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Management of Soybean (*Glycine max* L.) White Mold by Reducing *Sclerotinia sclerotiorum* Population Using Beneficial Microorganisms

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MANAGEMENT OF SOYBEAN (*GLYCINE MAX* L.) WHITE MOLD BY REDUCING *SCLEROTINIA SCLEROTIORUM* POPULATION USING BENEFICIAL MICROORGANISMS

By

Wenting Zeng

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ABSTRACT

MANAGEMENT OF SOYBEAN (*GLYCINE MAX* L.) WHITE MOLD BY REDUCING *SCLEROTINIA SCLEROTIORUM* POPULATION USING BENEFICIAL MICROORGANISMS

By

Wenting Zeng

White mold of soybean [*Glycine max* (L.) Merr.], caused by *Sclerotinia sclerotiorum* (Lib.) de Bary, is an important disease widely spread in the North Central region of the US. Effectiveness of biocontrol agents (BCAs) was evaluated in the laboratory, growth chamber and field at two locations in Michigan. The result showed that increased rates of BCAs decreased the sclerotial survival and carpogenic germination of *S. sclerotiorum*. *Coniothyrium minitans* CON/M/91-08 (Contans® WG) and *Streptomyces lydicus* WYEC 108 (Actinovate® AG) survived throughout the season and had the best efficacy on disease suppression among the BCAs tested. *C. minitans* did not fully eradicate infection by *S. sclerotiorum*, but over time it reduced the majority of inoculum densities in soil. *Coniothyrium minitans* strain W09 was isolated in Michigan. Morphological characteristics, effects of environmental factors on conidial production and mycelia growth, and colonization of sclerotia of *S. sclerotiorum* were compared between *C. minitans* W09 and CON/M/91-08. The optimal conditions were 20°C, pH 4.5 and 0 photoperiod h/d for mycelial growth, and 20°C, 24 photoperiod h/d for conidial production. W09 outperformed CON/M/91-08 in mycelia growth, conidial production/plate, and colonization of sclerotia at 10^4 and 10^6 conidia/ml.

To my love Yuanteng Pei, my mom Changbin Sun and dad Jinxiang Zeng,

I dedicate this thesis

谨以此献给我的爱人，我的母亲和父亲

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Wenting Zeng

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INTRODUCTION

Sclerotinia sclerotiorum (Lib.) de Bary is a destructive fungal plant pathogen which poses a threat to a great number of economically important crops worldwide (Abawi and Grogan 1979; Steadman 1979). White mold caused by *S. sclerotiorum* is ranked as the second most important disease of soybean in the United States next to soybean cyst nematode (*Heterodera glycines* Ichinohe) (Wrather *et al.* 2001). Annual loss of soybean production due to *S. sclerotiorum* is estimated to be $70 million in the United States (U. S. Canola Association 2005). Extensive economic losses have been a driving force for substantial research on this disease. This review focuses on the biology, physiology, pathogenicity of *S. sclerotiorum*, as well as disease development and management of soybean white mold.

SOYBEAN

Soybean [*Glycine max* (L.) Merr.] is derived from *Glycine ussuriensis* (Regel and Maack), a legume originating from Central China. Soybean has been cultivated as a food crop in Eastern China since the 11th century BC (Hymowitz and Shurtleff 2005). The crop was first introduced into the US in 1765 by Samuel Bowen (Hymowitz and Shurtleff 2005). At present, the United States, Brazil, China, Argentina, India, Canada, Australia, and South Africa produce over 90% of the worldís total soybean crop (Dill 2005). In 2010, 78.1 million acres of soybeans have been planted in the U.S., among which two million acres have been planted in Michigan (National Agricultural Statistics Service 2010). Soybean varieties grown in the United States are divided into indeterminate and determinant growth habit, and separated by 13 maturity groups (Tian *et al.* 2010). Soybean genome sequence has been released in public data base recently (Schmutz *et al.* 2010). Analysis from sequence will assist the identification of the genetic basis of traits and facilitate the creation of disease resistant varieties (Schmutz *et al.* 2010).

Soybean is an important crop, providing protein and oil. It is a high-quality food source because it contains high concentrations of fiber, vitamins, minerals, polyunsaturated fats, low concentrations of saturated fats, and all essential amino acids for humans (Sacks *et al.* 2006). Isoflavones from soybean help decrease low-density lipoprotein cholesterol concentrations (Sacks *et al.* 2006). Because of these constituents, soy products have been widely used in food supplies including cooking oil, meal, flour, infant formula, and dairy product substitutes. In addition, soybean is a major resource for biodiesel production in the United States. Over 80% of biodiesel in the U.S. is derived

from domestic soybean oil (Hill *et al.* 2006). However, only 6% of domestic diesel demand could be met if the entire U.S. soybean crop was processed (Hill *et al.* 2006). Therefore, the ability to produce sufficient biodiesel without affecting food supplies is urgently needed.

To increase yield and enhance the efficiency of soybean production, advanced technologies are the key to achieving disease and pest control and improving yield. For example, glyphosate-resistant soybeans released in 1996 have greatly impacted soybean production and weed management. Monsanto Company produces a genetically modified *Roundup Ready*® soybean with an inserted glyphosate-resistant gene CP4 EPSPS from *Agrobacterium tumefaciens* strain CP4. With these glyphosate-resistant soybeans, growers can spray the non-selective herbicide glyphosate without damaging the crop (Dill 2005). In 2005, more than 80% of soybeans grown in the U.S. were glyphosate-resistant (Dill 2005).

In spite of the success of weed control, disease control needs to be improved. Soybean is still prone to be attacked by a number of phytopathogens, including six bacteria, 38 fungi and oomycetes, 12 nematodes, and 15 viruses (Sinclair and Hartman 1999). Among them, *Sclerotinia sclerotiorum* (Lib.) de Bary is one of the most common and important fungal pathogens. Therefore, it is a research focus for plant pathologists.

SCLEROTINIA SCLEROTIORUM

Taxonomy

Sclerotinia sclerotiorum (Lib.) de Bary is characterized by the production of melanized tuber-like sclerotia, apothecia, ascospores and lack of conidia (Tourneau 1979). A sclerotium is a compact mycelial mass containing food reserves. Apothecia initials arise from sclerotia and form on stipes. Apothecia are brown and cup-shaped structures full of asci. An ascus contains eight ascospores which form after meiosis. At maturity, the ascospores are forcibly discharged into the air (Tourneau 1979).

Since *S. sclerotiorum* was first studied as a plant pathogen in 1837, its taxonomy and name have been changed several times (Purdy 1979). *Sclerotinia sclerotiorum* was first named as *Peziza sclerotiorum* in 1837 by Libert. Later in 1924, Fuckel renamed it as *Sclerotinia libertiania* Fuckel, which was in the same genus with *S. candolleana, S. fuckeliana, S. libertinia, S. tuberose,* and *S. baccata.* The binomial name stood until Wakefield demonstrated its conflict with the International Rules of Botanical Nomenclature. Waterfield incorrectly changed the name from *S. libertiania* Fuckel to *S. sclerotiorum* (Lib.) Massee. It wasnít until 1979 when Purdy recognized that de Bary used the name first in 1884 and gave the proper authority as *Sclerotinia sclerotiorum* (Lib.) de Bary. The detailed history of taxonomy is described by Purdy (1979). The classification of the organism was further confirmed by Kohn using restriction fragment length polymorphism finger printing (Kohn *et al.* 1988). *Sclerotinia sclerotiorum* is now classified in the kingdom Fungi, phylum Ascomycota, class Discomycetes, order Helotiales, family Sclerotiniaceae, and genus *Sclerotinia.*

Host range

The host range of *S. sclerotiorum* expanded from 64 plant families, 225 genera, 361 species in a total of 383 species compiled by Purdy (1979) to at least 75 families, 278 genera 408 species by Boland and Hall (1994). The hosts include both economically important dicotyledonous species, such as soybean, dry bean, peanut, canola, chickpea, lentils and many vegetables, and monocotyledonous species such as tulip and onion (Boland and Hall 1994). However, *S. sclerotiorum* does not infect corn, small grains or grasses (Rousseau *et al.* 2007). More than 60 diseases caused by *S. sclerotiorum* have been named for specific hosts, such as soybean white mold (Yang *et al.* 1999), lettuce drop (Chitrampalam *et al.* 2008), Sclerotinia wilt of sunflower (Eva 2003), head rot of sunflower (Huang *et al.* 2006), pod rot of pea (Huang *et al.* 2006), stem rot of common bean and tomato (Huang and Erickson 2007), watery soft rot of cabbage (Bolton *et al.* 2006), and Sclerotinia blight of peanut and canola (Huang *et al.* 2006).

Geographic distribution and diversity

Sclerotinia sclerotiorum usually occurs in cool (bellow 20°C) and moist areas of the world (Abawi and Grogan 1979). The growth of *S. sclerotiorum* is less active at temperatures below 0 or above 32°C than at temperatures in between (Abawi and Grogan 1979). These temperature ranges enable the fungus to have broad geographical niches across all continents (Purdy 1979). Whetzel (1945) believed that *S. sclerotiorum* originated from the Northern Hemisphere considering most of the sclerotiniaceous fungi are populated in this cool environment. However, DNA fingerprinting studies demonstrate that clones of *S. sclerotiorum* are scattered, dispersed and disconnected from

their points of origin; they are geographically mixed within fields and widely separated between fields (Anderson and Kohn 1995). Therefore, the precise geographical and ecological origin of *S. sclerotiorum* is unclear. Although *S. sclerotiorum* is more active in cool and moist areas, it does exist in hot and dry areas such as Florida, California, and Arizona (Purdy 1979; Subbarao 1998; Chitrampalam *et al.* 2008).

Genetic diversity of *S. sclerotiorum* is associated with geographic distribution. Sequence-related amplified polymorphism (SRAP) and random amplified polymorphic DNA (RAPD) have been applied to investigate the diversity of *S. sclerotiorum* isolates in China, Canada, and United Kingdom. The polymorphic loci of *S. sclerotiorum* isolates vary from 31% to 98% using SRAP (Li *et al.* 2009). These genetic variations can be considered as adaptation under environmental stress. Variations in oxalic acid production potential and mycelial compatibility of *S. sclerotiorum* isolates have also been observed from different geographic locations and hosts (Durman *et al.* 2005).

Sclerotia

Sclerotia are an overwintering structure of *S. sclerotiorum*. The development of sclerotia is practically divided into three phases: mycelial initiation, sclerotial development, and sclerotial maturation (Tourneau 1979). At the beginning, white round mycelial masses appear with tiny liquid droplets on the surface (Tourneau 1979). As this structure continues to expand, it forms a sclerotium initial. The medulla inside the sclerotium consists of compacted hyphae. Rind cells start to assemble beneath the sclerotial surface (Tourneau 1979). At maturity, a sclerotium is composed of black rind

cells, medulla of prosenchymatous tissues, fibrillar matrix and a cortical layer, which forms a black hardened compact mass of mycelia (Tourneau 1979).

The formation of sclerotia is influenced by the type of nutrition available and environmental factors (Humphersonjones and Cooke 1977). *Sclerotinia sclerotiorum* consumes organic compounds, such as carbohydrates and amino acids, to produce proteins, crude fat, and inorganic compounds required for growth and formation of sclerotia (Tourneau 1979). Different nutrition sources, for example, artificial media versus field soil, result in different biochemical composition of sclerotia (Tourneau 1979). Light, temperature, and pH value affect the sclerotial formation (Wong and Willetts 1974). Sclerotia are properly formed at temperatures between 0 to 30°C and at pH between 2.5 to 9.

Mature sclerotia are able to germinate either carpogenically or myceliogenically. For carpogenic germination, sclerotia first produce stipes from surface, followed by apothecia production on tip of stipes. Abawi and Grogan (1979) suggest that average three apothecia are produced from a single sclerotiorum, but the actual number of apothecia is highly correlated with sclerotial sizes (Hao *et al.* 2003).

The survival of *S. sclerotiorum* sclerotia is affected by many factors, such as moisture, temperature, depth of burial in soil, soil microflora, and soil profile. Soil moisture, temperature, and preconditioning are important factors that affect the carpogenic germination of *S. sclerotiorum*. The optimal condition for carpogenic germination in the laboratory is around 16°C, -0.03 MPa water potential for about 10 continuous days, and 24 hours of continuous light exposure/day (Hao *et al.* 2003; Harikrishnan and del Rio 2006). Temperature fluctuations of 8°C around 20°C lead to

maximum sclerotial germination (Mila and Yang 2008). On the contrary, fluctuations of water potential have negative effect on sclerotial germination and apothecial production (Mila and Yang 2008). Carpogenic germination can be enhanced by chilling sclerotia at 4°C for 2 weeks or longer at soil moisture near saturation and rinsing of sclerotia under running water prior to burial in soil (Dillard *et al.* 1995). In addition, soil texture and light intensity also affect sclerotial germination (Hao *et al.* 2003; Wu *et al.* 2008).

Not only do many abiotic factors affect survival of sclerotia in soil, microorganisms also play a critical role in sclerotia degradation (Lockwood 1977; Budge *et al.* 1995; Abdullah *et al.* 2008). Microorganisms in soil affect the germination of sclerotia. Lockwood (1977) explains this phenomenon as fungistasis. Mycoparasites of sclerotia such as *C. minitans* and *T. harzianum, T. koningii, T. gamsii, T. asperellum*, and *T. virens*, colonize sclerotia and therefore reduce the germination of *S. sclerotiorum* (Budge and Whipps 1991; Budge *et al.* 1995; Escande *et al.* 2002; Abdullah *et al.* 2008; Kim and Knudsen 2009). Bacteria such as *Bacillus subtilis,* have demonstrated the ability to degrade sclerotia (Yang *et al.* 2009). Volatile antifungal compounds produced by bacteria including aldehydes, alcohols, ketones, and sulfides inhibit carpogenic germination of *S. sclerotiorum* (Fernando *et al.* 2005). The soil bacterium *Serratia plymuthica* is able to completely suppress apothecial formation by producing a chlorinated macrolide (Thaning *et al.* 2001). In addition, the larvae of the fungus gnat (*Bradysia coprophila* Lintner) directly damage sclerotia by feeding and indirectly increase the colonization of sclerotia by mycoparasites (Gracia-Garza *et al.* 1997). A number of organisms that feed on or parasitize sclerotia include nematodes, earthworms,

centipedes, snails, mites may lead to the degradation of sclerotia as well (Coley-Smith and Cooke 1971).

SOYBEAN WHITE MOLD

Symptoms

The name white mold comes from the visual sign of the disease: white fluffy mycelia on leaves, stems, and pods. The earliest symptoms are necrotic foliage of soybean (Abawi and Grogan 1979). Water soaked lesions may be seen on leaves and leaves will turn brown and senesce. Stem nodes become bleached, which is a distinctive symptom of an infected host. At later stages of disease development, fluffy white mycelia may cover the bleached stem. Finally, the infection may result in death of the entire plant, leaving sclerotia formed in/on the pods and the stems of the plant debris.

Disease epidemiology

Sclerotia of *S. sclerotiorum* serve as survival structures and primary inocula. Sclerotia can survive for up to eight years in soil, but viability of sclerotia decrease over time (Coley-Smith and Cooke 1971). *Sclerotinia sclerotiorum* can initiate disease of soybean via two mechanisms. If sclerotia are in contact or in close proximity to soybean plants, mycelial germination may result in infection of the plant. However, this form of infection plays a minor role in disease development compared to carpogenic germination. Ascospores released by carpogenic germination of sclerotia account for the most common source of inoculum (Abawi and Grogan 1979). Apothecia are formed from sclerotia at germination and ascospores are forcibly discharged from matured apothecia into the air (Boland and Hall 1988). Under naturally infested field conditions, the density of apothecia in the field normally ranges from zero to six apothecia/m^2 (Gerlagh *et al.*

1999). However, up to 180 apothecia/ m^2 has been reported in artificially inoculated soil (Huang *et al.* 2006). Released ascospores are spread by precipitation (rain and irrigation) and air movement (wind). A single apothecium can produce as many as 3×10^7 ascospores with the maximum ascospore production occurring at days four to nine during ascospore release (Steadman 1979). Ascospores have been documented to travel at a speed of 1600 m/h (Clarkson *et al.* 2003) and travel up to four kilometers under field conditions, but 90% of ascospores are deposited within 100 to 150 m (Abawi and Grogan 1979; Steadman 1979). Ascospores can be trapped from neighboring fields as well (Hammond *et al.* 2008). Clearly, external sources of ascospores can lead to disease development in distant crops. Adjacent fields with plant debris are prone to sclerotial survival (Hammond *et al.* 2008). Ascospores exposed to field environment desiccate and lose their viability in three to four days (Olivier and Seguin-Swartz 2006). However, under optimal conditions at temperatures of 30°C and relative humidity over 90%, 50% of ascospores are able to last two to three weeks (Clarkson *et al.* 2003)

Ascospore infection includes ascospore germination, host penetration, and colonization (Jamaux *et al.* 1995). Abawi and Grogan (1979) demonstrated that ascospores require exogenous nutrients for germination and penetration of the host. For soybeans, senescent flower petals or necrotic tissues, serve as the nutrient resource for ascospore germination (Abawi and Grogan 1979; Bolton *et al.* 2006). Consequently, early reproductive stages are the most critical period for white mold development in soybean and many other crops (Boland and Hall 1988; Clarkson *et al.* 2003; Hammond *et al.* 2008). Ascospores can germinate with substantial moisture without nutrients in the environment, but the ability to form appresoria and to penetrate the host is reduced

(Harikrishnan and del Rio 2006). A period of approximately 48 to 72 hrs of free moisture is required for infection (Abawi and Grogan 1979), which may come from dew, fog, and rain (Young *et al.* 2004). In the field, a period of continuous wetness for 10 days is the key to initial infection and white mold development (Young *et al.* 2004).

Infection hyphae developed from ascospores grow through the flower pedicel and petals down to the stem and move all over the host tissue in the cortex (Lumsden and Dow 1973). The infection hyphae go intercellularly and intracellularly, consuming host nutrients from the senescent tissues (Lumsden and Dow 1973). The infected host tissues undergo histological changes including alteration in cell wall structure, hypersensitive response of cell, accumulation of fluids and phytoalexins, and other enzymes related to pathogenicity (Hancock 1972; Lumsden and Dow 1973).

Pathogenesis

Sclerotiorum sclerotiorum pathogenesis is associated with hydrolytic enzymes and toxins (Bolton *et al.* 2006). *Sclerotinia sclerotiorum* has several weapons to infect host plants, such as cell wall degrading enzymes (CWDEs), oxalic acid, and toxins (Maxwell and Lumsden 1970; Fraissinet-Tachet *et al.* 1995; Cessna *et al.* 2000; Rollins and Dickman 2001; Cotton *et al.* 2003; Guimaraes and Stotz 2004; Bolton *et al.* 2006). Plant cell walls consist of primary cell wall (cellulose, and pectin), the middle lamella (pectin), and secondary cell wall (cellulose). The enzyme complex produced by *S. sclerotiorum* facilitates cell wall degradation and tissue maceration, which includes pectinases, β-1, 3-glucanases, cutinases, and cellulases (Cotton *et al.* 2003). Pectinase breaks down the primary cell wall and the middle lamella (Cotton *et al.* 2003).

Sclerotinia sclerotiorum produces polygalacturonase (PG), a pectinase serving as a virulence factor (Bolton *et al.* 2006). PGs release oligo-galacturonides and degrade unesterified pectin (Fraissinet-Tachet *et al.* 1995). Based on their function, PGs are divided into endoPGs and exoPGs. Bolton (2006) has listed 18 genes encoding for CWDEs. The multiple copies of polygalacturonase isozymes of endoPGs produced by *S. sclerotiorum* give the pathogen flexibility and adjustability in the infection process to a wide range of hosts (Fraissinet-Tachet *et al.* 1995).

Oxalic acid (OA) is considered as a fundamental pathogenic factor that contributes to the early infection process of *S. sclerotiorum* (Maxwell and Lumsden 1970). OA targets host tissue and functions pathogenicity with several modes of action. (1) OA accumulates in early infected tissues; increased concentration of OA reduces the pH value of host tissue gradually (Maxwell and Lumsden 1970). Low pH values around 4 to 5 favor various cell wall degrading enzymes (Maxwell and Lumsden 1970). (2) OA activity is closely associated with chelation of Ca^{2+} (Bateman and Beer 1965). During penetration and infection, OA binds to the cell wall Ca^{2+}, resulting in host cell wall collapse and tissue maceration (Bateman and Beer 1965). (3) Oxidative burst is characterized by the release of O_2 and H_2O_2 at the site of pathogen infection and is determined by H_2O_2 and Ca^{2+} concentrations in most defense responses (Cessna *et al.* 2000). OA inhibits oxidative burst in soybean and tobacco (Cessna *et al.* 2000). (4) OA also functions as an elicitor of plant programmed cell death (PCD) which is responsible for induction of mammalian apoptotic-like features during white mold development (Kim *et al.* 2008). (5) In addition, OA inhibits polyphenol oxidase production, regulates pH-

regulated gene expression in molecular signaling pathways (Rollins and Dickman 2001), and operation of guard cells which control stomatal opening (Guimaraes and Stotz 2004).

DISEASE MANAGEMENT

Management of soybean white mold is based upon the epidemiology of the disease. Efficient management requires implementation of an integrated disease control approach. Strategies of disease management include a) pathogen refusal, such as using plant resistance; b) pathogen elimination, such as using chemicals, soil fumigation, biological control, cultural practice, and soil amendments; c) mistiming of pathogen germination and soybean flowering, such as rearranging planting dates and suppressing or stimulating sclerotial germination.

Disease resistance

The most effective way to manage white mold is to plant resistant or less susceptible soybean cultivars (Kim *et al.* 2000). To date, resistant germplasms to white mold have not been found, but the disease severity of soybean cultivars vary responding to *S. sclerotiorum* in controlled environments (Kim *et al.* 2000; Hoffman *et al.* 2002; Cober *et al.* 2003). For example, cultivars Corsoy, Corsoy 79, Hodgson 78, S19-90, and Asgrow A2506, demonstrate the greatest partial resistance to *S. sclerotiorum* (Kim *et al.* 1999; Yang *et al.* 1999; Kim *et al.* 2000).

The reason that immunity has not been developed for soybean is partially because the resistance is multigenic (Arahana *et al.* 2001; Vuong *et al.* 2008). Inconsistent field performance and undiscovered resistance in commercial cultivars support this conclusion. In recent years, quantitative molecular approaches have been developed. A number of resistance quantitative trait loci (QTLs) on molecular linkage groups (LGs) of soybean

have been identified by a large range of genetic markers such as RAPD, restriction fragment length polymorphism (RFLP), and simple sequence repeat (SSR) (Arahana *et al.* 2001; Guo *et al.* 2008; Vuong *et al.* 2008). These QTLs have been mapped and can provide valuable information for marker-assisted soybean breeding programs (Vuong *et al.* 2008). QTLs or specific resistant genes evaluation in controlled environment will make disease resistance evaluation more consistent.

Although genes for resistant to white mold have not been found, it is possible to develop resistance via genetic modification of soybean. For example, soybean is modified by gene transfer to obtain the oxalic acid degrading enzymes (Donaldson *et al.* 2001). Genes in soybean do not encode oxalic-acid-degrading enzymes. However, a number of microorganisms are capable of degrading oxalic acid, and therefore contain the genes encoding for oxalic acid degrading enzymes. Soybean introduced with an oxalate oxidase gene from wheat germin reduces disease incidence after cotyledon and stem inoculation of *S. sclerotiorum* (Donaldson *et al.* 2001). Oxygen oxidoreductase oxidase (OxO) is encoded to degrade oxalic acid and generate H_2O_2, which plays a role in oxidative burst and hypersensitive response (Donaldson *et al.* 2001). Transgenic tobacco plants over expressed an oxalate decarboxylase gene (oxdc) contain less OA, reduce colonization, and show resistance to *S. sclerotiorum* (Dias *et al.* 2006; Walz *et al.* 2008). Decarboxylase gene has been introduced in lettuce by *Agrobacterium*-mediated transformation and the transgenic lettuce was symptomless after inoculation of *S. sclerotiorum* (Dias *et al.* 2006).

Soybean produces polygalacturonase-inhibiting protein (PGIP) to target fungal PGs (Ferrari *et al.* 2003). PGIPs induce a number of defense responses. *Pgip* gene family

in *Arabidopsis thaliana* (L.) Heynh have been characterized by expressed sequence tag (EST) and genomic libraries (Ferrari *et al.* 2003). D'Ovidio (2006) compared four members of the legume *Pgip* gene family and determined the distinct regulation properties of each encoded protein product. These researches provide useful information for developing disease resistance.

Chemical control

Chemical fungicides play an important role in current management strategies for Sclerotinia diseases, although they are not fully effective. The efficacy of fungicides on white mold has been evaluated on snap bean (Hunter *et al.* 1978), soybean (Mueller *et al.* 2002), dry bean (Mueller *et al.* 1999), sunflower (Mueller *et al.* 1999), white bean (Morton and Hall 1989), canola (Bradley *et al.* 2006), and lettuce (Chitrampalam *et al.* 2008). Fungicides such as azoxystrobin (Quadris), benomyl (Benlate), boscalid (Endura), dicloran (Botran), iprodione (Rovral), prothioconazole (JAU6476), pentachloronitrobenzene (PCNB), tebuconazole (Folicur), trifloxystrobin (Gem), thiophanate-methyl (Topsin M), and vinclozolin (Ronilan) have provided moderate disease control with inconsistency (Hunter *et al.* 1978; Mueller *et al.* 2002; Bradley *et al.* 2006; Chitrampalam *et al.* 2008). Vinclozolin was the most effective fungicide in inhibiting mycelial growth of *S. sclerotiorum in vitro* (Mueller *et al.* 2002). At low disease incidence, foliar sprays of thiophanate methyl reduced the incidence of white mold by 50% (Mueller *et al.* 2002). However, high disease incidences result in non-consistent control (Mueller *et al.* 2002).

Thorough coverage of the canopy and timing of application are essential to improve the efficacy of fungicides (Mueller *et al.* 2002). Fungicides are recommended to be applied during the early reproductive stage of host plants, when plants are more susceptible to *S. sclerotiorum* (Mueller *et al.* 2002). However, the long period of soybean flowering (one to five weeks) limits the efficacy of foliar fungicides, and even two applications provide a maximum 28-day protection (according to manufactures' recommended application protocol) (Mueller *et al.* 2002). Because timing is critical, a forecasting system would be useful to maximize application efficacy, especially when disease risk is high.

Many other chemical compounds have been tested for managing white mold. Herbicides chlorsulfuron, cyanazine, metribuzin, triallate, and trifluraline significantly reduced carpogenic germination of sclerotia (Teo *et al.* 1992). A diphenyl ether herbicide such as lactofen can reduce white mold incidence from 40 to 60% (Dann *et al.* 1999). In addition, the essential oil *Orihanum syriacum* var. *bevanii* and *Foeniculum vulgare* were reported to have effect on apothecial germination of *S. sclerotiorum* sclerotia (Soylu *et al.* 2007).

Fungicide resistance in *S. sclerotiorum* remains a concern. Although no fungicide (benomyl) resistance was found based on 100 *S. sclerotiorum* isolates collected from a snap bean field in New York (Hunter *et al.* 1978), Michigan (Detweiler 1983), and Virginia (Smith *et al.* 1991), it may be possible that *S. sclerotiorum* also demonstrate resistance to these fungicides. However, further tests are needed.

Biological control

Biological control (biocontrol) has been used in the past decades for managing soybean white mold (Budge and Whipps 2001; Jones 2002; Abdullah *et al.* 2008). Beneficial microorganisms such as *Coniothyrium minitans* Campbell (syn. *Paraconiothyrium minitans*) can eliminate sclerotia of *S. sclerotiorum* via mycoparasitism (Campbell 1947; Budge *et al.* 1995; Gerlagh *et al.* 1999; Budge and Whipps 2001; Gerlagh *et al.* 2003; Li *et al.* 2005; Chitrampalam *et al.* 2008). Beside *C. minitans*, many other microorganisms have been demonstrated inhibitory effects on *S. sclerotiorum* (Table 1-1).

To date, a total of eight biocontrol products have been commercially available to control *Sclerotinia* diseases (Table 1-2). Among these formulations, products containing *C. minitans* have been well studied due to highly specialized mycoparasitism of *C. minitans* to sclerotia-forming fungi, including *S. sclerotiorum, S. minor, S. trifoliorum,* and *Sclerotium cepivorum* (Campbell 1947; Budge *et al.* 1995; Huang *et al.* 2000; Jones 2002; Jones *et al.* 2004a; Yang *et al.* 2007). Since its discovery from *S. sclerotiorum* (Campbell 1947), *C. minitans* has been evaluated as soil treatment on crops, such as dry bean, potato, oilseed rape, carrot, lettuce, celery, sunflower, bean, chicory and cowpea, and applied as a foliar spray on onion, kiwi fruit, rapeseed, and bean (Whipps *et al.* 2008).

Table 1-1. Beneficial microorganisms that affect *Sclerotinia sclerotiorum.*

Species	Mode of action	Reference
Coniothyrium minitans	Mycoparasitism	Campbell 1947; Budge 1995
Bacillus subtilis	Antibiosis	Schmiedeknecht *et al.* 2001
Bacillus amyloliquefaciens	Antibiosis	Abdullah 2008
Sporidesmium sclerotivorum	Mycoparasitism	del Rio *et al.* 2002
Trichoderma harzianum	Mycoparasitism, antibiosis, SAR	Menendez and Godeas 1998
Trichoderma koningii	Nutrient competition, antibiosis	Escande *et al.* 2002
Trichoderma aureoviride	Nutrient competition, antibiosis	Escande *et al.* 2002
Trichoderma gamsii	Nutrient competition, antibiosis	Van Beneden *et al.* 2010
Trichoderma asperellum	Nutrient competition, antibiosis	Van Beneden *et al.* 2010
Trichoderma longibrachiatum	Nutrient competition, antibiosis	Escande *et al.* 2002
Trichoderma hamatum	Mycoparasitism	Gracia-Garza *et al.* 1997
Trichoderma virens	Mycoparasitism	Tu 1980; Budge 1995
Ulocladium afrum	Unknown	Huang and Erickson 2007
Ulocladium atrum	Competitive colonization	Li *et al.* 2003
Pseudomonas fluorescens	Antibiosis	Ashofteh *et al.* 2009
Pseudomonas chlororaphis	Toxins	Selin *et al.* 2010
Ophiostoma mitovirus	Hypovirulence	Boland 2004
Cryptococcus albidus	Unknown	Reeleder 2004

The mechanisms of *C. minitans* attack on *S. sclerotiorum* are mycoparasitism and nutrient competition (Whipps *et al.* 2008). The latter is less important because *C. minitans* does not grow on/in plant tissue even with wound (Gerlagh *et al.* 1996). The suppressive effect on *S. sclerotiorum* has been studied using detailed co-culture methods to characterize the interaction (Smith *et al.* 2008). *Coniothyrium minitans* produces a wide range of cell wall degrading enzymes such as glucanases and chitinases that are associated with the mycoparasitic process (Whipps *et al.* 2008). *Coniothyrium minitans* is able to degrade oxalic acid produced by *S. sclerotiorum*, which interrupts the infection by *S. sclerotiorum* (Ren *et al.* 2007). *Coniothyrium minitans* can not only degrade oxalic acid but can also survive in the soil (Ren *et al.* 2007). The detection of antimicrobial metabolites in culture indicates that chemical compounds are associated with mycoparasitic processes (Tomprefa *et al.* 2009).

There is little information of molecular studies on mycoparasite-host interaction. Specific enzyme and metabolite production in the progress of mycoparasitism remains unclear. Several genes have been sequenced and 11 mutants of *C. minitans* were identified by *Agrobacterium tumefaciens*-mediated transformation (Whipps *et al.* 2008). A cosmid library of *C. minitans* has been established and pathogenic genes, *PKAC* and *PMK1* genes have been sequenced (Whipps *et al.* 2008). Muthumeenakshi (2007) conducted suppression subtractive hybridization (SSH) to determine genes regulating the mycoparasitism process using SSH, a cDNA library was established and 251 putative genes were identified (Muthumeenakshi *et al.* 2007). More than 20% of the genes were considered to have novel function during sclerotial mycoparasitism (Whipps *et al.* 2008).

Table 1-2. Commercialized biocontrol strains, product names and the producers.

Product name	Active ingredient	Company
Contans® WG	*C. minitans* CON/M/91-08	SipCamAdvan, Roswell, GA
Intercept® WG	*C. minitans* CON/M/91-08	Encore Technologies, Minnetonka, MN
Serenade® MAX	*B. subtilis* QST 713	AgraQuest, Davis, CA
Companion®	*B. subtilis*	Growth Products Ltd., White Plains, NY
PlantShield® HC	*T. harzianum* T-22	Bioworks, Victor, NY
Supresevit®	*T. harzianum*	Binab USA, Bridgeport, CT
Tenet®WP	*T. asperellum* and *T. gamsii*	SipCamAdvan, Roswell, GA
Soilgard®	*T. virens*	Certis U.S.A., Columbia, MD

In addition to *C. minitans*, a number of other species have been proved to be inhibitive to *S. sclerotiorum*. For example, *Trichoderma harzianum* is effective to control *S. sclerotiorum* (Kim and Knudsen 2009) with multiple modes of action including mycoparasitism, antibiosis, nutrient competition, and promotion of plant growth (Ousley *et al.* 1994); *Bacillus subtilis* is also effective in suppressing mycelia of *S. sclerotiorum* and reducing white mold incidence and severity in the field (Schmiedeknecht *et al.* 2001; Yang *et al.* 2009); *Streptomyces lydicus* demonstrates a strong antagonistic effect on *Pythium ultimum* and *R. solani* by producing extracellular antifungal metabolites (Yuan and Crawford 1995) and cell wall degrading enzymes (Mahadevan and Crawford 1997).

Application of biocontrol products is challenging as their efficacy can vary depending on environmental conditions. The microorganisms are affected by a number of abiotic and biotic factors. For example, soil moisture (Tu 1999), temperature (McQuilken

et al. 1997; Tu 1999), soil type, light (McQuilken *et al.* 1997), water tension (Ciotola *et al.* 2007), and pH (McQuilken *et al.* 1997) all affect the activity of biocontrol agents. The timing of application of biocontrol agents is important. Earlier application can benefit the strains that survive and establish better in soil (Jones *et al.* 2004b). Soil temperature is also an important factor to be considered prior to application. For example, *C. minitans* and *T. virens* were applied as integrated control for white mold. But *C. minitans* infected sclerotia at a lower temperature range (4 to 25°C) than *T. virens* (10 to 30°C) and therefore *C. minitans* had better efficacy (Budge *et al.* 1995).

Cultural practices and soil amendments

Cultural practices and cultivar selection can improve the management of *S. sclerotiorum*. Planting date, plant density, crop rotation, tillage, and weed management have significantly affected soybean white mold (Gracia-Garza *et al.* 2002; Rousseau *et al.* 2006). For example, early planting may result in lower disease incidence because of the mismatch in timing between the period covering soybean flowering and apothecial germination (Hammond *et al.* 2008). Increasing within-row distance of soybean results in decreased incidence of white mold due to improved air circulation and alteration of the microclimate (Vieira *et al.* 2010). Two or four years of soybean rotation with corn lowers the risk of white mold (Gracia-Garza *et al.* 2002; Rousseau *et al.* 2007). However, the longevity of sclerotia survival in soil limits the effect of rotation (Coley-Smith and Cooke 1971) and the effectiveness of cultural practices is influenced by inoculum density and environmental factors.

Soil amendments have been used in the field to control soybean white mold (Huang *et al.* 2006; Rousseau *et al.* 2006; Huang and Erickson 2007). Crop residues of mustard, wheat, broccoli, canola, barley, oat, and lentil have been amended in soil and reduced carpogenic germination of *S. sclerotiorum* (Huang and Sun 1991; Huang *et al.* 2007). Urban compost, mineral compost and fermented industrial wastes are also effective (Rousseau *et al.* 2006; Rousseau *et al.* 2007). Most of the soil amendments change the soil microbial community that favored disease suppression. However, all these soil amendments lack consistency, as changing the microbial community can result in either positive or negative effects (Rousseau *et al.* 2006). The results also vary depending on soil types, environmental conditions, and biochemical characteristics of pathogens (Rousseau *et al.* 2006). In addition, the large amount of soil amendment materials required for successful trails is often not practical for large-scale commercial application.

FUTURE PERSPECTIVES

Screening highly resistant varieties of soybean is the ultimate goal of disease management, although finding genes regulating resistance is challenging. In the future, more efforts need to be made on breeding programs for white mold resistance. Among the other research directions, utilization of oxalic acid degrading enzymes is attractive. Since the gene that encodes oxalic acid degrading enzymes has been transferred into soybeans, we anticipate that the efficiency and consistency of gene expression can be further improved. Alternatively, microorganisms that produce oxalic acid degrading enzymes can be directly used as biological control agents. Biocontrol research needs to focus on reproducibility and improved efficacy of disease control. Finding compounds or microorganisms that can stimulate or suppress the germination of *S. sclerotiorum* sclerotia is another possible way to manage the disease. In addition, a disease forecasting system is urgently needed to assess disease risk and suggest timings for chemical application, cutting down the cost of application and preventing severe diseases. The theme of the above researches is to help the establishment of sustainable agriculture.

OBJECTIVE OF THIS THESIS

White mold (caused by *Sclerotinia sclerotiorum*) significantly affects soybean production in Michigan. Since current strategies provide unsatisfactory control, better approaches are required for disease management. Studying the interactions between sclerotia of *S. sclerotiorum* and antagonistic biocontrol strains allows researchers to develop better strategies for disease control, optimize the efficacy of control, and reduce overall disease development.

Even though the successful biocontrol of white mold has been documented on various crops such as lettuce and dry bean, biocontrol of white mold on soybean have received mixed and inconsistent results. In addition, little information is available about the survival of biocontrol strains under environmental conditions in Michigan. Evaluating the effect of commercialized biocontrol products to control white mold on soybean, optimizing the application rates of biocontrol products, and studying the characteristics and survival of biocontrol strains in comparison to local strains will directly benefit Michigan soybean production in sustainable agricultural systems.

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CHAPTER TWO: EFFECTS OF BIOLOGICAL CONTROL AGENTS ON SCLEROTIAL SURVIVAL AND GERMINATION OF *SCLEROTINIA SCLEROTIORUM*

ABSTRACT

Biological control agents (BCAs) were evaluated on affecting sclerotial survival and germination of *Sclerotinia sclerotiorum* in the growth chamber. The BCAs included *Coniothyrium minitans* CON/M/91-08 [Contans® WG, 5.3% active ingredient (a.i.)] at rates of 0, 0. 001, 0.005, 0.025, 0.125, 0.625, 3.125 g/L soil, *Streptomyces lydicus* WYEC 108 (Actinovate® AG, 0.04% a.i.) and *Trichoderma harzianum* T-22 (PlantShield® HC, 1.15% a.i.) at 0, 0.025, 0.050, 0.100, 0.250, 0.500, 1.000 g/L soil, and *Bacillus subtilis* QST 713 (Serenade® MAX, 14.6% a.i.) at 0, 0.002, 0.010, 0.050, 0.250, 1.250, 6.250 g/L soil. Twenty-five sclerotia were buried in soil, followed by soil treatment with the BCAs. Five soybean seeds were sown in each pot. Presence and number of *S. sclerotiorum* apothecia were recorded daily. Sclerotia of *S. sclerotiorum* were retrieved after six weeks from soil treatment and viability was assessed on water agar plates. Increasing the rates of *C. minitans* and *T. harzianum* resulted in decreased *S. sclerotiorum* sclerotia survived. Increasing the rates of *C. minitans* and *S. lydicus* led to reduced apothecial production of *S. sclerotiorum*. The most effective rate for *C. minitans* was around 0.2 g/L soil with 90.1% apothecial reduction and 50.0% of sclerotial reduction. The most effective rate for *T. harzianum* was 0.2 g/L soil with 80.5% apothecial reduction and 31.7% of sclerotial reduction. The most effective rate for *S. lydicus* was 0.2 g/L soil with 100% apothecial reduction and 29.5% of sclerotial reduction. The effective rate for *B. subtilis* was 0.9 g/L soil with 81.24% reduction of apothecia and 29.6% of sclerotial reduction.

INTRODUCTION

Soybean white mold, caused by *Sclerotinia sclerotiorum*, can result in significant soybean yield losses (Abawi and Grogan 1979; Purdy 1979; Boland and Hall 1994; Bolton *et al.* 2006). Sclerotia of *S. sclerotiorum* serve as a survival structure that is resistant to drought, heat and remain viable in the soil for up to eight years (Coley-Smith and Cooke 1971). With continuous moisture, sclerotia germinate carpogenically and produce 10^7 ascospores per apothecium (Abawi and Grogan 1979; Clarkson *et al.* 2003), which may infect soybean. Currently, strategies for controlling white mold include use of partial resistant cultivars, foliar fungicides applications and cultural practices (Vuong *et al.* 2008, Mueller *et al.* 2004, and Gracia-Garza *et al.* 2002). However, no soybean cultivars are completely resistant to *S. sclerotiorum* (Vuong *et al.* 2008). Foliar fungicide sprays, even with multiple applications, cannot prevent the disease (Mueller *et al.* 2004). Cultural practices are inconsistent because of the variation of environmental conditions and the longevity of sclerotia in soil (Gracia-Garza *et al.* 2002). The desire for higher level of disease management and the concern for using chemicals drive the search for biological approaches as alternatives.

A range of commercially available biocontrol reagents (BCAs) were selected for screening their potential for control of white mold. The active ingredients of biocontrol agents are derived from fungal and bacterial strains. Specific antagonistic fungi have been reported to effectively suppress *S. sclerotiorum*. The mycoparasites *Sporidesmium sclerotivorum* and *Coniothyrium minitans* are able to specifically parasitize sclerotia and mycelia of *Sclerotinia* spp. (Budge *et al.* 1995; del Rio *et al.* 2002). *Coniothyrium*

minitans produces a broad range of cell wall degrading enzymes such as chitinases and glucanases as well as antifungal metabolites that enhance colonization and degradation of sclerotia of *S. sclerotiorum* (Hu *et al.* 2009). *Sporidesmium sclerotivorum* has been applied in the field to control white mold of soybean reducing 50% to 100% white mold incidence (del Rio *et al.* 2002). *Coniothyrium minitans* has been applied in soil to manage *S. sclerotiorum* sclerotia in lettuce reducing 50% disease incidence (Chitrampalam *et al.* 2008). It significantly reduced 90% of sclerotia in a snap bean field (Gerlagh *et al.* 2003). Foliar application of *C. minitans* on detached common bean leaves reduced lesion development from ascospore infection (Bremer 2000).

Besides specific mycoparasites of *S. sclerotiorum*, a number of non-specific bacterial and fungal antagonists have also shown to inhibit sclerotia or mycelia of *S. sclerotiorum*, such as *Bacillus amyloliquefaciens*, *B. subtilis*, *Trichoderma asperellum*, *T. aureoviride*, *T. gamsii*, *T. harzianum*, *T. koningii*, *T. longibrachiatum*, *T. virens*, *Ulocladium afrum*, and *Pseudomonas fluorescens* (Tu 1980; Budge *et al.* 1995; Menendez and Godeas 1998; Schmiedeknecht *et al.* 2001; del Rio *et al.* 2002; Escande *et al.* 2002; Huang and Erickson 2007; Abdullah *et al.* 2008; Ashofteh 2009; Van Beneden *et al.* 2010). *Trichoderma harzianum* is widely used to suppress various pathogens including *S. sclerotiorum* (Kim and Knudsen 2009), which has multiple modes of action including mycoparasitism, nutrient competition, antibiosis, and plant growth promotion (Ousley *et al.* 1994). *Bacillus subtilis* has broad inhibitory activities against pathogens such as *Rhizoctonia solani*, *Fusarium oxysporum, Gaeumannomyces graminis* var. *tritici*, and *S. sclerotiorum* (Schmiedeknecht *et al.* 2001; Yang *et al.* 2009).

Coniothyrium minitans, *T. harzianum, B. subtilis,* and *Streptomyces lydicus* have been registered as commercial products for biological control (Budge and Whipps 2001; Chitrampalam *et al.* 2008). High specificity of *C. minitans* to *S. sclerotiorum* has the advantages of avoiding undesirable side effects to human and the environment, although limits the market size and market expansion (Gerlagh *et al.* 2003). Although *T. harzianum and B. subtilis* have been used to control a range of diseases, little information is available for their efficacies on management of white mold on soybean. *Streptomyces lydicus* strongly inhibits *Pythium ultimum* and *R. solani* (Yuan and Crawford 1995) by producing cell wall degrading enzymes (Mahadevan and Crawford 1997) and extracellular antifungal metabolites (Yuan and Crawford 1995). With this strong antimicrobial activity, we were interested in testing it against *Sclerotinia sclerotiorum* on soybean.

The objectives of this study were to select effective BCAs on sclerotial and apothecial reduction of *S. sclerotiorum*, compare the efficacies of selected BCAs, and determine application rates for each product for suppressing *S. sclerotiorum* in soil. The efficacy of *C. minitans*, *T. harzianum*, *B. bacillus,* and *S. lydicus* was evaluated by measuring the sclerotial survival, and carpogenic germination of *S. sclerotiorum*.

MATERIALS AND METHODS

Isolate collection and inoculum production of *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum isolates, 1001, 1004 and 1005 were collected from infected soybean plants in Michigan and isolated in the laboratory. Sclerotia were surface sterilized for 3 min by submerging in 0.6% sodium hypochlorite, rinsed three times in sterile distilled water, and plated on water agar. A mycelial plug was excised from the edge of a 2-d-old culture using a 3 mm diameter cork borer, transferred to a new 100 mm diameter Petri plate containing potato dextrose agar (PDA), plates were then sealed with Parafilm.

Sclerotia of *S. sclerotiorum* were produced in quantity in the laboratory on peeled and cubed potato tubers. Tubers were cut into 1 cm^3 cubes, and placed in a flask (1 L) to a volume of approximately 25% flask capacity. The flask was covered with aluminum foil and twice autoclaved for 20 min with a 24 h rest period between. Three 3 mm diameter mycelial plugs from 2-3 day old *S. sclerotiorum* cultures on PDA were transferred onto the potato cubes. The flasks were incubated at 22°C for at least 15 d until sclerotia matured. Sclerotia were harvested by wet sieving (Hao *et al.* 2003). Briefly, soil was washed through constant water in series of three-layer sieves (U. S. standard sieves series 100-mesh, 200-mesh and 325-mesh, Fisher Scientific Inc., Pittsburgh, PA) to separate sclerotia and other soil particles. Harvested sclerotia were dried in a transfer hood on autoclaved paper towel and stored in plastic bags at 4°C until use.

Soil inoculation with *Sclerotinia sclerotiorum* and treatment with biocontrol agents

The products Actinovate® AG (*Streptomyces lydicus* strain WYEC 108, Natural Industries, Houston, TX), Contans® WG (*Coniothyrium minitans* strain CON/M/91-08, SipCamAdvan LLC, Roswell, GA), PlantShield® HC (*Trichoderma harzianum* strain T-22, Bioworks, Victor, NY), and Serenade® MAX (*Bacillus subtilis* strain QST 713, AgraQuest, Davis, CA) were used in this study. Products were applied based on manufactures' recommended rates (Table 2-1).

Plastic pots (1 L with 10 × 10 cm^2 opening), with bases lined with a single coffee filter paper (24.5 in diam.), were filled with potting mix (BACCTO Professional Planting Mix, Michigan Peat Company, Houston, TX; 70% - 80% sphagnum peat) and sandy soil (v:v = 1:2), and placed in two 1.3 m^2 growth chambers (Model: PGR14, Conviron, Winnipeg, Manitoba, Canada) using a completely randomized design. Twenty-five laboratorial produced *S. sclerotiorum* sclerotia (isolate 1005) were buried one centimeter below the surface, spread evenly in the pots. BCAs were suspended and applied in 100 ml distilled water per pot. Four weeks after the soil treatment, soybean seeds (cv. Olympus) were soaked for two hours in distilled water before sowing. Five seeds were individually placed in soil in the pot, two centimeters in depth from top, with even distance between.

Table 2-1. Biocontrol agents and their application rates in the study.

Biological control agents	Application rates (g/L soil)						Active ingredient (%)	No. of trials	Rec. rates (g/L)[a]
C. minitans	0 0.250	0.500	1.000	2.500	5.000	10.000	5.3	1[b]	0.008
C. minitans	0 0.001	0.005	0.025	0.125	0.625	3.125	5.3	3	0.008
B. subtilis	0 0.250	0.500	1.000	2.500	5.000	10.000	14.6	1	0.005
B. subtilis	0 0.002	0.010	0.050	0.250	1.250	6.250	14.6	3	0.005
T. harzianum	0 0.025	0.050	0.100	0.250	0.500	1.000	1.15	4	0.003
S. lydicus	0 0.025	0.050	0.100	0.250	0.500	1.000	14.6	4	0.001

[a] Recommended rates were converted from the rates on product labels for field application.

[b] Application rates of *C. minitans* and *B. subtilis* modified based on the results of first trial.

Apothecial observation and sclerotial retrieval

Numbers of apothecia of *S. sclerotiorum* were recorded every other day after the first apothecium was found. After six weeks from sclerotial placement in soil, soybean plants were removed, and total fresh weight of plants from each pot was recorded. To test viability, sclerotia of *S. sclerotiorum* were retrieved from each pot by wet sieving, surface sterilized (as described above) and placed on water agar medium in Petri plates, with ten sclerotia/plate. The plates were incubated at 20°C in the dark. Mycelial growth was observed after three to seven days of incubation (Budge and Whipps 1991).

Data

Data Analysis

The trials were divided into two groups for analyses: 1) no carpogenic germination occurred; 2) carpogenic germination was observed. If there was no interaction between trials and treatments, data were combined. Statistical analysis was performed using SAS package (v.9.2, SAS Institute, Cary, NC). PROC NLIN (non-linear model) was used to correlate between rates of BCA application and sclerotial survival or apothecial production. The curves were fit by the exponential model: $Y = a + b \times e^{(-x/c)}$, where x is rate of BCAs, Y is number of viable sclerotia or produced apothecia, and a, b, and c are parameters. Rates of BCAs were transformed to logarithmic form prior to analysis.

RESULTS

Effect of application rates of biological control agents on apothecial production of *Sclerotinia sclerotiorum*

Coniothyrium minitans, *T. harzianum*, *B. subtilis* and *S. lydicus* were all effective, at different levels of efficacy, to reduce the apothecial production of *S. sclerotiorum* (Fig. 2-1). Increasing the rates of BCA application resulted in an exponential decrease in apothecial production of *S. sclerotiorum* (Fig. 2-1). The rate at 0.22 g/L soil of *C. minitans* reached an efficacy plateau by 84.3% apothecial reduction of *S. sclerotiorum* (Fig. 2-1 A). *Trichoderma harzianum* reached the maximum apothecial reduction at 0.20 g/L soil (Fig. 2-1 B). *Bacillus subtilis* had the maximum reduction of apothecia at 0.15 g/L soil, and its highest efficacy was under 90% of apothecial inhibition (Fig. 2-1 C). *Streptomyces lydicus* reached the plateau of apothecial reduction around 0.25 g/L soil, where apothecial production was completely suppressed (Fig. 2-1 D). No further reduction in production of apothecia was observed at asymptotic application rates greater than 0.25 g/L of all the BCAs, indicating that rates above 0.25 g/L cannot further enhance the efficacy and therefore they are not recommended.

Effect of application rates of biological control agents on sclerotial survival of *Sclerotinia sclerotiorum*

Coniothyrium minitans, *T. harzianum*, and *B. subtilis* reduced the number of survived sclerotia in soil (Fig. 2-2, 2-3). The number of sclerotia was negatively correlated with application rates of BCAs. In group 1, where no carpogenic germination

occurred, the concentrations for the maximal efficacy were 0.15 g/L soil for *C. minitans* (Fig. 2-2A), 0.22 g/L soil for *T. harzianum* (Fig. 2-2B), and 0.89 g/L soil for *B. subtilis* (Fig. 2-2C). However, no 100% reduction of sclerotia was observed for all BCA treatments. The highest level of sclerotial reduction for each individual BCA was 60.9% for *C. minitans* (Fig 2-2 A), 30.9% for *T. harzianum* (Fig 2-2 B), and 38.7% for *B. subtilis* (Fig 2-2 C).

In group 2, where carpogenic germination occurred, the number of sclerotia continued to decrease with increased application rates of BCAs (Fig. 2-3). The maximum reduction of sclerotia for *C. minitans* was 84.5% (Fig. 2-3A); *T. harzianum* was 23.5% (Fig. 2-3 B); *B. subtilis* was12.3% (Fig. 2-3 C); and *S. lydicus* was 27.0% (Fig. 2-3 D) in this group of trials.

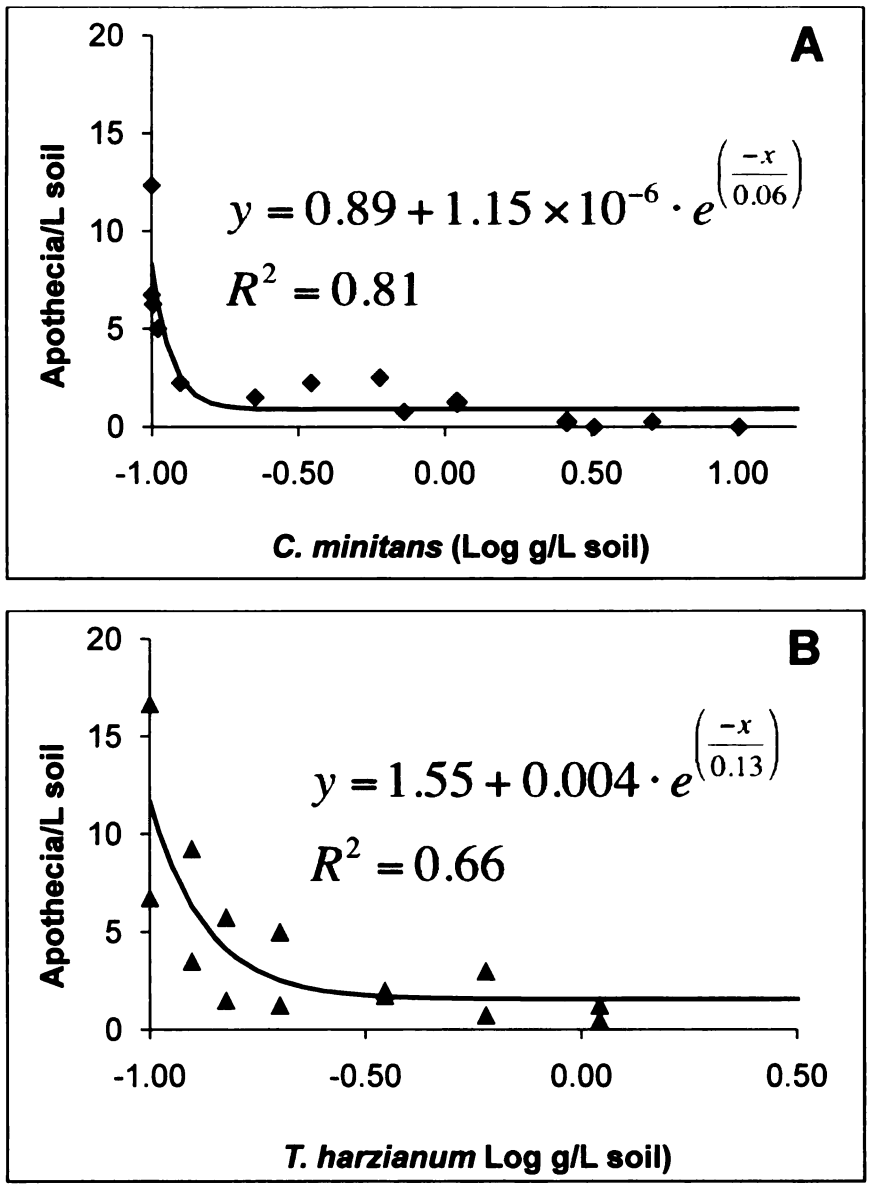
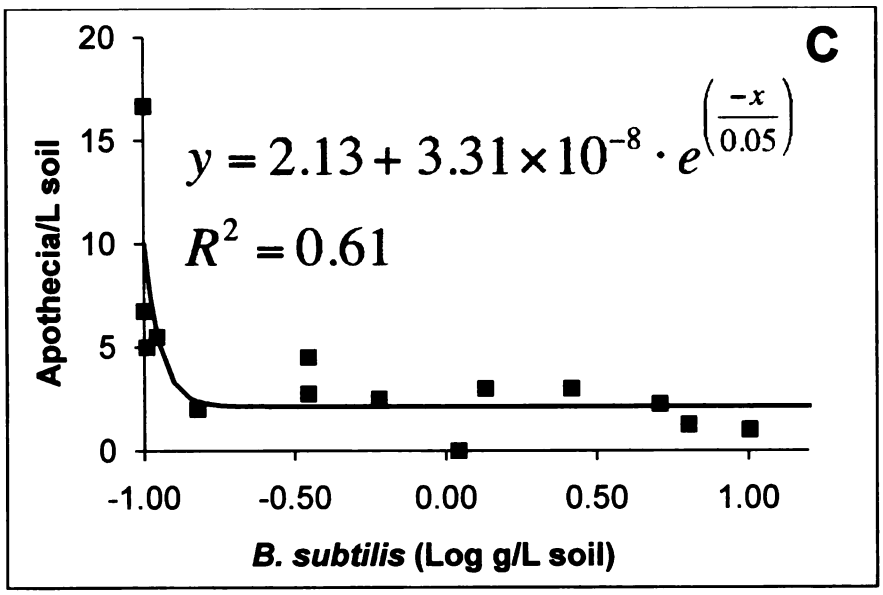


Figure 2-1. Effect of biological control agents on apothecial production of *Sclerotinia sclerotiorum*. Soil was treated with one of the products at various rates: *Coniothyrium minitans* (A), *Trichoderma harzianum* (B), *Bacillus subtilis* (C), and *Streptomyces lydicus* (D). Repeated trials were combined for regression analysis. In the equation, x = log (rate of application), and y = number of apothecia/L soil.



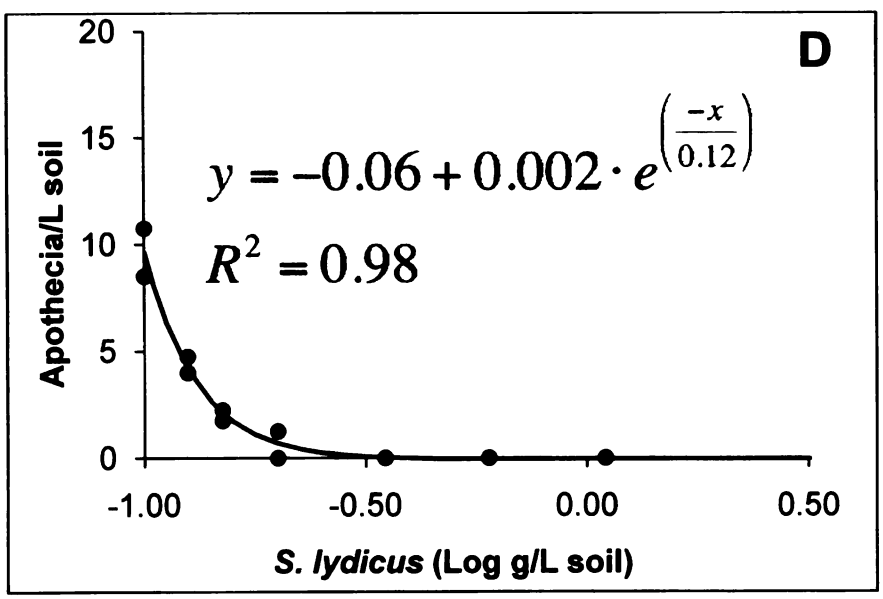


Figure 2-1continued.

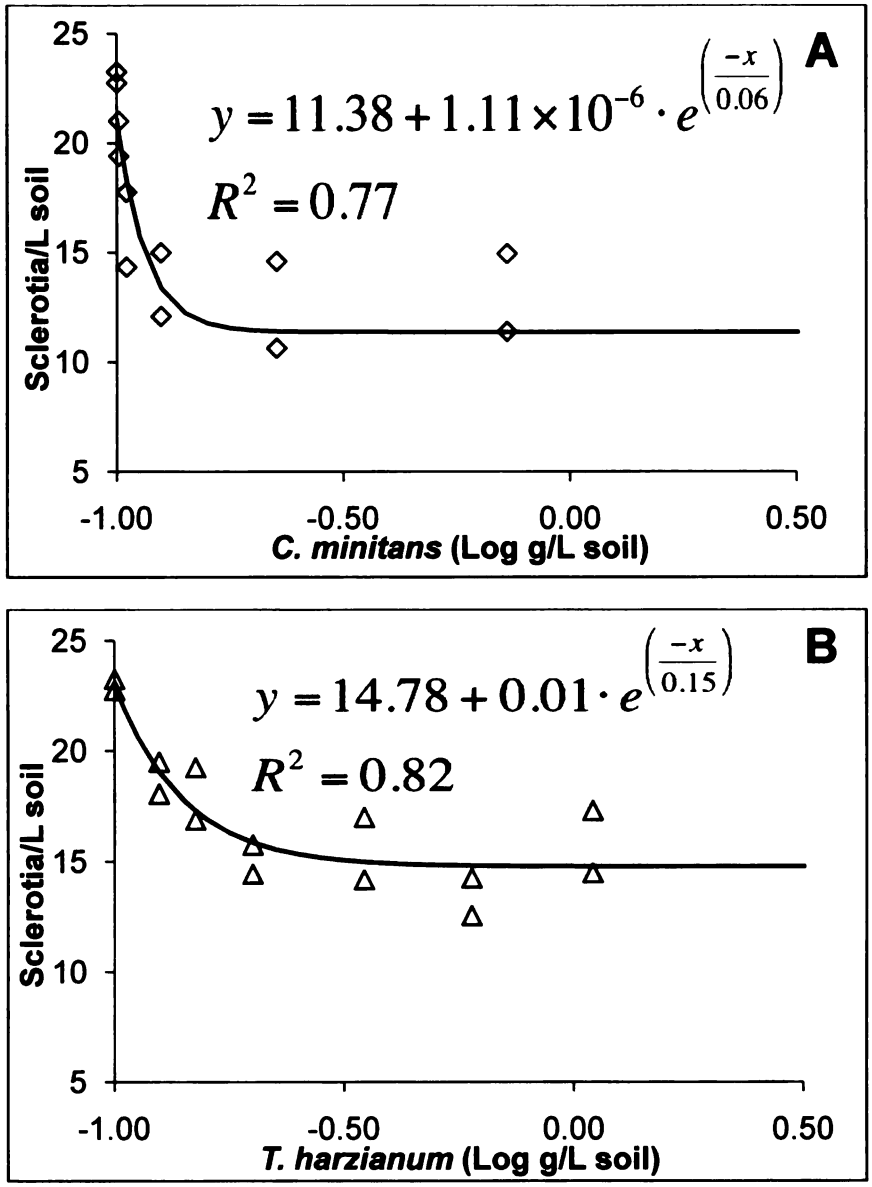


Figure 2-2. Effect of biological control agents on survival of *Sclerotinia sclerotiorum* sclerotia in group 1 trials (no carpogenic germination occurred). Soil was treated with one of the BCA products at various rates: *Coniothyrium minitans* (A), *Trichoderma harzianum* (B), and *Bacillus subtilis* (C). Data from all trials were averaged for regression analysis. In the equation, x = log (rate of application), and y = number of sclerotia/L soil.

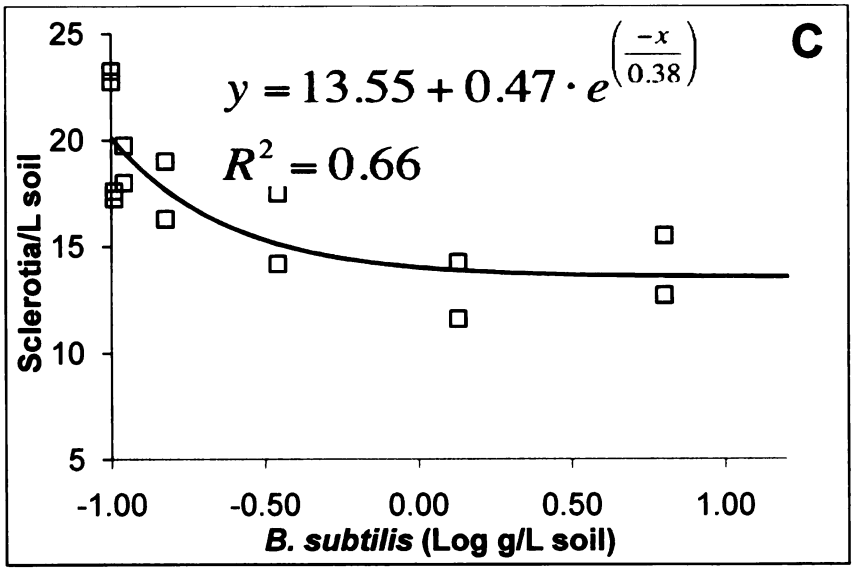


Figure 2-2 continued.

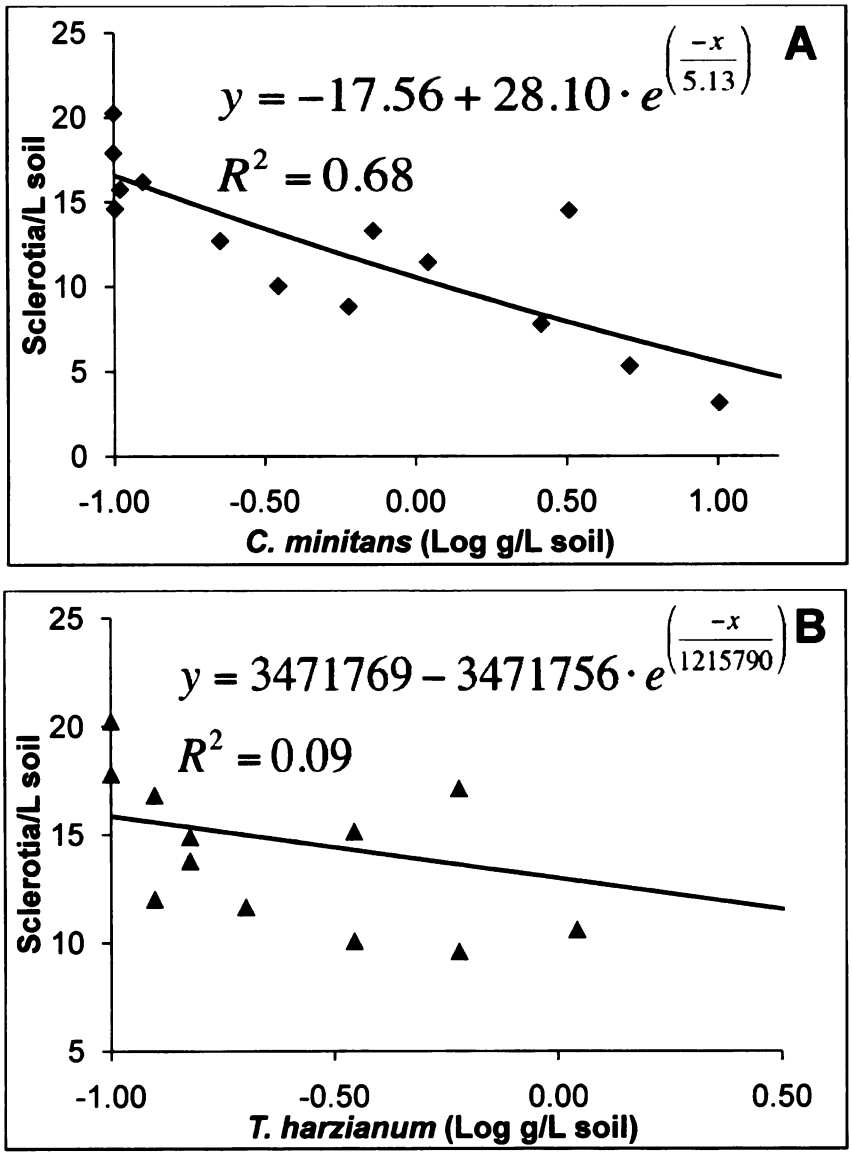


Figure 2-3. Effect of biological control agents on sclerotial survival of *Sclerotinia sclerotiorum* in group 2 (carpogenic germination occurred). Soil was treated with one of the BCA products at various rates: *Coniothyrium minitans* (A), *Trichoderma harzianum* (B), *Bacillus subtilis* (C), and *Streptomyces lydicus* (D). Data from all trials were averaged for regression analysis. In the equation, x = log (rate of application), and y = number of sclerotia/L soil.

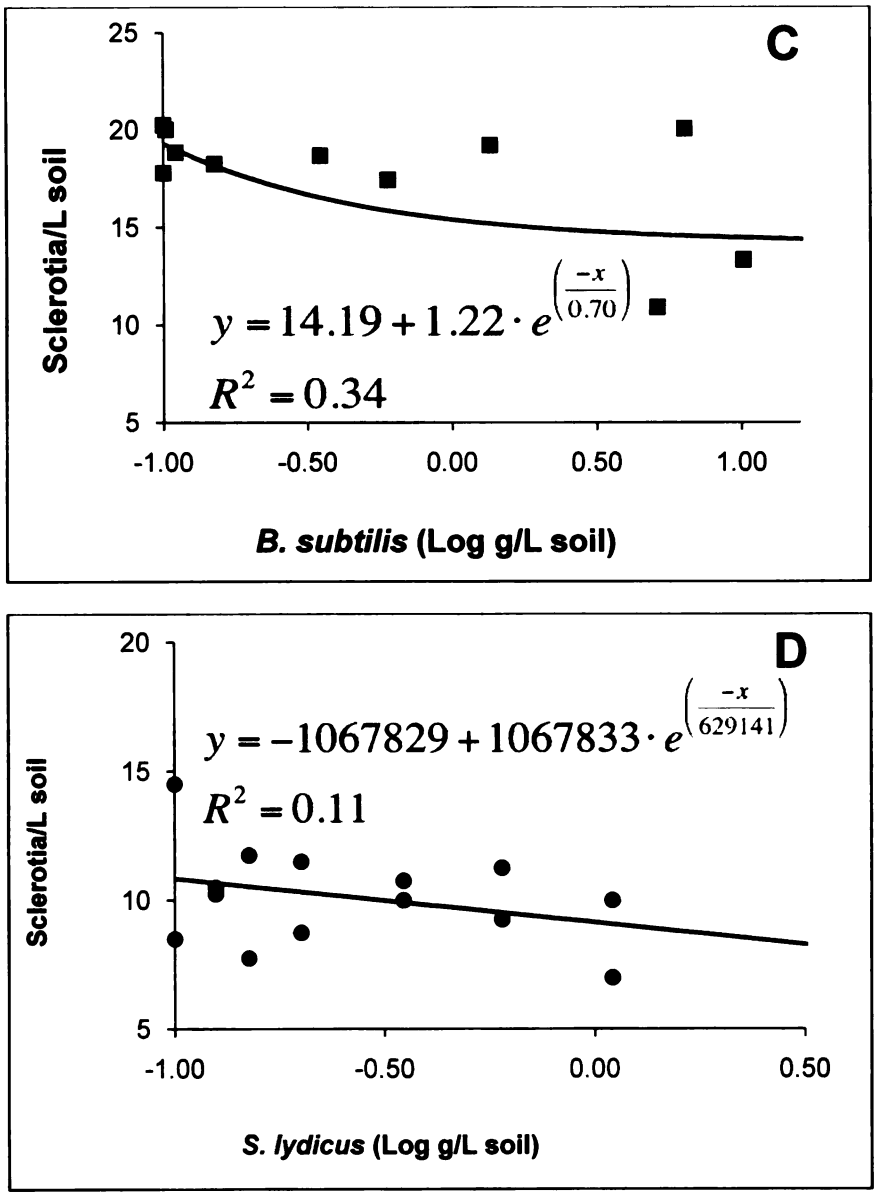


Figure 2-3 continued.

DISCUSSION

The results indicate that *B. subtilis*, *C. minitans*, *S. lydicus*, and *T. harzianum* can reduce soil population and suppress apothecial production by *S. sclerotiorum*, with varied efficacy depending on the biological control agent. The efficacy of BCAs is positively correlated with the rate of application. The results of *C. minitans* are consistent with previous study (Gerlagh et al. 2003). The manufacture recommended rates are less than the rates with best efficacy. This implies that increasing the rates for field application may reduce more primary inocula of white mold. However, once the rate passes certain level, increasing the amount of products will not give any benefit.

Mycoparasites will not degrade the pathogen unless they have direct contact (Adams and Fravel 1990). This can be done by active (extension of growing mycelia) or passive (carried and moved by other organisms or environment) modes (Williams *et al.* 1998). Lacking aggressive growth of *C. minitans* in the soil limits its efficacy (Adams and Fravel 1990). At this point, large amount of inocula and thorough mixing of BCAs in soil may help to enhance direct contact between sclerotia and BCAs. Fortunately, soil mesofauna can transport and relocate conidia of *C. minitans* in soil (Williams *et al.* 1998). Because of the transportation of conidia by soil mesofauna, the amount of inoculum may be reduced. This partially explains the efficacy of BCAs in this study reaches a limit of rates with no further reduction of apothecia or sclerotia.

Fungal (*C. minitans* and *T. harzianum*) and bacterial (*B. subtilis* and *S. lydicus*) BCAs have different mechanisms for affecting *S. sclerotiorum* sclerotia. Our data demonstrate that *C. minitans* and *T. harzianum* are more effective at reducing sclerotial

survival than *B. subtilis* and *S. lydicus* at comparable rates. Apparently, mycoparasitism is the key for the fungal BCA to colonize *S. sclerotiorum* sclerotia and eventually cause the sclerotia degraded. Although *S. lydicus* has no effect on sclerotial reduction, it greatly suppresses the carpogenic germination of *S. sclerotiorum* and its effectiveness is even superior to *C. minitans*. It is interesting to further study the mechanism how *S. lydicus* inhibits the carpogenic germination without the degradation of sclerotia.

For some unknown reasons, two trials had no carpogenic germination. This is not expected, but the results generate interesting information. In the group (1) with no carpogenic germination, the efficacy of BCAs reaches the plateau at certain application rates or above. The highest sclerotial reduction is less than 50%. While in the group (2) with carpogenic germination, the number of sclerotia continues to decrease as application rates of BCAs increases. In group 2 (sclerotia carpogenic germination occurs), sclerotial reduction is caused by both carpogenic germination and sclerotial degradation. We hypothesize that sclerotia in group 1 are in dormancy, which could be an indication why they are not germinated carpogenically. This status could prevent them from degradation by other parasitic microorganisms.

In summary, *C. minitans* and *T. harzianum* are effective in reducing sclerotial population of *S. sclerotiorum*. *Coniothyrium minitans and S. lydicus* are effective in reducing apothecia production. *Coniothyrium minitans* are more effective than other products on reduction of both sclerotia and apothecia. Its potential to reduce *S. sclerotiorum* population in soil over time can be an alternative strategy to chemical fungicides and play an important component in sustainable agriculture.

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CHAPTER THREE: MANAGEMENT OF SOYBEAN WHITE MOLD USING BIOLOGICAL CONTROL STRATEGIES IN THE FIELD

ABSTRACT

Biological control agents (BCAs) were evaluated for their efficacy on reducing soybean white mold and *Sclerotinia sclerotiorum* population in soil in Michigan. BCAs included *Coniothyrium minitans* CON/M/91-08 (Contans® WG), *Streptomyces lydicus* WYEC 108 (Actinovate® AG), *Trichoderma harzianum* T-22 (PlantShield® HC), and *Bacillus subtilis* QST 713 (Serenade® MAX). At two field locations, artificially infested soil with *S. sclerotiorum* sclerotia was treated by incorporating the above BCAs and boscalid (control) in the topsoil before planting. Populations of *Bacillus*, *Streptomyces*, *Trichoderma* spp., and *C. minitans* were monitored at 3, 28, 71, and 169 days after BCA application. *Coniothyrium minitans* had the best efficacy, reducing disease severity index (DSI) by 68.5% and sclerotia of *S. sclerotiorum* in soil by 95.3%. *Streptomyces lydicus* reduced DSI by 43.1% and sclerotia in soil by 90.6%. *Trichoderma harzianum* reduced DSI by 38.5% and sclerotia by 70.8%. *Bacillus subtilis* had marginal effect on *S. sclerotiorum*. The population of *Streptomyces*, *Trichoderma* spp., and *C. minitans* did not change significantly throughout the season. *Bacillus* spp. population was not significantly higher in *B. subtilis*-treated plots. In our trials, *C. minitans* was more effectively managed *S. sclerotiorum* in the field than other BCAs tested.

INTRODUCTION

White mold [*Sclerotinia sclerotiorum* (Lib.) de Bary] of soybean [*Glycine max* (L.) Merrill] is a devastating disease. It significantly affects the soybean industry, and is responsible for annual losses of about $70 million in the US (U. S. Canola Association 2005). *Sclerotinia sclerotiorum* has a wide host range; causing disease on a reported 408 plant species in 75 plant families and 278 genera of economically important crops worldwide (Bolton *et al.* 2006). Severe outbreaks of white mold are often associated with consistent cool and humid conditions at the time of soybean blossom (Abawi and Grogan 1979). In these conditions, sclerotia germinate and produce apothecia. Mature apothecia generate copious numbers of ascospores from in and outside soybean fields, causing initial infection (Abawi and Grogan 1979). If the peak time of ascospore release corresponds with soybean blossom, an outbreak of white mold may occur (Subbarao 1998; Bolton *et al.* 2006). Once epidemics initiate, white mold is difficult to manage. Sclerotia develop in infected pods and stems, leaving plant debris full of sclerotia in the field after harvest. The disease cycle results in accumulation of sclerotia in soil from season to season. The production of sclerotia is a key ecological stage in the life cycle of the pathogen for disease development. Therefore, reducing the sclerotial population may be the most efficient way to manage the disease, interrupting the disease cycle.

Crop rotation breaks down the disease cycle so as to reduce the rate of accumulation of sclerotia of *S. sclerotiorum* in the field, but one- or two-year rotations is unreliable and unpractical because *S. sclerotiorum* can survive in soil up to eight years and has a broad host range and (Coley-Smith and Cooke 1971). Fungicides such as

azoxystrobin, boscalid, thiophanate-methyl, iprodione and vinclozolin have provided moderate disease suppression (Bradley *et al.* 2006), but foliar spray on a 14-day interval is costly (Mueller *et al.* 2002; Bolton *et al.* 2006). Although soil fumigation can eliminate sclerotia in soil, it is expensive and may not meet expectations of environmental stewardship (Budge and Whipps 1991). Partially resistant soybean varieties have provided inconsistent protection under field conditions (Chen and Wang 2005). The concerns over environmental effects of chemical applications, the unsatisfied results from cultural practice or resistant cultivars to *S. sclerotiorum*, as well as the drive for higher efficiency and lower cost of disease control support the development of effective, consistent and durable biocontrol strategies.

Substantial studies have been conducted in evaluating biological control agents for controlling white mold in many cropping system such as lettuce (Budge and Whipps 1991; Chitrampalam *et al.* 2008), soybean (del Rio *et al.* 2002), sunflower (Eva 2003), dry bean (Huang *et al.* 2000), and celery (Budge and Whipps 1991). Biocontrol agents derived from antagonistic fungi and bacteria showed suppression of *S. sclerotiorum*. It was our interest to evaluate the performances of BCAs in Michigan and to determine the best candidates to manage soybean white mold. The objectives of this study were to evaluate the efficacy of the above BCAs to reduce the number of *S. sclerotiorum* sclerotia of and soybean white mold disease, and track the population density of BCAs in the soil after application. This may provide information for soybean white mold management in areas of North America with similar climates to Michigan.

MATERIALS AND METHODS

Field plots

Field trials were conducted at the Plant Pathology Research Farm (PLP) at Michigan State University, East Lansing, MI (N 42°41.477'; W 84°29.153') from 2007 to 2009, and at Clarksville (CLK) Horticulture Experiment Station, Clarksville, MI (N 42°42.626'; W 85°33.958') in 2008 and 2009. The soil texture of at PLP was a sandy loam, with 54.2% sand, 35% silt, and 10.8% clay, and a pH of 7.4. The trial at PLP was designed as a randomized complete block design (RCBD) with four replications (blocks). Plot size at PLP was 3.05 × 7.62 m^2 in 2007 and 6.10 × 9.14 m^2 in 2008 and 2009. The texture of soil at CLK was a sandy loam, with 70.2% sand, 25% silt, and 2.8% clay, and a pH of 6.8. Plots were established at CLK in a split-plot design with three replications. Each plot was split into sub-plots and treatments consisting of an application of *C. minitans* CON/M/91-08 applied on 1 Nov 2007 or no treatment were randomized over the sub-plots. No further treatments of *C. minitans* were applied in subsequent trial years 2008 and 2009. Individual sub-plot size was and 3.05 × 15.2 m^2 at CLK.

Soil infestation with *Sclerotinia sclerotiorum*

Sclerotia produced in the field and laboratory were used to inoculate the soil. *Sclerotinia sclerotiorum* isolates 1001, 1004, and 1005 from infected soybean plants in Michigan were used to produce sclerotia on autoclaved potato as described previously in chapter 2. Sclerotia produced in commercial soybeans were collected from a Michigan

grain elevator. Approximately 2.3 kg of field sclerotia and 3.3 kg of laboratory sclerotia were evenly distributed each year by hand or seed spreader onto the soil surface on 16 Apr 2007, 18 May 2008, and 21 Apr 2009 at PLP. *Sclerotinia sclerotiorum* density in soil was 2.25 sclerotia/L soil in 2007, and 2008, 2.0 sclerotia/L soil in 2009, respectively. Sclerotia were spread on 30 May 2008, and 24 Apr 2009 at CLK, and the final population was 1.25 sclerotia/L soil in 2008 and 2009, respectively. The sclerotial inoculum was incorporated into the top 10 cm of soil with a cultivator (2210 Field Cultivator, Deere & Company, Moline, IL).

Soil treatments

Three (in 2007) and four (2008 and 2009) BCAs were applied at PLP (Table 3-1). On 7 May 2007, *C. minitans* CON/M/91-08 (120 g a.i./ha, SipcamAdvan, Research Triangle Park, NC), *T. harzianum* T-22 (4 g a.i/ha, BioWorks, Inc., Victor, NY), *B. subtilis* QST 713 (60 g a.i./ha, AgraQuest, Inc., Davis, CA), and boscalid (180 g a.i./ha, Endura, BASF Corporation, Research Triangle Park, NC) were applied. On 7 May 2008 and 12 May 2009, *C. minitans* (240 g a.i./ha), *T. harzianum* (50 g a.i./ha), *B. subtilis* (60 g a.i./ha), *S. lydicus* (2 g a.i./ha) and boscalid (380 g a.i./ha) were applied in water suspension. The products were sprayed with a tractor-driven boom sprayer with TEEJET nozzles (model XR8008VS) set at xx cm apart. Applications were made at speed of 1.77 km/hr with a nozzle pressure of 103.4 kPa and final application volume rate of 280.6 L/ha. The treatment, application rate and date were summarized in Table 3-1.

Four BCAs were applied at CLK from 2008 to 2009 (Table 3-1). At CLK on 1 Nov 2007, half of the split-blocks were treated with *C. minitans* (240 g a.i./ha) as the fall

treatment. On 1 May 2008 *C. minitans* (240 g a.i./ha), *T. harzianum* (50 g a.i./ha), *B. subtilis* (60 g a.i./ha), and *S. lydicus* (2 g a.i./ha) were applied. On 8 Jul 2008, boscalid (380 g a.i./ha) was sprayed onto the foliage at 5% soybean bloom (stage R1 of soybean growth). On 15 May 2009, *C. minitans* (240 g a.i./ha), *T. harzianum* T-22 (50 g a.i./ha), *B. subtilis* (60 g a.i./ha), *S. lydicus* (2 g a.i./ha), and boscalid (380 g a.i./ha) were applied as described above (Table 3-1).

Table 3-1. Treatment arrangement, application rate and date in each field and year.

Field	Year	Fall treatment[a]	Treatment	Rate (g a.i./ha)	Date	DPP[b]
PLP	2007	None	*B. subtilis* QST 713	60	7-May	7
			C. minitans CON/M/91-08	120	7-May	7
			T. harzianum T-22	4	7-May	7
			Boscalid	180	7-May	7
	2008	None	*B. subtilis* QST 713	60	7-May	2
			C. minitans CON/M/91-08	240	7-May	2
			T. harzianum T-22	50	7-May	2
			S. lydicus WYEC 108	2	7-May	2
			Boscalid	380	7-May	2
			Control	0	7-May	2
	2009	None	*B. subtilis* QST 713	60	12-May	14
			C. minitans CON/M/91-08	240	12-May	14
			T. harzianum T-22	50	12-May	14
			S. lydicus WYEC 108	2	12-May	14
			Boscalid	380	12-May	14
			Control	0	12-May	14
CLK	2008	*C. minitans*	*B. subtilis* QST 713	60	1-May	9
		CON/M/91-08	*C. minitans* CON/M/91-08	240	1-May	9
			T. harzianum T-22	50	1-May	9
			S. lydicus WYEC 108	2	1-May	9
			Boscalid	380	8-Jul	-60
			Control	0	1-May	9
		None	*B. subtilis* QST 713	60	1-May	9
			C. minitans CON/M/91-08	240	1-May	9
			T. harzianum T-22	50	1-May	9
			S. lydicus WYEC 108	2	1-May	9
			Boscalid	380	8-Jul	-60
			Control	0	1-May	9
	2009	*C. minitans*	*B. subtilis* QST 713	60	15-May	7
		CON/M/91-08	*C. minitans* CON/M/91-08	240	15-May	7
			T. harzianum T-22	50	15-May	7
			S. lydicus WYEC 108	2	15-May	7
			Boscalid	380	15-May	7
			Control	0	15-May	7
		None	*B. subtilis* QST 713	60	15-May	7
			C. minitans CON/M/91-08	240	15-May	7
			T. harzianum T-22	50	15-May	7
			S. lydicus WYEC 108	2	15-May	7
			Boscalid	380	15-May	7
			Control	0	15-May	7

[a] Treatments were applied on 1 Nov, 2007 at 240 g a.i./ha. [b] DPP = days prior to planting.

Varieties M99927 (susceptible) and CL968413 (partially resistant) were planted at PLP on 20 April 2007. Olympus (susceptible) was used in 2008 and North Ripking (susceptible) in 2009 at PLP and CLK in early May. Soybean seeds were planted in 18-cm row space with average seeding rate around 444,790 seed/ha. A sprinkler irrigation system was set up to maintain canopy moisture in the canopy above about 80% RH and were run twice each day for 20 minutes at 11:00 am and 16:00 pm at a volume of 1000 L/ha/h. Irrigation was also applied when soil moisture was fell below 80% of field capacity as measured with soil moisture sensors placed at 6 and 12 cm below the soil surface (CR10X Measurement and Control System, Campbell Scientific, Logan, Utah).

Population of sclerotia in soil

Prior to soil inoculation, soil samples were obtained to determine the density of sclerotia in soil. Two liters of soil per sample from five random points to a depth of 10 cm in each plot were collected. Sampling was replicated three times for each plot. Soil samples were also collected at harvest. The samples were transported to the laboratory in an ice chest and stored at 4°C until processing. Sclerotia of *S. sclerotiorum* were retrieved by wet sieving described earlier in chapter two and viability was tested on water agar plates.

Disease evaluation

Counts of *S. sclerotiorum* apothecia were made when apothecia were visible. Counts were performed by randomly placing a 1-m^2 circles in each plot, counts were

replicated three times. The number of apothecia/m^2 of *S. sclerotiorum* was counted on 21 July and 28 July 2009 at PLP and 21 July 2009 at CLK (at bloom, stage R1 of soybean growth). Disease severity was measured using disease severity index (DSI), and the number of sclerotia/kg of harvested beans, depending on disease incidence each year. When disease incidence was low (<5%) in 2007 at PLP, sclerotia/kg bean was used and in 2008 at PLP and CLK, DSI, and sclerotia/kg bean were used. When disease incidence was moderate (5%-50%) in 2009 at PLP and CLK, sclerotia/kg harvested bean were used.

Disease severity index (DSI) (Kim *et al.* (2000) was determined at R7 stage of soybean growth as defined by the maturity of soybean pods on the main stem. Fifty plants from the center two rows were rated for DSI 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, and 3 = symptoms on main stem leading to plant death and poor or no pod fill (Kim *et al.* 2000). The DSI was calculated by the following formula:

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

Number of sclerotia per kg of harvested beans was recorded as a proxy for disease severity. Soybean samples were distributed in the Rubbermaid® white polypropylene pans (29.2 × 34.3 cm, Newell Rubbermaid, Atlanta, GA, 30328). Sclerotia were separated from beans by hand and the numbers of sclerotia were recorded.

Soybean yield evaluation

Soybeans were harvested with a small-plot combine harvester (Massey Harris 35 plot combine, with a 1.8 m cutting bar). Only 66% of plants in the center of each plot were harvested (the outside rows were not harvested and served as guard rows) and soybean yield was evaluated. Two to three liters of beans were sub-sampled from the harvested beans and sclerotia were retrieved. Soybean moisture content and 100 bean weight were determined from each plot with a Steinlite Moisture Tester SL95 (Seedburo Equipment Co. Chicago, IL) and Count-A-Pak Seed Totalizer (Seedburo Equipment Co. Chicago, IL), respectively.

Population dynamics of *Coniothyrium minitans*, *Trichoderma*, *Streptomyces* and *Bacillus* spp. in soil

Soil samples were collected from PLP and CLK in 2009 four times: 3, 28, 71 (soybean bloom), and 169 (harvest) days after application of BCAs, respectively. From each plot, three samples with 3 liters of soil for each were collected. Each sample contained soil cores from five arbitrarily selected locations in the plot. In the laboratory, soil samples were homogenized by breaking soil clods and shaking the sample bag for 1 min. To determine soil moisture content, 50 g of soil from each sample was weighed, placed in a glass Petri plate, and dried in an oven at 50°C for 48 h.

Soil dilution plating was conducted to enumerate the population of BCAs, measured as colony forming units (CFU)/g soil. Briefly, 10 g of soil was agitated in 90 ml of phosphate buffered saline (1.2 g Na_2HPO4, 0.18 g NaH_2PO4, 8.5 g NaCl, and adjusted pH to 7.4) for 10 min on a rotary shaker at 200 rpm. A series of dilutions were

prepared from 1:10 to $1:10^2$ for *C. minitans*, $1:10^2$ to $1:10^3$ dilutions for *Trichoderma* and *Streptomyces* spp., and $1:10^4$ to $1:10^5$ for *Bacillus* spp. *Streptomyces* spp. were detected on *Streptomyces* selective media (STR) (Conn *et al.* 1998). *Trichoderma* spp. were measured on *T. harzianum* selective media (THSM) (Williams *et al.* 2003). *Bacillus* spp. were detected on Tryptic Soy Agar (TSA): 3 g tryptic soy broth (EMD Chemicals, Gibbstown, NJ), and 17 g agar (EMD Chemicals, Gibbstown, NJ) amended with 100 mg cycloheximide (MP Biomedical, Solon, OH) and 100 mg Benlate (DuPont Co., Wilmington, DE). Aliquots of 100 µl were spread onto the three semi-selective media using Spiral Autoplate® 4000 (Advanced Instruments, Norwood, MA), using Even Deposition mode feature. Each plate was incubated at 20°C in the dark for 2 to 4 d for *Trichoderma* and *Streptomyces*, and 5 to 10 d for *C. minitans*, respectively. Individual colonies on each plate were enumerated twice within 7 d and CFU/g soil for each sample was calculated.

Data Analysis

Statistical analysis was performed using SAS package (v.9.2, SAS Institute, Cary, NC) with PROC GLIMMIX to analyze the effects of soil treatments. Treatments were compared using Tukey-Kramer multiple comparison at $P = 0.05$. Repeated measurements in Tukey-Kramer grouping were conducted to estimate the BCA population variation over time. Colony forming units (CFU/g soil) were log transformed to satisfy the assumption of homogeneity of variance.

RESULTS

Disease and yield evaluation

Soybean varieties M99927 (susceptible) and CL968413 (tolerant) demonstrated no significant difference in disease suppression. Variety M99927 had 9.2 sclerotia/kg harvested bean in control plots and CL968413 had 9.0 sclerotia/kg bean in the control. In 2009 at PLP, production of apothecia from boscalid treated plots significantly reduced 0.17 apothecia/m^2 in comparison to 4.58 apothecia/m^2 from control plots to lower number than the control (Table 3-2). The apothecia/m^2 in BCA treated plots were not significant from the control (Table 3-2). In 2009 at CLK, apothecia production is under detectable level, and only 0.33 apothecia/m^2 was observed in the non-treated plot (Table 3-3).

In 2007 at PLP, disease severity, measured as number of sclerotia/kg harvested bean was not significantly different between treated and non-treated plots (Table 3-2). However, BCAs and boscalid significantly reduced the DSI and sclerotia/kg harvested bean in 2008 and 2009 (Table 3-2). In 2008, *C. minitans*, *S. lydicus*, *T. harzianum*, *B. subtilis*, boscalid reduced DSI by 53.9%, 30.8%, 38.5%, 15.4%, 30.8% respectively compared to the control, and reduced sclerotia/kg harvested bean by 94.0%, 93.8%, 79.0 %, 77.5%, and 52.5% respectively, compared to the control. In 2009, with moderate disease pressure, the DSI was reduced by 68.5% for *C. minitans*, 43.2 % for *S. lydicus*, 4.7% for *T. harzianum*, 44.0% for *B. subtilis*, and 53.4% for boscalid. Sclerotia/kg harvested bean reduction was 80.5% for *C. minitans*, 85.8% for *S. lydicus*, 56.4% for *T.*

harzianum, 54.7% for *B. subtilis*, and 60.0% respectively (Table 3-2). Boscalid treated plots had significant lower number of apothecia than control plots. Soybean moisture content (15.3% in average) and 100 bean weight (69.8 kg/hL in average) were not significantly different between treatments. The yield of soybean (kg/ha) was not significant from different treatments in 2007 to 2009 (Table 3-2).

In 2008 at CLK, sclerotia/kg harvested bean and DSI were not different between treated and non-treated plots. However, in 2009, all treatments having fall application had a significant lower number of sclerotia/kg bean in comparison to the untreated control (Table 3-3). However, only sclerotia from *C. minitans* plots had significantly lower sclerotia/kg bean than the untreated control without fall treatment (Table 3-3). The yield of soybean (kg/ha) was not significant from different treatments in 2008 to 2009 (Table 3-3).

Population of sclerotia in soil

At PLP, the population of sclerotia in the soil, examined at harvest, was below 2 sclerotia/L soil for all the treatments for the three years, and not significantly different among the treatments in 2007 and 2009 (Table 3-2). However, in 2008, the number of sclerotia/L soil was significantly different, and reduced by 95.3.8%, 90.6%, 76.0%, 70.8%, and 59.1%, in *C. minitans*, *S. lydicus*, *B. subtilis*, *T. harzianum* and boscalid-treated plots respectively in comparison to the non-treated control (Table 3-2). In all years at CLK the number of sclerotia/L soil was also bellow 2 sclerotia/L soil, and not significantly different for BCA and boscalid-treated plots compared to the control (Table

3-3). Plots treated with *C. minitans* in the fall (1 Nov 2007) had no significantly lower number of sclerotia than plots without fall treatment.

Population dynamics of *Coniothyrium minitans*, *Trichoderma*, *Streptomyces* and *Bacillus* spp. in soil

Population of *C. minitans*, *Trichoderma*, and *Streptomyces* spp. was significantly higher ($P = 0.05$) in soil treated with *C. minitans* CON/M/91-08, *T. harzianum* T-22, and *S. lydicus* WYEC 108, respectively, compared with control plots without soil treatment (Fig. 3-1A, B, C). The population of the BCAs maintained the level above 10^3 CFU/g soil throughout the season (Fig. 3-1). The population of *C. minitans* changed from an average of 3.2 log CFU/g soil at application to 2.96 log CFU/g soil as measured at 169 days. Occasionally, one or two colonies were observed from non-treated soils, indicating that the majority of *C. minitans* colonies counted were from applied inocula (Fig. 3-1 A). *Trichoderma* spp. remained at 3.8 log CFU/g soil throughout the season in *T. harzianum* T-22-treated plots while the background population increased from 1.6 log CFU/g soil to 2.5 log CFU/g soil in untreated plots (Fig. 3-1 B). *Streptomyces* spp. decreased slightly during the season in *S. lydicus* WYEC 108-treated plots, but it was significantly higher than in non-treated plots, although the latter increased later in the season (Fig. 3-1 C). The population of *Bacillus* spp. remained at the same level in treated and non-treated soil (data not shown).

Table 3-2. Effects of biological control agents and fungicide at planting to soybeans on soil production of *Sclerotiorum sclerotiorum*, white mold severity and yield of soybean at PLP.

Year / Treatment	Sclerotia/ L soil[v]		DSI[w]		Apothecia/m^2[x]		Sclerotia/ kg bean[y]		Yield (kg/ha)	
2007										
B. subtilis	1.99	a[z]					16.5	a	2538	a
C. minitans	0.62	a					12.9	a	2514	a
T. harzianum	0.50	a					10.1	a	2392	a
Boscalid	1.42	a					12.8	a	2294	a
Control	0.80	a					7.5	a	2538	a
$HSD_{0.05}$	1.519						31.32		679.1	
2008										
B. subtilis	0.41	b	5.5	ab			15.2	bc	3214	a
C. minitans	0.08	b	3.0	b			4.1	c	3356	a
T. harzianum	0.50	ab	4.0	ab			14.2	bc	3681	a
S. lydicus	0.16	b	4.5	ab			4.2	c	3417	a
Boscalid	0.70	ab	4.5	ab			32.1	b	3417	a
Control	1.71	a	6.5	a			67.6	a	3315	a
$HSD_{0.05}$	1.388		2.78				27.51		1613.4	
2009										
B. subtilis	0.64	a	19.2	b	1.50	ab	74.3	b	2400	a
C. minitans	0.57	a	10.8	b	2.50	ab	31.9	b	2684	a
T. harzianum	0.70	a	32.7	a	1.75	ab	71.5	b	2725	a
S. lydicus	0.56	a	19.5	b	1.25	ab	23.3	b	2502	a
Boscalid	0.57	a	16.0	b	0.17	b	65.5	b	2359	a
Control	0.64	a	34.3	a	4.58	a	163.9	a	2481	a
$HSD_{0.05}$	0.742		20.0		3.378				315.7	

[v] Sclerotia were retrieved at harvest on 10 Oct 2007, 22 Oct 2008, and 26 Oct 2009.

[w] DSI = disease severity index, evaluated from 50 plants in the center row of plots 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, 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] Apothecia were observed during blossom stage of soybean. Number of apothecia/ m^2 was recorded in three sub-samples in each plot. [y] the number of sclerotia/kg harvested beans, evaluated at harvest.

[z] Tukey-Kramer multiple comparisons were conducted for each column each year in the table. Values followed by the same letter are not significantly different at $P = 0.05$.

Table 3-3. Effects of biological control agents and fungicide on soil population of *Sclerotinia sclerotiorum* and white mold severity and yield of soybean at CLK.

Year Treatment	Spring treatment	Sclerotia /L soil[w]		Sclerotia/ kg bean[x]		DSI[y]		Apothecia /m^2	Yield (kg/ha)	
2008										
C. minitans	*B. subtilis*	0.0	a	0.50	a	8.0	a		2702	a
	C. minitans	0.0	a	0.16	a	6.7	a		3743	a
	T. harzianum	0.0	a	1.13	a	6.0	a		3255	a
	S. lydicus	0.2	a	0.73	a	8.0	a		2832	a
	Boscalid	0.0	a	0.69	a	6.7	a		3157	a
	Control	0.0	a	0.16	a	6.7	a		2929	a
	$HSD_{0.05}$	0.27		2.146		6.77			2086.	
None	*B. subtilis*	0.28	a	3.14	a	6.7	a		4134	a
	C. minitans	0.00	a	0.45	a	4.7	a		2962	a
	T. harzianum	0.16	a	1.08	a	6.0	a		3808	a
	S. lydicus	0.13	a	1.91	a	8.0	a		3450	a
	Boscalid	0.49	a	0.88	a	6.0	a		3548	a
	Control	1.36	a	9.77	a	4.7	a		3548	a
	$HSD_{0.05}$	1.634		13.397		6.32			2670.	
2009										
C. minitans	*B. subtilis*	0.20	a	7.22	b			0.0	2995	a
	C. minitans	0.14	a	3.02	b			0.0	4036	a
	T. harzianum	0.33	a	7.17	b			0.0	4166	a
	S. lydicus	0.39	a	6.00	b			0.0	4492	a
	Boscalid	0.26	a	6.86	b			0.0	3437	a
	Control	0.42	a	30.50	a			0.0	3124	a
	$HSD_{0.05}$	0.423		11.937					2016.	
None	*B. subtilis*	0.10	a	9.51	ab			0.0	2376	a
	C. minitans	0.12	a	3.14	b			0.0	3776	a
	T. harzianum	0.19	a	10.95	ab			0.0	4459	a
	S. lydicus	0.32	a	10.93	ab			0.0	4166	a
	Boscalid	0.16	a	13.26	ab			0.0	4160	a
	Control	0.04	a	20.52	a			0.3	4069	a
	$HSD_{0.05}$	0.367		16.034					2345.	

[v] Treatment was applied on 1 Nov, 2007 at 0.24 kg a.i./ha. [w] Sclerotia were retrieved at harvest on 15 Oct 2007, 24 Oct 2008, and 28 Oct 2009. [x] the number of sclerotia/kg harvested beans. [y] DSI = disease severity index, evaluated 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, 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. [z] Tukey-Kramer multiple comparisons were conducted for each column each year in the table. Values followed by the same letter are not significantly different at $P = 0.05$.

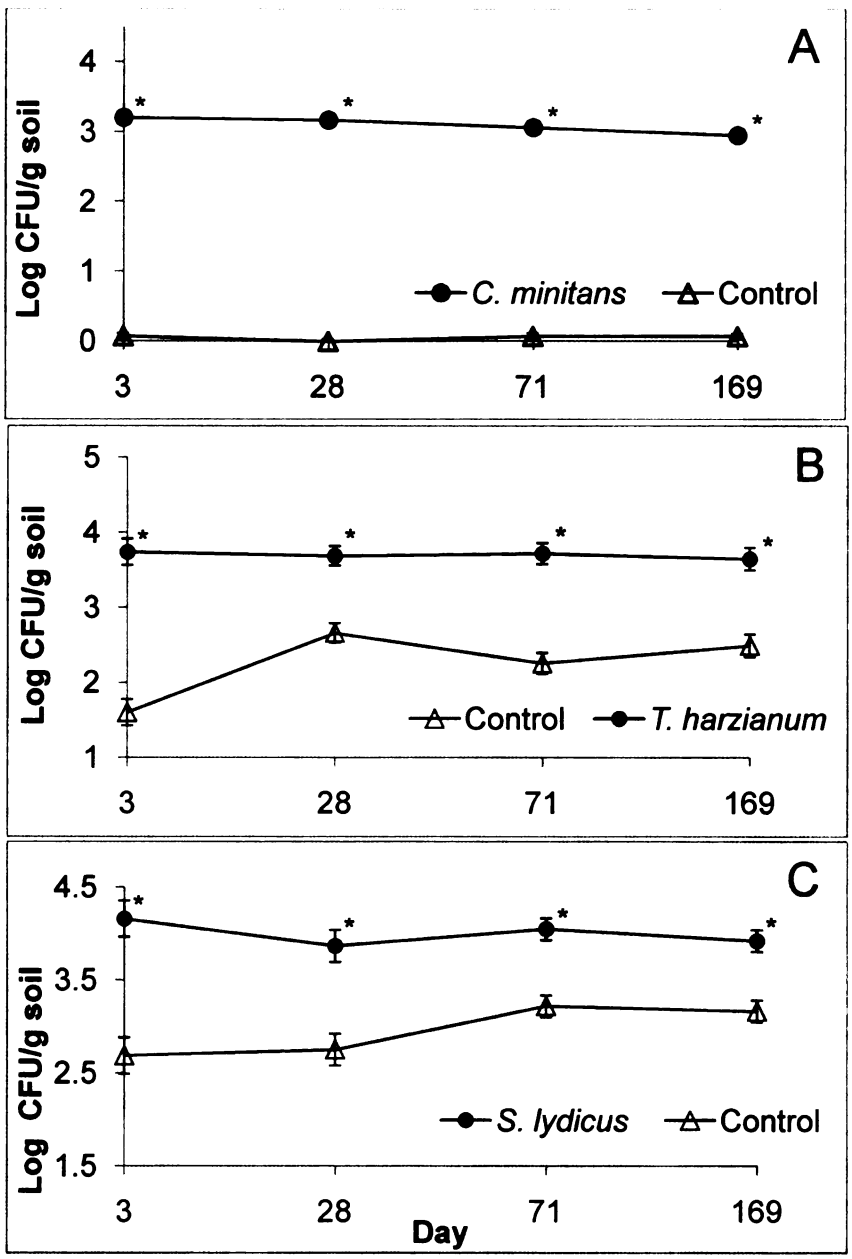

Figure 3-1. Population dynamics of *Coniothyrium minitans* in *C. minitans* CON/M/91-08-treated soil (A), *Trichoderma* spp. in *T. harzianum* T-22-treated soil (B), and *Streptomyces* spp. in *S. lydicus* WYEC 108-treated soil (C) in 2009. Each microorganism was detected and enumerated on semi-selective media. Soil samples were collected at 3, 28, 71, and 169 days after soil treatment. Error bars were determined by repeated measurements by Tukey-Kramer adjustment using RROC GLIMMIX at $P = 0.05$. * indicate significant difference between treated and control plots on the same sampling day.

DISCUSSION

The biological control agents (BCA) tested suppressed white mold caused by *Sclerotinia sclerotiorum* but the results were inconsistent over multiple years and locations. In our study, *C. minitans* significantly reduced DSI and the number of sclerotia/kg bean in 2008 and 2009 at PLP and reduced sclerotial density of *S. sclerotiorum* in soil in 2008 at PLP, this result is in agreement with previous studies on bean (Bremer 2000; Gerlagh *et al.* 2003), lettuce (Chitrampalam *et al.* 2008) and celery (Budge and Whipps 1991). However, disease severity and sclerotial density in soil in 2007 at PLP and in 2008 at CLK were not significantly different from the control. Hammond *et al.* (2008) had the same mixed results in potato field in the Columbia Basin and concluded that the ascospores originating external to fields were an abundant source of inoculum for white mold development. *Trichoderma harzianum* also reduced sclerotia/kg harvested bean in 2008 at PLP, as demonstrated by others on soybean (Menendez and Godeas 1998), celery (Budge and Whipps 1991), and lettuce. However, the efficacy of *T. harzianum* varied compared to *C. minitans*. Bacterial agents *Bacillus subtilis* and *Streptomyces lydicus* have different modes of action against *S. sclerotiorum*. *Bacillus subtilis* had only a marginal effect on reducing the sclerotia in soil and disease severity, and the suppression of carpogenic germination was sporadic. *Streptomyces lydicus* had a significant reduction of sclerotial density in 2008 at PLP, but the result was inconsistent across years and locations in our trials.

Efficacy of BCAs is affected by disease pressure, the timing of application and soil conditions. At PLP, the impact of BCAs was more significant in 2008 and 2009

when the disease pressure was higher than in 2007. In 2007, disease incidence was not significantly different due to a low disease pressure. Factors such as soil temperature, moisture, and microbial diversity can also affect the efficacy of BCAs (Hao *et al.* 2003; Reeleder 2003). Early application benefits the establishment and growth of biocontrol strains in soil. Application BCAs prior to tillage are preferred because tillage can enhance the even distribution of BCAs in soil and help direct contact between BCAs and pathogen. Pathogen population and non-target soil microbial diversity can be changed by the introduction of biocontrol strains (Cordier and Alabouvette 2009). The microbial diversity of total bacterial and fungal CFU/g dry soil was modified for a nine month period (Cordier and Alabouvette 2009). In addition, biocontrol agents are sensitive to some fungicides or bactericides that are applied in the field. For example, *C. minitans* are highly sensitive to iprodione (EC_{50} 7 to 18 μg a.i./ml) and moderately sensitive to thiram (EC_{50} 52 to 106 μg a.i./ml) (Budge and Whipps 2001). All these factors should be considered in integrated pest management programs.

Soybean yields in treated plots were not significantly different from untreated controls. This was partially due to the low incidence of white mold. According to del Rio *et al.*, no significant canola yield differences were detected between fungicide-treated and control plots when the average white mold incidence or severity was low (del Rio *et al.* 2007). Thus, evaluation of yield losses under low disease pressure could result in either overestimation underestimation of the damage.

Despite significant differences in DSI and the number of sclerotia per kg of harvested beans in 2009 at PLP, the number of sclerotia recovered from the soil was not significantly different between control and treated plots. Although the sample size may

need to be increased to detect significant differences in sclerotial density, it is possible that two different mechanisms of sclerotial degradation resulted in non-significant differences. Perhaps sclerotia in the control plot degraded predominantly via the production of apothecia, while those in treated plots were predominantly degraded by the BCAs. Although the number of apothecia recorded at PLP was not significantly different, the absolute number of apothecia recorded in the control was higher than those in the treated plots. Significant differences in DSI and the number of sclerotia per kg of harvested beans between control and treatments lend further support to this hypothesis, however further studies are needed to test this theory.

The populations of *Coniothyrium minitans*, *Trichoderma* and *Streptomyces* spp. maintained throughout the season. Maintenance of BCA populations is the key for effective control of sclerotia. In our greenhouse trials, a linear relationship between rates of BCA application and sclerotia/apothecia reduction was demonstrated (Chapter 2). The population of *C. minitans* decreased gradually over the season indicating that a single application may not be extended for more than one year. Since sclerotia are produced at the end of the crop season, either fall or spring application of *C. minitans* or other biocontrol agents may work. Therefore, multiple applications may not be necessary if a substantial population is established. The population of *Bacillus* spp. was not different between *B. subtilis*-treated and control plots. This is because the media is not highly selective and cannot separate the applied and the native *Bacillus* species, and high population of naturally occurred *Bacillus* spp. in the soil. This can be improved by finding a better selective media for *B. subtilis*.

In summary, the efficacy of BCAs varies. *Coniothyrium minitans* was more effective on reduction of disease severity and sclerotial density among the products tested. The key of this activity is to maintain a consistent high population of the biological control agents throughout the season. Multiple applications have a potential to improve the efficacy of *C. minitans* by increasing the total amount of applications.

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CHAPTER FOUR: GROWTH CHARACTERIZATION OF *CONIOTHYRIUM MINITANS* ISOLATES UNDER VARIOUS ENVIORNMENTAL CONDITIONS AND THEIR COLONIZATION ON *SCLEROTINIA SCLEROTIORUM*

ABSTRACT

Coniothyrium minitans has been shown to be an effective biological control agent against *Sclerotinia sclerotiorum*. To improve the efficacy of biological control agents in the area of application, biological characteristics of commercial and local strains of *C. minitans* were compared. Local strains of *C. minitans* were isolated from sclerotia of *S. sclerotiorum* found in Michigan. Eighty-eight percent of the Michigan isolates grew faster than the commercial strain. The isolate W09, with the fastest growth rate, was compared with commercial strain CON/M/91-08 for morphological and biological characteristics. Colonization rate of *C. minitans* isolates on *S. sclerotiorum* sclerotia was evaluated in the laboratory and growth chamber. Daily mycelial growth of *C. minitans* isolates was recorded at temperatures of 5, 10, 15, 20, 25, and 30°C; at pH values of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0; and at photoperiods of 0, 6, 12, 18 and 24 h in a day for eight consecutive days. Production of conidia of *C. minitans* was counted 3, 6, 9, and 12 days after incubation using a hemacytometer. The optimal conditions for mycelial growth of *C. minitans* W09 and CON/M/91-08 was 20°C, pH 4.5 in darkness. Moreover, the optimal condition for conidial production was at 20°C, pH 4.5 and a photoperiod of 12 or 24 h in a day. Strain W09 grew faster and produced more conidia than CON/M/91-08. W09 had relative similar colonization capability compared to CON/M/91-08. Therefore, commercial strain CON/M/91-08 has shown the ability of adaptation, although Michigan strain W09 has better overall growth characteristics.

INTRODUCTION

White mold [caused by *Sclerotinia sclerotiorum* (Lib.) de Bary] is a major disease of soybean in northern regions of the United States, including Michigan (Boland and Hall 1994; McQuilken *et al.* 1997; Workneh and Yang 2000; Mila *et al.* 2003). Overwintering sclerotia of *S. sclerotiorum* in the soil serve as the primary inocula for infection. Once sclerotia germinate and produce apothecia, millions of ascospores are released and under favorable environmental conditions for disease can infect plants (Abawi and Grogan 1979). The best strategy to manage the disease is to suppress sclerotia from germination or to eliminate their survival in soil (Coley-Smith and Cooke 1971; Adams and Ayers 1979; Subbarao 1998; Bardin and Huang 2001). Fungicides such as azoxystrobin, boscalid, iprodione, vinclozolin and herbicides including lactofen (Cobra®) have provided a moderate level of disease suppression (Dann *et al.* 1999; Matheron and Porchas 2004; Bradley *et al.* 2006). However, these chemicals are used for canopy sprays, which may require multiple applications. Crop rotation with non-host crops such as corn or cotton has a minor effect on suppressing white mold since sclerotia survive in soil for up to 8 years (Coley-Smith and Cooke 1971). The concerns over regular use of fungicides, the unsatisfactory cultural control practices, and an overall for higher level of disease control lead to the development of biological control alternatives.

Beneficial microorganisms have been used for decades to control plant diseases (Yuan and Crawford 1995; Subbarao 1998; Compant *et al.* 2005; Bolton *et al.* 2006; Whipps *et al.* 2008; Yang *et al.* 2009). Successful cases of biological control have been reported in different crop systems under laboratory conditions, controlled environments,

and in the field (Bolton *et al.* 2006; Whipps *et al.* 2008). For example, *Coniothyrium minitans* Campbell (syn: *Paraconiothyrium minitans*) has been used to control multiple diseases caused by *Sclerotinia sclerotiorum* (Campbell 1947; Jones *et al.* 2004; Chitrampalam *et al.* 2008), because *C. minitans* has been shown to effectively degrade sclerotia of *S. sclerotiorum* in the soil (Gerlagh *et al.* 1999; Jones 2002; Chitrampalam *et al.* 2008). Control of Sclerotinia diseases using *C. minitans* has been achieved on lettuce, celery, sunflower, bean, cowpea carrot, onion, kiwi fruit, rapeseed and alfalfa both in the greenhouse and field (Jones and Stewart 2000; Bardin and Huang 2001; Jones *et al.* 2004; Li *et al.* 2005; Whipps *et al.* 2008).

Since *C. minitans* is a strong mycoparasite of *S. sclerotiorum* (Campbell 1947; Budge and Whipps 1991; Jones *et al.* 2004; Chitrampalam *et al.* 2008), it has the advantage of removing *S. sclerotiorum* sclerotia from soil. For example, *C. minitans* at concentrations of 10^4 and 10^6 conidia/ml effectively controlled lettuce drop in the greenhouse, which can be enhanced by incorporating solid-substrate into soil (Jones 2002; Jones *et al.* 2004). *Coniothyrium minitans* strain CON/M/91-08 (active ingredient of biocontrol product Contans® WG, SipCamAdvan LLC, Roswell, GA) has been used in the US. The commercial strain was originally isolated from a natural sclerotium stored in the Deutsche Sammlumng von Microorganismen culture collection in Germany (Hutton 2007). As *C. minitans* has been developed to be a commercial product, it is important to know whether it can be adapted to different regions, and how environmental factors impact the efficacy of mycoparasitic activity of *C. minitans*. Microorganisms used as biological control strains may have the potential to establish better in the same areas where they are isolated. Therefore, local *C. minitans* isolates may serve as a good

comparison to the commercial strain CON/M/91-08 and may even provide additional isolates for future commercialization.

We have collected a number of *C. minitans* isolates from Michigan and selected one isolate (W09) with the fastest growth rate for this comparative study. The objectives of this study were to compare *C. minitans* commercial strain CON/M/91-08 and a Michigan isolate (W09) on their growth characteristics under various temperatures, pH, light regimes, and the colonization of *S. sclerotiorum.*

MATERIALS AND METHODS

Isolation of *Coniothyrium minitans* and the comparison of mycelial growth

Sclerotia of *Sclerotinia sclerotiorum* were collected from various locations in Michigan, including soybean fields in experimental plots at the Plant Pathology Farm (PLP) in East Lansing, MSU Horticulture Research Station at Clarksville (CLK), Lubeski Farm in Huron County, farms in Sanilac County, as well as a black bean field near Kinde from Huron County. From each field, 100 sclerotia were collected, surface sterilized for 3 min by submerging in 10% chlorine bleach solution (0.6% sodium hypochlorite) and rinsed three times in sterile distilled water. Sclerotia were dissected by a scalpel on sterile paper (Envision® Embossed, Georgia-Pacific Resins, Atlanta, GA), and placed on 25% strength potato dextrose agar (PDA, EMD Chemicals, Gibbstown, NJ): 10 g potato dextrose broth, and 15 g agar, amended with 100 mg tetracycline (MP Biomedical, Solon, OH) in 1 L volume. The sclerotia were incubated at 20°C in the dark for six to ten days. Fungi were isolated from the cultures and transferred to 25% strength PDA plates. Cultures were further purified by single-spore methods (Ho and Ko 1997). *Coniothyrium minitans* strain CON/M/91-08 was also revived and purified from commercial product Contans® WG by single-spore methods for this study.

Agar plugs (3 mm diam.) of *C. minitans* isolates were transferred to full strength PDA plates and the cultures were incubated at 20°C in the dark for four days. The diameters of *C. minitans* mycelia were recorded with three replicates.

Identification and confirmation of *Coniothyrium minitans*

Morphological characteristics of *C. minitans* strain CON/M/91-08 and isolate W09 were observed. The morphology of mycelia and pycnidia, and the size of conidia were observed with a dissecting microscope (Leica MZ 12.5 stereomicroscope, Leica Microsystems, Bannockburn, IL, 25 × magnification) and recorded with a microscope (Leica DM 2500, Leica Microsystems, Bannockburn, IL, 400× magnification). The sub-grouping of *C. minitans* was based on the classification scheme of Sandys-Winsch *et al.* (1993). Colony type based on the characteristics of top/reverse colors growing on media and distribution of pycnidia (Sandys-Winsch 1993) was recorded and conidia size of *C. minitans* was measured.

To further identify and confirm the taxonomy of W09, polymerase chain reaction (PCR) protocols were used. A mycelial plug of *C. minitans* culture was transferred into potato dextrose broth (PDB, same as PDA, but without agar) and incubated at 20°C for six days. The mycelia were collected on filter paper (12.5 cm in diam., Fisher Scientific Inc., Pittsburgh, PA) in a ceramic Büchner funnel (top diam. 83 mm and perf. area diam 60 mm, The Lab Depot, Dawsonville, GA). Mycelia were rinsed by sterilized distilled water using vacuum filtration. The mycelial mat was blotted dry on sterile paper, and ground into powder in liquid nitrogen with a mortar and pestle. Genomic DNA of the isolate was extracted using a DNeasy® Plant Mini Kit (GIAGEN, Valencia, CA) according to the manufacturer's protocol. The internal transcribed spacer (ITS) region of *C. minitans* was amplified using PCR with primers ITS1 and ITS4 (White *et al.* 1990). PCR products were purified using an UltraClean PCR Clean-up DNA Purification Kit (MO BIO, Carlsbad, CA), and sequenced at the Michigan State University Research

Technology Support Facility. The sequence was analyzed using the BLAST algorithm against the NCBI GenBank (http://blast.ncbi.nlm.nih.gov).

Effect of temperature on mycelial growth and conidial production of *Coniothyrium minitans*

Agar plugs (4 mm in diam.) of four-day culture of *C. minitans* isolates CON/M/91-08 and W09 were placed on PDA plates (pH 6.5), and incubated at 5, 10, 15, 20, 25, and 30°C in a temperature-controlled portable cabinet (PTC-1, Sable Systems International, Las Vegas, NV). Three replicated cultures were used for each treatment. Mycelial growth was determined by measuring the mycelial radius daily for eight consecutive days. Conidial production was determined using two methods: 1) calculating the total number of conidia from each Petri plate (90 mm in diam.) at 3, 6, 9, and 12 days; 2) calculating conidia per unit of mycelial area at the sixth day. To measure the number of conidia, the mycelial culture growing on PDA was cut into pieces and blended at 21,000 rpm in a Waring Laboratory Blender (The Lab Depot, Dawsonville, GA) for one minute with 40 ml sterile distilled water (Ooijkaas *et al.* 1999). Conidial concentration was counted using a hemacytometer. The experiment was repeated a second time as described.

Effect of pH on mycelial growth of *Coniothyrium minitans*

The pH values of PDA media were adjusted to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. The pH values were determined with a standard laboratory benchtop pH unit (UltraBasic, model UB-10, Denver Instrument, Bohemia, NY) before and after

autoclaving. Each treatment was replicated three times. Agar plugs (4 mm diam.) of four-day culture of *C. minitans* CON/M/91-08 and W09 were placed on the pH-adjusted PDA plates, and incubated at 20°C in the dark. Mycelial growth was determined by measuring colony diameter for eight consecutive days. The experiment was repeated twice as described.

Effect of light on mycelial growth and conidial production of *Coniothyrium minitans*

Cultures of *C. minitans* isolates CON/M/91-08 and W09 were grown on PDA (pH 6.5) as described above, incubated in the growth chamber at 20°C for 12 d. The photoperiods were: 0, 6, 12, 18 or 24 h/d with a light intensity of 153.96 μmol s^{-1} m^{-2} per μA. Each treatment was replicated three times. Mycelial growth was determined by measuring the culture colony diameter daily for eight consecutive days. The conidial production per plate was measured at days 3, 6, 9 and 12. Conidia per area of culture were estimated at the sixth day. Conidial concentration was determined using a hemacytometer. The experiment was repeated a second time as described.

Colonization of *Sclerotinia sclerotiorum* by *Coniothyrium minitans* on agar plates

Conidial suspensions of *C. minitans* isolates CON/M/91-08 and W09 were prepared by flooding 10-d-old cultures on PDA with 10 ml of sterile distilled water and gently scrapping the surface of the colony with a sterile bent glass rod. Conidia concentration was determined with a hemacytometer (Hausser Scientific, Horsham, PA) and adjusted to a final value of 10^8 conidia/ml prior to use.

Sclerotinia sclerotiorum isolates 1004, 1005 and 1006 collected from infected soybean plants in Michigan were used in the study. Nine sclerotia of each isolate were placed on water agar plates. This was repeated three times. A 5 µl aliquot of conidial suspension (10^8 conidia/ml) of the *C. minitans* isolates were placed on the top and center of each sclerotium. The plates were incubated at 20°C in the dark. Sclerotial colonization of *S. sclerotiorum* by *C. minitans* was observed for seven consecutive days after inoculation. Subsamples of sclerotia were collected on each day, surface sterilized, and cut into ten 0.5 mm slices with razor blade. To avoid possible cross contamination, sclerotia were washed individually (Jones *et al.* 2003). The sclerotial slices were incubated on 25% strength PDA at 20°C for six days. *Coniothyrium minitans* recovery was observed on each slice. The frequency of colonization was calculated by the number of infected slices divided by total number of slices. The experiment was repeated a second time as described.

In the second experiment, sclerotial colonization of *S. sclerotiorum* initiated by mycelia of *C. minitans* was observed. Nine sclerotia of each *S. sclerotiorum* isolate were placed on water agar with three plates as replications. A mycelial plug (4 mm diam.) of each *C. minitans* isolate was placed on water agar two mm from the sclerotium. The plates were incubated at 20°C in the dark and sclerotial colonization was observed as described above. The frequency of colonization was calculated by the number of infected slices divided by the number of total slices. The experiment was repeated a second time as described.

Colonization of *Sclerotinia sclerotiorum* sclerotia by *Coniothyrium minitans* in the growth chamber

A single coffee filter paper (62913 Junior Basket, 24.5 in diam., Melitta USA, Clearwater, Florida) was placed at the bottom of each pot (one liter with 10 × 10 cm^2 opening) prior to soil filling to avoid soil leakage. Each pot was filled with one liter of potting soil (BACCTO Professional Planting Mix, Michigan Peat Company, Houston, TX; ingredient: 70% - 80% horticultural sphagnum peat). Twenty-five laboratory-produced sclerotia from isolate 1006 (isolated from soybean in Michigan) were buried one centimeter below soil surface. The pots were placed in the 1.3 m^2 growth chamber (Model: PGR14, Conviron, Winnipeg, Manitoba, Canada). An 100 ml aliquot of conidial suspension of *C. minitans* CON/M/91-08 or W09 (10^8 conidia/ml) was evenly sprayed on the top soil, with three replications, at 20°C and a photoperiod of a 14:10 h light-dark circle. Light intensity was 73.56 μmol s^{-1} m^{-2} per μA. Control pots were applied with 100 ml of distilled water. Sclerotia were retrieved 1, 2, 3 and 4 wk after inoculation by wet sieving (Hao *et al.* 2003). The retrieved sclerotia were surface sterilized and placed on water agar at 20°C for three to seven days. Sclerotia that were viable were determined by mycelial growth on water agar cultures. Sclerotia infected by *C. minitans* were determined by growth of black pycnidia and conidia. The experiment was repeated a second time as described.

Effect of *Coniothyrium minitans* concentration on colonization of *Sclerotinia sclerotiorum* in the growth chamber

Sclerotia of *S. Sclerotiorum* (isolate 1006) were buried in pot soil as described above. Conidial concentrations of *C. minitans* isolates were 0, 10^2, 10^4, 10^6 and 10^8 conidia/ml. There were four replications for each treatment. The pots with sclerotia were placed in the growth chamber at 20°C and a photoperiod of 14/10 h. Light intensity was 73.56 μmol s^{-1} m^{-2} per μA. Inoculation, incubation, and sclerotial recovery were referred to the procedure as described above. The experiment was repeated a second time as described.

Statistical analysis

Statistical analysis was performed using SAS package (v.9.2, SAS Institute, Cary, NC). PROC GLM was used to compare mycelial growth rates and conidial sizes of *C. minitans* isolates, and analyze the effect of environmental factors (temperature, pH value, and light regime). Means were compared using least significant difference (LSD) at $P = 0.05$. Since there was no interaction between treatments and trials ($P>0.05$), data from two trials were combined. Because there was no difference among *S. sclerotiorum* isolates 1004, 1005 and 1006 ($P = 0.05$), data from the three isolates were pooled together for analysis. PROC NLIN was used to fit the curve for the colonization rates of *Sclerotinia sclerotiorum* sclerotia by *Coniothyrium minitans*. Conidial concentrations were transformed to a logarithmic scale prior to analysis. PROC REG was used to analyze the relationship between mycelial growth rates of *C. minitans* and colonization on sclerotia of *S. sclerotiorum* through time.

RESULTS

Identification of *Coniothyrium minitans* and the comparison of mycelial growth

The frequency of *C. minitans* colonization on sclerotia varied in different sampling locations (Table 4-1). A total of 108 *C. minitans* isolates were collected from 500 sclerotia, in which 20 isolates were from PLP, 9 from CLK, 22 from Lubeski, 28 from Kinde and 28 from Sanilac County. The mycelial growth of isolates collected from CLK, Lubeski, PLP and Sanilac County were significantly faster than the commercial strain CON/M/91-08 and isolates from Kinde. A total of 88% of Michigan isolates grew faster than CON/M/91-08. *Coniothyrium minitans* isolates from Michigan had similar morphology in culture (Fig. 4-2). Among all the isolates mycoparasitic to *S. sclerotiorum*, isolate W09 from Sanilac County grew the fastest (diam. 33.6 mm) was used for further study. W09 had similar morphology to *C. minitans* strain CON/M/91-08, with black pycnidia and conidia oozing out on the sclerotial surface in black, watery slime (Fig. 4-2). There was no significant difference in conidial length and width between W09 and CON/M/91-08 at $P = 0.05$ (Fisher's LSD, Table 4-2). However, W09 had different color on PDA agar in comparison to CON/M/91-08. The sequence of ITS gene of W09 was 100% identical to *C. minitans* on NCBI database (access number AJ293811.1).

Effect of temperature on mycelial growth and conidial production of *Coniothyrium minitans*

For both *C. minitans* isolates CON/M/91-08 and W09, temperature significantly affected daily mycelial growth (Fig. 4-3). At 20°C, both strains had the highest mycelial

growth rates: 4.10 and 8.43 mm/d for CON/M/91-08 and W09, respectively. At 15°C, the growth rate was the second highest: 2.69 and 6.08 mm/d for CON/M/91-08 and W09, respectively. At 5°C, limited mycelia growth (less than 1 mm) was observed. No growth occurred at 30°C. W09 had significantly ($P = 0.05$) faster growth than CON/M/91-08 at temperatures of 5, 10, 15, 20, and 25°C (Fig. 4-3).

Temperature significantly affected conidial production (Fig. 4-4, 4-5). *C. minitans* initiated conidia within the first three days at 10, 15, 20, and 25°C, but the conidial production was not observed until the ninth day at 5°C. In general, the overall conidial production /plate increased as incubation progressed, and the maximal conidial production occurred at 20°C. Both strains stopped producing conidia at 30°C as pycnidia were not produced at this temperature (Fig. 4-4, 4-5). At the sixth day, conidia/mm^2 mycelial area of CON/M/91-08 was greater ($P = 0.05$) at 10, 15, 20, and 25°C than W09, but there was no difference at 10°C (Fig. 4-5). However, conidia/plate of W09 was greater than CON/M/91 after six days (Fig. 4-4).

Effect of pH on mycelial growth of *Coniothyrium minitans*

Mycelial growth of *C. minitans* CON/M/91-08 and W09 occurred at pH values from 4 to 8, with the maximal growth rate at pH 4.5 (Fig. 4-6). The mycelial growth was gradually decreased when pH values increased from 5.0 to 8.0. W09 had higher rates of mycelial growth at pH values of 4.0 to 8.0 than CON/M/91-08 (Fig. 4-6).

Effect of light on mycelial growth and conidial production of *Coniothyrium minitans*

For both CON/M/91-08 and W09, mycelial growth was negatively correlated with the length of photoperiods (Fig. 4-7). The maximal mycelial growth of *C. minitans* occurred without light and the minimum growth rate with a 24 h photoperiod. At each point of all photoperiods, W09 had a significantly ($P = 0.05$) greater growth rate than CON/M/91-08 (Fig. 4-7).

Light also affected conidial production of *C. minitans* isolates. The highest conidia/mm^2 of mycelial area was observed with a 24 h photoperiod (Fig. 4-9), but the maximum conidia/plate was at 12 h/d photoperiod (Fig. 4-8). W09 produced more conidia/plate (Fig. 4-8), but fewer conidia/mm^2 mycelial area (Fig. 4-9) than CON/M/91-08. At day three, both CON/M/91-08 and W09 produced more conidia under photoperiod of 12, 18 and 24 h/d compared to 1 and 6 h/d. However, at the 6th, 9th and 12th day, the maximal conidial production was 6, 12 and 18 h/d. Under a photoperiod of 12 h/d, W09 had a significant greater number of conidia/plate than CON/M/91-08 at 3, 6, 9 and 12 d (Fig. 4-8, 4-9).

Colonization of Sclerotinia sclerotiorum by *Coniothyrium minitans* on agar plates

Coniothyrium minitans isolates W09 and CON/M/91-08 had a linear relationship between inoculation time and the rates of colonization, using either mycelia or conidia as inocula (Fig. 4-10). When mycelia were used as inocula, the coefficient of determination (R^2) in the regression equation was 0.9 for W09 and 0.85 for CON/M/91-08 at $P <$ 0.0001 (Fig. 4-10). Earliest colonization started within the first day by W09 and the

second day by CON/M/91-08. After five days, 100% of the sclerotia tested were infected by both W09 and CON/M/91-08. There was no significant difference between the rate of colonization between W09 and CON/M/91-08 except on the fifth day, when W09 had a higher colonization frequency (30.8%) than CON/M/91-08 (28.75%) (Fisher's LSD: P = 0.05, data not shown). When conidia were used as inocula, R^2 in the regression equation was 0.9 and 0.85 for W09 and CON/M/91-08, respectively, at $P < 0.0001$. The earliest colonization started within the first day by W09 and the second day by CON/M/91-08 while100% sclerotia were colonized by CON/M/91-08 after the fifth day and by W09 after the sixth day and. There was no significant difference of colonization rates between strains W09 and CON/M/91-08 except at the third day (Fisher's LSD: $P = 0.05$, data not shown).

Colonization of *Sclerotinia sclerotiorum* by *Coniothyrium minitans* in the growth chamber

Coniothyrium minitans isolates W09 and CON/M/91-08 were re-isolated from inoculated sclerotia. Colonization frequency was increased as time of incubation was extended (Fig. 4-11). In contrast, *C. minitans* was not recovered from sclerotia of control pots (Fig. 4-11). Colonization frequency and time of incubation had a curve linear relationship. The regression equation was $y = a \cdot \ln(x) + b$, where y is colonization rate, x is incubation time, a is slope, and b is the intercept of the curve. During the first week post inoculation, 47.0% of sclerotia were colonized by CON/M/91-08 and 49.0% by W09. In week two, 72.0% and 84.2% of sclerotia were colonized by CON/M/91-08 and W09, respectively. The portion of sclerotia colonized by W09 was higher than by CON/M/91-

08 in the second and third week (Fisher's LSD: $P = 0.05$, data not shown). In week four, all the sclerotia retrieved from soil were colonized. The frequency of colonized sclerotia was 63.6% from CON/M/91-08-treated pots and 65.2% from W09-treated pots (Fig. 4-11). Thus, W09 and CON/M/91-08 demonstrate similar efficacy for sclerotial colonization.

The survival of sclerotia from soil treated with W09 and CON/M/91-08 was significantly lower compared to control. When calculated in the equation: $y = a + b \cdot e^{x}$, y is the number of retrieved sclerotia, x is the time post inoculation, a and b are intercept and slope, respectively. In week one, 15.0% and 12.9% of sclerotia were reduced by CON/M/91-08 and W09, respectively. In week 2, 11.6% and 21.9% of sclerotia were reduced by CON/M/91-08 and W09, respectively. The number of retrieved sclerotia from the W09-treated pot was significantly lower than the number of sclerotia treated with CON/M/91-08 (Fisher's LSD: $P = 0.05$). The third week showed a similar trend as in week two: W09 had a significantly higher efficacy on sclerotial reduction than CON/M/91-08 (Fisher's LSD: $P = 0.05$). In week four, 63.1% and 70.8% of sclerotia were degraded by CON/M/91-08 and W09, respectively (Fig. 4-11). Overall, W09 and CON/M/91-08 demonstrated a similar trend for sclerotial reduction, but W09 was more aggressive in week two and three.

Effect of *Coniothyrium minitans* concentration on colonization of *Sclerotinia sclerotiorum* in the growth chamber

There was a linear relationship between conidial concentration and the rate of colonization for both *C. minitans* isolates CON/M/91-08 and W09. Increasing conidial

concentration of *C. minitans* enhanced the efficacy of colonization in *S. sclerotiorum* (Fig. 4-12). For CON/M/91-08, the colonization frequency was from 77.64% at concentration of 10^2 conidia/ml to 91.6% at 10^8 conidia/ml after four weeks. For W09, the colonization frequency ranged from 75.3% at 10^2 conidia/ml to 92.8% at 10^8 conidia/ml. There was no difference of colonization efficiency between the two *C. minitans* isolates, except W09 had a significant higher colonization frequency at 10^4 conidia/ml (Fig. 4-12). No *C. minitans* colonization of sclerotia was observed from control pots (Fig. 4-12).

There was a negative relationship between *C. minitans* concentration and survival of *S. sclerotiorum* sclerotia (Fig. 4-12). For both *C. minitans* isolates, lower numbers of sclerotia were retrieved from the soil treated with higher conidial concentration of *C. minitans*. The number of sclerotia from all treatments was significantly lower than the control ($P = 0.05$). For CON/M/91-08, *S. sclerotiorum* sclerotia were reduced by 27.7, 33.3, 47.6, and 65.7% at concentrations of 10^2,10^4, and 10^8 conidia/ml, respectively. For W09, sclerotia were reduced by 36.1, 47.6, 63.6, and 73.5% at concentration of *C. minitans* 10^2, 10^4, 10^6, and 10^8 conidia/ml, respectively. The CON/M/91-08 had a significant effect on the number of sclerotia retrieved from soil at concentrations of 10^4 and 10^6 conidia/ml. There was no difference between the two *C. minitans* isolates on sclerotial colonization at the highest concentrations (10^6 and 10^8 conidia/ml) (Fig. 4-12).

Table 4-1. Mycelial growth of *Coniothyrium minitans* isolated from different fields in Michigan on potato dextrose agar.

Location	Isolate	Mycelial colony (mm in diameter)[x]	Colony diam. (mm) range (%)			
			0-10.0	10.1-20.0	20.1-30.0	30.1-40.0
CLK	9	26.4 a[y]	0.0	0.0	88.9	11.1
Kinde	28	21.6 b	3.6	25.0	71.4	0.0
Lubeski	22	25.3 a	0.0	13.6	68.2	18.2
PLP	20	24.1 a	0.0	5.0	95.0	0.0
Sanilac	28	27.0 a	0.0	3.6	89.3	7.1
CON/M/91-08[z]	1	13.0 b	0.0	100.0	0.0	0.0

[z] Mycelial growth diameter were determined after four days of incubation at 20°C in the dark.

[y] Fisher's least significant difference (LSD) at $P = 0.05$was used to compare the average mycelial diameter of *C. minitans* isolates.

[z] Commercial strain of *C. minitans* CON/M/91-08 from product Contans® WG was listed to compare the mycelial growth rate with Michigan isolates.

Table 4-2. Origin, colony type and conidia size of *Coniothyrium minitans* isolates W09 and CON/M/91-08 on potato dextrose agar.

Isolate	Origin	Source of isolate	Colony type[x]	Conidia size (µm)[y, z]					
				Length			Width		
				Mean	Max	Min	Mean	Max	Min
CON/M/91-08	Germany	Sclerotia	Type I	5.9 a	7.7 a	4.1 a	4.4a	6.2 a	3.0 a
W09	Michigan	Sclerotia	Type II	5.9 a	7.6 a	3.9 a	4.2 a	5.3 a	3.5 a

[x] Colony type is described with top/reverse colors and distribution of pycnidia (Sandys-Winsch 1993). Type I: Isabelline (moderate yellowish brown)/Isabelline. Many black, mature pycnidia visible from top and reverse; type II: Hazel (light-moderate yellowish brown)/Isabelline. Many black, mature pycnidia were visible from both top and reverse sides of the media plate.

[y] Conidia size was measured under the microscope (Leica DM 2500, Leica Microsystems, Bannockburn, IL, 400 × magnification).

[z] Fisher's least significant difference (LSD) at $P = 0.05$was used to compare conidial sizes of *C. minitans* isolates

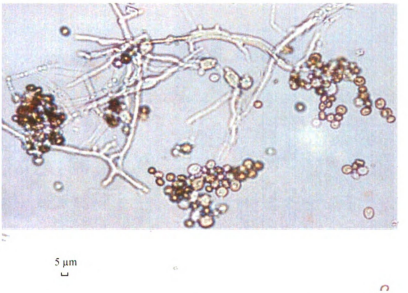

Figure 4-1. Morphology of *C. minitans* W09 (400 × magnification), showing mycelia and conidia.

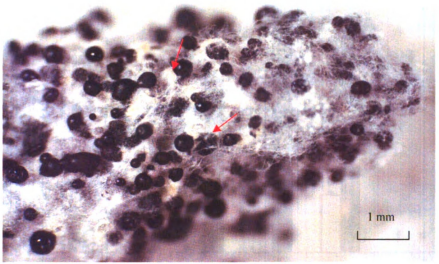

Figure 4-2. Colonization of *Sclerotinia sclerotiorum* sclerotia by *C. minitans* strain W09. Pycnidia (black spherical bodies) and conidia of *Coniothyrium minitans* oozing out of sclerotial surface (arrows) of *Sclerotinia sclerotiorum* under a dissecting microscope (25 × magnification).

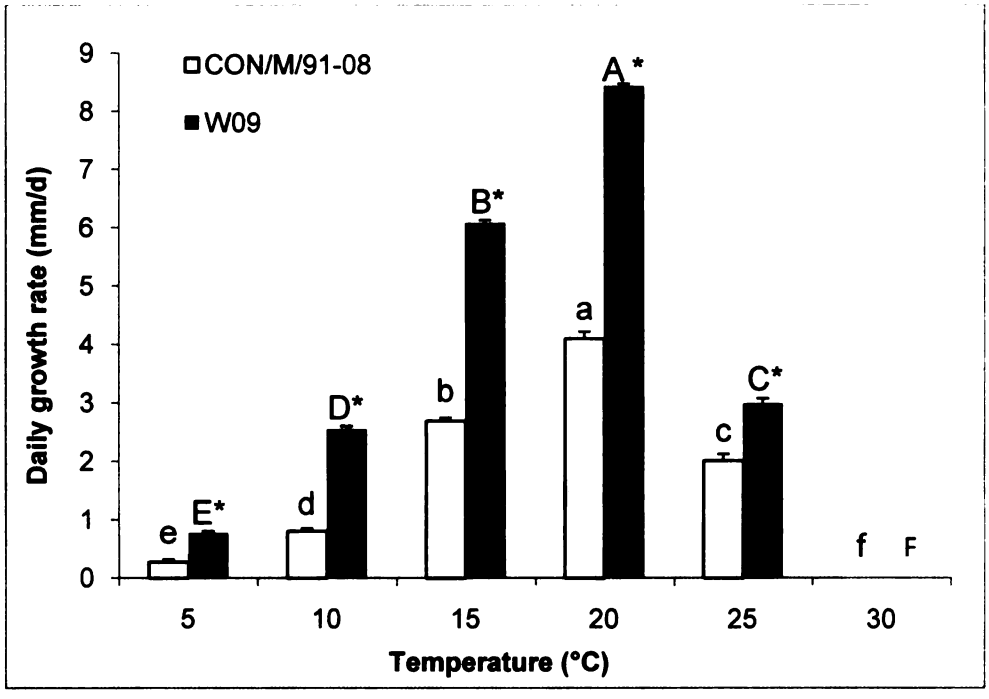

Figure 4-3. Effect of temperature on mycelial growth of *C. minitans* isolates, W09 and CON/M/91-08. Mycelial growth was measured for eight consecutive days. Growth rates were the slope of regression equations derived from eight-day mycelial growth. Bars on each column are standard deviation. Uppercase and lowercase letters are used for mean separation for *C. minitans* W09 and CON/M/91-08, respectively. Means with the same letters are significantly different. Growth at each temperature was compared between the two isolates, and * indicates significant difference of means between the two isolates at the same temperature.

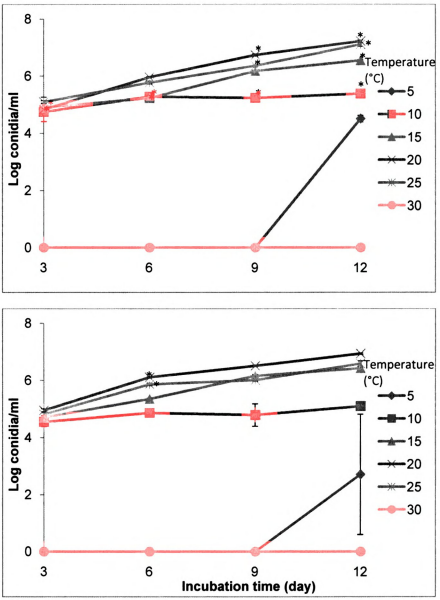

Figure 4-4. Effect of temperature on conidial production/plate (90 mm in diam.) of *C. minitans* isolates W09 (upper panel) and CON/M/91-08 (lower panel). Conidial production was determined at 3, 6, 9 and 12 d using a hemacytometer. Colored lines indicate different temperatures. Comparisons were performed using Fisher's least significant difference (LSD, $P = 0.05$) for each column. Bars on each column are standard deviation, and * indicates significant difference between the strains at the same temperature ($P = 0.05$).

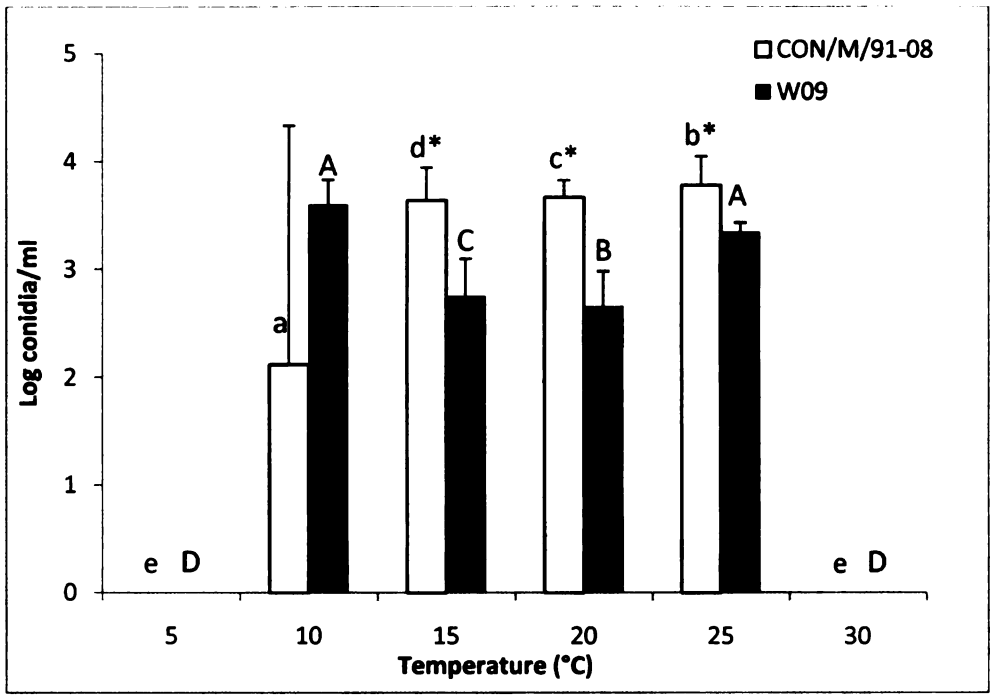

Figure 4-5. Effect of temperature on conidia production/mm^2 mycelial area of *C. minitans* isolates W09 and CON/M/91-08. Conidia were counted at the sixth day using a hemacytometer. Comparisons were conducted using Fisher's least significant difference (LSD, $P = 0.05$) for each column. Uppercase and lowercase letters are used for means separation of *C. minitans* W09 and CON/M/91-08, respectively. Means with same letters are not significantly different. Bars on each column are standard deviation, and * indicates significant difference between strains at the same temperature ($P = 0.05$).

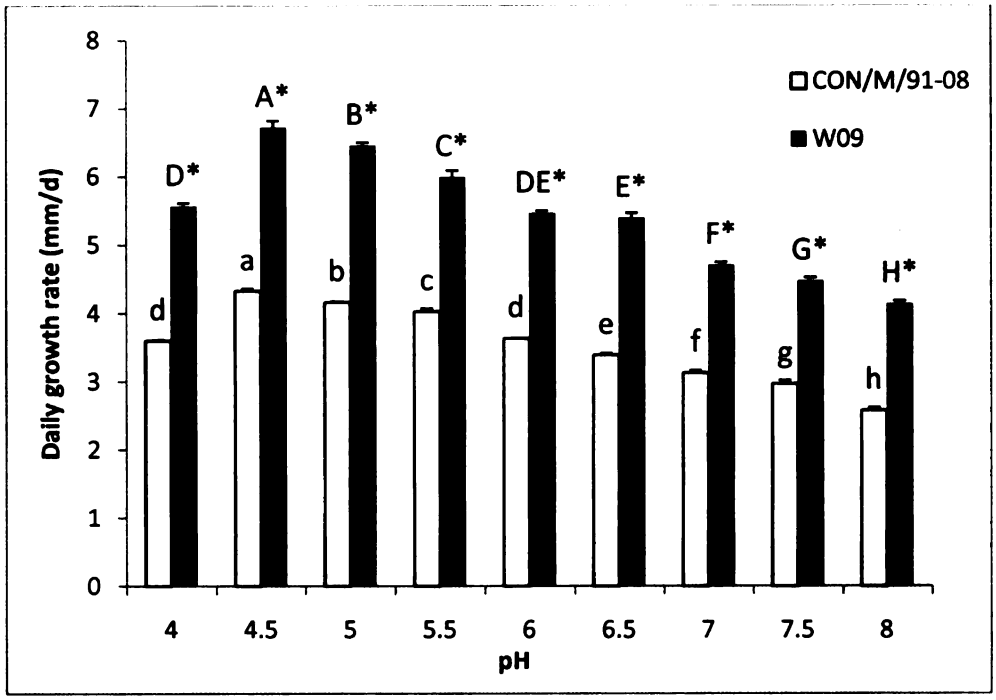

Figure 4-6. Effect of pH values on mycelial growth of *C. minitans* isolates W09 and CON/M/91-08. Mycelial growth was measured for eight consecutive days. Growth rates were determined by the slope of equations in linear regression based on the data of eight days. Growth rates of the two isolates were compared at each pH value. Uppercase and lowercase letters are used for means separation of *C. minitans* W09 and CON/M/91-08, respectively. Means with same letters are not significantly different. Bars on each column are standard deviation, and * indicates the significant difference between isolates at the same pH ($P = 0.05$).

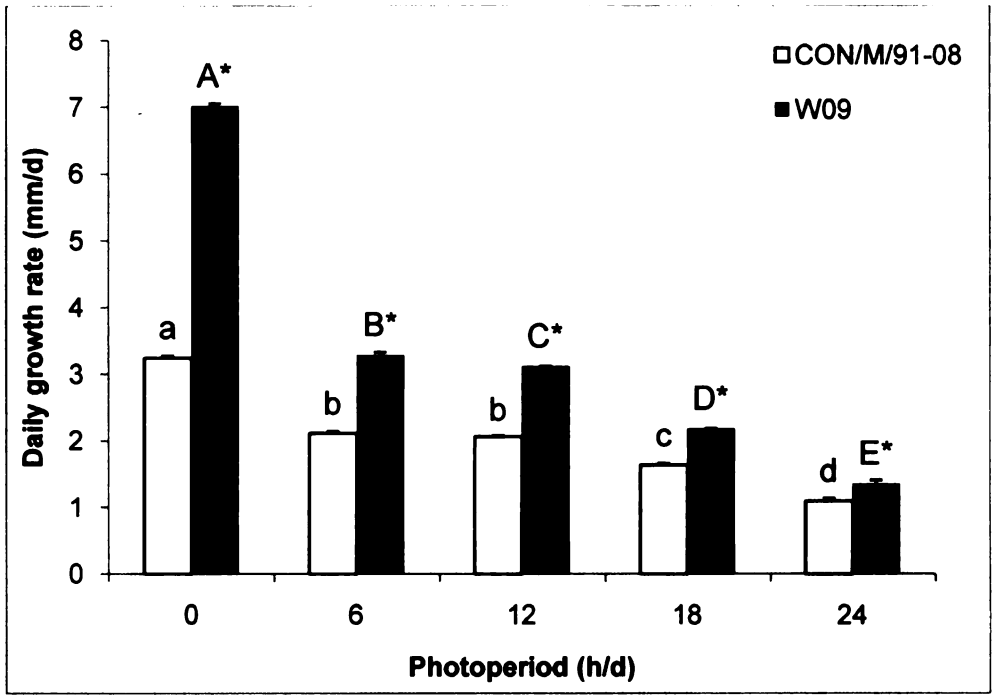

Figure 4-7. Effect of light on mycelial growth of *C. minitans* isolates W09 and CON/M/91-08. Mycelial growth was measured for eight consecutive days. Growth rate under each photoperiod was determined by the slope of equations in linear regression based on the growth data of eight days. Growth rates of the two isolates were compared at each photoperiod. Uppercase and lowercase letters are used for means separation of *C. minitans* W09 and CON/M/91-08, respectively. Means with same letters are not significantly different. Bars on each column are standard deviation, and * indicates the significant difference between isolates at the same photoperiod ($P = 0.05$).

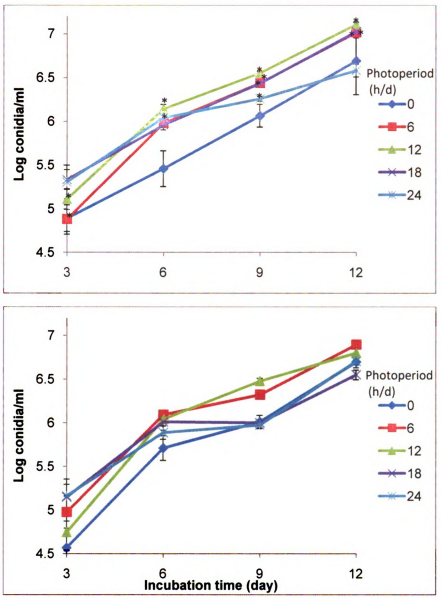

Figure 4-8. Effect of light on conidial production/plate (90 mm in diam.) of *C. minitans* isolates W09 (upper panel) and CON/M/91-08 (lower panel). Conidial production was determined at 3, 6, 9 and 12 d using the hemacytometer. Colored line indicated different photoperiod (h/d). Multiple comparisons were performed for each photoperiod using least significant difference (LSD, $P = 0.05$), and * indicates significant difference between the strains under the same photoperiod. Bars on each column are standard deviation.

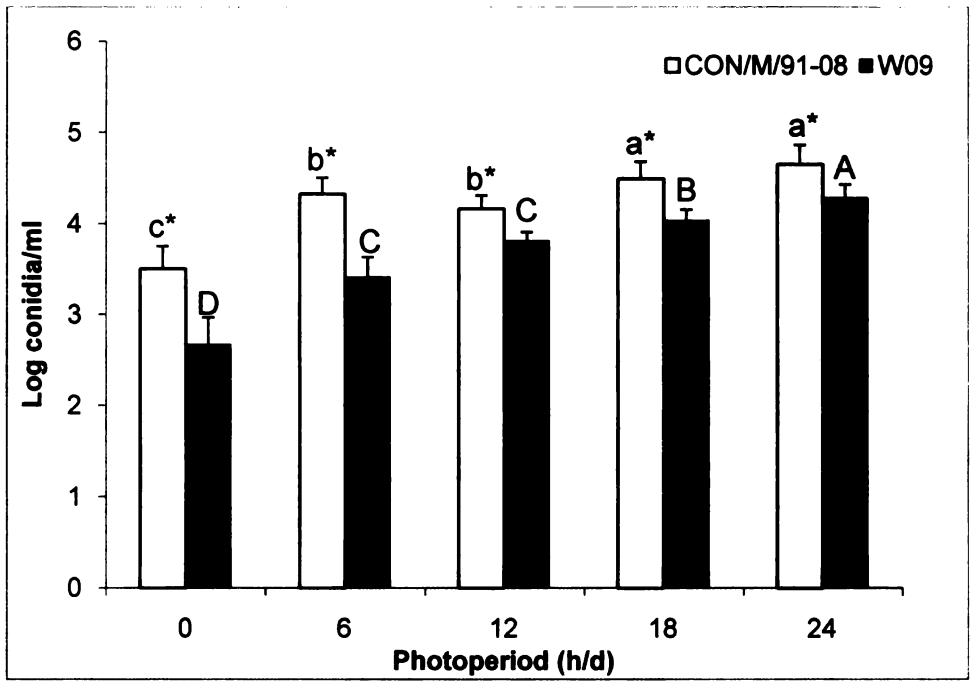

Figure 4-9. Effect of light on conidial production/mm^2 mycelial area of *C. minitans* isolates W09 and CON/M/91-08. Conidia were counted at the sixth day using a hemacytometer. Uppercase and lowercase letters are used for means separation of *C. minitans* W09 and CON/M/91-08, respectively. Means with same letters are not significantly different. Values of the two isolates were compared at each photoperiod using least significant difference (LSD, $P = 0.05$), and * indicates significant difference between the strains. Bars on each column are standard deviation.

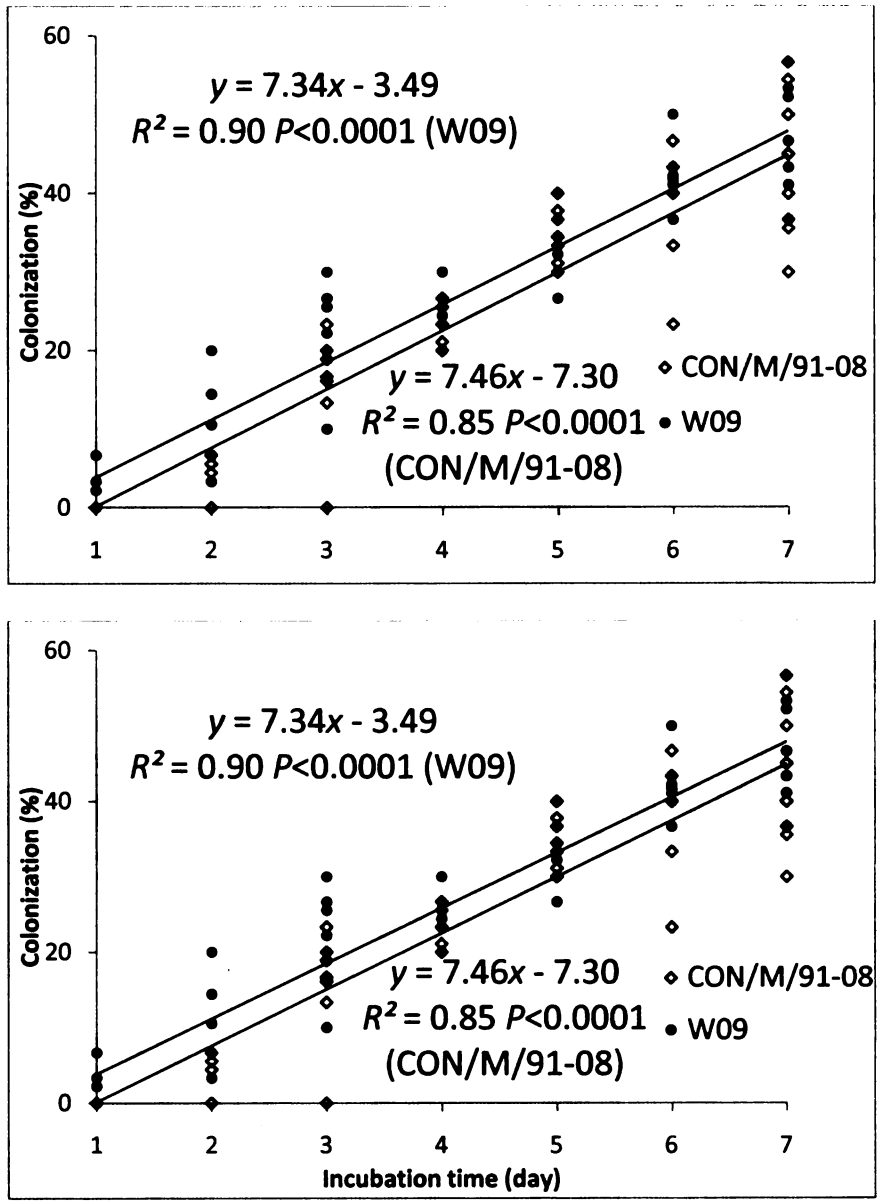

Figure 4-10. Colonization of *S. sclerotiorum* sclerotia by *C. minitans* isolates W09 and CON/M/91-08 via mycelia (upper panel) and conidial concentration of 10^8 conidia/ml (lower panel). The sclerotium was diced into slices with same thickness. Colonization was calculated as the number of infected sclerotial slices divided by total sclerotia slices examined. Linear regression estimated the correlation between time (day, *x* axis) of incubation and percentage of colonization (*y* axis).

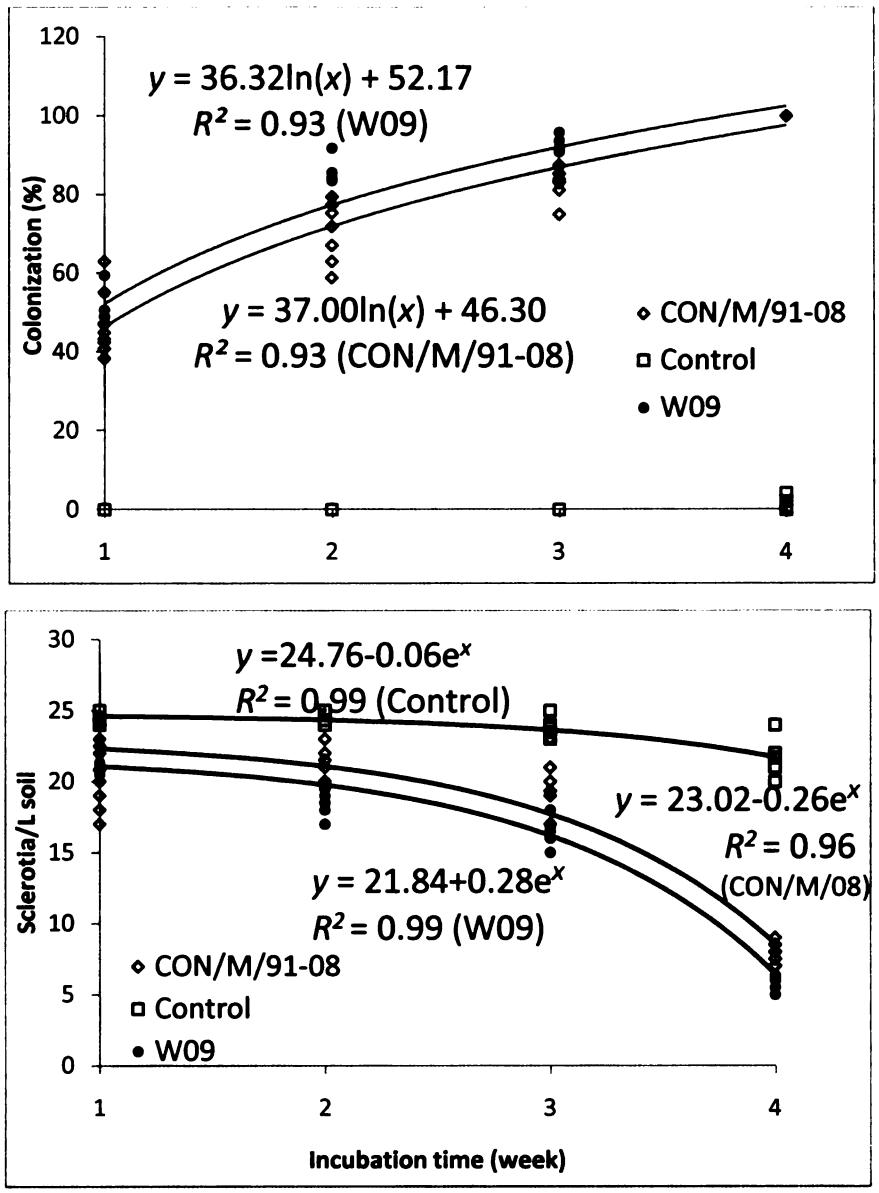

Figure 4-11. Effect of inoculation of *C. minitans* isolates W9 and CON/M/91-08 on colonization of sclerotia (upper panel) and sclerotial survival (lower panel) of *S. sclerotiorum* in the growth chamber. A total of 25 sclerotia were placed in each pot followed by 100 ml *C. minitans* conidial suspension (10^8 conidia/ml) was placed in each pot with three replications. The frequency of colonization was determined as the infected sclerotia divided by total sclerotia buried. The survival of sclerotia was recorded after four weeks. Non-linear regression determined the correlation between time (day, *x* axis) from inoculation and the percent of colonization or sclerotial number (*y* axis).

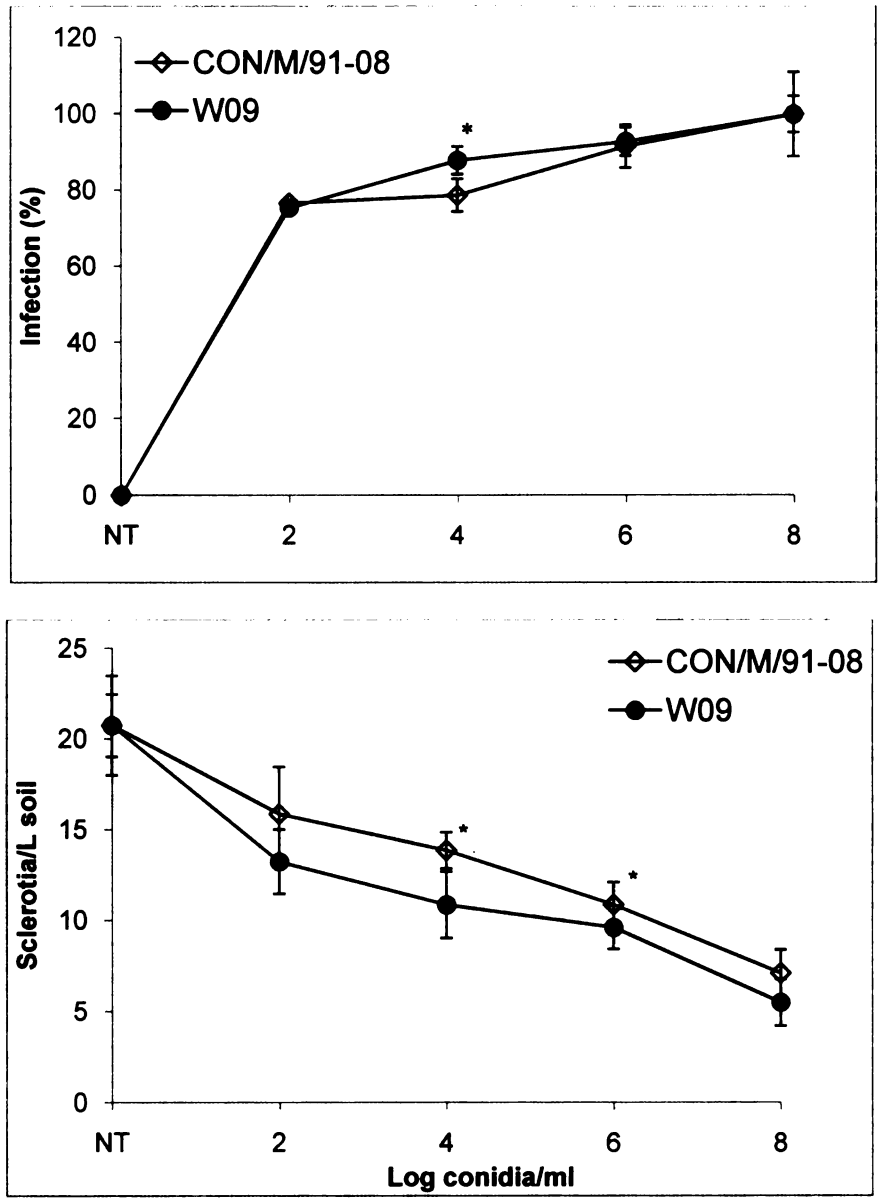

Figure 4-12. Effect of concentration of *C. minitans* isolates W09 and CON/M/91-08 on colonization of sclerotia (upper panel) and sclerotial survival (lower panel) of *Sclerotinia sclerotiorum*. A total of 25 sclerotia were placed in each pot. Conidial concentrations at 0 (NT), 10^2, 10^4, 10^6, 10^8 conidia/ml in a volume of 100 ml were inoculated in each plot with four replications. Sclerotial survival was determined after four weeks. Comparisons using Fisher's least significant difference (LSD) were performed for each week. Growth at each temperature was compared between the two isolates. Bars on each column are standard deviation, and * indicates significant difference between the means of isolates at the same temperature.

DISCUSSION

Coniothyrium minitans has a worldwide distribution (Sandys-Winsch 1993), and has been found in many regions, such as the United States, Australia, Belgium, Canada, Denmark, France, Germany, Israel, Japan, Korea, Netherlands, New Zealand, Portugal, South Africa, Sri Lanka, Sudan, Switzerland and the UK (Campbell 1947; Turner and Tribe 1976; Sandys-Winsch 1993; Jones and Stewart 2000; Budge and Whipps 2001; Ren *et al.* 2010). This report adds Michigan to this list.

Coniothyrium minitans isolates grew at temperatures between 5 and 30°C, with the optimum of 20°C for mycelial growth and conidial production. Similar results were found with European strains in previous studies (Campbell 1947; McQuilken *et al.* 1997). However, the optimal environmental temperature for mycoparasitic activity is higher than that for mycelial growth and conidial production. For example, the temperature for most efficient colonization on sclerotia of *Sclerotinia sclerotiorum* is 30°C (Tu 1999).

This study found that the optimal pH for mycelial growth is 4 to 5, which is also suggested by McQuilken (1997). Light was found to have a negative effect on the mycelial growth of *C. minitans*, which is different to a previous study that found that light has no effect (McQuilken *et al.* 1997). Regardless of this, light is necessary to promote the conidial production. Besides photoperiod, UV radiation and its effects on the survival of the fungus will be important in the future to determine viability in when employed under field conditions.

The colonization frequency of *C. minitans* W09 and CON/M/91-08 on *S. sclerotiorum* in the growth chamber was consistent with results from agar plate tests. Half

of the sclerotia were infected during the first week, indicating a strong mycoparasitic ability of *C. minitans* on sclerotia of *S. sclerotiorum*. After four weeks, sclerotia treated with both W09 and CON/M/91-08 were 100 percent colonized. This is in agreement with Turner and Tribe (1976), indicating that both W09 and CON/M/91-08 are more aggressive than other European isolates tested on sclerotial colonization.

Coniothyrium minitans W09 was similar in growth pattern with CON/M/91-08, but outperformed CON/M/91-08 on mycelial growth and conidial production/plate under most of the experimental conditions, including temperatures, pH and light. W09 had a lower conidia/mm^2 mycelial area than CON/M/91-08. However, since W09 grows faster, its overall conidial production/plate is higher than CON/M/91-08. From this, W09 is more efficient than CON/M/91-08 for large amounts of conidial production. The mycelial growth rate at 20°C for CON/M/91-08 was similar to many European isolates (McQuilken *et al.* 1997). However, W09 had a considerably higher daily growth rate, which doubled the growth rate of CON/M/91-08. At pH 4.5, the mycelial growth rate of W09 is 55.2% higher than CON/M/91-08. In dark conditions, W09 also doubled the average daily growth rate of CON/M/91-08. Therefore, under these artificial conditions W09 has a faster growth than CON/M/91-08.

Coniothyrium minitans W09 has a faster colonization at an early stage than CON/M /91-08. W09 can penetrate into *S. sclerotiorum* sclerotia and establish colonization within 24 h but CON/M/91-08 needs at least 48 h. W09 had significantly higher mycoparasitic activity in the growth chamber at moderate conidial concentrations (10^4 and 10^6 conidia/ml) than CON/M/91-08. This indicates that W09 may be more

efficient until its conidial concentrations reach the plateau. This may reduce the conidial concentration without scarifying the efficacy if it is used as a biocontrol product.

Sclerotinia sclerotiorum produces oxalic acid that decreases the pH in infected tissue and favors cell wall degrading enzymes to macerate tissue, and low pH (around 4.0 to 5.0) was the most favorable condition for colonization (Cessna *et al.* 2000). Interestingly, this range of pH values (4.0 to 5.0) is also favorable for mycelial growth of *C. minitans*. Oxalic acid produced by *S. sclerotiorum* can promote the growth of and be colonized by *C. minitans* (Ren *et al.* 2007). Its outstanding ability to grow in low pH could be one of the reasons that *C. minitans* colonizes sclerotia so successfully.

Care needs to be taken when implementing results from laboratory or growth chamber to the field. Several data showed the subtle differences under tested conditions although statistical significances appear. For example, mycelial growth rate of *C. minitans* W09 had significant difference ranging from pH 7.5 to 8.0. However, the absolute mycelial growth rate was 4.5 mm/day and 4.1 mm/day, respectively. This growth difference is hardly noticeable under field conditions. It is interesting to further evaluate the optimal growth and mycoparasitism of *C. minitans* under field conditions.

In conclusion, commercial strain CON/M/91-08 grows well in responding to various pH values and temperatures, and has a similar colonization ability to *C. minitans* W09, although the latter was more effective in mycelial growth, conidial production, and colonization frequency at certain conidial concentrations overall. This shows the advantage of using endemic isolates for effective disease control if they were developed into a biocontrol product.

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