fungicide seed treatments were examined for their management on SDS. Although none of them showed
efficacy against SDS, fluopyram (Ilevo®) reduced SDS with some inconsistency in its efficacy (Weem et al., 2015, Kandel et al., 2016).

Rhizobia

The order Rhizobiales belongs to alpha-Proteobacteria which is the largest class of Proteobacteria phylum. Rhizobiales is composed of a variety of bacteria strategically important for their diversity in function and in niche occupancy. Rhizobia is included of specific groups of gram negative soil bacteria capable to form symbiotic nitrogen fixing nodules on roots or stems of leguminous plants. The taxonomy and classification of rhizobia are becoming more complex because of new findings of new genera as well as new species and are revised periodically (Pongslip 2012). According to Shamseldin et al (2017), rhizobia consist of 238 species in 18 genera in which 16 genera belong to 6 families (including Rhizobiaceae, Phyllobacteriaceae, Bradyrhizobiaceae, Hyphomicrobiaceae, Methylbacteriaceae and Brucellaceae) of alphaproteobacteria. There are some criteria used to classify rhizobia. Legume rhizobia were firstly identified by their growth, production of acid or alkaline and host specificity. For example: fast-growing rhizobia is genus Rhizobium whereas Bradyrhizobium show slow growth (Summerfield and Roberts 1985; Somasegaran and Hoben 2012). The development of molecular biology and its tools had led the description of rhizobia in non-systemic way (Shamseldin et al., 2017). Multiple approaches such as guanine-cytosine (GC) content, 16S ribosomal RNA sequences, DNA hybridization, mass spectrometry, chromosomal housekeeping genes, multilocus sequence analysis, multilocus analysis typing, comparative genomics and average nucleotide identity had provided deeper information on rhizobia taxonomy (Pongslip 2012; Shamseldin et al., 2017. Although a few rhizobial species can nodulate soybean roots such as B
japonicum, B. elkanii, Sinorhizobium fredii), *Bradyrhizobium japonicum* were shown to be the best performer (Sessitsch et al., 2002).

Rhizobia bacteria can survive for a long time in the soil. Rao and Cooper (1995) found that they can persist in the soil without adding more for up to 54 years in very small numbers. The most problematic environments for rhizobia survival are low rain fall, low nutrient in acidic soils, extremes of temperature, and poor water-holding capacity (Bottomly, 1991). Rhizobia can be found in different climates, from desert to arctic. Zhang et al (1994) found that suboptimal temperatures in the soybean rhizosphere may reduce nodule formation and function. According to Hymowitz (1970), *B. japonicum* was firstly adapted to its hosts in warmer climates (in China) and hence it is not well adapted in cooler climates. Yang et al (2001) proved that inducing nodulation of rhizobia on soybean roots can be affected by soil pH. Also, Egamberdiyev et al (2004) concluded that decreased soil pH (increased acidity) leaded in poorer nodulation and reduces survival rates. Rhizobial populations can be found in soils with limiting moisture levels in which lowest population under the most desiccated conditions and increase when the moisture stress is relieved (Jenkins et al., 1989). Hunt et al (1981) suggested that low water content in soil was involved in the lack of success of soybean inoculation in soils with a high indigenous population of *B. japonicum*.

**Biocontrol potential of Bradyrhizobium japonicum**

*Bradyrhizobium japonicum* is a nitrogen-fixing symbiotic bacterium with the host legume soybean. *B. japonicum* also has been proven to have high potential to be used as Plant Growth Promoting Rhizobacteria (PGPR) on non-legumes such as radish (*Raphanus sativus* L.) (Antoun et al, 1998), tomato (*Solanum lycopersicum* Mill.), barley (*Hordeum vulgare* L.) (Carletti et al,
1994) and rice (*Oryza sativa* L.) (Biswas et al, 2000). These studies indicated that changes in growth physiology and root morphology were the main causes in higher yield due to increased biomass rather than biological nitrogen fixation. Chibeba et al. (2015) observed that co-inoculation of soybean with *Bradyrhizobium* spp. and *Azospirillum brasiliense* results in earlier nodulation which is important for crops with short growth cycles such as soybean. Leggert et al. (2018) showed that the greatest positive effects of *B. japonicum* inoculation on soybean were observed on late-planted soybean and was independent of soil organic matter (SOM).

The bicontrol potential of *B. japonicum* against some soilborne fungal pathogen has been examined. Chakraborty and Purkayasta (1983) proved soybean seeds coated with *B. japonicum* prior to sowing were protected from the charcoal rot pathogen, *Macrophomina phaseolina*, infection due to the production of rhizobitoxine. Tu (1978) reported *Phytophthora* root rot severity was decreased when *B. japonicum* was applied to the potted soybean. Buonassissi et al (1986) suggested that seed inoculation with *Rhizobium* strains could control *Fusarium* root rot pathogens. Ehteshamus and Ghaffar (1993) observed that in field conditions infection of *Macrophomina phaseolina*, *Rhizoctonia solani* and *Fusarium* spp. was reduced when *Rhizobium leguminosarum* and *B. japonicum* were used as seed treatments or soil drench in legumes including soybean and mungbean (*Vigna radiate* [L.] Wilczek); and in non-legume (sunflower (*Helianthus annuus* L.), okra (*Abelmoschus esculentus* [L.] Moench)). Siddiqui and Mahmood (1995) reported combined application of *B. japonicum*, *Bacillus subtilis* and *Glomus fasciculatum* on pigeonpea (*Cajanus cajan* [L.] Millsp.) increased nodulation and reduced significantly root infection by nematode and Fusarium wilt. *B. japonicum* were used as seed treatments and liquid inoculant can be good tools to control soybean root rot diseases caused by *Fusarium solani* and *Macrophomina phaseolina* (Al-Ani et al, 2012. Similarly, seed treatments

Different mechanisms may be involved in suppression of fungal pathogens by *Bradyrhizobium* strains. These mechanisms including antibiotic production (Chakraborty and Purkayasta, 1983), siderophore production (Nambiar 1987; Guerinot et al., 1990; Omar and Abd-Alla 1998; Siddiqui et al., 2000), hydrogen cyanide production (Antoun 1998), nodulation, seed germination and plant growth promotion (Chakraborty and Purkayasta 1983; Siddiqui et al., 2004; Al-Ani et al., 2012), improve dry plant biomass (Buonassisi, 1986; Dar et al., 1997) and induce plant defense system (Gao et al., 2012).

**Research Justification**

Graham and Vance (2003) named Leguminosae (Fabaceae) as the second most important plant family to humans. It is also the third largest flowering plants (Hirsch et al. 2001). In agriculture, legume play an important role; especially, soybeans are significant because the highest of 35% to 40% of the protein contents are carried among seed grains (Krishnan et al., 2005). In addition, soybeans as well as other legumes possess an big advantage over cereal grains as their symbiotic associations with rhizobia, symbiotic bacteria, allow them to fix nitrogen (Alexander 1984).
Soybean is a subtropical legume that requires root zone temperatures (RZTs) in the range of 25\(^0\)C to 30\(^0\)C for optimal symbiotic activity. The expression of the nod genes is suboptimal at lower temperatures, resulting in a delay in the onset of nodulation (Zhang 1996). Low soil temperature is considered one of the most important factors limiting soybean growth and nitrogen fixation. Lynch and Smith (1994) indicated that the onset of nitrogen fixation was delayed 2.5 days linearly for each decreased degree from 25\(^0\)C to 17\(^0\)C, and was 7.5 days delayed when soil temperature was below 17\(^0\)C.

Early planting can help increase yield, but also increase the risks of seedling diseases caused by pathogens favored by high soil moisture and cold temperatures such as Pythium damping off. These conditions also favor *Fusarium virguliforme*, the causal agent of soybean sudden death syndrome which is rapidly becoming a disaster for soybean production in the US especially in the Midwest. Leandro et al (2013) showed that yield losses by SDS in epidemic areas in Iowa were dependent on cool soil temperature and high precipitation in early-planting seasons. In fact, delaying planting is a recommendation to manage these diseases. It is reported that widespread use of chemicals to control plant diseases has contaminated soil environments and underground water, resulting in the development of many resistant pathogens and causing risks to human health (Atman and Campbell, 1977; Gressel, 2011). An alternative is to use a biological agent that is not expensive, ecological friendly and not harmful to human. However, there currently are no reports of using bacteria or fungi to control SDS. Chemical seed treatments have been used routinely to protect from early infection by root rot pathogens but most seed treatment fungicides are not effective against SDS (Weems et al., 2015). Although seed treatment with fluopyram currently shows the best efficacy to manage SDS, some inconsistency resulting from different disease levels of locations or years were reported (Kendel et al, 2016),
and baseline sensitivities to fluopyram were different for members of the *Fusarium* species in the complex causing SDS (Wang et al., 2017; Sang et al., 2018). Furthermore, the effects of crop rotation, tillage practices, and row spacing on SDS have proven to be inconclusive and only can reduce damage in some instances (Yang and Navi, 2006). Additionally, although there are many moderately resistant cultivars are being used, there are no soybean cultivars proven to be highly level resistant to SDS that are available commercially. Because none of these management approaches have provided acceptable levels of disease management, we need new management tools. Thus, we conducted this study to explore if biological control may help mitigate the damage caused by SDS.

Vessey (2003) recommended that inoculation of soybean with *B. japonicum* (BJ) can increase the effectiveness of nodulation and nitrogen fixation in order to maximize yield. For example, commercial BJ trains 532 C and USDA 110 are inoculants are widely used in Canada and in the US because of their good adaption under wide range of climates (Hume and Shelp, 1990; Lynch and Smith, 1993). A study conducted by Zhang et al. (2003) on using low temperature tolerant *B. japonicum* strains in the USDA collection confirmed that nitrogen fixation had increased under low temperature in field conditions when using these strains. Currently, there is no information to address the ability of *B. japonicum* against SDS. Hence, we will explore the potential for BJ to control SDS, particularly under low temperature conditions that are favorable for SDS infection in greenhouse and field conditions as well as a deeper insight view in this symbiont interaction on soybean.
Research Objectives

Based on the knowledge and the research needs stated previously, the objectives of this research were:

1. To determine the effects of seed treatment and soil inoculant using *Bradyrhizobium japonicum* on controlling soybean SDS under controlled conditions
2. To evaluate the effects of *B. japonicum* seed treatments on SDS under field conditions

To achieve the objectives, a BJ strain was used for efficacy tests in the greenhouse using distinct inoculation methods to evaluate its impact on SDS occurrence. With the results obtained, three USDA *B. japonicum* strains were used to test the effects of seed treatment against SDS under field conditions. Also, field experiments were run to test the seed treatment of *B. japonicum* either alone or in combination with Heads Up, a commercial product claimed as a biocontrol product against SDS. These trials were carried out twice. Disease incidence and severity were recorded periodically through growing seasons. For every trial, soybean nodulation was assessed, weights of fresh/dry shoots and roots also were determined collected at V4 stage. The results are discussed in terms of identifying the most effective treatments against SDS in both greenhouse and field conditions.
Literature cited


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CHAPTER 2.

TREATMENTS WITH *BRADYRhIZOBIUM JAPONICUM* ON GERMINATED OR NON-GERMINATED SEEDS REDUCE OCCURRENCE OF SOYBEAN SUDDEN DEATH SYNDROME ON EARLY-PLANTED SOYBEAN

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**Abstract**

Previous studies showed that cool soil temperatures are one of the favorable factors for infection of *Fusarium virguliforme* (FV), the causal agent of soybean sudden death syndrome (SDS). *Bradyrhizobium japonicum* (BJ), a nitrogen-fixing \(\alpha\)-Proteobacterium symbiont that forms nodules on soybean, is widely present in the United States soils. The minimum temperature for BJ to infect soybean has been reported to be 17.5°C and for *F. virguliforme* 15°C. Soybean planted early, into colder soil had greater incidence of SDS. In this study, a BJ strain applied to seeds before and after germination and was evaluated for SDS suppression in controlled conditions. Results showed that seed germination had significant influence on SDS
incidence under low temperature. BJ treated seeds planted in FV infested soil in clay pot or foam cup experiments significantly ($P<0.05$) reduced SDS incidence, both in germinated and non-germinated seed at 15°C compared to 25°C at V3 growth stage. BJ inoculated on vermiculite substrate as soil inoculant treatment showed the most effective treatment. Non-germinated treated seed had significantly higher nodule count than germinated ones at 20°C, but no significant effects were detected for plant biomass. This study suggested that BJ seed treatments or BJ soil inoculant could provide better nodulation as health benefits in early-planted soybean and may act as a biocontrol agent against SDS.

Keywords: Soybean, *Fusarium virguliforme*, *Bradyrhizobium japonicum*, seed treatments, early planting.

**Introduction**

Soybean sudden death syndrome (SDS) is among the most important soil-borne fungal diseases in US soybean production. SDS was ranked as one of the most damaging diseases on soybean from 2006 to 2009 (Koenning and Wrather 2010). In the US, SDS was first reported in Arkansas in 1971 (Roy 1997). After that, Hirrel (1987) named the disease SDS because of the sudden development of foliar symptoms. The SDS in North America is caused by *F. virguliforme* (Aoki et al. 2003), in South Africa by *F. virguliforme* (Tewoldemedhin et al. 2014) and *F. brasiliense* (Tewoldemedhin et al. 2017) and in South America SDS by a complex of *F. virguliforme*, *F. tucumaniae*, *F. brasiliense*, *F.cuneirostrum* (Aoki et al. 2005) and *F. crassistipitatum* (Aoki et al. 2012).

Numerous factors have been hypothesized to contribute to SDS prevalence and soybean yield decline, including biotic factors such as soybean cyst nematode (McLean and Lawrence 1993; Xing and Westphal 2006; Marburger et al. 2014). Wet and cool soil is believed to trigger
SDS development (Scherm and Yang 1996; Scherm et al. 1998). The greatest foliar symptoms of SDS were observed in soil temperature ranging from 22°C to 24°C, and root severity and temperature had a negative correlation in which the highest severity was observed at 15°C and the lowest at 30°C. In addition, increased SDS foliar symptoms were observed with increase in soil moisture (Scherm and Yang 1996).

In Iowa, SDS was first reported in 1993 (Yang and Rizvi 1994). After an SDS outbreak in 2010, *F. virguliforme* was reported to be extensively distributed in most Iowa soils (Leandro et al. 2013). Infection of *F. virguliforme* is generally favored by cold wet conditions which are very common in early planted soybean in Iowa and other Midwest regions. Leandro et al. (2013) showed that yield losses by SDS in epidemic years in Iowa were dependent on cool soil temperature and high precipitation in the early part of the growing season. Typical soil temperatures in Iowa in the early part of the season range from 10°C to 15°C (Leandro et al. 2013). Delayed planting has been one of the management tools to reduce SDS severity (Hershman et al. 1990; Rupe and Gbur 1995; Wrather et al. 1995). However, this practice may limit yield potential (Roy et al. 1997). A recent study (Marburger et al. 2016) on planting dates in Wisconsin, United States, suggested that planting in early May provided maximum yield potential despite leading to the greatest development of SDS symptoms.

Soybean requires 25°C to 30°C temperatures in the root zone for optimal symbiotic activity. According to Zhang et al. (1996), a delay in nodulation in the cold occurs because of inhibited expression of nod gene under suboptimal root zone temperature in soybean. Cool soil temperature is considered one of the most important factors limiting soybean growth and biological nitrogen fixation. Lynch and Smith (1993) indicated that the onset of nitrogen fixation was delayed 2.5 days linearly for each decreased degree from 25°C to 17°C, and was delayed 7.5
days when soil temperature was below 17°C. The sensitivity of atmospheric nitrogen fixation to low temperatures in soybean is a significant limitation when adopting this crop in a cool spring season (Zhang et al. 1995). Pre-incubation of rhizobacterial cells with plant-to-bacteria signal compounds as inducers of enhanced plant growth, photosynthetic rates, and nitrogen fixation, and could alleviate low root zone temperature stress on lentil (Lee 2009). A two-year study conducted by Zhang et al. (2003) indicated that a low-temperature tolerant *Bradyrhizobium japonicum* inoculant improved nodulation and nitrogen fixation in soybean under cooler growing temperatures.

Legume-Rhizobium mutualism has been widely documented especially for nitrogen fixation and plant health promotion. In soybean, *Rhizobia* play an important role as biocontrol agents against root rot diseases caused by *Macrophomina phaseolina, Fusarium solani,* and *Fusarium* spp. (Chakraborty and Purkayastha 1984; Buonassissi et al. 1986; Omar and Abd-Alla. 1998; Al-Ani et al. 2012;). Similarly, seed treatments with *Rhizobium* sp. significantly suppressed Fusarium wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (Arfaoui et al. 2005; Singh et al. 2010), root rot caused by *Rhizoctonia solani* (Hemissi et al. 2011) in chickpea and *Pythium* damping off of field pea and lentil (Huang and Erickson 2007). *B. japonicum* inoculum used either as soil inoculant or seed treatments may offer SDS management in early-planted soybeans. Therefore, the objectives of this study were (1) to evaluate the effects of *B. japonicum* inoculum and soil temperature on SDS occurrence and (2) to quantify the impacts of *B. japonicum* inoculation and soil temperature on nodule and plant biomass under SDS pressure.
Materials and methods

Soybean cultivar and isolate collections

Soybean cultivar P22T61RR (Pioneer Hi-Bred International, Inc., Johnston, IA) which is moderately resistant to SDS was used in all experiments carried out in greenhouse from 2014 to 2016.

A *B. japonicum* (ISU-BJ) strain isolated from healthy plants in an field experiment at Iowa State University in 2013 was used in experiments conducted in 2014, 2015 and 2016. Nodules were collected and rhizobia were isolated following methods described by Somasegaran and Hoben (2012). The ISU- BJ strain was cultured on Yeast Mannitol Agar (YMA), and was sub-cultured on yeast mannitol broth (Vincent 1970). Flasks (250 mL) were incubated on a shaker at 125 rpm at 25°C for 7 days. Later, the culture was diluted with sterile yeast mannitol broth to an OD£_{620}\text{nm}$ of 0.08, which was equivalent to about $10^8$ cells mL$^{-1}$. This concentration of BJ was used in all the experiments.

*F. virguliforme* isolate Fsg-ISU1 (Mbofung et al. 2012) originated from an field at Boone, Iowa was sub-cultured on potato dextrose agar (PDA) amended with 100 mg/L streptomycin. *F. virguliforme* (FV) inoculum was fermented on white sorghum grain following the methods of Hartman et al. (1997) and Navi and Yang (2016a).

Efficacy tests of ISU-BJ strain in 2014 and 2015

An experiment with eight treatments was conducted in the Plant Pathology Greenhouse of Iowa State University. Two separate runs were carried out. The first run was from March 2014 to May 2014 while the second was from April 2015 to June 2015. To prepare BJ seed treatments, commercially untreated seeds of P22T61RR were surface-sterilized following method described
by Somasegaran and Hoben (2012), and were placed in 10 cm diameter glass Pyrex® Petri dishes lined with one layer of moist sterilized germination sheets at 30 seeds per plate. The plates were incubated in the dark at 25°C for five days. Subsequently, uniformly germinated seeds were selected and were inoculated by submersion in BJ suspension (10⁸ cfu/mL) at 25°C for an hour. Similarly, surface sterilized seeds of P22T61RR were taken in a Ziploc bag and transferred 10 mL of a 10⁸ cfu/mL suspension to the bag, and mixed thoroughly and incubated at 25°C for an hour. FV seed treatments; the method described above for BJ seed treatments, was followed for FV seed treatments but with FV spore suspension at 10⁶ spores mL⁻¹. Control treatments included surface sterilized seeds not treated with BJ, but planted in FV-infested soil; seeds not infested with FV, but planted in BJ-treated soil. The controls were to assess the baseline effects of BJ treatment on growth of soybean seedlings, nodule development, FV infection and SDS foliar symptom development, and to create conditions where both microorganisms are initially present in soil. The details of seed treatments combinations provided in Table 1.

Soil treatment with BJ: Preparation of moist vermiculite inoculant of BJ was described by Pauu (1998). The vermiculite based inoculant was then incorporated into steam-pasteurized potting mixture (2 parts alfisol and 1 part sand) at a rate of 2% vermiculite (volume/volume). Soil treatment with FV: Steam-pasteurized soil was mixed with *F. virguliforme*-infested sorghum grain at 2% (volume/volume).

In summary, based on germinated or non-germinated seed, eight treatments were divided into two groups: germinated seed inoculation and non-germinated seed inoculation. Based on soil infestation either with *B. japonicum* and or with *F. virguliforme*, the inoculated seed (FV or ISU-BJ) and or un-inoculated seed were planted in those infested soil (Table 1). These treatments were evaluated for SDS incidence, severity and soybean plant nodulation.
Planting: Seeds of individual treatments were planted at seven seeds per 10-cm diameter clay pot (filled with potting mixture) at an approximate depth of 3 cm. Each treatment was planted in 10 clay pots and each pot was considered a replication. Treatments were randomly arranged and were incubated for 10 days in three growth chambers. Three set of treatments were placed in three growth chambers, which were set at 15°C, 20°C and 25°C with 90% relative humidity and 16h light/8 h dark cycle. Pots placed in growth chambers were watered once daily. After 10 days incubation in growth chambers, pots were shifted to greenhouse benches at 26 ± 2°C under 16 h light/8 h dark photoperiod using a 400W E-Ballast, Metal Halide type M59 bulb (Hydrofarm Inc. 2249 S. McDowell Ext., Petaluma, CA 94954). Plants in each pot were thinned to 5 plants, arranged in randomized complete block design and incubated for 40 days. Plants were watered twice daily to maintain saturated soil moisture.

Plants were rated for SDS infection when the first foliar symptom was observed and continued every 10 days until the V3 growth stage (30 days after planting). When plants were at V4 growth stage, 15 plants from three pots were randomly sampled from each treatment to determine total nodule number and number of nodules on primary roots.

Seed treatments with ISU-BJ strain in 2016

In 2016, two runs of greenhouse experiments with twelve treatments were conducted from March to July for additional evaluation of ISU-BJ inoculations in the presence of bioAPT® microbial carrier (American Peat Technology, LLC, 36203 350th Avenue Aitkin, MN 56431) treatment at 10 g/kg seed (Table 1). In treatment 1, uniformly germinated seeds were submerged-inoculated in ISU-BJ strain suspension (10^8 cfu/mL) and planted in 2% FV infested potting mixture. In treatment 2, germinated seed was treated with ISU-BJ by submerge-inoculation followed by sterile bioAPT microbial carrier added at 10g/1kg seed and planted in 2% FV
infested potting mixture. Germinated seeds inoculated with a FV-spore suspension at 10^6 spores/mL were planted in ISU-BJ infested soil (treatment 3). Treatments 4 and 5 were two positive controls. Treatment 4 included water-treated germinated seeds planted in 2 % FV infested potting mixture and treatment 5 with germinated seeds planted in BJ infested soil. Treatment 6 with water treated germinated seed planted in potting mixture without infestation served as a negative control. Treatment group (treatment 6 to treatment 12) was similar to the first set but with non-germinated seeds (Table 1). Each treatment was planted in 20 cups, as described below.

Planting: Seed of individual treatment were planted at seven seeds per 9-cm diameter foam cup (filled with potting mixture) using the planter developed by Navi and Yang (2016b). The incubation and evaluations were similar to experiments in 2014 and 2015 except at growth stage V4, 25 plants from 5 cups per treatment were randomly collected, washed and air-dried to determine the total nodules and fresh weights for roots and shoots. Dry weights of roots and shoots were determined after air drying on greenhouse benches for three weeks. Disease assessment was made until growth stage R1.

*Disease incidence and severity assessment*

Foliar disease incidence (DI) was determined as percentage of symptomatic plants in a pot or a cup. Disease severity (DS) was recorded on a 1-9 scale, where 1 = 0–10% Chlorotic (C), 1–5% Necrotic (N), 2 = 10–20% C, 6–10% N, 3 = 20–40% C, 10–20% N, 4 = 40–60% C, 20–40% N, 5 = greater than 60% C, greater than 40% N, 6 = up to 33% premature defoliation, 7 = up to 66% premature defoliation, 8 = greater than 66% premature defoliation, and 9 = premature death (Njiti et al. 1996; 1998).
Statistical analysis

The mixed model procedure (PROC MIXED) of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) was used to perform Analysis of Variance (ANOVA). The ANOVA contained fixed effects for the experimental run, seed condition and treatment level, and two and three way interactions. Data were analyzed separately by experimental temperatures (by 15, 20 or 25°C). Data of disease incidence, disease severity were analyzed only for either treatments with FV inoculated seeds or FV infested soil and ANOVA analyses were performed in Type 3 Tests of Fixed Effects. The LSMEANS procedure where Fisher’s least significant difference (LSD) test was applied to detect differences in means for foliar disease incidence and disease severity, nodule counts, fresh and dry weights with α = 0.05 (Steel and Torrie 1980). Data for nodule counts and plant biomass in fresh or dry weights (in 2016 experiment) were available for all treatments. Orthogonal contrasts were conducted to compare the effects of BJ seed treatments versus non-treated seeds planted in FV infested soil (control). These contrasts were for mean averaged for the two runs in 2016.

Results


Disease incidence and severity: Experiments conducted in 2014 and 2015 had two independent runs (run 1 in 2014 and run 2 in 2015). There was no significant difference in SDS incidence in treatments at 15°C (P= 0.2793) or 20°C (P=0.7695) between the two years. Although a significant difference was observed between two years for disease incidence at 25°C, year is not associated with interactions except in year × seed. Similarly, there was no significant difference in disease severity at 20°C (P=0.5791) or 25°C (P=0.3921) between the two years, but there was at 15°C (P=0.0494). However, interactions between year and other effect (treatment, seed) had
no significant effect on incidence and severity. Because the disease incidence (DI) and disease severity (DS) at each temperature generally did not differ between the two years, data from the two years were analyzed together as a blocked design with data from each year assigned as a block.

Differences among main effects on DI and DS were observed at each temperature. The only significant difference between non-germinated seeds and germinated seed treatments on DI and DS were for DI at 15°C ($P=0.0063$) and 25°C ($P=0.0059$). More specifically, in the presence of FV infested soil, germinated seed treated with BJ reduced SDS incidence by 19.3% at 15°C and 15.9% at 25°C compared with untreated seeds (52.5% at 15°C and 74% at 25°C) (Figure 1(a)). Similarly, in non-germinated treated seeds with BJ there was 32.8% reduction in SDS incidence at 15°C and 25.9% reduction at 25°C. The variables of treatment ($P<0.0001$) as well as the interaction of seed (i.e. seed state at inoculation) and treatment had significant effects on DI and DS. Except for interaction between seed × treatment level on DI ($P=0.3721$) and DS ($P=0.4167$) at 15°C. Seed treated with BJ on germinated in FV infested soil reduced 1.38; 1.3 and 1.58 point DS at 15°C, 20°C and 25°C compared with untreated seeds, respectively. Seed treated with BJ on non-germinated reduced 0.5; 0.6 and 1.0 point DS at 15°C, 20°C and 25°C compared with untreated controls, respectively.

Overall, significant reduction in DI and DS were observed in germinated seeds inoculated with FV and planted in BJ infested soil ($P<0.0001$). There were significant reductions ($P<0.01$) on SDS development in all three temperatures associated with BJ inoculation in germinated seed and non-germinated seed treated compared with non-treated seeds planted in FV infested soil (Figure 1), although treatment with BJ did not affect DS on non-germinated seeds at
15°C \((P=0.3505)\) and 25°C \((P=0.3116)\). Non-treated seeds planted in BJ infested soil did not show any SDS foliar symptoms (data not shown).

**Nodule number:** Main effects of seed germination state, treatment and interaction between seed × treatment were not detected on total nodule number at 15°C and 20°C, but were at 25°C (treatment \((P=0.0190)\) and seed state \((P=0.0333)\) (Figure 2(a), Figure 2(c)). Unlike total nodule number, number of nodules on primary roots at all three temperatures showed differences \((P=0.05)\) by treatment level and seed status. On non-germinated BJ inoculated seed planted in FV infested soil, the number nodules on primary roots produced were not different from non-treated seeds planted in FV infested soil at 15°C and 20°C but significantly higher at 25°C \((P<0.0001)\) (Figure 2(b)), while on germinated seed this number was not significant (Figure 2(d)).

Generally, presence of BJ increased nodules on pre-germinated seeds planted in FV infested soil more than that of non-germinated seeds. Mean number of total nodule (in 3 randomly sampled pots) was recorded lowest from water-treated germinated seeds planted in FV infested soil at 25°C, while the highest number recorded from FV treated seeds planted in BJ soil at 25°C (Figure 2). Difference between two years was found on nodule counts on primary root (supplemental Figure S1, Figure S2).

**Efficacy of ISU-BJ strain seed treatments on soybean sudden death syndrome in 2016.**

**Disease incidence and severity:** No significant effect was observed between two runs on DI at V1, V3 and R1 growth stages when data were analyzed separately by temperature at 15°C and 20°C, but significant difference on DI was observed at 25°C. However, two-way interactions between run × seed and three-way interactions of run × seed × treatment were not detected. There were no significant effect of seed germination on DI except for DI at V1 and V3 at 25°C.
(P=0.0457 and P= 0.0144, respectively). Treatment and interactions between treatment and seed germination state were detected at P<0.001. Generally, at growth stage V3, BJ treatment of germinated seeds reduced DI significantly at lowest temperatures (P<0.001). Non-germinated seeds inoculated with FV and planted in BJ infested soil had the lowest DI in all three temperatures (Figure 3). On average, at V3 growth stage, seed treatment with BJ reduced significantly DI by 32% at 15°C and only dropped by 5.3% at 25°C compared with untreated seed planted in FV soil (81.2% at 15°C and 67.5% at 25°C) (Figure 3(b)). Similarly, in non-germinated treated seeds with BJ there was 16.4% reduction in SDS incidence at 15°C and 22.1% reduction at 25°C (Figure 3(e)). Non-germinated seed treated with BJ had significantly lower DI at all stages in all three temperature (Figure 3(d), Figure 3(e), Figure 3(f)) except at V1 stage at 25°C (Figure 3(e)). On germinated seed, BJ treated without microbial carrier (bio APT) was not statistically different with treatment with microbial carrier at 15°C and 25°C. However, on non-germinated seed, BJ +bioAPT inoculated seeds reduced DI significantly at all temperature compared with BJ inoculated seeds without carrier.

Overall, foliar severity at different growth stages reduced significantly with BJ inoculated on germinated or non-germinated seeds compared with water-treated seed planted in FV soil except at V1 at 20°C and 25°C (Figure 4). Germinated seeds treated with BJ had significant effect on DS reduction only at 15°C (P≤0.05) but not at 20°C and 25°C (Figure 4(a), Figure 4(b), Figure 4(c)). Whereas in non-germinated seeds treated with BJ, DS decreased significantly compared with non-treated seed at all three temperatures (Figure 4(d), Figure 4(e), Figure 4(f)) (0.5, 0.58, and 1.03 point decrement on DS at 15°C, 20°C and 25°C, respectively). When compared between BJ only treat seeds and treatment with BJ treated together with carrier, there were significant differences on both seed germination states (P≤0.05). DS were lower on non-
germinated seed treated with BJ + bioAPT than BJ alone (Figure 4(d), Figure 4(e) and Figure 4(f)). Differences for DS between the two runs were detected. Results for each run were presented in supplemental Figure S3 and Figure S4.

**Nodule count and plant biomass:** There were no significant effects of seed status on total nodule number variable. However, significant effects of seed status on fresh shoot, fresh root, dry shoot and dry root weights were detected only on seeds planted at 25°C ($P \leq 0.05$) which those weights were larger on germinated seeds than non-germinated seeds. Treatment had significant effects on nodule counts and plant biomass ($P \leq 0.05$) except dry root weights at 20°C and 25°C. There were no significant effect of interactions between treatment level and seed status on nodule number and plant biomass (Figure 4). There were significant higher nodules formed on plants from non-germinated BJ treated seed at 20°C than plant from non-treated seeds at same planting condition ($P=0.0231$) (Figure 4(e)).

**Discussion**

Experiments conducted from 2014 to 2016 were to test seed treatments with BJ under different soil temperature on soybean sudden death syndrome occurrence, on development of nodules and plant biomass. We believe this is the first report of the effects of BJ treatments on SDS occurrence under different temperature regimes. Results of this study confirm for the first time in greenhouse conditions that BJ inoculation affected SDS foliar symptoms expressions as measured based on DI or DS. This study also seeks to answer the questions of how farmers can balance yield with control of the disease when early planting is optimal for both.

ISU-BJ strain used in this study was isolated from nodules collected from Iowa in 2013. In a previous study in 2013, plants with lower SDS severities showed greater number of nodules per plant compared with plants that had higher SDS severities in the greenhouse conditions. We
collected nodules from plants that had survived with light SDS symptoms at growth stage R1. Subsequently, an isolate of BJ was identified based on 16S rRNA sequence and results showed that the strain has 99% identity identical with USDA 6. In this study we used this strain to test our hypothesis. It was a challenge to interpret the differences among replicates; however, our data showed that seeds treated with BJ or infested in soil seedling stages suppression of SDS.

In this study, treatments were either designed to create conditions favorable for initial colonization of soybean roots by BJ or by FV via addition to seeds and subsequently exposure to soils infested with FV or BJ. Soybean seeds were either pre-germinated or not, in order to evaluate possible scenarios for initial root colonization by BJ or FV. Pre-germinated seeds were exposed to higher initial concentration of either BJ or FV inoculum (by virtue of increased seed surface area and the presence on radicles), whereas non-germinated seeds were exposed to lower initial concentrations of BJ or FV inoculum. It can be explained that pre-germinated seeds were overwhelmed by higher exposure to FV inoculum together with exudates from young roots so that BJ treatment on germinated seed performed their efficiency differently and unpredictably.

At each temperature, treatments with BJ inoculum reduced SDS occurrence compared with untreated controls. Treatments with FV-inoculated seed planted in BJ-infested soil showed the best results in controlling SDS at all three temperatures. This can be resulted from higher initial BJ inoculum level added in the soil than level used to treat directly on the seed. Particularly, clay pots used in experiments of 2014 and 2015 had better BJ efficiency of BJ infested soil on SDS than foam cups used in 2016. Additionally, BJ treatments showed reduction of SDS at 25°C compared to the other two temperatures when planted in foam cups (Figure 3). This can be explained by the different conditions when plants grow in greenhouse condition for example light, temperature or type of pot. This would lead to change in the performance of BJ.
strains which in turn results in nodulation and plant biomass differences between runs as difference of each biological replication. Although different growth chambers randomly selected to set up temperature for planting and random arrangement on greenhouse bench after shifting pots/cups from growth chambers may have changed performance of BJ treatment in experimental replications, the reduction of SDS occurrence in treatment with BJ in experiments confirmed that BJ can act against SDS pathogen.

Planting soybean in cool, wet soils in an early season increases the risk of SDS (Scherm and Yang 1996; Roy et al.1997; Leandro et al. 2013), suggesting that young roots in such conditions are most susceptible to FV infection. Our findings showed SDS incidence and severity were reduced significantly when BJ treated on seeds or adding in soil at lower soil temperature which favor for FV infection. The findings suggested that BJ infection can happen prior to FV or at the same time with FV infection. In addition, our observations suggested that better nodulations in BJ treatments could be the main effects on promoting plant health, and nitrogen fixation and increase metabolisms including secondary metabolites that can trigger plant defense to SDS infection. This was drawn based on the higher number of nodules in seed treated with BJ or BJ amended in soil, compared with the un-treated controls planted in FV-infested soils. Also number of nodules produced on primary roots assessed in 2014 and 2015 experiments were significantly different on BJ seed treatments. Different mechanisms may be involved in suppression of fungal pathogens by potential Bradyrhizobium strains. These mechanisms may include toxic metabolite production such as rhizobitoxine (Chakraborty and Purkayastha 1984), siderophore production (Nambiar and Sivaramakrishnan 1987; Guerinot et al. 1990), hydrogen cyanide production (Antoun et al. 1998) and plant growth promotion (Siddiqui and Mahmood 1995). BJ has been used as a plant growth promoting rhizobacteria (PGPR) on many row crops
and vegetables (Antoun et al. 1998, Carletti et al. 1994, Biswas et al. 2000). These studies indicated that changes in growth physiology and root morphology were the main causes in higher yields and increased dry matters rather than biological nitrogen fixation. A two year study conducted by Zhang et al. (2003) also indicated that using low temperature tolerant *B. japonicum* inoculant could improve nodulation and nitrogen fixation of soybean plant in a cool growing season. It has been suggested that induced systemic resistance (ISR) that results from a long-distance signaling mechanism is the cause of the colonization density of the symbionts.

In summary, our results showed that BJ incorporation as seed treatment or soil inoculant can suppress SDS on early planted soybean. Our results confirmed the findings by Gongora-Canul and Leandro (2011) that foliar severity is more severe when *F. virguliforme* is inoculated on early plant age. The results of the present study have indicated that it is important to introduce BJ initial populations by seed treatments or soil inoculant in a short growing season in Iowa to reduce SDS occurrence. A limitation of this study is that effects of BJ seed treatment against SDS were evaluated on foliar disease incidence and severity. Future work should investigate on root rot severity which can be measured under controlled conditions.

**Acknowledgments**

The authors thank Iowa Soybean Association, Iowa (USA) and Vietnam International Education Development (Viet Nam) for providing funding for this research. We thank Huaiqing Wu, Department of Statistics, and Sharon Eggenberger, Department of Plant Pathology and Microbiology, for their technical assistance in data analysis.
Literature cited


Table 1. Treatment descriptions in 2 experiments during 2014, 2015 and 2016 on effects of treatments with ISU-Bradyrhizobium japonicum strain on soybean sudden death syndrome

<table>
<thead>
<tr>
<th>Year</th>
<th>Seed inoculation</th>
<th>Soil infestation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2014 (run 1)</td>
<td>ISU BJ</td>
<td>FV</td>
<td>Germinated, ISU BJ-inoculated seeds planted in FV-infested soil</td>
</tr>
<tr>
<td></td>
<td>FV</td>
<td>ISU-BJ</td>
<td>Germinated, FV-inoculated seeds planted in ISU BJ-infested soil</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>FV</td>
<td>Germinated seeds planted in FV-infested soil</td>
</tr>
<tr>
<td></td>
<td>ISU-BJ</td>
<td>FV</td>
<td>Non-germinated, ISU BJ-inoculated seeds planted in FV-infested soil</td>
</tr>
<tr>
<td>2015 (run 2)</td>
<td>none</td>
<td>ISU-BJ</td>
<td>Germinated seeds planted in ISU BJ-infested soil</td>
</tr>
<tr>
<td></td>
<td>ISU-BJ</td>
<td>none</td>
<td>Non-germinated seeds planted in non-infested soil</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>ISU-BJ</td>
<td>Non-germinated seeds planted in ISU BJ-infested soil</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>none</td>
<td>Non-germinated seeds planted in non-infested soil</td>
</tr>
<tr>
<td>2016 (2 runs)</td>
<td>ISU-BJ</td>
<td>FV</td>
<td>Germinated, ISU BJ-inoculated seeds planted in FV-infested soil</td>
</tr>
<tr>
<td></td>
<td>ISU-BJ + bioAPT</td>
<td>FV</td>
<td>Germinated, ISU BJ + bioAPT-inoculated seeds planted in FV-infested soil</td>
</tr>
<tr>
<td></td>
<td>FV</td>
<td>ISU-BJ</td>
<td>Germinated, FV-treated seeds planted in ISU BJ-infested soil</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>FV</td>
<td>Germinated seeds planted in FV-infested soil</td>
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<td>Germinated seeds planted in non-infested soil</td>
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<tr>
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<td>ISU-BJ</td>
<td>FV</td>
<td>Non-germinated, ISU BJ-inoculated seeds planted in FV-infested soil</td>
</tr>
<tr>
<td></td>
<td>ISU-BJ + bioAPT</td>
<td>FV</td>
<td>Non-germinated, ISU BJ + bioAPT-inoculated seeds planted in FV-infested soil</td>
</tr>
<tr>
<td></td>
<td>FV</td>
<td>BJ</td>
<td>Non-germinated, FV-treated seeds planted in ISU BJ-infested soil</td>
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<td>none</td>
<td>FV</td>
<td>Non-germinated seeds planted in FV-infested soil</td>
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<td>ISU-BJ</td>
<td>Non-germinated seeds planted in ISU BJ-infested soil</td>
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<td></td>
<td>none</td>
<td>none</td>
<td>Non-germinated seeds planted in non-infested soil</td>
</tr>
</tbody>
</table>
\textit{Bradyrhizobium japonicum} isolate obtained from Iowa State University

\textit{Fusarium virguliforme}

Microbial Carrier (American Peat Technology®) used treated together with \textit{Bradyrhizobium japonicum}
Fig. 1 Effects of *Bradyrhizobium japonicum* seed treatments under different temperatures on soybean sudden death syndrome was measured by incidence (%) and severity (scale 1-9) at V3 growth stage in greenhouse conditions. Germinated seeds (a, b) or non-germinated seed (c, d) were treated either with ISU-BJ strain of FV in experiment with 2 runs (run 1 in 2014 and run 2 in 2015). Incidence (%) was calculated based on the percentage of plants in pot showed visible foliar symptoms. Incidence was assessed each pot (5 plants/pot) and 10 pots/treatment. Severity was scored on scale from 1 to 9 based on the percentage of leaf damage. Means were calculated based on Fisher’s protected least significance difference. Error bars are standard errors of the mean values. Treatments are including BJ inoc-FV soil (Seeds inoculated with BJ and planted in FV infested soil); FV inoc-BJ soil (Seeds inoculated with FV and planted in BJ infested soil) and FV soil (non-treated seeds planted in FV infested soil) as control treatment. An asterisk (*) above a bar indicates treatment was significantly different from control treatment (P≤0.05). Two asterisks (**) indicate significantly different at P<0.0001.
Fig. 2 Effects of *Bradyrhizobium japonicum* seed treatments under different temperatures on total nodule and nodule count on primary root per plant at V4 stage in greenhouse conditions. Treatments were applied on germinated (a, b) or non-germinated (c, d) seeds in experiment with 2 runs (2014, 2015). Fifteen plants in 3 pots per treatment were randomly collected, washed and air-dried to count nodule on primary root and total nodule. Means were calculated based on Fisher’s protected least significance difference. Error bars are standard errors of the mean values. An asterisk (*) above a bar indicates mean value was significantly different from mean value of water-treated seed planted in FV infested soil (P≤0.05). Two asterisks (**) indicate significantly different at P<0.0001.
Fig. 3 Efficacy tests of seed treatments with ISU strain of *Bradyrhizobium japonicum* on soybean sudden death syndrome incidence under different temperatures in experiment in 2016 (combined data from two independent runs). Germinated (panel A) or non-germinated (panel B) seeds were treated with BJ or BJ plus bio APT or FV. Disease incidence (%) was taken at V1, V3 and R1 growth stages. Incidence (%) was calculated based on the percentage of plants in cup showed visible foliar symptoms. Means were calculated based on Fisher’s protected least significance difference. Error bars are standard errors of mean values. An asterisk (*) indicates mean of treatment differed from mean of water-treated seed planted in FV infested soil (control treatment) at P≤0.05 while two asterisks (**) indicate differences at P<0.0001.
Fig. 4 Efficacy tests of seed treatments with ISU strain of *Bradyrhizobium japonicum* on soybean sudden death syndrome foliar severity under different temperatures in experiment in 2016 (combined data from two independent runs). Germinated (a, b and c) or non-germinated (d, e and f) seeds were treated with BJ or BJ plus bio APT or FV. Severity was taken at V1, V3 and R1 growth stages. Incidence was assessed on scale from 1 to 9 based on percentage of leaf damage in each cup. Means were calculated based on Fisher’s protected least significance difference. Error bars are standard errors of mean values. Asterisks indicate: ** significance at P<0.0001; * significance at P≤0.05.
Fig. 5 Effects of seed treatments with ISU-BJ strain on total nodule count, fresh shoot and fresh root weights (gram) at three different temperatures in experiment March 2016. Treatments were applied on germinated (a, b, c) or non-germinated (d, e, f) seeds. Samples were taken at growth stage V4. Means were calculated based on Fisher’s protected least significance difference. Asterisk (*) indicates significant differences (P≤0.05) between BJ treatment with non-treated seeds planted in FV soil.
Fig. 6 Effects of seed treatments with ISU-BJ strain on dry shoot and dry root weights (gram) at three different temperatures in experiment March 2016. Treatments were applied on germinated (a, b) or non-germinated (c, d) seeds. Samples were taken at growth stage V4. Means were calculated based on Fisher’s protected least significance difference.
Supplemental Fig. S1 Effects of *Bradyrhizobium japonicum* seed treatments under different temperatures on total nodule and nodule count on primary root at V4 stage in greenhouse conditions. Seeds were treated after germination (a, b) or before germination (c, d) in experiment in 2014 (run 1). Fifteen plants in 3 pots per treatment were randomly collected, washed and air-dried to count nodule on primary root. Means were calculated based on Fisher’s protected least significance difference. Error bars are standard errors of the mean values.
Supplemental Fig. S2 Effects of *Bradyrhizobium japonicum* seed treatments under different temperatures on total nodule and nodule count on primary root at V4 stage in greenhouse conditions. Seeds were treated after germination (a, b) or before germination (c, d) in experiment 2015 (run 2). Fifteen plants in 3 pots per treatment were randomly collected, washed and air-dried to count nodule on primary root. Means were calculated based on Fisher’s protected least significance difference. Error bars are standard errors of the mean values.
Supplemental Fig. S3 Effect of seed treatments with ISU-BJ strain on disease severity under different temperature in experiment in March 2016 (run1). Germinated (panel A) or non-germinated (panel B) seeds were treated either with BJ, BJ plus bio APT or FV. Severity was scored on scale from 1 to 9 based on the percentage of leaf damage. Means were calculated based on Fisher’s protected least significance difference. Error bars are standard errors of mean values.
Supplemental Fig. S4 Effect of seed treatments with ISU-BJ strain on disease severity under different temperature in experiment in March 2016 (run2). Germinated (panel A) or non-germinated (panel B) seeds were treated either with BJ, BJ plus bio APT or FV. Severity was scored on scale from 1 to 9 based on the percentage of leaf damage. Means were calculated based on Fisher’s protected least significance difference. Error bars are standard errors of mean values.
CHAPTER 3.

EFFECTS OF \textit{BRADYRHIZOBIUM JAPONICUM} SEED TREATMENTS ON SOYBEAN SUDDEN DEATH SYNDROME IN IRRIGATED AND NON-IRRIGATED FIELDS

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A manuscript to be submitted to Crop Protection

\textbf{Highlights}

- Seed treatments with \textit{Bradyrhizobium japonicum} (BJ) showed significant effects in reducing soybean sudden death syndrome disease index.
- Grain yield increased in BJ treated plots compared with untreated controls.
- Nodule counts and plant biomass weights on average were higher in BJ treated plots in irrigated than in non-irrigated fields.

\textbf{Abstract}

Seed treatments studies were conducted from 2015 to 2017 to investigate the efficacy of \textit{Bradyrhizobium japonicum} (BJ) seed treatments on soybean sudden death syndrome (SDS), caused by \textit{Fusarium virguliforme}, and grain yields as assessed based on disease in irrigated and
non-irrigated field conditions. Commercially untreated soybean seeds were treated separately with BJ strains ISU-BJ and USDA 110 in 2015 and USDA 30, USDA 31 and USDA 110, either alone or in combination with microbial carrier bioAPT or the commercial plant protectant HeadsUp in 2016 and 2017. Seed treatments trials were set up in randomized complete block designs in four replications with 3 m wide × 5.3 m long plots at Hinds Research Farm, Ames, IA. Plots were evaluated for stand counts, percent SDS incidence, severity, SDS foliar disease index (FDX) and grain yields. At R6 growth stage, BJ treated plots showed lower FDX than untreated plots and an average yield increase of 4.4% in irrigated plots and 0.8 % in non-irrigated plots. Our results also showed higher nodule counts, and shoot and root weights per plant at V5 in BJ-treated plots than in untreated plots in both the field conditions. The findings indicate that BJ seed treatment can be a good management approach to control SDS.

Key words: *Fusarium virguliforme, Bradyrhizobium japonicum*, soybean, seed treatment, irrigation

**Introduction**

Soybean [*Glycine max* (L.) Merr.] is an important oilseed crop in the United States. In 2018, the United States soybean production was 123.6 million metric tons worth $40.94 billion (FAS, USDA, 2019) with an average yield of 3.47 metric tons/hectare. Soybean sudden death syndrome (SDS) is one of the most significant soil-borne diseases in the United States soybean production. The SDS is caused by *F. virguliforme* (Aoki et al., 2003) in North America, *F. virguliforme*, *F. tucumaniae*, *F. brasiliense*, *F. cuneirostrum* (Aoki et al., 2005) and *F. crassistipitatum* (Aoki et al., 2012) in South America, and *F. virguliforme* (Tewoldemedhin et al., 2014) and *F. brasiliense* (Tewoldemedhin et al., 2017) in South Africa. In addition, Navi and
Yang (2016c) have reported SDS caused by *F. virguliforme* from Malaysia, and *F. azikicola* from Japan and an unconfirmed report from India.

Typical soil temperatures of Iowa in early soybean planting often range from 10 to 15°C (Leandro et al., 2013). Infection of *F. virguliforme* is favored by cool, wet soil conditions (Scherm and Yang, 1996) which are prevalent conditions in early planted soybean. A phytotoxin is produced after fungal colonization (Jin et al., 1996) and translocated in the plant through xylem vessels (Navi and Yang, 2008). This blue pigmented fungus on the external surface of tap roots causes reddish-brown discoloration in the internal surface of roots and significantly reduces nodulation and root biomass (Rupe, 1989; Roy et al., 1989). Soybean plants infected with SDS show symptoms both during the seedling stage and with the most dramatic symptoms, reproductive growth stages (Roy et al., 1997; Navi and Yang, 2016a). Chlamydospores, the survival structure of this fungus, can withstand wide soil temperature fluctuations and resist desiccation (Roy et al., 1989). Macro-conidia, the primary type of asexual spore of this fungal genus can be rain-splashed, scattered by water runoff, or dispersed by air during harvest, potentially resulting in the external infestation of seeds that can contribute to dissemination (Roy, 1997).

During 2007 to 2009, SDS was in the top five of soybean diseases causing yield losses, with losses estimated from 0.56 to 0.94 million metric tons (Koening and Wrather, 2010). Between 2010 and 2014, SDS was the fifth most destructive diseases in the twelve northern-most states in the United States and Ontario (Canada), causing estimated yield loss from 0.55 to 1.92 million metric tons (Allen et al., 2017). The SDS has been reported in 21 soybean-producing states in the U.S. and the corresponding economic losses from $15.7 million in 1988 to $669.2 million in 2010 (Navi and Yang, 2016c). In a recent review, several management options of SDS
have been compiled including screening techniques for resistance (Navi and Yang, 2016c). Integrating multiple management strategies is needed because of the destructive nature and wide geographical range of this disease. Many options tested to manage SDS such as crop rotation, soil tillage practices, and fungicide seed treatments; these have shown mixed results, making it difficult to provide appropriate recommendations to growers about how to manage SDS.

Cool soil temperatures and high soil moisture often are associated with early spring soil in Iowa. Infection of the roots early in the growing season under the right environmental conditions can bring about the most severe cases of SDS later in the season. Studies of Gongora-Canul and Leandro (2011) showed that fungal inoculation treatment at planting produced more severe foliar SDS symptoms than treatment weeks later, and that at warmer temperatures at planting foliar symptoms were less severe. Therefore, delayed planting is one of the options to reduce impact of SDS. However, this practice may limit yield potential (Roy et al., 1997) because it shortens the length of growing season for achieving maximum yield potential of soybean. High soil moisture is also associated with greater occurrence of SDS, severity and seed yield loss under field condition (Roy et al., 1997; Melgar et al., 1994; Scherm and Yang, 1996). Therefore, irrigation during reproductive stage that is important for toxin translocation from the roots to the foliage can help researcher to receive sufficient foliar symptoms (Farias Neto et al., 2006).

There are a few reports of using *Bradyrhizobium* spp. in management of row crop diseases (Chakraborty and Purkayasta, 1983; Tu, 1978; Buonassissi et al., 1986) considering it as a plant growth promoting rhizobacteria (Jordan, 1982; Kuykendall, 1992; Antoun et al., 1998, Carletti et al., 1994; Biswas et al., 2000). Studies conducted in the controlled environments on the effects of seed treatments with *Bradyrhizobium* strains showed suppression of SDS incidence
and severity (Huynh et al., 2016; Huynh et al. 2019 unpublished). Therefore, in this article, results of field studies using BJ as a potential biocontrol agent to suppress SDS in irrigated and non-irrigated field conditions provided. Also, field studies would complement the central hypothesis tested in greenhouse in the previous chapter namely that early infection of *B. japonicum* can enhance plant health and suppress early stage SDS infection.

**Materials and methods**

*Soybean cultivars, Bradyrhizobium and Fusarium isolates*

Commercially untreated roundup ready soybean varieties P22T61RR, and P22T73RR with moderately resistant SDS ratings were procured from DuPont Pioneer, Johnston, IA 50131-0184.

*Bradyrhizobium japonicum* (BJ)- ISU BJ strain was obtained from Dr. Xun Li and BJ strain USDA 110 was obtained from Dr. Gwyn Beattie, Iowa State University. These strains were used in 2015 field tests. In 2016 and 2017, USDA 30 and USDA 31 were received from USDA-ARS National Rhizobium Germplasm Resource Collection, Beltsville, Maryland, USA. These two strains and USDA 110 were used for additional field studies (Table 1). All BJ strains were maintained on Yeast Mannitol Agar (YMA) and were sub-cultured on yeast mannitol broth (YMB) as described by Vincent (1970). BJ strains were grown in flasks (250 mL) on VWR DS-500E Orbital Shaker at 125 rpm at 25°C for 7 days. Later, the culture was diluted with sterile yeast mannitol broth to an OD$_{620}$ of 0.08, which was equivalent to about $10^8$ cells mL$^{-1}$ (Bhuvaneswari et al. 1980).

*Fusarium virguliforme* isolate Fsg-ISU1 (Mbofung et al., 2012) originated from an Iowa field was sub-cultured on Potato Dextrose Agar (PDA) Petri dishes supplemented with 100 mg/L
streptomycin sulfate to suppress bacterial growth. *F. virguliforme* (FV) inoculum was fermented on white sorghum grains following the methods of Hartman et al. (1997) and Navi and Yang (2016a). The dried inoculum was transferred to brown paper bags and stored in at 4°C for use in field studies.

*Effects of Bradyrhizobium japonicum seed treatments on soybean sudden death syndrome in 2015*

Two studies, each with three treatments, were set up in a randomized complete block design (RCBD) with four replications. Individual plot had 3-m wide × 5.3 m long and were located at the Hinds Research Farm, Ames, IA (42° N latitude, 93.6° W longitude). Commercially untreated soybean seeds of P22T61RR (DuPont Pioneer, Johnston, IA) were treated separately with ISU-BJ and USDA 110 strains at 10 mL/kg seed (10⁸ cfu mL⁻¹ concentration); YMB- treated seed planted together with FV inoculated sorghum grain served as the control. The seeds were assigned at 700 seed/replication in 8.5 × 20.3 cm² envelopes (at 10 seed/linear foot) and *F. virguliforme* fermented on white sorghum grain was placed separately in another envelope (at 4cc/linear foot). Soybean seed were planted along with fermented sorghum grain using an ALMACO 4-Row SeedPro Precision Vacuum planter. In one trial, plots were irrigated on non-rainy days using overhead impact sprinklers from R1 to R6 to provide a total 2.5 cm/week or 6-hours/week while other trial was not irrigated.

*Effect of Bradyrhizobium japonicum seed treatments on SDS in 2016 and 2017*

Similar to studies in 2015, two seed treatment trials, each with eight treatments were set up in a RCBD at Hinds Research Farm, Ames, IA, during 2016 and 2017. Plot dimensions, seed rates, concentration of BJ strains in seed treatments, fermented sorghum grain rate, planter, and irrigation, were same as that of 2015 tests, except the variety P22T73RR (Table 1).
In 2016 and 2017 commercially-untreated seed of P22T73RR were treated separately with 1) USDA 30 at 10 mL/kg seed \((10^8 \text{ cfu mL}^{-1})\) mixed simultaneously with bioAPT at 10 g/kg, 2) USDA 31 at 10 ml/kg \((10^8 \text{ cfu mL}^{-1})\) together with bioAPT at 10 g/kg, 3) Heads Up® (Heads Up Plant Protectant Inc., 3002 Millar Avenue, Saskatoon, SK, Canada) seed treatments (at 1g mixed in 1 L water for 163.3 kg seed), 4) USDA 30 at 5 ml/kg \((10^8 \text{ cfu mL}^{-1})\) + HeadsUp, 5) USDA 31 at 5 ml/kg \((10^8 \text{ cfu mL}^{-1})\) + HeadsUp, 6) USDA 110 at 10 ml/kg \((10^8 \text{ cfu mL}^{-1})\), 7) YMB-treated seeds planted together with sorghum grains served as negative control and 8) YMB-treated seeds planted together with FV fermented on white sorghum grains served as negative control served as positive control (Table 1). Except treatment 7, all other treatments were infested with 4 cc linear foot of FV inoculum fermented on sorghum grain. Experiments were placed in non-irrigated and irrigated fields. Overhead sprinkler was run 6 hours/week in the irrigated field when soybean plants started flowering (R1) up to R6 growth stage.

**Data collection**

In each trial, soil temperature (°C), soil moisture at planting and monthly precipitation data were obtained from the nearest weather station (Ames - Horticulture ISU-RDF, State ID AEE14) through a public service website (https://mesonet.agron.iastate.edu) and https://w2.weather.gov/climate/. Stand count was recorded 25 days after planting and was then determined every 10 days until V3 growth stage every year. To measure nodule numbers, shoot and root weights, in 2016 and 2017, five plants were uprooted with a shovel from the center two rows at V5 growth stage (Fehr et al., 1971). The uprooted plants were rinsed in low pressure running tap water, and nodule numbers were recorded on a plant basis. Subsequently, plants were cut into shoot and root and fresh weights (g) was recorded. After drying plants on greenhouse benches for 2-3 weeks, dry weight (g) was recorded.
Sudden death syndrome (SDS) disease incidence and severity (Njiti et al., 1996) were recorded for each plot both in vegetative starting at growth stage V3 and continuing to reproductive growth stages up to R7. The disease incidence (DI) was calculated as the percentage of the SDS symptomatic plants out of the total plants in a plot. The SDS disease index (DX) was calculated as DI × DS/9 (Njiti, 1998). At maturity, plots were harvested using an ALMACO Research Plot Combine. Yields were adjusted to 13% grain moisture and measured in bushel per acre. Subsequently, yield in bushel/acre was converted to Kg/ha.

Statistical analysis

Results were analyzed statistically by analysis of variance using the Statistical Analysis System (version 9.4, SAS Institute Inc., Cary, NC, USA). Data for each trial in non-irrigated or irrigated field were analyzed separately. Data analysis was performed using the PROC ANOVA procedure. If the model was significant, the least significant difference (LSD) test was applied to make comparisons among the means at the 0.05 level of significance (Steel and Torrie, 1980). When differences occurred at levels of significance between 0.05 and 0.1, they are noted in the text.

Results

Effects of B. japonicum seed treatments on SDS in 2015.

This trial was established to test the efficacy of B. japonicum (ISU BJ, and USDA 110) seed treatments under field conditions. The stand count in the BJ treated plot differed from the untreated plots in the non-irrigated but not irrigated trial. Disease incidence (%) in vegetative growth stages (V3-V4) and reproductive growth stage R3 were significantly lower in BJ-treated plots compared with untreated plots both in non-irrigated trial and in irrigated trial (Table 2). In
reproductive stages (R4-R6), SDS incidence and foliar disease index (FDX) were not statistically different among the treatments in both fields. However, at R6 SDS incidence in BJ treated plots was 24% lower than untreated plots (Table 2). Also, at R6 FDX ratings in BJ treated plots were lower than untreated plots both in non-irrigated and in irrigated fields (Fig. 1). Generally, FDX values were higher in plots planted with FV infested sorghum grain (positive control) than BJ treated plots in both field trial, as expected (Fig. 1B).

There were no significant differences in grain yields among the treatments (Table 2). Grain yields in the non-irrigated trial were lower than in the irrigated trial; this was likely due to severe flooding in the non-irrigated plots toward maturity in 2015.

Effects of B. japonicum seed treatments on SDS in 2016 and 2017

In 2016 and 2017, no significant differences in stand counts were observed in seeds treated with BJ strains (either alone or in combination with HeadsUp) compared with untreated controls either in irrigated or in non-irrigated field trials (Tables 3).

Data for SDS disease incidence and FDX are presented for field trial in 2016. There was a significant effect of seed treatments on disease index at different stages under field conditions in field test in 2016 (Fig. 2). Foliar disease index were high in irrigated field (Fig. 2B). In irrigated field, FDX at growth stage R4 in BJ treated either alone or with Heads Up plots were 1% to 3% lower than untreated plot planted with FV sorghum grain (positive control); however, from R6 to R7 BJ treated plots had 10% to 15% FDX lower than positive control plots. Under irrigated conditions, seed treatment with USDA 30 and 31 showed a lower reduction of SDS than USDA 110 and Heads Up. In non-irrigated trial, FDX was low (from 0 to 2 point) in which USDA 31 treatment showed lowest disease incidence followed by USDA 30 seed treatment and their combination with Heads-Up. In 2017, we did not observe any SDS development.
In 2016, there were significant on nodule counts among treatments under non-irrigated field ($P=0.03$). Nodule count was obtained highest on positive control followed by USDA 31 seed treatment and USDA 31 and Heads-Up combination treatment in irrigated field while USDA 110 had lowest number (Table 4). In irrigated field, no significant effects on nodule counts was detected among treatment ($P=0.26$). Lowest nodule count was accounted for Heads up seed treatment followed by USDA 11 (Table 4). Overall, there were no consistent trends in the differences in nodule number among treatments across years or plots with distinct irrigation status.

Fresh and dry weights (g) of roots and shoots also were recorded in 2016 and 2017. Generally there were no significant effect of treatment on plant biomass ($P>0.05$) in irrigated or in non-irrigated trials (Table 5, 6, S1, S2).

Yield responses showed differently under field conditions across years. In 2016, under natural field, Heads Up seed treatments produced highest yield (4494 kg/ha) followed by USDA 31 (4461 kg/ha) and negative control (4401 kg/ha) (Table 7) while USDA 110 had smallest yield (4230 kg/ha). Treatments with BJ had a yield reduction by 1.5% compared with controls. On the other hand, under irrigated field, average yields in plot with USDA BJ seed treatments was greater by 4.3 % compared with controls. Seed treatment with USDA 30, 31 had significant effect on increasing grain yield in irrigated field compared with positive control ($P=0.01$). Negative control seed treatment obtained lowest yield (3813 kg/ha) (Table 7). In 2017 despite the fact that there was no significant effect of treatments on yield at $P=0.05$ among treatments, average yield was greater by 3% and 7.3 % in USDA BJ treatments compared with control treatments in non-irrigated and irrigated fields, respectively. Seed treatments with BJ combined Heads Up did not increase yields compared with BJ treated alone. In 2016 and 2017, BJ treated
seeds increased yields by 1.5 % and 4.95 % compared with BJ and Heads Up treatments in non-irrigated and irrigated fields, respectively.

Weather data.

Precipitations during growing seasons (from May 1st to October 1st) were different in three years (2015, 2016 and 2017) of the study. Ames location received higher precipitation in 2015 than in 2016 and 2017. Growing season in 2015 had 43% increase of precipitation level compared with an average level over 30 years; whereas in 2016 was 27% higher and 2017 season with 24% less precipitation compared with average precipitation over 30 years. Soil temperature at planting dates in three years ranged from 11.5°C to 17.5°C (Fig. 3).

Discussion

The current study confirms the findings of greenhouse study (Huynh et al., 2016) that seed treatment with BJ was effective in reducing soybean sudden death syndrome in the soil artificially infested with *Fusarium virguliforme*. It further demonstrates the effectiveness of BJ seed treatment against SDS occurrence under field conditions. Results from the study showed seed treatment with BJ did not affect stand count compared with untreated negative control.

Therefore, seed treatment with effective BJ strains may be good practice for field use. Further studies on using BJ treatment as other biocontrol agent are suggested because of the potential for the nitrogen-fixing bacteria to control SDS, improve soil fertility, increase crop productivity and reduce the negative environmental impact associated with chemical use.

Effects of BJ seed treatment on suppressing SDS under field conditions changed differently over years of the study. In 2015, treatments with ISU-BJ strain and USDA 110 strain had significant effects on reducing SDS in both fields. This can be explained by the tremendous
cumulative rainfall in late July and August during SDS onset at flowering stages on soybean. The performance of BJ in both field were the same in reducing SDS occurrence. Our data showed the performance of USDA 110 in greenhouse experiments exhibited lower nodulation and SDS suppression (unpublished, Huynh et al. 2019) compared with other USDA BJ strains; however, its effects under field condition on nodulation and yield was the same with other USDA strains even achieved higher yield in 2017 season compared with control. This result supports the finding that USDA 110 was the isolate growing well under lower temperature in field conditions (Zhang et al., 2003).

Results from our studies showed yield responses to BJ inoculum under SDS pressure was different in non-irrigated and irrigated systems especially that lower precipitation during growing season affected SDS occurrence. The changes of precipitation which are lower in recent years reflect a normal trend due to global climate changes. These changes, in turn, make indigenous BJ population no longer adapt on their soybean host which is also being changed in their genetic background. On the other hand, study by Yang and Feng (2001) predicted that highest soybean fungal disease diversity was on the regions which had most variable climatic parameters. Overall, there might be an explanation for yield responses on our experiments together with unpredictable changes on SDS occurrence.

In 2016, based on results of the previous experiments (in 2014, 2015 and 2016), we conducted experiments using only seed treatments with cold tolerant BJ strains obtained from USDA (unpublished data). The purpose of this study was to test the applicability of our findings in greenhouse studies to field conditions. The results showed that seed treatments with a microbial carrier should be an option as a management tool. Our findings also indicated that BJ seed treatments acted better than a commercial product on suppressing SDS. Our study is the
first comparing effects of a commercial product used as biological seed treatment with BJ strains on SDS development.

In 2016 trial, Heads-Up seed treatment was used as Systemic Acquired Resistance inducer to compare the different effects when using BJ strains alone or combination with commercial Heads-Up product. Overall, SDS occurrence difference among those treatments was not observed; however, there were differences on dry biomass and nodule counts in which treatments either with BJ alone or BJ with Heads-Up had higher weights than treatments with Heads-Up alone. This indicates seed treatments with BJ producing better nodulation and higher biomass.

In 2017 trial, eight treatments from 2016 at Hinds farm were repeated under 2 systems (natural and irrigated). We did not observed SDS occurrence during 2017 season. However, yield differences were observed in irrigated fields. The highest yield by USDA 110 treatment should explain for the adapted of this strain in the US soil. The results of the present study have indicated that the appropriate use of BJ inocula as seed treatments to introduce BJ population which can adapt in a short growing season in Iowa can be a good practice to reduce SDS. Further studies are recommended to explore the potential of control of BJ on other diseases caused by Fusarium spp. in other crops, control of other soilborne pathogens in legume and non-legume crops.

**Declarations of interest**

None
Acknowledgments

This study was funded by Iowa Soybean Association and VietNam International Education Development (VIED). We wish to thank Sharon Eggenberger, Department of Plant Pathology and Microbiology for her technical assistance in data analysis.

Literature cited


Table 1

Experiment descriptions, field number, planting and harvesting and SDS scoring dates at ISU Hinds Research Farm, Ames, Iowa.

<table>
<thead>
<tr>
<th>Year/Field</th>
<th>Date planted</th>
<th>Date harvested</th>
<th>Soil Temp&lt;sup&gt;b&lt;/sup&gt; (°C)</th>
<th>Seed treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>2015</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-irrigated</td>
<td>4 May</td>
<td>23 September</td>
<td>17.2</td>
<td>1. ISU- BJ&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Irrigated</td>
<td>4 May</td>
<td>26 September</td>
<td>17.2</td>
<td>2. USDA 110&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2016</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-irrigated</td>
<td>16 May</td>
<td>29 September</td>
<td>12.7</td>
<td>1. USDA 30&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Irrigated</td>
<td>14 May</td>
<td>29 September</td>
<td>11.5</td>
<td>2. USDA 31&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>2017</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-irrigated</td>
<td>9 May</td>
<td>19 October</td>
<td>15.8</td>
<td>3. Heads Up&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Irrigated</td>
<td>9 May</td>
<td>19 October</td>
<td>15.6</td>
<td>4. USDA 30 + Heads up&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5. USDA 31+ Heads up&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6. USDA 110&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7. Negative control&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8. Positive control&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Overhead sprinklers ran from growth stage R1 to R6 to provide 2.5 cm<sup>3</sup>/week.

<sup>b</sup>Soil temperature (10 cm depth) recorded at planting.

<sup>c</sup>Treated seeds planted with FV inoculated sorghum grain

<sup>d</sup>Untreated seeds planted together with FV fermented on white sorghum grain.

<sup>e</sup>Untreated seeds planted with white sorghum grain.
Table 2

Effects of ISU BJ seed treatments on stand count\(^a\) and sudden death syndrome (SDS) disease incidence in field trials in 2015. Values are means ± standard error of four replications. Means with the same letter within column are not significantly different at \(P<0.05\) according to Fisher’s protected least significant difference test.

<table>
<thead>
<tr>
<th></th>
<th>Stand count(^a)</th>
<th>SDS disease incidence (%)(^b)</th>
<th>Yield (kg/ha)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V3-V4</td>
<td>R3</td>
<td>R4-R4.5</td>
</tr>
<tr>
<td><strong>Non-irrigated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISU BJ</td>
<td>632.6±10.8(^a)</td>
<td>2.45±2.29(^b)</td>
<td>0.00±0.0(^b)</td>
</tr>
<tr>
<td>USDA 110</td>
<td>642.0±9.0(^a)</td>
<td>0.00±0.0(^b)</td>
<td>0.00±0.0(^b)</td>
</tr>
<tr>
<td>Positive control(^c)</td>
<td>648.6±12.6(^a)</td>
<td>8.41±1.15(^a)</td>
<td>2.86±0.6(^a)</td>
</tr>
<tr>
<td><strong>Irrigated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISU BJ</td>
<td>610±20.6(^b)</td>
<td>1.36±1.15(^b)</td>
<td>0.33±0.22(^ab)</td>
</tr>
<tr>
<td>USDA 110</td>
<td>689±17.7(^a)</td>
<td>0.53±0.18(^b)</td>
<td>0.00±0.0(^b)</td>
</tr>
<tr>
<td>Positive control(^c)</td>
<td>577.6±18.8(^b)</td>
<td>7.39±1.15(^a)</td>
<td>0.86±0.13(^a)</td>
</tr>
</tbody>
</table>

\(^a\) Stand counts recorded at V3 growth stage. Stand count mean value presented for averaged plot size of four rows with 5.3 m length with 75 cm row spacing.

\(^b\) SDS incidence was rated as the percentage of the plants in a plot that show visible foliar symptoms of SDS.

\(^c\) Untreated seeds planted together with FV inoculated sorghum grains.
Table 3.

Effects of seed treatments on stand counts at V3 growth stage recorded in non-irrigated and irrigated field trials in 2016 and 2017, ISU Hinds Farm, Ames, Iowa.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2016 Non-irrigated</th>
<th>2016 Irrigated</th>
<th>2017 Non-irrigated</th>
<th>2017 Irrigated</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA 30</td>
<td>639.75±12.3a</td>
<td>613±23.9a</td>
<td>639.25±61.5a</td>
<td>548±19.7a</td>
</tr>
<tr>
<td>USDA 31</td>
<td>647.5±24.7a</td>
<td>632.5±30.4a</td>
<td>591.75±46.7ab</td>
<td>485.25±27.3b</td>
</tr>
<tr>
<td>Heads Up® (HU)</td>
<td>689.75±25.5a</td>
<td>630.5±27.1a</td>
<td>657±54.2a</td>
<td>555.75±16.9a</td>
</tr>
<tr>
<td>USDA 30+HU</td>
<td>658.25±27.7a</td>
<td>673.5±14.6a</td>
<td>582.25±39.6ab</td>
<td>551.5±19.1a</td>
</tr>
<tr>
<td>USDA 31+HU</td>
<td>661.5±27.2a</td>
<td>643±34.3a</td>
<td>521.5±87b</td>
<td>506±23.6ab</td>
</tr>
<tr>
<td>USDA 110</td>
<td>620.75±39.9a</td>
<td>659±22.8a</td>
<td>579±47ab</td>
<td>510.25±26.2ab</td>
</tr>
<tr>
<td>Negative control</td>
<td>637.5±25.7a</td>
<td>633.5±47.8a</td>
<td>584±57.8ab</td>
<td>533.25±12.1ab</td>
</tr>
<tr>
<td>Positive control</td>
<td>601.5±32.9a</td>
<td>623.5±28.1a</td>
<td>603.5±35.5ab</td>
<td>524.5±13.7ab</td>
</tr>
</tbody>
</table>

Note: Values are means and standard errors of 4 replications. Means within each column followed by same letter are not significantly different at P <0.05 based on Fisher’s protected least significance difference. *Commercial product (Heads-Up® Plant Protectant Inc.)
Table 4. Effect of USDA strains either alone or in combination with Heads-Up and Heads-Up seed treatments on nodule number per plant under field conditions at Hinds Farm, Ames, Iowa in 2016 (A) and 2017 (B).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2016</th>
<th>2017</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-irrigated</td>
<td>Irrigated</td>
<td>Non-irrigated</td>
<td>Irrigated</td>
</tr>
<tr>
<td>USDA 30</td>
<td>32.7±4.01a</td>
<td>27.6±2.97ab</td>
<td>19.7±2.98b</td>
<td>27.5±3.63a</td>
</tr>
<tr>
<td>USDA 31</td>
<td>28.5±1.68abc</td>
<td>27.4±2.43ab</td>
<td>18.5±1.94b</td>
<td>22.8±2.13a</td>
</tr>
<tr>
<td>Heads Up (HU)</td>
<td>29.6±3.72ab</td>
<td>20.7±1.67b</td>
<td>21.7±3.21ab</td>
<td>26.4±2.38a</td>
</tr>
<tr>
<td>USDA 30+HU</td>
<td>31.2±2.51a</td>
<td>27.74±2.7ab</td>
<td>23.3±2.63ab</td>
<td>24.4±2.25a</td>
</tr>
<tr>
<td>USDA 31+HU</td>
<td>25.4±3.88abc</td>
<td>29.7±2.19ab</td>
<td>27.9±3.11a</td>
<td>24.0±2.34a</td>
</tr>
<tr>
<td>USDA 110</td>
<td>21.3±2.31bc</td>
<td>22.8±2.5ab</td>
<td>22.4±2.42ab</td>
<td>24.9±1.87a</td>
</tr>
<tr>
<td>Negative control</td>
<td>20.4±2.35c</td>
<td>29.9±4.61a</td>
<td>21.1±2.10ab</td>
<td>26.7±2.17a</td>
</tr>
<tr>
<td>Positive control</td>
<td>21.5±2.37bc</td>
<td>31.6±3.9a</td>
<td>23.8±2.05ab</td>
<td>24.5±2.35a</td>
</tr>
<tr>
<td>P value</td>
<td>0.03</td>
<td>0.26</td>
<td>0.3</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Note: Values are means and standard errors of 10 replicates (plants). Means within each column followed by the same letter are not significantly different at P <0.05 based on Fisher’s protected least significance difference.
Table 5.

Effect of USDA strains either alone or in combination with Heads-Up and Heads-Up seed treatments on fresh shoot weight (FSW), dry shoot weight (DSW), fresh root weight (FRW) and dry root weight (DRW) in non-irrigated field at ISU Hinds Farm, Ames, Iowa in 2016.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FSW (g)</th>
<th>DSW (g)</th>
<th>FRW (g)</th>
<th>DRW (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA 30</td>
<td>7.88a</td>
<td>1.77a</td>
<td>1.81a</td>
<td>0.36a</td>
</tr>
<tr>
<td>USDA 31</td>
<td>9.14a</td>
<td>2.28a</td>
<td>1.83a</td>
<td>0.37a</td>
</tr>
<tr>
<td>Heads Up (HU)</td>
<td>9.40a</td>
<td>2.31a</td>
<td>2.09a</td>
<td>0.46a</td>
</tr>
<tr>
<td>USDA 30+HU</td>
<td>7.59a</td>
<td>1.67a</td>
<td>1.72a</td>
<td>0.41a</td>
</tr>
<tr>
<td>USDA 31+HU</td>
<td>7.75a</td>
<td>1.87a</td>
<td>1.25a</td>
<td>0.29a</td>
</tr>
<tr>
<td>USDA 110</td>
<td>8.62a</td>
<td>1.91a</td>
<td>1.628a</td>
<td>0.34a</td>
</tr>
<tr>
<td>Negative control</td>
<td>8.89a</td>
<td>1.92a</td>
<td>1.71a</td>
<td>0.28a</td>
</tr>
<tr>
<td>Positive control</td>
<td>9.18a</td>
<td>1.98a</td>
<td>1.783a</td>
<td>0.33a</td>
</tr>
</tbody>
</table>

Note: Values are means of 10 plants for each treatment. Means within each column followed by same letter are not significantly different at P <0.05 based on Fisher’s protected least significance difference.
Table 6. Effects of seed treatments with USDA-BJ strains either alone or in combination with Heads-Up and Heads Up, on fresh and dry weights (g) of shoot and root in irrigated field in 2016 field tests. Means were calculated based on Fisher’s protected least significance difference.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FSW (g)</th>
<th>DSW (g)</th>
<th>FRW (g)</th>
<th>DRW (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA 30</td>
<td>10.85a</td>
<td>2.42a</td>
<td>1.86a</td>
<td>0.43a</td>
</tr>
<tr>
<td>USDA 31</td>
<td>9.24a</td>
<td>1.57a</td>
<td>1.89a</td>
<td>0.35a</td>
</tr>
<tr>
<td>Heads Up (HU)</td>
<td>7.93b</td>
<td>1.56a</td>
<td>1.57a</td>
<td>0.24a</td>
</tr>
<tr>
<td>USDA 30+HU</td>
<td>8.62a</td>
<td>1.97a</td>
<td>1.49a</td>
<td>0.28a</td>
</tr>
<tr>
<td>USDA 31+HU</td>
<td>9.66a</td>
<td>2.29a</td>
<td>2.14a</td>
<td>0.35a</td>
</tr>
<tr>
<td>USDA 110</td>
<td>8.07b</td>
<td>2.17a</td>
<td>1.41a</td>
<td>0.24a</td>
</tr>
<tr>
<td>Negative control</td>
<td>9.13a</td>
<td>2.12a</td>
<td>1.67a</td>
<td>0.39a</td>
</tr>
<tr>
<td>Positive control</td>
<td>8.15ab</td>
<td>2.1a</td>
<td>1.91a</td>
<td>0.41a</td>
</tr>
</tbody>
</table>

Note: Data presented the means of a sample size of 10 plants per plot. Means with the same letter(s) within each parameter are not significantly different according to Student’s t test at $P=0.05$
Table 7.
Effect of USDA strains either alone or in combination with Heads-Up and Heads-Up seed treatments on soybean yield (Kg/ha) under field condition at ISU Hinds Farm, Ames, Iowa in 2016 and 2017.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-irrigated</td>
<td>Irrigated</td>
</tr>
<tr>
<td>USDA 30</td>
<td>4251±31a</td>
<td>4081±181a</td>
</tr>
<tr>
<td>USDA 31</td>
<td>4461±157a</td>
<td>4079±133a</td>
</tr>
<tr>
<td>Heads Up (HU)</td>
<td>4494 ±162a</td>
<td>4038±111a</td>
</tr>
<tr>
<td>USDA 30+HU</td>
<td>4339±37a</td>
<td>3837±182a</td>
</tr>
<tr>
<td>USDA 31+HU</td>
<td>4264±245a</td>
<td>3957±181a</td>
</tr>
<tr>
<td>USDA 110</td>
<td>4230±189a</td>
<td>3984±46.82a</td>
</tr>
<tr>
<td>Negative control</td>
<td>4401±188a</td>
<td>3813±147a</td>
</tr>
<tr>
<td>Positive control</td>
<td>4362±138a</td>
<td>3994±107a</td>
</tr>
<tr>
<td>P value</td>
<td>0.87</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Note: Values are means and standard errors of 4 replicates (plots). Means within each column followed by same letter are not significantly different at P <0.05 based on Fisher’s protected least significance difference.
Fig. 1. Effects of Bradyrhizobium japonicum seed treatments on foliar soybean sudden death syndrome disease index in (A) non-irrigated and (B) irrigated trials in 2015. Foliar disease index (FDX) was calculated using the formula FDX = DI × DS/9 where, DI as percentage of diseased plants in plot; DS as visual estimation of leaf damage level in a plot scaled from 1 to 9.
Fig. 2. Effects of seed treatments with two *Bradyrhizobium japonicum* strains on foliar disease index in non-irrigated (A) and irrigated (B) trials in 2016. Soybean growth stages was recorded differently in each field. Foliar disease index (FDX) was calculated using the formula FDX = DI \times DS/9. (-) is negative control treatment; (+): positive control treatment.
Fig. 3 Weather data at Ames location (station AEEI4) during growing seasons 2015 (A), 2016 (B) and 2017 (C).
Supplemental Table S1.

Effects of seed treatments with USDA-BJ strains and Heads Up, on fresh and dry weights (g) of shoot and root in non-irrigated field in 2016 field tests. Means were calculated based on Fisher’s protected least significance difference. Data presented the means of a sample size of 10 plants per plot. Means with the same letter(s) within each parameter are not significantly different according to Student’s $t$ test at $P=0.05$.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FSW(g)</th>
<th>DSW(g)</th>
<th>FRW(g)</th>
<th>DRW(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA 30</td>
<td>6.8b</td>
<td>3.27b</td>
<td>1.45b</td>
<td>0.72a</td>
</tr>
<tr>
<td>USDA 31</td>
<td>7.0b</td>
<td>3.52b</td>
<td>1.63b</td>
<td>0.83a</td>
</tr>
<tr>
<td>Heads Up (HU)</td>
<td>7.7b</td>
<td>3.68b</td>
<td>1.68b</td>
<td>0.77a</td>
</tr>
<tr>
<td>USDA 30+HU</td>
<td>10.03ab</td>
<td>4.13ab</td>
<td>1.76ab</td>
<td>0.78a</td>
</tr>
<tr>
<td>USDA 31+HU</td>
<td>9.6ab</td>
<td>4.61ab</td>
<td>1.896ab</td>
<td>0.83a</td>
</tr>
<tr>
<td>USDA 110</td>
<td>10.3ab</td>
<td>3.25b</td>
<td>2.167a</td>
<td>0.98a</td>
</tr>
<tr>
<td>Negative control</td>
<td>10.1ab</td>
<td>3.96ab</td>
<td>1.778ab</td>
<td>0.75a</td>
</tr>
<tr>
<td>Positive control</td>
<td>12.17a</td>
<td>5.22a</td>
<td>2.22a</td>
<td>0.84a</td>
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</tbody>
</table>
Supplemental Table S2.

Effects of seed treatments with USDA-BJ strains and Heads Up, on fresh and dry weights (g) of shoot and root in irrigated field in 2016 field tests. Means were calculated based on Fisher’s protected least significance difference. Data presented the means of a sample size of 10 plants per plot. Means with the same letter(s) within each parameter are not significantly different according to Student’s t test at $P=0.05$.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FSW(g)</th>
<th>DSW(g)</th>
<th>FRW(g)</th>
<th>DRW(g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA 30</td>
<td>10.9a</td>
<td>2.38a</td>
<td>1.87a</td>
<td>0.73a</td>
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<tr>
<td>USDA 31</td>
<td>11.64a</td>
<td>2.79a</td>
<td>1.73a</td>
<td>0.70a</td>
</tr>
<tr>
<td>Heads Up (HU)</td>
<td>12.08a</td>
<td>3.07a</td>
<td>1.99a</td>
<td>0.7a</td>
</tr>
<tr>
<td>USDA 30+HU</td>
<td>12.82a</td>
<td>3.47a</td>
<td>2.07a</td>
<td>0.83a</td>
</tr>
<tr>
<td>USDA 31+HU</td>
<td>9.9a</td>
<td>3.12a</td>
<td>1.93a</td>
<td>0.8a</td>
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<tr>
<td>USDA 110</td>
<td>10.35a</td>
<td>2.89a</td>
<td>1.72a</td>
<td>0.79a</td>
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<tr>
<td>Negative control</td>
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<tr>
<td>Positive control</td>
<td>12.01a</td>
<td>3.03a</td>
<td>2.34a</td>
<td>0.82a</td>
</tr>
</tbody>
</table>
CHAPTER 5.

GENERAL CONCLUSIONS

Many studies have conducted on soybean sudden death syndrome management using different approaches such as crop rotation, SCN management, fungicide seed treatments. There is no information on using *Bradyrhizobium japonicum* on controlling SDS. The work contained in this dissertation investigated the effects of BJ seed treatments or inoculant on soybean SDS under controlled conditions and field environment.

Our results from experiments in controlled conditions showed that inoculum of *B. japonicum* applied either as seed treatments or soil inoculant reduced significantly SDS incidence and severity. Our results confirmed previous greenhouse observations suggesting high incidence and severity happened at early seedling stages. In addition, the effect of temperatures together with seed statuses (before or after germination) was investigated in soybean and SDS pathogen interaction to develop a better understanding of how this disease develops. We showed that a treatment with BJ can reduce SDS occurrence even with occurrence of lower temperatures. The BJ treatment did not improve plant biomass, suggesting that under controlled conditions treatments shared the same overwhelming pressure of SDS fungal pathogen inoculum. However, the higher nodule counts observed in BJ treated plants suggested an underlying mechanism of BJ on suppressing SDS. We concluded that treatment with BJ can be a management tool to protect early planted soybean from SDS.

Data from field experiments provides more evidence the effects of BJ treatment under field conditions. In both irrigated and non-irrigated field, BJ had significant effect on reducing
SDS index. Our results confirmed previous findings that SDS developed more severely in irrigation system or under high precipitation during reproductive stages. There were no differences on effect of BJ treated alone or BJ combined with Heads Up on reducing SDS. Soybean nodulation was detected higher in BJ plots in irrigated fields than non-irrigated fields while plant biomass showed non-significant differences. Yield increases were observed in BJ treated plots but not significantly different compared with untreated controlled.

To the best of our knowledge, the research presented in this dissertation is the first to investigate the effects of BJ on SDS occurrence and development. Findings from this study will be useful for researchers to better understand the impact of *Bradyrhizobium japonicum* on SDS disease development opened an avenue for SDS researchers to further investigate the use of BJ inoculum as a management strategy to control SDS.