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Efficacy of soybean seed treatments in Iowa and the effect of cold stress on damping-off caused by Pythium sylvaticum

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Efficacy of soybean seed treatments in Iowa and the effect of cold stress on damping-off caused by *Pythium sylvaticum*

by

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A dissertation submitted to the graduate faculty

in partial fulfillment of the requirement for the degree of

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ABSTRACT

Soybean (*Glycine max* (L.) Merr.) production is negatively affected by the occurrence of seedling diseases. In Iowa, the occurrence of seedling diseases is commonly associated with cool and wet weather soon after planting, and several *Pythium* spp. have been found as the predominant pathogens causing seedling disease. Increasing cost of the seed, risk of seedling diseases reducing stand in cold, wet soils, and the yield benefit sometimes provided by early planting have motivated an increase in the use of fungicide seed treatment. However, a positive effect of seed treatments on soybean plant stand and yield has not been clearly demonstrated in Iowa. This study was conducted to: (i) compare the effect of commercially available soybean seed treatments on plant stand and yield; (ii) assess the effect of cold stress at planting on the incidence of *Pythium* seedling disease and efficacy of a commercial seed treatment; and (iii) evaluate the effect of chilling temperatures after planting on the *Pythium* – soybean interaction.

Each year, three small plot field studies were conducted at different locations in Iowa in 2014, 2015 and 2016. Fifteen seed treatments were evaluated in 2014, and 13 seed treatments in 2015 and 2016. The experimental design was a randomized complete block with four replications. Seeding rate was 140,000 seeds per acre in 2014, and 120,000 seed per acre in 2015 and 2016. Periods of cool and wet weather soon after planting were observed in seven of nine trials within 4 to 7 days after planting. Enhancement of plant stand and yield by seed treatment was inconsistent among treatments and locations, even in trials where adverse weather conditions were observed soon after planting. Moreover, no bean leaf beetle
(Cerotoma trifurcate) injury was observed, low soybean cyst nematode (SCN) (Heterodera glycines) populations were measured at the trial sites, and sudden death syndrome (SDS) (Fusarium virguliforme) foliar disease index was in the trials was also low. Based on these findings, seed treatment may not be indispensable to achieve high plant stands in Iowa and should be regarded as a preventive control measure to protect soybean stand from seedling disease. Even though our results were inconclusive, as suggested by other authors farmers should consider the use of seed treatments in field has a history of SDS, SCN, or seedling disease particularly in early planting.

The effect of cold stress on efficacy of a seed treatment containing metalaxyl and ethaboxam (Intego Suite™) on soybean emergence was evaluated in a growth chamber experiment. A five-way factorial design with two cold stress temperatures (4°C and 10°C), two times for the initiation of cold stress (24 and 96 hours after planting), three cold stress durations (24, 48 and 96 hours), and two levels of seed treatments (Intego Suite™ and untreated) was conducted in cups inoculated with Pythium sylvaticum or a non-inoculated control. Emergence was reduced when the pathogen was present, and longer periods of cold stress further reduced emergence. No differences were observed between times for the initiation of cold stress. The cold stress had no effect on emergence when the pathogen was absent. Seed treatment protected soybean seedlings subjected to periods of cold stress. Seed treatment also resulted in improved shoot weight and reduced root rot severity. The results from this study demonstrated that seed treatment is a useful management
tool to protect seedlings from damping-off caused by *P. sylvaticum* when soybeans are planted under suboptimal temperatures.

To improve understanding of the effect of cold stress on *Pythium* damping-off, growth chamber and laboratory experiments were performed. A growth chamber study was conducted to test the effect of a 96-hour period of cold stress at different times after planting on soybean damping-off. The experimental design was a three way factorial with two cold stress temperatures (4°C and 10°C); seven levels of timing of the initiation of cold stress (no cold stress, 0, 1, 2, 4, 6, 8 days planting); and inoculation with *P. sylvaticum*-infested millet or sterile millet (non-inoculated control). Increased susceptibility to damping-off was observed when soybeans were subjected to cold stress. Emergence was assessed 21 days after planting and was particularly low when cold stress occurred 2 or 4 days after planting in inoculated cups. In the non-inoculated controls, no effect of cold stress on emergence was observed. Also, emergence and seedling growth was delayed when *P. sylvaticum* was present. In a laboratory experiment, mycelial growth of *P. sylvaticum* on diluted V8 media was assessed at different temperatures (4°C, 10°C and 18°C). Low temperatures delayed mycelial growth but the pathogen was still able to grow at 4°C. In another experiment, seed exudation was assessed by measuring electrical conductivity. Greater seed exudation was detected when soybean seeds were imbibed at 4°C compared to 10°C and 18°C. Sporangia of *P. sylvaticum* germinated in response to seed exudates, and sporangial germination increased when cultures were exposed to seed exudates from seeds imbibed at 4°C. These results demonstrate that the timing of cold stress during seed germination play an important
role in the occurrence of damping-off caused by *P. sylvaticum*. Moreover, low temperatures increase seed exudation and may enhance activity of *P. sylvaticum*.

The results from this study have improved our understanding of the soybean-*Pythium* interaction and conditions that favor disease development. These data will aid soybean farmers in Iowa in making their decisions whether or not to use a seed treatment.
CHAPTER 1.

GENERAL INTRODUCTION

Dissertation Organization

This dissertation is divided into five chapters. The first chapter includes a literature review, followed by a justification for the research conducted. The second chapter reports on a three-year field study evaluating the efficacy of commercially available seed treatments on plant stand and yield. The third chapter is a growth chamber study in which we evaluated the efficacy of soybean seed treatments on seedlings inoculated with *Pythium sylvaticum* and exposed to simulated periods of cold stress (chilling temperatures) soon after planting. The fourth chapter describes the effects of chilling temperatures at different times after planting on soybean emergence as well as the effect of chilling temperatures on soybean germination and pathogen growth. The last chapter presents the general conclusions for this dissertation.

Literature Review

**Soybean production in the United the States and Iowa**

Soybean is the most important oilseed crop produced and consumed in the world. The United States is the leading producer followed by Brazil, Argentina and China (www.fas.usda.gov/commodities/soybeans). Soybean (*Glycine max* (L.) Merr.) was introduced into the U.S. by Samuel Bowen in 1765; he obtained seed in China and planted them near Savannah, Georgia (Hymowitz and Shurtleff 2005). In 1804 Dr. James Mease coined the word “soybean” probably refering to the bean from
which soy sauce was produced (Hymowitz and Shurtleff 2005). Currently, soybean is produced extensively throughout the U.S. In 2016, a total of 83.4 million acres were planted in 31 states, including 9.5 million acres in Iowa (www.nass.usda.gov). In 2015, Iowa ranked first among the states producing soybeans in 2015 with 554 million bushels, equivalent to 14% of U.S. production (www.nass.usda.gov).

**Seedling diseases in U.S and Iowa**

Soybean is affected by more than 200 pathogens and at least 35 of them are economically important (Hartman et al. 2015). Seedling diseases were among the top three most damaging diseases across the U.S. from 2006 to 2009; it was estimated that 100 million bushels were lost in 2009 (Koenning and Wrather 2010). During this period (2006 to 2009) seedling diseases were observed in the both the northern and southern regions of the U.S. with the greatest yield losses reported in Illinois, Kansas, Minnesota, North Dakota, and Ohio (Koenning and Wrather 2010).

Seedling diseases are caused by *Pythium* spp., *Fusarium* spp., *Phythophthora sojae* and *Rhizoctonia solani*. All these pathogens are endemic to Iowa and cause seed rot, root rot, and seedling decay (Rizvi and Yang 1996; Arias et al. 2013; Matthiesen et al. 2016). *Pythium* spp. are the predominant pathogens causing seedling diseases in Iowa (Rizvi and Yang 1996; Murillo-Williams and Pedersen 2008); they have also been frequently isolated from diseased seedlings in recent surveys elsewhere in the North Central Region of the U.S. (Matthiesen et al. 2016; Rojas et al. 2017a; Rojas et al. 2017b). *Pythium sylvaticum* was the most frequently recovered species across the North Central Region of the U.S., while in
Iowa the most frequently recovered species was *P. oopapillum* (Rojas et al. 2017a; Rojas et al. 2017b). Other species such as *P. dissotocum*, *P. lutarium*, *P. sylvaticum* and *P. torulosum* were also frequently recovered in Iowa (Rojas et al. 2017a).

**Pythium spp. biology and phylogeny**

The genus *Pythium* was described in 1858 by Pringshame and placed in the Family Saprolegniaceae. In 1897, it was transferred to a new family, Pythiaceae, by Schroter (Hendrix and Campbell 1973). The genus *Pythium* is currently placed in the Family Pythiaceae, Order Peronosporales, and Phylum Oomycota in the Kingdom Chromista (=Stramenopila). Thus *Pythium* is an oomycete and distinct from true Fungi (Kingdom Eumycota) (Kendrick 2000). Oomycetes produce coenocytic hyphae that contain cellulose and β-glucans, and produce biflagellate zoospores that have one tinsel and one whiplash flagella (Kendrick 2000). The assimilative hyphae are diploid, and meiosis occurs in gametangia called oogonia (female) and antheridia (male) (Kendrick 2000; Schroeder et al. 2013). Although 116 species of *Pythium* that have been sequenced and identified (Lévesque and De Cock 2004), almost 300 species have been proposed in the literature (Schroeder et al. 2013).

*Pythium* is a polyphyletic genus, 11 clades have been identified based on phylogenetic analysis of the ITS region of ribosomal DNA (Cooke et al. 2000; Lévesque and De Cock 2004). Two major groups of clades that are phylogenetically separated according to the type of sporangia produced: Clades A to D produce filamentous sporangia, whereas most of the species in Clades E to J produces globose sporangia. The genus *Pythium* is considered polyphyletic because species
included in Clade K seems to be less related to the rest of the species in the genus *Pythium* (Lévesque and De Cock 2004). Sporangia type correlated with phylogenetic clades whereas other important characteristics such as ornamentation of oogonia and heterothallism did not correlate with clades and probably can be acquired or lost easily through evolution (Lévesque and De Cock 2004). Similar results were reported by Villa et al. (2006) who analyzed ITS regions as well as the coxII and beta-tubulin genes in isolates from 39 *Pythium* species and 9 *Phytophthora* species. Results revealed three major clades: Clade 1 grouped *Pythium* isolates with filamentous to lobulated sporangia; Clade 2 *Pythium* isolates produced globose to spherical sporangia; Clade 3 included *Phytophthora* isolates; and Clade 4 grouped *Pythium* species such as *P. undulata, P. helicoides, P. ostracodes, P. oedochilum* and *P. vexans* that were proposed to be intermediates in the *Pythium*-to-*Phytophthora* evolutionary line (Villa et al. 2006). Most of the species placed in Clade 4 by Villa et al. (2006) belong to the Clade K described previously by Lévesque and De Cock (2004).

**Seedling diseases caused by *Pythium* spp.**

*Pythium* spp. infect soybean at various growth stages, and cause seed rot, pre-emergence damping-off (death of seedlings prior to emergence), post emergence damping-off (death of seedlings after emergence) and root rot during advanced growth stages (Yang 1999). Seed rot occurs when seeds are attacked by the pathogen and the tissues become soft and mushy, turn brown and finally disintegrate (Agrios 2005). Symptoms of infection by *Pythium* include yellow to
brown lesions or general discoloration and rotting of the hypocotyls and roots (Hartman et al. 2015).

*Pythium* spp. cause severe symptoms, predominantly on germinating seeds and during early growth stages because they primarily infect young plant tissues since plants usually become more tolerant when they reach the V2 growth stage (Fehr et al. 1971; Yang 1997; Pedersen and Elbert 2004; Hartman et al. 2015). Secretome analysis revealed that *Pythium ultimum* is an opportunistic pathogen of young seedlings and infects roots that have no cuticle or heavily suberized tissue since it does not produce xylanase, pectin methylesterase or cutinase enzymes (Lévesque et al. 2010).

The effect on yield of rot root in advanced growth stages has not been precisely quantified, but occasionally root pruning and necrosis can cause stunting and wilting of soybean plants (Griffin 1990; Yang 1999; Hartman et al. 2015).

**Disease cycle of *Pythium* spp.**

*Pythium* spp. are soil inhabitants that live in plant debris as saprophytes or survive as oospores in soil (Yang 1999). The oospore is a thick-walled sexual spore that results from mating of antheridium and oogonium. Oospores germinate and produce a sporangium, which is the asexual spore within which zoospores are formed within a membrane-bound vesicle from where they are released in saturated soils. Zoospores swarm for a few minutes, round off to form a cyst and then germinate by producing a germ tube that penetrates host plant tissue (Agrios 2005; Hartman et al. 2015). In species such as *P. irregulare, P. ultimum* and *P. sylvaticum*
zoospores are not produced. Sporangia (also called hyphal swellings) are survival structures that germinate directly to infect plant tissues (Stanghellini and Hancock 1971b; Hartman et al. 2015). Oospores and sporangia are the primary inocula of these pathogens and the disease is considered monocyclic (Yang 1999). The pathogen grows fast, colonizing the tissue intercellularly and intracellularly and causing soft or wet rot. Saprophytic growth and parasitic activity leads to the production of more oospores and sporangia that will infect the host next season (Hartman et al. 2015).

**Pythium sylvaticum**

*Pythium sylvaticum* was described first by Campbell and Hendrix (1967a) and was the first heterothallic species described. Previously there had been no evidence of heterothallism in *Pythium* and the taxonomy of this genus relied on characteristics of oogonia and antheridia formation; however, an increasing number of described species lacked one of these structures or they developed too infrequently in culture, suggesting that heterothallism did occur (Hendrix and Campbell 1973). *Pythium sylvaticum* was the most frequently recovered species from forest and agricultural soils from all regions of U.S. (Campbell and Hendrix 1967a; Campbell and Hendrix 1967b; Hendrix and Campbell 1970). In a recent survey, *Pythium sylvaticum* was the most frequently recovered species from diseased soybean seedlings across the North Central Region of the U.S., and was recovered in 14% to 16% of the samples (Rojas et al. 2017b).
Colonies of *P. sylvaticum* on cornmeal agar have cottony aerial mycelium, intercalary or terminal sporangia (also called hyphal swellings) that germinate directly; sporangia are globose or limoniform (Campbell and Hendrix 1967a; Van der Plaats-Niterink 1981). *P. sylvaticum* sporangia germinate in response to seed exudates and volatile compounds (Nelson 1987; Nelson and Craft 1989). When referring to *Pythium* spp. asexual spores, some authors distinguish between sporangia (for zoospore producing species) and hyphal swellings (when zoospores are not produced) (Van der Plaats-Niterink 1981), whereas other authors use only the term sporangia (Campbell and Hendrix 1967a; Stanghellini and Hancock 1971b; Nelson 1987).

The optimum temperature for mycelial growth is 25ºC, but *P. sylvaticum* can also grow below 5ºC, and between 35ºC and 40ºC may stop growth (Van der Plaats-Niterink 1981). In a soybean seed rot assay Rojas et al. (2017b) determined higher aggressiveness of *P. sylvaticum* at 13ºC compared to 20ºC. On the other hand, Matthiesen et al. (2016) observed higher aggressiveness of *P. sylvaticum* at 23ºC and 18ºC in comparison to 13ºC in a seed rot assay and a root rot assay. This may be explained by the high variability within some species of *Pythium* (Schroeder et al. 2013). *P. sylvaticum* produces cell-wall degrading enzymes (polygalacturonase, polymethylgalacturonase and cellulase) and auxins (3-idoleacetic acid) that have been suggested to result in maceration of tissues, and abnormal and delayed root growth, respectively (Blok 1973; Posthumus 1973; Nemec 1974).
Conditions that favor occurrence of diseases caused by *Pythium* spp.

Emergence of soybeans is rapid and uniform when the soil temperature is above 18ºC, and planting is not recommended if soil temperatures are below 13ºC (Pedersen et al. 2004). In Iowa, the recommended planting date for northern Iowa is May 1st, and April 25th for central and southern Iowa to maximize soybean yield (De Bruin and Pedersen 2008). On these dates, soil temperatures average between 10.6 ºC and 14.8 ºC (De Bruin and Pedersen 2008). Moreover, soil temperatures often drop lower when cold fronts pass through the state (Robertson and Munkvold 2012).

Cool and wet soils are frequently associated with a higher occurrence of damping-off caused by *Pythium* spp. (Hartman et al. 2015). Low temperatures at planting keep seedlings at a susceptible stage for a longer period of time, providing greater chances for seedling infection (Martin and Loper 1999). In an experiment in a controlled environment, Thomson et al. (1971) demonstrated that periods of cold stress (4ºC) at planting increased soybean susceptibility to damping-off. Similarly, we observed in preliminary research that periods of cold stress after planting increased the susceptibility of soybean to *P. sylvaticum* and reduced emergence up to 70 % (Serrano and Robertson 2016).

*Pythium* sporangia germinate in response to seed exudates (Stanghellini and Hancock 1971a, b; Nelson and Craft 1989; Nelson 2004); under high soil moisture conditions, seedling infection is favored because of an increase in the size of the spermosphere and an increase in seed exudation (Kerr 1964; Stanghellini and Hancock 1971a). Furthermore, *Pythium* spp. are more tolerant of high levels of CO\(_2\) than many other soil microorganisms. As soil moisture increases, the concentration
of O₂ decreases and CO₂ increases, providing a low competition environment for

High disease incidence usually occurs on poorly drained soils with high clay
content and smaller pore sizes that promote water retention (Broders et al. 2009). In
flooded soybean fields, the frequency of recovery of \textit{Pythium} spp. from roots
increased compared to other filamentous microorganisms (Kirkpatrick et al. 2006). In
addition, flooding that occurred at emergence reduced plant stand, whereas flooding
that occurred at V4 stage increased root rot (Kirkpatrick et al. 2006).

Organic matter may affect the occurrence of diseases caused by \textit{Pythium}
spp. High levels of soil organic matter correlated with low disease incidence,
possibly because the organic matter favored a more competitive environment for
\textit{Pythium} species (Broders et al. 2009).

**Soybean germination at suboptimal temperatures**

Germination of soybean is very delayed at low temperatures. It has been
reported that 72 % of soybean seeds germinated after 36 hours at 23ºC, whereas at
10ºC, it took 144 hours for approximately 70 % of seed to germinate, and some
seeds never germinated (Duke et al. 1977). When soybean seeds were planted at 5
ºC and transferred to 25 ºC after 36 hours for 2 weeks, germination was reduced up
to 40 %, particularly when seed moisture was low since these conditions favored
imbibitional chilling injury (Hobbs and Obendorf 1972). Furthermore, exudation from
seeds increased when cold stress occurred, and in non-sterile soil, exudation
stimulated soilborne pathogenic organisms that further reduced seed germination (Hobbs and Obendorf 1972).

The vigor of all commercial soybean seed in the U.S. is tested prior to it being sold. Seed vigor involves a sum of properties that determines the performance of a seed lot in different environments, and includes the uniformity of seed germination and seedling growth, emergence ability under unfavorable environments and ability to germinate after storage (Powell 2006). The cold germination test is a standard test for seed vigor which subjects soybean seeds to 10°C for 7 days and 25°C for 7 days (AOSA 2009). The test reflects susceptibility to chilling injury and pathogen infections, and simulates wet and cold soils during early spring that may weaken and slow the activity of the germinating seed (AOSA 2009). Low vigor seeds show reduced emergence under adverse conditions and the cold germination test identifies problem seed lots, although soybean field emergence is not accurately estimated (Johnson and Wax 1978; Mason et al. 1982).

**Physical effects of cold stress on soybean seed and seedlings**

The optimal temperature for emergence of soybean seedlings ranges from 25°C to 35°C, whereas the optimal temperature for hypocotyl elongation is approximately at 30°C (Hatfield and Egli 1974). Cold stress occurs when the plant is subjected to suboptimal temperatures (usually less than 10°C) and restricts expression of the full genetic potential of the plant (Chinnusamy et al. 2007). Plants native to warm habitats, such as soybean, are often injured after exposure to low, nonfreezing temperatures and are thus considered chilling sensitive (Hopkins
Chilling injury results in physical damage because physiological and biochemical alterations are induced by suboptimal nonfreezing temperatures. Cell membranes are composed of a bi-layer of phospholipids in a liquid-crystalline state or the less fluid gel state. The relative proportion of saturated and unsaturated fatty acids determines fluidity of membranes and the transition temperature. Chilling-sensitive plants usually have higher proportion of saturated fatty acids and a correspondingly higher transition temperature, and when the plant is exposed to temperatures below the transition temperature, the phospholipids shift from liquid-crystalline to gel state (Hopkins 1999; Posmyk et al. 2005). After transition from liquid-crystalline to gel state the chilling-sensitive plants are not able to maintain membrane fluidity and it causes reduced integrity of membranes resulting in loss of compartmentation, solute leakage and impairment of other metabolic processes (Hopkins 1999).

Similarly, imbibitional chilling injury can occur during the seed imbibition process. Depending of the soybean cultivar imbibitional chilling injury can occur at temperatures between 7°C to 17°C (Bramlage et al. 1979) and is due to disruptive effects on the reorganization of cell membranes as water enters the dry seed (Leopold 1980; Bewley et al. 2013). Phospholipids may be in the less fluid gel state (dehydrated) or the liquid-crystalline state (hydrated) depending on the presence of water. When the seed imbibes water at chilling temperatures the membranes are not able to revert to the liquid crystalline state and consequently the disruption of membranes, leakage and cellular damage occurs (Bramlage et al. 1978; Bewley et al. 2013). Increased soybean leakage has been observed at temperatures below
15°C and is usually associated with reduction in germination and hypocotyl growth (Bramlage et al. 1978; Leopold 1980). High water uptake occurs within the first 35 minutes of the imbibition process and it is this time when the seed is particularly susceptible to chilling injury (Leopold and Musgrave 1979; Tully et al. 1981; Vertucci and Leopold 1983). Furthermore, soybean seeds with low seed moisture have been demonstrated to be more sensitive to chilling injury during imbibition (Obendorf and Hobbs 1970; Hobbs and Obendorf 1972).

The increased solute leakage as a result of chilling injury during imbibition can be quantitatively measured using the electrical conductivity test (Leopold 1980). The conductivity of seed soak water is increased by electrolytes leaked from the seeds such as inorganic ions, proteins and organic acids (AOSA 2009). Thus, the measured conductivity reflects membrane integrity since disorganized membranes fail to provide selective permeability and impair the membrane’s ability to function as a barrier to cellular solute leakage (AOSA 2009).

Chilling temperatures also affect respiration. The respiratory rate of soybean seed was reduced more than 60% when subjected to 15 minutes of chilling treatment during the initial stages of imbibition (Leopold and Musgrave 1979). This reduction in respiration resulted from a decline in the cytochrome oxidase pathway within the mitochondria and engagement of the alternative pathway (Leopold and Musgrave 1979; Purvis and Shewfelt 1993). Once the seed has germinated, electron microscopy studies have revealed that mitochondrial structures may also be damaged when soybean radicles were exposed to chilling temperatures (Chabot and Leopold 1985). Moreover, malfunction of the electron transport chain in the
mitochondria as a result of chilling temperatures can enhance the production of reactive oxygen species (ROS) that further damage cell membranes (Purvis and Shewfelt 1993).

Other detrimental effects of chilling temperatures on young seedlings include reduced leaf expansion, wilting and chlorosis (Hopkins 1999). Soybean hypocotyl growth was extremely low or abnormal at 10ºC (Hatfield and Egli 1974; Duke et al. 1977). Moreover, some undesirable effects of chilling injury are evident only after the plants have been transferred to non-chilling temperatures. When soybean seedlings were transferred from chilling to warm conditions, post-chilling oxidative stress reduced root and hypocotyl growth at warm temperatures (Posmyk et al. 2005).

**Soybean seed treatments for seedling diseases**

Managing seedling diseases may include the use of crop protection pesticides. However, due to environmental concerns the use of seed treatments is preferred over soil applications of pesticides (Munkvold 2009). The use of seed treatments on soybean in the U.S. has increased considerably over the past few decades. It is estimated that 8% of the soybean seed were treated in 1996, compared to 30% in 2008 and it is likely to continue increasing (Munkvold 2009).

One reason for the increase in the use of seed treatments is the increasing cost of the seed that motivates farmers to apply a seed treatment to protect their investment (Basol et al. 2013; Plastina 2016). According to USDA estimations between 2001 and 2009 soybean seed prices increased 108 % (Neuman 2010). This increase matches with a higher use of genetically engineered seed which
comes with a higher price tag. More than 85% of both crops were grown from genetically engineered seed in 2010. The seed-producing companies have claimed that the increasing seed price is justified because the quality of the seed is higher, and new biotech traits have been added that enable farmers to save money due to higher efficiency in cropping operations such as weed control and reduced chemical spraying for pest control (Neuman 2010).

Commercial seed treatments usually contain at least two fungicides each with a different mode of action that against targeted soilborne fungi (Fusarium and Rhizoctonia species) and oomycetes (Pythium and Phytophthora species) (Mueller et al. 2013). Phenylamide fungicides, such as metalaxyl and mefenoxam, inhibit RNA synthesis (FRAC 2016) and are commonly applied for control of Pythium and Phytophthora species (Dorrance and McClure 2001; Dorrance et al. 2004; Hartman et al. 2015). Metalaxyl contains the R and S enantiomers of metalaxyl whereas mefenoxam contains only the R form, which is more active and consequently provides the same efficacy at half the application rate (Monkiedje and Spiteller 2005). For fungal pathogens such as Fusarium and Rhizoctonia species, phenylpyrrol (e.g. fludioxonil) and DMI fungicides (e.g. Ipconazole and prothioconazole) are used (Dorrance et al. 2003; Ellis et al. 2011; FRAC 2016; Ajayi-Oyetunde et al. 2017) The phenylpyrrol fungicide fludioxonil was originally synthesized from pyrrolnitrin, an antibiotic compound produced by Pseudomonas pyrrocinia (Paranjape et al. 2014), which interferes with the signal transduction involved in fungal response to osmotic stress (Zhang et al. 2002). Demethylation inhibitor (DMI) fungicides interfere with the biosynthesis of ergosterol, which is a
component of fungal cell membranes (Siegel 1981; FRAC 2016). Another fungicide class that is common in contemporary seed treatments is the strobilurins which are quinone outside inhibitor (QoI) fungicides that interfere with respiration at cytochrome b of the complex III in the inner mitochondrial membrane (Bartlett et al. 2002). Strobilurins such as pyraclostrobin and trifloxystrobin provide some control against *Fusarium graminearum*, but their efficacy is lower than fludioxonil (Ellis et al. 2011). Mycelial growth of *Pythium* and *Phytophthora* species is also affected by strobilurin fungicides but oomycetes are usually less sensitive to strobilurins than to metalaxyl (Broders et al. 2007b; Matthiesen et al. 2016; Radmer et al. 2017).

Some species and isolates of fungi and oomycetes may have reduced sensitivity to fungicides; therefore, the use of a fungicide does not guarantee complete protection for all the *Pythium* or *Fusarium* species prevalent at each specific location. Phenylamide and QoI fungicides belong to groups at high risk for fungicide resistance (FRAC 2016) and *Pythium* isolates with reduced sensitivity have been reported (Dorrance et al. 2004; Broders et al. 2007b). Broders et al. (2007b) observed isolates of various species of *Pythium* that had reduced sensitivity to mefenoxam, azoxystrobin and trifloxystrobin; thus, individual use of any of these fungicides may not protect seedlings from all *Pythium* populations present in Ohio soils. In addition, Matthiesen et al. (2016) reported that *Pythium* species from Iowa showed reduced sensitivity to fungicides in a temperature dependent manner. Reduced sensitivity to fludioxonil and ipconazol in four *Fusarium* species from Iowa and surrounding states has also been reported (Cruz et al. 2014); moreover, these fungicides are differentially effective against various species. Fludioxonil inhibited *F.*
graminearum more than ipconazole, whereas ipconazole was more effective against 
F. oxysporum, F. acuminatum and F. solani than F. graminearum (Cruz et al. 2014). 
Fusarium graminearum isolates with reduced sensitivity to fludioxonil have been 
reported (Broders et al. 2007a).

Recently, active ingredients belonging to the succinate dehydrogenase inhibitor fungicide (SDHI) group have been labeled for use as soybean seed treatments. The target of this group of fungicides is the succinate dehydrogenase complex in the respiratory chain, also known as complex II in the mitochondrial electron transport system. SDHI fungicides block the electron transport at complex II and consequently interrupt fungal respiration (Avenot and Michailides 2010). Penflufen and sedaxane are SHDI fungicides and have shown high efficacy for Rhizoctonia control (Ajayi-Oyetunde et al. 2017). Fluopyram is an SDHI fungicide that is highly effective against Fusarium virguliforme, the causal agent of sudden death syndrome (Kandel et al. 2016a).

Ethaboxam, a thiazole carboxamide fungicide that interferes with microtubule assembly in Oomycetes and has efficacy against Pythium and Phytophthora species (Uchida et al. 2005; Dorrance et al. 2012), was recently registered on soybean.

In recent years, seed treatments have also provided an opportunity to protect against yield losses caused by two other economically important pathogens in Iowa and the Midwest: soybean cyst nematode, caused by Heterodera glycines (Ichinohe), and sudden death syndrome caused by Fusarium virguliforme O'Donnell and Aoki (Aoki et al. 2003). Thus there is further the interest in using soybean seed treatments to manage these pathogens (Zaworski 2014; Beeman and Tylka 2015;
Soybean cyst nematode is considered the most important cause of yield loss in the U.S. (Koenning and Wrather 2010). The nematode is a parasite of soybean plants. Juveniles penetrates and feed on soybean roots, and females produce and egg mass in a gelatinous matrix. When the dead adult changes to a darker color it is referred as a cyst, which is filled with eggs that may be viable for more than 10 years (Niblack et al. 2006). Significant yield loss can occur with no visually detectable symptoms on the plant (Wang et al. 2003). Sudden death syndrome was ranked fourth among the causes of soybean yield loss in the U.S. from 2006 to 2009 (Koenning and Wrather 2010). *F. virguliforme* colonizes soybean root tissues, producing brown discoloration of vascular tissue and leaf interveinal chlorosis. Symptoms in the leaves may advance, producing necrosis and premature defoliation with a consequent reduction in yield (Navi and Yang 2016).

**Efficacy of seed treatments**

The efficacy of soybean seed treatments in controlled environments has been demonstrated consistently for control of pathogens causing seedling disease such as *Phytophthora sojae* (Dorrance and McClure 2001), *Rhizoctonia solani* (Dorrance et al. 2003), *Fusarium graminearum* (Ellis et al. 2011), *Pythium* spp. and *Pythium sylvaticum* (Urrea et al. 2013; Serrano and Robertson 2016). In field trials, while seed treatments have been demonstrated to protect yield, their effect on yield is often negligible. In North Dakota, seed treatments protected stand when cool and wet conditions occurred at planting, but there was no yield improvement with the use
of seed treatments in years with low precipitation and warm temperatures at planting (Bradley 2008). Similarly, in a multi-state field experiment, Dorrance et al. (2009) detected efficacy of seed treatments in field trials in Ohio, South Dakota, and Ontario when heavy precipitation occurred within two weeks after planting, but no effect of seed treatment was detected at Iowa, Nebraska, Wisconsin and Ohio where no heavy precipitation occurred during the planting season. In Wisconsin Esker and Conley (2012) detected an increase in plant stand and yield with the use of seed treatments when soybeans were planted early, but an interaction of soybean cultivar with location affected the economic benefit in the use of seed treatment at each location.

Field trials in Iowa that evaluated commercially available seed treatments from 2010 through 2012 were inconclusive regarding the economic benefit of seed treatments in Iowa. Although a few seed treatments protected stand or improved yield, there was variation among products, locations and years (Robertson et al. 2012; Robertson et al. 2013). Since seed treatments are an added expense to production, most growers would like to see a return on investment in terms of yield. Seed treatments however, can also be considered as insurance to reduce the risk of re-planting, which adds to production costs. It is possible to reduce economic risk and increase profit with the use of seed treatments. However, to maximize profit farmers may need to consider lowering their seeding rates (Gaspar et al. 2015). Historically, seeding rates for soybean have ranged from 180,000 to 240,000 seeds per acre (Basol et al. 2013). In some environments, the economically optimal
seeding rate (based on yield, grain sale price, seed cost, and seed treatment cost) may be as low as 94000 seed per acre (Gaspar et al. 2015).

**Justification**

Soybean production is negatively affected by the occurrence of seedling diseases. The increasing cost of the seed, the yield benefit of early planting and the risk of seedling diseases are factors that should be considered when deciding whether to use a seed treatment. Nevertheless the benefit of the use of seed treatments on plant stand and yield has not been clearly determined in Iowa. Damping-off is predominantly caused by *Pythium* spp. in Iowa and *P. sylvaticum* is the most prevalent species in the North Central Region of the U.S. Higher disease incidence is associated with periods of cool and wet weather however it is not well understood how periods of chilling temperature affect the soybean plant and pathogen and result in increased disease incidence. Furthermore, the efficacy of seed treatments protecting seedlings subjected to periods of cold stress is unknown. To address these questions regarding the occurrence of seedling diseases caused by *Pythium* spp. in Iowa and the use of seed treatments the objectives of this research project were to:

(i) Compare the effect of commercially available soybean seed treatments on plant stand and yield.

(ii) Assess the effect of cold stress at planting on the incidence of *Pythium* damping-off and the efficacy of commercial seed treatments.
(iii) Evaluate the effect of chilling temperatures on the *Pythium* – soybean interaction.

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CHAPTER 2.
EFFECT OF COMMERCIAL SOYBEAN SEED TREATMENTS ON PLANT STAND 
AND YIELD IN IOWA

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Abstract

The use of fungicide seed treatments on soybean in Iowa has increased considerably over the past decade but their impact on early plant stand and yield is not clear. A three-year small-plot field trial was conducted at three locations in Iowa in 2014, 2015 and 2016 to evaluate the effect of commercial soybean seed treatments on plant stand and yield. Fifteen seed treatments were evaluated in 2014, and 13 seed treatments in 2015 and 2016, in a randomized complete block design with 4 replications. Seeding rate was 140,000 seeds per acre (56,656 seed per hectare) in 2014, and 120,000 seed per acre (48,562 seed per hectare) in 2015 and 2016. Data were collected for plant stand, presence of bean leaf beetle (BLB) 35 days after planting, soybean cyst nematode (SCN) (eggs/100 cm$^3$) at planting and harvest, foliar symptoms of sudden death syndrome (SDS) at R6 growth stage

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and yield data. Efficacy of seed treatments in improving stand was inconsistent among treatments and locations. Of the 123 treatment-site-years evaluated over 3 years, only two of them significantly improved yield. No BLB injury was observed, and pressure from SCN and SDS was low. The results from this study suggest that with seeding rates of 120,000 to 140,000 seed per acre, small reductions in plant stand were compensated by soybean and no effect on yield was observed. Unless the field has a history of seedling disease, SDS or SCN, the use of seed treatments may not result in improved yield in Iowa.

Introduction

In 2016 soybean (\textit{Glycine max} (L.) Merr.) was, 9.5 million acres were planted to soybean and the crop was worth $9.2 million dollars to the state. \url{www.nass.usda.gov}.

Numerous factors may affect soybean emergence including soil crusting, herbicide injury, flooding, soil compaction, insect damage and seedling disease (damping-off) (Rathore et al. 1981; Bradley et al. 2002; Kirkpatrick et al. 2006; Hyatt et al. 2007; Bradley 2008). Damping-off was ranked as the 2\textsuperscript{nd} to 4\textsuperscript{th} most destructive disease of soybean in 12 states of northern U.S. from 2010 to 2014 (Allen et al. 2017). Several species of \textit{Pythium} and \textit{Fusarium}, as well as \textit{Phythophthora sojae} and \textit{Rhizoctonia solani} have been associated with damped-off soybean seedlings in Iowa (Rizvi and Yang 1996; Dorrance et al. 2009; Arias et al. 2013; Matthiesen et al. 2016; Rojas et al. 2017). Damping-off of soybean seedlings often occurs when soil
conditions are cool (<15°C) and wet (Hartman et al. 2015). Fungicide seed treatments are recommended for control of seedling diseases (Munkvold 2009).

For optimal emergence, soybeans are planted when soil temperatures reach 18°C (65°F), and planting is not recommended when temperatures are <13°C (55°F) (Pedersen et al. 2004). Early planting maximizes yield but also increases the chances of suboptimal temperatures at planting (De Bruin and Pedersen 2008). In Iowa, cold fronts are common during planting and soil temperatures often fluctuate between 4°C (40°F) and 18°C (65°F) with extreme precipitation events (> 25 mm of rain) that occur over a few days (Serrano et al. 2015). Consequently, emerging seedlings may be at risk for infection by soilborne pathogens (Robertson and Munkvold 2012).

Bean leaf beetle *Cerotoma trifurcata* (Förster) (Coleoptera: Chrysomelidae) may affect plant stand because adults and larvae injure soybean seedlings by feeding on roots, leaves and stem (Higley and Boethel 1994). Moreover, BLB is a vector of *Bean pod mottle virus* (BPMV) (Giesler et al. 2002). During the spring, overwintering adults move from wild hosts and colonize soybeans in early vegetative stages. Greater colonization has been observed in early planted soybeans. The use of neonicotinoid insecticide seed treatments have been shown to reduce BLB colonization and incidence of *Bean pod mottle virus* (Bradshaw et al. 2008).

Two pathogens that do not affect plant stand but impact yield loss are the soybean cyst nematode (SCN) (*Heterodera glycines* (Ichinohe)) and sudden death syndrome (SDS) caused by *Fusarium virguliforme* (O’Donnell and Aoki). SCN, the most important cause of yield loss in the US and is prevalent in Iowa and many other
soybean producing states (Tylka and Marett 2014; Allen et al. 2017). This nematode paratizes soybean roots, and significant yield reduction can occur without readily detectable symptoms (Wang et al. 2003). Crop rotation, the use of resistant varieties, and more recently seed treatment have been recommended to suppress SCN-incited yield loss (Niblack 2005; Zaworski 2014; Tylka et al. 2015). SDS was ranked between the second and fifth most important cause of soybean yield loss in 12 northern states U.S. states from 2010 to 2014 (Allen et al. 2017). This pathogen infects soybean roots soon after planting and produces brown discoloration of vascular tissue, interveinal chlorosis of leaves that progresses to necrosis, and premature defoliation with a consequent reduction in yield (Navi and Yang 2016).

To reduce the risk of stand loss due to pests and pathogens, soybean seed is often treated with various pesticides. Seed treatments are a rapidly growing market in Iowa and the Midwest. It is estimated that approximately 8% of soybean seed was treated in 1996, whereas 30% of seed was treated in 2008 (Munkvold 2009). The increasing cost of soybean seed may motivates farmers to apply a seed treatment to protect their investment (Basol et al. 2013; Gaspar et al. 2015; Plastina 2016). Seed treatment usually include fungicides, an insecticide and more recently a nematicide. The fungicides protect the germinating seed against fungal and oomycete pathogens; the insecticide is active against early season pests, such as the BLB, and the nematicide protects against SCN.

Soybean seed treatments protected plant stand and yield when cool and wet conditions occurred at planting; however when precipitation was low and soil temperatures were above 13°C, seed treatments had no effect on stand (Bradley
These data suggest the environment, soybean cultivar, inoculum level and prevalent pathogen complex influence the benefits of seed treatments (Dorrance et al. 2009; Esker and Conley 2012). In Iowa, the benefit of soybean seed treatments to farmers is not well understood, although grower interest in new seed treatments for control of SCN and SDS is increasing.

In this study we report the results of field experiments at three locations of Iowa over three years to evaluate the effect of commercially available soybean seed treatments on stand and yield. Our objectives were to (i) determine if commercial seed treatments protected plant stand and contributed to greater yields; (ii) evaluate the effect of seed treatments that include a nematicide on the population of SCN; and (iii) evaluate the effect of commercial seed treatments on SDS.

**Materials and Methods**

In 2014, 2015 and 2016, field experiments were planted annually at three locations in Iowa (Table 1). The experimental design was a randomized complete block with four replications. Plot sizes were 10 feet wide (3.05 m) by 17.5 feet (5.33 m). Each plot had 4 rows spaced 30 inch (76 cm) apart. The seeding rate was 140,000 seed per acre (56,656 seed per hectare) in 2014, and was reduced to 120,000 seed per acre (48,562 seed per hectare) in 2015 and 2016 to increase the chances of detect an effect of seedling diseases on yield. Seed treatments for each year are indicated in Table 2. Seed treatments varied among years and are representative of the treatments that were sold by the agrochemical companies in
the year they were tested. Seed was sent to each participating for application of the seed treatment, and then treated seed was shipped back to Iowa State University for planting.

In each trial, soil temperature (°C), soil moisture (percent water content), air temperature (°C) and air moisture (%) data were recorded using a WachtDog 1000 Series data logger (Spectrum Technologies Inc, Aurora, IL). Daily precipitation data were obtained from the nearest weather station.

Stand count data in the central two rows of each plot were collected 35 days after planting at growth stage V1 to V3 (Fehr et al. 1971; Pedersen et al. 2004). Insect incidence was also assessed in the center rows by counting the number of adult bean leaf beetles at growth stage V1 to V3 (35 days after planting) (Bradshaw et al. 2008). SCN populations were assessed in plots of seed treatments that contained a nematicide, as well as the untreated control. Soil cores were collected within 7 days of planting and within 7 days after harvest. For each plot, 10 soil cores were collected at arbitrary locations on either side of the center two rows of each plot and combined in one plastic bag to represent a single sample (Beeman et al. 2016). Soil samples were processed using standard methods (Niblack et al. 1993; Wang et al. 2003) and the population of SCN in each soil sample was determined by counting the number of eggs per 100 cm$^3$ of soil. The Reproductive Factor (RF) was calculated by dividing the final SCN population density by the average initial SCN population. In 2015 and 2016, SDS severity was assessed in the center two rows of each plots when the plants approached growth stage R6 using a Foliar Disease Index (FDX) scale outlined in (Njiti et al. 1996). Foliar disease index (FDX) is
calculated as $FDX = DI \times DS/9$ where $DI$ (disease incidence) is the percent of plants in the plot with visible leaf symptoms and $DS$ (disease severity) is the severity of disease on a 1 to 9 scale where 1 = 0 to 10% chlorosis or 1 to 5% necrosis, and 9 = premature death of plant. Foliar disease indices can range from 0 (no disease) to 100 (all plants prematurely dead at or before R6). The center two rows of each plot were harvested at full maturity (R8) using an Almaco Plot Combine Harvester (Nevada, Iowa, USA), and yield data were adjusted to 13 % moisture.

Analysis of variance was performed using PROC GLIMMIX and LSD test at alpha = 0.1 in SAS (version 9.3; SAS Institute Inc.) for plant stand, yield, and SDS disease index; PROC GLM and LSD test was performed at alpha = 0.1 for SCN reproductive factor.

**Results**

**Weather data.** Planting dates varied across years from very early at all locations in 2015 to late at Nashua and Crawfordsville in 2014 and close to recommended planting dates in 2016 (Table 1). Soil temperatures at planting also varied considerably (Table 1, Figures 1, 2 and 3). In 2015, soil temperatures at planting were the coolest, 10°C, 10°C and 13°C (55°F) at Nashua, Kanawha and Crawfordsville, respectively and they dropped to less than 5°C 4 to 5 days after planting when more than 30 mm of precipitation occurred (Figure 2).

**Plant stand and yield.** In 2014 an effect of seed treatment on stand count was detected at Crawfordsville ($P = 0.0597$) and seed treatment resulted in lower stand than the untreated control. No differences in yield were detected amongst treatments at this location. At Nashua, no differences among treatments were
detected in plant stand but there were differences in yield (P=0.0951). One treatment increased and one treatment decreased yield at this location (Table 3).

In 2015 we detected lower stand at Nashua and higher stand at Crawfordsville with the use of several seed treatments (P = 0.0222 and P = 0.0323, respectively). No effect of seed treatments on yield was observed at any of the locations (Table 4).

In 2016 no effect of seed treatment on plant stand was observed at any of the three locations. In Kanawha, two seed treatments improved yield (P = 0.0330; Table 5).

The mean yield response of all the seed treatments from 9 trials presented in this 3-year study was 0.5 bu/ac and the frequency of a positive yield difference was 62%. Of 123 seed-treatment-site-year evaluated, a significant yield increase was detected in only 2 products (Figure 4).

**Soybean cyst nematode.** In 2014 an effect of seed treatments on SCN populations was detected at Roland (P = 0.0753; Table 6). Four seed treatments reduced SCN populations although two did not include a nematicide. In 2015 and 2016, initial SCN populations were between 338 and 71 egg per 100cm³ soil, and no significant effect of seed treatments was observed.

**Sudden death syndrome.** Disease pressure was low at all locations in all years and no effect of seed treatment was observed. In some locations the disease was not observed at all (Tables 7 and 8).

**Bean leaf beetle.** No insect feeding damage was observed in any of the trials and consequently no bean leaf beetle data were collected.
Discussion

No product consistently protected stand or resulted in a yield improvement over the 9 site years. Furthermore, we did not observe a consistent improvement of seed treatments on plant stand and yield. Our data are similar to previous research from Iowa that reported seed treatments had minimal effect on soybean stands and yield (Dorrance et al. 2009; Robertson et al. 2012; Robertson et al. 2013; Gaspar et al. 2017).

Reductions in plant stand may not affect yield because soybeans are able to compensate for a small decrease in population with the production of new branches, pods, and seeds (Carpenter and Board 1997a, b; Cox and Cherney 2011). In a highly productive environment, 95% of the maximum yield can be achieved with 75,000 plants per acre at harvest (185 300 plants per Ha) (De Bruin and Pedersen 2008; Basol et al. 2013). In our trials, despite sowing at comparatively low populations (120,000 to 140,000 seed per acre), we were rarely observed reduced plant stands and emerged plants compensated for any small decreases in stand.

In 7 site years, periods of cold stress and precipitation occurred soon after planting, yet we rarely detected an effect of seed treatment on stand count or yield response. Preliminary research suggested that soybean seedlings were more susceptible to infection by Pythium after a period of cold stress that occurred 1 day after planting (Serrano and Robertson 2016). In many of our field trials we observed suboptimal soil temperatures for soybean germination, accompanied by rain within 4 to 7 days after planting but with no significant reductions in plant stand. Our data
differ from Bradley (2008) who determined a positive effect on stand and yield at 4 of 8 locations in North Dakota when high rainfall and soil temperatures below 15ºC (50F) at planting in all locations. Similarly, Dorrance et al. (2009) reported the occurrence of heavy rains within 1 or 2 weeks after planting, increased the occurrence of seedling diseases. Further research is required to determine whether there is a differential effect of the time when precipitation and cold stress occurs.

Since cold, wet conditions have been reported to increase seedling disease, in 2015 our trials at all three locations were planted 1 to 2 weeks before the recommended planting dates for Iowa. Nevertheless, we did not observe yield increase with the use of seed treatments. Esker and Conley (2012) found a positive yield response with the use of seed treatments when soybeans were planted early, although not at all locations or over all varieties.

*Pythium* spp. are the predominant pathogens associated with seedling diseases in Iowa (Rizvi and Yang 1996; Murillo-Williams and Pedersen 2008; Rojas et al. 2017). Poorly drained soils with high clay content and flooded soils are favorable for the occurrence of *Pythium* damping-off (Martin and Loper 1999; Kirkpatrick et al. 2006; Broders et al. 2009). Despite the fact that our trials at Crawfordsville, Roland, and Kanawha were planted in poorly drained clay loam soils with periods of high rainfall after planting, we did not observe damped-off seedlings in our trials. Other factors prevalent in Iowa soils such as high soil organic matter content and competing microorganisms, may reduce the development of disease compared to regions with dissimilar soils. For example, high levels of soil organic matter has been reported to correlate with low disease incidence, possibly by
providing a competitive environment for *Pythium* spp. (Broders et al. 2009). Our trials were planted in Mollisols that represent 67% of the soils in Iowa; these soils have high soil organic matter content in the thick, dark-colored, humus and base-rich surface horizon (mollic epipedon) (Liu et al. 2012; ISPAID 2013). Furthermore, low inoculum levels of the pathogen in our trials may explain the inconsistent effect of seed treatments on plant stand. Further research is required to determine whether field inoculum levels correlate with the probability of a positive response of the seed treatment.

Historically, seeding rates for soybean have ranged from 180,000 to 240,000 seeds per acre (Basol et al. 2013). Increased cost of the seed, however, has motivated farmers to reduce seeding rates and apply seed treatment to protect their investment and prevent replanting costs (Basol et al. 2013; Plastina 2016). In addition to increased production costs, replanting also results in significant loss of yield potential because of the delayed planting date (Whigham et al. 2000). In Wisconsin, Gaspar et al. (2015) reported that seeding rates can be reduced with the use of a seed treatment (CruiserMaxx®) and that the economically optimal seeding rate (based on yield, grain sale price, seed cost, and seed treatment cost) may be as low as 94,000 seed per acre (Gaspar et al. 2015). In a similar multistate study in Wisconsin, Iowa, Indiana, Michigan, and Ontario, Gaspar et al. (2017) found seed rates could be reduced to approximately 104,000 seeds per acre with the use of ILeVO (contains fluopyram for SDS control) in fields with prevalence of SDS. Interestingly, the control base seed treatment (No-ILeVO) had higher yield than the untreated control in all locations except Iowa (Gaspar et al. 2017). This corresponds
with previous reports of minimal effect of seed treatments on yield in Iowa (Dorrance et al. 2009; Robertson et al. 2012; Robertson et al. 2013), however when SDS is present yield benefit is likely to be observed using ILeVO (Kandel et al. 2016b; Gaspar et al. 2017).

Many soybean seed treatments contain an insecticide and in some cases a nematicide or fungicide for specific targets. In our trials the SDS disease pressure was low and therefore it was difficult to compare our results with those reported by others. For example, field trials with ILeVO®, which has efficacy against both SDS and SCN (Zaworski 2014; Kandel et al. 2016b), showed a yield increase between 7% to 20% compared to the control base seed treatment in SDS-infested fields in Iowa and Wisconsin (Kandel et al. 2016a; Gaspar et al. 2017). In addition, in most of our trials the initial SCN population was low and no effect of nematicide seed treatments was detected. A reduction in SCN population was observed in 2014 at Roland, even with the use of no-nematicide seed treatments. Flooding is detrimental for nematodes and we hypothesize the heavy rains and waterlogged soils observed in June 2014 affected SCN population (Figure 5) (Trivedi and Barker 1986).

We did not observe BLB injury in our trials even in our non-treated control plots. In Iowa, high mortality of overwintering BLB adults was predicted for 2014 and 2015 because of extremely low temperatures during the winter (Hodgson and Sisson 2014). In 2016 predicted mortality was lower than 2014 and 2015 (Hodgson and Sisson 2016), but precipitation and low temperatures observed within 2 weeks after planting likely affected activity of BLB (Lam et al. 2001). Consequently, weather conditions throughout winter and at planting probably reduced the pressure of insect
pests in our trials, and we were not able to detect an effect of seed treatment on BLB damage. Field trials in Wisconsin showed yield improvement with the use of an insecticide-fungicide seed treatment (CruiserMaxx) in comparison with a fungicide (ApronMaxx) only (Gaspar et al. 2015).

Over the 9 site years of this study, we did not observe an effect of seed treatment on plant stand or yield, even when the trials were seeded at lower than recommended seeding rates, planted 10 days to 2 weeks before recommended planting dates, and when cool and wet temperatures were observed soon after planting. Consequently, soybean seed treatments in Iowa may play more of a role as a preventive control measure against replanting rather than increasing yield, except when SDS is present in the field. The disease history of the field is key when making a seed treatment decision, particularly if damping-off or SDS have been observed in previous seasons. Furthermore, farmers may consider applying a seed treatment and reducing seeding rates to increase profit as suggested by Gaspar et al. (2015). Based on our research, we do not discourage the use of a seed treatment in Iowa if adverse weather conditions are expected soon after planting and/or the fields have a history of damping-off. A clearer understanding of the interaction between the environment, specifically the occurrence of periods of rain and cold temperatures after planting, inoculum levels and soybean genotype is needed to identify conditions under which seed treatments may be beneficial. In addition, the study of the role of soil organic matter and communities of microorganisms prevalent in Iowa soils may provide additional details about the occurrence of seedling diseases and the need for seed treatments.
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Table 1. Description of field experiments evaluating commercial seed treatments in Iowa in 2014, 2015 and 2016.

<table>
<thead>
<tr>
<th>Year</th>
<th>Location</th>
<th>Coordinates</th>
<th>Soil&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Thickness of mollic epipedon (cm)&lt;sup&gt;y&lt;/sup&gt;</th>
<th>Clay (%)&lt;sup&gt;y&lt;/sup&gt;</th>
<th>Soybean variety</th>
<th>Maturity group</th>
<th>Planting</th>
<th>Harvest</th>
</tr>
</thead>
<tbody>
<tr>
<td>2014</td>
<td>Nashua</td>
<td>42°56'8.66&quot;N 92°34'20.61&quot;W</td>
<td>Kenyon loam</td>
<td>25-49</td>
<td>20-30</td>
<td>IA 2094</td>
<td>2.4</td>
<td>May 20</td>
<td>Oct 10</td>
</tr>
<tr>
<td></td>
<td>Roland</td>
<td>42° 7'59.79&quot;N 93°30'4.19&quot;W</td>
<td>Okoboji silty clay loam</td>
<td>60-150</td>
<td>35-40</td>
<td>IA 2094</td>
<td>2.4</td>
<td>May 9</td>
<td>Oct 18</td>
</tr>
<tr>
<td></td>
<td>Crawfordsville</td>
<td>41°11'35.00&quot;N 91°28'50.57&quot;W</td>
<td>Taintor silty clay loam</td>
<td>35-60</td>
<td>38-42</td>
<td>IA 3014</td>
<td>3.0</td>
<td>May 21</td>
<td>Oct 22</td>
</tr>
<tr>
<td>2015</td>
<td>Nashua</td>
<td>42°56'29.34&quot;N 92°34'3.45&quot;W</td>
<td>Readlyn loam</td>
<td>30-49</td>
<td>22-30</td>
<td>IA 2094</td>
<td>2.4</td>
<td>Apr 16</td>
<td>Oct 5</td>
</tr>
<tr>
<td></td>
<td>Kanawha</td>
<td>42°54'42.61&quot;N 93°47'29.58&quot;W</td>
<td>Canisteo clay loam</td>
<td>25-60</td>
<td>20-35</td>
<td>IA 2094</td>
<td>2.4</td>
<td>Apr 16</td>
<td>Oct 5</td>
</tr>
<tr>
<td></td>
<td>Crawfordsville</td>
<td>41°11'51.36&quot;N 91°29'12.74&quot;W</td>
<td>Taintor silty clay loam</td>
<td>35-60</td>
<td>38-42</td>
<td>IA 3014</td>
<td>3.0</td>
<td>Apr 17</td>
<td>Oct 2</td>
</tr>
<tr>
<td>2016</td>
<td>Nashua</td>
<td>42°55'54.50&quot;N 92°34'41.32&quot;W</td>
<td>Clyde silty clay loam</td>
<td>30-60</td>
<td>24-30</td>
<td>IA 2094</td>
<td>2.4</td>
<td>May 7</td>
<td>Oct 10</td>
</tr>
<tr>
<td></td>
<td>Kanawha</td>
<td>42°54'56.83&quot;N 93°47'27.06&quot;W</td>
<td>Nicollet clay loam</td>
<td>25-61</td>
<td>24-35</td>
<td>IA 2094</td>
<td>2.4</td>
<td>May 7</td>
<td>Nov 1</td>
</tr>
<tr>
<td></td>
<td>Crawfordsville</td>
<td>41°12'0.05&quot;N 91°29'42.11&quot;W</td>
<td>Mahaska silty clay loam</td>
<td>36-61</td>
<td>35-42</td>
<td>IA 3014</td>
<td>3.0</td>
<td>May 6</td>
<td>Oct 19</td>
</tr>
</tbody>
</table>

<sup>a</sup>Soil data was obtained from Web Soil Survey of USDA Natural Resources Conservation Service. https://websoilsurvey.sc.egov.usda.gov

<sup>y,z</sup>Range in characteristic for the soil series was obtained from Web Site for Official Soil Series Descriptions and Series Classification of USDA Natural Resources Conservation Service. https://soilseries.sc.egov.usda.gov
Table 2. Description of the seed treatments evaluated in field experiment in Iowa in 2014, 2015 and 2016.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Active ingredient</th>
<th>Manufacturer</th>
<th>2014</th>
<th>2015</th>
<th>2016</th>
</tr>
</thead>
<tbody>
<tr>
<td>CruiserMaxx Advanced + Vibrance</td>
<td>thiamethoxam</td>
<td>Syngenta</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mefenoxam</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>fludioxonil</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>sedaxane</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CruiserMaxx + Vibrance</td>
<td>thiamethoxam</td>
<td>Syngenta</td>
<td>X</td>
<td>X</td>
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</tr>
<tr>
<td></td>
<td>mefenoxam</td>
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<td></td>
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</tr>
<tr>
<td>Clariva Complete Beans</td>
<td>thiamethoxam</td>
<td>Syngenta</td>
<td>X</td>
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<td>X</td>
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<td>mefenoxam</td>
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<td>fludioxonil</td>
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<td>sedaxane</td>
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<td>fludioxonil</td>
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<td></td>
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<tr>
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<td>sedaxane</td>
<td>Pasteuria nishizawae thibendazole</td>
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</tr>
<tr>
<td>EvergolEnergy + Gaucho + Allegiance</td>
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<td>Bayer</td>
<td>X</td>
<td></td>
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</tr>
<tr>
<td></td>
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<td></td>
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</tr>
<tr>
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<td>imidacloprid</td>
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<td>EvergolEnergy + Pocho/Votivo + Allegiance</td>
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<td>Bayer</td>
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<tr>
<td></td>
<td>metalaxyl</td>
<td>Bacillus firmus</td>
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<td></td>
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<tr>
<td></td>
<td>clothianidin</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Proline + Trilex Flowable + Allegiance + Poncho/Votivo</td>
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<td>Bayer</td>
<td>X</td>
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<td></td>
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<tr>
<td></td>
<td>metalaxyl</td>
<td>Bacillus firmus</td>
<td>flupyrind</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>clothianidin</td>
<td></td>
<td></td>
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<tr>
<td>Proline + Trilex Flowable + Allegiance + Poncho/VOTIVO + ILeVO</td>
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<td>Bayer</td>
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<td>X</td>
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<td>Bacillus firmus</td>
<td>flupyrind</td>
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<td>clothianidin</td>
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<td></td>
<td>fluopyram</td>
<td></td>
<td></td>
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<tr>
<td>EverGol Energy + Allegiance + Poncho/VOTIVO + ILeVO</td>
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<td>Bayer</td>
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<td>X</td>
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<td>penflufen</td>
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<td></td>
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<td>Bacillus firmus</td>
<td>flupyrind</td>
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<td>clothianidin</td>
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<td></td>
<td>fluopyram</td>
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<tr>
<td>Proline + Fluxastrobine + Allegiance + Poncho/VOTIVO + ILeVO</td>
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<td>Bayer</td>
<td>X</td>
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</tr>
<tr>
<td></td>
<td>fluxastrobine</td>
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<td>metalaxyl</td>
<td>Bacillus firmus</td>
<td>flupyrind</td>
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<td></td>
<td>clothianidin</td>
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<td></td>
</tr>
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<td></td>
<td>fluopyram</td>
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<td></td>
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</tr>
<tr>
<td>Intego Suite</td>
<td></td>
<td>Valent</td>
<td>X</td>
<td>X</td>
<td>X</td>
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</table>
Table 2 continued.

<table>
<thead>
<tr>
<th>Treatment Description</th>
<th>Active Ingredients</th>
<th>Manufacturer</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warden CX</td>
<td>thiamethoxam&lt;br&gt;mefenoxam&lt;br&gt;fludioxonil&lt;br&gt;sedaxane</td>
<td>Winfield</td>
<td>X</td>
</tr>
<tr>
<td>Acceleron</td>
<td>pyraclostrobin&lt;br&gt;metalaxyl&lt;br&gt;fluxapyroxad&lt;br&gt;imidacloprid</td>
<td>BASF</td>
<td>X       X</td>
</tr>
<tr>
<td>Acceleron + Vault HP Integral</td>
<td>pyraclostrobin&lt;br&gt;metalaxyl&lt;br&gt;fluxapyroxad&lt;br&gt;imidacloprid&lt;br&gt;Bacillus subtilis&lt;br&gt;Bradyrhizobium japonicum</td>
<td>BASF</td>
<td>X       X</td>
</tr>
<tr>
<td>Acceleron + Vault HP Integral + FloRite</td>
<td>pyraclostrobin&lt;br&gt;metalaxyl&lt;br&gt;fluxapyroxad&lt;br&gt;imidacloprid&lt;br&gt;Bacillus subtilis&lt;br&gt;Bradyrhizobium japonicum</td>
<td>BASF</td>
<td>X       X</td>
</tr>
<tr>
<td>Acceleron</td>
<td>pyraclostrobin&lt;br&gt;metalaxyl&lt;br&gt;fluxapyroxad&lt;br&gt;imidacloprid</td>
<td>Monsanto</td>
<td>X</td>
</tr>
<tr>
<td>PPST 2030 + EvergolEnergy + Allegiance + Gaucho</td>
<td>prothioconazole&lt;br&gt;penfluaten&lt;br&gt;metalaxyl&lt;br&gt;imidacloprid&lt;br&gt;Bacillus subtilis&lt;br&gt;Bacillus pumilis</td>
<td>Pioneer</td>
<td>X       X</td>
</tr>
<tr>
<td>Rancona 3.8FS + Belmont + Attendant</td>
<td>ipconazole&lt;br&gt;metalaxyl&lt;br&gt;imidacloprid</td>
<td>Arysta</td>
<td>X       X</td>
</tr>
<tr>
<td>Rancona 3.8FS + Belmont + Attendant + ALS-1006</td>
<td>ipconazole&lt;br&gt;metalaxyl&lt;br&gt;imidacloprid</td>
<td>Arysta</td>
<td>X</td>
</tr>
<tr>
<td>Rancona CTS + Belmont + Attendant</td>
<td>ipconazole&lt;br&gt;metalaxyl&lt;br&gt;imidacloprid</td>
<td>Arysta</td>
<td>X</td>
</tr>
<tr>
<td>Rancona V100ProFS + Belmont + Attendant</td>
<td>carboxin&lt;br&gt;ipconazole&lt;br&gt;metalaxyl&lt;br&gt;imidacloprid</td>
<td>Arysta</td>
<td>X       X</td>
</tr>
<tr>
<td>Rancona V100ProFS + Belmont + Attendant + Thiabendazole</td>
<td>carboxin&lt;br&gt;ipconazole&lt;br&gt;metalaxyl&lt;br&gt;imidacloprid thiaubendazole</td>
<td>Arysta</td>
<td>X</td>
</tr>
</tbody>
</table>
Table 3. Effect of 15 commercial seed treatments on soybean stand (% emergence) and yield (Bushels/Acre) at three locations in Iowa in 2014.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stand (% emergence 35 dap)</th>
<th>Yield (Bushels/Acre)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nashua</td>
<td>Roland</td>
</tr>
<tr>
<td>CrusierMaxx Advanced + Vibrance³</td>
<td>91.4</td>
<td>79.5</td>
</tr>
<tr>
<td>Clariva Complete Beans⁸</td>
<td>83.7</td>
<td>89.4</td>
</tr>
<tr>
<td>EvergolEnergy + Gaucho + Allegiance¹</td>
<td>82.2</td>
<td>89.5</td>
</tr>
<tr>
<td>EvergolEnergy + Pocho/Votivo + Allegiance⁹</td>
<td>92.5</td>
<td>83.3</td>
</tr>
<tr>
<td>Prolin + Trilex Flowable + Allegiance + Poncho/Votivo⁰</td>
<td>90.4</td>
<td>92.1</td>
</tr>
<tr>
<td>Intego Suite⁶</td>
<td>95.7</td>
<td>90.6</td>
</tr>
<tr>
<td>Warden CX⁵</td>
<td>77.6</td>
<td>91.8</td>
</tr>
<tr>
<td>Acceleron⁷</td>
<td>69.9</td>
<td>92.3</td>
</tr>
<tr>
<td>Acceleron + Vault HP Integral¹</td>
<td>66.2</td>
<td>84.9</td>
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<tr>
<td>Acceleron + Vault HP Integral + FloRite⁵</td>
<td>70.6</td>
<td>79.7</td>
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<tr>
<td>Acceleron²</td>
<td>90.6</td>
<td>82.9</td>
</tr>
<tr>
<td>PPST 2030 + EvergolEnergy + Allegiance + Gaucho⁴</td>
<td>90.4</td>
<td>85.5</td>
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<tr>
<td>Rancona 3.8FS + Belmont + Attendant³</td>
<td>82.7</td>
<td>87.3</td>
</tr>
<tr>
<td>Rancona CTS + Belmont + Attendant⁸</td>
<td>85.0</td>
<td>88.6</td>
</tr>
<tr>
<td>Rancona V100ProFS + Belmont + Attendant⁸</td>
<td>74.2</td>
<td>78.9</td>
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<tr>
<td>Untreated</td>
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<td>91.2</td>
</tr>
<tr>
<td>P value</td>
<td>0.6122</td>
<td>0.7863</td>
</tr>
</tbody>
</table>

¹Contains thiamethoxam 0.0755 mg, mfenoxam 0.0056 mg, fludioxonil 0.0037 mg, sedaxane 0.0038 to 0.0076 mg of active ingredient per seed.
²Contains thiamethoxam 0.0755 mg, mfenoxam 0.0056 mg, fludioxonil 0.0037 mg, sedaxane 0.0038 to 0.0076 mg of active ingredient per seed, and *Pasteuria nishizawai* 0.015 to 0.044 mg of active ingredient per seed.
³Contains prothoconazole 0.008 mg, fenpropimorph 0.004 mg, imidacloprid 0.100 mg, and metalaxyl 0.025 mg of active ingredient per seed.
⁴Contains prothoconazole 0.008 mg, fenpropimorph 0.004 mg, clothianidin 0.110 mg, Bacillus firmus 0.020 mg, and metalaxyl 0.025 mg of active ingredient per seed.
⁵Contains prothoconazole 0.018 mg, trifloxystrobin 0.009 mg, metalaxyl 0.007 mg, clothianidin 0.110 mg, and Bacillus firmus 0.020 mg of active ingredient per seed.
⁶Contains prothoconazole 0.018 mg, clothianidin 0.081 mg, ethaboxin 0.012 mg, ipconazole 0.004 mg, metalaxyl 0.0032 mg of active ingredient per seed.
⁷Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed.
⁸Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed, *Brassica subtilis*, and *Bradyrhizobium japonicum*.
Table 3 continued.

Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed, Bacillus subtilis, and Bradyrhizobium japonicum.

Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed.

Contains prothioconazole 0.008 mg, penflufen 0.004 mg, metalaxyl 0.025 mg, and imidacloprid 0.101 mg of active ingredient per seed.

Contains ipconazole 0.004 mg + metalaxyl 0.025 mg + imidacloprid 0.101

Contains ipconazole 0.004 mg + metalaxyl 0.025 mg + imidacloprid 0.101

Contains carboxin 0.063 mg, ipconazole 0.004 mg, metalaxyl 0.025 mg, and imidacloprid 0.101 mg active ingredient per seed.

Days after planting

Asterisk indicates the mean is significantly different to untreated control according to LSD test alpha = 0.1.
Table 4. Effect of 13 commercial seed treatments on soybean stand count (%) and yield (Bushels/Acre) at three locations in Iowa in 2015.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nashua % emergence 35 dap</th>
<th>Kanawha</th>
<th>Crawfordsville</th>
<th>Yield (Bushels/Acre)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CruiserMaxx + Vibrance(^l)</td>
<td>83.1(^z)</td>
<td>91.2</td>
<td>93.3*</td>
<td>85.1</td>
</tr>
<tr>
<td>Clariva Complete Beans(^m)</td>
<td>84.7</td>
<td>84.1</td>
<td>89.7</td>
<td>77.8</td>
</tr>
<tr>
<td>Clariva Complete Beans + Mertect(^n)</td>
<td>77.4*</td>
<td>87.8</td>
<td>91.9*</td>
<td>73.0</td>
</tr>
<tr>
<td>Proline + Trilex Flowable + Allegiance + Poncho/VOTIVO + ILeVO(^o)</td>
<td>85.1</td>
<td>87.1</td>
<td>93.1*</td>
<td>78.5</td>
</tr>
<tr>
<td>EverGol Energy + Allegiance + Poncho/VOTIVO + ILeVO(^p)</td>
<td>80.3*</td>
<td>86.8</td>
<td>93.1*</td>
<td>81.0</td>
</tr>
<tr>
<td>Intego Suite(^q)</td>
<td>91.4</td>
<td>87.2</td>
<td>95.1*</td>
<td>82.0</td>
</tr>
<tr>
<td>Warden CX(^l)</td>
<td>89.5</td>
<td>86.9</td>
<td>93.4*</td>
<td>83.1</td>
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<tr>
<td>Acceleron(^s)</td>
<td>86.6</td>
<td>86.0</td>
<td>88.9</td>
<td>81.5</td>
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<td>Acceleron + Vault HP + Integral(^t)</td>
<td>81.6*</td>
<td>88.3</td>
<td>87.9</td>
<td>79.9</td>
</tr>
<tr>
<td>Acceleron + Vault HP + Integral + Flo-Rite(^u)</td>
<td>79.9*</td>
<td>87.4</td>
<td>89.2</td>
<td>77.3</td>
</tr>
<tr>
<td>PPST 2030 + EverGol Energy + Allegiance + Gauch(^v)</td>
<td>81.7*</td>
<td>82.2</td>
<td>87.8</td>
<td>81.5</td>
</tr>
<tr>
<td>Rancona V100ProFS + Belmont + Attendant(^w)</td>
<td>85.1</td>
<td>87.9</td>
<td>90.8</td>
<td>80.6</td>
</tr>
<tr>
<td>Rancona V100ProFS + Belmont + Attendant + Thiabendazole(^x)</td>
<td>86.0</td>
<td>90.6</td>
<td>90.0</td>
<td>83.7</td>
</tr>
<tr>
<td>Untreated</td>
<td>89.0</td>
<td>87.0</td>
<td>86.6</td>
<td>80.1</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.0222</td>
<td>0.6007</td>
<td>0.0323</td>
<td>0.1502</td>
</tr>
</tbody>
</table>

\(^l\)Contains thiamethoxam 0.0755 mg, mefenoxam 0.0056 mg, fludioxonil 0.0037 mg, and sedaxane 0.0038 to 0.0076 mg of active ingredient per seed.

\(^m\)Contains thiamethoxam 0.0755 mg, mefenoxam 0.0056 mg, fludioxonil 0.0037 mg, sedaxane 0.0038 to 0.0076 mg, and Pasteuria nishizawai 0.015 to 0.044 mg of active ingredient per seed.

\(^n\)Contains thiamethoxam 0.0755 mg, mefenoxam 0.0056 mg, fludioxonil 0.0037 mg, sedaxane 0.0038 to 0.0076 mg, Pasteuria nishizawai 0.015 to 0.044 mg of active ingredient per seed.

\(^o\)Contains prothiocarbazole 0.018 mg, trifloxystrobin 0.009 mg, metalaxyl 0.007 mg, clothianidin 0.11 mg, B. firmus 0.02 mg, and fluopyram 0.15 mg of active ingredient per seed.

\(^p\)Contains prothiocarbazole 0.008 mg, penflufen 0.004 mg, metalaxyl 0.014 mg, clothianidin 0.11 mg, Bacillus firmus 0.02 mg, and fluopyram 0.15 mg of active ingredient per seed.

\(^q\)Contains clothianidin 0.081 mg, ethaboxam 0.012 mg, ipconazole 0.004 mg, metalaxyl 0.0032 mg of active ingredient per seed.

\(^r\)Contains pyraclostrobin 0.008 mg, mefenoxam 0.025 mg, flupyradolu 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed.

\(^s\)Contains pyraclostrobin 0.008 mg, mefenoxam 0.025 mg, flupyradolu 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed, Bacillus subtilis, and Bradyrhizobium japonicum.
Table 4 continued.

\textsuperscript{4}Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed, \textit{Bacillus subtilis}, and \textit{Bradyrhizobium japonicum}.

\textsuperscript{v}Contains prothioconazole 0.008 mg, penflufen 0.004 mg, metalaxyl 0.025 mg, and imidacloprid 0.10 mg of active ingredient per seed.

\textsuperscript{w}Contains carboxin 0.063 mg, ipconazole 0.0093 mg, metalaxyl 0.025 mg, and imidacloprid 0.1013 mg of active ingredient per seed.

\textsuperscript{x}Contains carboxin 0.063 mg, ipconazole 0.0093 mg, metalaxyl 0.025 mg, imidacloprid 0.101 mg, and thiabendazole 0.008 mg of active ingredient per seed.

\textsuperscript{y}Days after planting.

\textsuperscript{z}Asterisk indicates the mean is significantly different to untreated control according to LSD test alpha = 0.1.
Table 5. Effect of 13 commercial seed treatments on soybean stand count (%) and yield (Bushels/Acre) at three locations in Iowa in 2016.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stand (% emergence 35 dap)</th>
<th>Yield (Bushels/Acre)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nashua</td>
<td>Kanawha</td>
</tr>
<tr>
<td>CruiserMaxx + Vibrance(^1)</td>
<td>87.7</td>
<td>92.1</td>
</tr>
<tr>
<td>Clariva Complete Beans(^m)</td>
<td>92.1</td>
<td>86.6</td>
</tr>
<tr>
<td>Clariva Complete Beans + Mertect(^n)</td>
<td>86.8</td>
<td>88.3</td>
</tr>
<tr>
<td>Proline + Trilex Flowable + Allegiance + Poncho/VOTIVO + ILeVO(^o)</td>
<td>91.8</td>
<td>93.2</td>
</tr>
<tr>
<td>Evergol Energy + Allegiance + Poncho/VOTIVO + ILeVO(^p)</td>
<td>91.5</td>
<td>91.1</td>
</tr>
<tr>
<td>Proline + Fluoxastrobin + Allegiance + Poncho/VOTIVO + ILeVO(^q)</td>
<td>89.9</td>
<td>90.6</td>
</tr>
<tr>
<td>Intego Suite(^r)</td>
<td>91.6</td>
<td>88.7</td>
</tr>
<tr>
<td>Acceleron(^s)</td>
<td>88.3</td>
<td>89.1</td>
</tr>
<tr>
<td>Acceleron + Vault HP + Integral(^t)</td>
<td>86.5</td>
<td>89.4</td>
</tr>
<tr>
<td>Acceleron + Vault HP + Integral Flo Rite(^u)</td>
<td>89.7</td>
<td>85.1</td>
</tr>
<tr>
<td>Rancona 3.8FS + Belmont + Attendant(^v)</td>
<td>90.5</td>
<td>92.2</td>
</tr>
<tr>
<td>Rancona 3.8FS + Belmont + Attendant + ALS-1006(^w)</td>
<td>86.9</td>
<td>92.0</td>
</tr>
<tr>
<td>Rancona V100Pro + Belmont + Attendant(^x)</td>
<td>90.7</td>
<td>90.9</td>
</tr>
<tr>
<td>Untreated</td>
<td>90.7</td>
<td>91.4</td>
</tr>
</tbody>
</table>

P value

<table>
<thead>
<tr>
<th></th>
<th>Nashua</th>
<th>Kanawha</th>
<th>Crawfordsville</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3382</td>
<td>0.3725</td>
<td>0.2566</td>
</tr>
<tr>
<td></td>
<td>0.3117</td>
<td>0.0330</td>
<td>0.1359</td>
</tr>
</tbody>
</table>

\(^1\)Contains thiamethoxam 0.0755 mg, mefenoxam 0.0056 mg, fludioxonil 0.0037 mg, and sedaxane 0.0038 to 0.0076 mg of active ingredient per seed.

\(^m\)Contains thiamethoxam 0.0755 mg, mefenoxam 0.0056 mg, fludioxonil 0.0037 mg, sedaxane 0.0038 to 0.0076 mg, and Pasteuria nishizawae 0.015 to 0.044 mg of active ingredient per seed.

\(^n\)Contains thiamethoxam 0.0755 mg, mefenoxam 0.0056 mg, fludioxonil 0.0037 mg, sedaxane 0.0038 to 0.0076 mg, Pasteuria nishizawae 0.015 to 0.044 mg, and thiabendazole 0.030 mg of active ingredient per seed.

\(^o\)Contains prothiocarbazole 0.018 mg, trifloxystrobin 0.009 mg, metalaxyl 0.007 mg, clothianidin 0.11 mg, B. firmus 0.02 mg, and fluopyram 0.15 mg of active ingredient per seed.

\(^p\)Contains prothioconazole 0.008 mg, penflufen 0.004 mg, metalaxyl 0.014 mg, clothianidin 0.11 mg, Bacillus firmus 0.02 mg, and fluopyram 0.15 mg of active ingredient per seed.

\(^q\)Contains prothiocarbazole 0.008 mg, fluoxastrobin 0.008 mg, metalaxyl 0.007, clothianidin 0.11 mg, Bacillus firmus 0.02 mg, and fluopyram 0.15 mg of active ingredient per seed.

\(^r\)Contains clothianidin 0.081 mg, ethaboxam 0.012 mg, ipconazole 0.004 mg, metalaxyl 0.0032 mg of active ingredient per seed.

\(^s\)Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed.

\(^t\)Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed.

\(^u\)Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed, Bacillus subtilis, and Bradyrhizobium japonicum.
Table 5 continued.

\(^1\)Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed, \textit{Bacillus subtilis}, and \textit{Bradyrhizobium japonicum}.

\(^2\)Contains ipconazole 0.004 mg, metalaxyl 0.025 mg, and imidacloprid 0.101 mg of active ingredient per seed.

\(^3\)Contains ipconazole 0.004 mg, metalaxyl 0.025 mg, and imidacloprid 0.101 mg of active ingredient per seed.

\(^4\)Contains carboxin 0.063 mg, ipconazole 0.004 mg, metalaxyl 0.025 mg, imidacloprid 0.101 mg, and thiabendazole 0.008 mg of active ingredient per seed.

\(^7\)Days after planting.

\(^2\)Asterisk indicates the mean is significantly different to untreated control according to LSD test alpha = 0.1.
Table 6. Effect of seed treatments on Soybean Cyst Nematode (factor (RF) at three locations of Iowa in 2014, 2015, and 2016.

<table>
<thead>
<tr>
<th>Year</th>
<th>Treatment</th>
<th>Nashua</th>
<th>Roland</th>
<th>Crawfordsville</th>
</tr>
</thead>
<tbody>
<tr>
<td>2014</td>
<td>CruiserMaxx Advanced + Vibrance</td>
<td>0.2</td>
<td>0.8 bc</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Clariva Complete Beans</td>
<td>0.2</td>
<td>1.0 bc</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Evergol Energy + Gaucho + Allegiance</td>
<td>0.1</td>
<td>0.9 bc</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Evergol Energy + Pocho/Votivo + Allegiance</td>
<td>0.0</td>
<td>1.3 ab</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Proline + TrilexFlowable + Allegiance + Poncho/Votivo</td>
<td>0.0</td>
<td>0.6 c</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>Untreated Control</td>
<td>0.0</td>
<td>1.7 a</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td><strong>P value</strong></td>
<td>0.4116</td>
<td>0.0753</td>
<td>0.6190</td>
</tr>
<tr>
<td>2015</td>
<td>CruiserMaxx + Vibrance</td>
<td>20.5</td>
<td>3.0</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Clariva Complete Beans</td>
<td>5.8</td>
<td>3.4</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Clariva Complete Beans + Mertect</td>
<td>4.3</td>
<td>3.2</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Proline + Trilex Flowable + Allegiance + Poncho/VOTiVO + ILeVO</td>
<td>4.7</td>
<td>2.9</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>EverGol Energy + Allegiance + Poncho/VOTiVO + ILeVO</td>
<td>18.2</td>
<td>4.4</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>Untreated Control</td>
<td>3.2</td>
<td>2.7</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td><strong>P value</strong></td>
<td>0.2207</td>
<td>0.8933</td>
<td>0.1516</td>
</tr>
<tr>
<td>2016</td>
<td>CruiserMaxx + Vibrance</td>
<td>3.7</td>
<td>.</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Clariva Complete Beans</td>
<td>4.0</td>
<td>.</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Clariva Complete Beans + Mertect</td>
<td>6.3</td>
<td>.</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Proline + Trilex Flowable + Allegiance + Poncho/VOTiVO + ILeVO</td>
<td>4.4</td>
<td>.</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Evergol Energy + Allegiance + Poncho/VOTiVO + ILeVO</td>
<td>2.4</td>
<td>.</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Proline + FluoXastrobil + Allegiance + Poncho/VOTiVO + ILeVO</td>
<td>2.8</td>
<td>.</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Untreated Control</td>
<td>6.0</td>
<td>.</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td><strong>P value</strong></td>
<td>0.5897</td>
<td>0.8147</td>
<td>0.8147</td>
</tr>
</tbody>
</table>

*In 2014 initial SCN population density was 1838 egg per 100 cm³ soil at Roland, 3271 egg per 100 cm³ soil at Nashua and 83 egg per 100 cm³ soil at Crawfordsville.*
Table 6 continued.

1In 2015 initial SCN population density was 338 egg per 100 cm³ soil at NRF, 117 egg per 100 cm³ soil at NERF and 71 egg per 100 cm³ soil at SERF.

2In 2016 initial SCN population density was 218 egg per 100 cm³ soil at Nashua and 275 egg per 100 cm³ soil at Crawfordsville.

3Contains thiamethoxam 0.0755 mg, mefenoxam 0.0056 mg, fludioxonil 0.0037 mg, and sedaxane 0.0038 to 0.0076 mg of active ingredient per seed.

4Contains thiamethoxam 0.0755 mg, mefenoxam 0.0056 mg, fludioxonil 0.0037 mg, sedaxane 0.0038 to 0.0076 mg, and *Pasteuria nishizawai*ae 0.015 to 0.044 mg of active ingredient per seed.

5Contains prothioconazole 0.008 mg, penflufen 0.004 mg, imidacloprid 0.100 mg, and metalaxyl 0.025 mg of active ingredient per seed.

6Contains prothioconazole 0.008 mg, penflufen 0.004 mg, clothianidin 0.110 mg, *Bacillus firmus* 0.020 mg, and metalaxyl 0.025 mg of active ingredient per seed.

7Contains prothioconazole 0.018 mg, trifloxystrobin 0.009 mg, metalaxyl 0.007 mg, clothianidin 0.110 mg, and *Bacillus firmus* 0.020 mg of active ingredient per seed.

8Contains thiamethoxam 0.0755 mg, mefenoxam 0.0056 mg, fludioxonil 0.0037 mg, and sedaxane 0.0038 to 0.0076 mg of active ingredient per seed.

9Contains thiamethoxam 0.0755 mg, mefenoxam 0.0056 mg, fludioxonil 0.0037 mg, sedaxane 0.0038 to 0.0076 mg, *Pasteuria nishizawai*ae 0.015 to 0.044 mg of active ingredient per seed.

10Contains prothioconazole 0.018 mg, trifloxystrobin 0.009 mg, metalaxyl 0.007 mg, clothianidin 0.11 mg, B. firmus 0.02 mg, and fluopyram 0.15 mg of active ingredient per seed.

11Contains prothioconazole 0.008 mg, penflufen 0.004 mg, metalaxyl 0.014 mg, clothianidin 0.11 mg, *Bacillus firmus* 0.02 mg, and fluopyram 0.15 mg of active ingredient per seed.

12Contains prothioconazole 0.008 mg, fluoxastobin 0.008 mg, metalaxyl 0.007, clothianidin 0.11 mg, *Bacillus firmus* 0.02 mg, and fluopyram 0.15 mg of active ingredient per seed.

13RF is the final SCN egg population density (eggs per 100 cm³ soil at harvest) divided by the initial SCN population density.

14Same letters indicate no significant differences with LSD test alpha = 0.1.

15Samples not taken at this location.
Table 7. Effect of 13 commercial soybean seed treatments on Sudden Death Syndrome (SDS) Foliar Disease Index (FDX) at three locations in Iowa in 2015.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Foliar Disease Index (FDX)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nashua</td>
</tr>
<tr>
<td>CruiserMaxx + Vibrance™</td>
<td>0</td>
</tr>
<tr>
<td>Clariva Complete Beans™</td>
<td>0</td>
</tr>
<tr>
<td>Clariva Complete Beans + Mertect°</td>
<td>0</td>
</tr>
<tr>
<td>Proline + Trilox Flowable + Allegiance + Poncho/VOTiVO + ILeVO®</td>
<td>0</td>
</tr>
<tr>
<td>EverGol Energy + Allegiance + Poncho/VOTiVO + ILeVO®</td>
<td>0</td>
</tr>
<tr>
<td>Intego Suite®</td>
<td>0</td>
</tr>
<tr>
<td>Warden CX®</td>
<td>0</td>
</tr>
<tr>
<td>Acceleron†</td>
<td>0</td>
</tr>
<tr>
<td>Acceleron + Vault HP + Integral™</td>
<td>0</td>
</tr>
<tr>
<td>Acceleron + Vault HP + Integral + Flo-Rite®</td>
<td>0</td>
</tr>
<tr>
<td>PPST 2030 + EverGol Energy + Allegiance + Gaucho™</td>
<td>0</td>
</tr>
<tr>
<td>Rancona V100ProFS + Belmont + Attendant®</td>
<td>0</td>
</tr>
<tr>
<td>Rancona V100ProFS + Belmont + Attendant + Thiabendazole®</td>
<td>0</td>
</tr>
<tr>
<td>Untreated</td>
<td>0</td>
</tr>
</tbody>
</table>

P value 0.3550

*=Contains thiamethoxam 0.0755 mg, mefenoxam 0.0056 mg, fludioxonil 0.0037 mg, and sedaxane 0.0038 to 0.0076 mg of active ingredient per seed.

**Contains thiamethoxam 0.0755 mg, mefenoxam 0.0056 mg, fludioxonil 0.0037 mg, sedaxane 0.0038 to 0.0076 mg, and Pasteuria nishizawai 0.015 to 0.044 mg of active ingredient per seed.

°Contains thiamethoxam 0.0755 mg, mefenoxam 0.0056 mg, fludioxonil 0.0037 mg, sedaxane 0.0038 to 0.0076 mg, Pasteuria nishizawai 0.015 to 0.044 mg, and thiabendazole 0.030 mg of active ingredient per seed.

*Contains prothioconazole 0.018 mg, trifloxystrobin 0.009 mg, metalaxyl 0.007 mg, clothianidin 0.11 mg, B. firmus 0.02 mg, and fluopyram 0.15 mg of active ingredient per seed.

Contains prothioconazole 0.008 mg, flufen 0.004 mg, metalaxyl 0.014 mg, clothianidin 0.11 mg, Bacillus firmus 0.02 mg, and fluopyram 0.15 mg of active ingredient per seed

†Contains clothianidin 0.081 mg, ethaboxam 0.012 mg, ipconazole 0.004 mg, metalaxyl 0.0032 mg of active ingredient per seed
Table 7 continued.

<table>
<thead>
<tr>
<th>Seed Treatment</th>
<th>Active Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>thiamethoxam 0.0756 mg, mefenoxam 0.0226 mg, fludioxonil 0.0038 mg, and sedaxane 0.0038 mg of active ingredient per seed.</td>
</tr>
<tr>
<td>b</td>
<td>pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed.</td>
</tr>
<tr>
<td>c</td>
<td>pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed, <em>Bacillus subtilis</em>, and <em>Bradyrhizobium japonicum</em>.</td>
</tr>
<tr>
<td>d</td>
<td>pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed, <em>Bacillus subtilis</em>, and <em>Bradyrhizobium japonicum</em>.</td>
</tr>
<tr>
<td>e</td>
<td>prothioconazole 0.008 mg, penflufen 0.004 mg, metalaxyl 0.025 mg, and imidacloprid 0.10 mg of active ingredient per seed.</td>
</tr>
<tr>
<td>f</td>
<td>carboxin 0.063 mg, ipconazole 0.0093 mg, metalaxyl 0.025 mg, and imidacloprid 0.101 mg of active ingredient per seed.</td>
</tr>
<tr>
<td>g</td>
<td>carboxin 0.063 mg, ipconazole 0.0093 mg, metalaxyl 0.025 mg, imidacloprid 0.101 mg, and thiabendazole 0.008 mg of active ingredient per seed.</td>
</tr>
<tr>
<td>h</td>
<td>Data not taken</td>
</tr>
</tbody>
</table>

"Contains thiamethoxam 0.0756 mg, mefenoxam 0.0226 mg, fludioxonil 0.0038 mg, and sedaxane 0.0038 mg of active ingredient per seed.

"Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed.

"Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed, *Bacillus subtilis*, and *Bradyrhizobium japonicum*.

"Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed, *Bacillus subtilis*, and *Bradyrhizobium japonicum*.

"Contains prothioconazole 0.008 mg, penflufen 0.004 mg, metalaxyl 0.025 mg, and imidacloprid 0.10 mg of active ingredient per seed.

"Contains carboxin 0.063 mg, ipconazole 0.0093 mg, metalaxyl 0.025 mg, and imidacloprid 0.1013 mg of active ingredient per seed.

"Contains carboxin 0.063 mg, ipconazole 0.0093 mg, metalaxyl 0.025 mg, imidacloprid 0.101 mg, and thiabendazole 0.008 mg of active ingredient per seed.

"Data not taken"
Table 8. Effect of 13 commercial soybean seed treatments on Sudden Death Syndrome (SDS) Foliar Disease Index (FDX) at three locations in Iowa in 2016.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nashua</th>
<th>Kanawha</th>
<th>Crawfordsville</th>
</tr>
</thead>
<tbody>
<tr>
<td>CruiserMaxx + Vibrance&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>6.6</td>
</tr>
<tr>
<td>Clariva Complete Beans&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>10.6</td>
</tr>
<tr>
<td>Clariva Complete Beans + Mertect&lt;sup&gt;p&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>21.4</td>
</tr>
<tr>
<td>Proline + Trilex Flowable + Allegiance + Poncho/VOTiVO + ILeVO&lt;sup&gt;q&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>32.8</td>
</tr>
<tr>
<td>Evergol Energy + Allegiance + Poncho/VOTiVO + ILeVO&lt;sup&gt;r&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>18.3</td>
</tr>
<tr>
<td>Proline + Fluoxastrobin + Allegiance + Poncho/VOTiVO + ILeVO&lt;sup&gt;s&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Intego Suite&lt;sup&gt;i&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>26.4</td>
</tr>
<tr>
<td>Acceleron&lt;sup&gt;u&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>15.3</td>
</tr>
<tr>
<td>Acceleron + Vault HP + Integral&lt;sup&gt;v&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>23.5</td>
</tr>
<tr>
<td>Acceleron + Vault HP + Integral Flo Rite&lt;sup&gt;w&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>7.3</td>
</tr>
<tr>
<td>Rancona 3.8FS + Belmont + Attendant&lt;sup&gt;x&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>9.9</td>
</tr>
<tr>
<td>Rancona 3.8FS + Belmont + Attendant + ALS-1006&lt;sup&gt;y&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>22.5</td>
</tr>
<tr>
<td>Rancona V100Pro + Belmont + Attendant&lt;sup&gt;z&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>37.5</td>
</tr>
<tr>
<td>Untreated</td>
<td>0</td>
<td>0</td>
<td>31.4</td>
</tr>
</tbody>
</table>

P value: 0.1939

<sup>a</sup>Contains thiamethoxam 0.0755 mg, mfenoxam 0.0056 mg, fludioxonil 0.0037 mg, and sedaxane 0.0038 to 0.0076 mg of active ingredient per seed.

<sup>b</sup>Contains thiamethoxam 0.0755 mg, mfenoxam 0.0056 mg, fludioxonil 0.0037 mg, sedaxane 0.0038 to 0.0076 mg, and *Pasteuria nishizawai* 0.015 to 0.044 mg of active ingredient per seed.

<sup>p</sup>Contains thiamethoxam 0.0755 mg, mfenoxam 0.0056 mg, fludioxonil 0.0037 mg, sedaxane 0.0038 to 0.0076 mg, *Pasteuria nishizawai* 0.015 to 0.044 mg, and thiabendazole 0.030 mg of active ingredient per seed.

<sup>q</sup>Contains prothioconazole 0.018 mg, trifloxystrobin 0.009 mg, metalaxyl 0.007 mg, clothianidin 0.11 mg, *B. firmus* 0.02 mg, and fluopyram 0.15 mg of active ingredient per seed.

<sup>r</sup>Contains prothioconazole 0.008 mg, penflufen 0.004 mg, metalaxyl 0.014 mg, clothianidin 0.11 mg, *Bacillus firmus* 0.02 mg, and fluopyram 0.15 mg of active ingredient per seed.

<sup>s</sup>Contains prothioconazole 0.008 mg, fluoxastobin 0.008 mg, metalaxyl 0.007, clothianidin 0.11 mg, *Bacillus firmus* 0.02 mg, and fluopyram 0.15 mg of active ingredient per seed.
### Table 8 continued.

1. Contains clothianidin 0.081 mg, ethaboxam 0.012 mg, ipconazole 0.004 mg, metalaxyl 0.0032 mg of active ingredient per seed.

2. Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed.

3. Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed, *Bacillus subtilis*, and *Bradyrhizobium japonicum*.

4. Contains pyraclostrobin 0.008 mg, metalaxyl 0.025 mg, fluxapyroxad 0.008 mg, and imidacloprid 0.118 mg of active ingredient per seed, *Bacillus subtilis*, and *Bradyrhizobium japonicum*.

5. Contains ipconazole 0.004 mg, metalaxyl 0.025 mg, and imidacloprid 0.101 mg of active ingredient per seed.

6. Contains ipconazole 0.004 mg, metalaxyl 0.025 mg, and imidacloprid 0.101 mg of active ingredient per seed.

7. Contains carboxin 0.063 mg, ipconazole 0.004 mg, metalaxyl 0.025 mg, imidacloprid 0.101 mg, and thiabendazole 0.008 mg of active ingredient per seed.
Figure 1. Weather data during the planting season at 3 locations in Iowa in 2014.
Figure 2. Weather data during the planting season at 3 locations in Iowa in 2015.
Figure 3. Weather data during the planting season at 3 locations in Iowa in 2016.
Figure 4. Yield difference (Bu/Ac) to the untreated control of all seed treatments evaluated in field experiments in Iowa in 2014, 2015 and 2016. Asterisks indicate significant differences to untreated control with LSD test alpha = 0.1.

Figure 5. Water-logged soil in field trial at Roland, IA. Picture was taken on June 27th, 2014.
CHAPTER 3.

SEED TREATMENT REDUCES DAMPING-OFF CAUSED BY *Pythium sylvaticum* ON SOYBEANS SUBMITTED TO PERIODS OF COLD STRESS

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Abstract

Soybean damping-off caused by *Pythium* spp. is frequently associated with the occurrence of cold fronts soon after planting. In this study we evaluated the efficacy of a commercial seed treatment with metalaxyl and ethaboxam on emergence during various simulated cold stress conditions. A growth chamber experiment utilized five-way factorial design with two cold stress temperatures (4°C and 10°C), two cold stress timings (initiated 24 and 96 hours after planting), three durations of cold stress (24, 48 and 96 hours), and two levels of seed treatment (Intego Suite™ and untreated). Cups planted with soybean seeds IA 2094 were inoculated with *P. sylvaticum* or not inoculated. Cups were incubated at 18°C and evaluated 21 days after planting. Emergence was reduced when the pathogen was present compared to the non-inoculated controls. In untreated seed, cold stress duration reduced emergence, but no differences in emergence were detected in
response to either initiation of cold stress or cold stress temperature. The seed treatment improved emergence, reduced root rot severity, and increased shoot weight. There was no significant effect of seed treatment on root weight. Data from this study confirms that cold stress soon after planting can increase the risk of reduced crop stands in Iowa and suggest seed treatment with metalaxyl and mefenoxam protect seedlings when cold conditions are expected soon after planting.

Keywords: damping-off, oomycete, *Pythium sylvaticum*, seed treatment, soybean

**Introduction**

Soybean (*Glycine max* (L.) Merr.) is an important oilseed crop in the United States. In 2016 a total of 83.4 million acres were planted in the U.S. with 9.5 million acres planted in Iowa (www.nass.usda.gov/Statistics_by_State/Iowa/). Seedling diseases are the second most damaging disease of soybean; it is estimated that up to 55 million bushels were lost to seedling diseases between 2006 to 2009 (Koenning andWrather 2010). The predominant pathogens causing seedling disease in Iowa and the North Central Region of the U.S. are *Pythium* spp.; *P. sylvaticum* was the most frequently recovered *Pythium* species from diseased seedlings in recent surveys (Rizvi and Yang 1996; Murillo-Williams and Pedersen 2008; Matthiesen et al. 2016; Rojas et al. 2017).

*Pythium* spp. are oomycetes that reduce crop stands due to “damping-off” (death) of developing seedlings. Symptoms on affected seedlings may include general discoloration, yellow to brown lesions, root rot and seed rot (Hartman et al.
Replanting of the field is sometimes needed because severe reduction in plant stand can result in poor crop yield (Whigham et al. 2000; Hartman et al. 2015).

Climate change concerns in the Midwest include the occurrence of extreme events of temperature and precipitation (Easterling et al. 2000) that have negative effects on crop production. Springs weather is projected to become wetter, which not only delays planting but also increases the risk for occurrence of seedling diseases, particularly those caused by oomycetes (Rosenzweig et al. 2002; Wuebbles and Hayhoe 2004). In Iowa, weather conditions at planting vary considerably. Although soil temperatures at planting may be >15°C, it is not uncommon for cold fronts, accompanied by rainfall >20 mm and soil temperatures dropping below 10°C to occur during the planting period (mid-April through mid-May). Moreover, early planting is recommended to maximize yield in the Midwest and consequently soybeans are often exposed to cool spring temperatures (De Bruin and Pedersen 2008). Emergence of soybeans is optimal when soil temperatures are >18°C and consequently planting is not recommended if soil temperatures are below 13°C (Pedersen et al. 2004). Moreover, low soil temperatures at planting keep seedlings at a germination stage at which they are susceptible to infection by soilborne pathogens for a longer period of time and consequently increase the risk of occurrence of damping-off (Martin and Loper 1999). In controlled environments emergence was significantly reduced by Pythium spp. when seeds were exposed to cold stress at planting (Thomson et al. 1971). The effect of periods of cold stress that occur within a week after planting on emergence of soybean is unknown. Our preliminary research suggested that periods of cold stress within 1 to 5 days after
planting may reduce plant stand particularly when the *Pythium sylvaticum* is present (Serrano and Robertson 2016).

Seed treatments are often recommended to protect stand by managing seedling disease, and their use in the U.S. has increased. It is estimated that 8% of soybean seeds were treated in 1996, compared to 30% in 2008 (Munkvold 2009). The benefit of soybean seed treatments protecting plant stand and yield particularly under cool and wet conditions at planting has been documented (Bradley 2008; Dorrance et al. 2009). Seed treatments that contain metalaxyl or mefenoxam and, more recently, ethaboxam are usually applied for control of *Pythium* spp. (Dorrance et al. 2012; Hartman et al. 2015). Metalaxyl is a phynlamide fungicide which inhibits RNA synthesis in oomycetes (FRAC 2016), and ethaboxam is a thiazole carboxamide fungicide that interferes with oomycete microtubule assembly and has shown efficacy against *Pythium* and *Phytophthora* species (Uchida et al. 2005; Dorrance et al. 2012)

In this study we simulated periods of cold stress soon after planting to (i) determine if cold stress soon after planting increase the risk of damping-off; and (ii) evaluate the efficacy of a seed treatment at protecting seedlings subjected to cold stress. We hypothesize that cold stress increases susceptibility to damping-off and consequently the use of a fungicide seed treatment in adverse conditions may be an important management tool to protect seedlings. Results provided in this study may be useful for disease management of seedling diseases caused by *Pythium* spp. particularly when soybeans are planted early.
Materials and Methods

Plant material and seed treatment. Soybean cultivar IA 2094, relative maturity 2.4 (Weidenbenner et al. 2014), was used in this study. Seed (454 g) was treated in a plastic Ziploc® bag with 1 ml of the commercial product INTEGO SUITE™ (Valent USA) according to label recommendations for soybean: clothianidin 0.081 mg, ipconazole 0.004 mg, ethaboxam 0.012 mg, and metalaxyl 0.0032 mg of active ingredient per seed. The seed was thoroughly mixed with the seed treatment to ensure good seed coverage.

Inoculum production. Autoclavable bags with a filter patch (Myco Supply Company Inc., Pittsburgh, PA) were filled with approximately 600 cm³ of millet seed that had been soaked overnight and autoclaved twice, with 24 hours between each cycle. *Pythium sylvaticum* isolate IASO 2-8.18 (Matthiesen et al. 2016) was grown on dilute V8 juice media with antibiotics (40 mL V8 juice, 0.6 g CaCO₃, 0.2 g Bacto yeast extract, 1 g sucrose, 0.01 g cholesterol, 20 g Bacto agar, 0.05 g neomycin sulfate, 0.01 g chloramphenicol and 1.0 L distilled water) in the dark at room temperature. Each bag was inoculated with 20 pieces (1 cm²) of media colonized with 3-day-old mycelium, and kept in the dark at room temperature (20°C) for 10 days. The bags were shaken once per day to ensure colonization of all millet seed.

Growth chamber experiment. Polystyrene cups (237 ml) with 3 holes (2 mm diameter) punctured through the base were filled with 90 ml of vermiculite. A layer of either inoculum or sterile millet (5 ml per cup) was placed on top of the vermiculite and a second layer of 40 ml vermiculite was added. After soybean seed (10 seeds
per cup) were placed on top of the second layer of vermiculite, a final layer of vermiculite (90 ml) was used to cover the seed. The cups were watered daily until runoff and kept in a growth chamber with 12 hours day light:dark cycle at 18ºC. Depending on the treatment, at either 24 or 96 hours after planting, cups were moved to growth chambers at 4ºC or 10ºC for various periods (24, 48 and 96 hours) before they were returned to 18ºC. Emergence, root rot severity, fresh root weight and fresh shoot weight were evaluated 21 days after planting. Emergence was assessed by counting the amount of seedlings with cotyledons completely emerged from the vermiculite. Root rot severity was evaluated in a 0 to 3 scale modified from Balk (2014) where 0 = all roots healthy; 1 = 1 to 20 % root system has visible lesions on lateral roots; 2 = 21 to 75 % of roots show visible symptoms, with symptoms beginning to show on tap root; 3 = 76 to 100 % of roots infected with symptoms on lateral roots and tap roots, short or very few lateral roots observed. Average fresh root weight per plant and average fresh shoot weight per plant were calculated by measuring the weight divided by the number of emerged seedlings in each cup.

The experimental design was a five-way factorial design with 2 levels of cold stress temperature (4ºC, 10ºC), 3 levels of cold stress duration (24, 48 and 96 hours), 2 levels of timing of cold stress occurrence (24 hours and 96 hours after planting), 2 levels of pathogen (inoculated and a non-inoculated control), and 2 levels of seed treatment (Intego Suite™ and untreated seed). There were four reps (cups) per treatment and the experiment was repeated once.

**Data analysis.** All data were analyzed using SAS 9.3 (SAS Institute, Cary, NC). Emergence was analyzed with PROC LOGISTIC to determine the probability of
emergence as a function of treatments. A contrast analysis with the addition of the EXP statement was performed to compare the odds of success of emergence. Root rot severity, fresh root weight and fresh shoot weight were analyzed with PROC GLIMMIX and a contrast analysis for means comparison.

Results

**Emergence.** No difference between runs of the experiment was observed (P=0.7837) and therefore data from both runs were pooled. Neither cold stress temperature nor the timing at which cold stress was imposed affected emergence (P=0.5052 and P=0.6590, respectively). Interaction pathogen x seed treatment was detected (P<0.0001) (Table 1). Emergence was reduced in cups inoculated with *Pythium sylvaticum* when compared to non-inoculated cups (P <0.0001). Use of the seed treatment increased the odds of successful emergence ratio by 175.9 % (P<0.0001) (Table 2) (Fig. 1). A longer period of cold stress duration reduced emergence (P=0.0443) (Table 1). Emergence of untreated seed was lower when subjected to 96 hours of cold stress compared to 24 hours of cold stress (P=0.0067); in contrast, the duration of cold stress had no effect on treated seed (P=0.4363) (Table 2).

**Root rot severity.** No difference between runs of the experiment were observed (P=0.4812) so the data for both runs were pooled. Presence of *P. sylvaticum* and longer cold stress duration increased root rot severity (P<0.0001, P=0.0020, respectively), and the seed treatment reduced root rot severity (P<0.0001). Interaction pathogen x seed treatment was detected (P=0.0018) (Table
1). Lower root rot severity was observed in treated seed and non-inoculated controls (Fig. 2). Root rot severity increased with longer cold stress duration in untreated seed (P=0.0030 and P=0.0017 for 48 and 96h, respectively) (Table 2) but no effect of cold stress duration was detected in treated seed (Table 2). No difference between cold stress timing (P=0.3209) or cold stress temperatures (P=0.4235) was detected on the severity of root rot (Table 1).

**Root weight.** *P. sylvaticum* and cold stress duration reduced root weight (P<0.0001 and P<0.0001, respectively), but cold stress temperature, cold stress timing and seed treatment had no effect on root weight (P=0.5457, P=0.0908 and P=0.7087, respectively). Interaction pathogen x seed treatment was detected (P<0.0001) (Table 1). Cold stress duration reduced root weight in treated and untreated seed (Fig. 3). Lower root weight after 96 hours of cold stress was detected when compared to 24 and 48 hours of cold stress duration in untreated seed (P<0.0001, P<0.0001, respectively) and treated seed (P=0.0002, P=0.0008, respectively) (Table 2). Difference between runs was detected (P<0.0001, Table 1) and higher values of root weight were observed in run 2. Results from run 1 and run 2 are presented in supplemental figs 1 and 2.

**Shoot weight.** Shoot weight was reduced by the presence of the pathogen (P<0.0001) and longer cold stress duration (P<0.0001). Unlike root weight, seed treatment improved shoot weight (P<0.0001). Interaction pathogen x seed treatment was detected (P<0.0001) (Table 1). Overall, shoot weight was greater with the use of seed treatment when the pathogen was present but no improvement was observed in non-inoculated treatments (Fig. 4). Lower shoot weight was detected
after 96 hours of cold stress when compared to 24 and 48 hours of cold stress duration in untreated seed (P<0.0001, P<0.0001, respectively) and with the use of seed treatment (P=0.0009, P=0238, respectively) (Table 2). No difference between cold stress temperatures and timing of cold stress was detected on shoot weight (P=0.7862, P=0.5570, respectively) (Table 1). Difference between runs was detected (P<0.0001) and higher shoot weight values were observed in run 2. Results from run 1 and run 2 are presented in supplemental figs 3 and 4.

**Discussion**

In this study we confirmed under controlled conditions previous reports that cold stress increased the occurrence of seedling diseases and stand problems in Iowa (Robertson and Munkvold 2012). In previous preliminary research we observed a significant effect of *P. sylvaticum* on emergence when seedlings were subjected to cold stress (4ºC) at planting (Serrano and Robertson 2016). In this study we evaluated temperatures, timings and cold stress durations that were more representative of those that would occur in the field. Cold stress duration affected emergence more than the temperature or the timing at which the cold stress occurred. Our data suggest that 24 or more hours of cold stress is deleterious to emergence of soybean in presence of inoculum of *P. sylvaticum*. The occurrence of suboptimal temperatures delays emergence, providing increased chances for seedling infection (Martin and Loper 1999).

Other authors have also suggested a role for cold stress increasing the occurrence of seedling diseases. Bradley (2008) reported that seedlings diseases
were more prevalent in North Dakota when the seed was planted in temperatures <15°C and when more than 100 mm of rain occurred during the period from 1 week before planting to 3 weeks after planting. Seedling diseases were less prevalent when warmer temperatures and low precipitation occurred at planting (Bradley 2008). Similarly, Dorrance et al. (2009) observed reduced plant stand in field trials in Ohio, South Dakota, and Ontario when heavy precipitation occurred within 2 weeks after planting, but no difference in plant stand were detected at Iowa, Nebraska, Wisconsin and Ohio where no heavy precipitation occurred during the planting season.

Our observations are similar to those of Thomson et al. (1971) who also demonstrated that cold stress increased damping-off caused by *Pythium* spp. In their experiments, Thomson and his colleagues investigated cold stress only at planting. They also observed reduced emergence in their non-inoculated control probably caused by chilling injury during imbibition that occurs at temperatures below 15°C (Thomson et al. 1971; Bramlage et al. 1978; Leopold 1980). Seed imbibition is the first event in the germination process and can be completed in few hours at optimal temperatures (Tully et al. 1981; Vertucci and Leopold 1983). We planted soybean seeds at an optimal soil temperature (18°C) and then we subjected the seedlings to cold stress 24 and 96 hours after planting. These treatments are more representative of what occurs at planting in Iowa because it is very unlikely that a soybean farmer would plant when soil temperatures were less than 10°C. Recommended planting dates for soybean in Iowa are April 25 in the southern two-thirds of the state and May 1 in the northern third of the state when the average soil
temperature is normally between 10.6ºC and 14.8ºC (De Bruin and Pedersen 2008). However, most farmers wait until soil temperatures are above 13ºC before planting. It is not uncommon for soil temperatures in Iowa to fluctuate considerably during this period because when cold fronts pass across the state, soil temperatures often drop below 10ºC for a few days (Serrano et al. 2015). Thus, in our experiments we probably avoided the detrimental effect of chilling injury during imbibition. Consequently the reductions in emergence that we observed in our inoculated treatments can be attributed to the detrimental effect of the pathogen, since we observed no effect of cold stress on our non-inoculated controls.

The efficacy of soybean seed treatments such as metalaxyl, mefenoxam and ethaboxam under controlled conditions has been demonstrated (Dorrance et al. 2012; Urrea et al. 2013). In this study we demonstrate that seed treatments are highly effective at protecting seedlings that are subjected to periods of cold stress soon after planting. Seed treatments are an important disease management tool that should be considered if suboptimal temperatures at or soon after planting are expected, particularly in fields with history of damping-off. Moreover, since early planting increases the chances that seedlings may be exposed to cool and wet soils soon after planting, using a seed treatment may help to protect plant stand.

Although we observed reduced root rot severity when the seed was treated with fungicides, no effect of the seed treatment on root biomass was detected in inoculated plants. Dorrance et al. (2009) suggested that the fact that the soybean seed coat containing the seed treatment is pushed out of the ground during germination process (epigeal germination), shortens the effective period of the seed
treatment. In our study we observed poor lateral root development in our inoculated cups even with the use of a seed treatment, probably because of infection of the lateral roots by *Pythium*. We hypothesize that because of the shorter effective period, the lateral roots were not adequately protected by the seed treatment, and consequently we measured lower root biomass.

In this study we demonstrated under controlled conditions that cold stress increases susceptibility of soybean to damping-off caused by *P. sylvaticum*, thus supporting previous suggestions that reduced plant stand due to increased seedling disease occurs with cold stress that is imposed soon after planting (Bradley 2008; Robertson and Munkvold 2012). Furthermore, we showed that a seed treatment was effective at protecting soybean seedlings under these adverse conditions and that the efficacy was not affected by the duration or timing of imposition of the cold stress period. Consequently, seed treatments can be used to protect soybean stand particularly in areas with a history of seedling disease and where extreme fluctuations in weather conditions occur during or soon after planting.

**Acknowledgments**

The authors thank Iowa Soybean Association, North Central Soybean Research Program and Valent USA Corporation for providing funding for this research.
References


FRAC. 2016. FRAC Code List 2016: Fungicides sorted by mode of action (including FRAC code numbering). Fungicide Resistance Action Committee


http://www.extension.iastate.edu/Publications/PM1851.pdf.

**Table 1.** P-values from analyses of variance for effect of cold stress treatments on soybean seedling probability of emergence, root rot severity, and root and shoot weight.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Probability of Emergence</th>
<th>Root rot severity</th>
<th>Root weight</th>
<th>Shoot weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run</td>
<td>0.7837</td>
<td>0.4812</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Timing of the initiation of cold stress (hap)</td>
<td>0.6590</td>
<td>0.3209</td>
<td>0.0908</td>
<td>0.5570</td>
</tr>
<tr>
<td>Duration of cold stress (h)</td>
<td>0.0443</td>
<td>0.0020</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Cold stress temperature (t)</td>
<td>0.5052</td>
<td>0.4235</td>
<td>0.5457</td>
<td>0.7862</td>
</tr>
<tr>
<td>Fungicide seed treatment (st)</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.7087</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Presence of pathogen (p)</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>hap x h</td>
<td>0.0128</td>
<td>0.0132</td>
<td>&lt;.0001</td>
<td>0.2129</td>
</tr>
<tr>
<td>hap x t</td>
<td>0.0057</td>
<td>0.3872</td>
<td>0.1375</td>
<td>0.0760</td>
</tr>
<tr>
<td>h x t</td>
<td>0.4873</td>
<td>0.7128</td>
<td>0.2384</td>
<td>0.1739</td>
</tr>
<tr>
<td>hap x st</td>
<td>0.6509</td>
<td>0.8979</td>
<td>0.4205</td>
<td>0.0682</td>
</tr>
<tr>
<td>h x st</td>
<td>0.2937</td>
<td>0.3310</td>
<td>0.7626</td>
<td>0.2849</td>
</tr>
<tr>
<td>t x st</td>
<td>0.6851</td>
<td>0.9491</td>
<td>0.7397</td>
<td>0.6955</td>
</tr>
<tr>
<td>hap x p</td>
<td>0.1054</td>
<td>0.0013</td>
<td>0.0044</td>
<td>0.6939</td>
</tr>
<tr>
<td>h x p</td>
<td>0.6960</td>
<td>0.0027</td>
<td>0.2162</td>
<td>0.0086</td>
</tr>
<tr>
<td>t x p</td>
<td>0.3813</td>
<td>0.3874</td>
<td>0.0062</td>
<td>0.1444</td>
</tr>
<tr>
<td>p x st</td>
<td>&lt;.0001</td>
<td>0.0018</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
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</tbody>
</table>

\(^2\)Pooled data from two independent runs of the experiment.
Table 2. Contrast analysis for probability of emergence, root rot severity, and root and shoot weight of soybean seedlings subjected to cold stress.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Difference in the odds of successful emergence (%)</th>
<th>Odds ratio of emergence</th>
<th>P-value of Emergence</th>
<th>P-value root rot severity</th>
<th>P-value root weight</th>
<th>P-value shoot weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>All treatments</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INTEGO SUITE™ vs untreated</td>
<td>175.9</td>
<td>2.7593</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
<td>0.7087</td>
<td>&lt;.0001</td>
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<tr>
<td>Untreated seed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hap™ vs 96 hap</td>
<td>0.2</td>
<td>1.0017</td>
<td>0.9941</td>
<td>0.4285</td>
<td>0.0782</td>
<td>0.3813</td>
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<tr>
<td>24h duration™ vs 48h duration</td>
<td>28.9</td>
<td>1.2886</td>
<td>0.4123</td>
<td>0.0017</td>
<td>0.5317</td>
<td>0.8113</td>
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<tr>
<td>24h duration vs 96h duration</td>
<td>98.2</td>
<td>1.9819</td>
<td>0.0067</td>
<td>0.8676</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
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<tr>
<td>48h duration vs 96h duration</td>
<td>53.8</td>
<td>1.5379</td>
<td>0.1343</td>
<td>0.0030</td>
<td>&lt;.0001</td>
<td>&lt;.0001</td>
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<tr>
<td>4°C vs 10°C</td>
<td>17.6</td>
<td>1.1761</td>
<td>0.4842</td>
<td>0.5416</td>
<td>0.5085</td>
<td>0.6399</td>
</tr>
<tr>
<td>Seed treatment</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>24 hap vs 96 hap</td>
<td>-12.7</td>
<td>0.8735</td>
<td>0.4869</td>
<td>0.5402</td>
<td>0.5293</td>
<td>0.0878</td>
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<tr>
<td>24h duration vs 48h duration</td>
<td>22.0</td>
<td>1.2203</td>
<td>0.4070</td>
<td>0.2436</td>
<td>0.7407</td>
<td>0.2849</td>
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Table 2 continued.

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</thead>
<tbody>
<tr>
<td>24h duration vs 96h duration</td>
<td>20.8</td>
<td>1.2084</td>
<td>0.4363</td>
<td>0.8025</td>
<td>0.0002</td>
<td>0.0009</td>
</tr>
<tr>
<td>48h duration vs 96h duration</td>
<td>-1.0</td>
<td>0.9903</td>
<td>0.9663</td>
<td>0.1570</td>
<td>0.0008</td>
<td>0.0238</td>
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<tr>
<td>4°C vs 10°C</td>
<td>4.0</td>
<td>1.0403</td>
<td>0.8392</td>
<td>0.6018</td>
<td>0.8471</td>
<td>0.9322</td>
</tr>
</tbody>
</table>

<sup>1</sup>odds ratio of treatments compared in the contrast.

<sup>2</sup>odds ratio difference expressed as percentage calculated with the formula % = (odds ratio – 1)*100.

<sup>3</sup>seed treatment INTEGO SUITE™ contains clothianidin 0.081 mg, ipconazole 0.004 mg, ethaboxam 0.012 mg, and metalaxyl 0.0032 mg of active ingredient per seed.

<sup>4</sup>timing of cold stress initiation (hours after planting).

<sup>5</sup>only untreated seed was included in this contrast.

<sup>6</sup>cold stress duration period.

<sup>7</sup>only seed treatment was included in this contrast.
Fig. 1 Emergence (%, predicted means) 21 days after planting for soybean seedlings planted at 18°C and subjected to three periods of cold stress 48 hours after planting (48 hap) or 96 hours after planting (96 hap). A, 4°C *Pythium sylvaticum*-inoculated cups; B, 4°C non-inoculated cups; C, 10°C *P. sylvaticum* inoculated cups; D, 10°C non-inoculated cups. Error bars indicate confidence interval 95%. Emergence of inoculated control at 18°C was 75.0 ± 8.0 %.
Fig. 2 Root rot severity rating 21 days after planting for soybean seedlings planted at 18°C and subjected to three periods of cold stress 48 hours after planting (48 hap) or 96 hours after planting (96 hap). A, 4°C *Pythium sylvaticum*-inoculated cups; B, 4°C non-inoculated cups; C, 10°C *P. sylvaticum*-inoculated cups; D, 10°C non-inoculated cups. Error bars indicate confidence interval 95%. A score of 0 to 3 was used to assess root rot severity where 0 = all roots healthy; 1 = 1 to 20% root system has visible lesions on lateral roots; 2 = 21 to 75% of roots show visible symptoms, with symptoms beginning to show on tap root; 3 = 76 to 100% of roots infected with symptoms on lateral roots and tap roots, short or very few lateral roots are observed.
Fig. 3 Fresh root weight per plant (mg) measured 21 days after planting for soybean seedlings planted at 18ºC and subjected to three periods of cold stress 48 hours after planting (48 hap) or 96 hours after planting (96 hap). A, 4ºC *Pythium sylvaticum*-inoculated cups; B, 4ºC non-inoculated cups; C, 10ºC *P. sylvaticum* inoculated cups; D, 10ºC non-inoculated cups. Error bars indicate confidence interval 95%.
Fig. 4 Fresh shoot weight per plant (mg) 21 days after planting for soybean seedlings planted at 18ºC and subjected to three periods of cold stress 48 hours after planting (48 hap) or 96 hours after planting (96 hap). A, 4ºC *Pythium sylvaticum*-inoculated cups; B, 4ºC non-inoculated cups; C, 10ºC *P. sylvaticum*-inoculated cups; D, 10ºC non-inoculated cups. Error bars indicate confidence interval 95%.
Supplemental Fig. 1 Results for Run 1 of fresh root weight per plant (mg) measured 21 days after planting for soybean seedlings planted at 18°C and subjected to periods of cold stress 48 hours after planting (48 hap) or 96 hours after planting (96 hap). A, 4°C Pythium sylvaticum-inoculated cups; B, 4°C non-inoculated cups; C, 10°C P. sylvaticum inoculated cups; D, 10°C non-inoculated cups. Error bars indicate confidence interval 95%.
Supplemental Fig. 2 Results for Run 2 of fresh root weight per plant (mg) measured 21 days after planting for soybean seedlings planted at 18ºC and subjected to periods of cold stress 48 hours after planting (48 hap) or 96 hours after planting (96 hap). A, 4ºC *Pythium sylvaticum*-inoculated cups; B, 4ºC non-inoculated cups; C, 10ºC *P. sylvaticum* inoculated cups; D, 10ºC non-inoculated cups. Error bars indicate confidence interval 95%.
Supplemental Fig. 3 Results of Run 1 of fresh shoot weight per plant (mg) 21 days after planting for soybean seedlings planted at 18°C and subjected to periods of cold stress 48 hours after planting (48 hap) or 96 hours after planting (96 hap). A, 4°C *Pythium sylvaticum*-inoculated cups; B, 4°C non-inoculated cups; C, 10°C *P. sylvaticum*-inoculated cups; D, 10°C non-inoculated cups. Error bars indicate confidence interval 95%.
Supplemental Fig. 4 Results of Run 2 of fresh shoot weight per plant (mg) 21 days after planting for soybean seedlings planted at 18°C and subjected to periods of cold stress 48 hours after planting (48 hap) or 96 hours after planting (96 hap). A, 4°C *Pythium sylvaticum*-inoculated cups; B, 4°C non-inoculated cups; C, 10°C *P. sylvaticum*-inoculated cups; D, 10°C non-inoculated cups. Error bars indicate confidence interval 95%.
CHAPTER 4.
THE EFFECT OF COLD STRESS ON DAMPING-OFF OF SOYBEAN, SEED EXUDATION AND SPORANGIA GERMINATION OF *Pythium sylvaticum*

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**Abstract**

Damping-off of soybean caused by *Pythium* spp. is frequently associated with cold, wet soils during germination. To improve our understanding of the effect of the timing of cold stress on *Pythium* damping-off, we performed growth chamber and laboratory experiments. In the growth chamber, we tested the effect of a 96 hour period of cold stress at different times after planting on soybean damping-off. A three-way factorial experiment was performed with two cold stress temperatures (4°C and 10°C); seven levels of cold stress timing (no cold stress, and initiation of cold stress 0, 1, 2, 4, 6, 8 days after planting); and inoculation with *P. sylvaticum*-infested millet or sterile millet (non-inoculated control). We observed increased susceptibility to damping-off with cold stress, particularly when cold stress began 2 or 4 days after planting. In the non-inoculated controls, no effect of cold stress on emergence was observed. Emergence and seedling growth was delayed when *P.*
sylvaticum was present. In the laboratory, we evaluated seed exudation, mycelial growth and sporangia germination of *P. sylvaticum* at 4ºC, 10ºC and 18ºC. When soybean seeds were imbibed at 4ºC, 10ºC and 18ºC, we detected the highest level of seed exudation at 4ºC. Low temperatures delayed mycelial growth of *P. sylvaticum* although the pathogen was still able to grow at 4ºC. More sporangia germinated in response to seed exudates when they were incubated at 24ºC, compared to 4ºC and 10ºC. Moreover, more sporangia germinated in seed exudates from seeds imbibed at 4ºC compared to those in seed exudates from seeds imbibed at 10ºC and 18ºC. Results from this study demonstrate that cold stress events that occur 2 to 4 days after planting increases soybean susceptibility to damping-off, presumably because of increased seed exudation that stimulates germination of sporangia of *P. sylvaticum* in the vicinity of the seed.

**Introduction**

Soybean (*Glycine max* (L.) Merr.) is an important oilseed crop in Iowa and United States. A total of 83.4 million acres were planted in US in 2016. In Iowa, 9.5 million acres were planted to soybean in 2016 and the crop value was estimated at $5.4 billion dollars. ([www.nass.usda.gov](http://www.nass.usda.gov)).

Once a soybean field is planted, the occurrence of seedling diseases may affect soybean production. Seedling diseases resulted in losses of up to 55 million bushels annually between 2006 and 2009, which is equivalent to $558 million, and were the second most damaging disease of soybean in the U.S. during that period (Koenning and Wrather 2010). *Pythium* spp. are the predominant pathogens causing
seedling diseases in Iowa and the North Central Region of the U.S (Rizvi and Yang 1996; Murillo-Williams and Pedersen 2008; Rojas et al. 2017). These oomycetes cause “damping-off” of developing seedlings. Symptoms on affected seedlings may include general discoloration, yellow to brown lesions, root rot and seed rot (Hartman et al. 2015).

*Pythium sylvaticum*, first described by Campbell and Hendrix (1967), was one of the most frequently isolated species recovered from diseased seedlings in recent surveys in Iowa and the North Central Region of the U.S. (Matthiesen et al. 2016; Rojas et al. 2017). *P. sylvaticum* was the first known heterothallic species of *Pythium* (Campbell and Hendrix 1967). It produces intercalary or terminal globose sporangia (hyphal swellings), and sporangia germinate directly in response to seed exudates and volatile compounds, (Campbell and Hendrix 1967; Van der Plaats-Niterink 1981; Nelson 1987; Nelson and Craft 1989).

Cool and wet soils are frequently associated with higher occurrence of damping-off caused by *Pythium* spp. because low temperatures at planting keep seedlings at a susceptible stage for a longer period of time, providing greater chances for seedling infection (Martin and Loper 1999). Moreover, field observations have suggested a role of cold stress increasing the occurrence of seedling diseases and poor plant stand (Bradley 2008; Robertson and Munkvold 2012). In controlled environments, Thomson et al. (1971) demonstrated that periods of cold stress (4°C) at planting increased soybean susceptibility to damping-off. Similarly, in preliminary research we observed that cold stress (4°C) that occurred 1 day after planting
increased the susceptibility of soybean to *P. sylvaticum* and reduced the emergence by up to 70% (Serrano and Robertson 2016).

Planting is not recommended if soil temperatures are below 13 ºC (Pedersen et al. 2004). In Iowa, soybeans are usually planted in late April through May when soil temperatures are 13 ºC and warming. However, it is not uncommon for cold fronts to pass through the state and for soil temperatures to drop below 10 ºC for a few days (Serrano et al. 2015). It is unknown how cold stress that occurs a few to several days after planting affects the susceptibility of soybean to damping-off.

Cold stress has a detrimental effect on soybean germination and growth. For example, 50% of soybean seeds germinated after 24 hours at 23 ºC whereas at 10 ºC 120 hours were required for 50% of seed to germinate (Duke et al. 1977). Once the seed has germinated, hypocotyl elongation is extremely slow at 10 ºC and below (Hatfield and Egli 1974). When seed is planted at temperatures below 15 ºC, there is a high risk of chilling injury during imbibition, and there is more seed exudation than at higher temperatures (Bramlage et al. 1978; Leopold 1980). It is possible that increased seed exudation increases *Pythium* sporangia germination, and consequently risk of infection

*P. sylvaticum* may cause more disease at lower temperatures. Rojas et al. (2017) found greater seed rot at 13 ºC than at 20 ºC. Although mycelial growth is optimal at 25 ºC, the pathogen can grow at temperatures below 5 ºC (Van der Plaats-Niterink 1981). An improved understanding of how low temperatures affect the soybean-*P. sylvaticum* interaction may help to explain the higher occurrence of damping-off after periods of cold stress.
The objectives of this study were to: (i) determine how cold stress imposed at different times after planting affects the susceptibility of soybean to damping-off; (ii) measure the effect of cold stress on mycelial growth and sporangia germination of *P. sylvaticum*, (iii) evaluate the effect of cold stress on soybean seed exudation during imbibition, and (iv) compare sporangia germination in response to seed exudates from seeds imbibed at different temperatures. We hypothesized that since *P. sylvaticum* is able to grow at low temperatures, and soybean germination and growth is slowed during periods of cold stress, exudates released from the germinating seed stimulate the pathogen and increase damping-off. The data from this study will improve our understanding of how periods of cold stress increase the occurrence of damping-off, and this in turn will help to improve disease management recommendations.

**Materials and Methods**

**Plant material and oomycete culture.** All experiments were done using soybean cultivar IA 2094, which has a relative maturity of 2.4 and was susceptible to *Pythium* damping-off in previous experiments (Weidenbenner et al. 2014; Serrano and Robertson 2016). *Pythium sylvaticum* isolate IASO 2-8.18 (Matthiesen et al. 2016) was used for inoculum production, mycelial growth and sporangia germination experiments.

**Cold stress timing assay.** Ninety milliliters of vermiculite per cup was added to foam cups (237 ml) and three 2-mm-diameter holes were punched through the base of the cup. A layer of pathogen-infested or sterile millet (5 ml per cup) was
placed on top of the vermiculite and another layer of 40 ml vermiculite covered the millet. Ten soybean seeds per cup were placed on the second layer of vermiculite, and a final layer of vermiculite (90 ml) was used to cover the seed.

*Pythium sylvaticum*-infested millet was used as a source of inoculum. Autoclavable bags with a filter patch (Myco Supply Company Inc., Pittsburgh, PA) were filled with approximately 600 cm$^3$ of millet seed that had been soaked overnight were autoclaved twice with 24 hours between each cycle. *Pythium sylvaticum* was grown on dilute V8 juice media (Stewart and Robertson 2012) and were kept in the dark at 20°C. Each bag was inoculated with 20 pieces (1 cm$^2$) of media with 3-day-old mycelium, and kept in the dark at 20°C for 10 days; the bags were shaken once per day to ensure colonization of all millet seed. The experimental design was a three-way factorial with two cold stress temperatures (4°C or 10°C, for 96 hours); seven levels of cold stress timing (no cold stress (18°C), or imposition of cold stress beginning 0, 1, 2, 4, 6, 8 days after planting). Five individual cups (replications) were inoculated with *P. sylvaticum*-infested millet or sterile millet (non-inoculated control).

Emergence, root fresh weight and shoot fresh weight data were taken 21 days after planting. Average fresh root weight and average fresh shoot weight per plant was calculated by measuring the total tissue weight divided by the number of emerged seedlings in each cup.

Seedling growth and emergence was assessed in soybeans planted at 18°C. Emergence was assessed 3, 6, 9, 12, 15, 18, and 21 days after planting by counting the number of seedlings with cotyledons completely pushed out of the ground. Additional replications were planted for use in measuring seedling length 1, 2, 4, 6
and 8 days after planting. Seedling length was measured from the proximal end of the cotyledon to the root tip as described in Ellis et al. (2011). All experiments were repeated once.

**Mycelial growth of *P. sylvaticum***. Plugs (3 mm in diameter) of *P. sylvaticum* -colonized diluted V8 media were placed 2 mm from the edge of a 90-mm-diameter petri dish plates filled with dilute V8 juice media and incubated in the dark at 4°C, 10°C, or 18°C. Mycelial growth from the edge of the plug to the furthest edge of the colony was measured 48, 72, and 96 hours after inoculation. The experimental design was completely randomized with five replicates. The experiment was repeated once.

**Solute leakage after imbibition at different temperatures**. Solute leakage was measured with the Electrical Conductivity Test modified from AOSA (2009). Soybean seeds were inspected visually for damage to the seed coat and undamaged seed were selected for the test. Four reps of 50 seeds per treatment were weighed and then imbibed for 24 hours in 75 ml of distilled sterile water at 4 °C, 10 °C and 18 °C. The water was equilibrated for at least 4 hours in growth chambers at each temperature before imbibition. After imbibition the seeds were removed and the remaining solution was equilibrated at 25°C for at least 4 hours. The electrical conductivity of each rep of each solution was measured with an Electrical Conductivity meter (Solution Analyzer, Amber Science Inc., Model 4603, San Diego, CA) that had been calibrated with a potassium chloride conductivity standard solution (718 µS/cm) at 25°C (Ricca Chemical Company, Arlington, TX). The conductivity of a water control (blank) was subtracted from each reading before
calculations of electrical conductivity were done. Data for electrical conductivity were calculated per gram of seed weight for each replicate. The experiment was repeated once.

**Production of sporangia for sporangia germination assays.** *Pythium sylvaticum* was grown on dilute V8 juice plus neomycin sulfate (0.05 g/L) and chloramphenicol (0.01 g/L) (Stewart and Robertson 2012) at 24°C for 48 hours in the dark. A method modified from Nelson and Craft (1989) was used for sporangia production. Briefly, colonized disks (3 mm diameter) from 2-day-old cultures of the pathogen were placed in sterile petri dishes and submerged for two consecutive 10-minutes periods in approximately 20 ml of leaching buffer (pH 5.8) containing 0.01 M Ca(NO₃)₂·4H₂O, 0.004 M MgSO₄·7H₂O, and 0.005 M KNO₃, followed by one 3-hours submergence period. The buffer was replaced with fresh buffer after each 10-minutes period of submergence. Finally, the disks were rinsed with sterile distilled water and incubated in a petri dish at 24°C for 48 hours to facilitate sporangia production.

**Effect of exudates from seeds imbibed at cold temperatures on sporangia germination.** A modification of the method described by Nelson and Craft (1989) was used to assess sporangia germination. Prepared culture disks containing sporangia were placed approximately 10 mm apart on sterile glass slides (three culture disks per slide). Ten microliters of either seed exudate from seeds imbibed at 4°C, 10°C, 18°C (as described above) or sterile water were pipetted carefully on top of each culture disk so that the disk was submerged in the solution. The slides of each culture disk were incubated for 2 and 3 hours at 24°C in the dark,
and then the culture disks were stained with 0.03% acid fuchsin in 85% lactic acid and examined microscopically (X200). The number of germinated and non-germinated sporangia were recorded. The experimental design was a randomized complete block randomized with 3 imbibition-temperature treatments and 3 replicates. The experiment was repeated once.

**Effect of cold temperatures on sporangia germination.** Soybean seeds were imbibed at 18°C, as described above, and 10 microliters of the seed exudate solution or sterile water was pipetted on top of culture disks as described above. The slides were incubated for 3 hours at 4°C, 10°C and 18°C in the dark. The culture disks were stained and sporangia were counted as described above. The experimental design was a randomized complete block with 3 incubation-temperature treatments and 3 replicates. The experiment was repeated once.

**Data analysis.** All data were analyzed using SAS 9.3 (SAS Institute, Cary, NC). Emergence 21 days after planting was analyzed with PROC LOGISTIC to determine the probability of emergence as a function of treatments. Emergence over time, root weight, shoot weight, mycelial growth, solute leakage and sporangia germination data were analyzed with PROC GLIMMIX. When a treatment effect was detected (P<0.05), the Tukey test (alpha = 0.05) was used for mean comparisons. In root and shoot weight the Tukey test did not reflect all significant comparisons and therefore LSD test (alpha = 0.05) was used.
Results

Effect of cold stress at different times after planting on soybean growth and susceptibility to *P. sylvaticum*. Significant differences in emergence among treatments were detected for cold stress temperatures (P=0.0006), cold stress timing (P<0.0001) and presence of the pathogen (P<0.0001). A temperature x pathogen interaction (P=0.0073) and timing x pathogen (P<0.0001) interaction was detected (Table 1). Cold stress and presence of *P. sylvaticum* increased damping-off. Emergence was lowest in inoculated cups exposed to either 4°C or 10°C at 2 and 4 days after planting (Figure 1). The emergence was lower in inoculated cups exposed to 4°C than 10°C, and this difference was not observed in non-inoculated cups. When cold stress was absent, no differences in emergence were observed between inoculated and non-inoculated treatments (Figure 1). However, in the absence of cold stress emergence was reduced 6, 7, 8 and 10 days after planting in the inoculated cups compared to the non-inoculated cups (P<0.0001) (Figure 2).

Differences between runs in seedling length at 18°C were detected (P=0.0135) and results from each run are presented separately (Figure 3). Seedlings in *P. sylvaticum*-inoculated cups were shorter than in control cups in both runs at 4, 6, and 8 days after planting (P<0.0001) (Figure 3).

The presence of *P. sylvaticum* reduced root weight (P<0.0001), and significant effects were detected for temperature and timing of cold period initiation (P<0.0001, P=0.0012, respectively) (Table 1). Lower root weight was also observed in cups subjected to 4°C in comparison to 10°C (Figure 4). Lower root weight was observed in all the inoculated treatments (18°C and subjected to cold stress either
4°C or 10°C) when compared to non-inoculated treatments. In inoculated cups, root weight was further reduced with cold stress at 4°C but not with cold stress at 10°C when compared to the inoculated control at 18°C (Figure 4). Overall, in non-inoculated treatments cold stress did not reduce root weight when compared to 18°C controls, except when 4 °C occurred 0 and 6 days after planting. Differences between runs in root weight were detected (P=0.0003); data from each run are presented in Supplemental Table 1. No root weight mean was calculated in the second run of the inoculated-cold stress (4°C) 4 days after planting because no plants germinated (Supplemental Table 1).

The presence of *P. sylvaticum* reduced shoot weight (P<0.0001), and a significant effect of cold stress timing and interaction of temperature x timing was detected (P=0.0005, P=0.0158, respectively) (Table 1). Cold stress at 10°C reduced shoot weight in all the inoculated treatments when compared to the 18°C control (Figure 5). In inoculated cups, shoot weight was reduced with cold stress (4°C) 4 days after planting, but not at 2 days after planting or in the control at 18°C. In most of the non-inoculated treatments cold stress did not reduce shoot weight, except when 4°C was imposed immediately after planting (Figure 5).

**Effect of temperature on mycelial growth of *P. sylvaticum***. There were significant differences in mycelial growth of *P. sylvaticum* (P<0.0001) at different temperatures. Growth was greater at 18°C, compared to 10°C, and at 18°C compared to 4°C at each time point assessed (Figure 6).
Effect of temperature on solute leakage during imbibition of soybean seed. Higher solute leakage occurred when seeds were imbibed at 4 ºC in comparison to 10 ºC and 18 ºC (P<0.0001; Table 2).

Effect of seed exudates from seeds imbibed at cold temperatures on sporangia germination. P. sylvaticum sporangia germinated after exposure to seed exudate solutions but not when they were exposed to sterile water (P<0.0001) (Table 3) (Figure 7). After 3 hours of exposure to seed exudates from seeds imbibed at 4ºC, more sporangia germinated compared to those exposed to a solution of seed exudates from seeds imbibed at 10 ºC and 18 ºC (P<0.0001).

Sporangia germination after incubation at 4 ºC, 10 ºC or 18 ºC. We detected differences in sporangia germination between runs (P=0.0086) (Table 4). In both runs, however, greater sporangial germination occurred at 18 ºC than at 10ºC or 4ºC (P<0.0001). No sporangia germinated in sterile water.

Discussion

In this study we demonstrated that periods of cold stress that occurred between 2 and 4 days after planting increased the susceptibility of soybean to damping-off under controlled conditions. To our knowledge, this study is the first to evaluate the effect of cold stress after planting on emergence. Previous studies evaluating the effect of suboptimal temperatures on germination of soybeans have been done to study chilling injury during imbibition or when screening of cold tolerant varieties in field trials and laboratory experiments (Hobbs and Obendorf 1972; Littlejohns and Tanner 1976; Ismail et al. 1989; Cabane et al. 1992). The cold stress
treatments that we tested mimic what occurs in the field soon after planting in Iowa when cold fronts pass through the region for a few days resulting in suboptimal soil temperatures for germination and seedling development. Previous research suggested that cold soils delay emergence and thus provide a greater chance for seedling infection (Martin and Loper 1999). In this study we demonstrated that the timing at which cold temperatures are initiated can have substantial impact on the incidence of damping-off.

Similarly, Thomson et al. (1971) reported that cold stress increased susceptibility to damping-off, but in their experiments cold stress (4°C) was applied only at planting. They also observed reduced emergence in their non-inoculated control that may have been a consequence of chilling injury during imbibition which is common at temperatures below 15°C (Bramlage et al. 1978; Leopold 1980). In our study, although we observed reduction in root and shoot weight in soybeans subjected to cold stress (4°C) immediately after planting (0 dap), we did not observe reduced emergence, possibly because it took 3 to 4 hours to equilibrate the temperature inside the cup and the growth chamber temperature. (Supplemental Figure 1). Seed imbibition is the first event in the germination process in which water uptake occurs within the first 35 minutes. During this time seeds are particularly susceptible to imbibitional chilling injury since rearrangement of cell membranes occurs during the first few minutes of imbibition (Parrish and Leopold 1977; Bramlage et al. 1978; Vertucci and Leopold 1983). In the first 30 minutes after planting in our study, the temperature of our cups was 19°C and it took 3 to 4 hours
for our cups to reach 4°C. Thus the risk of imbibitional chilling injury in our experiments was likely low.

No significant reduction in emergence was observed in the cold stress treatments when the pathogen was absent. High-vigor soybean seed usually germinates well in prolonged cold conditions. In the standard cold germination test, in which soybean seeds are subjected to 7 days of cold stress at 10°C, seed lots with high vigor show high germination rates (AOSA 2009). Low quality seed has greater seed exudation and thus stimulates soilborne pathogens to attack germinating seedlings (Hobbs and Obendorf 1972; AOSA 2009). The seed we used in this study was high quality with a 97% germination rate (Supplemental Figure 2) and consequently the effect of soilborne pathogens on emergence was not confounded by characteristics of poor quality seed.

Cold stress under the conditions we used favored damping-off when the pathogen was present. We hypothesized that higher susceptibility to damping-off would occur in seeds subjected to cold stress at planting because of chilling injury and greater solute leakage that would attract the pathogen. Unexpectedly, soybean was more susceptible to damping-off caused by *P. sylvaticum* when cold stress occurred 2 and 4 days after planting, which is well after imbibition when chilling injury usually occurs. Still soybean seedlings may be particularly susceptible to cold stress and other stresses initiated several days after imbibition. Wuebker et al. (2001) reported that soybean susceptibility to flooding damage was highest at 3 days after planting, suggesting that soybean seedlings may be more susceptible to stress at this developmental stage. Interestingly, isoflavone levels in the hypocotyl
undergo a programmed decrease between 2 and 4 days after planting (Graham 1991). Isoflavonoids are phenylpropanoid-derived metabolites that have been involved in resistance to *Pythium aphanidermatum* and *Phytophthora sojae* (Bhattacharyya and Ward 1986; Morris et al. 1991; Avanzato and Rupe 2011).

Isoflavonoid levels in the hypocotyl and roots of 3-day old soybean seedlings were subjected to several days of cold stress at 1°C and subsequently returned to 25°C for 3 days compared to seedlings that were not cold stressed (Posmyk et al. 2005). Further research is required to elucidate whether the increased susceptibility observed in our study when cold stress occurred at 2 to 4 dap is related to decreased levels of isoflavonoids.

Hatfield and Egli (1974) reported that hypocotyl elongation was minimal at temperatures below 10°C. Furthermore, when soybeans seedlings were placed at 1°C for 2 days, Posmyk et al. (2005) observed post-chilling stress in that seedling growth was slower over the following 3 days at 25°C. It is possible that cold stress not only results in increased risk of seedling infection but may also favor colonization of the seedling by the pathogen since growth of the plant is slower when it is moved back to more favorable conditions. Further research is needed to test this hypothesis.

Although *P. sylvaticum* prefers warmer soil temperatures, we observed the pathogen was still able to grow at 4°C and 10°C. Moreover, our data suggest that *P. sylvaticum* grows faster than soybean roots in the soil at these low temperatures. We also observed that root growth and emergence of soybean was delayed in the presence of the pathogen even at favorable temperatures (18°C) for soybean
growth. Although we did not do disease assessments, we propose that some infection had occurred and consequently seedling growth was reduced.

Sugars, amino acids and unsaturated fatty acids that are present in seed and root exudates stimulate sporangia germination (Nelson 1990; Ruttledge and Nelson 1997). We observed sporangia germination only when sporangia were flooded with soybean seed exudate solution. Moreover, temperature played a role in sporangia germination. The optimal temperature for sporangia germination in our study was 18ºC which was the closest to optimal temperature for mycelial growth (25ºC) (Van der Plaats-Niterink 1981).

In this study we detected greater solute leakage when soybean seeds were imbibed at 4ºC in comparison to 10ºC and 18ºC. These data are similar to those reported by Bramlage et al. (1978), in which more solutes leaked from seeds imbibed at 10ºC and lower temperatures compared to seeds imbibed at 20ºC. In our study, we also observed greater germination of *P. sylvaticum* sporangia in the presence of seed exudates from seeds imbibed at 4ºC. Since sporangia germination is stimulated by seed exudates it is possible that the quantity of the seed exudates varied at each temperature at which the seed was imbibed.

In our study however we measured exudation at 24 hours after imbibition and evaluated percent sporangia germination in the exudate. Exudates are continually released from plants and their quantity and composition can vary over time depending on plant age, and can influence the composition and activity of microbial communities (Gransee and Wittenmayer 2000; Han et al. 2017). Moreover, cold stress may have affected exudation. Vancura (1967) analyzed exudates from maize
and cucumber seedlings planted at 19°C and subjected to cold stress (5°C) 48 hours after planting, and found that cold stress increased the quantity of exudates, and substantially increased exudation of amino acids. Fructose, saccharose and other three oligosaccharides were detected only when maize seedlings where subjected to cold stress. In our study we did not specifically quantify the amount of exudates or determine the composition of exudates. Consequently we do not know if the increased germination of sporangia in exudates from seed imbibed at 4°C for 24 hours was due to a higher concentration of exudates, or a different composition of exudates. Further research is needed to determine if the quantity and composition of soybean seed exudates produced at 2 and 4 days after planting differs, if cold stress affects the composition of exudates, and the effect of the exudates on sporangia germination.

For this study we selected \textit{Pythium sylvaticum} as the pathogen causing damping-off on soybeans. Although \textit{P. sylvaticum} is the most frequently recovered species in U.S., there is a great diversity of \textit{Pythium} species causing soybean seed rot and root rot (Rojas et al. 2017). Moreover, the aggressiveness of some pathogenic \textit{Pythium} spp. can be enhanced or reduced at low temperatures (Thomson et al. 1971; Matthiesen et al. 2016; Rojas et al. 2017). Further research is required to determine whether the results observed in this study change with other \textit{Pythium} spp.

In this study we found that cold stress increased soybean susceptibility to damping-off caused by \textit{Pythium sylvaticum}, particularly when cold stress occurred 2 and 4 days after planting. \textit{Pythium sylvaticum} delayed seedling root growth and
emergence at 18°C. Our data suggest that the increase in damping-off that we observed after a period of cold stress could be due to several factors including the apparent growth rate advantage of the pathogen at low temperatures, and increased solute leakage that stimulates germination of sporangia of \textit{P. sylvaticum}. We also hypothesize that susceptibility to damping-off may be increased by physiological and metabolic changes that occur during germination and seedling development. Further research is required to elucidate why soybean seedlings are more susceptible to damping-off when cold stress occurs 2 and 4 days after planting.

\textbf{Acknowledgements}

The authors thank Iowa Soybean Association, North Central Soybean Research Program and Valent USA Corporation for providing funding for this research, and Iowa State University Seed laboratory for help provided with solute leakage tests.

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Casteel, S. N., Davis, V. M., Diers, B. W., Esker, P. D., and Specht, J. E.
2014. Fungicide management does not affect the rate of genetic gain in
effects on soybean germination. Crop Science 41:1857-1861.
Table 1. Effect of different variables on probability of emergence, root weight and shoot weight of soybeans (IA 2094).

<table>
<thead>
<tr>
<th>Effect</th>
<th>P-value probability of emergence</th>
<th>P-value root weight</th>
<th>P-value shoot weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run</td>
<td>0.4769</td>
<td>0.0003</td>
<td>0.2339</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.0006</td>
<td>&lt;0.0001</td>
<td>0.8015</td>
</tr>
<tr>
<td>Timing</td>
<td>&lt;0.0001</td>
<td>0.0012</td>
<td>0.0005</td>
</tr>
<tr>
<td>Pathogen</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Temperature x Timing</td>
<td>0.3128</td>
<td>0.5979</td>
<td>0.0158</td>
</tr>
<tr>
<td>Temperature x Pathogen</td>
<td>0.0073</td>
<td>0.5029</td>
<td>0.7458</td>
</tr>
<tr>
<td>Timing x Pathogen</td>
<td>&lt;0.0001</td>
<td>0.4036</td>
<td>0.1555</td>
</tr>
<tr>
<td>Temperature x Timing x Pathogen</td>
<td>0.0066</td>
<td>0.2168</td>
<td>0.1152</td>
</tr>
</tbody>
</table>

Table 2. Solute leakage (µS/cm g) of soybean seed IA 2094 after incubation for 24 hours at different temperatures.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Leakage (µS/cm g)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>65.9 a²</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>10</td>
<td>59.6 b</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>56.9 b</td>
<td></td>
</tr>
</tbody>
</table>

Different letters indicate significant differences according to Tukey test alpha = 0.05.
Table 3. Sporangia germination (%) of *Pythium sylvaticum* after exposure to different seed exudates and incubation at 24°C for 2 and 3 hours.

<table>
<thead>
<tr>
<th>Seed exudate</th>
<th>2h</th>
<th>3h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeds imbibed at 4°C</td>
<td>35.5 a²</td>
<td>65.2 a</td>
</tr>
<tr>
<td>Seeds imbibed at 10°C</td>
<td>32.0 a</td>
<td>42.9 b</td>
</tr>
<tr>
<td>Seeds imbibed at 18°C</td>
<td>31.9 a</td>
<td>48.2 b</td>
</tr>
<tr>
<td>Sterile water</td>
<td>0.6 b</td>
<td>0.5 c</td>
</tr>
</tbody>
</table>

P value: <0.0001  <0.0001

³Three seed exudates were prepared by imbibition of soybean seed in sterile water at 4°C, 10°C or 18°C for 24 hours.

²Different letters indicate significant differences according to Tukey test alpha = 0.05.

Table 4. Sporangia germination (%) of *Pythium sylvaticum* after exposure to seed exudate or sterile water and incubation for 3 hours at different temperatures.

<table>
<thead>
<tr>
<th>Incubation temperature (°C)</th>
<th>Germinated sporangia (%)</th>
<th>seed exudate</th>
<th>sterile water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>9.6 b</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>18.4 b</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>55.3 a</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.0001</td>
<td>0.8088</td>
<td></td>
</tr>
<tr>
<td>Run 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.1 b</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9.0 b</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>48.2 a</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.0001</td>
<td>0.2913</td>
<td></td>
</tr>
</tbody>
</table>

³Seed exudate was prepared by imbibition of soybean seeds in sterile water at 18°C for 24 hours.

²Different letters indicate significant differences according to Tukey test alpha = 0.05.
Figure 1. Probability of emergence (%) of soybeans IA 2094 non-inoculated (control) and inoculated with *Pythium sylvaticum* subjected to 96 hours of cold stress (4 °C or 10°C) at different days after planting (dap). Different letters indicates significant differences according to Tukey test alpha = 0.05; uppercase letters for cold stress 10°C, lowercase letters for cold stress 4°C.
Figure 2. Emergence (%) of soybeans IA 2094 non-inoculated (control) and inoculated with *Pythium sylvaticum* evaluated at different days after planting at 18ºC. Error bars indicate confidence interval 95%. Asterisk indicates a significant difference with respect to the control.
Figure 3. Seedling length (mm) of soybeans IA 2094 non-inoculated (control) and inoculated with *Pythium sylvaticum* evaluated at different times after planting at 18°C. Two independent runs of the experiment are presented. **A**, run 1; **B**, run 2. Error bars indicate confidence interval 95%. Asterisk indicates a significant difference with respect to the control.
Figure 4. Root weight (mg) of soybeans IA 2094 non-inoculated (control) and inoculated with *Pythium sylvaticum* subjected to 96 hours of cold stress (4 °C or 10°C) at different days after planting (dap). Different letters indicate significant differences according to Tukey test alpha = 0.05; uppercase letters for cold stress 10°C, lowercase letters for cold stress 4°C.
Figure 5. Shoot weight (mg) of soybeans IA 2094 non-inoculated (control) and inoculated with *Pythium sylvaticum* subjected to 96 hours of cold stress (4°C or 10°C) at different days after planting. Different letters indicates significant differences according to Tukey test alpha = 0.05; uppercase letters for cold stress 10°C, lowercase letters for cold stress at 4°C.
Figure 6. Mycelial growth (mm) of *P. sylvaticum* at three different temperatures on diluted V8 media plus antibiotics. Error bars indicate standard deviation.

Figure 7. *Pythium sylvaticum* sporangia (X200). A, germinated sporangium with a germ tube 3 hours after exposure to seed exudate. B, non-germinated sporangium 3 hours after exposure to sterile water.
**Supplemental Table 1.** Root fresh weight (mg) of soybeans IA 2094 subjected to 96 hours of cold stress.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Pathogen</th>
<th>Cold stress timing</th>
<th>Root weight (mg) Run 1</th>
<th>Root weight (mg) Run 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ºC</td>
<td>Inoculated</td>
<td>No Cold Stress</td>
<td>385 d&lt;sup&gt;v&lt;/sup&gt;</td>
<td>356 defghi</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 dap&lt;sup&gt;x&lt;/sup&gt;</td>
<td>306 g</td>
<td>298 ghijk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 dap</td>
<td>270 i</td>
<td>419 cdefg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 dap</td>
<td>254 i</td>
<td>407 cdefgh</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 dap</td>
<td>265 i</td>
<td>252 hijk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 dap</td>
<td>245 hi</td>
<td>324 fghijk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 dap</td>
<td>311 fgh</td>
<td>337 efghijk</td>
</tr>
<tr>
<td></td>
<td>Non-inoculated</td>
<td>No Cold Stress</td>
<td>534 a</td>
<td>601 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 dap</td>
<td>469 abc</td>
<td>511 abcd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 dap</td>
<td>482 ab</td>
<td>507 abcd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 dap</td>
<td>521 a</td>
<td>508 abcd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 dap</td>
<td>514 a</td>
<td>596 ab</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 dap</td>
<td>511 a</td>
<td>478 abcdef</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 dap</td>
<td>513 a</td>
<td>487 abcd</td>
</tr>
<tr>
<td>4 ºC</td>
<td>Inoculated</td>
<td>No Cold Stress</td>
<td>302 gh</td>
<td>363 efghij</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 dap</td>
<td>156 j</td>
<td>234 jk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 dap</td>
<td>252 hi</td>
<td>235 ijk</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 dap</td>
<td>231 i</td>
<td>145 k</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 dap</td>
<td>174 ji</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 dap</td>
<td>248 hi</td>
<td>382 cdefghi</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 dap</td>
<td>197 ji</td>
<td>300 ghijk</td>
</tr>
<tr>
<td></td>
<td>Non-inoculated</td>
<td>No Cold Stress</td>
<td>426 bcd</td>
<td>511 abcd</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 dap</td>
<td>353 efg</td>
<td>372 dfghij</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 dap</td>
<td>437 bcd</td>
<td>535 abc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 dap</td>
<td>410 cde</td>
<td>442 bcdefg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 dap</td>
<td>472 abc</td>
<td>465 abcdef</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 dap</td>
<td>383 de</td>
<td>391 efghi</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 dap</td>
<td>378 def</td>
<td>471 abcdef</td>
</tr>
</tbody>
</table>

<sup>x</sup>Days after planting

<sup>y</sup>Different letters indicate significant differences according to LSD test alpha = 0.05.

<sup>z</sup>No germinated plants in this treatment.
**Supplemental Figure 1.** Soil temperature (°C) of soybeans IA 2094 planted and incubated immediately in growth chambers at 4°C and 10°C.
Supplemental Figure 2. Seed quality analysis provided by the Seed Testing Laboratory of Iowa State University for the seed lot of soybeans IA 2094 used in this study.
CHAPTER 5.
GENERAL CONCLUSIONS

In Iowa, cold fronts soon after planting have been associated with higher occurrence of soybean seedling disease that may result in a reduction in crop stand and yield loss. Seedling disease can be managed using seed treatments. In previous field studies, however, results were inconclusive regarding crop stand and yield improvement when a seed treatment was applied. The work contained in this dissertation further investigated the effects of commercial seed treatments on soybean stand and yield in Iowa and the efficacy of seed treatments when soybean was subjected to cold stress and inoculated with \textit{P. sylvaticum} in controlled environments. In addition, the effect of cold stress temperatures and time after planting that cold stress occurred was investigated in soybean-\textit{P. sylvaticum} interaction to develop a better understanding of how this disease develops.

Our results from a three-year field study showed the effect of seed treatments on plant stand were inconsistent among treatments and locations. In 7 of 9 trials cool and wet periods occurred several days after planting; however, this had no impact on plant stand or yield, and we did not observe a consistent effect of seed treatment. Of 123 seed-treatment-site-year evaluated through this study only 2 significantly improved yield. Seeding rates in this study were 120,000 to 140,000 seeds per acre which is below rates commonly used in this crop. Even with these low seeding rates, our results suggests small reductions in stand were compensated by soybean and no effect on yield was observed. The inconsistent effect of seed treatments on improving plant stand suggests that low inoculum levels of soilborne pathogens or
other factors that reduce seedling disease are prevalent in Iowa soils and this could be further investigated. Low populations of Bean leaf beetle and Soybean cyst nematode, and low prevalence of SDS meant it was not possible to evaluate the efficacy of seed treatments containing insecticides, nematicides and fluopyram for SDS control. Thus, disease history of each field is key when making a seed treatment decision. Moreover, in Iowa soils the use of seed treatments should be regarded as a preventive control measure to protect seedlings from seedling diseases rather than an input to improve yield, except in fields with history of SDS.

Data from experiments in controlled environments showed that inoculum of *P. sylvaticum* reduced soybean emergence in untreated seeds, and that a longer duration of cold stress period further reduced emergence in the presence of the pathogen. Our results confirmed previous field observations suggesting high soybean susceptibility to damping-off when cold stress occurs soon after planting. We showed that a seed treatment containing metalaxyl and ethabuxam improved emergence when soybean was subjected to 96 hours of cold stress at 4°C and 10°C. Seed treatment protected seedlings and high emergence was observed even with occurrence of cold stress. Root rot severity was reduced and shoot weight was increased by the seed treatment. The seed treatment did not improve root weight, suggesting that the seed treatment had a restricted efficacy period with limited protection of lateral roots. We concluded that periods of cold stress soon after planting increase the risk of damping-off, and that seed treatments are a management tool to protect soybean seedlings when adverse conditions are expected soon after planting.
We provided evidence of the role of periods of cold stress in the occurrence of damping-off caused by *P. sylvaticum*. The time when cold stress occurred had a pronounced impact on disease incidence. We observed increased soybean susceptibility to damping-off when cold stress occurred 2 and 4 days after planting. It is unknown why soybeans were particularly susceptible to damping-off at this stage, and this could be an avenue of research in the future. Mycelial growth of *P. sylvaticum* on media culture was greater at 18°C than at lower temperatures; however, the pathogen was still able to grow at 10°C, and even 4°C. This may be an advantage for *P. sylvaticum* since soybean germination and growth is likely to be very restricted at these cool temperatures. Furthermore, higher seed exudation was detected when soybean seeds are imbibed at 4°C in comparison to 10°C and 18°C. Thus, low temperatures at planting may be a factor in increasing the activity of the pathogen since higher seed exudation corresponded with higher sporangia germination of *P. sylvaticum*. It remains to be determined if changes in the quantity or quality of components in seed exudates are responsible of the increase in sporangia germination. Furthermore, seed exudation of germinating seedlings 2 and 4 days after planting needs to be investigated in the future to determine if cold stress at these stages affects seed exudation and sporangia germination.
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