Anaerobic Soil Disinfestation Reduces Germination and Affects Colonization of *Sclerotium rolfii* Sclerotia

Utsala Shrestha, Mary E. Dee, Bonnie H. Ownley, and David M. Butler†

First and fourth authors: Department of Plant Sciences, and second and third authors: Department of Entomology and Plant Pathology, University of Tennessee, Knoxville.

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ABSTRACT

Growth chamber and field studies were conducted with organic amendment mixtures of carbon (C) and nitrogen (N) at C:N ratios 10:1, 20:1, 30:1, and 40:1 and amendment rates of C at 2, 4, 6, and 8 mg/g of soil (C:N ratio 30:1) to evaluate anaerobic soil disinfestation (ASD) effects on germination and colonization of *Sclerotium rolfii*. In the growth chamber, sclerotial germination was reduced in all ASD treatments regardless of C:N ratio (0.6 to 8.5% germination) or amendment rate (7.5 to 46%) as compared with nonamended controls (21 to 36% and 61 to 96%, respectively). ASD treatment increased *Trichoderma* spp. colonization of sclerotia, with consistently higher colonization in ASD treatments

with amendment rates of C at 2 or 4 mg/g of soil (>87% colonization) compared with nonamended controls (<50% colonization). In the 2014 field study, sclerotial germination was reduced by 24 to 30% in ASD treatments when compared with the nonamended control. Sclerotial colonization by *Trichoderma* spp. was predominant; however, other potential mycoparasites (i.e., *Aspergillus* spp., *Fusarium* spp., zygomycetes, and other fungi) were present in the field study. Amendment C:N ratios in the range of 10:1 to 40:1 were equally effective in reducing sclerotial germination and enhancing colonization by potentially beneficial mycoparasites of sclerotia.

With the mandated phase-out of the ozone-depleting fumigant methyl bromide (MeBr), vegetable and small fruit growers need alternative methods to control soilborne plant pathogens. This has led to the introduction of several alternative fumigants for these high-value crops. However, these alternative methods do not control all pathogens and pests targeted by MeBr (Belova et al. 2013) and all soil fumigants face regulatory restrictions to protect human health (Csinos et al. 2002; Martin 2003). In this scenario, anaerobic soil disinfestation (ASD) has potential as a nonchemical alternative to soil fumigants (Butler et al. 2012b; Roskopf et al. 2015; Shennan et al. 2014). Initiated in Japan and the Netherlands in the 2000s, ASD has evolved as an effective measure to control soilborne pathogens and nematodes (Shrestha et al. 2016). Significant decreases in disease were observed with initial ASD studies (Blok et al. 2000; Momma et al. 2006). Application of organic amendments to soil to suppress disease has been widely studied also (Bonanomi et al. 2010, 2013). ASD emphasizes using readily available organic amendments as a source of labile carbon (C) in saturated and covered soil to create anaerobic conditions, enhance populations of microbial biocontrol agents, and release decomposition byproducts (volatile fatty acids and other compounds) to suppress soilborne pathogens (Hewavitharana et al. 2014; Roskopf et al. 2015), suggesting that the rate of added C is a key determinant of ASD treatment efficacy. Anaerobic conditions are achieved by filling soil pore space with water, reducing air exchange to the atmosphere by tarping, and microbial respiration of available soil oxygen during decomposition of the added organic amendment. Thus far, ASD effectiveness has been most well documented against soilborne fungal pathogens, although there are also a limited number of studies on oomycetes and bacterial pathogens (Shrestha et al. 2016).

Sclerotium rolfii Sacc. (current name: *Athelia rolfii* (Curzi) C. C. Tu & Kimbr.) is a sclerotia-forming, necrotrophic soilborne pathogen known to cause seedling damping-off, root rot, and stem rot. The disease is commonly called southern blight. *S. rolfii* is economically important because it infects more than 500 plant species. In the United States, *S. rolfii* is reported mainly in the southern region with tropical, subtropical, and warm temperate climates (Punja 1985; Xu et al. 2009). *S. rolfii* is also an active saprophyte, surviving several years (Aycock 1966; Coley-Smith and Cooke 1971) in a wide range of soil temperature and soil pH (Coley-Smith et al. 1974).

A comprehensive meta-analysis showed that use of various types of amendments with ASD reduces viability of many sclerotial pathogens, including *S. rolfii* (Shrestha et al. 2016). Amendments, including agricultural byproducts, cereal brans, crop residues, and animal manures, were incorporated in soil to test ASD effectiveness on suppression of sclerotia or microsclerotia (Roskopf et al. 2015; Strauss and Kluepfel 2015) of *Verticillium dahliae* (Blok et al. 2000; Goud et al. 2004; Shennan et al. 2007; Thanning and Gerhardson 2001), *Macrophomina phaseolina* (Roskopf et al. 2010, 2014), and *Rhizoctonia solani* (Blok et al. 2000; Hewavitharana and Mazzola 2013; McCarty et al. 2014). All of these pathogens were well suppressed by ASD treatment. Only a few studies on ASD techniques have been conducted in controlled environments (i.e., growth chamber and greenhouse studies) to observe impacts on *S. rolfii*; with warm-season cover crops in Florida (Butler et al. 2012b) and cool-season cover crops in Tennessee (McCarty 2012). Reduction in sclerotial germination was reported in Florida trials with liquid molasses; however, the result was inconsistent between trials (Butler et al. 2012b). Similarly, in Tennessee trials, only cereal rye as a carbon amendment resulted in a consistent reduction in sclerotial germination in both trials, indicating that type of C amendment may have an effect on sclerotial survival and germination (McCarty 2012). Soil amendments play a critical role in determining microbial population structure (Fernandez et al. 2016; Reardon and Wuest 2016; Tao et al. 2015), decomposition rates (Akhtar and Malik 2000), plant growth (Rodríguez-Kábana et al. 1987), and, collectively, the effectiveness of ASD (Butler et al.

†Corresponding author: D. M. Butler; E-mail: dbutler@utk.edu

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2012b). Thus, it is imperative to identify the properties and rates of C amendment that are needed to suppress germination of *S. rolf sii*.

The ratio of C to nitrogen (N) (C:N) in organic soil amendments is known to be important for shifts in microbial population structure and decomposition of organic matter (Leite et al. 2017). At the same time, both high C:N ratio amendments (e.g., ethanol, glucose, and molasses) and low to moderate C:N ratio amendments (cover crop residue, grape pomace, wheat bran, and rice bran) have been successfully used as ASD amendments (Shennan et al. 2014). Physiologically, soil microbial biomass stoichiometry is fairly constrained within a C:N ratio range of approximately 5:1 to 7:1 (Spohn 2015), with a threshold for N limitation on growth at approximately 20:1 to 25:1 (Sinsabaugh et al. 2013). Accordingly, C use efficiency and soil microbial decomposition of amendments at relatively high C:N ratios (i.e., low N concentration) is reduced due to N limitation. Although aerobic microbial communities may “dispose” of excess C through increased respiration or metabolic activity and release of dissolved organic C compounds (Hessen and Anderson 2008; Spohn 2015), anaerobic microbes are metabolically constrained by reduction-oxidation reactions. However, certain anaerobic microbes are known to change fermentation pathways due to changes in substrate availability and nutrient limitation, resulting in differing profiles of anaerobic decomposition byproducts (Streekstra et al. 1987; Thomas et al. 1979) which, in the case of ASD treatment, could alter treatment mechanisms and efficacy in given pathosystems.

In this study, the efficacy of ASD using organic amendments of C at rates of 2, 4, 6, and 8 mg/g of soil (constant C:N ratio of 30:1) and C:N ratios of 10:1, 20:1, 30:1, and 40:1 (constant C rate of 4 mg/g of soil) was evaluated for germination suppression of sclerotia of *S. rolf sii*. The specific aims were to (i) evaluate cumulative redox potential of soils treated with C amendments at a range of C rates and C:N ratios for ASD treatment, (ii) examine germination and colonization of introduced sclerotia of *S. rolf sii* using ASD amendment mixtures at a range of C:N ratios (10:1 to 40:1) in growth chamber and field conditions, and (iii) examine germination and colonization of introduced sclerotia of *S. rolf sii* using ASD amendments of C at rates from 2 to 8 mg/g of soil in growth-chamber conditions.

MATERIALS AND METHODS

Sclerotia production and inoculum preparation. *S. rolf sii* was isolated from hybrid field tomato crops grown at the University of Tennessee (UT) Organic Crops Unit, East Tennessee Research and Education Center, Knoxville. *S. rolf sii* was cultured on potato dextrose agar (PDA) for 7 days, and plugs were transferred to the center of freshly prepared PDA plates. Cultures were incubated at room temperature. After 4 to 6 weeks, mature sclerotia of consistent large size and dark color were harvested, dried overnight under a laminar flow hood, and stored in sterile glass vials until use in the experiment. Ten sclerotia were sealed in each 5-by-5-cm permeable packet (aperture Delnet polyolefin fabric; DelStar Technologies, Austin, TX) and string was attached for easy retrieval of the packet from soil. Although the use of culture-produced sclerotia rather than sclerotia formed on soil has been debated in the literature (Punja

1985), the consistency of culture-produced sclerotia and lack of microbial contamination was deemed important to study objectives. Although some physiological and morphological differences have been reported among sclerotia production methods (Linderman and Gilbert 1973; Punja et al. 1984), it is unclear as to how sclerotia production methods affect survivability, germination, and colonization during soil disinfestation methods in field conditions, and the results of the present study should be interpreted in that context.

Growth chamber or pot trials 1 and 2: ASD amendment type and C:N ratio effect. A Dewey silt loam (fine, kaolinitic, thermic, Typic Paleudult) was collected from a field at the UT Organic Crops Unit, sieved through a 10-mm aluminum mesh, and mixed with fine sand (1:1 [vol/vol]). The soil and sand mixture was placed in tall black plastic pots (12 cm in diameter, 23 cm in height, 2,500 cm³ in volume) with two pathogen packets placed at 5- and 15-cm depths. Initial total soil C content was 10 g/kg of soil, with a soil C:N ratio of 11.9. Soil treatments consisted of two main treatments: (i) dry molasses-based mixture and (ii) wheat bran-based mixture at four C:N ratios: 10:1, 20:1, 30:1, and 40:1 (Tables 1 and 2). Control pots without amendments were included for each main treatment. All pots were saturated with tap water to replace air-filled pore space with water. Pots were irrigated and then covered with standard mulch polyethylene, which was secured to the pot with a heavy-duty rubber band. No further irrigation was applied during treatment. Pots were randomly positioned on a wire rack in an environmental growth chamber (TC2 Controller; ECG, Chagrin Falls, OH) with temperature at 25 and 15°C day and night, respectively, to approximate environmental conditions typical during spring soil treatment in warm temperate to subtropical climates (McCarty et al. 2014). The study was a completely randomized design with four replications. Trials were conducted on 30 April 2013 (trial 1) and repeated on 22 May 2013 (trial 2).

Growth chamber and pot trials 3 and 4: ASD amendment rate effect. To determine the effect of ASD amendment rates on sclerotial germination and colonization by potential microbial antagonists, growth chamber experiments were initiated on 17 June 2014 (trial 3) and 29 May 2015 (trial 4), as described for growth chamber pot study trials 1 and 2. Initial soil C content was 5.5 g/kg of soil, with a soil C:N ratio of 12.8. Treatments included ASD amendment rates of C at 2, 4, 6, and 8 mg/g of soil, created by mixing dry molasses with corn starch at a C:N ratio of 30:1 (Tables 1 and 2). Two nonamended control treatments, one with and one without polyethylene mulch, were included. The control without polyethylene mulch was irrigated initially as other treatments with polyethylene mulch. The design was completely randomized, with four replications.

Field study. A field study was established at the UT Plateau Research and Education Center in Crossville on 16 May 2013 and 13 May 2014. Four ASD treatments were established at four amendment C:N ratios (10:1, 20:1, 30:1, and 40:1), at a total amendment rate of C at 4 mg/g of soil, with dry molasses-based mixtures used as the ASD amendment (Tables 1 and 2). In addition, an amendment C:N ratio of 30:1, at a lower rate of C at 2 mg/g of soil, and a nonamended untreated control were included. Amendment rates were calculated based on soil mass calculated for the 0-to-20-cm horizon based on measured soil bulk density. Research was conducted in different sites of the same field in each

TABLE 1. Anaerobic soil disinfestation amendment properties

Soil amendment	Particle size (mm)	pH	Moisture (%) ^x	N (%) ^y	C (%)	C:N	Mineral content (g/kg)					
							DO C ^z	Ca	K	Mg	P	S
Dry molasses	0.2–4.5	6.5	0.06	1.3	38.7	29.7	10.6	8.8	40.3	4.6	0.9	7.4
Soybean meal	0.15–5	7.4	0.08	8.8	42.6	4.8	4.8	4.5	22.2	3.7	7.0	4.1
Wheat bran	0.2–4.5	7.3	0.05	3.1	40.3	13.3	2.9	1.0	12.5	6.4	12.5	1.8
Corn starch	<0.03	5.5	0.15	0.0	41.7	N/A	0.1	0.1	0.2	0.1	0.2	0.2

^x Gravimetric moisture content.

^y Elemental content presented on dry matter basis.

^z Dissolved organic (DO) C, hot and cold water extractable.

year. In 2013, the initial gravimetric soil moisture content was 2.5 to 2.7 g/g of soil, total C content was 11 g/kg of soil, and soil C:N ratio was 12.9. In 2014, the initial gravimetric soil moisture content was 2.7 to 3.1 g/g of soil, C content was 12 g/kg of soil, and soil C:N ratio was 13.4. The experiment was a randomized complete block design with four replications. Each plot was 7.6 by 1.8 m. The soil type was a Lily series (fine-loamy, siliceous, semiactive, mesic Typic Hapludult). Amendments were applied in each plot using a drop fertilizer spreader and were thoroughly incorporated in soil with a rotovator to approximately 20 cm in depth. Raised beds (approximately 5 cm) were formed and mulched with standard black polyethylene. Two packets of sclerotia were inserted in soil at a depth of 10 cm in the field to assess the effect of ASD on sclerotial germination and colonization by potential microbial antagonists. Beds were irrigated with 5 cm of water at the initiation of ASD treatment based on the amount of water needed to saturate soil to a depth of approximately 20 cm. Irrigation was applied for 9.3 h using a single drip irrigation line installed in the center of each bed under the polyethylene mulch.

Assessment of sclerotia following ASD treatment. After ASD treatment (3 weeks), packets were collected from the pots in growth chamber studies and fields and stored in sealed bags at 4°C until examined. Packets containing sclerotia were washed with tap water to remove adhering soil, sonicated for 1 min, and surface sterilized in 10% commercial bleach for 1 min followed by 1 min in 10% ethanol. Sclerotia were carefully removed from packets, plated onto PDA amended with fenpropathrin at 6.9 mg/liter (Danitol 2.4 EC; Valent Chemical, Walnut Creek, CA) and rifampicin at 10 mg/liter (Sigma-Aldrich, St. Louis), and incubated at room temperature for 4 to 6 weeks. Sclerotia that were soft at plating and failed to germinate were recorded as decomposed. Germination of sclerotia was confirmed by mycelial growth at 3 to 5 days. Production of new sclerotia and colonization by *Trichoderma* spp. and other microbial antagonists were observed and identified over a 4- to 6-week period.

Soil moisture, temperature, pH, and anaerobic conditions. Each pot in the growth chamber study was equipped with an oxidation-reduction electrode (Combination ORP Electrode; Sensores Corp., Garden Grove, CA) at a 15-cm depth to measure cumulative redox potential. The temperature and volumetric moisture content (only for trials 3 and 4) of the soil mixture of each

representative treated pot (two replicates) were recorded using temperature-moisture sensors at 10 cm (5TM Soil Moisture Probe; Decagon Devices, Pullman, WA). Soil samples from each pot were collected from depths of 0 to 10 cm at ASD termination using a clean plastic spoon and composited for gravimetric soil moisture content; then, air-dried soil was used to determine soil pH, which was completed with a 2:1 ratio of 0.01 M CaCl₂/soil, and pH was determined with a pH electrode (Orion 3-Star Plus pH Benchtop Meter; Thermo Scientific, Waltham, MA). The soil pH values in CaCl₂ were adjusted to soil pH in water by adding 0.6 U to approximate equivalent values of soil pH determined in soil and deionized water suspension while reducing measurement interference due to varying soil electrolyte concentrations (Kissel et al. 2009). The cumulative anaerobic conditions of soil over a 3-week treatment period was calculated as described by Butler et al. (2012a). In field trials, anaerobic condition was determined by an iron-oxyhydroxide-coated indicator of reduction in soils (IRIS) tubes (Castenson and Rabenhorst 2006). Tubes were inserted in each plot at depths of 5 to 15 cm before irrigation and retrieved after the 3-week treatment and cleaned with tap water, and removal of paint was assessed as described by Rabenhorst (2012). Ten soil cores from depths of 0 to 15 cm were collected from each plot and combined for determination of soil moisture and soil pH.

Statistical analysis. Data were analyzed with mixed model analysis of variance using SAS 9.4 (SAS Institute, Cary, NC) and differences between means were determined with Tukey's test ($P < 0.05$). In repeated trials in the pot study, ASD amendment type and ASD amendment C:N ratio effects were analyzed separately as completely randomized factorial analysis designs, where ASD amendments, amendment C:N ratios, and depth were treated as fixed factors. The amendment C rate study was analyzed as a completely randomized design, with amendment C rate as a fixed effect. Burial depth data were pooled because the results from repeated trials were consistent, with no significant interactions between depth and amendment C rate effect. Field studies were analyzed separately each year. Within field experiments, ASD amendment C:N ratio was treated as a fixed effect and replication as a random effect. For all studies, data were checked for outliers before analysis, and nonnormal and unequal variances were transformed using arcsine square root or rank transformations as appropriate. Nontransformed means and standard error of the mean are

TABLE 2. Anaerobic soil disinfestation treatment carbon to nitrogen (C:N) ratios, amendment types, and amendment rates in pot trials 1 and 2 (amendment and C:N ratio study), pot trials 3 and 4 (C rate study), and field trials (C:N ratio study)

Trials, treatment label	Primary amendment	Amendment C:N ratio	C (mg/g of soil)	Primary amendment rate (g/kg of soil)	Primary amendment rate (kg/m ²)	Adjustment amendment rate (g/kg of soil) ^y
Pot 1 and 2 (C:N ratio)						
10	Wheat bran	10:1	4	7.8	...	1.8 (SM)
20	Wheat bran	20:1	4	6.4	...	3.3 (CS)
30	Wheat bran	30:1	4	4.2	...	5.6 (CS)
40	Wheat bran	40:1	4	3.2	...	6.7 (CS)
10	Dry molasses	10:1	4	6.4	...	3.6 (SM)
20	Dry molasses	20:1	4	9.4	...	0.9 (SM)
30	Dry molasses	30:1	4	10.3	...	0.1 (CS)
40	Dry molasses	40:1	4	7.7	...	2.6 (CS)
Pot 3 and 4 (C rate)						
2	Dry molasses	30:1	2	5.1	...	0.04 (CS)
4	Dry molasses	30:1	4	10.3	...	0.09 (CS)
6	Dry molasses	30:1	6	15.4	...	0.13 (CS)
8	Dry molasses	30:1	8	20.5	...	0.17 (CS)
Field trials (C:N ratio)						
10	Dry molasses	10:1	4	...	0.86	0.48 (CS)
20	Dry molasses	20:1	4	...	1.26	0.12 (CS)
30	Dry molasses	30:1	4	...	1.38	0.01 (CS)
40	Dry molasses	40:1	4	...	1.03	0.35 (CS)
LC30 ^z	Dry molasses	30:1	2	...	0.69	0.01 (CS)

^y Primary amendment adjusted with corn starch (CS) or soybean meal (SM).

^z LC = low carbon.

reported. Relationships between soil moisture, cumulative anaerobic conditions, and rates of germination and colonization of sclerotia were assessed with correlation analysis at $P < 0.05$. Pearson (r) or Spearman (r_s) correlation coefficients are reported when the correlation was significant.

TABLE 3. Effect of anaerobic soil disinfestation (ASD) amendment on mean posttreatment gravimetric soil moisture, posttreatment soil pH, and anaerobic conditions from pot trials 1 and 2 (amendment and C:N ratio study)^y

Trial, amendment ^z	Gravimetric soil moisture (g/g)	Soil pH (units)	Soil anaerobic condition (V h)
1			
Control	0.22 ± 0.0	6.0 ± 0.0	42.7 ± 15.3 b
Dry molasses based	0.22 ± 0.0	5.9 ± 0.1	185.3 ± 10.6 a
Wheat bran based	0.22 ± 0.0	5.8 ± 0.1	157.6 ± 12.8 a
<i>P</i> value	0.607	0.492	<0.0001
2			
Control	0.24 ± 0.0 b	5.3 ± 0.0	82.3 ± 20.3 b
Dry molasses based	0.27 ± 0.0 a	5.4 ± 0.1	196.0 ± 10.4 a
Wheat bran based	0.26 ± 0.0 a	5.3 ± 0.1	165.2 ± 10.0 a
<i>P</i> value	0.003	0.188	<0.0001

^y For each trial, within a column, values = least square means ± standard error of four replicate pots. Values followed by different letters are significantly different according to Tukey's test at $P < 0.05$.

^z Control = covered, nonamended control.

RESULTS

ASD amendment C:N ratio effect on anaerobic conditions, and germination and colonization of sclerotia in pot trials 1 and 2. A significant but moderate negative correlation between sclerotial germination and cumulative anaerobic conditions ($r_s = -0.4$, $P < 0.01$) was observed in trial 1. No relationship between sclerotial germination and cumulative anaerobic conditions was observed in trial 2, with a correlation value of 0.27 ($P = 0.09$). No interactions among ASD amendment type, ASD amendment C:N ratio, or soil depth were observed for sclerotial germination, decomposition, or colonization in either trial.

Mean soil temperatures did not differ across treatments and ranged from 28.4 to 29.4°C during the day and 18.4 to 19.3°C during the night. The volumetric water content of soil was higher in nonamended control treatments and lowest in treatments amended with wheat bran (data not shown). In contrast, gravimetric soil moisture content was slightly lower in the control than in ASD treatments in trial 2, possibly due to the moisture-holding capacity of added organic amendments (Table 3). Gravimetric soil moisture content was lower in trial 1 than trial 2. Soil pH was measured after ASD treatment and did not differ across ASD amendment type but differed among ASD amendment C:N ratios. The lowest soil pH was recorded in ASD-treated pots with an amendment C:N ratio of 10:1 (4.9 and 5.3, trial 1 and trial 2, respectively) (Fig. 1A). An interaction between ASD amendment type and ASD amendment C:N ratio was observed for soil pH but was only

Fig. 1. Effect of anaerobic soil disinfestation (ASD) amendment carbon to nitrogen (C:N) ratio on **A**, soil pH; **B**, cumulative anaerobic condition; **C**, percent sclerotial germination; and **D**, percent decomposed sclerotia during ASD treatment, pot study. Bars indicated by different letters are significantly different at $P < 0.05$ according to Tukey's test. Capital letters are used to compare the respective means of trial 1 and small letters are used to compare respective means of trial 2. Error bars indicate standard error with four replicates; 10 = C:N ratio 10:1, 20 = C:N ratio 20:1, 30 = C:N ratio 30:1, 40 = C:N ratio 40:1, and Control = nonamended control.

significant in trial 1 ($P < 0.04$), with higher pH for soil treated with dry molasses. Soil pH increased as ASD amendment C:N ratio increased from 10:1 to 30:1 for dry molasses. Mean cumulative anaerobic conditions did not differ among ASD amendment type in either trial but values for ASD treatments were significantly higher than the

nonamended control (Table 3, Fig. 1B). No interaction was observed between ASD amendment type and ASD amendment C:N ratio. Across both trials, the highest mean anaerobic condition values were recorded for ASD treatments with dry molasses amendment (191 V h), with an ASD amendment C:N ratio of 10:1 (210 V h) (data not shown).

TABLE 4. Effect of anaerobic soil disinfestation amendment on sclerotia of *Sclerotium rolfsii* in pot trials 1 and 2 (amendment and C:N ratio study)^y

Trial, amendment ^z	Sclerotial germination (%)	<i>Trichoderma</i> colonization of sclerotia (%)	<i>Fusarium</i> colonization of sclerotia (%)	Sclerotial decomposition (%)	Colonization by unidentified fungi (%)	Colonization by fungi other than <i>Trichoderma</i> (%)
1						
Control	21.5 ± 5.0 a	81.0 ± 4.3 b	2.5 ± 1.4	2.5 ± 1.9	1.9 ± 1.0	4.4 ± 1.6
Dry molasses based	11.2 ± 3.2 b	91.8 ± 2.6 a	0.7 ± 0.5	4.1 ± 1.4	4.5 ± 1.4	5.2 ± 1.4
Wheat bran based	2.5 ± 1.1 c	96.1 ± 1.7 a	3.6 ± 2.1	2.2 ± 1.2	1.6 ± 0.8	5.2 ± 2.4
<i>P</i> value	<0.0001	0.002	0.44	0.38	0.14	0.41
2						
Control	38.5 ± 9 a	54.2 ± 9.2 b	8.8 ± 2.8	5.0 ± 2.2	3.2 ± 1.5	12.0 ± 3.2
Dry molasses based	3.8 ± 1.5 b	77.4 ± 5.2 a	11 ± 3.1	5.3 ± 2.3	13.3 ± 3.7	24.2 ± 5.2
Wheat bran based	3.4 ± 2.6 b	84.8 ± 4.5 a	12.6 ± 3.7	4.1 ± 1.8	5.9 ± 1.8	18.5 ± 4.4
<i>P</i> value	<0.0001	0.004	0.90	0.60	0.10	0.49

^y Values are least square means ± standard error of four replicate pots. For each trial, within columns, values followed by different letters are significantly different according to Tukey's Test at $P < 0.05$.
^z Control = covered, nonamended control.

Fig. 2. Effect of anaerobic soil disinfestation (ASD) amendment carbon to nitrogen (C:N) ratio on percentage sclerotia colonization by **A**, *Trichoderma* spp.; **B**, *Fusarium* spp.; **C**, unidentified fungi; and **D**, *Fusarium* spp. and other fungi after ASD treatment, pot study. Bars indicated by different letters are significantly different at $P < 0.05$ according to Tukey's test. Capital letters are used to compare the respective means of trial 1 and small letters are used to compare respective means of trial 2. Error bars indicate standard error with four replicates; 10 = C:N ratio 10:1, 20 = C:N ratio 20:1, 30 = C:N ratio 30:1, 40 = C:N ratio 40:1, and Control = nonamended control.

ASD treatment reduced sclerotial germination compared with nonamended controls in both trial 1 and trial 2 ($P < 0.0001$). In trial 1, the lowest sclerotial germination was observed in the ASD treatments with wheat-bran-based amendment (Table 4). Sclerotial germination was lower in all ASD treatments (0.6 to 8.4%) compared with the nonamended control (22 to 39%), and this difference was significant with the 10:1 amendment C:N ratio in trial 1 and all amendment C:N ratios in trial 2 (Fig. 1C). However, sclerotial decomposition was not significantly affected by ASD amendment type, ASD amendment C:N ratio (Fig. 1D), or depth of burial. Both ASD amendment types had a significant positive impact on colonization of sclerotia by *Trichoderma* spp., with higher colonization in ASD treatments (93 to 95%, trial 1 and 80 to 84%, trial 2) compared with nonamended controls (81%, trial 1 and 54%, trial 2) (Fig. 2A). Colonization by other potential mycoparasites (*Fusarium* spp. and other unidentified fungi) was observed but was not different among ASD amendment types (Table 4) or ASD amendment C:N ratios (Fig. 2B, C, and D), except for unidentified fungi in trial 1 (Fig. 2C).

Sclerotial germination assessed for pathogen packets retrieved from burial depths of 5 and 15 cm was lower for ASD treatments compared with nonamended controls in trial 2, whereas sclerotial germination did not differ for the ASD treatments with dry molasses-based amendment and nonamended controls in trial 1 (Fig. 3A). At the 5-cm burial depth, sclerotial colonization by *Trichoderma* spp. was higher in ASD treatments (94 to 98% in trial 1 and 95 to 97% in trial 2) compared with nonamended controls (75% in trial 1 and 64% in trial 2) but did not differ at the 15-cm depth (Fig. 3B). Higher percentages of *Fusarium* spp. and other fungi were generally observed on sclerotia

retrieved from the 15-cm depth (19 to 23%) compared with the 5-cm depth (2 to 3%) in ASD treatments in trial 2 but no differences were observed between ASD treatments and the nonamended controls (Fig. 3C and D).

ASD amendment rate effect on anaerobic conditions, and germination and colonization of sclerotia in pot trials 3 and 4. There was a significant but moderate negative correlation of sclerotial germination with anaerobic condition for both trials 3 and 4 ($r_s = -0.6$ to -0.7 , $P < 0.0001$). Strong negative correlation ($r_s = -0.9$, $P < 0.0001$) was observed between sclerotial germination and *Trichoderma* colonization in trial 4 and moderate negative correlation observed in trial 2 ($r_s = -0.6$ at $P < 0.005$), indicating that lower sclerotial germination is associated with higher *Trichoderma* colonization. There was a negative, moderate correlation between percent sclerotial colonization with zygomycetes and sclerotial germination in both trials ($r_s = -0.5$, $P < 0.05$). There was no significant correlation of germination of sclerotia with colonization of sclerotia by *Fusarium* spp. and bacteria.

The data for the two depths of sclerotial burial were pooled because there was no effect of depth of sclerotial burial on sclerotial germination or colonization by *Trichoderma* spp. in trials 3 and 4. Average volumetric soil moisture in trial 3 was 0.27 to 0.28 cm³/cm³ and, in trial 4, soil moisture was 0.23 to 0.27 cm³/cm³ in covered pots, with uncovered pots at 0.01. Mean soil temperature did not differ among treatments during the treatment period, ranging from 21.3 to 26.2°C for trial 3 and 19.9 to 22°C for trial 4. Average volumetric soil moisture content differed among treatments in both trials, ranging from 0.2 to 0.3 cm³/cm³. Soil pH was relatively higher in trial 4 than

Fig. 3. Effect of anaerobic soil disinfestation (ASD) amendments at depths of 15 and 5 cm on **A**, percentage sclerotial germination; percentage sclerotial colonization by **B**, *Trichoderma* spp.; **C**, *Fusarium* spp.; and **D**, unidentified fungi after ASD treatment, pot study. Bars indicated by different letters are significantly different at $P < 0.05$ according to Tukey's test. Capital letters are used to compare the respective means of trial 1 and small letters are used to compare respective means of trial 2. Error bars indicate standard error with four replicates. Control = nonamended control.

trial 3 but no significant differences were observed among ASD amendment C rates for soil pH (Fig. 4A). Cumulative anaerobic conditions were not affected by ASD amendment rate differences from 2 to 8 mg/g; however, anaerobic conditions were significantly higher in ASD treatments (141 to 158 V h in trial 3 and 107 to 150 V h in trial 4) than in the covered and uncovered nonamended controls (0 to 18 V h) (Fig. 4B).

All ASD treatments were effective in suppressing sclerotial germination compared with the nonamended controls. In both trial 3 and 4, sclerotial germination was not significantly reduced by increasing ASD amendment rates of C above 2 mg/g of soil (Fig. 4C). However, germination with ASD amendment rate of C at 4 mg/g of soil may be more consistently low at this soil temperature given the narrower standard error. Sclerotial colonization by *Trichoderma* spp.

was higher at all ASD amendment rates compared with nonamended controls in both trials 3 and 4 (Fig. 4D). Sclerotial colonization by *Fusarium* spp., zygomycetes, and bacteria was observed in all ASD treatments, with the highest percentage of colonization by all organisms observed for ASD amendment rates of C at 6 and 8 mg/g (Fig. 4E), indicating that, in addition to *Trichoderma* spp., colonization of sclerotia by other diverse microbes increased at higher ASD amendment C rates.

ASD amendment effect on anaerobic conditions, and sclerotial germination and colonization in field studies. In field trials, no significant correlations of sclerotial germination with soil moisture, soil pH, and microbial colonization were observed in 2013. However, there was a moderate negative relationship between *Trichoderma* colonization with IRIS tube paint removal ($P < 0.04$) and

Fig. 4. Effect of anaerobic soil disinfestation (ASD) amendment carbon (C) rates on A, soil pH; B, cumulative anaerobic condition; C, percentage sclerotial germination; and percentage sclerotial colonization by D, *Trichoderma* spp. and E, *Fusarium* spp., zygomycetes, bacteria and other fungi during ASD treatment, pot study. Bars with different letters are significantly different at $P < 0.05$ according to Tukey's test. In E, different letters with a line above the error bars indicates significant differences for total sclerotial colonization by *Fusarium* spp., zygomycetes, bacteria, and other fungi. Capital letters are used to compare the respective means of trial 3 and small letters are used to compare respective means of trial 4. Error bars indicate standard error with four replicates. C amendment rates were 2, 4, 6, and 8 mg/g of soil; Control (+p) = polyethylene covered, nonamended control and Control (-p) = uncovered, nonamended control.

a positive relationship with soil moisture, initial soil pH, other fungi, and *Aspergillus* colonization of sclerotia in 2013 ($r_s = 0.4$, $P < 0.04$). In 2014, sclerotial germination had a moderate negative correlation with *Trichoderma* colonization of sclerotia ($r_s = -0.7$, $P < 0.001$). In addition, soil pH and IRIS tube paint removal were moderately correlated ($r_s = 0.4$, $P < 0.01$).

Average soil temperatures did not differ among ASD treatments in either field study. In 2013, mean soil temperatures ranged from 22 to 25°C and, in 2014, slightly higher mean soil temperatures were observed, with a range between 24 and 25°C. Before treatment

TABLE 5. Effect of anaerobic soil disinfestation (ASD) amendment carbon/nitrogen (C:N) ratio on soil gravimetric moisture content (g/g) in field trials^y

Treatment ^z	Field trial 1, 2013		Field trial 2, 2014	
	Pre ASD	Post ASD	Pre ASD	Post ASD
10	2.5 ± 0.16	2.9 ± 0.05 ab	2.7 ± 0.19	3.4 ± 0.24
20	2.6 ± 0.09	3.1 ± 0.1 a	2.9 ± 0.11	2.9 ± 0.29
30	2.7 ± 0.07	2.8 ± 0.15 b	3.1 ± 0.26	3.9 ± 0.55
40	2.5 ± 0.05	2.5 ± 0.17 cd	3 ± 0.08	2.9 ± 0.35
LC30	2.7 ± 0.1	2.9 ± 0.08 ab	2.8 ± 0.11	3.1 ± 0.31
Control	2.5 ± 0.05	2.8 ± 0.03 bc	2.8 ± 0.1	3.2 ± 0.29

^y Within each column, values are least square means ± standard error of four replicate plots. Values followed by different letters are significantly different according to Tukey's test at $P < 0.05$.

^z Treatments are 10 = C:N ratio 10:1, 20 = C:N ratio 20:1, 30 = C:N ratio 30:1, 40 = C:N ratio 40:1, LC30 (low carbon) = C:N ratio 30:1 and C rate at 2 mg/g of soil, and Control = nonamended control.

application, gravimetric soil moisture ranged from 2.5 to 2.7 g/g in 2013 and was slightly higher in 2014, ranging from 2.7 to 3.1 g/g (Table 5). In 2013, after a 3-week treatment period, soil moisture content was significantly higher with an ASD amendment C:N ratio of 20:1 (C at 3.1 g/g of soil) when compared with C:N ratio of 30:1, 40:1, and the nonamended control (>2.8 g/g of soil). There was no significant influence of ASD treatment on soil pH at pretreatment (5.8 to 6.0 during 2013 and 5.4 to 5.5 during 2014) and posttreatment (5.8 to 6.0 during 2013 and 5.9 to 6.1 during 2014) (data not shown). No decline in soil pH among treatments was observed from pretreatment to posttreatment in either field study, although soil pH was not monitored during treatment. Cumulative anaerobic conditions indicated by the IRIS tubes showed significantly enhanced anaerobic conditions in ASD treatments with C at 4 mg/g of soil ($P < 0.05$). The maximum iron solubilization by reduction (i.e., paint removal rates) in ASD-treated plots amended with C at 4 mg/g of soil, regardless of C:N ratio, were 31 to 42% in 2013 and 23 to 33% in 2014. The lowest rates of iron solubilization were observed for the ASD treatment with an amendment rate of C at 2 mg/g of soil (6 to 28%) and the nonamended control (1 to 16%).

In 2013, sclerotial germination was relatively low among ASD treatments, ranging from 1% in amendment C:N ratio 40:1 to 11% in C:N ratios 10:1 and 20:1, compared with the nonamended control (27%), although this difference was not significant ($P = 0.06$). However, in 2014, the germination of sclerotia was significantly lower in all ASD treatments (1 to 7%, $P < 0.0001$) as compared with the nonamended control (31%) (Fig. 5A). Relatively high sclerotial colonization by *Trichoderma* spp., *Fusarium* spp., zygomycetes,

Fig. 5. Effect of dry molasses anaerobic soil disinfestation (ASD) amendment at different carbon to nitrogen (C:N) ratios on **A**, percentage sclerotial germination; **B**, percentage sclerotial colonization by *Trichoderma* spp. in the year 2013 (field trial 1) and 2014 (field trial 2); and percentage sclerotial colonization by microorganisms other than *Trichoderma* spp. in **C**, field trial 1 and **D**, field trial 2 after ASD treatment. Bars indicated by different letters are significantly different at $P < 0.05$ according to Tukey's test. Error bars indicate standard error with four replicates; 10 = C:N ratio 10:1, 20 = C:N ratio 20:1, 30 = C:N ratio 30:1, 40 = C:N ratio 40:1, LC30 = C:N ratio 30:1 and C rate of 2 mg/g of soil (LC = low carbon), and Control = nonamended control.

and other fungi was observed (Fig. 5B, C, and D) but generally did not differ among treatments. *Aspergillus* colonization of sclerotia was observed in 2013 and bacterial colonization in 2014; however, the results were not significant in either study ($P = 0.07$ for *Trichoderma* spp. and $P > 0.1$ for all fungal colonization). The only significant colonization difference was observed for zygomycetes in 2014 ($P < 0.02$) (Fig. 5D), where the lowest parasitism was observed from the control and the highest from amendment C:N ratios of 20:1 and 40:1.

DISCUSSION

Effectiveness for control of sclerotia was measured in terms of sclerotial germination and sclerotial colonization by various culturable soil microorganisms. The majority of sclerotia recovered from ASD treatments failed to germinate and were colonized by potential mycoparasites. The presence of endemic *Trichoderma* and *Fusarium* spp. and other fungi known to be mycoparasites of sclerotia (Coley-Smith and Cooke 1971; Elad et al. 1984; Hu et al. 2013; Rai and Saxena 1975) does suggest that sclerotia were either weakened or lysed during ASD treatment. At the same time, many sclerotia that germinated were also colonized by potential mycoparasites, suggesting that colonization alone does not explain ASD treatment effects on sclerotia germination. Sclerotial colonization by *Trichoderma* spp. was abundant in all studies; however, *Aspergillus* colonization was observed in field trial 1 (2013) and bacterial colonization in field trial 2 (2014). *Trichoderma* spp. are the most studied mycoparasites that are known to produce bioactive metabolites (Ownley et al. 2010) and produce enzymes such as β -1,3-glucanase and chitinase as a means to degrade the protective outer layer of sclerotia (Elad et al. 1984) or reduce hyphal growth (Madi et al. 1997). It has been reported that eruptive germination of sclerotia in soil or weakening of the rind due to treatment effects can release nutrients that stimulate the colonization of sclerotia (Smith 1972). Colonization of sclerotia by zygomycetes (e.g., *Rhizopus* and *Mucor* spp.) was also observed. Zygomycetes are known to produce chitosan, a substance derived from chitin with antimicrobial activity (Gharieb et al. 2015). However, significant colonization of sclerotia by other mycoparasites as compared with the nonamended control was not observed in the present study. In addition, it is also expected that sclerotia are targeted by anaerobic bacteria during ASD treatment, especially Clostridia (Adandonon et al. 2015), during the highly anaerobic phase soon after amendment incorporation (Huang et al. 2015); however, the present study did not evaluate sclerotial decomposition by anaerobic bacteria.

With the application of amendments, regardless of amendment C:N ratio and C rates, high cumulative anaerobic conditions were recorded within 3 weeks of ASD treatment, which was similar to previous studies (McCarty et al. 2014). Consistent relationships among anaerobic conditions, sclerotial germination, and *Trichoderma* colonization of sclerotia were observed only in the pot studies with varying C rates. Endemic *Trichoderma* spp. also colonized a higher number of sclerotia in ASD treatments compared with the nonamended control. However, under field conditions, sclerotial colonization by *Trichoderma* spp. was lower than in pot studies and not significantly different between ASD treatments and the nonamended control. Greater diversity in populations of microorganisms present in field soil and less available nutrients could lead to more competition for sclerotial colonization or parasitism. In addition, no significant differences among ASD amendment C:N ratios in sclerotial germination or colonization by *Trichoderma* spp. were observed in either pot or field conditions, although *Trichoderma* populations are reported to increase in soils amended to obtain high C:N ratios (e.g., 40:1) (Shaban et al. 1998). Overall, ASD was effective in reducing sclerotial germination compared with controls.

An ASD amendment rate of C at 4 mg/g of soil was optimal to suppress sclerotial germination due to more consistent suppression of sclerotial germination under the environmental conditions of this study. However, given no statistical differences observed in sclerotial

germination between amendment rates of C at 2 and 4 mg/g of soil (at C:N ratio of 30:1), it may be possible to use rates lower than 4 mg/g of soil to reduce amendment costs. Likewise, using rates of C at 2 or 4 mg/g of soil with ASD treatment resulted in the highest sclerotial colonization by *Trichoderma* spp. (Fig. 4D). Studies have suggested that increased depth of sclerotial burial increases mortality of sclerotia (Imolehin and Grogan 1980; Smith et al. 1989) because increased soil pressure increases sclerotial substrate leakage (Punja et al. 1984). However, in this study, there was no significant difference in mortality of sclerotia between two depths across ASD and control treatments. At the 15-cm depth for ASD treatments in trial 1, there was no difference in sclerotial mortality when compared with the nonamended control (Fig. 3A). In contrast, at 15 cm, higher sclerotial mortality for ASD treatments than the nonamended control in trial 2 may be attributable to exposure to greater soil moisture (Abawi et al. 1985; Moore 1949) with lower soil pH and high soil anaerobic conditions (Table 3). Overall, ASD treatment using either dry molasses or wheat bran as C amendments was more effective than the nonamended control in inducing sclerotial mortality.

In short, ASD can effectively reduce percent sclerotial germination. With ASD treatments, the majority of sclerotia failed to germinate. For those that did germinate, there was a high rate of colonization, mainly by *Trichoderma* spp. This phenomenon was more prevalent at shallow (5 cm) than deep (15 cm) soil depths. However, no differences were observed among ASD amendment C:N ratios for germination of sclerotia. The addition of dry molasses or wheat bran as organic amendments not only reduces pathogen inoculum but also likely contributes to increased populations of beneficial organisms that suppress *S. rolfii* in the soil (Beute and Rodríguez-Kábana 1981). Moreover, organic matter enrichment, obtained with the addition of ASD amendments, improves soil physical, chemical, and biological properties (Bailey and Lazarovits 2003; Blum and Rodríguez-Kábana 2004; Bulluck et al. 2002) that can benefit crop production.

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