Anaerobic Soil Disinfestation Reduces Viability of *Sclerotinia sclerotiorum* and *S. minor* Sclerotia and Root-Knot Nematodes in Muck Soils

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ABSTRACT

Experiments were conducted to evaluate potential functional and mechanistic differences in the suppression of *Sclerotinia sclerotiorum* and *S. minor* and root-knot nematodes in muck soils by anaerobic soil disinfestation (ASD) using different carbon source amendments. Volatile compounds produced during ASD in muck soil amended with molasses, wheat bran, or mustard greens at 20.2 Mg/ha or a 2% ethanol solution significantly reduced the mycelial growth and number of sclerotia produced by both *Sclerotinia* spp. compared with the anaerobic control. In amended soils, acetic and butyric acids were detected in concentrations that reduced the viability of sclerotia of both pathogens. Higher concentrations of carbon dioxide were observed in ASD-treated soils, regardless of the amendment, than in the nonamended anaerobic control. Only amendment with wheat bran did not increase the production of

methane gas during ASD compared with the controls. *Meloidogyne hapla* survival was completely suppressed in soils treated with ASD regardless of carbon source. Field trials were conducted in Ohio muck soil to assess survival of sclerotia of both *Sclerotinia* spp. The viability of sclerotia of both *Sclerotinia* spp. was significantly reduced in soil subjected to ASD amended with wheat bran (20.2 Mg/ha), molasses (10.1 Mg/ha), or wheat bran (20.2 Mg/ha) plus molasses (10.1 Mg/ha) compared with the controls. A consistent negative correlation between soil reduction and viability of sclerotia of both pathogens was observed. Wheat bran and molasses are both widely available amendments that can be used as ASD carbon sources for the management of soilborne pathogens in muck soils.

Keywords: biological control, mycology, nematology

Leafy green vegetables, including several types of noniceberg lettuce, are produced primarily on muck soils in Ohio. Muck soils contain 20 to 80% organic matter, compared with mineral soils that generally contain less than 5% organic matter in this growing region (Gohil et al. 2014). Crop production on muck soils is intensive, with multiple crops produced in a single season leading to rapid increases in pathogen populations. Lettuce is susceptible to various soilborne pathogens that cause damage and reduce yields. The two causal agents of lettuce drop, *Sclerotinia minor* and *S. sclerotiorum*, constitute a major problem and lead to the death of the plants (Hayes et al. 2010). These pathogens produce sclerotia that can survive in the field for more than 5 years, serving as primary inoculum for new epidemics (Wu and Subbarao 2006). Another soilborne pathogen that limits lettuce production is the root-knot nematode, *Meloidogyne* spp. In northern Ohio, *M. hapla* is most commonly encountered; in southern Ohio, *M. incognita* has been found (Testen and Miller

2018). Members of the genus *Meloidogyne* are key nematode pests that cause major economic losses in the cultivation of vegetables (Koenning et al. 1999). Both aforementioned soilborne fungi cause monocyclic diseases, whereas root-knot nematodes cause polycyclic disease. Consequently, management that focuses on the reduction of primary inoculum (sclerotia and nematode eggs) will have an impact in decreasing the intensity of epidemics caused by these pathogens (Kora et al. 2003; Kratochvil et al. 2004).

Research on generating sustainable alternatives for soil disinfestation has been very active since the legal use of methyl bromide was curtailed (Ristaino and Thomas 1997). Researchers in the Netherlands (Blok et al. 2000) and Japan (Momma et al. 2013) simultaneously developed a soil disinfestation technique known as anaerobic soil disinfestation (ASD) that combines soil amendment with a labile carbon source together with irrigation that brings water up to saturation, followed by covering with plastic mulch for a period of 2 to 15 weeks to generate anaerobic conditions in the soil (Blok et al. 2000; Momma et al. 2010).

Changes in microbial activity during ASD lead to the development of anaerobic conditions with associated increases in the production of organic acids and gases (hydrogen sulfide [H₂S], methane [CH₄], carbon dioxide [CO₂], nitrous oxide, and ammonia) that can be deleterious to soilborne pathogens (Runia et al. 2014). Acetic acid and butyric acid are recognized as toxic to soilborne pathogens in certain concentrations based on previous ASD studies (Katase et al. 2009; Momma et al. 2006). Different carbon sources may induce the production of different types and quantities of these compounds during ASD and consequently lead to fluctuations in effectiveness (Hewavitharana et al. 2014) and differential sensitivity of soilborne pathogens to ASD (Testen and Miller 2018). Furthermore, during the anaerobic process, it is likely that gases like CH₄ and CO₂ are produced in different quantities depending on the carbon source used (Arriaga et al. 2011; Ludeking et al. 2011).

Many investigations still focus on optimizing ASD to control different pathogens across different environmental conditions and

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on determining the key mechanism of action (Shennan et al. 2010). In the United States, ASD has been implemented in fruit and vegetable production fields in the states of Florida (Butler et al. 2012), Tennessee (McCarty et al. 2014), Washington (Mazzola and Hewavitharana 2014), and California (Achmon et al. 2016; Browne et al. 2018). In Ohio, research and implementation of this technique began in 2015 with field applications beginning soon after (Testen and Miller 2018).

The first objective of this study was to identify the carbon sources most effective in reducing the viability of *S. sclerotiorum* and *S. minor* sclerotia and of *M. hapla* during ASD in Ohio muck soils. A second objective was to identify and quantify organic acids and gases produced during ASD with different carbon source amendments and their effects on mycelial growth and viability of sclerotia of both *Sclerotinia* species. Various ASD carbon sources were evaluated under growth chamber conditions to assess impacts on soil reducing conditions, gas and organic acid production, sclerotia viability, and nematode survival. The most promising ASD carbon sources were assessed in field trials in muck soil.

MATERIALS AND METHODS

Inoculum production. *S. minor* isolate *SM16-10* (isolated from lettuce plants grown in muck soil in Stark County, Ohio) and *S. sclerotiorum* isolate *SM15-08* (isolated from tomato plants in Stark County, Ohio) were used in growth chamber and field trials in Ohio. Sclerotia were produced using the protocol of Matheron and Porchas (2005) with some minor modifications. Potato tubers were peeled and sliced, placed into glass Petri plates, and autoclaved at 121°C. Agar plugs from the margins of actively growing colonies were placed on potato slices in each Petri plate, followed by incubation at room temperature for 4 weeks. Sclerotia of each isolate were separated from the potato pieces, dried, and stored at room temperature until further use.

M. hapla inoculum was extracted from tomato roots with galling symptoms from Erie County, Ohio. Eggs of *M. hapla* were produced through successive inoculations of tomato 'Moneymaker' (Everwilde Farms, Bloomer, WI), which were grown in 10.2-cm-diameter pots in Baccto Grower's Mix (Michigan Peat Co., Houston, TX). Immediately prior to experiment establishment, nematode eggs were extracted from the roots of these plants using the methodology described by Coolen and D'Herde (1972).

Soil. Muck soil was collected to a depth of 15 cm from the Ohio Agricultural Research and Development Center (OARDC) Muck Crops Agricultural Research Station in Huron County, Ohio (N 41° 0' 36.414'' W 82° 44' 2.8608'') from fields with a history of leafy vegetable production but not lettuce drop or root-knot. Soil was homogenized using a cement mixer, sealed in buckets, and stored at 4°C until use in the experiments. Soil bulk density was assumed to be 0.3 g/cm³ (Zobeck et al. 2013), and moisture was determined to be 12.5 ± 5%.

Culture media. Half-strength potato dextrose agar (PDA; IBI Scientific, Dubuque, IA) amended with rifampicin (150 ppm) and bromophenol blue (1A ppm) was used to assess the viability of sclerotia. This neon-blue PDA allows for detection of *Sclerotinia* sclerotia viability as the medium changes in color from blue to yellow as a consequence of oxalic acid production during sclerotial germination. For experiments evaluating the effects of ASD-generated volatile compounds, full-strength PDA amended with 75 ppm of lactic acid (APDA) was used.

Effects of different carbon sources on production of volatile compounds inhibitory to *S. minor* and *S. sclerotiorum*. Molasses, wheat bran, mustard greens, and ethanol were evaluated for production of volatile compounds during ASD that reduce mycelial growth and sclerotia production of *Sclerotinia* spp. in vitro. Mustard greens (*Brassica juncea* 'Southern Giant Curled', Thiram treated seed; Seedway, Hall, NY) were grown in plastic trays containing Baccto Grower's Mix. Stems and leaves of 8-week-old mustard greens plants were chopped and macerated using a Waring blender

(model 33BL79; Dynamics Corp., New Hartford, CT). Experiments were conducted in 90-mm-diameter glass Petri dishes. Carbon sources were mixed with 65 g of muck soil in the following concentrations: molasses, 10.1 Mg/ha; wheat bran and mustard greens, 20.2 Mg/ha; and ethanol, 2% solution, 0.30 ml per g of soil. Molasses, ethanol, and mustard greens were applied with water (20 ml per dish), while wheat bran was applied first and then water was added. Amended and control soils were placed in the bottom of the Petri dishes, saturated, and covered with another 90-mm Petri dish bottom containing 20 ml of APDA medium and an inoculum plug from a 3-day-old colony of *S. sclerotiorum* or *S. minor*. The two facing Petri dishes were sealed with two layers of Parafilm M (Bemis Company, Neenah, WI) and electrical tape (TradeGear, Radcliff, KY) and placed inside individual polyethylene bags (17.7 × 18.8 cm, Ziploc; S.C. Johnson & Son, Racine, WI) to prevent gas exchange with the external environment. Anaerobic controls were not amended but were saturated, covered, and sealed as described above. Aerobic control soils were not amended, but they were saturated and maintained unsealed and placed inside open bags to allow air exchange. Petri dishes were incubated in an unlit growth chamber at 30°C for 4 weeks. Mycelial growth was assessed by measuring colony area (in square centimeters) using the free image processing software ImageJ (Schneider et al. 2012) and the number of sclerotia was counted. Experiments were conducted using a completely randomized design with 10 replicates of six treatments (four carbon sources and two controls). The experiment was performed twice for each species.

Effects of carbon source amendment on production of organic acids and gases, soil reducing conditions, and pathogen suppression during ASD. An experiment was designed to identify inhibitory compounds produced during ASD using different carbon sources and to compare the relative effects of each treatment on survival of *S. sclerotiorum*, *S. minor*, and *M. hapla*. Treatments were the following: ethanol (2% solution), molasses (10.1 Mg/ha), and wheat bran and mustard greens (20.2 Mg/ha), with five replicates of each treatment. Control treatments were not amended but were saturated and either covered (anaerobic control) or maintained uncovered (aerobic control). Experiments were organized in a completely randomized design, and trials were conducted twice.

Fresh stems and leaves of 8-week-old mustard greens, 'Southern Giant Curled', were prepared as previously described. Molasses, ethanol, and mustard greens were applied to 650 g of muck soil together with water (195 ml per 1-liter Erlenmeyer flask), while wheat bran was applied first and then water was added. Soils were immediately thoroughly mixed manually with a sterile stir rod. Two mesh packets, each containing 16 sclerotia of *S. minor* or *S. sclerotiorum*, were placed in the bottom of a flask together with a vertically positioned 15-cm-long, 2-cm-diameter Indicator of Reduction in Soils (IRIS) tube (Rabenhorst 2010). Each flask was filled with the soil previously mixed with one of the carbon sources. For *M. hapla*, a suspension containing 1,000 eggs was dispensed into soil in each flask and mixed thoroughly. Flasks were sealed with rubber stoppers to prevent gas exchange with the environment. Aerobic control flasks were saturated with water but not amended, inoculated or not inoculated, and maintained unsealed. Anaerobic control flasks were similarly prepared but sealed. Gases generated during ASD were collected using 5-liter Tedlar gas sampling bags (Restek, Lancaster, PA) connected to rubber stoppers by a short length of tubing. Gases were not collected for the aerobic control. Flasks were incubated in a growth chamber in the dark for 4 weeks at 30°C, then 50-ml samples were taken from each gas sampling bag, and gas composition was determined using gas chromatography (GC), with an HP 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a ValcoPlot VP-Alumina column (Valco Instruments, Houston, TX) and an isothermally operated (40°C) thermal conductivity detector. The carrier gas was helium at a flow rate of 5.2 ml/min. The temperature for the injector was

150°C, while the detector temperature was 200°C (Wan et al. 2011). CH₄ and CO₂ content was measured and expressed in terms of percentage per volume.

Once ASD was completed, a 40-g soil sample was collected from each flask, mixed with 60 ml of distilled water in 250-ml centrifuge bottles, and shaken on an orbital shaker for 15 min, followed by centrifugation at 3,000 × g for 5 min. The supernatant was filtered through 0.2-µm filter paper (Mansion Labs, Austin, TX) attached to a syringe and the filtrate was added to 2-ml glass vials (Restek, Lancaster, PA). The remainder of this soil suspension was used to determine the pH using a Thermo Orion pH meter (model 410; Thermo Scientific Orion, Beverly, MA). The organic acid concentrations in the soil supernatant were analyzed with a GC-2014 gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) and an automatic injector and sampler arm. The temperature for the column was 150°C, while the temperature for the FID was 250°C. The carrier gas used was helium at a flow rate of 20 ml/min (Lin et al. 2014). Acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids were enumerated and values were expressed in milligrams per kilogram of soil solution.

IRIS tubes were removed from the soil, rinsed in tap water, and allowed to dry. The percentage of paint loss, an indicator of soil anaerobicity/reducing conditions, was assessed using the following formula:

$$\text{IRIS tube paint removal(\%)} = 100\% - ((\text{PA} - \text{PR}) / \text{PA} \times 100)$$

in which PA is painted area and PR is the area where paint was removed.

Mesh bags containing sclerotia were recovered following treatment and sclerotia were washed with tap water and dried overnight. Sclerotia were then surface disinfested for 60 s with a solution of 70% ethanol, followed by 60 s in 5% sodium hypochlorite and three 60-s rinses in sterile distilled water. Disinfested sclerotia were then placed on neon-blue PDA medium and incubated at room temperature until all sclerotia in the aerobic control germinated.

ASD-treated and control soils (325 g) were each placed in two 10.2-cm round plastic pots (Pöppelmann Teku, Claremont, NC) per experimental unit and maintained at rest for 2 weeks to allow any phytotoxic compounds to dissipate. Two-week-old butterhead lettuce seedlings ‘Mirlo’ (Johnny’s Selected Seeds, Winslow, ME), previously sown in plastic trays containing Baccto Grower’s Mix, were transplanted into each pot (one seedling per pot). The plants were maintained in the greenhouse for 40 days at 28 ± 7°C with a 12-h photoperiod. The roots of these plants were then washed with tap water, and nematode eggs were extracted using the methodology described by Coolen and D’Herde (1972). Fresh root weight, number of galls, and number of eggs per gram of root were evaluated.

Effect of organic acids on sclerotia of *S. minor* and *S. sclerotiorum*. In vitro experiments were performed to examine the effect of pure acetic and butyric acids on the viability of *S. sclerotiorum* and *S. minor* sclerotia. Acetic acid (1,572 mg/kg of solution; Mallinckrodt, St. Louis, MO) and butyric acid (2,555 mg/kg of solution; Alfa Aesar, Ward Hill, MA) were tested individually and combined (acetic [1,572 mg/kg of solution] plus butyric [2,555 mg/kg of solution]) using concentrations (EC₉₅) previously reported to inhibit sclerotia germination (Tenuta et al. 2002). Ten sclerotia of each pathogen were placed inside Falcon tubes (Falcon Plastics, Oxnard, CA) containing pure acetic, butyric, or mixed acetic plus butyric acids in distilled water (pH 5.5). Controls were placed in distilled water. Falcon tubes plus sclerotia were incubated in a controlled environment at 30°C for 2 weeks. For each treatment, five repetitions were laid out in a completely randomized design, and the trial was performed twice for each isolate. Sclerotia were removed, rinsed, and plated on neon-blue PDA medium to assess germination as described above.

Effect of ASD carbon source on viability of *S. minor* and *S. sclerotiorum* sclerotia under field conditions. Experiments were conducted at the OARDC Muck Crops Agricultural Research Station in Huron County, Ohio, during the months of July and September 2017 to assess the effect of different carbon source amendments during ASD on the viability of *S. sclerotiorum* and *S. minor* sclerotia under field conditions. ASD amendments were wheat bran (20.2 Mg/ha), molasses (10.1 Mg/ha), and wheat bran (20.2 Mg/ha) plus molasses (10.1 Mg/ha). Controls were non-amended, uncovered, with saturated soil (aerobic control) and nonamended, covered, with saturated soil (anaerobic control). The first trial was arranged in a Latin-square design with five treatments with plots 0.91 m × 3 m with 3-m buffers within rows and 0.91-m buffers between rows (Testen and Miller 2019). A tractor-mounted rototiller was used to incorporate the carbon source 15 cm into the soil. The second trial was a Latin-square design with five treatments with plots of 1 m × 1 m with a buffer zone of 1 m between the rows. Carbon sources were incorporated into the soil by hand to a depth of 15 cm. In both trials, soils were saturated with water using overhead irrigation and plots were then covered with black plastic mulch, sealing the edges with excess soil to trap gases produced during ASD. The ASD treatments lasted 4 weeks.

Mesh packets containing 16 sclerotia of *S. minor* and 10 sclerotia of *S. sclerotiorum* were buried in the soil in each plot 6 cm deep prior to soil saturation. Soil temperatures were monitored using Hobo pendant data loggers (Onset Computer, Bourne, MA). IRIS tubes (30-cm long) were inserted into guide holes prepared using 2-cm-diameter PVC pipes before covering the plots with plastic mulch (Castenson and Rabenhorst 2006). IRIS tubes were removed immediately after the plastic mulch was removed and paint loss percentage was quantified as described above.

Sclerotia in the mesh packets were recovered and surface disinfested as described previously and plated on neon-blue PDA medium to assess their viability, which was expressed as the percentage of the 16 sclerotia of *S. minor* or 10 sclerotia of *S. sclerotiorum* that germinated.

Statistical analyses. All statistical analyses were carried out in R version 3.4.4 (R Core Team 2019) using statistical packages *agricolae* (de Mendiburu 2015) and *epiR* (Stevenson et al. 2015). Differences between treatment variances in each trial were checked using Levene’s test. When nonsignificant differences were found, data for both trials were combined for further analysis. Only data from ASD-produced volatile compounds analysis and field trials were analyzed and presented separately for each trial. Analysis of variance was performed for the data obtained and treatment means were compared using Tukey’s test with a family-wise error rate of 5%. For all studies, normality of distributions was checked before analysis and data were transformed using the arcsine square root if appropriate. Nontransformed means and standard deviations are reported. Principal component analysis (PCA) was performed using the viability of *S. sclerotiorum* and *S. minor* sclerotia and *M. hapla* survival (root-knot nematode) data obtained from the growth chamber trials. Results from PCA were displayed as a biplot to illustrate the correlation of pathogen viability and the predictor variables: soil solution (pH), soil reduction (IRIS paint removal), concentration of organic acids in soil solution (acetic, propionic, isobutyric, butyric, isovaleric), and total concentration of organic acids (total acids) after ASD treatment.

RESULTS

Effects of different carbon sources on production of volatile compounds inhibitory to *S. minor* and *S. sclerotiorum*. Significant reductions in colony growth were observed in mycelial cultures of both *Sclerotinia* spp. incubated over ASD-treated soil. During trial 1, no significant differences in colony area of *S. sclerotiorum* between the aerobic and anaerobic control were observed (Table 1). ASD with any of the carbon source amendments significantly reduced

colony area compared with both controls (60.0 to 55.2 cm²); the wheat bran-based ASD treatment was the most suppressive (3.2 cm²) ($P < 0.0001$). However, colony growth in the anaerobic control (26.3 cm²) in trial 2 was significantly lower than in the aerobic control (31.0 cm²). ASD reduced colony growth compared with that of the aerobic control regardless of carbon source. ASD treatments with mustard greens (25.0 cm²) and ethanol (24.0 cm²) did not significantly reduce colony diameter compared with the anaerobic control. The least colony growth was observed in wheat bran- and molasses-based ASD treatments (13.2 to 17.4 cm²). No differences in colony growth of *S. minor* between the aerobic (16.9 cm²) and anaerobic control (25.1 cm²) and mustard greens-based ASD treatment (17.4 cm²) were observed during trial 1, the most suppressive amendments being wheat bran (4.7 cm²), molasses (2.6 cm²), and ethanol (6.1 cm²). During trial 2, colony area of *S. minor* in the anaerobic control (4.2 cm²) was significantly lower than in the aerobic control (7.1 cm²). However, colony growth in the anaerobic control was similar to growth in the ethanol-based (4.5 cm²) or mustard greens-based (4.1 cm²) ASD treatments. ASD with any of the carbon sources led to a significant reduction of mycelial area compared with aerobic control, with the most effective being wheat bran (2.2 cm²) and molasses (2.4 cm²).

The number of sclerotia produced by *S. sclerotiorum* ($P < 0.0001$) and *S. minor* ($P < 0.0001$) was significantly reduced when mycelial cultures were incubated above soils treated with ASD regardless of the carbon source, compared with the anaerobic and aerobic controls, with the exception of mustard greens-based ASD treatment during trial 1 (Table 1). The number of sclerotia in the anaerobic control was significantly reduced compared with the aerobic control for *S. sclerotiorum* but not for *S. minor*.

Identification and quantification of organic acids and gases produced during ASD with different carbon source amendments. The pH values of aerobic (pH 6.1) and anaerobic

(pH 6.4) control soils were statistically similar in this experiment. A significant ($P < 0.0001$) decrease in soil pH was observed following ASD treatment with molasses (pH 5.2) or wheat bran (pH 5.6) as a carbon source compared with both nonamended controls, while the pH of ethanol-amended soil (pH 5.9) was significantly lower than that of the anaerobic control soil only (Table 2). The pH of soil treated with ASD using mustard greens as a carbon source (pH 6.6) was similar to that of the anaerobic control but significantly higher than the pH of the aerobic control.

The organic acids produced in highest concentrations were acetic acid (up to 1,442.1 mg/kg of soil solution) and butyric acid (up to 2,729.4 mg/kg of soil solution), whereas the least abundant organic acid over the detection limit was isovaleric acid (up to 152.2 mg/kg of soil solution) (Table 2). The highest concentration of propionic acid was 333.5 mg/kg of soil solution, whereas isobutyric acid was 308.1 mg/kg of soil solution. The concentrations of all six organic acids were statistically similar between anaerobic and aerobic controls. Soil treated with ASD using molasses accumulated the highest concentration of butyric acid among all treatments (2,729.4 mg/kg of soil solution), while ASD/wheat bran-treated soils had the second highest concentration (1,795.5 mg/kg of soil solution). The butyric acid concentration was significantly lower in ethanol-amended ASD-treated soils (708.1 mg/kg of soil solution) compared with wheat bran and molasses. Soil treated with ASD using molasses (1,340.1 mg/kg of soil solution) did not have significantly different concentrations of acetic acid than ASD-treated wheat bran-amended soils (1,442.1 mg/kg of soil solution) ($P < 0.0001$). The total organic acid concentration was significantly higher in ASD-treated soils than in anaerobic and aerobic control soils ($P < 0.0001$). Mustard greens-amended soils had the lowest total organic acids concentration (1,283.7 mg/kg of soil solution) among the amended, ASD-treated soils, whereas the

TABLE 1. Effect of volatile compounds produced during anaerobic soil disinfestation (ASD) conducted with different carbon source amendments on colony growth and sclerotia formation of *Sclerotinia sclerotiorum* and *S. minor*^a

ASD carbon source ^z	Colony area (cm ²)				Sclerotia (n)			
	<i>S. sclerotiorum</i>		<i>S. minor</i>		<i>S. sclerotiorum</i>		<i>S. minor</i>	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Aerobic control	60.0 C	31.0 c	16.9 B	7.1 c	17.0 C	13.0 b	135.0 B	113.0 b
Anaerobic control	55.2 C	26.3 b	25.1 B	4.2 b	8.0 B	4.0 a	128.0 B	111.0 b
Ethanol	9.2 B	24.0 b	6.1 A	4.5 b	0.0 A	0.0 a	0.0 A	0.0 a
Molasses	18.6 B	17.4 a	2.6 A	2.4 a	0.0 A	0.0 a	0.0 A	0.0 a
Mustard greens	26.1 B	25.0 b	17.4 B	4.1 b	2.0 AB	0.0 a	96.0 B	0.0 a
Wheat bran	3.2 A	13.2 a	4.7 A	2.2 a	0.0 A	0.0 a	0.0 A	0.0 a
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

^a Values in a column indicated by different letters are significantly different at $P < 0.05$ according to Tukey's test. Values are means with 10 replications. Uppercase letters are used to compare the means of trial 1, and lowercase letters are used to compare means of trial 2.

^z Aerobic control = nonamended saturated control, anaerobic control = nonamended saturated plastic covered control, ethanol = ethanol 2% solution amended, molasses = molasses amended (10.1 Mg/ha), mustard greens = mustard greens amended (20.2 Mg/ha), and wheat bran = wheat bran amended (20.2 Mg/ha).

TABLE 2. Soil pH and concentration of organic acids and gases produced during anaerobic soil disinfestation (ASD) using different carbon sources in growth chamber trials^a

ASD carbon source ^z	pH	Concentration of organic acids (mg/kg of soil solution)						Concentration of gases (% in vol)	
		Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Total acids	Carbon dioxide	Methane
Aerobic control	6.1 CD	560.3 A	24.4 A	0.0 A	79.7 A	0.0 A	764.4 A	—	—
Anaerobic control	6.4 DE	487.7 A	0.0 A	12.2 AB	256.4 A	15.6 A	771.8 A	2.7 A	1.3 A
Mustard greens	6.6 E	682.6 A	122.1 B	59.9 B	419.2 AB	0.0 A	1,283.7 B	31.5 BC	39.7 B
Ethanol	5.9 C	1,046.9 B	224.5 C	135.3 C	708.1 B	30.3 A	2,162.1 C	44.8 C	32.3 B
Wheat bran	5.6 B	1,442.1 C	296.4 CD	252.9 D	1,795.5 C	152.2 B	3,994.1 D	27.1 B	9.0 A
Molasses	5.2 A	1,340.1 C	333.5 D	308.1 E	2,729.4 D	62.8 A	4,718.6 E	27.3 B	31.3 B
P value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

^a Values in a column indicated by different letters are significantly different at $P < 0.05$ according to Tukey's test. Values are means with five replications. Data represent combined results of two trials.

^z Aerobic control = nonamended saturated control, anaerobic control = nonamended saturated plastic covered control, ethanol = ethanol 2% solution amended, molasses = molasses amended (10.1 Mg/ha), mustard greens = mustard greens amended (20.2 Mg/ha), and wheat bran = wheat bran amended (20.2 Mg/ha).

highest concentration of organic acids was observed in soils treated with molasses (4,718.6 mg/kg of soil solution). Significantly lower total organic acid concentrations were observed in ASD-treated soils amended with wheat bran (3,994.1 mg/kg of soil solution) or ethanol (2,162.1 mg/kg of soil solution) compared with molasses-amended soils.

The percentage of CO₂ among gases recovered from ASD-treated soils was significantly ($P < 0.0001$) higher than from the anaerobic control soil, regardless of carbon source (Table 2). CO₂ concentrations from ASD-treated soils amended with wheat bran (27.1%) or molasses (27.3%) were significantly lower than CO₂ concentrations from ASD-treated soils amended with ethanol (44.8%) as the carbon source. CO₂ concentrations from soils amended with mustard greens and subjected to ASD were highly variable across replications.

CH₄ production was significantly higher in soils treated with ASD after amendment with mustard greens, ethanol, or molasses than in soils amended with wheat bran prior to ASD or not amended (anaerobic control) (Table 2). ASD with wheat bran as the carbon source did not increase CH₄ production compared with the anaerobic control.

Effects of different ASD carbon source amendments on soil reducing conditions. The percentage of iron oxide paint lost from IRIS tubes was significantly higher in soils amended with any of the carbon sources tested and subjected to ASD compared with either aerobic or anaerobic control soils ($P < 0.0001$; Fig. 1A). Iron oxide paint loss was >40% higher in soils treated with ASD regardless of the carbon source used than in the anaerobic control. Reducing conditions were higher in the anaerobic control than in the aerobic control.

Effects of different carbon source amendments on soilborne pathogen survival and plant health. The viability of *S. sclerotiorum* sclerotia when treated with ASD in growth chamber trials, regardless of the carbon source used, was significantly reduced compared with viability of sclerotia in anaerobic and aerobic control soils ($P < 0.0001$; Fig. 1B). Sclerotia viability was high and did not differ significantly between aerobic (96%) and anaerobic (91%) control soils. Sclerotia viability was lowest in soils amended with molasses (10%) or wheat bran (10.2%) prior to ASD. Viability of sclerotia in soils amended with ethanol (34.2%) and mustard greens (43.8%) was similar but higher than that of soils amended with molasses or wheat bran and subjected to ASD.

For *S. minor*, sclerotia viability was significantly reduced in the anaerobic control (75% viable) compared with the aerobic control (98.7% viable) (Fig. 1B). Sclerotia viability in all amended, ASD-treated soils was significantly lower than in both control soils. Significantly fewer sclerotia were viable from soils amended with ethanol (23.8%), molasses (6.3%), or wheat bran (8%) than in mustard greens-based ASD-treated soils (45%).

No significant differences were observed for fresh root weight of lettuce plants ($P = 0.7093$, Table 3), while root-knot nematode galling differed significantly between plants produced in amended, ASD-treated soils and nonamended aerobic and anaerobic control soils. No galls were observed on roots of plants transplanted into any of the amended, ASD-treated soils. Plants grown in anaerobic control soils had significantly fewer galls than plants grown in aerobic control soils. Similarly, the number of eggs per gram of roots was significantly higher for lettuce planted in aerobic control soils compared with plants grown in anaerobic control soils ($P < 0.0001$). No eggs of *M. hapla* were found following extractions from plant roots grown in any amended, ASD-treated soils ($P < 0.0001$).

PCA of soilborne pathogen viability after ASD. The viability of *S. sclerotiorum* and *S. minor* sclerotia was negatively correlated with the concentration of organic acids in soil solution ranging from $r = -0.69$ to -0.84 ($P < 0.0001$), whereas isovaleric acid was less strongly negatively correlated ($r = -0.43$, $P = 0.0007$ for *S. minor* and $r = -0.46$, $P = 0.0002$ for *S. sclerotiorum*) (Fig. 2).

Similarly, *M. hapla* survival was also negatively correlated with the concentration of organic acids in soil solution ranging from $r = -0.50$ to -0.61 ($P < 0.0001$) with isovaleric acid concentration weakly correlated ($r = -0.32$, $P = 0.0114$). Soil solution pH was positively correlated with sclerotia viability ($r = 0.57$, $P < 0.0001$) but weakly correlated to *M. hapla* survival ($r = 0.29$, $P = 0.0224$). IRIS paint removal was strongly negatively correlated with sclerotia viability ($r = -0.80$, $P < 0.0001$ for *S. minor* and $r = -0.78$, $P < 0.0001$ for *S. sclerotiorum*) and to *M. hapla* survival ($r = -0.85$, $P < 0.0001$).

The first principal component (PC1) accounted for 69.6% of the variance, whereas the second component (PC2) accounted for 11.5% of the variance; together both accounted for 81.1% of the variance during the growth chamber experiments (Fig. 2).

Effect of organic acids on *S. minor* and *S. sclerotiorum* sclerotia in vitro. When sclerotia of *S. minor* and *S. sclerotiorum* were exposed to acetic acid (1,572 mg/kg of solution) or butyric acid (2,555 mg/kg of solution) in concentrations similar to those produced during ASD in soils amended with wheat bran or molasses (Table 2), a significant reduction of sclerotia viability was observed

Fig. 1. Effect of anaerobic soil disinfestation treatment using different carbon sources during growth chamber trials. **A**, Percentage of iron oxide paint removed from Indicator of Reduction in Soils (IRIS) tubes. Higher values for paint removal indicate higher anaerobicity and reducing conditions in the soil. **B**, Viability of *Sclerotinia sclerotiorum* and *S. minor* sclerotia. Error bars indicate standard deviation of the mean of five replicates, and bars indicated by different letters are significantly different at $P < 0.05$ according to Tukey's test. Data represent combined results of two trials. A = aerobic control (nonamended saturated noncovered), AN = anaerobic control (nonamended saturated covered control), E = ethanol 2% amended, M = molasses amended (10.1 Mg/ha), MG = mustard greens amended (20.2 Mg/ha), and W = wheat bran amended (20.2 Mg/ha).

compared with sclerotia exposed to water ($P < 0.0001$; Fig. 3). Fewer sclerotia of *S. sclerotiorum* and *S. minor* remained viable after exposure to acetic and butyric acids in combination than those exposed to acetic acid alone. The percentage of viable sclerotia of either pathogen exposed to butyric acid alone did not differ significantly from the percent viability of sclerotia exposed to both acids combined. Butyric acid reduced sclerotial viability significantly more than acetic acid for *S. sclerotiorum* but not for *S. minor*.

Effect of ASD carbon source on survival of *S. minor* and *S. sclerotiorum* sclerotia under field conditions. Mean soil temperatures were lower in the second field trial compared with the first field trial (Table 4). Soil reducing conditions indicated by iron oxide paint removal from IRIS tubes were significantly higher in soils amended with molasses or wheat bran alone or in combination and subjected to ASD (Table 4) than in aerobic and anaerobic controls in both trials. The effects of the blocking factors on iron

TABLE 3. Root-knot nematode (*Meloidogyne hapla*) survival and root health of lettuce grown in anaerobic soil disinfestation (ASD)-treated and nontreated control soils^y

ASD carbon source ^z	<i>M. hapla</i>		
	Root (g)	Galls	Eggs/g of roots
Aerobic control	11.2	139.0 C	7,413.0 C
Anaerobic control	11.3	71.0 B	4,485.0 B
Wheat bran	9.8	0.0 A	0.0 A
Mustard	11.0	0.0 A	0.0 A
Ethanol	10.9	0.0 A	0.0 A
Molasses	12.1	0.0 A	0.0 A
<i>P</i> value	0.7093	<0.0001	<0.0001

^y Values in a column indicated by different letters are significantly different at $P < 0.05$ according to Tukey's test. Values are means with five replications. Data represent combined results of two trials.

^z Aerobic control = nonamended saturated control, anaerobic control = nonamended saturated plastic covered control, ethanol = ethanol 2% solution amended, molasses = molasses amended (10.1 Mg/ha), mustard greens = mustard greens amended (20.2 Mg/ha), and wheat bran = wheat bran amended (20.2 Mg/ha).

Fig. 2. Biplot from principal component analysis of the viability of sclerotia of *Sclerotinia sclerotiorum* and *S. minor* and root-knot nematode (RKN) survival considering soil solution (pH), soil reduction (IRIS paint removal), concentration of organic acids in soil solution (acetic, propionic, isobutyric, butyric, isovaleric), and total concentration of organic acids (total acids) after anaerobic soil disinfestation treatment. The x-axis represents projections of the first principal component (PC1) and the y-axis the second component (PC2); the two account for 81.1% of the variance during the growth chamber experiments. IRIS = Indicator of Reduction in Soils.

oxide paint removal were not significant for either trial. No differences in the percentage of paint removed were observed between aerobic and anaerobic controls, with only approximately 1% of paint lost from the tubes. In contrast, iron oxide paint loss was >60% when soils were treated with ASD using molasses plus wheat bran. No significant differences in iron oxide paint loss were observed among ASD carbon sources ($P < 0.0001$).

Viability of *S. sclerotiorum* sclerotia was significantly reduced when packets were placed in plots amended with any carbon source and subjected to ASD compared with sclerotia placed in anaerobic control soils in both trials. However, in trial 2, sclerotia viability from aerobic control soils was relatively low (80%) and not significantly different from viability of sclerotia buried in amended, ASD-treated soils (Table 4). No significant effect of the blocking factors was observed for either trial. No significant differences were observed among ASD treatments with any of the carbon sources in sclerotia viability during both trials. Sclerotia viability ranged from 14 to 56.3% in soils amended with molasses, 16 to 60% wheat bran, or 2 to 61.3% molasses plus wheat bran in trial 1 and trial 2, respectively.

Viability of *S. minor* sclerotia was significantly lower in soils treated with ASD, regardless of the carbon source, compared with

Fig. 3. Effect of organic acids produced during anaerobic soil disinfestation on sclerotia viability. **A**, *Sclerotinia sclerotiorum*. **B**, *S. minor*. Error bars indicate the standard deviation of the mean of five replicates. Bars indicated by different letters are significantly different at $P < 0.05$ according to Tukey's test. Data represent combined results of two trials. A = acetic acid (1,572 mg/kg of solution), A+B = acetic (1,572 mg/kg of solution) plus butyric acid (2,555 mg/kg of soil solution), B = butyric acid (2,555 mg/kg of solution), and W = sterile distilled water.

the aerobic control in both trials (Table 4). Differences in sclerotia viability were not significant between the aerobic and anaerobic controls. Only in trial 1, sclerotia viability in soils amended with wheat bran and subjected to ASD was not significantly different from that of the anaerobic control. There were no significant differences in the viability of *S. minor* sclerotia in soils amended with any of the three carbon sources and subjected to ASD.

A significant negative correlation between sclerotia viability and soil reduction indicated by iron oxide paint removal from IRIS tubes in ASD-treated soils was observed for *S. minor* ($r = -0.75$, $P < 0.0001$) and *S. sclerotiorum* ($r = -0.81$, $P < 0.0001$). Coefficients of determination (R^2) were lower than 0.70 for both pathogens.

DISCUSSION

Experiments were conducted to evaluate potential functional and mechanistic differences in ASD using different carbon source amendments in the suppression of *Sclerotinia* spp. and root-knot nematodes in muck soils. Root-knot nematodes were highly sensitive to ASD, regardless of carbon source; both root galling and egg recovery was reduced to zero in lettuce planted in ASD-treated soils. Overall, ASD using either molasses or wheat bran was consistently effective in reducing the viability of *Sclerotinia* spp. sclerotia in growth chamber assays and field trials. Although ASD utilizing ethanol and mustard greens as carbon sources also reduced sclerotia survival, these carbon sources were not always as effective in ASD as molasses and wheat bran in growth chamber trials. These results support the findings of Testen and Miller (2018), which indicate differential sensitivity of soilborne pathogens to carbon sources in ASD. Ethanol has been reported as a highly effective ASD carbon source for reducing a broad spectrum of soilborne pathogens (Momma et al. 2010), but results were variable in our study against *Sclerotinia* spp. This carbon source is also difficult to obtain, and legal regulations may limit its use in ASD as a soil amendment. ASD using mustard greens as a carbon source was significantly less effective in reducing survival of sclerotia than using wheat bran or molasses amendments. It is likely that this inconsistency was attributable to the higher variability in the composition of this carbon source, probably owing to phenological differences in the mustard greens used in this study (Warmington and Clarkson 2016). Such phenological differences in nutrient composition of cover crops may limit their usefulness as ASD carbon sources.

Different mechanisms are thought to be responsible for pathogen suppression during ASD, such as changes in microbial communities (Huang et al. 2016), reduction of oxygen availability (Runia et al. 2014), and production of organic acids (Katase et al. 2009). In our study, the production of volatile fatty acids during ASD led to

deleterious effects on both mycelial growth and sclerotia production of *S. minor* and *S. sclerotiorum*. During ASD, microorganisms have been shown to produce toxic byproducts (e.g., acetic or butyric acids) (Momma et al. 2006). Similar to our observations during in vitro trials, Hewavitharana et al. (2014) reported that volatile compounds emitted during ASD treatment significantly inhibited mycelial growth of the fungal pathogens *Rhizoctonia solani*, *Fusarium oxysporum*, and *Pythium ultimum*. The production of “odorous” compounds during ASD inhibited mycelial growth of *F. oxysporum* f. sp. *lycopersici* (Momma 2008). The same author reported that these “odorous scents” appeared to be the consequence of volatile organic acid production by anaerobic microorganisms that metabolized the carbon sources provided during the ASD process. Moreover, Warmington and Clarkson (2016) indicated that during in vitro experiments, mycelial growth and carpogenic germination of sclerotia of *S. sclerotiorum* were directly inhibited by the biofumigant volatiles produced by mustard varieties.

Organic acids are commonly produced by anaerobic microorganisms as a result of soluble substrate hydrolyzation (Horiuchi et al. 2002) and are thought to be a key mechanism of ASD (Katase et al. 2009). Use of different ASD carbon sources led to differential production of organic acids. Acetic (maximum 1,442 mg/kg of soil solution) and butyric (maximum 2,729 mg/kg of soil solution) acids were the most abundant among the organic acids detected in this study. Other organic acids, including propionic, isobutyric, and isovaleric acids, were present but in lower concentrations. Previous research reported that when wheat bran was used as a carbon source for ASD, concentrations of acetic and butyric acid of up to 2,000 and 1,900 mg/kg of dry soil, respectively, were observed. Moreover, when *Ralstonia solanacearum* and *F. oxysporum* f. sp. *lycopersici* were exposed to these concentrations of these organic acids, their survival significantly decreased (Momma et al. 2006). In our study, the carbon sources that induced the highest production of organic acids were molasses and wheat bran followed by ethanol. The concentrations required to decrease the viability of microsclerotia of *Verticillium dahliae* by 95% (EC₉₅) were 1,572 and 2,555 mg/liter of solution of acetic and butyric acids, respectively (Tenuta et al. 2002). In our work, similar concentrations were detected in ASD-treated soils amended with wheat bran or molasses.

Several authors reported that the toxicity of organic acids to pathogens is a pH-dependent phenomenon (Conn et al. 2005; Katase et al. 2009; Momma et al. 2006; Tenuta et al. 2002). Only nonionized forms of organic acids suppressed the viability of microsclerotia of *V. dahliae* in solutions with acid pH (Tenuta et al. 2002). In this study, a consistent decrease in soil pH after ASD was observed in soils amended with molasses or wheat bran, whereas the pH did not decrease significantly in soils amended with ethanol and mustard greens compared with the aerobic or anaerobic control.

TABLE 4. Effect of anaerobic soil disinfestation (ASD) on soil temperature, reduction in soil, and sclerotia viability of *Sclerotinia sclerotiorum* and *S. minor* during trials in the Muck Crops Agricultural Research Station, Huron County, Ohio^v

ASD carbon source ^w	Soil temperatures (°C)		IRIS paint removed (%) ^x		Sclerotia viability (%)			
					<i>S. sclerotiorum</i>		<i>S. minor</i>	
	Trial 1 ^y	Trial 2	Trial 1 ^y	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Aerobic control	23.6	20.4	0.2 A	0.0 a	96.0 B	80.0 ab	83.3 C	88.8 b
Anaerobic control	24.6	20.0	0.9 A	0.0 a	96.0 B	97.5 b	63.7 BC	86.3 b
Wheat bran	ND ^z	20.2	65.3 B	26.0 b	16.0 A	60.0 a	21.7 AB	51.3 a
Molasses	25.1	20.2	43.6 B	17.3 b	14.0 A	56.3 a	16.7 A	52.2 a
Wheat bran plus molasses	25.3	20.4	57.6 B	23.5 b	2.0 A	61.3 a	13.3 A	43.8 a
P value	—	—	<0.0001	<0.0001	<0.0001	0.0014	0.0002	0.0002

^v Values in a column indicated by different letters are significantly different at $P < 0.05$ according to Tukey's test. Values are means with five replications. Uppercase letters are used to compare the means of trial 1, and lowercase letters are used to compare means of trial 2.

^w Aerobic control = nonamended saturated control, anaerobic control = nonamended saturated plastic covered control, ethanol = ethanol 2% solution amended, molasses = molasses amended (10.1 Mg/ha), mustard = mustard greens amended (20.2 Mg/ha), and wheat bran = wheat bran amended (20.2 Mg/ha).

^x Higher values for paint removal indicate higher anaerobicity and reducing conditions in the soil.

^y Values for soil temperature and Indicator of Reduction in Soil (IRIS) paint removed from trial 1 were published in Testen and Miller (2019).

^z ND = not determined as a result of probe failure.

Momma et al. (2006) also observed a decrease in soil pH to 5.5 when soil was treated with ASD using wheat bran as a carbon source. The change in the pH of soils treated with ASD depends on the carbon source used. For example, the pH of soils amended with ethanol can be higher than that of nonamended soils (Hewavitharana et al. 2014), although we did not observe this in our study. In our experiments, sclerotia viability was lower in soils following ASD with wheat bran or molasses than with mustard greens (both *Sclerotinia* spp.) or ethanol (*S. sclerotiorum* only). ASD with wheat bran or molasses resulted in not only the highest concentration of organic acids produced but also the lowest pH among the ASD-treated soils. A possible mechanism by which organic fatty acids are toxic to pathogens is that nonionized forms of these compounds can penetrate cell membranes (Conn et al. 2005) and interfere with the cellular uptake of other molecules. If *Sclerotinia* sclerotia are affected in this manner, these sclerotia would not germinate or produce oxalic acid.

Gases such as CO₂ and CH₄ are also produced during ASD (Runia et al. 2014) and were evaluated in this study. The concentration of both gases was significantly higher for ASD-treated soils than for the anaerobic control soils. However, high variability in the production of these gases was observed across trials. The production of these gases during anaerobic conditions is considered an indicator of the microbial utilization of the carbon sources (Achmon et al. 2016). Biogas usually consists of 55% CH₄ and 35% CO₂, with H₂S and water vapors making up the remainder (Ryckebosch et al. 2011; Wu et al. 2010). Similarly, when soils are subjected to anaerobic conditions (commonly in flooded soils), production of these gases is observed (Pezeshki 2001; Wang et al. 1993). The production of biogas is also observed during anaerobic digestion of organic residues under controlled conditions (Jang et al. 2014; Lehtomäki et al. 2007) and specific methanogenic bacteria are inoculated in the reactors in order to obtain a homogenous production of CH₄ (Harada et al. 1994). ASD can be considered a technique that combines flooding with anaerobic digestion, with effectiveness dependent on microbial communities already present in soils to carry the anaerobic process (Testen and Miller 2018). Therefore, the production of these gases depends strongly on the composition the carbon source used (Lehtomäki et al. 2007) and on the type of soil and the microbial communities present (Le Mer and Roger 2001).

Nematodes are very sensitive to ASD. *M. hapla* populations were completely suppressed during our growth chamber trials when soils were treated with ASD regardless of the carbon source. Similar strong nematocidal effects of ASD attributable to organic acid production were observed for control of *M. incognita* juveniles (Katase et al. 2009). Likewise, Runia et al. (2014) observed a very strong reduction in the survival of potato cyst nematode (*Globodera pallida*) in soils treated with ASD, but treatment did not completely eliminate these nematodes. ASD treatments nearly eliminated all golden nematode (*G. rostochiensis*) populations but soil flooding without amendments only reduced populations by 45 to 50% (Ebrahimi et al. 2016). Unlike eggs of *Globodera* spp., eggs of *M. hapla* are not protected by a cyst, so it is likely that the latter are more sensitive to ASD than those of the former. Using naturally infested Ohio soils, significantly fewer root galls (caused by either *M. hapla* or *M. incognita*) were observed on tomatoes grown in ASD-treated soils, regardless of the carbon source used, than from tomato plants from nonamended control soils (Testen and Miller 2018). In our experimental conditions, eggs were applied artificially and mixed homogeneously with the carbon source without variation in soil depth. Under field conditions, the effectiveness of ASD to completely suppress *M. hapla* will depend on how deep the disinfection is accomplished in the soil. In a recent separate field study, galling of lettuce roots by *M. hapla* was significantly reduced in muck soils amended with wheat bran or wheat bran plus molasses and subjected to ASD in each of 2 years compared with the aerobic or anaerobic control (Testen and Miller 2019).

Soil anaerobiosis has been reported to be fundamental to the disinfection process (Min et al. 2007) and depending on the composition and/or combination of carbon sources incorporated into the soil, these anaerobic conditions may vary (Hewavitharana and Mazzola 2016). During our growth chamber trials, a significant amount of iron oxide paint loss was consistently observed on IRIS tubes placed in soils treated with ASD, regardless of the carbon source used. In addition, a negative correlation between soil anaerobic conditions and pathogen viability was observed. Similarly, a significant negative correlation between anaerobic conditions indicated by IRIS tubes and sclerotial germination of *Sclerotium rolsii* ($r = -0.6$ to -0.7 , $P < 0.0001$) was observed during growth chamber ASD trials (Shrestha et al. 2018). In addition, a negative correlation between iron oxide paint loss and root-knot severity ($r = -0.581$, $P < 0.0001$) was reported during growth chamber trials using naturally infested soil from Ohio (Testen and Miller 2018).

According to our PCA, all of the variables measured during the growth chamber trials were negatively correlated with viability of *Sclerotinia* spp. and *M. hapla*, except for soil pH, which was positively correlated (Fig. 3). These results suggest that during ASD, carbon sources are broken down by soil microbes, increasing anaerobic conditions (measured as IRIS paint removal) and the concentration of organic acids (measured as acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids and total acid concentration). As each of these conditions progresses during ASD, the pH decreases, acidifying the soil. Organic fatty acids are deleterious to cells only in their nonionized form and only when the pH is acidic. If the pH is not acidic, nonionized forms will not be available in the medium. The organic fatty acid nonionized forms are small enough to pass through the cell membrane and interfere with vital processes of living cells, causing deleterious effects (Conn et al. 2005; Favre-Bonvin et al. 1991; Huang et al. 2015). As a result, the viability of the *Sclerotinia* spp. and *M. hapla* decreases during ASD treatment (measured as the percentage of viable sclerotia/eggs). This knowledge can be useful when screening carbon sources for ASD using small-scale experiments because, ideally, a carbon source should be selected that is broken down rapidly enough to generate highly anaerobic conditions and yield high concentrations of organic acids (Kleerebezem et al. 2015; Parawira et al. 2004).

Sclerotia exposed to ASD in the field responded similarly to those treated in growth chambers, indicating that this technology could be easily transferred to the field. Lower reduction in sclerotia viability was observed during our second field trial compared with the first, most likely owing to cooler temperatures during the second trial. Lower temperatures during ASD lead to less effective pathogen control (Butler et al. 2014). Degradation of the melanized outer layer of the sclerotia was observed in sclerotia from ASD-treated soils (Supplementary Fig. S1). This outer layer provides considerable resistance against unfavorable environmental conditions and antagonistic microorganisms (Butler et al. 2009). Moreover, recent research showed that clostridial microorganisms isolated from ASD-treated soil degraded fungal cell walls, completely inhibiting the growth of *F. oxysporum* f. sp. *spinaciae* (Ueki et al. 2017). Molasses and wheat bran were equally effective as carbon sources in ASD in reducing germination of *Sclerotium rolsii* sclerotia and enhancing colonization by potentially beneficial mycoparasites (Shrestha et al. 2018). Soil amendment with fresh white mustard or oats and covering the soil was effective in reducing the germination of sclerotia of *S. sclerotiorum* and other sclerotial pathogens, with the exception of *Sclerotium cepivorum*. The reduction of viability of *S. sclerotiorum* was partially explained by the colonization of antagonist sclerotial parasites, such as *Coniothyrium minitans* and *Trichoderma* spp. (Thaning and Gerhardson 2001).

This work provides evidence that ASD reduces *Sclerotinia* spp. sclerotia viability in soil and that carbon source influences the degree of pathogen suppression. In addition, concentrations of

organic acids produced during ASD are deleterious to these sclerotial pathogens and vary by carbon source. *M. hapla* is highly sensitive to ASD, and there were no differences among carbon sources in root-knot suppression. Whereas pH and organic acid production were correlated with reduced sclerotial viability, mechanisms for pathogen suppression by ASD are likely complex. Future research will focus on characterizing other possible mechanisms of suppression such as the role of microbial communities in the production of other compounds deleterious to pathogens. Wheat bran and molasses are potential carbon sources for ASD that can be used for soilborne pathogen management by not only Ohio farmers but also farmers all over the world. Further work will be done to optimize ASD for soilborne pathogen management in different soils and with additional readily available organic amendments.

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