STUDY OF CHEMICAL AND MICROBIAL-MEDIATED STRATEGIES FOR MANAGING SCLEROTINIA STEM ROT OF SOYBEAN (*GLYCINE MAX* L.)

By

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ABSTRACT

STUDY OF CHEMICAL AND MICROBIAL-MEDIATED STRATEGIES FOR MANAGING SCLEROTINIA STEM ROT OF SOYBEAN (*GLYCINE MAX* L)

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The management of Sclerotinia stem rot of soybean caused by [*Sclerotinia sclerotiorum* (Lib) de Bary] integrates several tactics that may include biological control. However, the successful incorporation of biocontrol agents into the management program might be hindered by agrochemical application. To determine the compatibility of potential biocontrol agents with agrochemicals, growth of *Trichoderma asperellum*, *T. gamsii* and *Coniothyrium minitans* was tested with selected chemicals on agar plates. Effects of selected agrochemicals on *C. minitans* growth and biocontrol activity were also tested in soil and in water. On amended agar media, *Trichoderma* spp. and *C. minitans* were insensitive to the herbicide lactofen and were inhibited to varying degrees by some of the tested fungicides. The survival and the biocontrol activity of *C. minitans* in the soil was not impaired by propamocarb HCl, tetraconazole or lactofen treatments. When mixed in a water suspension, none of the selected the herbicides or a biofertilizer affected *C. minitans* population in the suspension over a 4 h period, suggesting that they can be applied in a tank mix. Under field conditions, *Trichoderma* spp. and *C. minitans* reduced the disease severity and viable sclerotial population of *S. sclerotiorum*, but their efficacy varied across years and location. Co-application with lactofen enhanced the efficacy of disease suppression and yield in some cases but reduced the efficacy in others. The effect of the biological control agents or chemicals evaluated on yield during three years of trials was inconsistent.

To my mother Martha for showing me the value of hard work and discipline, to my father Alfonso for teaching me to pursue my dreams with optimism, to my brother Mario for being my confidence provider, to my husband Jose Luis for his love and unconditional support, and to Jeronimo for being my motivation now and forever, I dedicated this thesis.

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CHAPTER ONE: LITERATURE REVIEW

SOYBEAN

The soybean (*Glycine max* L. Merr.) is an important crop globally that is cultivated widely in countries such as Brazil, Argentina, China, India, and the United States (Singh and Hymowitz 1999). Soybeans were first domesticated in Northeast China around 1500 B.C. (Hymowitz 2004, Stacey 2008) and introduced in the United States (U.S.) in 1765 but were not formally recognized as a crop until the early 19th century (Singh and Shivakumar 2010). The U.S. now leads the world in soybean production followed by Brazil (Singh and Hymowitz 1999).

From 2000 to 2008, soybeans were grown on an average of more than 29 million hectares in 31 states occupying approximately 22% of the roughly 138 million hectares of harvested cropland in the U.S. This makes soybean the second largest crop in acreage after corn (USDA-NASS 2008). In 2012, Michigan reported 809,371.3 hectares planted to soybean, with approximately 2.3 billion of kg harvested. This is worth about $1.2 billion (Heatherly et al. 2009, USDA-NASS 2012).

Soybeans have been called the “miracle golden bean” because of high mineral (P, K, Ca, and Zn) and protein (42 to 45%) content (Rahman et al. 2011). In the U.S., soybeans also are a major resource for biodiesel production, as soybean has the advantage of lower nitrogen input as compared to crops such as sunflower (Pimentel and Patzek 2005).

An important agronomic trait associated with soybean physiology is the maturity groups that determine its geographical adaptation (Zhang et al. 2007). Soybeans have been classified into 13 maturity groups (MG) of which I and II are typically grown in the northern region of the U.S., whereas higher maturity groups (III- VI) are grown farther south (Heatherly et al. 2009) . This pattern of adaptation from north to south is due primarily to differences in sensitivity to

photoperiod (Scott and Aldrich 1970). To maximize yield, one must tailor the physiological events of plant development with favorable environmental conditions (Singh and Shivakumar 2010).

The modern soybean germplasm collection generated through U.S.breeding programs contains cultivars and breeding lines with a relatively low genetic diversity (Zhu et al. 2003). This lack of diversity increases the potential vulnerability of soybean to plant diseases (Sleper and Poehlman 2006). The most important soybean diseases in the U.S. are soybean cyst caused by *Heterodera glycines* Ichinoche (Heatherly et al. 2009), seedling diseases, Phytophthora root and stem rot caused by *Phytophthora sojae* Kaufman & Gerdemann (Wrather and Koenning 2006), and sudden death syndrome caused by *Fusarium virguliforme* Akoi, O'Donnell, Homma & Lattanzi (Koenning and Wrather 2010). Sclerotinia stem rot (also known as white mold), caused by *Sclerotinia sclerotiorum* (Lib.) de Bary has caused significant yield and economic losses of soybean, particularly in northern states (Wrather et al. 2001). In the U.S. the Sclerotinia stem rot epidemic in 2009 was ranked as the second most damaging disease following by soybean cyst (reviewed in Peltier et al. 2012). Yield losses have been estimated at 60 million bushels (Sleper and Poehlman 2006, Wrather et al. 2001). Thus, many efforts have aimed to reduce the impact of the disease through appropriate disease management. Disease management practices include cultural control (Boland and Hall 1988a, Gracia-Garza et al. 1998, Mueller et al. 2002b) and chemical control (Dann et al. 1998, Dann et al. 1999, Mueller et al. 2002a, Mueller et al. 2004). Biological control practices have been also evaluated (Del Rio et al. 2002, Huang et al. 2000a, Zeng et al. 2012a, Zhang et al. 2011).

SCLEROTINIA SCLEROTIORUM

Biology and life cycle

Sclerotinia sclerotiorum is a fungus belonging to the family Sclerotiniaceae, the order Helotiales, and the phylum Ascomycota (Whetzel 1945). Like all other species in the Helotiales, *S. sclerotiorum* forms cup- or disk-shaped apothecia from sclerotia; the apothecia produce inoperculate asci which contain ascospores (reviewed in Bolton et al. 2006, Whetzel 1945). A sclerotium is a hyphal aggregate with an outer black rind composed of thick cells containing melanin, a compound that is believed to play an important role in protection from adverse conditions and microbial degradation (Butler et al. 2001, reviewed in Henson et al. 1999). Sclerotia of *S. sclerotiorum* serve as survival structures that can remain viable for 3-5 years in soil under natural conditions (Ayers and Adams 1979, Cook et al. 1975). Changes in environmental conditions such as pH can prevent sclerotial formation. This was demonstrated with a culture media assay showing that at pH 5 complete development and maturation of sclerotia were observed in 4 d. However, under controlled conditions, when neutral pH was evaluated, complete inhibition of sclerotia development was obtained (Rollins and Dickman 2001). Therefore the manipulation of external factors may provide alternatives to the control of the Sclerotinia stem rot through disease cycle disruption.

Sclerotinia sclerotiorum overwinters as sclerotia in soil (Cook et al. 1975, Merriman 1976), as mycelium in infected seeds (Cook et al. 1975, Nicholson et al. 1972) or as mycelia in dead or living hosts other than soybean (Boland and Hall 1994). When environmental conditions are favorable, sclerotia germinate carpogenically and produce apothecia. Over a period of 2-3 weeks, large numbers of ascospores are discharged from mature apothecia into the air (Clarkson et al. 2003). These ascospores can be spread by wind (Williams and Stelfox 1979) to nearby

hosts and serve as primary inoculum for infection (Abawi and Grogan 1975, Boland and Hall 1988b). It has been documented that ascospores can travel up to four kilometers under field conditions; however, the majority are deposited within 100 to 150 m (Abawi and Grogan 1979, Steadman 1979). In addition to production of ascospores, myceliogenic germination of sclerotia results in the production of mycelial strands which can directly infect soybean seedlings (Grau and Hartman 1999). The greatest potential for long distance dissemination of *Sclerotinia* spp. is by transportation of seeds infested with mycelia or by sclerotia mixed with seeds (Mueller et al. 1999).

Host range

Sclerotinia sclerotiorum is a necrotrophic pathogen that has a wide host range. Host include cultivated plants such as canola (oilseed rape, *Brassica napus* L.) (Morrall and Dueck 1982), soybean (*Glycine max* L. Merr.) (Boland and Hall 1988a), bean (*Phaseolus vulgaris* L.) (Abawi and Grogan 1975), and sunflower (*Helianthus annuus* L.) (Hoes and Huang 1976). Wild plants species are also reported as *S. sclerotiorum* host and this is considered to be an important factor in Sclerotinia stem rot epidemiology (Boland and Hall 1994). A survey in 1994 reported 408 plant species from 278 genera and 75 families are susceptible to *S. sclerotiorum*. Most of hosts belonging to the Dicotyledoneae subclass of Angiospermae (Boland and Hall 1994). The wide host range of *S. sclerotiorum* often limits the opportunities for disease management through crop rotation (Krupinsky et al. 2002, Tu 1989) but when combines with zero-tillage an effective strategy is obtained, reducing the primarily inoculum of *S. sclerotiorum* in infested soybean fields (Garza et al. 2002).

Molecular mechanisms of pathogenicity

Disease development by *S. sclerotiorum* requires secretion of virulence factors like oxalic acid (Godoy et al. 1990, Magro et al. 1984), extracellular hydrolytic enzymes such as cellulases, hemicellulases, pectinases, aspartyl protease, and/or endopolygalacturonases, and acidic protease (Li et al. 2004, Marciano et al. 1982, Martel et al. 2006, Riou et al. 1992). Pectinolytic enzymes and pectin- methyesterases (Lumsden 1976) are highly active under acidic conditions such as those provided by oxalic acid (Marciano et al. 1983). The synergistic relation between the oxalic acid and endopolygalacturonases (pectinolytic enzymes) promote the disruption of the calcium-pectate complex in the middle lamella and primary cell wall of the host. (Bateman and Beer 1965). Other polymers such as cellulose and hemicelluloses in the cell wall are more exposed to other enzymes (i.e. cellulases, hemicellulases, and xylanases) that contribute to tissue maceration and pathogen invasion (reviewed in Lagaert et al. 2009). At early stages of disease development polygalacturonases (PGs) are associated with pathogenicity in *S. sclerotiorum* (Lumsden 1976).

Oxalic acid has three modes of action with respect to pathogenesis (Cessna et al. 2000, Marciano et al. 1983). First, oxalic acid directly blocks host-defense by suppressing the oxidative burst and callose deposition (Cessna et al. 2000, Kim et al. 2008, Williams et al. 2011) at early stages of infection, thus gaining important time for *S. sclerotiorum* establishment prior to plant recognition. Second, the secretion of oxalic acid may be toxic to host plants, leading to the loss of plant tissue integrity (Noyes and Hancock 1981). It was shown that oxalic acid moves systemically to the leaves where it apparently accumulates to levels that produce wilt in infected plants (Noyes and Hancock 1981). Third, the oxalic acid can chelate the cell wall Ca^{2+}, thereby destabilizing the cell wall host integrity and favoring the action of polygalacturonases (Bateman and Beer 1965, Marciano et al. 1983).

Once *S. sclerotiorum* is established in the host tissue, the induction of an apoptotic-like programmed cell death of host tissue occurred through a manipulation of plant defense response. The nutrients that are derived from the dying plant cells benefit the pathogen (Kabbage et al. 2013, Williams et al. 2011). Overall, the infection process by this necrotrophic fungus is complex.

SCLEROTINIA STEM ROT

Symptoms

In soybeans symptoms appear after crop flowering and canopy closure and are observed on the main stem 15 to 40 cm above the soil surface and also appear on lateral branches (Boland and Hall 1988a). The initial symptoms of Sclerotinia stem rot in soybean consist of brown and water-soaked spots located at the junction of the petiole and the stem , similar to that described in dry beans (Tu 1989) and the lower branches of the plant where senescent leaves or flower petals have fallen and adhered (Natti 1971). Leaves of infected branches turn yellow and wilt. As the infection progresses, the affected tissue show discoloration and a soft rot results in dieback of the branches (Tu 1989). The lesions generally turn into necrotic tissues that show patches of fluffy white mycelium, often with sclerotia, which is a typical sign of plants infected with *S. sclerotiorum* (reviewed in Bolton et al. 2006).

Disease cycle

The major infection propagules for *S. sclerotiorum* on soybean are the ascospores derived from carpogenic germination of sclerotia (Boland and Hall 1988a, Le Tourneau 1979). The ascospores can germinate on the surface of healthy tissues but cannot infect the plant without an exogenous nutrient source (Abawi and Grogan 1975, Sutton and Deverall 1983). Initial

colonization of dead or senescing tissue is required for successful infection of the plant organs such as blossoms, cotyledons, leaves, seeds or injured plant tissue (Abawi and Grogan 1979, Purdy 1979).

Details of early events of infection by the scanning electron microscopy have shown that the formation of appressoria is a prerequisite to penetration of healthy plant tissues by *S. sclerotiorum* (Tariq and Jeffries 1984). The development and complexity of the appresoria at early infection stage depends on the nutritional status of the inoculum and the physical resistance of the surface under attack (Tariq and Jeffries 1984). The formed appresorium is usually complex, multicelled, dome-shaped structure (reviewed in Lumsden 1979, Lumsden and Dow 1973). The complex appressoria were observed in bean hypocotyls 6 hours after inoculation. They are formed by three different types of hyphae which are involved in penetration and infection of bean tissue formed via dichotomous branching from a single hyphae, with terminal swelling of the apex followed by repeated branching and secretion of adhesive mucilage (Lumsden and Dow 1973, Tariq and Jeffries 1984). Pore-like infection pegs formed at the tips of appresoria allow the ingress of *S. sclerotiorum* into the host tissue and their development contributes to the cellular disorganization of the leave (Abawi and Grogan 1975). Penetration of the host surface may be mediated by the secretion of enzymes and toxins in addition to the mechanical force exerted by the specialized hyphae when a tight interface is formed with the host surface (Abawi and Grogan 1975, Tariq and Jeffries 1987). Once the stems and petioles are infected, vascular tissues are disrupted, and stems, pods, or leaves beyond the site of infection die. After consumption of plant nutrient, fungal mycelia aggregate into sclerotia, which form both inside and outside the stem. These sclerotia then fall to the ground with the potential to remain viable for an extended period of time (Coley-Smith and Cooke 1971).

Epidemiology

The release of ascospores from apothecia of *Sclerotinia sclerotiorum* is considered the initial step for Sclerotinia stem rot epidemics (Abawi and Grogan 1975, Cook et al. 1975, Schwartz and Steadman 1978) and environmental conditions constituted an important factors for its occurrence (Boland and Hall 1988a, Pennypacker and Risius 1999).

Studies on the conditions for carpogenic germination indicated that different ranges of temperatures and soil moistures impact the development of apothecia (Abawi and Grogan 1975, Hao et al. 2003 , Mila and Yang 2008). The range of temperature for sclerotial germination indicates an adaptation pattern of the pathogen to different geographic regions (Mila and Yang 2008, Phillips 1987). Size of sclerotia (Hao et al. 2003) and sclerotial burial (Mitchell and Wheeler 1990, Wu and Subbarao 2008) appear to contribute to apothecia development. The information from such studies may lead to a better understanding of the epidemiology of the Sclerotinia stem rot and thus provide better strategies for its management.

Sclerotinia stem rot development on soybean and beans is also associated with canopy closure (Boland and Hall 1988a, Natti 1971, Schwartz and Steadman 1978) that provides the moisture conditions for carpogenic germination of sclerotia (Weiss et al. 1980). However, the high relative humidity provided by canopy closure is not the only factor required for an effective disease development (Boland and Hall 1988a). The amount and source of inoculum of *S. sclerotiorum* are also critical factors. Boland et al. (1988) indicated that Sclerotinia stem rot incidence was determined mainly by inoculum produced within the field. It is evident that more than one factor is required for the onset of infection under field conditions, making the prediction of this disease difficult.

DISEASE MANAGEMENT

The management of Sclerotinia stem rot involved the integration of several measures (Abawi and Grogan 1979 , Boland and Hall 1988a, Kurle et al. 2001, Whipps et al. 2007).Strategies of disease management can include a) eradication through the reduction of inoculum density of sclerotia in the soil, such as cultural practices, and soil amendments. b) selection of tolerant varieties; and c) protection through chemical application, impairing the pathogen ability to infect the susceptible soybean stage.

Tillage and crop rotation. Integration of zero-tillage with crop rotation can reduce Sclerotinia stem rot (Garza et al. 2002). Under zero-tillage, *S. sclerotiorum* sclerotia are generally located close to the soil surface (2 to 3 cm in depth) favoring the rapid degradation of sclerotia mainly by soil microflora (Ayers and Adams 1979, Merriman 1976, Tu 1997). In contrast, tilled soils promote the burial of sclerotia providing conditions for the sclerotia to survive for several years (Duncan et al. 2006, Subbarao et al. 1996). Thereby, reduction of the inoculum potential can be achieved through zero-tillage and subsequent infection of host plants by the pathogen may be minimized (Tu 1986).

Cultivation of non-host crops is recommended to reduce sclerotia populations in soil (Kurle et al. 2001, Steadman 1979); however, the dissemination of ascospores from field to field by wind (Williams and Stelfox 1979) or long distance dissemination through contaminated seed with sclerotia (Ayers and Adams 1979) might limit the efficacy of this strategy.

Resistant cultivars. Plant breeding for resistance to Sclerotinia stem rot has had limited success (Cober et al. 2003). Genetic resistance to *S. sclerotiorum* is difficult to identify and evaluate because both physiological resistance (i.e. incomplete resistance conditioned by multiple genes of partial effect) and escape mechanisms (i.e. agronomic traits or modification in physiological

plant events that are involved in halt pathogen infection) contribute to differences in plant disease responses under field conditions (Boland and Hall 1987, Grau et al. 1982, Kim and Diers 2000). Advances in genetic engineering, however, have allowed the development of Sclerotinia stem rot-resistant soybean through the introduction of defense genes, such as oxalate oxidase, an oxalic-acid degrading enzyme (Cober et al. 2003). Although the biotechnology approach may provide potentially useful management tools, to date there is no transgenic commercial soybean variety resistant to Sclerotinia stem rot or any fungal diseases (Hudson et al. 2013). In general, some factors to be considered for commercialization of new improved varieties include: the acceptance of farmers, society and regulatory agencies as well as the cost required during the experimental and regulatory stages. Altogether, these factors may prevent the practical application of these new technologies in a short term (Sleper and Shannon 2003).

Chemical control. According to Peltier et al. (2012), three different classes of fungicides are registered for Sclerotinia stem rot control on soybean: methyl benzimidazole carbamate, succinate dehydrogenase inhibitors, and demethylation inhibitors. These fungicides have limited movement, and none of them move down in the plant (Yuan and Crawford 1995). In this regard, Natti (1971) showed the importance of the systemic activity of a fungicide to achieve an effective method of Sclerotinia stem rot control, as well as, the permanence of fungicidal activity in senescent and dead bean blossoms.

For Sclerotinia stem rot control on bean and canola, foliar applications of fungicides have been used but have not proven economically feasible on soybeans (Mila et al. 2003, Steadman 1979). In soybean good canopy penetration and foliar coverage with fungicides along with appropriate application timing are critical for effective Sclerotinia stem rot control (Mueller et al. 2002a). This is particularly true for indeterminate soybean growth pattern, in which there are

many infection courts because of extended flowering (1-5 wk). This increases the opportunity for pathogen infection over the time and more than two fungicide applications may be required (Mueller et al. 2002a).

Chemicals are used for seed treatment against Sclerotinia stem rot in different crops (Herd and Phillips 1988, Mueller et al. 1999, Tu 1989). In beans (Tu 1988) and soybeans (Mueller et al. 1999) the sclerotia produced from the mycelium-infected seed may increase the amount of inoculum for the next season. Even though the sclerotia from contaminated seeds may occur in low proportion for both crops, under conducive environmental conditions the potential for high disease incidence can increase (Mueller et al. 1999, Tu 1988). This indicates that seed treatment may be an appropriate measure to prevent the introduction of *S. sclerotiorum* into uncontaminated fields.

Herbicides constitute a class of chemicals that is routinely used in soybean production for weed control. As well as their impact on weeds control, some herbicides affect *S. sclerotiorum* directly (Altman and Campbell 1977, Radke and Grau 1986). The soil-applied herbicides pendimethalin and trifluralin increased the number of apothecia produced per sclerotia of *S. sclerotiorum*, whereas chloramben, alachlor and DNBP suppressed apothecia production (Radke and Grau 1986). Casale et al. (1986) reported that soil amended with atrazine and simazine induced abortion of apothecial disk with subsequent production of stipes or apothecia abnormally shaped without ascospore production. Huang et al. (1995) confirmed that the triazine family of herbicides enhanced branching of stipes and induced malformation of the apothecia of *S. sclerotiorum.* These results provide evidence that it is possible that the routine application of some herbicides can potentially alter the carpogenic germination of *S. sclerotiorum* sclerotia, contributing to the reduction of a primary source of inoculum under field conditions.

Resistance inducing chemicals. The induction of plant resistance to pathogens is classified as systemic acquired resistance (SAR) (reviewed in Hammerschmidt 1999a) or induced systemic resistance (ISR) (reviewed in Van Loon et al. 1998). Both types of responses can be induced by chemical or synthetic elicitors and may result in promising strategies for managing crop pest.

Systemic acquired resistance can be induced by biotic factors (Caruso and Kuć 1979, Cohen and Kuc 1981) or chemical inducers of SAR such as 2,6-dichloro-isonicotinic acid (INA) (Dann et al. 1998) or S-methyl benzo [1,2,3] thiadiazole-7-carbothioate (ASM) (Dann et al. 1998, Friedrich et al. 1996). Both INA and ASM are considered as functional analogs of salicylic acid (reviewed in Vallad and Goodman 2004). In soybeans, multiple applications of both chemicals (INA and ASM) results in a significant reduction of Sclerotinia stem rot severity especially in susceptible cultivars compared to partially resistant varieties (Dann et al. 1998). However reductions of Sclerotinia stem rot varied among field and years which might influence its adoption in commercial soybean production.

Some herbicides modify the plant defense response either by inducing antimicrobial compounds (e.g. phytoalexins) to stop the pathogen growth or conversely, reducing the plant defense response making it vulnerable to diseases and pests (reviewed in Duke et al. 2012, Johal and Huber 2009). In soybean, the diphenyl-ether, lactofen, has been shown to reduce Sclerotinia stem rot severity under field conditions (Dann et al. 1999). High phytoalexin content (glyceollin) in lactofen-treated leaves was associated with reduction in lesion size caused by *S. sclerotiorum* (Dann et al. 1999). The increase in phytoalexin induction by lactofen treatment is restricted to treated leaves with a systemic resistance response that protects the untreated leaves after challenge with the pathogen (Nelson et al. 2002b). The mechanism associated with the increase in glyceollin is the inhibition of protoporphyrinogen IX oxidase (protox), an enzyme involved in

the biosynthesis of heme (Ahrens and Edwards 1994). Although lactofen has been shown to reduce disease severity in some cases, it needs further study as soybean cultivars vary in their response to the chemical (Dann et al. 1999, Nelson et al. 2002a).

Biological control. Difficulties in disease control related with long-term survival structure, sporadic occurrence and widespread distribution (Zhou and Boland 1998) of sclerotia-forming pathogens in different crops have been promote studies on biological control (Adams and Ayers 1982, Budge and Whipps 2001, Campbell 1947, Huang 1980).

Several mechanisms have been associated with biological control including but not limited to competition for space and nutrients (Baker 1968, Handelsman and Stabb 1996), parasitism (Papavizas and Lumsden 1980, Whipps 2001), siderophore production (Duffy and Défago 1999, Kloepper et al. 1980), synthesis of antibiotics (reviewed in Baker 1968, Fravel 1988), and induced resistance (reviewed in Handelsman and Stabb 1996, reviewed in Whipps 2001). The mechanisms of biocontrol associated with *S. sclerotiorum* that have been reported include: 1) mycoparasitism (Budge et al. 1995, Campbell 1947, Del Rio et al. 2002, Gracia-Garza et al. 1997, Trutmann and Keane 1990), 2) antibiosis (Abdullah et al. 2008, Fernando et al. 2007, McLoughlin et al. 1992) and 3) nutrient competition (Huang et al. 1993, Savchuk and Fernando 2004, Yuan and Crawford 1995).

Even though some microorganisms have proven to be effective biocontrol agents against *S. sclerotiorum* under controlled conditions, their application under field conditions were not always effective (reviewed in Alabouvette et al. 2006, Spadaro and Gullino 2005, reviewed in Van Veen et al. 1997). Since the soil is a dynamic and heterogeneous system in which diverse forms of organisms interact under variable conditions (reviewed in Van Veen et al. 1997), a

complete understanding of the mode(s) of action(s) of biocontrol agents in an ecological context is required to improve the reproducibility of disease control.

According to Whipps et al (2007), few commercial biological control agent products are available in the market for Sclerotinia-induced diseases. Those available include Contans®, Intercept® and KONI® (*Coniothyrium minitans* Campbell) and BioTrek ®, Harzian-10®, Rootshield®, and T-22G® (*Trichoderma* species). Among these biological control agents, *C. minitans* with mycoparasitic activity on *Sclerotinia* spp. has been the focus of extensive research (Campbell 1947, Sandys-Winsch et al. 1993, Turner and Tribe 1976). This biological control agent has been used successfully under controlled and field conditions, suppressing *S. sclerotiorum* in numerous crops (Budge et al. 1995, Budge and Whipps 1991, Gerlagh et al. 1999, Huang 1977, Huang et al. 2000a, McQuilken et al. 1995). Despite the good performance in preliminary trials, different factors may constrain the adoption of commercial biological control in commercial crop systems: the levels of suppression may not be below to the economical threshold for growers (Gnanamanickam 2002), the inconsistencies in disease control associated with environmental factors and high pathogen inoculum levels (Whipps et al. 2007), and the lack of effective technology transfer to growers (Glare et al. 2012).

The mechanism of sclerotia parasitism by *C. minitans* starts with the formation of appressorium-like hyphae on *S. sclerotiorum* hyphae or sclerotia, follow by inter-and intracellular growth within subcortical layers of the target pathogen (Grau and Radke 1984). Since no depression was observed at every point of penetration, Tu (1984) suggested the action of cell wall degrading enzymes were involved in the initial stage of *S. sclerotiorum* infection by *C. minitans*. This observation was later confirmed by the detection and identification of a gene encoding an exo-β-glucanases during sclerotia parasitism by *C. minitans* (Giczey et al. 2001).

Chitinases and exo-β-1,3-glucanases have been directly involved in the mycoparasitism interaction between *Trichoderma* species and its hosts (Harman et al. 2004, Howell 2003). Evidence of the wide range of enzymes produced by *C. minitans* can be supported by expression analysis, which showed that the wide range of hydrolytic enzyme genes identified are likely to be important in fungal-fungal interaction (Muthumeenakshi et al. 2007). The same study revealed that nearly 20% of the identified unisequences may have potential novel function during sclerotial colonization. The possibility to explore novel functions of promising biocontrol agents through comparative genomics or other molecular tools, may allow a better exploitation of these microorganism for more successful plant disease management.

Similarly, *Trichoderma* spp. have shown biological control properties associated with the successful reduction of disease incidence caused by *Sclerotinia* spp. (Abd-El-Motty and Shatla 1981, Clarkson et al. 2006). The process of mycoparasitism exerted by *Trichoderma* spp. is complex and involves a sequence of events. It has been shown that *Trichoderma* secretes exochitinases constitutively at low levels while growing towards the target fungus (Chet et al. 1981, Haran et al. 1996, Inbar and Chet 1995). When chitinases degrade fungal cell walls, the target fungus releases oligomers that induce the expression of these enzymes by *Trichoderma* spp. and the attack of the host begins. (reviewed in Benitez et al. 2004). Once in contact with the target fungus, the *Trichoderma* spp. produce several hydrolytic enzymes (e.g. β-1,3-glucanase, chitinase, protease and cellulase) which initiate the fungal cell wall degradation (Elad et al. 1982, Rey et al. 2001). Examination of sclerotia colonization by fluorescent microscopy revealed intracellular growth of the antagonist in the medulla *S. sclerotiorum* (Sarrocco et al. 2006). The uniform distribution of *T. virens* Miller, Giddens & Foster strain I10 just beneath the sclerotia ring suggests that the sclerotia became infected randomly without any preferential point of entry

(Sarrocco et al. 2006). In the same study, after 9 and 14 d of colonization, it was demonstrated that *T. virens* strain I10 was growing in the medulla and that the colonization was characterized by intercellular growth. At 20 days complete colonization and formation of chlamydospores was observed (Sarrocco et al. 2006). The time required for partial or complete degradation of sclerotia by *Trichoderma* spp. and the sequential events that might occur during the colonization and infection process by the antagonist constitute important information in terms of the practical application and improvement of the antagonist activity for an effective disease control.

Some bacteria have been tested experimentally for managing Sclerotinia stem rot (Boland 1997, Huang et al. 1993, Thaning et al. 2001, Tu 1997). In soybean, the effect of different *Bacillus subtilis* (Ehrenberg) Cohn strains on control has been variable. Zeng et al. (2012) found that treatment with *B. subtilis* strain QST 713 (Serenade MAX®) resulted in a modest disease severity index (DSI) reduction of Sclerotinia stem rot compared to other biological control agents and reduced the disease severity 15.4% compared with the control treatment. Cell suspension of *Bacillus subtilis* strains SB01 or SB24 successfully reduced Sclerotinia stem rot severity and their effectiveness was maintaining during 15 days of evaluation (Zhang et al. 2011).

Fluorescent *Pseudomonas* spp. have been the subject of detailed studies on antibiotic production (Duffy and Défago 1999, Keel et al. 1996, Raaijmakers et al. 1997) for disease control (Raaijmakers et al. 2002, reviewed in Whipps 1997). Preliminary studies on detached canola petals identified *Pseudomonas chlororaphis* Guignard & Sauvageau strains PA-23 and DF41 as potential effective inhibitors of *S. sclerotiorum* mycelial growth and germination of sclerotia and ascospores (Savchuk and Fernando 2004). The results showed significant disease severity reduction when the bacterial strains were co-applied with the pathogen and subsequent

inhibition of ascospore germination compared to control treatments (Savchuk and Fernando 2004). Overall, these results clearly show that biological control by bacterial strains may be a viable measure in addition to *S. sclerotiorum* control. However more efforts are needed to evaluate these candidates on soybean and under field conditions, identifying the different factors that might prevent an effective disease control to improve their application.

Integrated disease management. Integrated pest management (IPM) is a systems approach that combines different crop protection practices with careful monitoring of pests (Chandler et al. 2011, Flint and Bosch 1981) looking to manage disease below levels that cause economic damage (Chandler et al. 2011).

Synthetic pesticide in combination with biological control agents have been reported to successfully manage *Sclerotinia* spp. in diverse cropping systems such as lettuce (Budge and Whipps 2001), witloof chicory (Benigni and Bompeix 2010), peanut (Partridge et al. 2006), garlic (Clarkson et al. 2006) and bean (Li et al. 2002). This demonstrated that integrated control of *S. sclerotiorum* may be an additional tool to other more conventional disease management measures.

The combined use of biocontrol agents and chemical pesticides has gained much attention (Abd-El Moity et al. 1982, Spadaro and Gullino 2005) aimed at obtaining synergistic or additive effects in the control of soil-borne pathogens (Sarkar et al. 2010). For controlling sclerotia-producing fungi, combination of pesticides with biological control agents may have benefits such as reduction in the use of synthetic pesticides (Budge and Whipps 2001). It is also possible that using more than one biocontrol agent may prove effective (Jain et al. 2012). Combining biocontrol agents with cultural practices such as adding organic matter, compost or the use of soil steaming may provide other management approach (Whipps et al. 2007). For example, the

combination of *T. viride* with either tebuconazole or onion waste compost in greenhouse tests enhanced disease control in comparison with the individual treatments with complete suppression of *Allium* white rot caused by *S. cepivorum* Berk. (Clarkson et al. 2006). The integration of partial soil sterilization (pasteurization) with a subsequent *C. minitans* application for a sustainable control of *S. sclerotiorum*, contribute to a rapid colonization of sclerotia compared to non-pasteurized soil (Bennett et al. 2005). Nevertheless, consideration of the economic benefit for growers needs to be evaluated before the adoption of any of the disease control measures mentioned above.

OBJECTIVES OF THIS STUDY

The integration of different management practices has the potential to provide an effective strategy for Sclerotinia stem rot control. Even though some biological control agents have shown as a promising additional tool to disease management, their use in practice is limited due to inconsistent results under field conditions that prevent their practical application in commercial soybean production. The successful integration of biological control agents for disease management would be achieved, if under a routine regimen of agrochemicals, the introduced soil microorganisms maintain their biological control properties as well as adequate population levels in the soil. Thereby, a significant reduction of the inoculum levels of *S. sclerotiorum* might be expected. Since any escaped sclerotia from biological control agent action can potentially become a threat for the plant, an additional treatment during the soybean growing season may strengthen the disease control.

The combination of biological control agents with chemicals, can be an additional measure to enhance the levels of Sclerotinia stem rot control. To achieve this goal, the following objectives were proposed:

1. Assess the sensitivity of *Coniothyrium minitans* (CON/M/91-08), *Trichoderma asperellum* Samuels, Lieckf. & Nirenberg (ICC 012); and *T. gamsii* Samuels & Druzhin (ICC 080) to select agrochemicals *in vitro.*
2. Determine the efficacy of biological control agents combined with chemical applications for managing Sclerotinia stem rot of soybean under field conditions.

CHAPTER TWO: SENSITIVITY OF BIOCONTROL AGENTS TO PESTICIDES

ABSTRACT

Trichoderma asperellum (strain ICC 012), *T. gamsii* (ICC 080), and *Coniothyrium minitans* (CON/M/91-08), which have been studied by others for managing Sclerotinia stem rot (*Sclerotinia sclerotiorum*) were examined for their compatibility with pesticides used in soybean production. This was accomplished by determined *in vitro* sensitivities to 15 conventional pesticides used in soybean production. The herbicide lactofen, and the fungicides propamocarb HCl, mefenoxam and mandipropamid had no inhibitory effects on mycelial growth of *Trichoderma* spp. and *C. minitans*. At the tested levels, *Trichoderma* spp. were additionally insensitive to boscalid, cyazofamid, and thiram. Based on responses in agar plate tests, propamocarb HCl, tetraconazole, and lactofen were applied at 1,166.2; 58.5 and 105.2 g a.i./ha respectively in a soil-mixture containing *S. sclerotiorum* sclerotia and *C. minitans* under growth chamber conditions to assess their effects on survival and biocontrol activity of *C. minitans*. *Coniothyrium minitans* growth was evaluated at1, 2 and 7 days after being inoculated into the soil. The tested chemicals did not significantly reduce the survival as determined by *C. minitans* growth on agar plates ($P = 0.541$) or biocontrol activity as determined by viability of infected sclerotia ($P = 0.601$). The *C. minitans* population decreased significantly as the time of incubation extended in the soil-mixture whether or not they were treated with the chemicals ($P < 0.001$). To test for the potential to tank mix biological control agents, a suspension of five selected herbicides and a biofertilizer were incubated with *C. minitans* for up to 4 hours. No significant change in *C. minitans* growth was observed ($P = 0.944$). These results indicated that some chemicals used in soybean production could be co-applied with *C. minitans*.

INTRODUCTION

The management of Sclerotinia stem rot of soybean relies on multiple strategies including biological control agent application (Del Rio et al. 2002). However, its practical implementation against soilborne pathogens has not been consistently effective, and alternatives aimed to improve the efficacy of biocontrol have been explored (reviewed in Spadaro and Gullino 2005). These alternatives include combinations with other biological control agents, with chemicals, or different agronomic o cultural practices (reviewed in Fravel 2005).

Currently in the U.S, the soybean production include the application of different fungicides for managing different fungal diseases (NCRA 2013) as well as herbicides for weed control (USDA-NASS 2012). The registered fungicide for Sclerotinia stem rot management included thiophanate methyl, boscalid, tetraconazole and prothioconazole (reviewed in Peltier et al. 2012). Accordingly with a recent survey, the used of herbicides are widely extensive compared to compared to other agrochemicals (USDA-NASS 2012). Some of the most commonly applied in soybean crop are: glyphosate potassium salt, glyphosate isopropylamine salt, chlorimuron-ethyl, and flumioxazin (USDA-NASS 2012).This list of pesticides provide possibilities to explore alternatives for the combined application of biological control agents with pesticides or their integration with different agronomical practices aimed to promote plant health, towards to a practical application of the biological control agents.

Evidence of the combination of pesticides and biological control agents are documented for management of diseases caused by *Sclerotinia* spp. These are aimed at reducing fungicide application and promoting an eco-friendly environment (Abd-El Moity et al. 1982, Budge and Whipps 2001, Van Beneden et al. 2010). In a greenhouse experiment, *Trichoderma viride* Pers. strains L14 and S17A combined with a tebuconazole-based seed treatment reduced *Allium* white

rot caused by *S. cepivorum* Berk. compared with using either *T. viride* or tebuconazole alone (Clarkson et al. 2006). Lettuce drop suppression caused by *S. minor* Jagger. was achieved with a single foliar application of iprodione combined with *C. minitans* similar to that obtained with prophylactic sprays with iprodione every 2 weeks. This indicates that reduction foliar iprodione applications could be a feasible option for lettuce drop control (Budge and Whipps 2001). Nevertheless, when studies are extended to field conditions (for example, Clarkson et al., 2006), the level of *Allium* white rot control was less consistent than in the glasshouse assays. These inconsistencies might constitute one of the principal limitations for adoption of biological control agents in commercial crop production.

An additional approach to improve biocontrol efficacy for Sclerotinia stem rot management is the integration of *C. minitans* with other cultural control measures. For instance, in oilseed rape, the combination of *C. minitans* with a N–P–K compound fertilizer application reduced carpogenic germination of *S. sclerotiorum in vitro* (Yang et al. 2011). However, in field the experiments, the application of *C. minitans* either alone or in combination with the compound fertilizer had limited effect on disease suppression, despite the fact that such applications significantly reduced carpogenic germination of *S. sclerotiorum* sclerotia (Yang et al. 2011).

Agronomical practice techniques such as tank mixes ("the associations among two or more chemical products (pesticides) or among chemical products and fertilizers in a unique tank for application in crops") (Tornisielo et al. 2013), could be used as a means to combine biological control agents with other management tools. This may help to the adoption of biological control agents by farmers.

It is essential to recognize the limitations of biological control agents as living organisms and explore different approaches to Sclerotinia stem rot management. Thus, attempts towards the

integration of biological, chemical and cultural methods for successful disease control (Warrior 2000) need to be considered. The objectives of this study were 1) to test the sensitivity of the commercial biocontrol agents *T. asperellum*, *T. gamsii*, and *C. minitans* to selected pesticides in vitro; 2) to assess the effect of selected pesticides on the survival and mycoparasitism activity of *C. minitans* in a potting mix-natural field soil trial in controlled conditions; and 3) to test the survival of *C. minitans* with herbicides or a biofertilizer under tank mix conditions.

MATERIALS AND METHODS

Sensitivities of biological control agents to pesticides *in vitro*

Isolation of biological control agents from commercial products. Isolates of *Trichoderma asperellum* (ICC 012), *T. gamsii* (ICC 080) and *Coniothyrium minitans* (CON/M/91-08), were obtained by plating out the commercial products Tenet® WP (Isagro USA, Inc., Morrisville, NC) and Contans® (Prophyta Biologischer Pflanzenshutz GmbH, malchow/Poel, Germany) on potato dextrose agar (PDA; EMB Chemical Inc., Gibbstown, NJ, USA) at room temperature for 3 d under constant fluorescent light for *Trichoderma* spp. and for 5-10 d in the dark for *C. minitans*. The colonies selected by morphological features were isolated from single conidia and grown in potato dextrose broth (PDB, EMB Chemical Inc., Gibbstown, NJ, USA) at 20°C on a rotatory shaker at 120 rpm for 3 d for *Trichoderma* spp. and 7 d for *C. minitans*. Mycelia were harvested by vacuum filtration through sterile filter paper (Whatman # 1, Clifton, NJ, USA) and used for genomic DNA extraction using the DNeasy Plant Mini kit (QIAGEN, Inc. Valencia, CA. USA) according to the manufacturer's instructions. Fungal identity was confirmed by amplification of the internal transcribed spacer region (ITS) using universal primers ITS 1 and ITS 4 (White et al. 1990). Visualization of PCR products was confirmed by electrophoresis on a 1.5% (w/v) agarose gel containing Tris-Acetate-EDTA [(TAE) 40mM Tris acetate, pH 8.0, 1mM ethylenediaminetetractic acid (EDTA)] as the running buffer, and stained with GelGreen (Biotium Inc., Hayward, CA, USA). Purification of PCR products was done using a PCR purification kit (Denville Scientific Inc., Metuchen, NJ, USA) and products were sequenced at the Michigan State University Genomic Technology Support Facility (East Lansing, MI, USA). The sequences were analyzed using the BLAST algorithm against the National Center of Biotechnology Information (NCBI) GenBank database.

Preparation of inoculum of biological control agents and pesticides. *Trichoderma asperellum* and *T. gamsii* strains were cultured on malt extract agar (MEA; Difco Laboratories, Detroit MI, USA) under constant fluorescent light exposure for 5 d to enhance sporulation (Schrüfer and Lysek 1990). *Coniothyrium minitans* was grown on PDA for 10 d in the dark at 20°C. The plates were flooded with 5 ml of sterile water and the surface was gently scraped using a plastic rod to dislodge the conidia. The resulting suspensions were collected in a 1.8 ml sterile tube for each fungal isolate.

Fourteen pesticides, selected based on their routine use in soybean production in the U.S. (NCRA 2013), and one herbicide reported to reduced Sclerotinia stem rot severity (Dann et al. 1999) were included and evaluated with each fungal isolate (Table 2-1).

Table 2-1. Pesticides used in the soybean cropping system in the U.S. included for the evaluation of commercial biological control agents sensitivity test. All chemicals are fungicides with the exception of lactofen, a post emergence herbicide.

Compounds	Trade name	Manufacturer	Active ingredient (%)
Fludioxonil	Apron maxx	Syngenta crop protection Greensboro, NC	2.30
Mefenoxam	Apron XL	Syngenta	33.3
Metconazole	Caramba	BASF Corp., Research Triangle Park, NC	8.6
Trifloxystrobin	Compass	Bayer Corporation, Kansas City, MO	50
Cyazofamid	Ranman	Ishihara Sangyo Kaisha, Ltd	34.5
Tetraconazole	Domark	Valent USA Corp., Walnut Creek, CA	20.5
Boscalid	Endura	BASF	70
Tebuconazole	Folicur	Bayer	38.7
Azoxystrobin	Heritage 50WG	Syngenta	50
Mandipropamid	Revus	Syngenta	23.3
Propamocarb HCl	Previcur flex	Bayer	66.5
Pyraclostrobin	Pristine	BASF	12.8
Propiconazole	Stratego	Bayer	11.4
Thiram	Thiram	Bayer	42
Lactofen	Cobra	Valent	24

Sensitivity of biological control agents to pesticides using a spiral gradient dilution method

The effective concentration leading to a 50% reduction in mycelial growth (EC_{50}) was determined for all fungal isolates. The spiral gradient endpoint (SGD) method was used as described by Förster et al. (2004). Fifty ml of PDA was poured into 15 cm diameter petri dishes at least 24 h before fungicide solution were applied. Stock concentrations of each of the pesticides (Table 2-1) were prepared at 1,000 mg/L of active ingredient (a.i) in sterile distilled water. A total of 50 µl of pesticides suspension was applied individually to each plate with a Spiral Autoplate ® 4000 (Advanced Instruments, Norwood, MA, USA) using the exponential deposition mode. No salicylhydroxamic acid (SHAM) as inhibitor of alternative respiratory pathway (Olaya et al. 1998) was added to the agar plates for QoI fungicide (azoxystrobin, pyraclostrobin and trifloxystrobin) evaluation.

The plates were incubated at room temperature in a horizontal-flow transfer hood, for 3 h to allow the diffusion of the chemical into the medium, forming a gradient of concentrations along the radius of the plate. The untreated center portion of the plate was removed with a sterile cork borer (25 mm diameter) to avoid growth of the fungus across the plate's center. A droplet of conidial suspension of each isolate was spread along the radial lines using a template at predetermined positions with a sterile glass rod. Duplicate samples for each *Trichoderma* spp. were placed at opposite sides of the plate. *Coniothyrium minitans* was evaluated on different plates due to its slow growth rate (Whipps and Gerlagh 1992) compared to the *Trichoderma* spp. The plates were incubated at 20°C for 3 d and 5-7 d for *Trichoderma* spp. and *C. minitans*, respectively.

The growth of the fungus along the pesticide gradient concentration was recorded based on two parameters: ending radius (ER) (point at which mycelia starts to grow) and the end of

mycelial growth, defined as the tail-ending radius (TER). The mid-distance point between the ER and the TER was calculated for each fungal strain on each treatment. The incubation option in the SGD software was used for calculation of the local concentrations at which 50% growth inhibition was observed. Isolates were rated qualitatively and classified based on SGD software calculations as highly sensitive (if the obtained EC_{50} is below the calculated range), highly insensitive (if the obtained EC_{50} is above the calculated range) or moderate (if the obtained EC_{50} within the calculated range) to the tested pesticide at the concentration evaluated (Wharton 2010). The experiment was repeated with four replicates per isolate.

Survival of *Coniothyrium minitans* with herbicides and fertilizers under tank mix conditions

Inoculum preparation. Conidial suspensions of *C. minitans* (CON/M/91-08) were prepared from cultures grown on PDA for 10 d in the dark at 20°C as described above. The conidial concentration was estimated with a hemocytometer and adjusted approximately to 1×10^7 conidia/ml with sterile water. The conidial suspension (5 ml) was placed in 15 ml centrifuge tubes (Denville, Scientific Inc., Metuchen, NJ).

Herbicides and fertilizer tested. Commercial formulations of herbicides commonly used in the U.S. soybean production (Heatherly et al. 2009). These included: metolachlor (Stalwart® 86.4% a.i., Sipcam Agro USA, Inc., Roswell, GA; field rate 2.3 Kg a.i/ha); sulfentrazone and chlorimuron ethyl (Authority XL® 62.22% a.i, 7.78% a.i., FMC Corporation Agrochemical Products Group, Philadelphia, PA; field rate 0.4 Kg a.i./ha); sulfentrazone and cloransulam-methyl (Sonic®, 62.1% a.i.,7.9% a.i., Dow AgroSicence LLC, Indianapolis, IN; field rate 0.2 Kg a.i./ha), simazine (Simtrol® 42.8% a.i., Sipcam Agro USA, Inc., Roswell, GA; field rate 4.6 Kg a.i./ha), glyphosate (Roundup Power Max®, 48.7% a.i., Monsanto Co., St Louis, Mo; field rate

1.6 Kg a.i./ha) and a biofertilizer Accomplish LM® (Loveland Products Inc. Loveland, CO; field rate 4.6 Kg a.i./ha). The chemicals were prepared according to the recommended field rate and adjusted to a final volume of 5 ml.

Evaluation of *Coniothyrium minitans* survival. A volume of 2.5 ml of the conidial suspension prepared as described was mixed thoroughly with 500 µl of one of the prepared chemical solutions with each chemical tested separately. Suspensions were transferred to 15 ml sterile plastic centrifuge tubes. The prepared suspensions were incubated at 23˚C for 0, 1, 2, and 4 hours, followed by centrifugation at 7,600 × g for 10 min. The conidial pellet was washed three times by centrifugation with sterile water to remove chemicals and resuspended in 1 ml of sterile water. An aliquot of 100 µl of the spore suspension was added to 900 µl of sterile water in a 1.5 ml tube. Serial dilutions with three replications were made on PDA and colony forming units (CFU) ml $^{-1}$of *C. minitans* were estimated after incubation of 10 d at 20°C. Sterile water was used as a control.

Effect of pesticides on *Coniothyrium minitans* survival and its biocontrol activity under growth chamber conditions

Pesticides and commercial biological control preparation. The commercial formulation of propamocarb HCl (Previcur flex® 66.5% a.i. Bayer CropSience, Research Triangle Park, NC) at 1,166.2 g/ha; tetraconazole (Domark® 20.5% a.i. Valent Bioscience Corporation) at 58.5 g/ha and lactofen (Cobra® 24% a.i. Valent U.S.A Corporation, Walnut Creek, CA) at 105.2 g/ha, were selected based on the *C. minitans* sensitivity test. The chemicals were prepared in a water solution and adjusted to the appropriate volume based on the surface area of the treated pots. A commercial formulation of *C. minitans* (CON/M/91-08) ContansWG® was used at the

recommended field rate (2,241.2 g/ha) and applied as an aqueous suspension in sterile distilled water based on the surface area as above.

Soil infestation with pesticides and *Coniothyrium minitans*. Plastic pots (1L with 15 x 15 cm^2 opening) were filled with a mix of: potting mix (BACCTO Professional Planting Mix, Michigan Peat Company, Houston TX 70-80% sphagnum peat) and a mixture of natural field soil with sand and bailed peat (v:v=1:1) obtained from Michigan State University greenhouse facilities. Sclerotia of *S. sclerotiorum* were produced artificially using the method of Jones et al. (2003). Briefly, mushroom spawn bags containing 150 g of wheat and 400 ml of distilled water were autoclaved, allowed to cool for 6 h and inoculated with 5 discs (3 mm diameter) cut from the colony margin of a 5 day-old *S. sclerotiorum* culture grown on PDA. The spawn bags were incubated at room temperature for 4 weeks and exposed to ambient light. Ten sclerotia between approximately 2 to 5 mm long visually were selected and buried at 1 cm depth from the surface of each pot and placed approximately 1 cm apart. The treatments are described in Table 2-2. The pots were placed in a growth chamber (Model: PGR14, Conviron, Winipeg, Manitoba, Canada) adjusted to 20˚C with a 14:10 h light-dark circle. Light intensity was 73.56 µmol $^{-1}$ m $^{-2}$ per µA.

Soil sample collection and processing. Samples were collected three times: 1, 2 and 7 d after the *C. minitans* application. Dilution plating was conducted to enumerate *C. minitans* colony forming units (CFU/g of soil). Briefly, 1 g of soil randomly selected from the pots was agitated in 9 ml of phosphate buffered saline (1.2 g Na_2HPO_4, 0.18 g NaH_2PO_4, 8.5 g NaCl, adjusted to pH 7.4 with 1M HCl) for 10 min on a rotary shaker at 150 rpm. A dilution series was prepared from 1:10 to 1:10^2 and plated onto PDA amended with chlortetracycline (25µg/ml) (AMRESCO, Solon, OH) and Triton X-100 (2 ml/l) (Research Product International Corp, Mt. Prospect, IL). Aliquots of 50 µl were spread onto a semi selective media for *C. minitans* isolation (Bennett et

al. 2003) using the Spiral Autoplate ® 4000 with the even deposition mode feature. Each plate was incubated at 20°C in the dark for 5-10 d. Each dilution was done in duplicate and the experiment was repeated twice.

Table 2-2. Description of treatments combinations included to evaluate the effect of two selected fungicides and one herbicide on a commercial formulation of *Coniothyrium minitans* (CON/M/91-08) survival and its biological control activity against *Sclerotinia sclerotiorum* under 20°C, 14h light:10 h dark.

Treatment 1	Treatment 2	Treatment 3
Sclerotia[5]	Sclerotia	Sclerotia
Sclerotia + *C. minitans* [1]	Sclerotia + *C. minitans*	Sclerotia + *C. minitans*
Sclerotia + Propamocarb HCl [2]	Sclerotia + Tetraconazole [3]	Sclerotia + Lactofen [4]
Sclerotia + *C. minitans* + Propamocarb HCl	Sclerotia + *C. minitans* + Tetraconazole	Sclerotia + *C. minitans* + Lactofen

[1] *Coniothyrium minitans* was applied at the recommended field rate based on the surface area treated. A total volume of 50 ml was applied to the corresponding treated pots.[2, 3, 4] Pesticide treatments were applied at the equivalent field rate of the treated surface area at a total volume of 10 ml per pot. [5] Pots with only sclerotia of *S. sclerotiorum* were treated with sterile water (10 ml) as a control.

Sclerotia viability. Three days after the last sample collection sclerotia were removed from all the evaluated treatments, surface disinfested for 3 min by submerging in 0.6% sodium hypochlorite, rinsed three times in sterile distilled water and placed onto 1.5 % water agar plates (15-mm). The viability of the sclerotia was assessed visually by production of typical mycelia after 3 d of incubation at room temperature in the dark.

Data Analysis

Statistical analysis was performed with SAS Version 9.2 (SAS Institute, Inc., 100 SAS Campus Drive, Cary NC). The tank mix experiment was analyzed with PROC MIXED to test for the significance of the treatments for the main effects and their interaction. Significant difference between treatment means were compared using Tukey-Kramer multiple comparison at $\alpha = 0.05$.

Repeated measurements in Tukey-Kramer grouping were conducted to estimate the *C. minitans* population variation over time. Colony forming units (CFU per gram of soil-mix) were log transformed to satisfy the assumption of homogeneity of variance. The effect of pesticides on *C. minitans* survival experiment was analyzed with PROC MIXED. Significant differences between treatment means were compared using Tukey-Kramer multiple comparison at $\alpha = 0.05$. Analysis of variance for *C. minitans* biocontrol activity and sclerotia viability were evaluated with PROC GLIMMIX. Significant differences between treatment means were compared using Tukey-Kramer multiple comparison at $\alpha = 0.05$. For all the experiments the residual analysis performed by PROC UNIVARIATE confirmed the hypothesis that the experimental errors were normally distributed with a common variance.

RESULTS

Sensitivity of biological control agents to pesticides *in vitro*

Of the fifteen pesticides evaluated, three fungicides mefenoxam, propamocarb HCl, mandipropamid, and the herbicide lactofen did not inhibit mycelial growth of any of the biological control agents evaluated. Although no inhibition of mycelial grown with lactofen treatment was detected, a delay (3-5 d) in conidia production was observed for *C. minitans* compared with the untreated control. *Trichoderma* spp. were highly insensitive to cyazofamid or thiram (Table 2-3). *Coniothyrium minitans* and *Trichoderma* spp. showed moderate sensitivity to fludioxonil, metconazole, trifloxystrobin, tetraconazole, tebuconazole, azoxystrobin, pyraclostrobin and propiconazole (Table 2-3). Sensitivities of the biological control agents to the fungicides mentioned above at the level tested varied depending on the fungus but overall the range was between 0.05 to 1 µg/ml.

Survival of *Coniothyrium minitans* with herbicides and fertilizers under tank mix condition

No significant differences were found between the pesticides (P= 0.944), time of incubation (P= 0.993) or agrochemical × time effect interaction (P= 0.425) on the survival of *C. minitans* in the simulate tank mix conditions (Figure 2-1). Similar levels of *C. minitans* were recovered with simazine, sulfentrazone and cloransulam- methyl, sulfentrazone and chlorimuron-ethyl and the bio-fertilizer booster product (Accomplish LM) compared with the control treatment (Figure 2-1).

Table 2-3. Effective concentration for 50% reduction of mycelial growth (EC_{50}) values for fifteen fungicides and one herbicide. Data are for three biological control fungi as determined by the spiral gradient dilution (SGD) method.

Compound	Dilution range (SGD) [b]	EC_{50} (µg/ml)		
		T. asperellum	*T. gamsii*	*C. minitans*
Fludioxonil	0.03 – 4.34	0.35 [a]	0.06	0.10
Mefenoxam	0.03 – 4.33	≥[c] 4.33	≥ 4.33	≥ 4.33
Metconazole	0.02 – 4.32	0.06	0.07	0.09
Lactofen	0.02 – 4.20	≥ 4.20	≥ 4.20	≥ 4.20
Trifloxystrobin	0.02 – 4.20	0.08	0.12	0.07
Cyazofamid	0.02 – 4.31	≥ 4.31	≥ 4.31	0.09
Tetraconazole	0.02 – 4.27	0.27	0.19	0.05
Boscalid	0.02 – 4.30	≥ 4.30	≥ 4.30	0.10
Tebuconazole	0.02 – 4.32	0.14	0.10	0.08
Azoxystrobin	0.02 – 4.25	0.19	0.21	0.12
Mandipropamid	0.02 – 4.24	≥ 4.24	≥ 4.24	≥ 4.24
Propamocarb HCl	0.03 – 4.28	≥ 4.28	≥ 4.28	≥ 4.28
Pyraclostrobin	0.02 – 4.26	0.43	0.10	1.00
Propiconazole	0.02 – 4.30	0.2	0.71	0.05
Thiram	0.03 – 4.34	≥ 4.34	≥ 4.34	0.24

[a] Each number is the average of four replicates per each fungal isolate per pesticide. The experiment was repeated twice and combined. [b] Range of pesticide concentrations (µg/ml) calculated by the spiral plate dilution (SGD) method. [c] The (≥) indicates that the EC_{50} value is greater than the concentration evaluated and this was classified as highly insensitive.

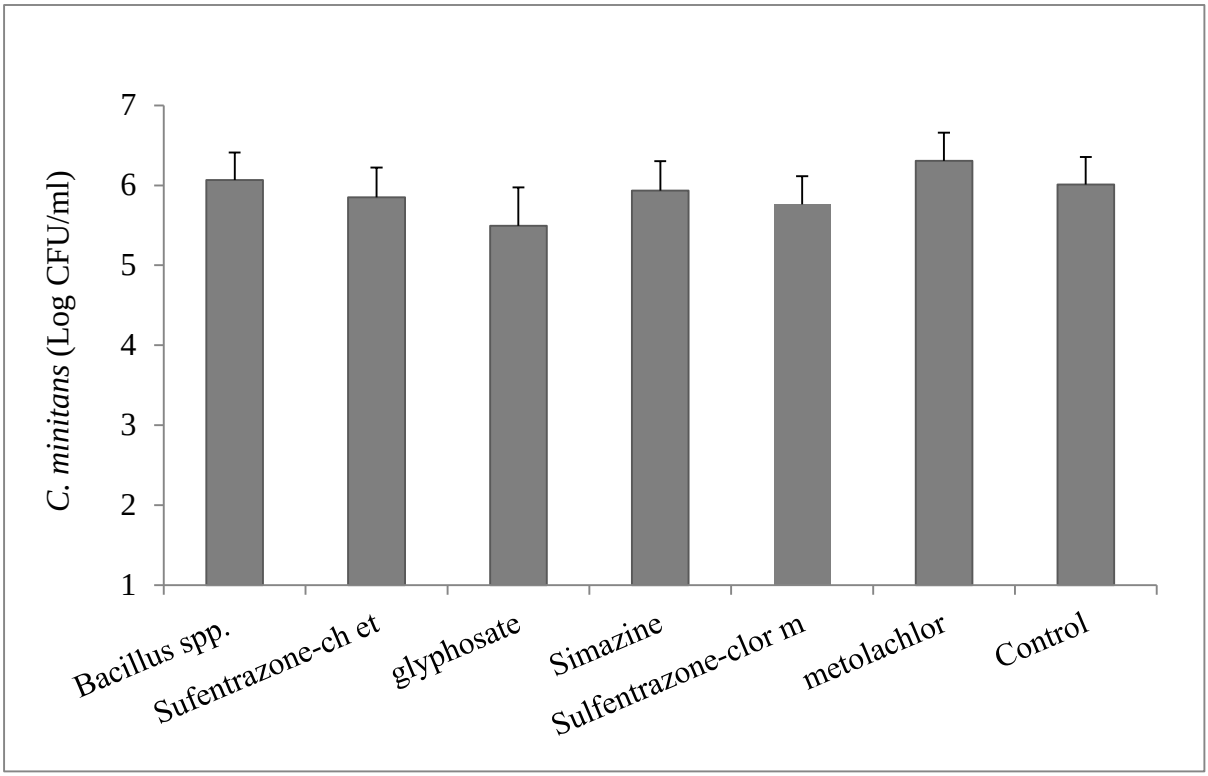


Figure 2-1. Effect of 5 herbicides (below) and a bio-fertilizer (Accomplish LM®) on *Coniothyrium minitans* population under tank mix conditions. Active ingredient, commercial name, percentage of active ingredient (a.i.) and field rate are listed below: metolachlor (Stalwart® 86.4% a.i., 4,677 g/ha), sulfentrazone and cloransulam -methyl (Sonic®, 62.1% a.i., 7.9% a.i., 210.1g/ha), sulfentrazone and chlorimuron-ethyl (Authority XL® 33.33% a.i, 6.67% a.i.; field rate 451.83 g/ha), simazine (Simtrol® 42.8% a.i., 4,677 g/h), glyphosate (Roundup Power Max®, 48.7% a.i., 607.8 g/ha), *Bacillus* spp., *Acidovorax facilis* and *Rhodococcus rhodochorus* (Accomplish LM®, Loveland Products Inc. Loveland, CO; 4,677 g/ha). Sterile water was used as a control. Each column is the average of 32 observations (mean of three replicates per isolate) and the experiment was conducted twice. Bars on each column are standard deviation.

Effect of pesticides on *Coniothyrium minitans* survival and its biocontrol activity under growth chamber conditions

Three pesticides with differing effects on *C. minitans* growth in the plate test were evaluated. These included the herbicide lactofen and the fungicides propamocarb HCl (no inhibitory effect on mycelial growth) and the fungicide tetraconazole (inhibitory effect on mycelial growth). No significant differences in the survival of *C. minitans* were found for the three treatments ($P = 0.5409$) compared to the levels for the biological control agent applied alone**.**

The interaction of the pesticide treatments with the time of exposure did not significantly affect *C. minitans* population ($P = 0.5762$). However, the main effect of time on the BCA population was statistically significant ($P < 0.001$).

The initial level of *C. minitans* before any pesticide application was 4.5 log CFU/g soil. By the second day after its incorporation into the soil and immediately after pesticide application a significant reduction in the level of *C. minitans* (4.12 Log CFU/g soil) was observed compared to the initial population ($P < 0.001$). At the 7 d sample, *C. minitans* was lower than the initial levels (4.32 Log CFU/g soil) ($P =0.007$). At the second day of sampling, lower levels of the biological control agent was found compared with the 7 d ($P = 0.0024$) (Figure 2-2).

Biocontrol activity of *C. minitans* was evaluated after pesticide application and compared with the untreated control. There was a significant reduction in sclerotial viability of *S. sclerotiorum* with *C. minitans* application alone (without pesticide application) or in combination with pesticides ($P < 0.001$) (Figure 2-3). No significant difference between biological control agent alone ($P = 0.601$) or BCA in combination with pesticide treatments ($P = 0.522$) was found.

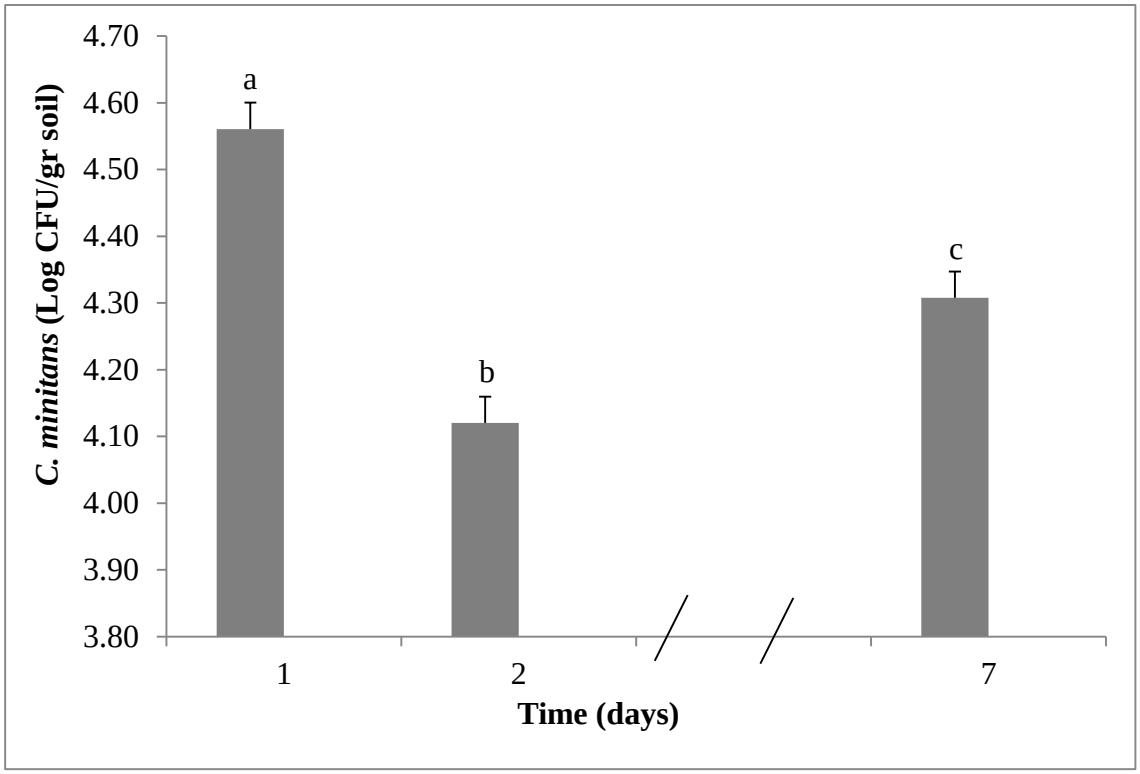


Figure 2-2. Time effect on the survival of *Coniothyrium minitans*. Samples were collected at 1, 2, and 7 days after treatment. *Coniothyrium minitans* population was detected and enumerated by plating on semi-selective media. Columns represent the average of 32 observations of combined experiments. Error bars indicate standard deviation. Mean of *C. minitans* growth followed by same letter are not significantly different at $\alpha = 0.05$ (Tukey-Kramer HSD).

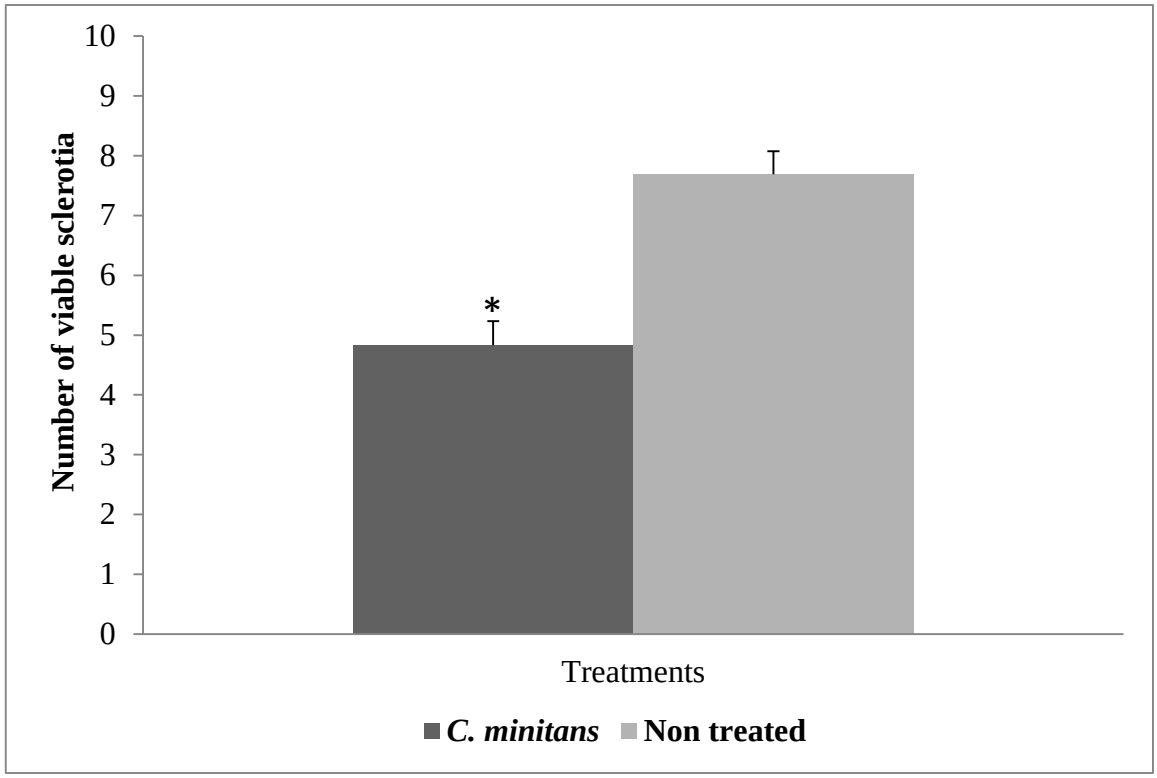


Figure 2-3. Biological control activity of *Coniothyrium minitans* on viability of sclerotia of *Sclerotinia sclerotiorum* in a mixture of potting mix (BACCTO Professional Planting Mix, Michigan Peat Company, Houston TX 70-80% sphagnum peat) and a natural field soil with sand and bailed peat (v:v=1:1). Ten sclerotia were used per pot. The sclerotia were buried in potting mix: soil mixture and retrieved 10 days after *C. minitans* application. Their viability was determined by observing mycelial germination on water agar. Since no significant difference between C. *minitans* alone or in combination with pesticide treatments were found ($P = 0.601$ or $P = 0.522$ respectively), the treatments were combined over *C. minitans* treated and non-treated pots. Columns represent the average of 48 observations of combined experiments. Error bars indicate standard deviation. Means of number of viable sclerotia in each treated and non-treated control were compared by Tukey-Kramer HSD at $\alpha = 0.05$. "*" Significant difference at $P < 0.001$**.**

DISCUSSION

In this study, *Coniothyrium minitans* (CON/M/91-08), *Trichoderma asperellum* (ICC 012) and *T. gamsii* (ICC 080) showed different ranges of sensitivities to the selected pesticides at the level tested (Table 2-3). The commercial biological control agents were classified as insensitive (range above the threshold calculated by SGD) to lactofen, mefenoxam, propamocarb HCl, and mandipropamid. This result was expected since these chemicals are registered either for weed control (lactofen) (Vidrine et al. 1993) or for control of oomycetes (Cohen et al. 2007, Papavizas et al. 1978, Taylor et al. 2002). In addition, other researchers have demonstrated that propamocarb HCl does not have an adverse effect on beneficial microorganisms such as mycorrhizae and *Trichoderma* spp., so it is considered a good component of IPM programs that use biological control (Hu et al. 2007, May and Kimati 2000).

A moderate sensitivity of mycelial growth of *Trichoderma* spp. and *C. minitans* was observed to the quinone outside inhibitors (QoI) (i.e. trifloxystrobin, azoxystrobin, pyraclostrobin), and sterol demethylation inhibitors (DMI) (i.e. metconazole, tetraconazole, tebuconazole and propiconazole) (Table 2-3). The field rates of these fungicide are higher when compared to the EC_{50} concentrations for mycelial growth for both of the biological control agent evaluated. The fungicides are applied in the field in a range of 258 to 1520 µg a.i./ml. This suggests that *Trichoderma* spp. and *C. minitans* might not be combined with the tested fungicides. Although, since this biocontrol agents are applied before planting at the soil level, the detrimental effect of late season foliar applications of these fungicides might be lower than early sprays. Factors such as rainfall or irrigation can contribute to reduction pesticide concentration at the soil surface (Moorman 1989).

Fungicides used as a soybean seed treatment, fludioxonil and thiram, (Mueller et al. 1999) showed different effects on mycelial growth of *C. minitans* whereas *Trichoderma* spp. were inhibit only by fludioxonil under level tested (Table 2-3). A complete inhibition of fludioxonil on *C minitans* mycelial growth was reported previously (Benigni and Bompeix 2010). Similarly, fludioxonil was proved to have an inhibitory effect on mycelial growth for *T. virens* strain G-6 (Howell 2007). Inhibitory effect of thiram on mycelial growth of *C. minitans* was reported as moderate by Budge et al. (2001). An *in vitro* study showed that the combination of thiram with *T. harzianum* and *T. viride* was feasible, since no significant inhibition was detected (Bagwan 2010). These observations might open the possibility to use *Trichoderma* spp. as a soybean seed treatment in combination with thiram to control *S. sclerotiorum* in contaminated seeds. In addition, since a pesticide is concentrated near the treated seed and not in the bulk soil around (Moorman 1989), it might be also possible the soil application of *C minitans* after sowing the treated seeds.

The mycelial growth of *C. minitans* was not inhibited by the herbicide lactofen but a delay in the conidiation of the fungus was observed. Partridge et al. (2006) found that the herbicide flumioxazin reduced the spore germination, growth and biocontrol activity of *C. minitans.* Since flumioxazin and lactofen belong to the same group of diphenyl-ether herbicide, it might be expected to have the same effect on fungal growth; however, unlike the lactofen, the flumioxazin did reduce mycelial growth and conidia germination of *C. minitans* while lactofen only retarded the conidiation without any restriction in vegetative growth. It may be useful to determine the effects of the different components in commercial formulations of pesticides as some of these may interfere with different biological properties of beneficial microorganisms.

This is the first report that the mycelial growth of at least two *Trichoderma* spp. is not inhibited by lactofen. A similar result has been reported for oxyflorofen (diphenyl-ether herbicide, belonging to the same group of herbicides as lactofen), for which no inhibition of *T. viride* occurred (Madhavi et al. 2011). The compatibility of the *Trichoderma* spp. evaluated with lactofen, indicate the potential combination of these treatments for Sclerotinia stem rot of soybean management as new additional strategy.

Propamocarb HCl, tetraconazole, and lactofen did not affect mycelial growth or biocontrol activity of *C. minitans* population in a soil potting mixture. The persistence of lactofen in the soil is considered low (Wauchope et al. 1992) and herbicides of the diphenyl ether family can be degraded by soil microorganism (Chakraborty et al. 2002, Keum et al. 2008). This may explain why it did not have any effect on the biological properties on *C. minitans* in pot trials. Studies with fungicides in the triazole (Strickland et al. 2004, White et al. 2010) and carbamate families (Knowles and Benezet 1981, Myresiotis et al. 2012) have shown no effect on microbial communities in the soil. Some of the other factors involved in the impact of pesticides on soil microbial communities include physical, chemical (Beulke and Malkomes 2001) and biochemical properties of the soil (Monkiedje and Spiteller 2002), and the nature and concentration of the chemicals applied (Kah et al. 2007). All of these factors may play an important role in the potential effect of pesticides on biological control agent's survival when applied to soil environments.

In this study, the levels of *C. minitans* decreased in soil over time (Figure 2-2). Since the sclerotia of *S. sclerotiorum* serve as a reservoir of *C. minitans* and the biocontrol agent has been shown to survive up to 10 months (Bennett et al. 2006), it might expect a progressive increase in the *C. minitans* levels as the sclerotia were infected by the mycoparasite (Gerlagh and Vos 1991,

Jones et al. 2003). However, Jones and Stewart (2011) reported a significant decrease in *C. minitans* recovery from soil immediately after application and during the subsequent evaluation from 1 up to 24 weeks. In other words, there was no return to the initial population levels. In contrast, in other studies the *C. minitans* population did not change during the testing period (Jones et al. 2004, McQuilken et al. 1995). For example, the population remained at the same level (1×10^6 CFU/g) for 30 d, whether the soil was sterile or not (Bennett et al. 2003). In contrast, at the lowest rate (1×10^3 CFU/g) the recovery of *C minitans* in sterile soil versus non-sterile soil was significantly different. This suggests that the successful establishment and survival of *C. minitans* in the soil might be affected either by the interaction with other indigenous soil microorganisms or by the rate of application.

The main purpose of applying herbicides is to eliminate weeds that compete with crop growth and, in particular, for the Sclerotinia stem rot epidemiology because some weeds may serve as potential hosts of *S. sclerotiorum* (Boland and Hall 1994). A secondary advantage may be that some herbicides, such as lactofen, can reduce the severity of Sclerotinia stem rot under field conditions (Dann et al. 1999, Nelson et al. 2002a). This study showed that *C. minitans* is compatible with lactofen, allowing them to be applied together, which might be an advantage in terms of reduced cost of applications. It means that with a single application it may be possible to obtain a dual-purpose treatment, hence reducing the operational cost. A specific cost analysis is required to determine the actual impact to farmers in the adoption of this particular practice.

No inhibitory effect on *C. minitans* growth was observed with the biofertilizer tested under tank mix conditions. It has been documented that the antimicrobial compounds produced by *Bacillus* spp. may inhibit the growth of some fungal pathogens (Fernando et al. 2007, He et al. 1994). However, this was not the case in this study. A possible explanation is that the

production of some secondary metabolites may be modified by external factors such as carbon source, mineral concentration, and environmental conditions (Duffy and Défago 1999, Haas and Keel 2003). It is also possible that the simulated tank mix conditions were not conducive to the production of antimicrobial compounds. However, it would require additional evidence to understand the microbial metabolism under this particular environment. Production of an antimicrobial compound (macrosphelide A) by *C. minitans* is also dependent on other factors: temperature, pH and fungal strain (Tomprefa et al. 2011). This antimicrobial compound has an inhibitory effect to some *Bacillus* spp. (Tomprefa et al. 2009). This suggests that a contrary effect might occur and it may be interesting to establish if is there any negative effect caused by *C. minitans* against *Bacillus* spp. under tank mix conditions.

In conclusion, the *in vitro* study provide preliminary evidence towards the incorporation of the *Trichoderma* spp. and *C. minitans* into an integrated Sclerotinia stem rot management program with some pesticide evaluated. Simulated soil and tank mix conditions indicate that *C. minitans* can be applied together with selected chemicals without limiting its biocontrol ability and growth respectively. Future studies may include the *in vitro* test on *Trichoderma* spp. and *C. minitans* conidial germination with the selected pesticides as well as their effect on the antagonistic activity of *Trichoderma* spp. under soil environment context.

CHAPTER THREE: MANAGEMENT OF SCLEROTINIA STEM ROT BY INTEGRATION OF BIOCONTROL AND CHEMICAL STRATEGIES UNDER FIELD CONDITION

ABSTRACT

Bacillus amyloliquefaciens (BAC03) and the commercial biological control products Contans WP® (*Coniothyrium minitans* CON/M/91-08), Tenet® (mixture of *Trichoderma asperellum* ICC 012 and *T. gamsii* ICC 080), Actinogrow ® (*Streptomyces lydicus* WYEC 108), and Regalia® (*Reynoutria sachalinensis*) were evaluated for their efficacy in reducing *Sclerotinia sclerotiorum* sclerotial populations and Sclerotinia stem rot severity in field trials at two locations in Michigan from 2010 to 2012. The biocontrol materials were either applied to the soil alone or co-applied with the herbicide lactofen, which has been shown to reduce Sclerotinia stem rot severity in some soybean cultivars. Two fungicides, boscalid and thiophanate methyl, were included as foliar treatments. The biological control agents were applied to soil at planting in field plots artificially infested with *S. sclerotiorum* sclerotia. Reductions in the severity of Sclerotinia stem rot by 78 %, 71 % and 70 % were obtained with the co-application of *C. minitans, Trichoderma* spp., and *S. lydicus* with lactofen respectively at Clarksville Horticulture Experimental Station (CLK) under high disease pressure in 2011 and 2012. *Coniothyrium minitans* and *Trichoderma* spp. reduced the sclerotia viability in the soil by 90 % and 86 % respectively at CKL in 2011 and 2012. The co-application of lactofen with *Trichoderma* spp. and *C. minitans* significantly increased the yield compared to the non-treated control in some cases but results depended on the year and location. In 2010, no significant difference in Sclerotinia stem rot reduction was observed with boscalid compared with the non-treated control. Thiophanate methyl reduced the severity of Sclerotinia stem rot at CKL in 2012.

INTRODUCTION

Sclerotinia stem rot of soybean is a threat to soybean production and is a major concern in the north central region of the United States (Grau and Radke 1984, Mueller et al. 2002b, Workneh and Yang 2000).Both the severity of Sclerotinia stem rot and the frequency of outbreaks have increased after the 1990s (Kurle et al. 2001). The adoption of management practices intended to increase soybean yield have been associated with the increase of Sclerotinia stem rot prevalence in north-central region of the U.S. (Mila et al. 2003).

Cultural and management practices associated with reduction of incidence and severity of Sclerotinia stem rot include planting tolerant cultivars (Boland and Hall 1987), zero-tillage (Kurle et al. 2001), wider row spacing and reduction of irrigation regimens (Grau and Radke 1984), and fungicide application in situations where susceptible cultivars must be grown, or where modification of cultural practices is not an option (Mueller et al. 2004). Fungicides such as azoxystrobin, boscalid, thiophanate-methyl, and iprodione have been used against *S. sclerotiorum* with moderate disease suppression (Bradley et al. 2006).

The diphenyl ether herbicides, such as lactofen, may be used as an additional strategy to fungicides. The reduction in Sclerotinia stem rot severity after lactofen application has been associated with the induction of phytoalexins, modification of canopy structure and delay in reproductive development (Dann et al. 1999, Nelson et al. 2002a). In field trials, lactofen treatment resulted in a significant reduction in Sclerotinia stem rot severity of 40-60% (Dann et al. 1999) and higher levels of glyceollin accumulation compared with other herbicides of the same class. However, the reduction in disease severity does not necessarily imply an increase in yield (Nelson et al. 2002a, Nelson et al. 2002b). According to da Rocha and Hammerschmidt

(2005), the poor effect on yield would be the result of the plant's energy resources invested in defense rather than development and reproduction.

Studies have been conducted in evaluating biological control agents to reduce the inoculum population of *S. sclerotiorum* in soil (McQuilken et al. 1995, Santos and Dhingra 1982, Trutmann et al. 1980). Different biocontrol agents derived from antagonistic fungi with diverse modes of action have been shown to suppress *S. sclerotiorum*. Fungal antagonists such as *Coniothyrium minitans* Campbell, have been tested on different crops as a means of reduction of sclerotia levels in soil (Budge and Whipps 1991, Huang 1977, McQuilken et al. 1995, Zeng et al. 2012a). Similarly, *Trichoderma* spp. also have the ability to directly parasitize and degrade the sclerotia of *S. sclerotiorum* (Menendez and Godeas 1998, Santos and Dhingra 1982, Trutmann and Keane 1990). Although successful reduction of *S. sclerotiorum* inoculum has been observed, a better understanding of the ecological context of the fungal antagonist and the pathogen, might contribute to enhance the performance of biological control.

Filamentous bacteria in the genus *Streptomyces*, such as *S. lydicus* have been attractive as biocontrol agents because of the ability to produce secondary antifungal compounds (Yuan and Crawford 1995). Field evaluation showed that *Streptomyces lydicus* WYEC-108 reduced Sclerotinia stem rot severity of soybean by 43.1% and sclerotia levels in soil by 90.6% (Zeng et al. 2012a). This indicated its potential as an effective biological control under natural conditions. Similarly, *Bacillus* spp. also have been recognized for the production of a vast array of biologically active molecules potentially inhibitory to phytopathogen growth (Emmert and Handelsman 1999) such as a novel class of antibiotics (He et al. 1994, Silo-Suh et al. 1994).

Biological control agents including *C. minitans*, *Trichoderma harzianum* Rifai, and *Streptomyces lydicus* WYEC-108 have been previously studied to manage Sclerotinia stem rot in

Michigan (Zeng et al. 2012). The results showed a potential use of some biological control agents as additional strategy to Sclerotinia stem rot management. Although, it may be advantageous to combine different strategies for disease management. For instance, the use of biological control agents that reduce pathogen inoculum in soil (Gerlagh et al. 1999, Santos and Dhingra 1982) and the chemical (lactofen) that is correlated with the induction of defense response in soybean (Dann et al. 1999). Therefore, to enhance the disease management, the objective of this study was to examine the efficacies of these products individually or in combination to affect Sclerotinia stem rot of soybean under field conditions.

MATERIALS AND METHODS

Field plots

Field trials were conducted at the Michigan State University Plant Pathology Research Farm (PLP) (N 42°41.477'; W 84°29.153') in East Lansing, and at the Clarksville Horticulture Experiment Station (CLK) (N 42°42.626'; W 85°33.958'), Clarksville, MI in 2010, 2011 and 2012. The soil at PLP is a sandy loam, with 54.2% sand, 35% silt, and 10.8% clay, and a pH of 7.4. At CLK the soil is also a sandy loam, with 70.2% sand, 25% silt, and 2.8% clay, and a pH of 6.8. In 2010 the plots were arranged as a randomized complete block design (RCBD) with a plot size of 6.1 × 9.1 m^2 at PLP and 9.1× 15.2 m^2 at CKL. In 2011 and 2012 the trials were arranged in a split-plot design. At PLP the plot sizes were 13.7 × 9.14 m^2 in 2011 and 9.1 × 9.1 m^2 in 2012; at CKL the plot sizes were 10.6 × 12.2 m^2 and 9.14 x 9.14 m^2 in 2011 and 2012 respectively. There were four replications at PLP and three replications at CLK.

Soil infestation with *Sclerotinia sclerotiorum*

Sclerotia from commercial soybean production fields were collected from a Michigan grain elevator for the 2010 and 2011 trials, and from D.F Seed Inc., Dansville, MI in 2012. The sclerotia were separated from the seed by a gravity table and collected in burlap seed sacks and stored until used for soil infestation. Approximately 6 kg of sclerotia were evenly distributed each year by hand, onto the soil surface of plots. This was done on the 23rd of April 2010, the 5th of May 2011, and the 11th of May 2012 at PLP. Sclerotia were spread on the 20th of April 2010, the 7th of May 2011, and the 2nd of May 2012 at CLK. The sclerotia were incorporated into the top 10 cm of soil with a cultivator (2210 Field Cultivator, Deere & Company, Moline, IL).

Soil and foliar treatments

The treatments for PLP and CLK trials were applied at the manufacturers recommended field rates. *Coniothyrium minitans* strain CON/M/91-08 (Contans WG, SipcamAdvan, Research Triangle Park, NC), *Trichoderma asperellum* ICC 012 -*T. gamsii* ICC 080 (Tenet WP, SipcamAdvan), *Streptomyces lydicus* WYEC 108 (Actinogrow, SipcamAdvan), *Reynoutria sachalinensis* (Regalia, Marrone Bio Innovations Inc., Davis, CA). *Bacillus amyloliquefaciens (*BCA03) were applied either to the soil or as foliar treatment at soybean R1growth stage as water suspension (Table 3-1).

The chemical fungicides boscalid (Endura, BASF Corp., Research Triangle Park, NC) and thiophanate-methyl (Topsin M 70 WP, Nisso TM LLC, New York, NY) and the herbicide lactofen (Cobra, Valent U.S.A. Corp., Walnut Creek, CA) (Table 3-1) were applied foliarly to the plants and arranged in split-plot designs. All the treatments were applied with a tractor-driven boom sprayer with TEEJET nozzles (model XR11003VS) set 18 cm apart and 45 cm above the soil. Applications of biocontrol agents and chemicals (at rates shown in Table 3-1) were made at speed of 1.77 km/hr with a nozzle pressure of 103.4 kPa.

In 2010, soybean cultivar Northup King S-20 p3 was planted on the 24th of May at PLP and on the 19th of May at CLK; In 2011 and 2012, Great Lakes hybrids GL2449R2 was planted the 12th of May at PLP and the 4th of June at CLK; In 2012, the GL2449R2 was planted on the 29th of May at PLP and the 6th of June at CLK. Soybean seeds were planted in 20 cm row spacing with average seeding rate around 444,790 seed/ha. A sprinkler irrigation system was used to maintain canopy moisture by watering twice each day for 20 minutes at 11:00 and 16:00 h at a volume of 1000 L/ha/h. Irrigation was also applied when soil moisture was below 80% of

field capacity, as measured with soil moisture sensors (CR10X Measurement and Control System, Campbell Scientific, Logan, Utah) placed at 6 cm and 12 cm below the soil surface.

Viability of sclerotial population in soil

Sclerotia were counted at both pre-planting and at harvest. Prior to soil treatment, soil samples were obtained to determine the base line density of *S. sclerotiorum* sclerotia in soil. Four liters of soil were collected from each plot using a trowel. Each bag contained soil samples randomly collected from each plot to a depth of 10 cm. Soil samples were also collected at harvest using the same method. The samples were transported to the laboratory in an ice chest and stored at 4°C until processed. Sclerotia of *S. sclerotiorum* were harvested by wet sieving (Hoes and Huang 1975). The soil was rinsed through a series sieves (U. S. standard sieves series 100-mesh, 200-mesh and 325-mesh, Fisher Scientific Inc., Pittsburgh, PA) using cool running tap water to separate sclerotia. A subsample of ten sclerotia were taken and surface disinfested with 0.6 % sodium hypochlorite for 3 min, and rinsed three times with sterile water for 1min each. The disinfested sclerotia were air-dried on sterile filter paper. Once dried, sclerotia were placed on water agar (1.5%) plates with a sterile forceps and incubated at room temperature in the dark for 5 d. Sclerotia that germinated were considered viable.

Disease evaluation

Disease severity was estimated using a disease severity index (DSI). Plots were rated for disease severity based on the rating system of Grau et al. (1982) at approximately the R7 stage of soybean growth (Fehr et al. 1971) as defined by the maturity of soybean pods on the main stem. Fifty plants from the center two rows were rated on a scale of 0 to 3, with 0 = no symptom, 1 = symptoms on lateral branches, 2 = symptoms on the main stem with little or no damage on pods,

Table 3-1. Biocontrol agents and chemical treatments used in this study with rates of application and dates used to test the efficacy against Sclerotinia stem rot of soybean at the Plant Pathology Farm (PLP) and Clarksville Horticultural Experimental Station (CKL) from 2010, 2011 and 2012.

Year	Treatments	Rate [a]	Date (day-month) [b]	Date (day-month) [c]
2010	*Coniothyrium minitans*	2.2×10^{12} cfu/ha	28-April	4-May
	Streptomyces lydicus	1.1×10^{10} cfu/ha	28-April	4-May
	Trichoderma asperellum + *T. gamsii*	2.8×10^{10} cfu/ha	28-April	4-May
	C. minitans + *T. asperellum* + *T. gamsii*	1.1×10^{12} cfu/ha + 1.4×10^{10} cfu/ha	28-April	4-May
	Reynoutria sachalinensis extract	9,354 ml/ha	19-July	16-July
	Boscalid	1,525 g/ha	19-July	16-July
	Non-treated control			
2011	*C. minitans*	2.2×10^{12} cfu/ha	15-Nov	16-Nov
	C. minitans	1.1×10^{12} cfu/ha	15-Nov -9-May	16-Nov 11-May
	C. minitans	2.2×10^{12} cfu/ha	9-May	11-May
	S. lydicus	1.1×10^{10} cfu/ha	9-May	11-May
	T. asperellum + *T.gamsii*	2.8×10^{10} cfu/ha	9-May	11-May
	Lactofen	105 g/ha	20-July	3-August
	Non-treated control			
2012	*C. minitans*	2.2×10^{12} cfu/ha	17-May	26-May
	C. minitans	1.1×10^{12} cfu/ha	17-May	26-May
	B. amyloliquefaciens BAC03	8×10^{11} cfu/ha	19-July	24-July
	S. lydicus	1.1×10^{10} cfu/ha	17-May-19-July	26-May-24-July
	T. asperellum + *T.gamsii*	2.8×10^{10} cfu/ha	17-May-19-July	26-May-24-July
	Lactofen	105 g/ha	12-July	18-July
	Thiophanate-methyl	1,000 g/ha	27-July	1-August
	Non-treated control			

[a] Biological control agents and chemicals were applied as the rate given in 280 L/ha of water . [b] Dates for treatments application at Plant Pathology Farm (PLP). [c] Dates for treatments application at Clarksville Horticultural Experimental Station (CLK).

and 3 = symptoms on main stem leading to plant death and poor or no pod fill. The DSI was calculated by the formula:

$$\text{DSI} = \frac{\Sigma \text{ disease rating of each plant}}{3 \times \text{number of plants rated}} \times 100$$

Soybean yield evaluation

Soybeans were harvested with a small-plot combine harvester (Massey Harris 35 plot combine, with a 1.8 m cutting bar in 2010 or ALMACO Small Plot Combine (SPC20) with a 1.5 m cutting bar in 2011 and 2012). To avoid plot interactions, only the center of each plot was harvested (one run of the harvester, covering about 66% of the width of the plot). The collected soybean samples were weighed and yield. The yield was calculated as amount of soybeans expressed in kg per harvested area (hectare). Two to three liters of beans were collected from the pile of harvested beans from each plot and transported to the laboratory for examination.

Soybean moisture content and 100 bean weight were determined with a Steinlite Moisture Tester SL95 (Seedburo Equipment Co. Chicago, IL) and seed counter (Count-A-Pak Seed Totalizer Seedburo Equipment Co).

Number of sclerotia in harvested beans

Soybean samples were distributed in white polypropylene pans (29 × 34 cm, Newell Rubbermaid, Atlanta, GA, 30328). Sclerotia were separated from beans by hand and the numbers of sclerotia per kg of harvested beans was recorded.

Data analysis

Statistical analysis was performed using the SAS statistical package Version 9.2 (SAS Institute, Inc., Cary, NC). Analysis of variance was performed using PROC GLM to test the significance of the treatments for main effects and their interaction. Significant difference

between treatment means were compared using the Ryan Einot -Gabriel-Welsh Multiple Range Test with $\alpha = 0.05$. The residue analysis was performed by PROC UNIVARIATE and confirmed the hypothesis that the experimental errors are normally distributed with a common variance.

RESULTS

Disease severity index (DSI) and density of sclerotia in beans

The treatment effects on the DSI were significant at both CLK and PLP ($P < 0.001$) in 2010. At PLP, the DSI was reduced by 75, 77, and 81 % with the *S. lydicus,* the *Trichoderma* spp. /*C. minitans* combined treatment, and the extract of *R. sachalinensis,* respectively, as compared to the non-treated control (Table 3-2). At CLK, the extract of *R. sachalinensis* and the *Trichoderma* spp. /*C. minitans* combined treatment were the most effective, reducing the DSI by 55 % and 53 % respectively compared to the control (Table 3-2). In contrast, in the *Trichoderma* spp. treatment an increase of the disease severity was observed when compared with the non-treated control (Table 3-2).

Significant effects of the treatments on the number of sclerotia in harvested beans were observed at CLK ($P = 0.006$) and PLP ($P = 0.027$), in 2010 (Table 3-2). At PLP, the *C. minitans* applied as single treatment had the highest number of sclerotia/kg of bean and differed significantly from the non-treated control as well as *S. lydicus* and *Trichoderma* spp. /*C. minitans* (Table 3-2). No significant differences were observed between the non-treated control with boscalid, extract of *R. sachalinensis, Trichoderma* spp. (Table 3-2). At CLK, the *C. minitans,* the *Trichoderma* spp. individually applied, *Trichoderma* spp. /*C. minitans* combined application, *S. lydicus* and extract of *R. sachalinensis* had lower sclerotia numbers compared to the non-treated control (Table 3-2). No difference was observed in number of sclerotia/kg of bean with boscalid compared to the non-treated control (Table 3-2).

The combination of the biological control agents and lactofen significantly impacted the DSI at PLP ($P < 0.0004$) and CLK ($P < 0.0001$) in 2011. At PLP, none of the treatments were significantly different from the non-treated control in the reduction of Sclerotinia stem rot severity except the *C. minitans/*Fall treatment in the absence of lactofen. In this treatment, the DSI was higher than the non-treated control (Table 3-3). At CLK, significant DSI reductions were obtained with all the treatments when compared with the non-treated control with the exception of the *Trichoderma* spp. individually applied (Table 3-4).

The effect of the interaction between biological control agents and pesticide treatments on the numbers of sclerotia in the beans was significant ($P < 0.001$*)* at CLK and PLP ($P = 0.003$) in 2011. At PLP, a reduction in the number of sclerotia in the beans was significant for lactofen/*Trichoderma* spp., lactofen/*C. minitans/*Spring combined application, and the *S. lydicus* individually applied compared to the non-treated control (Table 3-3). The highest numbers of sclerotia in the harvested beans were observed in the *C. minitans*/Fall-Spring and *Trichoderma* spp. alone application compared to the non-treated control (Table 3-3). At CLK, significant reduction in the number of sclerotia in harvested beans were found in the lactofen/*C. minitans*/Fall, lactofen/*S. lydicus* and lactofen/*Trichoderma* spp. treatments compared to the same treatment applications without lactofen. No significant difference in sclerotia density were observed when compared the lactofen treatment and the non-treated control (Table 3-4).

A significant chemical and biological control agent interaction for the severity of Sclerotinia stem rot was observed at PLP and CLK ($P < 0.001$) in 2012. At PLP, the most effective treatment was *Trichoderma* spp. alone reducing the severity of Sclerotinia stem rot by 84% followed by 42% for the combined treatment of lactofen/*C. minitans* at full rate (2.2×10^{12} cfu/ha) compared to the non-treated control (Table 3-5)**.** The highest levels of DSI were observed

for BCA03 and lactofen/*Trichoderma* spp. when compared to the non-treated control (Table 3-5). Significant reductions in the DSI at CLK were observed for the *C. minitans* full rate (2.2×10^{12} cfu/ha) and BAC03 in individual or combined application with lactofen when compared to non-treated control. Similarly, *S. lydicus* applied individually, *C. minitans* applied alone at 1.1×10^{12} cfu/ha or combined with thiophanate methyl and lactofen/*Trichoderma* spp. significantly reduced the DSI compared with the non-treated control (Table 3-6).

In 2012 at PLP, the interaction of the biological control agent treatments with the chemicals was significant ($P = 0.037$) for the number of sclerotia in the harvested beans. Significant differences were observed in thiophanate methyl application; foliar application of *S. lydicus* combined with lactofen; and one test with *C. minitans* alone applied at 1.1×10^{12} cfu/ha compared to the non-treated control. No differences were detected among the other evaluated treatments compared with the non-treated control (Table 3-5). At CLK, the interaction of biological control agents with chemical treatments was significant ($P < 0.001$). Application of thiophanate methyl significantly reduced the sclerotia in harvested beans compared to the non-treated control (Table 3-6). Individual application of *S. lydicus*, BCA03 and *C. minitans* at 1.1×10^{12} cfu/ha significantly increased the number of sclerotia in harvested beans compared with the non-treated control. No significant differences were found among the other treatments (Table 3-6).

Table 3-2. Effect of biological control agents *Coniothyrium minitans*, *Trichoderma asperellum*, *T, gamsii, Streptomyces lydicus*; a plant extract from *Reynoutria sachalinensis* or a fungicide boscalid on *Sclerotinia sclerotiorum* viable sclerotia in soil and harvested beans, severity of Sclerotinia stem rot as expressed as a disease severity index (DSI) and yield of soybean in 2010 at two field locations: the Plant Pathology Farm (PLP) and the Clarksville Horticulture Experimental Station (CLK).

Location Treatment	Viable Sclerotia/L soil [v]	DSI [w]	Sclerotia/Kg bean [x]	Yield (Kg/ha)
PLP				
Coniothyrium minitans	0.4 a [y]	18.2 a	3.0 a	4521.0 ab
T. asperellum and *T.gamsii*	0.4 a	14.2 a	1.8 ab	3888.0 b
Trichoderma asperellum +T. gamsii (50%) + C. minitans (50%)	0.4 a	4.2 b	1.1 b	5018.3 a
S. lydicus	0.4 a	4.7 b	1.4 b	4656.3 ab
Reynoutria sachalinensis extract	0 b	3.6 b	2.0 ab	5154.0 a
Boscalid	0.5 a	13.9 a	1.9 ab	3797.7 b
Control	0.5 a	15.9 a	1.5 b	4385.3 ab
CLK				
C. minitans	0.2 b	21.1 b	3.8 c	2414.3 a
T. asperellum and *T.gamsii*	0.2 b	35.1 a	3.8 c	2821.0 a
Trichoderma spp. + *C. minitans*	0.2 b	16.4 c	4.4 bc	2889.0 a
S. lydicus	0.4 a	23.6 b	4.1 bc	2522.3 a
Reynoutria sachalinensis extract	0 c	15.8 c	4.3 bc	2495.3 a
Boscalid	0 c	23.8 b	5.2 ab	2604.0 a
Control	0 c	23.8 b	5.6 a	2522.7 a

[v] Sclerotia were retrieved by washing thorough sieves at harvest on 6 Oct and 7 Oct at PLP and CLK respectively. [w] DSI = disease severity index of Sclerotinia stem rot, evaluated from 90 plants in the plot center on a scale of 0 to 3, with 0 = no symptoms, 1 = symptoms on lateral branches, 2 = symptoms on the main stem with little or no damage on pods, and 3 = symptoms on main stem leading to plant death; poor or no pod fill. The DSI was calculated by [(the sum of disease scale rating of each plant)/ (3 × total plants rated)] × 100. [x] Sclerotia were counted at harvest. [y] The Ryan Einot -Gabriel-Welsh Multiple Range test was conducted for each category of data (in columns). The statistical analysis was done separately for each location.Values followed by the same letter are not significantly different at α= 0.05.

Table 3-3. Effect of biological control agents and the herbicide lactofen applied individually or in combination on severity of Sclerotinia stems rot, number of sclerotia in harvested beans and yield of soybean. Different times of application were included for the *C. minitans* treatments: Fall, Spring and Fall-Spring. The experiment was conducted at the Plant Pathology Farm in 2011.

Treatment	Timing of application[v]	DSI[w]	Sclerotia/ Kg bean[x]	Yield (Kg/ha)
C. minitans	Fall			
Lactofen[u]		3.2 cd[y]	2.9 cd	2936.0 a
no-lactofen		15.0 a	2.0 cd	2634.5 abc
C. minitans	Spring			
Lactofen		4.3 bcd	0 d	2652.7 abc
no-lactofen		3.8 bcd	3.2 cd	2762.3ab
C. minitans	Fall + Spring			
Lactofen		4 bcd	17.5 bcd	2483.5 bc
no-lactofen		0 d	37.2 a	2401.3 c
***Trichoderma* spp.**	Spring			
Lactofen		2.7 cd	0.9 d	2524.7 bc
no-lactofen		0 d	30.7 ab	2506.3 bc
S. lydicus	Spring			
Lactofen		10.3 ab	4.2 cd	2812.8 ab
no-lactofen		8.7 abc	1.3 d	2469.7 bc
No BCA	Spring			
Lactofen		2.0 cd	11.3 cd	2671.0 abc
non-treated control		3.5 bcd	19.2 bc	2579.5 bc

[u] The herbicide lactofen was applied as a split treatment at the R1 growth stage of soybean. [v] The BCAs were applied as a complete plot treatment before planting as a spray suspension on the soil surface. [w] DSI = disease severity index, evaluated from 150 plants in the plot center on a scale of 0 to 3, with 0 = no symptoms, 1 = symptoms on lateral branches, 2 = symptoms on the main stem with little or no damage on pods, and 3 = symptoms on main stem leading to plant death and poor or no pod fill. The DSI was calculated by (the sum of disease scale rating of each plant)/ (3 × total plants rated) × 100. [x] Sclerotia were counted and evaluated at harvest. [y] The Ryan Einot - Gabriel-Welsh Multiple Range test was conducted for each category of data (in columns). Values followed by the same letter are not significantly different at α=0.05.

Sclerotia viability in the soil

In 2010 at PLP, a significant reduction in sclerotia viability was observed with the extract of *R. sachalinensis* treatment compared with the non-treated control and the other treatments evaluated (Table 3-2). In 2010 at CLK, the number of viable sclerotia in the soil with *S. lydicus* application was significantly higher compared to the extract of *R. sachalinensis*, boscalid and the non-treated control (Table 3-2). Similarly, viability of sclerotia in soil was higher for the *C. minitans*, *Trichoderma* spp. and *C. minitans*/*Trichoderma* spp. treatments compared with the non-treated control (Table 3-2). In 2011 at PLP, no sclerotia were found from any plots evaluated. At CLK, the interaction of chemical with biological control agents was significant ($P < 0.001$). A significant reduction of 98 % was observed for the Fall application of *C. minitans* in the absence of lactofen, compared to lactofen applied alone and the non-treated control (Table 3-3). *Coniothyrium minitans* applied at Fall or Spring combined with lactofen or in individual application reduced the sclerotia viability in the soil by 98, 91, and 90%, respectively (Table 3-4).

In 2012, the biocontrol and chemical treatments interaction for the reduction of sclerotia viability in soil was significant at PLP ($P < 0.002$). *Coniothyrium minitans* at the full rate (2.2×10^{12} cfu/ha) combined with lactofen reduced sclerotial viability in soil by 82 % when compared to the same treatments applied individually. The highest number of viable sclerotia in soil was found in the lactofen/*Trichoderma* spp. combined treatment. No significant differences were found between the other treatments evaluated compared to the non-treated control (Table 3-5). The biocontrol agent treatments effect was significant for viability of sclerotia in the soil at CLK ($P<0.001$). Significant reductions by 79 % and 86 % for foliar applied *S. lydicus* and *Trichoderma* spp. respectively were observed compared to *C. minitans* applied at 2.2×10^{12}

cfu/ha and 1.1×10^{12} cfu/ha of the recommended field rate; BCA03; *S. lydicus* and non-treated control. *Trichoderma* spp. ground application combined with lactofen had the highest number of viable sclerotia in soil (Table 3-6).

Table 3-4. Effect of biological control agents applied at varying times in absence/combination with the herbicide lactofen on soil levels of *Sclerotinia sclerotiorum* viable sclerotia, severity of Sclerotinia stems rot, number of sclerotia in harvested beans and yield of soybean at Clarksville Horticulture Experiment Station in 2011.

Treatment [t]	Timing of application[v]	Viable Sclerotia/L soil[w]	DSI[x]	Sclerotia/ Kg bean[y]	Yield (Kg/ha)
C. minitans	Fall				828.3 a
Lactofen[u]		0.7 de[z]	14.1 ef	2.9 bc	
no-lactofen		0.1 f	23.7 d	55.9 a	
C. minitans	Spring				723.8 ab
Lactofen		0.4 ef	16.1 ef	2.0 bc	
no-lactofen		0.5 ef	16.0 ef	19.1 bc	
C. minitans	Fall + Spring				585.6 b
Lactofen		0.9 cde	13.0 f	2.7 bc	
no-lactofen		2.1b	37.7 c	22.4 b	
***Trichoderma* spp.**	Spring				688.6 ab
Lactofen		2.3 b	18.8 de	7.2 bc	
no-lactofen		1.1cd	65.1 a	63.8 a	
S. lydicus	Spring				803.5 a
Lactofen		1.1 cd	19.5 de	0.6 c	
no-lactofen		1.9 b	19.9 de	64.2 a	
No BCA	Spring				743.2 ab
Lactofen		5.3 a	10.7 f	8.2 bc	
non-treated control		1.2 c	52.7 b	4.4 bc	

[u] The herbicide lactofen was applied as a split treatment at the R1 growth stage of soybean. [v] The biological control agents were applied as a complete plot treatment before planting as spray suspension on the top soil surface. [w] Sclerotia were retrieved by washing through sieves at harvest on 15 Nov. [x] DSI = disease severity index, evaluated from 150 plants in the plot center on a scale of 0 to 3, with 0 = no symptoms, 1 = symptoms on lateral branches, 2 = symptoms on the main stem with little or no damage on pods, and 3 = symptoms on main stem leading to plant death and poor or no pod fill. The DSI was calculated by (the sum of disease scale rating of each plant)/ (3 × total plants rated) × 100. [y] Sclerotia were counted and evaluated at harvest. [z] The Ryan Einot -Gabriel-Welsh Multiple Range test was conducted for each category of data (in columns). Values followed by the same letter are not significantly different at α=0.05.

Yield evaluation

A significant effect of the interaction between biological control agent and chemical application on yield was observed at PLP in 2010 ($P = 0.006$). Yield was significantly higher with the extract of *R. sachalinensis* and the combination of *C. minitans* and *Trichoderma* spp. compared with *Trichoderma* spp. alone and boscalid. No significant differences were found between *S. lydicus*, and *C. minitans* compared with the non-treated control (Table 3-2). At CLK, no significant effect of biological control agent or fungicide was observed ($P = 0.802$) on the yield (Table 3-2). At PLP the effect of biological control agent and chemical interaction on yield was significant ($P = 0.02$) in 2011. The Fall application of *C. minitans* had a higher yield than the non-treated control. No significant differences were observed on yield among the other evaluated treatments (Table 3-3). At CLK the effect of biological control agent was significant ($P = 0.02$). The Fall application of *C. minitans* and *S. lydicus* was significantly different from Fall/Spring application of *C. minitans.* None of the evaluated treatments were significantly different from the control (Table 3-4). In 2012 at PLP, no significant differences were found among the treatments compared with the non-treated control except the thiophanate-methyl application compared with *Trichoderma* spp. alone application (Table 3-5). At CLK, there was an interaction between biological control agent and chemical treatments for soybean yield response ($P = 0.024$). The yield was increased with *Trichoderma* spp. in combination with lactofen when compared to the non-treated control. No significant differences were observed among the other treatments (Table 3-6).

Table 3-5. Effect of biocontrol agents with foliar or ground application in absence/presence of the herbicide lactofen or with thiophanate-methyl on soil levels of *Sclerotinia sclerotiorum* viable sclerotia, severity of Sclerotinia stem rot, number of sclerotia in harvested beans and yield of soybean at the Plant Pathology Farm in 2012.

Treatment[t]	Type of application[u]	Viable Sclerotia /L soil [v]	DSI [w]	Sclerotia/ Kg bean [x]	Yield (Kg/ha)
C. minitans 2.2 $\times 10^{12}$cfu/ha	Ground				
Lactofen		0.7 c[y]	39.3 e	13.3 abc	4478.7 ab
no-lactofen		3.2 ab	73.8 b	11.7 bcd	4326.0 ab
C. minitans 1.1 $\times 10^{12}$cfu/ha	Ground				
Thiophanate-methyl		1.6 abc	55.1 cd	13.5 abc	4610.3 ab
no-thiophanate-methyl		1.4 abc	52.1 d	3.0 e	4563.0 ab
BCA03	Ground				
Lactofen		1.7 abc	86.8 a	8.3 cde	4501.7 ab
no-lactofen		2.0 abc	94.7 a	13.0 abc	4336.7 ab
Trichoderma spp.	Foliar				
Lactofen		2.0 abc	63.3 bcd	12.8 abc	4599.5 ab
no-lactofen		1.6 abc	66.8 bc	20.1 a	4282.0 ab
Trichoderma spp.	Ground				
Lactofen		3.9 a	86.7 a	9.3 cde	4167.5 ab
no-lactofen		1.5 abc	14.8 f	5.4 cde	4065.0 b
S. lydicus/**lactofen**	Foliar	1.0 bc	64.0 bcd	3.6 e	4565.0 ab
S. lydicus/**no-lactofen**	Ground	1.0 bc	64.3 bcd	18.4 ab	4682.3 ab
Thiophanate methyl	Foliar	2.0 abc	53.7 cd	4.7 de	4896.3 a
Non-treated control		1.2 bc	66.5 bc	13.3 abc	4467.0 ab

[t] Chemicals were applied as subplot treatments at the V6 and R1 stages of soybean growth for lactofen and thiophanate methyl respectively. [u] Foliar application of biological control agents was done at the R1 stages of soybean growth and ground application was done before planting as a spray suspension on the soil surface. [v] Sclerotia were retrieved by washing through sieves at harvest on 2 Nov. [w] DSI = disease severity index, evaluated from 150 plants in the plot center on scale of 0 to 3, with 0 = no symptoms, 1 = symptoms on lateral branches, 2 = symptoms on the main stem with little or no damage on pods, and 3 = symptoms on main stem leading to plant death and poor or no pod fill. The DSI was calculated by [(the sum of disease scale rating of each plant)/ (3 × total plants rated)] × 100. [x] Sclerotia were counted and evaluated at harvest. [y] The Ryan Einot -Gabriel-Welsh Multiple Range test was conducted for each category of data (in columns). Values followed by the same letter are not significantly different at $\alpha = 0.05$.

Table 3-6. Effect of biocontrol agents with foliar or ground application in absence/presence of the herbicide lactofen or with thiophanate-methyl on soil levels of *Sclerotinia sclerotiorum* viable sclerotia, severity of Sclerotinia stem rot, number of sclerotia in harvested beans and yield of soybean at the Clarksville Horticulture Experiment Station in 2012.

Treatment[t]	Type of application[u]	Viable Sclerotia/ L soil [v]	DSI [w]	Sclerotia/ Kg bean [x]	Yield (Kg/ha)
C. minitans 2.2 ×10^{12}cfu/ha	Ground	9.2 bcd[y]			
Lactofen			36.5 f	69.0 bcd	1147.5 b
no-lactofen			38.7 ef	60.0 cde	2252.5 ab
C. minitans 1.1 ×10^{12}cfu/ha	Ground	8.4 cd			
Thiophanate-methyl			86.1 bc	66.2 cd	1718.5 ab
no-thiophanate-methyl			71.3 d	164.1 a	845.0 b
BCA03	Ground	8.1 d			
Lactofen			47.0 ef	31.8 ef	1733.0 ab
no-lactofen			51.0 e	94.6 b	1515.0 ab
Trichoderma spp.	Foliar	2.2 e			
Lactofen			99.0 a	42.4 de	1725.0 ab
no-lactofen			89.8 abc	78.7 bc	2939.0 ab
Trichoderma spp.	Ground	14.6 a			
Lactofen			40.8 ef	51.8 cde	3536.0 a
no-lactofen			97.0 ab	56.5 cde	1208.0 b
S. lydicus/**lactofen**	Foliar	3.0 e	91.2 abc	42.0 de	1781.5 ab
S. lydicus/**no-lactofen**	Ground	11.3 b	45.0 ef	95.7 b	2196.5 ab
Thiophanate methyl	Foliar	11.0 bc	82.5 c	14.0 f	1006.5 b
Non-treated control		8.2 cd	100.0 a	51.0 cde	1421.0 b

[t] Chemicals were applied as subplot treatments at the V6 and R1stages of soybean growth for lactofen and thiophanate methyl respectively. [u] Foliar application of biological control agents was done at the R1soybean growth stage and ground application of was done before planting as a spray suspension on the soil surface. [v] Sclerotia were retrieved by washing through sieves at harvest on 2 Nov. [w] DSI = disease severity index, evaluated from 150 plants in the plot center on scale of 0 to 3, with 0 = no symptoms, 1 = symptoms on lateral branches, 2 = symptoms on the main stem with little or no damage on pods, and 3 = symptoms on main stem leading to plant death and poor or no pod fill. The DSI was calculated by [(the sum of disease scale rating of each plant)/ (3 × total plants rated)] × 100. [x] Sclerotia were counted at harvest. [y] The Ryan Einot - Gabriel-Welsh Multiple Range test was conducted for each category of data (in columns). Values followed by the same letter are not significantly different at $\alpha = 0.05$.

Table 3-7. Summary effects of the biological control agents evaluated at different time, mode of application or in absence/combination with the herbicide lactofen on Sclerotinia stem rot severity and yield of soybean at Plant Pathology Farm in 2010, 2011 and 2012.

Treatment	2010		2011		2012	
	DSI	Yield	DSI	Yield	DSI	Yield
Streptomyces lydicus	+	0				
C. minitans + *T. asperellum* + *T. gamsii*	+	0				
Reynoutria sachalinensis extract	+	0				
C. minitans Fall						
Lactofen			0	+		
no-lactofen			-	0		
C. minitans 2.2 $\times 10^{12}$cfu/ha						
Lactofen					+	0
C. minitans 1.1 $\times 10^{12}$cfu/ha						
no-thiophanate-methyl					+	0
BCA03						
Lactofen					-	0
no-lactofen					-	0
Trichoderma spp.						
Lactofen					-	0
no-lactofen					+	0

(+) Reduction of Sclerotinia stem rot severity or yield increase by the biological control agents, chemical treatment or their combination compared to the non-treated control. (0) No significant effect of the biocontrol, chemical treatment or their combination in reduction Sclerotinia stem rot severity or yield increase compared to the non-treated control. (-) Increase in Sclerotinia stem rot severity of soybean by the biological control agents, chemical treatment or their combination compared to the non-treated control.

Table 3-8. Summary effects of the biological control agents evaluated at different time, mode of application or in absence/combination with the herbicide lactofen on Sclerotinia stem rot severity and yield of soybean at Clarksville Horticultural Experimental Station in 2010, 2011 and 2012.

Treatment	2010		2011		2012	
	DSI	Yield	DSI	Yield	DSI	Yield
Trichoderma asperellum + *T. gamsii*	-	0				
C. minitans + *T. asperellum* + *T. gamsii*	+	0				
Reynoutria sachalinensis extract	+	0				
C. minitans Fall				0		
Lactofen			+			
no-lactofen			+			
C. minitans Spring				0		
Lactofen			+			
no-lactofen			+			
C. minitans Fall + Spring				0		
Lactofen			+			
no-lactofen			+			
Trichoderma spp.				0		
Lactofen			+			
no-lactofen			-			
S. lydicus				0		
Lactofen			+			
no-lactofen			+			
Lactofen			+	0		
C. minitans 2.2 $\times 10^{12}$cfu/ha						
Lactofen					+	0
no-lactofen					+	0
C. minitans 1.1 $\times 10^{12}$cfu/ha						
Thiophanate-methyl					+	0
no-thiophanate-methyl					+	0
BCA03						
Lactofen					+	0
no-lactofen					+	0
Trichoderma spp.						
Lactofen					+	+
S. lydicus/no-lactofen					+	0
Thiophanate methyl					+	0

(+) Reduction of Sclerotinia stem rot severity or yield increase by the biological control agents, chemical treatment or their combination compared to the non-treated control. (0) No significant effect of the biocontrol, chemical treatment or their combination in reduction Sclerotinia stem rot severity or yield increase compared to the non-treated control. (-) Increase in Sclerotinia stem rot severity of soybean by the biological control agents, chemical treatment or their combination compared to the non-treated control.

DISCUSSION

Coniothyrium minitans, *Trichoderma* spp. and *S. lydicus* can be effective in reducing the severity of Sclerotinia stem rot, viability of sclerotia populations in the soil and sclerotia density in harvested beans, but their performance varied among the years and locations. The lack of consistency of microbial antagonist for disease suppression has been previously reported (reviewed in Adams 1990, Boland 1997, Inglis and Boland 1992). It is not always clear which factors may account for inconsistencies or which factors cause an antagonist to be effective in biological control (Stolk et al. 1998). This situation may limit the adoption of these products in commercial soybean production.

Effective reductions in the DSI and density of viable sclerotia in soil by *Coniothyrium minitans* was observed in some instances, depending on the location and on the years with particularly high disease levels (Table 3-4, 3-5 and 3-6). Other reports have shown that the effective disease suppression by *C. minitans* was observed under low disease pressure (Budge et al. 1995, Budge and Whipps 1991, Huang et al. 2000b). The extra 'nutrient source' provided by the artificial soil infestation with sclerotia before planting during the current study might stimulate the spore production of *C. minitans* fostering further propagation in the treated plots. Similar observations was made by del Rio et al. (2002) with the mycoparasite *Sporidesmium sclerotivorum* Uecker, Adams et Ayers in a plots with high disease pressure.

A significant reduction in sclerotia viability of *S. sclerotiorum* was observed at Fall and Spring application of *C. minitans* compared with the non-treated control (Table 3-4). Under controlled conditions, *C. minitans* strain CON/M/91-08 has a maximum growth rate at 18 to 20ºC (Zeng et al. 2012b). Temperatures either at fall or spring might provide the optimum growth conditions and establishment for of *C. minitans* in the soil. The similarity between *C.*

minitans treated plots and the non-treated in regard of sclerotia viability in the soil, may be related with the reported dispersal mechanisms of *C. minitans* such as irrigation water (Williams et al. 1998a), and soil mesofauna (Williams et al. 1998b). The combination of any of these dissemination factors with *C. minitans* application may improve the conidial dissemination efficiency in the soil. An experiment with oilseed rape has reported a promissory result using water-assisted application of *C. minitans* (Yang et al. 2009). Evaluation of this alternative mode of *C minitans* application in soybean production may be useful to achieve consistent levels of Sclerotinia stem rot control.

Trichoderma spp. reduced the DSI in 2011 and 2012 at CKL and 2012 only at PLP (summary Table 3-8). Their role in reduction of viable sclerotia density in soil was minimal. Infection of sclerotia under controlled conditions (Clarkson et al. 2004, Inbar et al. 1996, Knudsen et al. 1991) and suppression of Sclerotinia diseases have been documented in other crop-systems by *Trichoderma* spp. (Huang et al. 2000a, Zeng et al. 2012a), though under field conditions the efficacy may be reduced (Budge and Whipps 1991, Ojaghian 2011). Different abiotic factors (Duffy et al. 1997, Kredics et al. 2003) and biotic factors (Bae and Knudsen 2005, Simon and Sivasithamparam 1988) may reduce growth and establishment of *Trichoderma* spp. in soil ecosystems. The difference in clay content at PLP and CLK (10.8% and 2.8% respectively), might impacted the biocontrol activity of the tested *Trichoderma* spp. Although, a report with *T. koningii* indicated a positive correlation of clay contend with its biocontrol activity (Duffy et al. 1997). In fact, different mechanisms act synergistically to achieve disease control in some *Trichoderma* spp. (reviewed in Howell 2003). According to Duffy et al. (1997), the relative importance of individual mechanisms depending on the strain, environmental conditions, and pathosystems; factors that might account for the results observed in the current study.

A relatively limited impact in reduction of sclerotia viability in the soil was observed with *Trichoderma* spp. when compared with non-treated control for 2011 and 2012 at PLP. The spatial pattern distribution of *S. sclerotiorum* sclerotia in the soil seems to play an important role for a successful colonization of *T. harzianum*. Sclerotia in highly aggregated spatial pattern were significantly colonized by the biocontrol in the soil compared to sclerotia in random distributions (Bae and Knudsen 2007). It is possible that the even artificially infested sclerotia in the soil may impact the biocontrol efficacy of *Trichoderma* spp. as a result of the modification of the natural spatial pattern arrangement of sclerotia. This could be particularly important in terms of the cultural practices that involved soil disturbance and the biocontrol application.

The bacterial strains included in this study had limited effect in Sclerotinia stem rot suppression, although *S. lydicus* reduced the DSI in 2010 at PLP and 2012 at CLK and reduced the sclerotial viability at CLK in 2012. Under growth chamber condition a complete reduction of apothecia formed was observed with *S. lydicus* WYEC108 (Zeng et al. 2012b), which may account for its effect in disease suppression, albeit the efficacy of *S. lydicus* was not consistent under field conditions.

The co-application of biological control agents and the herbicide lactofen might be advantageous strategy for disease control since they focus on different targets: the biocontrol agents infect the primary inoculum of *S. sclerotiorum* (Abdullah et al. 2008, Froes et al. 2012, McQuilken et al. 1995) and lactofen is associated with a plant defense response (Dann et al. 1999). The results were variable and dependent on the location and the year. Lactofen did strengthen the disease suppression when was combined with *Trichoderma* spp., and *C. minitans* particularly in years with high disease pressure (2011 and 2012) (summary Table 3-7 and 3-8). In some cases the individual application of the biocontrol agents also reduced the Sclerotinia stem

rot severity compared the non-treated control. Based on these results it is difficult to provide any conclusive recommendation due to the fluctuating results over the years evaluated. Many factors, including biotic and abiotic environment (Bostock 2005), host genotype (reviewed in Da Rocha and Hammerschmidt 2005, Herman et al. 2007) and the extent to which plants in the field are already induced (reviewed in \Walters and Fountaine 2009) might modify or influence the response of a resistance activators such as the herbicide lactofen.

Interestingly, disease reduction and yield increase was observed if *Trichoderma* spp. were combined with lactofen at CKL in 2012 (summary Table 3-8). According with Nelson et al. (2002a), the treatment of different soybean cultivars with postemergence herbicide such lactofen reduced the number of flower per plant and influenced the peak flowering for all cultivars. This could have direct implication in disease severity, as was indicated by Nelson and co-workers, since it reduces the number of infection court (flowering stage) and convenient time (delay peak of flowering) for *S. sclerotiorum* infection. The treatment of lactofen in combination with *C. minitans* in 2011 at PLP (summary Table 3-7) did not reduced the Sclerotinia disease severity but did increase the yield. The variation in the relationship between yield loss and disease incidence in soybean may be the result of plant spatial compensation capacity. This correlation between yield and disease is also affected by the environmental conditions after the infection process has stared (Yang et al. 1999). Thus, recommendation of Sclerotinia stem rot management with combined application of lactofen and biological treatment needs to be cautious given that disease control and ultimately yield depend on multiple factors.

The fungicide application in the years evaluated also were inconsistent in the Sclerotinia stem rot reduction and yield. Application of boscalid at PLP and CLK in 2010 (Table 3-2) did not reduce the disease severity compared with the non-treated control. According with Mueller et

al. (2002a), poor coverage of the fungicide on the existing infection sites as well as additional blossoms developing after that the fungicide has been applied might impact the efficacy of fungicide. The application of thiophanate methyl at CLK in 2012, a year with a high disease level, was effective in the diseases reduction but did not showed any benefits in yield when compared with the non-treated control plot (summary Table 3-8). It is possible that although the thiophanate methyl application reduced disease other factor such as environmental conditions, or the presence of other soybean pathogen under field conditions might potentially limit the grain yield.

In summary, the results indicated that reductions in the inoculum potential in the soil and in Sclerotinia stem rot severity could be achieved by *C. minitans* and *Trichoderma* spp. However, the effect varied over the years and locations. Similarly, combination of biocontrol agents with lactofen provided effective reduction of Sclerotinia stem rot in some cases but not others. The yield did not increase in all cases where disease severity was reduced. This variable result emphasizes the need of evaluating different approaches that help to increase the stability of disease suppression. For instance the selection of biocontrol strains natural adapted to soybean-soil ecosysistems. Further studies might evaluate the co-application of lactofen with selected fungicides that may offer less variation in Sclerotinia stem rot management.

APPENDIX

THE EFFECT OF LACTOFEN ON REDUCTION OF SCLEROTINIA STEM ROT UNDER CONTROLLED CONDITIONS

INTRODUCTION

In conventional agriculture synthetic chemical activators of resistance have been tested in different pathosystems (Dann and Deverall 1996, Faoro et al. 2008, Katz et al. 1998), providing a different approach to conventional disease management. Chemical activators such as 2,6-dichloro-isonicotinic acid (INA) or S-methyl benzo [1,2,3] thiadiazole-7-carbothioate (ASM) induced the defense response by mimicking the signal molecule salicylic acid (SA) and inducing pathogenesis-related proteins (PR-proteins) gene expression (Friedrich et al. 1996, Uknes et al. 1993). In soybean multiple applications of these compounds under field and greenhouse conditions resulted in a reduction on Sclerotinia stem rot severity by 70 % and 60% with INA and ASM in susceptible varieties respectively (Dann et al. 1998). The adoption of these materials in commercial soybean production may be hindered by their variable disease suppression over the period of time and fields evaluated.

Variations in disease incidence and severity by the induction of defense response in plants have been also documented with synthetic herbicides (reviewed in Altman and Campbell 1977, reviewed in Johal and Huber 2009). In soybean, lactofen, a postemergence herbicide of the diphenyl ether class, has shown a protective effect against Sclerotinia stem rot under field conditions (Dann et al. 1999). According to this study 40% to 60% disease reduction was observed when lactofen was applied at an early soybean growth stage (V3-third trifoliolate- three unfolded trifoliolate leaves) or at a later growth stage close to flowering. High levels of the soybean phytoalexins were detected in herbicide injured leaves and a significant reduction in lesion size of *S. sclerotiorum* were observed on plants treated at the V4 growth stage compared

with the untreated plant's leaves (Dann et al. 1999). Subsequent studies of the effect of lactofen on Sclerotinia stem rot revealed that the impact of the herbicide treatment is cultivar dependent and the increase of phytoalexin production was detected up to 26 days after treatment (Nelson et al. 2002a). Another study indicated that levels of glyceollin were observed in the leaves but were not detected in the stem of the treated plants (Nelson et al. 2002b). This may suggest that an additional mechanism(s) could be involved in the plant defense response of soybean treated with lactofen.

The expression of plant induced resistance can be influenced by host genotype, biotic and abiotic and environmental conditions (reviewed in Vallad and Goodman 2004). Hence, the objective of this experiment are: 1. Evaluate the effect of Cobra® (a.i lactofen) on Sclerotinia stem rot in a commercial soybean variety Great Lakes Hybrids GL2449R2 which belong to maturity group 2.5 very suitable for Michigan condition, 2. Test two different growth stages at the V2 and the V6 under greenhouse conditions to define the best time for an effective disease reduction. 3. Evaluate three different concentrations of lactofen to determine the extent of effective disease response.

MATERIALS AND METHODS

Plant material and growth conditions.

Glyphosate-resistant soybean (variety GL2449R2, Great Lakes® Hybrids) seeds with partial resistance to Sclerotinia stem rot were planted in plastic pots 15.5 cm (height) x 20 cm (diameter of top opening) filled with potting mix (BACTO Professional Planting Mix, Michigan Peat Company, Houston, TX; 70%-80% sphagnum peat) sown at a 2.5 cm depth. Plants were grown in a 1.3 m^2 growth chamber (PGR14, Conviron, Winipeg, Manitoba, Canada) adjusted to 20°C and a 16:10 h light: dark cycle with light intensity of 73.56 µmol/m^2/µA. At emergence of cotyledons, seedlings were transferred to the greenhouse where the temperature ranged from 15° C to 20° C. A 10 h photoperiod of natural sun light and supplemental lighting for 6 hours was provided. Pots were watered on a regular basis as needed. Starting four weeks after planting, soybeans were fertilized bi-weekly with a blood meal organic nitrogen supplement 12-0-0 (Scotts Miracle-Gro Products Inc., Marysville, OH).

Lactofen treatment

The application of lactofen was made at the V2 (second trifoliolate - two sets of unfolded trifoliolate leaves) and the V6 (six trifoliolate – six unfolded trifoliolate leaves) growth stages of soybean development. Treatments included water-treated control or lactofen (Valent, USA Corporation, Walnut Creek, CA) treatments at 0.052 kg/ha, 0.11 kg/ha (field rate), and 0.22 kg/ ha. Ten ml of herbicide solution was applied per pot with a plastic household sprayer delivering a fine mist to the leaf surface, covering the entire plant. A total of thirty plants were treated for each herbicide concentration.

Sclerotinia sclerotiorum inoculum preparation

An isolate of *Sclerotinia sclerotiorum* originating from a single sclerotioum was collected from an experiment plot with a history of Sclerotinia stem rot at the Pathology Farm (East Lansing, MI) in 2011. A modification of the method of Bastien et al. (2011) for inoculum production was used. *Sclerotinia sclerotiorum* was first growth on potato dextrose agar (EMB Chemical Inc., Gibbstown, NJ, USA) at room temperature for 5 d. A pre-culture was initiated by transferring three mycelial plugs (3 mm) to 200 ml of potato dextrose broth. After 5 days on a rotary shaker at 110 rpm and 20°C the pre-culture was transferred to a 2 L flask containing 1 L of potato dextrose broth and agitated on a rotary shaker at 150 rpm for 3 d at 20°C. The mycelial suspension was homogenized for 30 s in a blender (Waring® Commercial New Hartford, CT). Pieces (3 x 8 cm) of cotton pad (Top Care® Skokie, IL) were soaked in the fresh suspension and immediately used for plant inoculation. Five ml of mycelial suspension was collected to determine the fungal inoculum concentration by serial dilution plating. Approximately 5 ml of fungal suspension was absorbed in the cotton pad as determined by estimating the remaining volume in a 50 ml plastic centrifuge tube.

The inoculation was made when more than 80% of the plants reached the beginning of the flowering stage (R1). The inoculum suspension was applied on the petiole of the lowest node bearing flowers. The cotton pad covered the flower bud at the node but not the main stem (Figure 3-1). The inoculated plants were covered with plastic bags held to the pot with a rubber band. After 5 d the plastics bags were removed along with the cotton pad. The disease was evaluated approximately 20 d to 25 d after inoculation when the plants were at the R2-R3 (full flowering-beginning pod) growth stage. A disease severity index (DSI) was calculated, using a subjective disease scale from 0 to 5, where 0 = no disease, 1 = inoculated nodes with bleached lesion, 2 =

extension of bleached lesion to lateral branches, 3 = bleached lesion on the main stem, 4 = progression of bleached lesion along the stem, 5 = general wilting/plant dead. The DSI was calculated by the following formula:

$$DSI = \frac{\Sigma \text{ disease rating of each plant}}{5 \times \text{number of plants rated}} \times 100$$

Each experiment for the two soybean growth stage (V2 and V6) were done separately and arranged as a two-factorial design with ten replications per treatment with pots as experimental units for each the stage tested. Each experiment was repeated.

Data analysis

Statistical analysis was done separately for each soybean growth stage (the V2 and the V6 using the SAS statistical package Version 9.2 (SAS Institute, Inc., Cary, NC). The analysis of variance was performed to test for significance of the treatments main effects. Means were compared by the Ryan Einot -Gabriel-Welsh Multiple Range Test with $\alpha = 0.05$. The residue analysis was tested by PROC UNIVARIATE and confirmed the hypothesis that the experimental errors were normally distributed with a common variance.

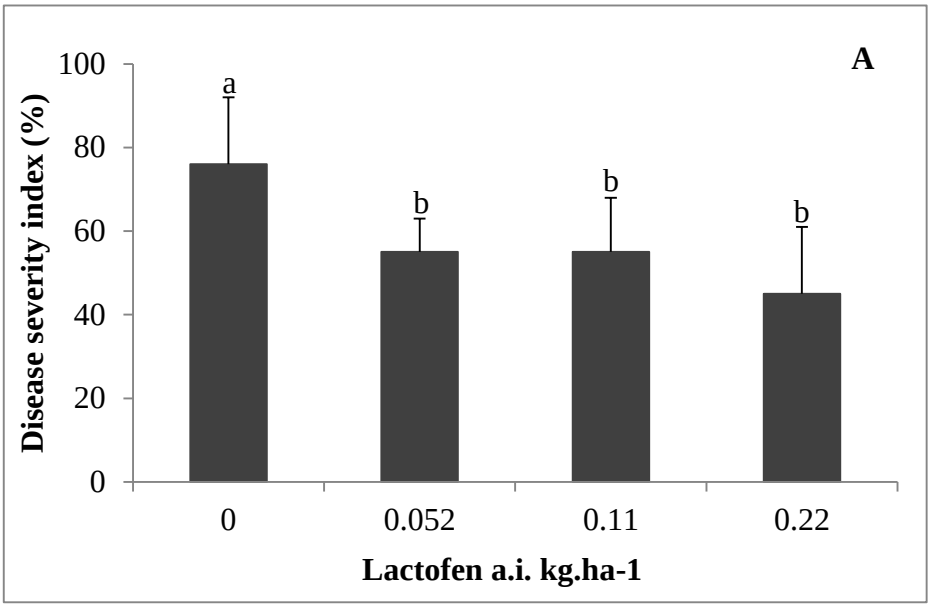
RESULTS

Sclerotinia stem rot evaluation

A statistically significant effect of lactofen on the Sclerotinia stem rot severity was observed for the V2 and the V6 growth stages ($P < 0.001$). Lactofen application reduced Sclerotinia stem rot severity at the V2 and the V6 soybean developmental stages, an average of 58.7 and 12.8 % respectively. The different lactofen concentrations evaluated did not differ statistically in their effect on disease severity at the V2 soybean growth stage (Figure 3-2A). Significant differences were found among lactofen concentrations applied at the V6 growth stage

(Figure 3-2B). All the treatments were significantly different from the non-treated control. No symptoms of Sclerotinia stem rot were observed in plants treated with the highest lactofen concentration at the V6 (Figure3-2B).

Figure 4-1. Cotton pad inoculation method and Sclerotinia stem rot symptoms. **A.** The inoculation was done on the petiole of the lowest node bearing flowers of the soybean cv. GL2449R2 at the R1 growth stage with a cotton pad saturated with a mycelial suspension of *S. sclerotiorum* (10^4 cfu/ml). **B.** Typical symptoms of Sclerotinia stem rot: stem discoloration and leaf wilting 5 d after inoculation.



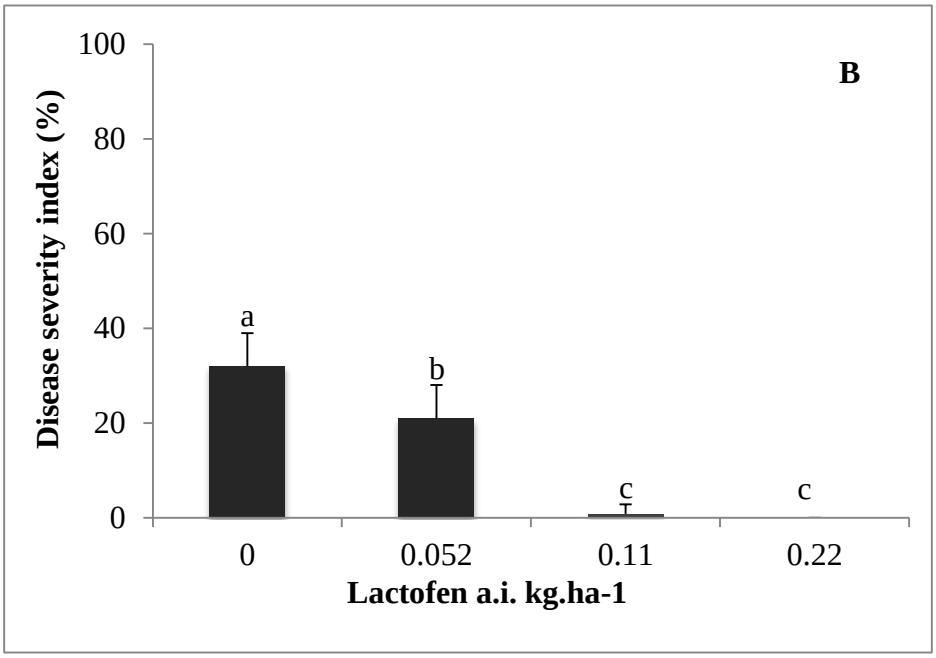


Figure 4-2. Panel A and Panel B. Effect of the herbicide lactofen application at the V2 (A) and the V6 (B) soybean growth stages on Sclerotinia stem rot severity in soybean (cv. GL2449R2). The plants were all inoculated with Sclerotinia sclerotiorum at the R1 growth stage and were rated 20 to 25 days after inoculation. Disease severity was evaluated on a 0 to 5 scale (0 = no symptoms and 5 = general wilting/plant dead). A disease severity index (DSI) was calculated using the formula: DSI (%) = Σ (scores of all plants)/[5 × (total number of plants)] × 100. A total of 30 plants were rated per lactofen concentration and the experiments were repeated. Soybean plants treated with destilled water were used as control for both trials. Bars indicate standard deviation. Means followed by same letter are not significantly different at $\alpha = 0.05$ (Ryan-Gabriel-Welsh-Multiple Range Test).

DISCUSSION

The severity of Sclerotinia stem rot was significantly reduced by lactofen application at the V2 and V6 growth stages at the three concentrations evaluated for cv. GL2449R2. Similar results were observed in soybean plants treated with lactofen under field conditions at early growth stages (V3) or at flowering (R1) with a reduction of 40 to 60% in Sclerotinia stem rot severity (Dann et al. 1999). In a previous report, lactofen treatment on soybean before flowering resulted in a reduction of Sclerotinia stem rot but the extent of control varied among the cultivars evaluated (Nelson et al. 2002a).This is particularly important in terms of recommendation for growers since the impact of herbicide treatments on the incidence of disease may depend, among other factors, on the cultivar (Nelson et al. 2002a, reviewed in Vallad and Goodman 2004). In this study a reduction of Sclerotinia stem rot severity in the soybean variety Great Lakes Hybrids GL2449R2 after lactofen treatments, indicate the potential applicability of the herbicide treatment under field condition for the disease management.

Phytoalexin production is a good indicator of defense expression induced by a herbicide treatment (Dann et al. 1999, Hammerschmidt 1999b). The levels of glyceollin induction depend on seedling age, the chemical elicitor, and its concentration (Liu et al. 1992, Stössel 1982). Preliminary results, using thin layer chromatography (TLC), suggest that the treatment with the different lactofen rates (0.052; 0.11 and 0.22 kg/ha) at V6 induced glyceollin up to 20 days after lactofen treatments compared with the glyceollin induction only with the highest rate when lactofen was applied at the V2 growth stage (data not shown).

In summary, the application of lactofen to soybean cv. GL2449R2 at an early growth stage or before flowering at the recommended field rate or even at half of the rate provided significant reductions in Sclerotinia stem rot severity under controlled conditions when

compared with the non-treated plants. These results may contribute to appropriate recommendations for Sclerotinia stem rot management with lactofen application under field conditions for this variety.

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LITERATURE CITED

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