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Time and burial depth influencing the viability and bacterial colonization of sclerotia of *Sclerotinia sclerotiorum*

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Abstract

Sclerotia are the primary over wintering inoculum of *Sclerotinia sclerotiorum* (Lib.) de Bary. The effects of tillage on the primary inoculum are not well understood. The purpose of this research was to study sclerotial viability over time and between burial depths in soil, to identify bacteria colonizing and degrading the sclerotia, and determine whether these bacteria may be utilized as biological control agents. Correlation analysis indicated that a significant negative relationship existed between sclerotial viability and elapsed temporal factors ($R^2 = -0.68$, $P < 0.0001$), and depth of burial ($R^2 = -0.58$, $P < 0.0001$). After twelve months, sclerotia on the soil surface had the highest viability (57.5%), followed by those at the 5 cm depth (12.5%), and only 2.5% of those placed at the 10 cm depth remained viable. A significant negative relationship between sclerotial viability and bacterial populations also existed ($R^2 = -0.60$, $P < 0.0001$). Two hundred and sixty-eight bacteria were isolated from sclerotia, 29 of which showed strong in vitro antagonism to the mycelial growth of *S. sclerotiorum*. Biodiversity of the inhibitory bacterial isolates was minimal on sclerotia from the soil surface and within all depths sampled at three months (i.e. in January). All burial depths within the April and July sampling dates produced bacterial diversities that were distinct from each other.

Keywords: *Sclerotinia sclerotiorum*; Sclerotia viability; Bacteria colonization; Tillage; Biological control

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a devastating pathogen affecting yield and product quality of a vast number of susceptible hosts. *Sclerotinia sclerotiorum* is one of the most nonspecific and successful of plant pathogens (Purdy, 1979), with a host range of over 400 species, 100 of which are found in Canada (Boland and Hall, 1994). Purdy (1979) reported that *S. sclerotiorum* is the causal agent of more than 60 diseases, including stem rot, stalk rot, head rot, pod rot, and wilt. This broad range of diseases occurs in almost every country of the world from the cool, moist regions to the hot, dry areas (Purdy, 1979), but more often in temperate regions (Reichert, 1958).

Sclerotinia sclerotiorum can cause disease through two distinct mechanisms, either germinating carpogenically to produce airborne ascospores, or myceliogenically to infect roots of hosts such as sunflowers and carrots (Bardin and Huang, 2001). These two modes of action cause infection of plants through a range of tissues, including, sunflower heads (Huang, 1983), canola stems and leaves (Gugel and Morrall, 1986), pea pods (Huang and Kokko, 1992), tubers of Jerusalem artichoke (Laberge and Sackston, 1987), and alfalfa blossoms (Gossen and Platford, 1999). In addition to the physiological mechanisms of disease spread, *S. sclerotiorum* can spread between diseased and healthy plants by direct contact (Huang and Hoes, 1980), by transportation of infected pollen grains (Stelfox et al., 1978), and through infected seed lots (Mueller et al., 1999).

Biological control of *S. sclerotiorum* has received considerable attention over the last few decades because of the ineffectiveness of other management practices including chemical fungicides (Bardin and Huang, 2001). Registered biocontrol products such as Intercept (Prophyta Biologischer Planzenschutz, Malchow, Germany) seem

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effective in decreasing in-field inoculum (Sayler, 2003). The active ingredient in Intercept is *Coniothyrium minitans*, a naturally-occurring fungus that can decrease the germination of sclerotia. Imperative to the effectiveness of Intercept is a light tillage operation following Intercept application. The purpose of the light tillage is to incorporate *C. minitans* into the soil to induce colonization. However, light tillage alone has actually increased the density of sclerotia in the soil surface and increased apothecial formation (Mueller et al., 2002). Mueller et al. (2002) showed that deep plowing decreases sclerotinia infection, apothecial formation, and the density of sclerotia in the soil. The decrease in infection is due mainly to the burial of sclerotia deeper in the soil profile where germination conditions are inappropriate. However, previous studies have reported plowing is not effective in reducing disease caused by sclerotinia stem rot in soybean (Kurle et al., 2001). Similarly, no-till has been suggested as more effective than tillage because no-till soils have higher microbial activity, encouraging sclerotial degradation (Workneh and Yang, 2000). Keeping the sclerotia in the upper soil profile has been shown to increase sclerotial degradation (Cook et al., 1975).

Soil microbial activity is instrumental in the degradation of *S. sclerotiorum* sclerotia. Positive correlation has been found between the colonization of sclerotia (*Sclerotium rolfsii*) with *Trichoderma virens* and a decrease in sclerotial germination (Papavizas and Collins, 1990). Sclerotia of *S. rolfsii* have also been antagonized by *Trichoderma harzianum* hyphae, which colonized the sclerotial surface and actually penetrated the rind (Benhamou and Chet, 1996). Likewise, *Talaromyces flavus* application decreased the germination of microsclerotia of *Verticillium dahliae* from 84% to 17% in only 14 d, in comparison to the control where germination was only reduced to 74% (Fahima et al., 1992). Similarly, an isolate of *Trichoderma hamatum*, TMCS-3, also reduced the viability of *S. sclerotiorum* sclerotia (Gracia-Garza et al., 1997). There has been limited progress in research related to bacterial effects on sclerotia of *S. sclerotiorum*; however, bacterial colonization has been reported to be negatively correlated with *Rhizoctonia solani* sclerotial germination (Gupta et al., 1995). Further information is required on the effects of bacterial colonization of sclerotia of *S. sclerotiorum*, as the majority of previous biocontrol research has concentrated on fungal antagonists (Oedjijono et al., 1993).

The objectives of this study were: (1) to determine the effects of sclerotia placement at different depths within the soil over time, investigating the effect of depth and time on sclerotial germination by omitting any soil disturbance caused by tillage; (2) to establish the relationship between sclerotial germination and bacterial colonization of sclerotia, and determine whether the bacterial populations interrelate with the burial depth and time; (3) to isolate bacterial populations and assess their in vitro inhibition of *S. sclerotiorum* mycelial growth; and (4) to analyze the biodiversity of the inhibitory bacterial populations.

2. Materials and methods

2.1. Sclerotia burial

On October 1, 2001 sclerotia were collected from a sunflower field north of Sanford, Manitoba, Canada. All sclerotia from sunflower basal stalk rot infections were collected from a localized area (20 m^2) within the field. Single uniform-shaped sclerotia averaging 10×6 mm in size, weighing approximately 0.05 g each, were placed in ten separate compartments (5×5 cm) made from nylon window screening (14 mesh).

The experiment was initiated on October 23, 2001, at the Department of Plant Science Field Station, on the University of Manitoba Fort Gary Campus, Winnipeg, Manitoba. The physical and aggregate soil properties for this location consist of 34.5% silt, 50.0% clay, and 15.5% sand, with a pH of 7.7, and an organic matter content of 5.6%. No crop was grown the previous season on the trial location. The mean soil temperature at the sclerotial burial site was 6.4 °C, with a total precipitation of 456.7 mm over the twelve-month experimental period.

The experiment was designed as a repeated measure randomized complete block design, with burial depth representing the main plots and harvest date representing the sub-plots. The trial contained four replications. Sclerotia were placed at three burial depths: on the soil surface (0 cm), 5, and 10 cm. For the 0 cm depth, sclerotia packets were pinned down to the soil so that environmental conditions would not relocate the mesh bags. Sclerotia that were buried at the 5 and 10 cm depths were placed in level excavations and covered with the soil profile that was initially extracted. Within each main plot, four mesh bags were placed, each containing ten separately packaged sclerotia, representing the four sampling dates: January 23, 2002 (three mo), April 23, 2002 (six mo), July 23, 2002 (nine mo), and October 23, 2002 (twelve mo). An initial sampling analysis took place on a representative sample of sclerotia collected directly from the host. On the appropriate sampling date, the mesh bags containing ten separate sclerotia were recovered from the 0, 5, and 10 cm depths for each replication. The mesh bags collected on each sampling date were placed separately in 10 lbs poly plastic bags (Unisource, Winnipeg, MB, Canada) and placed at 4 °C for approximately two weeks until analysis could take place.

2.2. Sclerotial germination

Sclerotia from all sampling periods were analyzed for their percent germination. Percent germination was defined as the number of sclerotia that germinated myceliogenically divided by the total number of sclerotia sampled for each treatment ($n=40$). Each sclerotium was cut in two so that one half of the sclerotium could be tested for viability and the other half stored in 1.5 ml polypropylene micro centrifuge tubes (Fisher Scientific, Ottawa, ON, Canada)

for isolation and identification of colonizing bacteria. The sclerotia halves analyzed for germination were surface sterilized for three min with store brand bleach having an initial concentration of 4.0% NaOCl, diluted in distilled water to a concentration of 1.0% NaOCl. Surface sterilization removed fungal populations colonizing the sclerotia so *S. sclerotiorum* mycelial growth could confidently be identified. Sclerotia were allowed to air dry and plated on potato dextrose agar (PDA, Beckton/Dickinson, Sparks, MD, U.S.A.) until *S. sclerotiorum* mycelial growth was present. *Sclerotinia sclerotiorum* mycelial growth was the indicator of germination. A sclerotium was regarded as non-viable if mycelial growth was not morphologically identified as that of *S. sclerotiorum*. Subsequent sclerotial and apothecial formation were not measured, as viable sclerotia do not consistently form new sclerotia or apothecia (Abawi and Grogan, 1979).

2.3. Bacterial colonization

The sclerotia halves for bacterial analysis were sonicated (Branson Ultrasonic cleaner™, Branson Cleaning Equipment Company, Shelton, Conn, U.S.A.) for 20 s in sterile distilled water. Viable sclerotia were analyzed together, and sclerotia that did not germinate were analyzed individually. Serial dilutions were prepared using standard dilution plating techniques, and plated on half nutrient agar (11.5 g Nutrient Agar and 10.0 g Agar Technical (Becton/Dickinson, Sparks, MA, U.S.A.), amended with Nyastatin (Sigma Chemical CO., St. Louis, MO, U.S.A.). Bacterial colonies were enumerated after 72 h, counting plates with 20–200 colonies and determining the colony forming units (cfu) per ml. The mean colony count per sclerotia is reported ($n=40$).

2.4. In vitro screening of inhibitory bacteria

Visually distinct bacteria were isolated from the dilution plates onto Luria Bertani agar (LBA, 15.0 g agar, technical (Difco Laboratories, Detroit, Mich., U.S.A.), 10.0 g tryptone peptone (Becton/Dickinson, Sparks, MA, U.S.A.) , 5.0 g yeast extract (Sigma Chemical CO., St. Louis, MO., U.S.A.), 5.0 g NaCl and assessed for purity through morphological characteristics, then stored in LB broth amended with 20% glycerol at −80 °C. All isolated bacteria were plated for percent inhibition of *S. sclerotiorum* mycelium growth on 30% Tryptic Soy Broth/70% Potato Dextrose Broth (TSA/PDA), [9 g Tryptic Soy Broth, 16.8 g Potato Dextrose Broth, and 17.0 g Agar Technical] and PDA (Wood, 1951; Fernando and Pierson, 1999; Savchuck, 2002). A loop of bacteria removed from a 24 h sub-culture was placed in LB broth on an incubator shaker at 28 °C for 16 h at 160 rpm. Five micro liters of the bacterial suspension were pipetted onto both TSA/PDA and PDA in 15× 100 mm petri plates at four equidistant points near the periphery of the plate. Bacteria were allowed to grow for 24 h at 21 °C (±2 °C). Mycelial plugs, 5 mm in diameter, were taken from the actively growing margin of *S. sclerotiorum* cultures and placed onto the center of each plate, and incubated for 14 d. *Sclerotinia sclerotiorum* mycelial cultures for in vitro biocontrol assessment were produced from the initial sclerotia collection. Measurements of radial mycelial growth were recorded at 48 h (mycelial growth had reached the circumference of the plate in the control, 80 mm), and after 14 d. The percentage of mycelial inhibition was calculated with the formula 100(R1-R2)/R1 where R1 is the maximum radius of growth (80 mm) and R2 is the radius directly opposite the bacterial cultures (Fernando and Pierson, 1999; Savchuck, 2002). Only the 14 d assessment will be reported in this study, as it best represents the in vitro biological control activity of each bacterium. Each bacterium was replicated three times for the initial plate inhibition assays. Any bacterial isolate that produced greater than 40% mycelial inhibition in the initial screening was repeated in ten replications.

Bacterial isolates that produced over 40% mycelial inhibition were also assessed for volatile production (Fernando and Linderman, 1994). Bacteria were streaked onto one half of a divided plate containing Tryptic Soy Agar (TSA), 30.0 g TSA and 10.0 g Agar Technical (Becton/Dickinson, Sparks, MA, U.S.A.), and then sealed with parafilm® (Fisher Scientific, Nepean, ON, Canada). After 72 h of incubation at room temperature, 5 mm plugs of *S. sclerotiorum* mycelial cultures were placed on the other half of the plate containing PDA, and the plates re-sealed. After 48 h, mycelial growth had reached the furthest circumference of the plate (80 mm) in the control (no bacteria on the TSA half). Measurements of mycelial growth were recorded in a fashion similar to that described above for the mycelial inhibition study. Three replications were used for the initial screening of volatile production, and each bacterium producing volatiles that inhibited mycelial growth was replicated ten times.

2.5. Bacterial identification

All inhibitory bacteria were identified using standard Gram stain techniques followed by the use of the Biolog© Microstation, utilizing the Biolog Microlog™ 3, Version 4.2 software (Microlog, Hayward, CA, U.S.A.). Single colonies were obtained and the following steps were involved in the process of identification: (i) Gram stain (Biolog Inc.) rated as Gram-positive or Gram-negative; (ii) bacteria were streaked onto Biolog universal growth (BUG) agar medium (Biolog Inc.); (iii) approximate bacterial concentration was quantified with a turbidimeter, and 150 µl of the bacterial suspension was pipetted into each of the 96 wells in the Biolog microplates; (iv) the plates were incubated at 32 °C for 16–24 h and scanned with an automated plate reader (Biolog Inc.), assessed visually, and identified to genus or species level. Each inhibitory bacterium isolated was

identified once and identification was repeated if confirmation was necessary.

2.6. Data analysis

Experiments were analyzed using analysis of variance (ANOVA), a mean separation test (Fisher's Least Significant Difference) ($P=0.05$), and coefficient of variance computed with the Analyst procedure of SAS, Version 8.1 (SAS Institute, Cary, NC, U.S.A.). Correlation coefficients using Pearson's Rank Correlation were determined using the Descriptive procedure of SAS. Population biodiversity for the inhibitory bacteria using bacterial identities was compared for each sampling date and depth using the NTSYSpc, Numerical Taxonomy System, Version 2.1 software (Exeter Software, Setauket, NY, U.S.A.). The genetic distance between treatments was determined using the bacterium presence or absence for each sampling date and depth. This genetic distance between treatments was utilized to produce the dendogram using SAHN clustering and a UPGMA clustering method.

3. Results

3.1. Sclerotial germination

The mean germination from the initial sclerotia collection was 80% (i.e. October, 2001). By January (3 mo), viability of sclerotia had increased for all three depths (Fig. 1). For the January sampling period, a significant difference was expressed between the 0 and 5 cm depths compared to the 10 cm depth. All three burial depths were significantly different from each other for the April sampling period. In July (9 mo), sclerotial viability on the soil surface was still greater than the initial viability (80.0%), and did not decrease significantly from the April sampling period. After 12 mo in the field (i.e. October, 2002), sclerotial viability significantly decreased to 57.5, 12.5, and 2.5% for the 0, 5, and 10 cm depths respectively. All germination data was significantly different in October, in comparison to any previous sampling period (Fig. 1). A significant negative relationship existed between sclerotial viability and elapsed temporal factors ($R^2=-0.68$, $P<0.0001$), and burial depth ($R^2=-0.58$, $P<0.0001$) (Table 1).

3.2. Bacterial colonization

No distinction between burial depths was made for the initial sclerotia collection when comparing bacterial populations. The initial bacterial population was 3.98 log10 (cfu/ml/sclerotia) (Fig. 2). In January, the bacterial populations on sclerotia at the soil surface significantly decreased to 2.7 log10 (cfu/ml/sclerotia). In April, all bacterial populations significantly increased in comparison to samples from the same depth from the previous sampling period. The July sampling period produced bacterial populations within the same depth not significantly greater than the April sampling period. Bacterial populations at

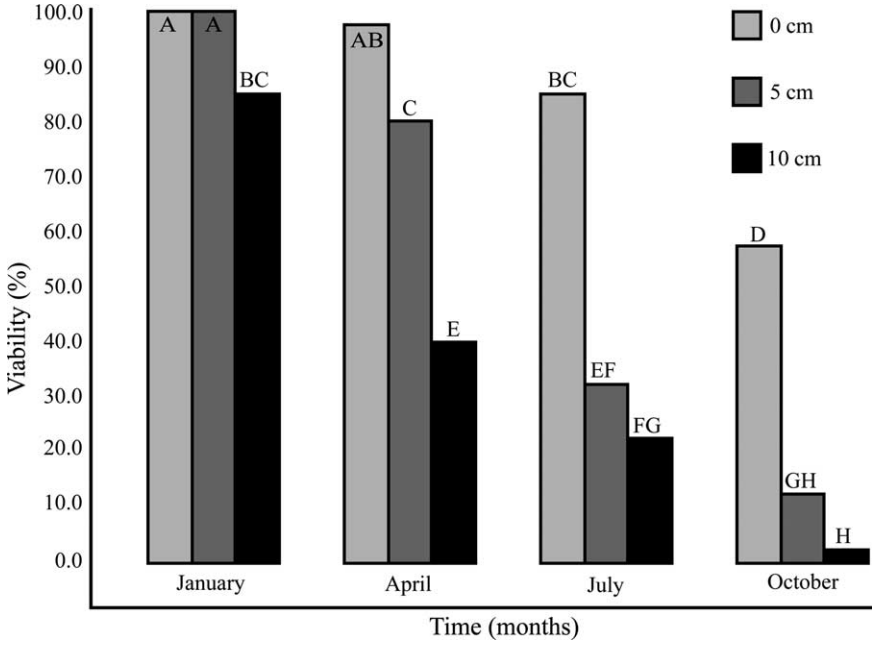

Fig. 1. Viability of *S. sclerotiorum* sclerotia buried at 0, 5, and 10 cm depths, sampled in January, April, July, and October 2002 from Winnipeg, Manitoba, Canada. Initial viability for the sclerotia collection was 80.0% (October 2001). Each bar represents the mean sclerotial viability of four replications with ten sclerotia analyzed per replication. No significant difference between replications was produced ($P=0.90$). Letters denote significance for Fisher's LSD value of 14.3 ($P=0.05$).

Table 1
Correlation coefficients of Pearson's Rank Correlation for sclerotial germination and bacterial colonization levels from a sclerotial burial study at Winnipeg, Manitoba, Canada, from 2001 to 2002

Variable	Variable	
	Sclerotial germination	Bacteria colonization levels
Time	−0.68	0.56
Depth	−0.58	0.49
Bacteria Colonization Levels	−0.60	–

All correlation coefficients shown are significant at $P<0.0001$.

5 and 10 cm were not significantly different from each other but were significantly higher than the surface sclerotia bacterial populations. A reverse trend in bacterial populations occurred for the October analysis. The surface sclerotia had the greatest bacterial populations, followed by the 5 and 10 cm depths; however, the bacterial populations were not significantly different among the three depths. Bacterial populations were significantly correlated with time ($R^2=0.56$, $P<0.0001$) and depth ($R^2=0.49$, $P<0.0001$) (Table 1).

3.3. Bacterial isolation

Over the one-year sampling period, 268 morphologically different bacterial isolates were collected. Twenty-nine isolates were inhibitory in vitro to mycelial growth of *S. sclerotiorum* (Table 2). Of those 29 isolates, there were only 15 different bacterial species. *Bacillus* spp. outnumbered the rest of the genera (>50%). Different isolates of the same species often produced differing mycelial growth inhibition results. *Bacillus amyloliquefaciens* strain 2033 caused the greatest in vitro inhibition on TSA/PDA, reducing *S. sclerotiorum* mycelial growth by 77.3%. This was significantly greater inhibition than all other bacterial isolates except *B. amyloliquefaciens* strain 268, which produced 73.8% mycelial inhibition on TSA/PDA. Four out of the top seven inhibitory bacteria on TSA/PDA were *B. amyloliquefaciens* isolates. Other bacteria that were effective on TSA/PDA were *Staphylococcus sciuri* strain 3055, *Bacillus licheniformis* strain 266, and *Mannheimia haemolytica* strain 230, which reduced mycelial growth by 72.6, 72.5, and 71.9%, respectively. Sixteen isolates on TSA/PDA caused greater in vitro inhibition than the overall mean of all 29 bacteria.

Inhibition tests on PDA produced similar results, with isolates of *B. amyloliquefaciens* again producing four of the top seven in vitro inhibition results. The most inhibitory bacterium on PDA was *B. licheniformis* strain 223, which caused 88.5% inhibition, significantly greater than all other isolates. However, on TSA/PDA, strain 223 caused only 53.3% mycelial inhibition. Sixteen isolates were more inhibitory in reducing mycelial germination than the overall mean of all 29 isolates on PDA; however, some of the 16 inhibitory isolates differed from the 16 isolates effective on TSA/PDA. *Kocuria rosea* strain 41, *B. cereus/thuringiensis* strain 54, and *B. amyloliquefaciens* strain 4078 produced

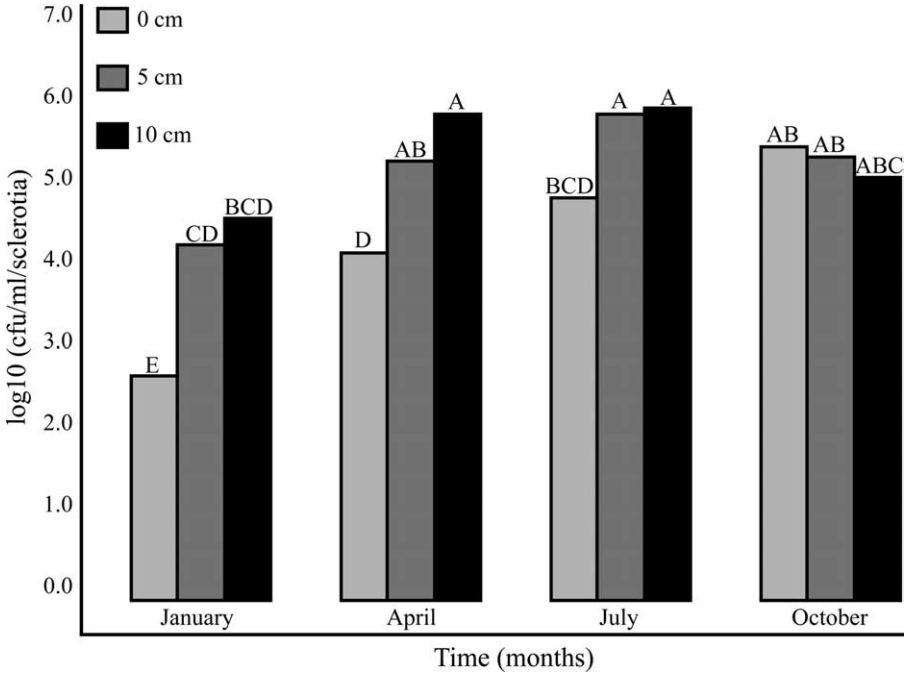

Fig. 2. Bacterial populations colonizing sclerotia buried at 0, 5, and 10 cm, sampled in January, April, July, and October 2002 from Winnipeg, Manitoba, Canada. Initial bacterial populations (in October 2001) for the sclerotia collection were 3.98 log10 (cfu/ml/sclerotia). Each bar represents the mean log10 colony forming units (cfu)/ml/sclerotia of four replications with ten sclerotia analyzed per replication. A significant difference between replications was produced ($P=0.02$) with a coefficient of variance of 12.79. Letters denote significance for Fisher's LSD value of 0.8454 ($P=0.05$).

Table 2
Twenty-nine inhibitory bacteria to *Sclerotinia sclerotiorum* isolated from sunflower, 0, 5, and 10 cm depths, during the initial sclerotial collection (October 2001) and in January, April, July, and October of 2002 in Winnipeg, Manitoba, Canada

				Inhibition (%)		Volatile Inhibition (%)
Strain	Location (cm)	Sampling Date	Identity	TSA/PDA	PDA	TSA/PDA
73	Host	Initial collection	*Hafnia alvei*	59.4	52.6	0.0
54	5	Jan	*Bacillus cereus/thuringiensis*	46.6	0.0	0.0
29	10	Jan	*Bacillus amyloliquefaciens*	62.1	54.9	0.0
41	10	Jan	*Kocuria rosea*	42.9	2.8	0.0
67	10	Jan	*Staphylococcus lentus*	62.5	72.0	0.0
207	0	Apr	*Brevibacterium otitidis*	52.5	71.4	0.0
240	0	Apr	*Bacillus subtilis*	68.4	78.1	0.0
2031	0	Apr	*Bacillus subtilis*	66.2	66.5	0.0
2033	0	Apr	*Bacillus amyloliquefaciens*	77.3	78.5	0.0
223	5	Apr	*Bacillus licheniformis*	53.3	88.5	0.0
248	5	Apr	*Bacillus amyloliquefaciens*	72.4	79.5	0.0
2056	5	Apr	*Pseudomonas corrugata*	50.0	56.9	28.6
226	10	Apr	*Bacillus amyloliquefaciens*	54.4	67.6	0.0
230	10	Apr	*Mannheimia haemolytica*	71.9	73.9	0.0
265	10	Apr	*Bacillus amyloliquefaciens*	71.7	82.6	0.0
266	10	Apr	*Bacillus licheniformis*	72.5	74.1	0.0
268	10	Apr	*Bacillus amyloliquefaciens*	73.8	76.4	0.0
2090	10	Apr	*Bacillus subtilis*	65.0	70.6	0.0
3055	10	Jul	*Staphylococcus sciuri*	72.6	76.3	0.0
3057	10	Jul	*Bacillus subtilis*	65.6	78.2	0.0
3060	10	Jul	*Staphylococcus sciuri*	50.4	63.6	0.0
3073	10	Jul	*Pseudomonas corrugata*	56.0	49.8	35.8
3008	10[a]	Jul	*Pseudomonas fluorescens*	31.9	47.3	17.8
3020	10[a]	Jul	*Macrococcus equipercicus*	56.9	55.9	0.0
3039	10[a]	Jul	*Bacillus licheniformis*	16.3	56.0	0.0
3045	10[a]	Jul	*Staphylococcus aureus ss aureus*	10.2	37.6	0.0
4076	10	Oct	*Bacillus licheniformis*	50.0	57.7	0.0
4078	10	Oct	*Bacillus amyloliquefaciens*	65.1	0.0	0.0
4079	10	Oct	*Bacillus mycoides*	47.6	55.5	43.2
			Mean (10)	56.8	59.5	4.3
			LSD $P=0.05$	4.5	3.3	3.7

[a] Similar morphological strains also found at 0 cm for strains 3008 and 3020, and at 5 cm for 3020, 3039, and 3045.

extremely low inhibition of *S. sclerotiorum* mycelial growth on PDA, but were inhibitory on TSA/PDA media. Twenty-two of the 29 bacteria were more inhibitory at reducing *S. sclerotiorum* mycelial growth on PDA than on TSA/PDA.

Only four of the 29 bacterial isolates caused reduced mycelial growth when tested for volatile production. All four bacteria produced *S. sclerotiorum* mycelial growth inhibition that was significantly different from each other isolate. Volatiles produced by *Bacillus mycoides* strain 4079 caused the strongest inhibition (43.2%), followed by *Pseudomonas corrugata* strain 3073 (35.8%), *P. corrugata* strain 2056 (28.6%), and *P. fluorescens* strain 3008 (17.8%). Volatiles of *Pseudomonas* spp. were more inhibitory that those of *Bacillus* spp. (Table 2).

3.4. Inhibitory bacterial population diversity

The sclerotia burial treatments were compared by analyzing the presence or absence of the inhibitory bacteria in each treatment. The inhibitory bacteria isolated from the initial sclerotia collection were similar to inhibitory bacteria isolated from the 0, 5, and 10 cm depths in January, the 0 cm depth in July, and the 0 and 5 cm depths in October (Fig. 3). This similarity is due to the fact that few inhibitory bacteria were isolated from these treatments. The inhibitory bacterial populations isolated from 10 cm in July, 5 cm in April, 10 cm in April, and 10 cm in October were all distinct from all other bacterial populations isolated from other sampling treatments (Fig. 3). Inhibitory bacteria isolated from the surface in April, and from 5 cm in July, were similar to each other; however, these two treatments produced inhibitory populations that were distinct from all other treatments.

Inhibitory populations isolated from the initial collection and the January sampling date were all similar, regardless of the sampling depth. Only the 0 cm inhibitory populations isolated in April were distinct from all other 0 cm depth isolations. All inhibitory populations sampled within April and July were distinguished by sampling depth (Fig. 3).

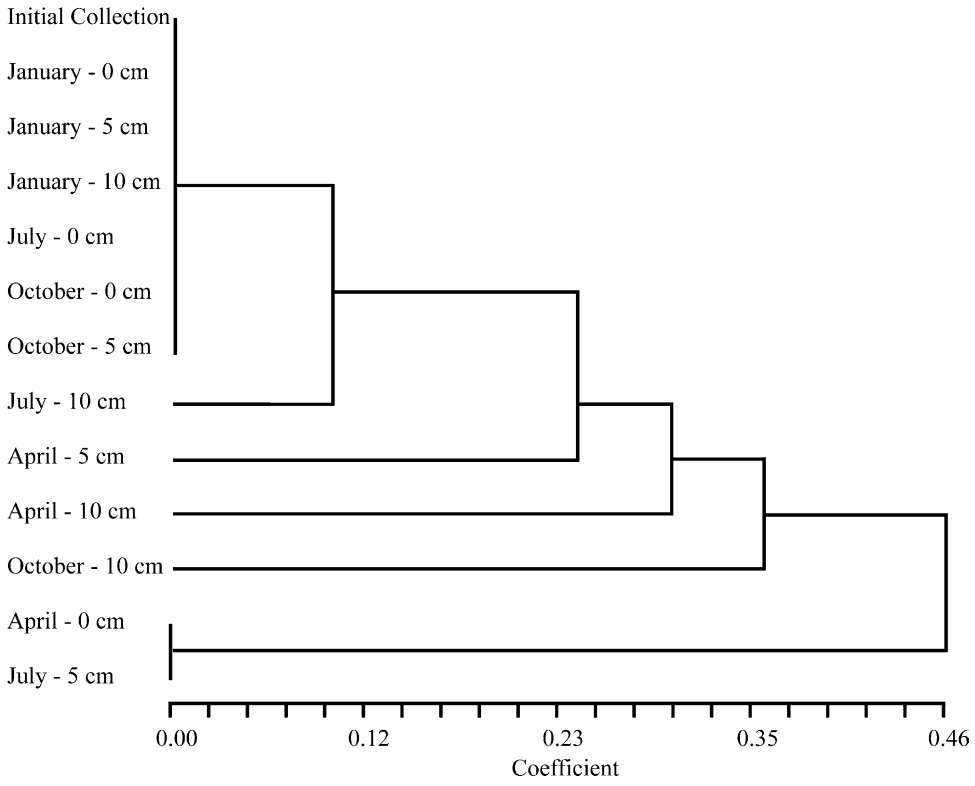

Fig. 3. Inhibitory bacterial population diversity analysis for the 0, 5, and 10 cm depths, sampled initially (October 2001), in January, April, July, and October 2002 from Winnipeg, Manitoba, Canada. Each isolation depth and time composed a treatment and the 13 treatments were compared by the presence of the bacterial identities isolated from within each treatment.

4. Discussion

Sclerotial viability was the lowest for all sampling dates at the 10 cm depth, followed by the 5 cm depth, while the sclerotia that were placed on the soil surface had the highest viability for all sampling treatments. Sclerotial germination increased early in the winter when sclerotia were exposed to seasonal temperatures, similar to results found in Beltsville, MD (Adams, 1975). As the temperatures begin to increase in the spring, along with the activity of soil microorganisms, sclerotial germination begins to decrease, just as mycelial viability decreases when temperatures increase (Huang and Kozub, 1993). Merriman (1976) reported that viability of sclerotia remaining on the soil surface is the least affected, perhaps due to the low bacterial colonization. Huang and Kozub (1993) reported similar results when examining the survival of *S. sclerotiorum* mycelium, and stated that buried mycelia have low viability, possibly because of the microorganisms in the soil. However, Cook et al. (1975) stated that sclerotia remaining in the upper soil profile degrade rapidly in comparison to sclerotia deeper in the soil profile. Imolehin and Grogan (1980b) recovered *S. minor* sclerotia from 0, 5, 10, and 20 cm, finding similar results in regard to depth, but no viable sclerotia were found in the soil after 3 mo. Kurle et al. (2001) also indicated that sclerotia viability decreased with increasing depth under chisel plow and no-tillage cultivation systems. Kurle et al. (2001) stated that sclerotia within the upper 5 cm soil profile will carpogenically germinate. Depths greater than 5 cm would not be conducive to carpogenic germination.

The sclerotial viability results were negatively correlated with the bacterial populations colonizing the sclerotia ($R^2 = -0.60$, $P < 0.0001$). The 10 cm depth produced the highest bacterial populations, while the lowest sclerotial viability was also found at these sampling depths. Sclerotia placed on the soil surface consistently had the lowest bacterial colonization levels and greatest viability for each sampling date, probably due to periodic drying (Kurle et al., 2001). Only in October did bacterial populations change, when sclerotia on the soil surface had the greatest bacterial colonization, followed by the 5 and 10 cm depths. This inversion in bacterial colonization is due to partially- or completely-degraded sclerotia at the 5 and 10 cm depths. Sclerotial remnants were even difficult to locate in the mesh bag compartments at the depths of 5 and 10 cm. Kurle et al. (2001) indicated that germination was reduced at 10–20 cm, but conceded that these results were produced by a low number of sclerotia found at this depth. Results from the present study suggest that the reason a low number of sclerotia were found at this depth is not due to the fact that less sclerotia were located in this depth, but due to the high level of sclerotial degradation influenced by fungi, soil characteristics, sclerotial characteristics, and bacterial populations. Fungal populations (Hoes and Huang, 1975;

Imolehin and Grogan, 1980b) actinomycetes, and fauna all will undoubtedly interact with the bacterial community and sclerotia themselves to impact sclerotial degradation over time and between depths. Soil characteristics that affect the viability of sclerotia at different depths over time are soil type and pH (Merriman, 1976), tillage (Kurle et al., 2001), moisture (Moore, 1949; Hao et al., 2003), humidity (Huang and Kozub, 1993), temperature (Workneh and Yang, 2000), and gases (Imolehin and Grogan, 1980a). While sclerotia survival is also affected by sclerotia size and shape (Hoes and Huang, 1975; Hao et al., 2003) and sclerotia source (Merriman, 1976). Nonetheless, it is important to recognize the influence that the present bacteria community has contributed to sclerotial degradation in this experiment ($R^2 = -0.60$, $P < 0.0001$). Whether this bacterial contribution to sclerotial degradation is a result of a single antagonistic bacteria or the communication of the bacterial community colonizing the sclerotia is yet to be determined. These inhibitory bacterial populations need to be assessed individually for biocontrol activity under controlled conditions to determine the extent of their activity in the soil. The in vitro assay is simply an identification tool to characterize the biocontrol potential of candidate bacteria, and their true influence on sclerotial viability cannot be realized until their biocontrol activity is assessed in a natural soil environment. The sclerotial bacterial community may even include non-culturable bacteria and the influence of these species on sclerotia survival is unknown, though probable because of their 'contribution to ecosystem functioning' (Kennedy, 1999).

Kurle et al. (2001) suggests that reduced sclerotia viability in chisel plow and moldboard plow tillage may be due to increased parasitism. In contrast to these findings, Gracia-Garza et al. (2002) reported that no-till may increase microbial activity, thus increasing degradation of sclerotia. No-tillage has been shown to impact physical and chemical soil factors, affecting microorganisms that decompose organic material (Lafond and Derksen, 1996), supporting the principle that tillage affecting sclerotial location will impact sclerotial viability.

Out of 268 bacteria, twenty-nine provided positive inhibition to *S. sclerotiorum* mycelial growth. Of the 29 inhibitory bacteria, 24 were Gram-positive, 17 of which were spore-forming bacteria. This is in contrast to bacterial populations found in the rhizosphere and phylloshere of a canola crop where they were heavily colonized and dominated by *Pseudomonas* spp. (Savchuck, 2002). The morphological ability of these spore-forming bacteria and the previous industrial uses of these species supports their potential biocontrol success (Emmert and Handelsman, 1999). Fifteen distinct bacterial species were present within the 29 isolates, with *Bacillus* spp. the most effective at inhibiting *S. sclerotiorum* mycelial growth. The *Bacillus* spp. identified has potential for commercialization, as *B. thuringiensis* (Bt) comprises 90% of the bio-insecticides market (Emmert and Handelsman, 1999). Strains of *B. amyloliquefaciens*, *B. licheniformis*, and *B. subtilis* all provided over 70% inhibition on either TSA/PDA or PDA. *Bacillus amyloliquefaciens* has been reported to be effective on *Botrytis cinerea* in tomato (Mari et al., 1996) and against anthracnose (*Colletotrichum dematium*) on mulberry leaves (Yoshida et al., 2001). Likewise, *B. licheniformis* has been effective against *Pyrenophora tritici-repentis* in wheat (Mehdizadegan and Gough, 1987), and *Pyrenophora teres* of barley (Scharen and Bryan, 1981). *Bacillus subtilis* was reported to be effective against *Fusarium*, *Rhizoctonia*, and *Sclerotinia* pathogens, as well as stimulating plant growth (Turner and Backman, 1991; Kondoh et al., 2000; Estevez et al., 2002). *Bacillus mycoides* also provided positive inhibition of *S. sclerotiorum* mycelial growth, mostly due to volatile production in the present study. Similarly, *B. mycoides* was effective against *Botrytis cinerea* on strawberry leaves, and volatile production was also detected (Guetsky et al., 2002). *Pseudomonas chlororaphis*, *P. corrugata*, *P. aurantiaca* and *P. fluorescens* strains isolated from the canola and soybean rhizosphere produced organic volatiles such as nonanal, cyclohexanol, benzothiazole, 2-ethyl, 1-hexanol, *n*-decanal and dimethyl trisulfide that were inhibitory to the mycelial growth, and sclerotia and ascospore germination of *S. sclerotiorum* (Fernando et al., 2005).

The bacteria with the greatest biocontrol potential were isolated in April from sclerotia that were in the soil or on the soil surface. Effective biocontrol bacteria on PDA were also isolated from sclerotia sampled in July. Thirteen of the 29 inhibitory bacteria were isolated from the April sampling date, followed by eight bacteria from the July sampling date. Sclerotia sampled in January and October had few inhibitory bacteria colonizing the sclerotia, with six of the seven bacteria from these two sampling dates isolated from the 10 cm depth. Twenty of the 29 inhibitory bacteria were isolated from the 10 cm depth, which had the highest bacterial populations for all sampling dates, except in October, when sclerotia were heavily degraded. Only seven inhibitory bacteria were isolated from the 5 cm depth, followed closely by six bacteria from the surface. Only one inhibitory bacterium was directly isolated from sclerotia retrieved from sunflower (initial sclerotia collection). Plants influence the biodiversity of bacteria in soils (Dunfield and Germida, 2001) due to the release of amino acids, sugars and root exudates (Rovira, 1956a), impacting the species of bacteria present at depths within the rhizosphere (Rovira, 1956b). Root exudates affecting the bacterial biodiversity in this way may explain the bacterial biodiversity between sclerotial depths within the soil.

Inhibitory bacterial population biodiversity was evident between different treatments in this study. Bacterial populations were similar for all 0 cm depths excluding the April analysis. This exception at 0 cm was most likely due to the spring conditions of the sampling period, causing greater bacterial populations. The January and October sampling dates were similar, due to no isolation of

inhibitory bacteria from either of these dates. All 10 cm sampling dates were dissimilar from each other, as over 66% of the bacteria were isolated from the 10 cm depth. Distinction in time was also clear when comparing the biodiversity of inhibitory bacteria. All depths within the April sampling date as well as the July sampling date contained inhibitory bacterial populations that were distinct.

5. Conclusions

This is the first study to compare inhibitory bacteria populations between depths, over time, and their effect on sclerotial germination. The results in this study indicate that sclerotial longevity is negatively correlated with time of burial and depth within the soil. Sclerotial longevity is often over-estimated, but sclerotia can remain viable if located on the soil surface. Reducing the initial inoculum level is one strategy of managing *S. sclerotiorum*, and it is suggested that bacterial populations will influence the sclerotia degradation rate. Soil cover of sclerotia, promoting bacterial colonization, will increase the sclerotial degradation rate. Tillage needs to be manipulated so subsequent soil disruption does not simply recover previously buried sclerotia to the soil surface. With well characterized information on sclerotial germination and bacterial colonization, tillage practices can be properly implemented and integrated with bacterial biocontrol agents to manage *S. sclerotiorum*.

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