Research

Effects of Anaerobic Soil Disinfestation on *Sclerotinia sclerotiorum* in Paraguay

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Abstract

The effects of anaerobic soil disinfestation (ASD) on *Sclerotinia sclerotiorum* sclerotia viability was tested in four field trials in Paraguay. Plots were 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), saturated with water, and covered with black plastic mulch for 3 weeks. Control plots were not amended but were saturated and either covered (anaerobic control) or maintained uncovered (aerobic control). Tubes painted with iron oxide paint were placed in soils to assess soil reducing conditions. Sclerotia were buried 6 cm deep in treated and control soils along with temperature data loggers. After 3 weeks, the viability of sclerotia was significantly lower in all ASD-treated soils (4 to 52%) compared with the aerobic control soil (100%), regardless of the

carbon source used. Sclerotial viability was also significantly reduced compared with anaerobic controls at three sites, depending on the carbon source used. A significant negative correlation between soil reducing conditions and sclerotia viability was observed at all sites. Wheat bran and molasses are widely available and inexpensive in Paraguay, and ASD with these carbon sources provides smallholder South American vegetable farmers with a new option for sustainable management of *Sclerotinia* and potentially other soilborne pathogens.

Keywords: anaerobic soil disinfestation (ASD), *Sclerotinia sclerotiorum*, white mold, cultural practices, sustainable agriculture, vegetable crops

Anaerobic Soil Disinfestation as a Sustainable Management Technique for *Sclerotinia sclerotiorum* in Paraguayan Soils

One of the most economically important and widely distributed soilborne diseases in South America is white mold caused by the fungal plant pathogen *S. sclerotiorum*, causing yield losses in a wide range of crops (Durman et al. 2003; Lobo et al. 2000; Wrather et al. 1997). In Paraguay, white mold threatens high-value vegetable crops such as tomato, pepper, and lettuce (Arrúa and Aquino 2013). This disease causes wilting of plants, and as it progresses, cottony white mycelium is formed and sclerotia are produced on, in, and around infected plants. Diseased plants eventually die, returning the sclerotia to the soil and increasing the amount of inoculum (Bardin and Huang 2001).

White mold disease management can be quite challenging for farmers, given the wide host range of the pathogen that limits the effectiveness of crop rotation and the general lack of resistant varieties (Subbarao 1998). In addition, *S. sclerotiorum* sclerotia can

survive for more than 5 years in the soil without a host (Ben-Yephet et al. 1993). When environmental conditions are conducive (20 to 25°C and 93% humidity), sclerotia can produce apothecia in which ascospores are formed (Matheron and Porchas 2005). These ascospores can be blown by the wind and land on susceptible tissue, causing disease (Kohli et al. 1995). Plants may be directly infected by mycelia that germinated eruptively from sclerotia as well (Patterson and Grogan 1985).

Sclerotia are the primary source of inoculum and play the main role in the disease cycle of *S. sclerotiorum*, because epidemics caused by this pathogen are monocyclic (Abawi and Grogan 1979). Therefore, management practices targeted to reducing the viability and number of sclerotia will result in a significant reduction in disease intensity (Ben-Yephet 1988). However, sclerotial pathogens can be particularly difficult to manage because inoculum is heterogeneously distributed in the field and difficult to reach (Hao and Subbarao 2005). In addition, few products are currently available for management of white mold, and many of them provide inconsistent results (Fernando et al. 2004). For this reason, more sustainable and appropriate methods for South American smallholder farmers that can reduce the inoculum in the soil are required.

A promising soil disinfestation technique was developed simultaneously in Japan (Momma et al. 2013) and the Netherlands (Blok et al. 2000) in the late 1990s called anaerobic soil disinfestation (ASD). The ASD process involves the application of soil amendments such as molasses, seed brans, or cover crops into the soil. Once soil amendments are incorporated, the soil is saturated with water and covered with plastic mulch for a period of 3 to

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15 weeks (Momma et al. 2013). During this process, microorganisms break down the carbon sources, producing toxic compounds deleterious to pathogens (Huang et al. 2016b) and leading to temporary anaerobic conditions in the soil (Shennan et al. 2018).

Soil treatment using ASD has been shown to reduce the viability of pathogens that produce sclerotia, such as *Sclerotium rolfsii* (Shrestha et al. 2018), as well as reducing the viability of micro-sclerotia of *Verticillium dahliae* (Goud et al. 2004; Wei et al. 2016) and *Macrophomina phaseolina* (Roskopf et al. 2014). In addition, *Rhizoctonia solani* was significantly suppressed in soils treated with ASD regardless of amendment type, compared with a nonamended control (Huang et al. 2016a). However, Butler et al. (2014) reported that carbon source rates less than 1 mg of carbon per gram of soil at moderate soil temperatures did not reduce the viability of *S. sclerotiorum* sclerotia or Fusarium root rot of common bean compared with the nontreated control. This suggests that ASD treatments have different effects on plant pathogens depending on the type and amount of carbon source used. Furthermore, the effectiveness of the disinfestation may vary considerably depending on environmental conditions and soil types (Testen and Miller 2018).

In Paraguay, environmental conditions and dominant soil types (Oxisols) (Amado and Eltz 2008) are different from those found in areas of the world in which ASD has been conducted previously (Shrestha et al. 2016). The humid tropical climate of the southeastern region is quite different from the more temperate climate in which ASD was tested previously for control of *S. sclerotiorum* (Thaning and Gerhardson 2001). In these tropical conditions, soils are likely to experience higher temperatures and moisture during summer (Amado and Eltz 2008), which could potentially improve ASD performance.

ASD appears to be a promising management technique for smallholder farmers in developing countries. However, before recommending this management practice, it is necessary to carry out trials in tropical soils with carbon sources locally available. The main objective of this study was to evaluate the effects of ASD using different carbon sources (molasses, wheat bran, and the combination of molasses and wheat bran) on *S. sclerotiorum* sclerotia under field conditions at four different sites in Paraguay. We evaluated the effect of ASD on soil temperature, quantified soil surface CO₂ concentration for each carbon source during ASD, quantified soil reducing conditions during ASD, and evaluated the effects of ASD carbon sources (alone and combined) on sclerotia viability.

ASD Treatments

The experiments were performed at four different sites in the southern region of Paraguay (Fig. 1): Paraguayan Center of Agrarian Technologies (CETAPAR), Paraguayan Institute of Agrarian Technologies (IPTA), National University of Asuncion (UNA), and a smallholder farm. The characteristics of the sites are shown in Table 1; three of the locations were research institutions and one a small vegetable farm.

The experimental period was from December 2017 to January 2018. The trials were laid out in a Latin square design with five replications for each treatment with plots of 1 m by 1 m with a buffer zone of 1 m between the rows. Treatments 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), and controls were nonamended and saturated and were either covered (anaerobic control) or uncovered (aerobic control) (Testen and Miller 2019). Carbon sources were manually incorporated into the soil with agricultural tools to a depth

of 15 cm. Molasses was diluted in tap water (1:2), thoroughly mixed, and applied with watering cans. Following amendment incorporation, fields were irrigated overnight using impulse sprinklers (Tramontina, Lambare, Central, Paraguay). As described by Momma et al. (2013), when the soil reached field capacity, plots were covered with black plastic mulch (1.5 mm thickness), making sure the edges were tightly sealed to avoid gas exchange with the environment (Fig. 2). Plots remained covered for 3 weeks, at which time the plastic mulch was removed and evaluations were made.

Data Collection and Analysis

The experimental designs used for data collection during the ASD trials are described in Table 2. Analysis of variance was used to analyze the data, and treatment means were compared using Tukey's honestly significant difference with a 5% family-wise error rate. Statistical analyses were carried out in R version 3.4.4 (R Core Team 2018) using statistical packages *agricolae* (de Mendiburu 2015) and *eRiR* (Stevenson et al. 2015). Data were analyzed separately by location, except for soil temperature, for which data were pooled. For all studies, normality of distributions was checked before analysis, and data were transformed using arcsine square root if appropriate. Nontransformed means and standard deviations were reported. Relationships between soil reducing conditions, measured as percentage of paint removed from indicator of reduction in soil (IRIS) tubes, and sclerotia viability were evaluated using correlation analysis at $P < 0.05$. Pearson (r) coefficients were reported when the correlation was significant.

Soil Temperatures During ASD

Soil temperatures were monitored using Hobo pendant data loggers (Onset Computer Co., Bourne, MA). Data loggers were wrapped in latex to avoid direct contact with the soil and placed

FIGURE 1

Experimental sites for anaerobic soil disinfestation trials in Paraguay. CETAPAR = Paraguayan Center of Agrarian Technologies; IPTA = Paraguayan Institute of Agrarian Technologies; UNA = National University of Asuncion; and smallholder = smallholder farm.

6 cm deep in the center of the plots before covering with plastic mulch. Five data loggers were used in each location (one per treatment). Temperature readings were taken every 30 min for 3 weeks, and mean temperatures were reported.

The effect of location as a blocking factor was significant ($P = 0.0001$). At CETAPAR, the mean soil temperature across treatments was 30°C, and in UNA the mean temperature was 30.2°C.

The mean soil temperature for IPTA was the 30.9°C, whereas at the smallholder farm it was 32.2°C.

Mean soil temperatures across all four sites differed significantly by carbon source ($P = 0.0001$) during the ASD trials. Significantly lower mean soil temperatures were observed in aerobic control plots (29.5°C) than in the anaerobic control and ASD-treated plots (Fig. 3). Mean soil temperatures in wheat bran-amended (31.6°C) and

Trial location	Description	Department	Previous crops	Soil type ^a	Annual precipitation ^b	Coordinates
CETAPAR	Paraguayan Center of Agrarian Technologies	Alto Parana-Yguazu	Peppers, cucumbers	Oxisol	1,600–1,800	55° 31' 44.5919" S, 26° 4' 18.1834" W
IPTA	Paraguayan Institute of Agrarian Technologies	Cordillera-Caacupe	Onions, beans	Entisol	1,400–1,600	56° 30' 0.0194" S, 25° 0' 25.8181" W
UNA	National University of Asuncion	Central- San Lorenzo	Onions, sweet corn	Alfisol	1,400–1,600	57° 29' 24.0004" S, 25° 49' 1.8336" W
Smallholder farm	Small private vegetable farmer	Central- Nemby	Tomatoes, peppers, lettuce	Mollisol	1,400–1,600	59° 17' 20.4" S, 25° 18' 25.818" W

^a USDA soil taxonomy classification.

^b Millimeters.

FIGURE 2

Anaerobic soil disinfestation (ASD) trial setup in Paraguay. **A**, Packets containing 10 sclerotia of *Sclerotinia sclerotiorum* were placed 6 cm deep in the center of soil beds. **B**, Indicator of reduction in soil (IRIS) tubes were placed in the center of soil beds to measure anaerobic conditions during the soil treatment. **C**, Different carbon sources and their combinations were applied and mixed into the beds. **D**, Soil beds were saturated with water and covered with black plastic, and edges were sealed with soil to trap gases produced during ASD.

wheat bran plus molasses-amended (31.9°C) plots were significantly higher than in anaerobic control plots (30.2°C). The mean soil temperature in plots amended with molasses alone was similar to temperatures in the anaerobic control and wheat bran-amended plots and was significantly lower than the temperature of plots amended with wheat bran plus molasses.

Soil Reducing Conditions During ASD

The development of anaerobic conditions in soils can be measured using iron oxide-painted tubes to indicate extent of reducing conditions in soil (Rabenhorst 2010). The reduction of Fe (III) to Fe (II) during anaerobic conditions causes the paint to become soluble, allowing the quantification of areas where paint was removed from the tube (Castenson and Rabenhorst 2006). One IRIS tube (30 cm long, 1.27 cm diameter polyvinyl chloride piping) was inserted into a guide hole in the center of each plot. At the end of the field trials, tubes were removed and washed carefully with tap water to remove soil debris. Areas where the paint was removed were quantified using ImageJ (National Institutes of Health, Bethesda, MD).

Only low levels (0 to 11%) of iron oxide paint were removed from IRIS tubes from control plots across all four sites, and no differences in the percentage of paint removed were observed between tubes placed in either aerobic or anaerobic control soils (Fig. 4). The effects of blocking factors were not significant for any site. At three sites out of four (CETAPAR, IPTA, and smallholder farm), iron oxide paint removal was significantly higher for IRIS tubes placed in ASD-treated soils regardless of the carbon source compared with the aerobic control. However, iron oxide paint removal from IRIS tubes placed in soils amended with molasses and subjected to ASD did not differ significantly from paint removal from tubes placed in aerobic and anaerobic control plots at the UNA site (Fig. 4). The percentage of paint loss on IRIS tubes was not significantly different among ASD-treated soils regardless of the carbon source at three of four sites (CETAPAR, IPTA, and UNA). At the smallholder farmer site, IRIS tube paint removal was significantly lower in soils amended with wheat bran alone compared with those amended with wheat bran plus molasses (Fig. 4).

Carbon Dioxide Concentration After ASD

Soil surface carbon dioxide concentrations were measured using Dräger tubes (Dräger Safety, Lübeck, Germany). Soil air samples were taken from the center of the beds by making small holes in the middle of the plastic mulch and carefully introducing the Dräger tubes to avoid air exchange with the environment. Soil air was drawn through the Dräger tube once using a 100-ml syringe. A calibration scale of CO₂ percent printed on the side of each tube allowed the measurement of the length of coloration showing the CO₂ percentage by volume of sampled air (Hamada and Tanaka 2001).

Soil air CO₂ concentrations differed significantly among treatments in all four sites (Fig. 5). The effect of blocking was not

significant for any site. Significantly lower concentrations of CO₂ were detected in aerobic control soils (0.18 to 0.56% in vol.) than in plastic covered soils (with or without amendment) at three of four sites (CETAPAR, UNA, and smallholder farm). Significant differences in CO₂ production were not observed at the IPTA site between the aerobic and anaerobic controls. Soil CO₂ concentrations ranged from 2.3 to 2.8% in anaerobic control plots. At every site, CO₂ concentration was higher in ASD plots amended with wheat bran plus molasses (5.5 to 6% in vol.) than in the anaerobic control plots. The concentration of CO₂ in plots amended with molasses alone was similar to that of the anaerobic control in three of four sites (CETAPAR, smallholder farmer, and IPTA); CO₂ concentration in plots amended with molasses alone was higher than in the anaerobic control at the UNA site. The CO₂ concentration in ASD-treated plots amended with wheat bran alone was only higher than in the anaerobic control at CETAPAR. No differences in soil CO₂ concentration were observed between soils treated with ASD using molasses alone (3.5 to 5.3% in vol.) or wheat bran alone (3.3 to 4.5% in vol.) at any site (Fig. 5).

Effect of ASD on Sclerotia Viability of *S. sclerotiorum*

The isolate FCQ40-SCSCCOP16 of *S. sclerotiorum* isolated from a pepper production field in Paraguay was used in the field trials (Insaurralde et al. 2017). To produce sclerotia, potato tubers

FIGURE 3

Mean soil temperatures over 3 weeks of anaerobic soil disinfestation (ASD) treatment with different carbon sources at four sites in Paraguay. Error bars indicate standard deviations with four replicates over four sites, and means indicated by different letters are significantly different according to Tukey's test ($P = 0.0001$). A = aerobic control (nonamended, saturated, noncovered); AN = anaerobic control (nonamended, saturated, plastic covered); M = molasses amended (10.1 Mg/ha); W = wheat bran amended (20.2 Mg/ha); and W+M = wheat bran (20.2 Mg/ha) plus molasses (10.1 Mg/ha) amended.

TABLE 2
Experimental design used for data analysis for anaerobic soil disinfestation trials in Paraguay

Variable	Model	Treatment ^a	Replicates	Blocking factor(s)	Experimental units
Soil temperature (°C)	Complete block design	A, AN, W, M, W+M	4	Experimental site	20 in total
Iron oxide paint loss (%)	Latin square design	A, AN, W, M, W+M	5	Row and column	25 per site
CO ₂ concentrations (% in vol.)	Complete block design	A, AN, W, M, W+M	4	Row	20 per site
Sclerotia viability (%)	Latin square design	A, AN, W, M, W+M	5	Row and column	25 per site

^a A = aerobic control (nonamended, saturated, noncovered); AN = anaerobic control (nonamended, saturated, plastic covered); M = molasses amended (10.1 Mg/ha); W = wheat bran amended (20.2 Mg/ha); and W+M = wheat bran (20.2 Mg/ha) plus molasses (10.1 Mg/ha) amended.

were peeled and cut into round slices, placed in glass Petri plates, and autoclaved once for 20 min. Agar plugs from the margins of 3-day-old *S. sclerotiorum* cultures were removed and placed on the autoclaved potato slices, and plates were incubated at room temperature for 4 weeks. Sclerotia were separated from the potato pieces, dried, and stored at room temperature until further use (Matheron and Porchas 2005).

Mesh packets containing 10 sclerotia of *S. sclerotiorum* were placed 6 cm deep in the center of each plot prior to soil saturation.

Once ASD treatment concluded, the sclerotia in the mesh packets were washed with tap water and dried for 24 h. Sclerotia were then surface disinfested with a solution of 70% ethanol (60 s) and another wash with 5% sodium hypochlorite (60 s), followed by a triple rinse in sterile distilled water (60 s each). Neon-blue potato dextrose agar (PDA) medium, which is PDA amended with 100 ppm of rifampicin and 100 ppm of bromophenol blue, was prepared to assess the viability of sclerotia. This medium is semiselective for detection of *Sclerotinia* spp. and changes color from blue to yellow as a result of

FIGURE 4

Indicator of reduction in soil (IRIS) iron oxide paint removal percentage during anaerobic soil disinfestation treatment with different carbon sources evaluated in four sites in Paraguay. Higher values of paint removal indicate higher anaerobicity. Error bars indicate standard deviation with five replicates, and means indicated by different letters are significantly different according to Tukey's test. CETAPAR = Paraguayan Center of Agrarian Technologies ($P = 0.0069$); IPTA = Paraguayan Institute of Agrarian Technologies ($P = 0.0001$); UNA = National University of Asuncion ($P = 0.0004$); and smallholder = smallholder farm ($P \leq 0.0001$). A = aerobic control (nonamended, saturated, noncovered); AN = anaerobic control (nonamended, saturated, plastic covered); M = molasses amended (10.1 Mg/ha); W = wheat bran amended (20.2 Mg/ha); and W+M = wheat bran (20.2 Mg/ha) plus molasses (10.1 Mg/ha) amended.

oxalic acid production upon germination of viable sclerotia (Kawasaki and Machado 2013). Sclerotia were then placed on neon-blue PDA medium to assess their viability. Sclerotia producing white, fluffy mycelia and/or producing oxalic acid (changing the medium from blue to yellow) were considered viable.

All *S. sclerotiorum* sclerotia placed in aerobic control plots at all four sites were viable (Fig. 6) following the 3-week treatment period. Sclerotia viability ranged from 44 to 80% in anaerobic control plots. At the IPTA and UNA sites, significantly fewer sclerotia survived in anaerobic control plots than in aerobic control plots, whereas sclerotia survival did not differ significantly between

the two controls at the CETAPAR and smallholder farmer sites. Sclerotia viability was significantly reduced in soils treated with ASD regardless of the carbon source compared with the aerobic control at all experimental sites (Fig. 6). Differences between carbon source effectiveness in reducing sclerotial survival were observed at two (UNA and smallholder farm) sites.

Viability ranged from 6 to 20% across sites for sclerotia recovered from ASD-treated soils amended with wheat bran alone or in combination with molasses, whereas for soils amended with molasses alone, viability ranged from 22 to 52%. At three sites (CETAPAR, UNA, and IPTA), the viability of sclerotia removed

FIGURE 5

Soil CO₂ concentration (%) by volume measured after anaerobic soil disinfestation treatment at four sites in Paraguay. Error bars indicate standard deviations with five replicates, and means indicated by different letters are significantly different according to Tukey's test. CETAPAR = Paraguayan Center of Agrarian Technologies ($P < 0.0001$); IPTA = Paraguayan Institute of Agrarian Technologies ($P = 0.0004$); UNA = National University of Asuncion ($P < 0.0001$); and smallholder = smallholder farm ($P < 0.0001$). A = aerobic control (nonamended, saturated, noncovered); AN = anaerobic control (nonamended, saturated, plastic covered); M = molasses amended (10.1 Mg/ha); W = wheat bran amended (20.2 Mg/ha); and W+M = wheat bran (20.2 Mg/ha) plus molasses (10.1 Mg/ha) amended.

from wheat bran-amended soils did not differ from that of sclerotia recovered from molasses-amended soils (Fig. 6). However, at the smallholder farm, significantly more viable sclerotia were recovered from plots amended with molasses alone than from wheat bran-amended plots. Sclerotia viability in soils treated with ASD using the combination of wheat bran plus molasses (4 to 20%) did not differ significantly from viability in ASD-treated soils amended with wheat bran alone across all sites. Significantly more sclerotia recovered from molasses-amended plots were viable than those

removed from plots amended with wheat bran plus molasses at two (UNA and smallholder farm) sites (Fig. 6).

A significant negative correlation was observed between sclerotia viability and soil reducing conditions as measured by iron oxide paint loss across the four sites with correlation values (r) ranging from 0.58 to 0.76 (Fig. 7). Determination coefficients (R^2) were lower than 0.60 for all of the sites. This negative relationship suggests that as soils become more anaerobic, the viability of the sclerotia of *S. sclerotiorum* decreases.

FIGURE 6

Effect of anaerobic soil disinfestation using different carbon sources on the viability of sclerotia of *Sclerotinia sclerotiorum* in four different sites in Paraguay. Error bars indicate standard deviation with five replicates, and means indicated by different letters are significantly different according to Tukey's test. CETAPAR = Paraguayan Center of Agrarian Technologies ($P = 0.0003$); IPTA = Paraguayan Institute of Agrarian Technologies ($P \leq 0.0001$); UNA = National University of Asuncion ($P < 0.0001$); and smallholder = smallholder farm ($P \leq 0.0001$). A = aerobic control (nonamended, saturated, noncovered); AN = anaerobic control (nonamended, saturated, plastic covered); M = molasses amended (10.1 Mg/ha); W = wheat bran amended (20.2 Mg/ha); and W+M = wheat bran (20.2 Mg/ha) plus molasses (10.1 Mg/ha) amended.

ASD: A Promising Tool for Paraguayan Smallholder Farmers for the Sustainable Management of *S. sclerotiorum* and Potentially Other Soilborne Pathogens

ASD is a soilborne disease management practice that is appropriate for smallholder farmers. In most cases, this technique has been reported to be effective for the control of soilborne pathogens in developed countries such as the United States of America, Japan, the Netherlands, Italy, Spain, the United Kingdom, and Sweden (Guerrero-Díaz and Lacasa-Martínez 2013; McCarty et al. 2014; Momma et al. 2010; Paudel et al. 2018; Runia et al. 2014; Shrestha et al. 2016; Thaning and Gerhardsson 2001). Only one study on the efficacy of ASD in South America has been reported, in an Argentinian greenhouse cut flower production system (Yossen et al. 2008). Smallholder farmers in Paraguay, as in other developing countries, have few options for soilborne disease management besides the use of chemical fumigants (Parsa et al. 2014). For this reason, ASD has high potential as a management practice for

reduction of *S. sclerotiorum* viable inoculum, being relatively easy and inexpensive for Paraguayan farmers.

During ASD, an increase in soil temperature is observed as a result of the metabolization of carbon sources by soil microorganisms (Achmon et al. 2016). In the present study, mean soil temperatures in ASD-treated soils were significantly higher than in the aerobic control by 1.4 to 2.4°C. Similarly, during ASD trials in Ohio muck soil (Huron and Stark county), mean soil temperature of ASD-treated plots increased by 1.5 up to 6.3°C compared with aerobic control plots (Testen and Miller 2019). For all sites in this study, the mean soil temperatures across treatments ranged from 30 to 32.2°C. These mean soil temperatures can be considered moderate for ASD according to the classification proposed by Shrestha et al. (2016). In contrast to soil solarization, ASD does not rely on high temperatures to reduce the viability of soilborne pathogens. However, ASD effectiveness decreases at lower temperatures, leading to longer tarping period requirements (Butler et al. 2014).

FIGURE 7

Pearson's correlation value (r) for the relation between indicator of reduction in soil (IRIS) paint removed and *Sclerotinia sclerotiorum* sclerotia viability evaluated in four sites in Paraguay. CETAPAR = Paraguayan Center of Agrarian Technologies ($P = 0.0049$); IPTA = Paraguayan Institute of Agrarian Technologies ($P < 0.0001$); UNA = National University of Asunción ($P = 0.00252$); and smallholder = smallholder farm ($P = 0.0001$). Higher values for paint removal indicate higher anaerobicity of the soil.

Higher microbial activity was indicated by higher amounts of CO₂ emitted from soils treated with ASD using the combination of wheat bran plus molasses than from aerobic and anaerobic control soils. During the initial phase of ASD, bacterial oxidation of organic matter has the potential to raise the concentration of CO₂ within the soil (Achmon et al. 2016). Runia et al. (2014) showed that CO₂ concentrations increased with time during ASD treatment, indicating the breakdown of organic matter through microbial activity. High soil CO₂ levels reflect the change in microbial biomass, indicating a shift to a bacterial-dominated community (van Agtmaal et al. 2015). It is likely that by combining amendments and increasing amendment rates, specific members of microbial communities could have greater access to utilizable carbon and produce higher amounts of byproducts, such as organic acids and gases deleterious to sclerotia. Runia et al. (2014) developed a “cocktail” of ASD carbon sources called “Herbie.” When different soils were treated with “Herbie,” higher concentrations of acetic and butyric acids that correlated negatively with the survival of *Globodera pallida* were observed compared with nontreated soils.

Low oxygen levels (2%) and high concentrations of carbon dioxide (19%) have been shown to inhibit the germination of *S. minor* (Imolehin and Grogan 1980). However, the mechanisms of pathogen suppression in ASD are more complex than soil anaerobicity, and other factors such as microbial activity (Mowlick et al. 2013), production of organic acids (Momma et al. 2006) and deleterious gases (Runia et al. 2014), degradation of fungal cell walls by anaerobic bacteria (Ueki et al. 2017), and enhancement of mycoparasitic antagonism (Shrestha et al. 2018; Thaning and Gerhardson 2001) may be involved in reducing sclerotia viability. Although these other factors affecting sclerotia survival were not evaluated during this study, soil anaerobicity is related to these pathogen suppression factors (Shennan et al. 2018). Moreover, increased soil anaerobicity has been linked to higher levels of control of *Fusarium oxysporum* (Momma et al. 2011), *V. dahliae* (Shennan et al. 2018), *Meloidogyne* spp. (Testen and Miller 2018), and *S. rolfii* (Shrestha et al. 2018).

Sclerotia from molasses-amended ASD-treated soils were not less viable than those from anaerobic control soils at three of four sites. Similarly, ASD using molasses alone did not reduce *R. solani* propagules in the soil compared with the nonamended control (McCarty et al. 2014). Similarly, no significant differences in anaerobic soil conditions were observed between molasses-amended soils compared with nonamended control soils during 2 years of field trials (Butler et al. 2012a). Honma et al. (2012) reported that the application of molasses in paddy soil did not lead to the development of anaerobic conditions owing to its limited percolation. Limited percolation of molasses was observed in our study as well. However, this restraint can be overcome by molasses dilution in water followed by thorough mixing and immediate application (Momma 2008). Also, the rate of molasses applied was half that of wheat bran. It is possible that increasing the rate of molasses may increase pathogen suppression in ASD treatments in which this carbon source is used.

In the present study, wheat bran-amended ASD was as effective in reducing sclerotial survival of *S. sclerotiorum* as the combination of wheat bran plus molasses in all the sites. The use of wheat bran as a carbon source for ASD led to the production of acetic and butyric acids up to 2,000 and 1,900 mg/kg of dry soil, respectively, concentrations that were deleterious to *Ralstonia solanacearum* and *F. oxysporum* f. sp. *lycopersici* (Momma et al. 2006). Moreover, the incidence of Fusarium wilt (caused by *F. oxysporum*) in a carnation crop was significantly reduced (by 84 to 97%) in ASD-treated soils using wheat bran as an amendment (Yossen et al. 2008).

Likewise, soil populations of *V. dahliae* were significantly reduced by wheat bran-based ASD treatments during bioassays (Testen and Miller 2018).

Although wheat bran alone and wheat bran plus molasses were equally effective in ASD for reducing viability of *S. sclerotiorum* sclerotia, it may be advantageous to use the combination to improve overall suppression of a range of soilborne pathogens (Katase et al. 2009; Momma et al. 2006; Mowlick et al. 2012; Shrestha et al. 2018; Yossen et al. 2008). These organic amendments are inexpensive and readily available to Paraguayan farmers. Wheat is broadly cultivated and processed in Paraguay, and wheat bran is a byproduct of milling whole grains (Alegre 2017). Similarly, molasses is a byproduct obtained from the processing of sugarcane, which is also widely grown in Paraguay (Hira and Torres-Garcete 2011). Even though these organic amendments are relatively inexpensive, costs related to transportation and labor could potentially increase ASD costs. Ideally, in order to reduce costs, cover crops could be grown and incorporated into the soil as potential carbon sources for ASD (Butler et al. 2012b). However, more research is needed on optimum carbon source rates, tarping period, and plastic covers before farmers are likely to adopt ASD as a common practice in Paraguay.

In summary, ASD reduced the viability of sclerotia of *S. sclerotiorum* after 3 weeks of treatment in different soil types and environments in Paraguay. Differences between carbon source effectiveness to reduce sclerotia survival were observed in two out of four sites. More research will be required to achieve the same effectiveness regardless of potential differences in sites. Future work should examine different rates and carbon source combinations and their effect on different pathogens (bacteria, plant parasitic nematodes, and oomycetes), considering various depths of disinfection. The use of on-farm trials can help in disseminating this technology to farmers in Paraguay and in other South American countries. In addition, an evaluation of the economic impact of this management tool on crop profitability considering the time and inputs necessary in each region will be necessary to promote adoption of ASD by smallholder farmers.

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