UPCYCLING ORGANIC WASTE FOR THE SUSTAINABLE MANAGEMENT OF SOILBORNE PESTS AND PATHOGENS IN AGRI-FOOD SYSTEMS

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Editorial: Upcycling organic waste for the sustainable management of soilborne pests and pathogens in agri-food systems

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circular economy, soil disinfestation, biofumigation and organic amendment, sustainable pest control management, soil microbial communities, organic acids

Editorial on the Research Topic
Upcycling organic waste for the sustainable management of soilborne pests and pathogens in agri-food systems

Introduction

Agricultural and food supply systems face the challenge to meet a delicate balance between supporting the increasing demand for food and minimizing their environmental impact. Two main challenges dominate these impacts. First, the accumulation of organic wastes at each stage of the whole food chain process. Second, to protect crops against pests and pathogens without applying hazardous chemicals. This is translated in stricter regulation. Organic wastes are being banned from landfill disposal (i.e., California Senate Bill 1383 or Directive EU 2018/851 of the European Parliament and of the Council of 30 May 2018 amending Directive 2008/98/EC on waste); and hazardous chemicals such as methyl bromide are being banned worldwide (i.e., Montreal protocol). To guarantee the success of these regulations without disrupting the demanding growing population economy and environment, the development of novel technologies to upcycle organic residues is an urgent need. This aligns with the increasing consumer's demand for sustainable food products, which offers a unique opportunity to develop more sustainable agricultural practices by revalorizing agri-food by-products. Composting, soil bio-fumigation, biosolarization and anaerobic soil disinfestation (ASD) are some of the practices included in this topic that can turn agri-food by-products into substitutes for chemical fumigants as well as improve soil health. This Research Topic covers a

TABLE 1 Summarizing table of key information of the Research Topic articles.

Topic reference	Technology	Organic wastes	Soilborne pests and pathogens	Cropping system
Axelrod et al.	Biosolarization	Insect compost		Lettuce
Gandariasbeitia et al.	Soil biodisinfestation	Beer bagasse Defatted rapeseed cake Fresh cow manure	*Meloidogyne incognita*	Lettuce
Guerrero et al.	Soil biodisinfestation	Wheat husk Fresh sheep manure Sunflower pellets	*Phytophthora capsici*	Bell pepper
Khadka and Miller	ASD*	Wheat bran Molasses Chicken manure Mustard greens	*Rhizoctonia solani*	Radish
Larregla et al.		Fresh sheep manure Poultry manure	*Phytophthora capsici*	Protected pepper crops
Muramoto et al.	Integrated Soil Health Management	NA		Strawberries
Serrano Perez et al.	Biofumigation	Brassica carinata	*Phytophthora nicotianae*	Paprika Pepper
Vincent et al.	ASD	Molasses Composted poultry litter	NA	Tomato
Shrestha et al.	ASD	Molasses Soybean hulls Wheat bran Corn starch	NA	Bell pepper Tomato Eggplant
Swilling et al.	ASD	NA	*Sclerotium rolfsii*	NA
Testen et al.	ASD	Corn gluten meal Distillers dried grains Soybean meal Wheat bran Dry sweet whey Cover crops	*Pyrenochaeta lycopersici, Colletotrichum coccodes, Verticillium dahliae, Meloidogyne spp.*	Tomato
Avidov, Varma, Saadi, Hanan, et al. Avidov, Varma, Saadi, Khoury, et al.	Composting	Broiler litter	*Salmonella*	NA

*Anaerobic Soil Disinfestation.

wide range of articles dealing with the challenges of organic by-products amendment-based approaches to understand the main mechanisms of soilborne pests and pathogens inactivation. Table 1 summarizes the wide diversity of by-products, pathogens, and crop systems where these technologies can be applied, highlighting their potential impact. Being technologies based on the biological degradation of exogenous labile organic matter, many of the published articles focused on the characterization of the microbial communities. Realistically, up to now, there is no silver bullet. Therefore, Muramoto et al., explores the concept of Integrated Soil Health Management (ISHM) to better address management strategies for soil-borne disease and overall soil and plant health.

Outcomes and challenges on organic wastes

There is no one stop solution for managing organic waste meaning a constant optimization process is needed. Despite the consensus regarding their adverse effects, landfilling and incineration are still the waste disposal techniques most used around the world (Siddiqua et al., 2022). In the European Union, incineration is considered adequate only if it delivers the best environmental outcome possible, such as energy recovering (Directive 2008/98/EC on waste). The term "organic waste" being phased out and replaced by "recycled organic resources" should be the short/mid-term inspiring goal.

Scientific research is the basis for any future implementation of new solutions, and the rapid advancement in the areas of big data, artificial intelligence, "omics" should also lead the way in organic waste upcycling. An ecological approach should be considered as the way to move forward, where farms becomes a "niche" that must be balanced in term of inputs and outputs of resources (Oren et al., 2018). Replacing hazardous chemicals by sustainable biological treatments is a priority. The challenge is that often agrochemicals, including synthetic soil fumigants are cheaper and more reliable than biological solutions. In this Research Topic readers can identify the diversity of by-products employed to face agronomical challenges (Table 1) and their potential application as future "weapons" in the constant "arms race" against soilborne pests and pathogens.

Outcomes and challenges on mechanisms of soilborne pests and pathogens management

The present Research Topic also describes some biological, chemical and physical mechanisms involved in pest inactivation. A better understanding of their mode of action can contribute to improve the efficacy. This includes understanding how abiotic factors such as soil temperature, pH, redox conditions directly or indirectly affect pests and pathogens or increase their susceptibility to bioactive compounds. High soil temperature is one of the most direct mechanisms in pests/pathogens inactivation particularly during solarization and biosolarization (Dahlquist et al., 2007). Higher temperatures also have shown to make weeds more susceptible to organic acids (Fernández-Bayo et al., 2020). Understanding the role temperature plays is particularly important to manage pathogens in regions or seasons characterized by milder temperatures (Henry et al., 2020; Vecchia et al., 2020). The temporary development of anaerobic conditions is also key to manage soil borne pest and pathogens directly due to the lack of oxygen (Khadka et al.). Indirectly, anaerobic conditions promote the shift of soil microbiome (Hewavitharana et al., 2019); the reduction of metal ions such as Fe^{2+} and Mn^{2+} (Momma, 2015); or the fermentation of the added labile organic carbon and the generation of organic acids (Momma, 2008; Fernández-Bayo et al., 2020). One goal should be therefore maximizing the generation of well-established bioactive compounds such as volatile fatty acids (Momma e al., 2006), glucosinolates (Gimsing and Kirkegaard, 2009), and other compounds that need to be studied. In this context, understanding the soil biological activity is key. On the one hand, there is a need to understand the short-term interactions of the soil microbial community with the exogenous microorganisms and nutrients applied with the organic by-products (Fernández-Bayo et al., 2019). On the other hand, microbes can also directly suppress the target pest and pathogen (Mazzola, 2007). This requires understanding microbial community changes and their resilience and legacy effect in the soil post-treatment. Ultimately, only treatments capable of preventing pathogen recolonization in the long-term (Rosskopf et al., 2020) will guarantee the sustainability of these technologies and their adoption by farmers. Among these mechanisms, this Research Topic highlights some promising information on the promotion of soil suppressiveness after ASD (i.e. Gandariasbeitia et al.) as well as the key role of organic acids on pest inactivation (i.e. Swilling et at.).

Trends on sustainable soilborne pests and pathogens management

The use of organic amendments for the management of soilborne pests and pathogens has a long history; however, only after the phase out of methyl bromide, we have seen a renewed interest and a more consistent research effort toward the development of biological soil disinfestation methods (Rosskopf et al., 2020). The present Research Topic introduces the importance of employing ISHM strategies and provides important updates on some of the most promising amendment-based biological techniques available for the management of soil health with a focus on the management of soilborne pests and pathogens. The updated state of the art on the development and optimization of soil managements biotechnologies such as ASD, biosolarization, biofumigation and composting presented in this Research Topic contributes to disclose the great potential of employing by-products of the agri-food industry, otherwise considered waste, as a resource for the management of soilborne pests and pathogens. The evaluation of the performance of such biotechnologies employing a variety of organic by-products to manage different pathosystems under different environmental conditions, is proof of a new trend and of a renewed research effort aimed at developing more sustainable soil health management practices. However, none of this will be successfully implemented without proper involvement and training of farmers. Furthermore, the enhanced capacity to analyze the soil microbiome, is greatly expanding our understanding of the key role the soil microbiome plays in determining soil health in an agroecosystem. The possibility to characterize the soil microbiome and correlate shift of the soil microbial communities to specific soil inputs and to the suppression of specific pathosystems represents a great opportunity for the advancement of the biotechnologies considered in this Research Topic. The gain of such perspective further highlights the risks associated with the employment of soil disinfestation practices that indiscriminately reduce the soil microbial population. Overall, this Research Topic contributes to enhance our awareness of the need for more sustainable and integrated soil health management practices.

Author contributions

JF-B has led, written and reviewed this manuscript. YA, MG, and FD have written and reviewed this manuscript. All authors contributed to the article and approved the submitted version.

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Synergy of Anaerobic Soil Disinfestation and *Trichoderma* spp. in Rhizoctonia Root Rot Suppression

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Potential synergy between anaerobic soil disinfestation (ASD) and *Trichoderma* spp. in suppression of Rhizoctonia root rot in radish was evaluated. A split-plot design with three replications was used; main plots were *Trichoderma harzianum* T22, *Trichoderma asperellum* NT25 and a non-*Trichoderma* control. Subplots were ASD carbon sources wheat bran, molasses, chicken manure, and mustard greens and two non-amended controls: anaerobic (covered and flooded) and aerobic (not covered or flooded). Carbon sources and *Rhizoctonia solani* inoculant were mixed with soil, placed in pots, and flooded, followed by drenching *Trichoderma* spore suspensions and sealing the pots in zip-lock bags. After 3 weeks, bags were removed, soil was aired for 1 week and radish "SSR-RR-27" was seeded. Rhizoctonia root rot severity and incidence were lowest in radish plants grown in ASD-treated soil amended with wheat bran, molasses, or mustard greens across all *Trichoderma* treatments. Disease severity was lower in radish plants treated with NT25 than with T22 or the non-*Trichoderma* control across all ASD treatments, and in radish grown in ASD-treated soil amended with wheat bran plus NT25 compared to ASD-wheat bran or NT25 alone. *Rhizoctonia solani* populations were significantly reduced by ASD treatment regardless of carbon source, while *Trichoderma* populations were not affected by ASD treatment with the exception of ASD-mustard greens. The interactions of either *Trichoderma* isolate and ASD with most carbon sources were additive, while T22 with ASD-molasses and NT25 with ASD–wheat bran interactions were synergistic in reducing disease severity. One interaction, T22 with ASD-chicken manure was antagonistic. Enhancement of ASD efficacy in suppressing soilborne diseases such as Rhizoctonia root rot by additional soil amendment with *Trichoderma* spp. during the process appears to be dependent on both *Trichoderma* isolate and ASD carbon source.

Keywords: reductive soil disinfestation, biocontrol, soilborne pathogen, radish, anaerobic soil disinfestation, *Trichoderma* spp., Rhizoctonia root rot

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INTRODUCTION

Rhizoctonia solani is an important soilborne plant pathogen that causes diseases including root rot, crown rot, damping off, and foliar blight in numerous economically important plant species (Ajayi-Oyetunde and Bradley, 2018). *Rhizoctonia solani* frequently produces highly resilient sclerotia, has a wide host range, and is composed of diverse groups (anastomosis groups), making it difficult to

manage (Ohkura et al., 2009). These diseases are particularly challenging in vegetable production systems because of the succulent nature of the plants, lack of resistant cultivars, and limited fungicide efficacy. Emerging and young seedlings are particularly susceptible to *R. solani* (Jaiswal et al., 2019).

Anaerobic soil disinfestation (ASD) is a promising tool to manage soilborne diseases in vegetable crops (Shennan et al., 2014; Testen and Miller, 2019). The broad-spectrum efficacy of ASD against nematodes, plant pathogens, and weeds is attractive to growers (Butler et al., 2012). Anaerobic soil disinfestation (ASD) treatment includes the incorporation of easily decomposable carbon sources into the soil, irrigation to saturation, and covering with plastic to create anaerobic conditions (Butler et al., 2014). During decomposition of carbon sources, microbial activities increase and organic acids and volatile compounds are released, which results in significant changes in soil pH, metal ion availability, and microbial community composition (Momma et al., 2005; Momma, 2015). These changes and their interaction with other soil and environmental factors have negative impacts on plant pathogens in soil (van Agtmaal et al., 2015). However, considerable increase in fungal diversity and microbial activity in soil after ASD treatment have been reported (Zhao et al., 2018). Microbial community shifts in ASD-treated soils are particularly driven by carbon source inputs (Mazzola et al., 2018; Testen and Miller, 2018).

Trichoderma spp. are widely studied and commonly used beneficial fungi (Benítez et al., 2004; Singh et al., 2014; Harman et al., 2019). They have multifaceted benefits in crop production including growth promotion (Altomare et al., 1999), disease suppression (Vinale et al., 2009; Widmer, 2014), soil remediation (Vankar and Bajpai, 2008; Tripathi et al., 2013), and nutrient mobilization in soil (Khalili et al., 2012). *Trichoderma* spp. suppress soilborne diseases through mechanisms including mycoparasitism, production of antibiotics, induced systemic resistance, and competitive rhizosphere colonization (Benítez et al., 2004). Some *Trichoderma* isolates have been reported to grow normally up to 37–40°C (Pedreschi et al., 1997; Poosapati et al., 2014), producing stress protectant sugars such as trehalose, mannose, and raffinose under high temperatures to adapt to extreme conditions (Poosapati et al., 2014). *Trichoderma* isolates have been shown to grow in conditions of extremely high and low pH (Chovanec et al., 2005) and salinity (Gal-Hemed et al., 2011) by utilizing diversified secondary metabolic processes. Chovanec et al. (2005) also reported that some *Trichoderma* isolates survived under oxygen-deficient conditions using fermentative metabolism.

Several *Trichoderma* isolates naturally parasitize fungal sclerotia, which leads to a substantial mortality of these structures in soil (Geraldine et al., 2013). Production of cell wall-degrading enzymes by *Trichoderma* spp., stimulated in the presence of fungal sclerotia, is responsible for their mortality in soil (Geraldine et al., 2013). Anaerobic soil disinfestation (ASD) treatment has been shown to increase both endemic and artificially inoculated populations of *Trichoderma harzianum* in soil (Shrestha et al., 2019). However, they found no added benefit of combining *T. harzianum* or *Trichoderma asperellum* inoculation and ASD in increasing mortality of sclerotia of *Sclerotium rolfsii*.

The present study was designed to determine if carbon source differentially affects the survival of two *Trichoderma* species isolates during ASD, and if the isolates act synergistically with ASD to suppress Rhizoctonia root rot of radish caused by *R. solani*. *Trichoderma asperellum* NT25 is a native isolate of Nepal effective in reducing Rhizoctonia root rot disease caused by *R. solani* in radish and clubroot caused by *Plasmodiophora brassicae* in mustard greens (unpublished data). We tested *T. asperellum* NT25, isolated from the mid-hill region of Nepal in 2016 and commercial isolate *T. harzianum* T22 in this study. T22 is a commercially well-established strain of *Trichoderma* developed by protoplast fusion of two *T. harzianum* isolates, which are reported as benomyl-resistant, rhizosphere-competent, and suppressive to several fungal and oomycete pathogens (Sivan and Harman, 1991).

MATERIALS AND METHODS

Fungal Isolates and Inoculum Preparation

Rhizoctonia solani SAM-RS-33.1-2016 isolated from radish and previously determined to be pathogenic on radish was used. The pathogen was retrieved from long term storage on twice-autoclaved winter rye seed by culturing on acidified potato dextrose agar medium (aPDA; 39 g PDA (IBI Scientific, Dubuque, IA), 750 µl lactic acid per L).

Inoculum was prepared in soil potato mix (Ko, 1971) with minor modifications. One hundred twenty-five milliliter of sandy soil was mixed with 13 g peeled and chopped potato and 25 ml distilled water in a 250 ml Erlenmyer flask. The mixture was autoclaved (121°C, 16 PSI for 30 min) twice at 24 h intervals. Pieces ≈1–2 cm in size were cut from the edge of one 7-day-old *R. solani* culture on PDA medium per flask and added to the soil-potato mixture. The mixture was agitated every 3 days by hand. After 21 days, the *R. solani*-inoculated soil-potato mixture was stirred thoroughly with a glass rod, vortexed briefly, poured onto paper towels and allowed to dry in a laminar flow hood overnight. The dry mixture was passed through a 2 mm mesh sieve followed by a 0.59 mm mesh sieve. The inoculum was stored at 4°C. Soil was inoculated at the rate of 0.6 g per liter of soil.

Trichoderma harzianum T22 was applied as the commercial formulation of the biocontrol product RootShield-WP (BioWorks, Victor, New York, USA; Sivan and Harman, 1991). *Trichoderma asperellum* NT25 maintained on silica gel (Samuels, 2015) was grown on PDA plates for 7 days at 25 ± 2°C with a 12 h light/dark cycle. Ten milliliters of sterile water was poured in each *Trichoderma* culture plate and a sterile plastic inoculating loop was used to dislodge the conidia. The suspensions were placed into 5 ml test tubes and vortexed briefly, then passed through four layers of sterilized cheesecloth to remove hyphae and mycelia. Conidia were counted with a hemocytometer and the final concentrations were adjusted to 10^5 conidia ml^{-1} by addition of sterile deionized water.

Soil Attributes

Certified organic field soil was collected from Badger Farm, OSU CFAES Wooster Campus in November 2016 and sealed in plastic bags. Soil was dried, ground, homogenized, and screened through 1 cm mesh before storing at 10°C until use. Soil pH was 6.8, organic matter was 1.7% and cation exchange capacity was 10.7 (med/100 g; Spectrum Analytic Inc., Washington Court House, OH).

Evaluation of ASD and *Trichoderma* for Suppression of *R. solani*

Experiments were established in a split plot design in which *Trichoderma* isolates were the main plot treatments and carbon sources were subplot treatments. Soils were treated with ASD and/or *Trichoderma* in 10-cm-diameter (350 ml) plastic pots with drainage holes in the bottom. Carbon sources were raw chicken manure, wheat bran, molasses, and mustard greens biomass (**Table 1**). Wheat bran, chicken manure, and mustard greens biomass were mixed with soil before placement in the pots. Molasses was mixed with an equal volume of water and poured onto soil in pots. Mustard greens "Southern Giant Curled" seeds (Thiram® treated seed, Seedway, Hall, NY, USA) were sown into 50-cell plug trays containing Baccto Professional Grower Mix (Houston, TX) and grown for 30–40 days under greenhouse conditions programmed at 25°C and 14-h light. Plants were uprooted and washed in tap water followed by chopping and maceration of entire plants in a blender (Waring Commercial Blender, Waring Commercial, Torrington, CT). Mustard greens biomass was applied at 10 g dry matter kg^{-1} soil equivalent to ≈100 g fresh biomass kg^{-1} soil, and chicken manure, wheat bran and molasses were applied at 10 g kg^{-1} soil.

Pots were flooded with ≈300 ml tap water and allowed to drain for about 2 h. One IRIS (Indicator of Reduction in Soils; Rabenhorst, 2012) tube, a 1.3 cm diameter polyvinyl chloride (PVC) pipe painted with iron oxide paint (Rabenhorst, 2008), was inserted into the soil through a guide hole in the center of each pot to measure reducing conditions in the soil during treatment. For treatments including *T. asperellum* NT25, a 525 µl suspension of 1.5×10^6 conidia ml^{-1} of the isolate was pipetted into each pot. Rootshield WP (6% v/v suspension) was inoculated at 1 ml kg^{-1} soil. Pots were then double-bagged with (946 ml) zipper plastic bags (Ziploc®, S.C. Johnson and Son, Racine, WI), sealed and incubated in growth chambers for 25 days on a 12 h light/30°C−12 h dark/25°C cycle. Soil samples (≈100 g) were collected in paper bags immediately after removal of the plastic bags for identification and enumeration of microorganisms. Two non-amended control treatments—anaerobic and aerobic controls were also included. Anaerobic control pots received 300 ml water and were sealed within plastic bags, while aerobic control pots received 300 ml water but not sealed.

TABLE 1 | Characteristics of carbon sources for anaerobic soil disinfestation used in the experiments.

Carbon source[z]	Rate (g kg^{-1} soil)	C:N ratio	Source
Raw chicken manure	10	9.5	Poultry Research Farm, OSU CFAES Wooster Campus
Wheat bran	10	17	The Mennel Milling Company, Fostoria, OH
Molasses	10	81	Golden Barrel Blackstrap Molasses, Good Food, Inc., Honey Brook, PA
Mustard greens biomass	10	12	"Southern Giant Curled" grown for 30–40 days in greenhouse

[z] *Mustard greens were used on a dry matter basis equivalent to 100 g fresh weight.*

Soil Attributes After ASD Treatment

Soil moisture percentage affects soil reducing conditions, therefore soil gravitational moisture was determined. Soil samples of ≈50 g were placed in paper envelopes immediately after removal of plastic bags from ASD-treated and anaerobic control pots, as well as aerobic control pots, and soil weights were recorded. Then soil samples were dried at 60°C in a hot air oven for 48 h and final weights recorded. The soil moisture percentage was calculated by using the following formula:

$$\frac{(Weight\ of\ the\ soil\ before\ drying - Weight\ after\ drying)}{Soil\ weight\ before\ drying} \times 100$$

Soil reducing conditions in each pot were determined based on the loss of iron oxide paint on IRIS tubes inserted in soil in pots prior to treatment. The percentage of paint removal from pipes was assessed visually using a grid after rods were removed from the pots.

Enumeration of *Trichoderma* in ASD-Treated Soil

Additional soil samples (total ≈50 g) removed from three locations in each pot using a metal spatula immediately after termination of ASD and control treatments were dried at room temperature for1 week in a paper envelope. Soil samples were then broken up by pounding the envelope with a rubber mallet followed by thorough mixing by gently shaking the envelope. Five grams of soil were taken from each sample and mixed with 45 ml sterile deionized water and vortexed briefly. One hundred microliters of this suspension were added to 900 µl sterile water in a 1.2 ml well of a 96 deep well plate (Uniscience Corporation, Miami Lakes, FL). The 10^{-2} suspension was serially diluted to 10^{-4} and 200 µl suspensions from the 10^{-3} and 10^{-4} dilutions were spread-plated onto 85 mm plates containing *Trichoderma* selective medium (Askew and Laing, 1993). Total *Trichoderma* spp. colonies were counted 10 days after plating and colony forming units (CFU) g^{-1} soil were calculated (Foght and Aislabie, 2005).

Quantitative PCR Assay for *R. solani* Population Quantification in ASD-Treated Soil

Rhizoctonia solani populations in soil were quantified by using a SYBR Green-based qPCR assay (Lievens et al., 2006) targeting the *R. solani* rDNA internal transcribed spacer (ITS) region. Soil DNA was extracted using the DNeasy® PowerSoil® Kit (Qiagen Hilden, Germany) following the manufacturer's instructions. The qPCR assay was performed in a total volume of 20 μl as follows: 2 μl of target DNA, 10 μl SYBR Green premix Ex Taq (2×, Takara Bio Inc., Otsu, Shiga, Japan), 1 μl of 10 μM forward primer (ST1—AGTGTTATGCTTGGTTCCACT), 1 μl of 10 μM reverse primer (ITS4—TCCTCCGCTTATTGATATGC), and 6 μl nuclease free water. The PCR cycle was set at 95°C for 2 min, followed by 95°C for 10 s and 60°C for 34 s, for 40 cycles in a thermal cycler (Bio-Rad C100 Touch Thermal Cycler, Bio-Rad, Hercules, CA, USA). A melting curve analysis was generated at the end of the qPCR assay by monitoring fluorescence from the PCR solution during heating to 95°C, cooling to 60°C, and slowly heating to 95°C at 0.1°C s^{-1} to evaluate the amplification specificity.

Genomic DNA was extracted from 7-day-old colonies of *R. solani* SAM-RS-33.1-2016 grown from a single hyphal tip on PDA at room temperature in the dark. After grinding the mycelia in liquid nitrogen, nucleic acid was extracted using a Promega Wizard genomic DNA purification kit (Promega Corporation, Madison, WI) following the manufacturer's instructions. Concentration and quality of the DNA were measured with a NanoDrop ND 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Three subsets of eight-fold dilutions of genomic DNA (74 ng μl^{-1} to 7.4 fg μl^{-1}) were also run in the same qPCR assay to generate a standard curve. ITS copy numbers were calculated by using the following formula (http://scienceprimer.com/copy-number-calculator-for-realtime-PCR).

$$\textit{Number copies (molecules)} = \frac{Xng \times 6.0221 \times 10^{23} molecules/mole}{(N \times 660\, g/mole \times 1 \times 10^{9}\, ng/g)}$$

Where X = amount of amplicon (ng), N = length of ds DNA amplicon, which is 187 bp for ST1, 660 g/mole = average mass of 1 bp ds DNA.

Rhizoctonia Root Rot Bioassay With Radish (*Raphanus sativus* L.)

After ASD treatment, soil was allowed to air for 1 week to dissipate volatile compounds generated during the ASD process. Sixteen hybrid radish seeds cv. SSR-RR-27 (Seed Science, Salinas, CA) were planted per ASD-treated and control pot. Light irrigation was provided after sowing and pots were maintained in a growth chamber with the light and temperature regime mentioned above. Relative humidity of the chamber was maintained above 85% to facilitate infection. Plants were irrigated daily with tap water and no additional nutrients were supplied. Radish plants were uprooted 14 days after sowing and washed with tap water. Disease incidence was calculated by using the following formula: number of plants with symptoms × 100/total number of plants assessed. Root rot severity was scored using a 0 to 4 scale, in which 0 = 0%; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; and 4 = 76–100% root rot. The disease severity index was calculated by using the mid-point value of the percentage range according the following formula: [Σ(mid-point value × number of plants in category)] × 100/number of plants assessed. After disease assessment, fresh whole plant biomass was measured.

The presence of *R. solani* in symptomatic roots was confirmed by plating samples (cut into 2–3 cm pieces that were surface disinfected with 0.8% sodium hypochlorite solution for 30 s and rinsed twice in sterilized distilled water) on aPDA medium.

Data Analysis

Differences between treatments were evaluated using the linear model function "lmr" in R Studio (R-3.2.5; RStudio Team, 2019) where exp (experimental run), exp:rep, and exp:rep:isolate were treated as random factors and isolate and isolate:carbon source were treated as fixed factors. All experiments were conducted twice. Data were subjected to the Shapiro-Wilk test for normality followed by the Bartlett test to check the homogeneity of variance before doing analysis of variance (ANOVA). Data that deviated from a normal distribution were either square root, log or arcsine-square-root transformed before proceeding with ANOVA. When there was a significant difference between treatment means, the Fisher test of least significant difference (LSD) was applied in the Agricolae package (De Mendiburu, 2016).

The Bliss independence model was applied assuming ASD carbon source and *Trichoderma* isolate act independently to suppress the root rot severity in radish (Yan et al., 2010; Willyerd et al., 2011; Xu et al., 2011). The combined effect of *Trichoderma* and ASD carbon source on root rot severity indicates the union of two probabilistically independent events. The combined effects (F_{UA}) were calculated as the product of individual effects of *Trichoderma* isolates (F_{UA1}) and ASD carbon source (F_{UA2}).

$$F_{UA} = F_{UA1} \times F_{UA2}$$

Where F_{UA} is the remaining fraction of severity control relative to non-ASD and non-*Trichoderma* treatments (unaffected fraction of disease severity reduction, for example if disease severity reduction is 0.17, F_{UA} will be 1–0.17 = 0.83). According to the Bliss independence assumption, F_{UA} is the expected effect of combined treatments; synergistic, additive and antagonistic relationships between the treatments were determined as follows:

1. If the observed combined effect of *Trichoderma* isolate and ASD-carbon source is equal to $F_{UA,}$ the relationship is additive and there is no interaction between ASD-carbon sources and *Trichoderma i*solates
2. If the observed combined effect is greater than $F_{UA,}$ the relationship is synergistic
3. If the observed combined effect is less than $F_{UA,}$ the relationship is antagonistic.

RESULTS

Soil Attributes After ASD Treatment

Soil inoculation with *Trichoderma* isolates had no effect on soil gravitational moisture after ASD (**Table 2**). Soil gravitational moisture percentage after ASD treatment was not significantly different between the anaerobic control soils and soils amended with mustard greens, molasses, wheat bran or chicken manure, ranging from 24.9 to 25.9% (anaerobic control). Soil reducing conditions as indicated by iron oxide paint loss from IRIS tubes were not significantly affected by *Trichoderma* inoculation ($P = 0.3$; **Figure 1A**). Reducing conditions developed in anaerobic control but not in aerobic control soils (**Figure 1B**). Paint loss was higher on IRIS tubes in ASD-treated soil regardless of type of amendment than in either non-amended control soil ($P = 0.01$). However, paint loss was higher on IRIS tubes in ASD-treated soils amended with molasses, mustard greens, or wheat bran than in soil amended with chicken manure. There were no significant differences in IRIS tube paint loss among soils amended with molasses, mustard greens, or wheat bran during ASD.

Rhizoctonia Root Rot Incidence and Severity in ASD- and *Trichoderma*-Treated Soils

Combined analysis of two independent experiments indicated that inoculation of soil with either of the *Trichoderma* isolates did not significantly ($P = 0.5$) reduce Rhizoctonia root rot incidence in radish plants compared to the non-inoculated controls across all ASD subplot treatments and controls (**Figure 2A**). However, Rhizoctonia root rot incidence was significantly lower ($P = 0.01$) in radish plants grown in molasses-, mustard greens-, or wheat bran-amended, but not chicken manure-amended, ASD-treated soil than in radish plants grown in aerobic or anaerobic control soils across the *Trichoderma* main plot treatments (**Figure 2B**). Rhizoctonia root rot incidence was reduced by 36.6, 31.7, and 44.5% in ASD-treated soils amended with molasses, mustard greens and wheat bran, respectively, compared to the aerobic control across the *Trichoderma* main plot treatments (**Supplementary Table 1**). Specifically, ASD with molasses, mustard greens, or wheat bran carbon sources reduced root rot incidence significantly compared to the non-*Trichoderma* inoculated, aerobic control regardless of *Trichoderma* inoculant (none, *T. asperellum* or *T. harzianum*; **Supplementary Table 2**).

Rhizoctonia root rot severity was marginally significantly ($P = 0.1$) lower in radish plants grown in *T. asperellum*-inoculated soil than in radish plants grown in ASD-treated soils without *Trichoderma* inoculum or inoculated with *T. harzianum* across all carbon sources (**Figure 2C**). Disease severity was reduced by 24.6% in *T. asperellum*-inoculated soils compared to aerobic control soils not inoculated with *Trichoderma* (**Supplementary Table 3**). Root rot severity was significantly lower ($P < 0.001$) in radish plants grown in molasses-, mustard greens-, or wheat bran-amended, ASD-treated soil than in radish plants grown in chicken manure-amended, ASD-treated soil and both aerobic and anaerobic control soils across the *Trichoderma* main plot treatments (**Figure 2D**). Mean disease severity ranged from 14.4% in radish plants grown in wheat bran-amended, ASD-treated soil to 58.1% in plants grown in anaerobic control soil, across all *Trichoderma* treatments (**Supplementary Table 1**). Disease severity was significantly lower in radish plants grown in ASD-treated soil amended with wheat bran, mustard greens (21.0%) or molasses (24.1%) than in ASD-treated soil amended with chicken manure (48.7%) or in the aerobic (49.3%) or anaerobic control soils. Rhizoctonia root rot severity was reduced by 51.1%, 57.4, and 70.9% in ASD-treated soils amended with molasses, mustard greens and wheat bran, respectively, compared to the aerobic control across the *Trichoderma* main plot treatments (**Supplementary Table 1**). Disease severity was significantly lower ($P < 0.001$) in radish plants grown in soils treated with any combination of *Trichoderma* inoculum (*T. asperellum* or *T. harzianum*) and ASD with any carbon source except chicken manure compared to non-*Trichoderma*-inoculated aerobic control soils (**Supplementary Table 2**).

Synergy analysis using the Bliss independence model indicated that the combination responses between ASD treatment with any of the carbon sources and either of the two *Trichoderma* isolates were additive in suppressing Rhizoctonia root rot severity in radish, with the exception of *T. asperellum* with ASD-wheat bran, *T. harzianum* with ASD-molasses and *T. harzianum* with ASD-chicken manure (**Table 3**). The combination responses of *T. harzianum* inoculation with ASD-molasses and *T. asperellum* inoculation with ASD-wheat bran amendment were synergistic in suppressing Rhizoctonia root rot in radish. However, the combination response of *T. harzianum* inoculation with ASD-chicken manure was antagonistic toward suppression of disease severity in radish.

TABLE 2 | Gravitational moisture percentage of soil after anaerobic soil disinfestation (ASD) with different carbon source amendments and with or without *Trichoderma* spp.

Isolate	Soil gravitational moisture (%)[xyz]
Non-inoculated control	25.0 ± 3.8
T. harzianum T-22	24.8 ± 2.3
T. harzianum NT25	24.2 ± 4.8
P-value	0.75
Carbon source	
Aerobic control	20.1 ± 7.7b
Anaerobic control	25.9 ± 0.6a
Mustard greens	24.9 ± 0.6a
Molasses	25.6 ± 0.9a
Chicken manure	25.5 ± 0.6a
Wheat bran	25.8 ± 0.8a
P-value	0.004

[x] *Values in a column followed by different letters are significantly different at $P \leq 0.05$ according to Fisher's LSD test after square root transformation. The values after ± indicate standard error of the mean.*

[y] *Average of two experiments each with three replications per treatment.*

[z] *Gravitational moisture percentage was determined by drying soil collected just after removal of plastic covering from ASD-treated or anaerobic control pots in an oven at 80° C for 48 h.*

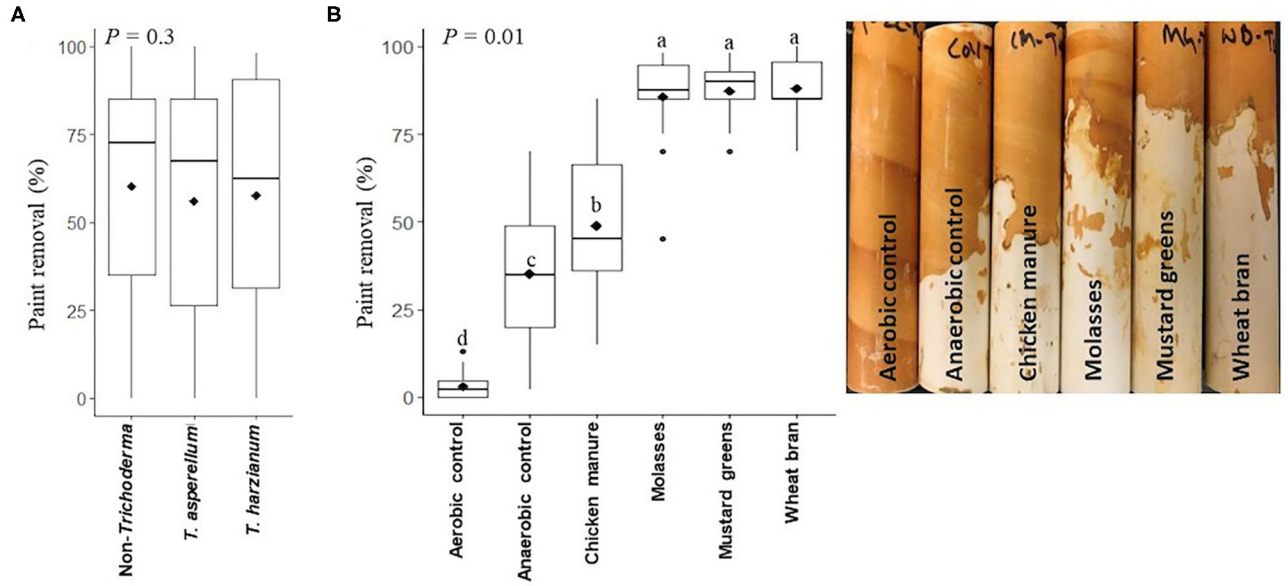

FIGURE 1 | Iron oxide paint loss from IRIS (Indicator of Reduction in Soil) tubes buried in soils amended with different carbon sources prior to anaerobic soil disinfestation affected by **(A)** Trichoderma isolate inoculation (main plots), **(B)** anaerobic soil disinfestation (ASD) carbon sources (subplots). The Y-axis is the percentage of iron oxide paint loss, an indicator of soil reducing conditions; in the boxes, black lines represent median, black diamonds represent mean values and black dots are outliers. *P*-value is the analysis of variance probability value after square-root transformation. Means followed by same letter(s) do not differ significantly at the indicated level of probability. Data presented are from two combined independent experiments each with three replications.

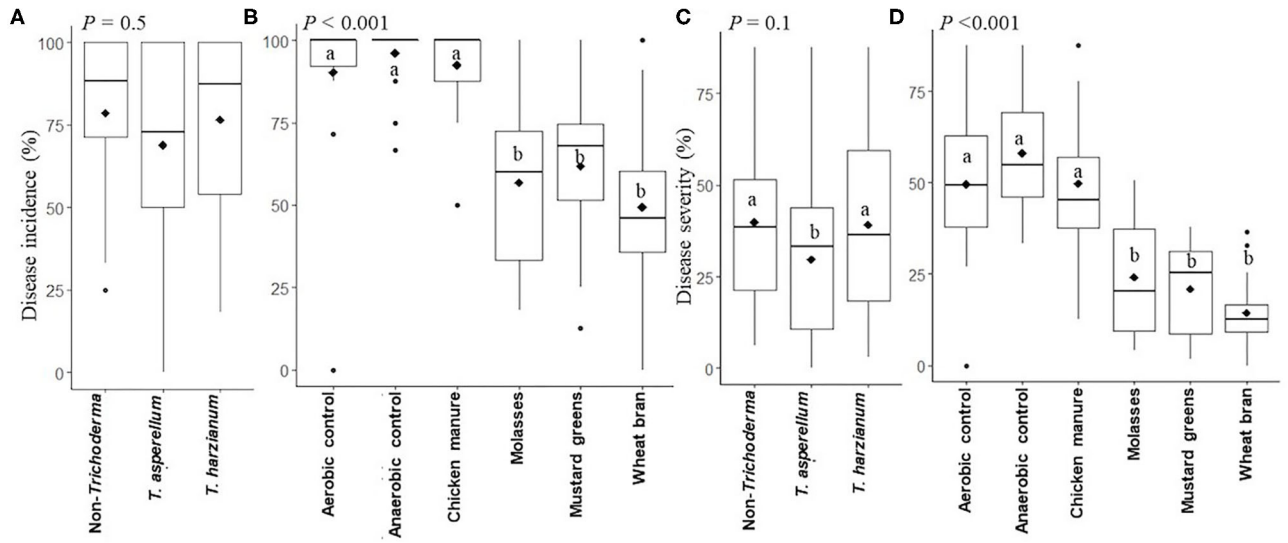

FIGURE 2 | Rhizoctonia root rot incidence **(A,B)** and severity **(C,D)** in radish affected by *Trichoderma* isolate inoculation (main plots) **(A,C)** and anaerobic soil disinfestation (ASD) carbon sources (subplots) **(B,D)** presented in box plots; black lines indicate median, black diamonds indicate mean values and black dots are outliers, *P*-value is the analysis of variance probability value after arcsine-square-root) transformation. Means followed by same letter(s) do not differ significantly at the indicated level of probability. Data presented are from two combined independent experiments each with three replications.

Effect of *Trichoderma* and ASD Treatment on Radish Biomass

Inoculation of soil prior to ASD with either *Trichoderma* isolate did not significantly ($P = 0.2$) affect the fresh biomass of radish plants grown in these soils across ASD subplot treatments (**Figure 3A**). However, soil treatment by ASD with chicken manure, mustard greens, or wheat bran amendments resulted in significantly ($P < 0.001$) higher radish biomass than ASD treatment of soils amended with molasses and the aerobic and anaerobic controls across

TABLE 3 | Synergy and additivity analysis using the Bliss independence model between carbon source and *Trichoderma* spp. amendments in suppression of Rhizoctonia root rot in radish plants grown in anaerobic soil disinfestation (ASD)-treated soil.

Biocontrol	ASD carbon source	Disease severity (%)	Reduction[x] (observed)[u]	F^{v}_{UA1}	F^{w}_{UA2}	Expected disease control (E) (F_{UA})[x]	O-E[y]	Remarks[z]
Non-*Trichoderma* control	Anaerobic control	67.28	0					
T. harzianum	Anaerobic control	55.63	0.17	0.83				
T. asperellum	Anaerobic control	51.30	0.24	0.76				
Non-*Trichoderma* control	Chicken manure	49.27	0.27		0.73			
Non-*Trichoderma* control	Molasses	29.17	0.57		0.43			
Non-*Trichoderma* control	Mustard greens	21.57	0.68		0.32			
Non-*Trichoderma* control	Wheat bran	20.87	0.69		0.31			
T. asperellum	Chicken manure	35.13	0.48			0.44	0.04	Additive
T. asperellum	Molasses	27.88	0.59			0.67	0.08	Additive
T. asperellum	Mustard greens	18.98	0.72			0.76	–0.04	Additive
T. asperellum	Wheat bran	8.52	0.87			0.76	0.11	Synergistic
T. harzianum	Chicken manure	61.67	0.08			0.39	–0.31	Antagonistic
T. harzianum	Molasses	15.32	0.77			0.64	0.13	Synergistic
T. harzianum	Mustard greens	22.42	0.67			0.73	–0.07	Additive
T. harzianum	Wheat bran	13.72	0.80			0.74	0.05	Additive

[u] *Percent reduction in Rhizoctonia root rot severity in radish compared to non-Trichoderma inoculated anaerobic control.*

[v] $F_{UA1} = 1$ *– Percent reduction in Rhizoctonia root rot severity for Trichoderma isolate under anaerobic control conditions compared to non-Trichoderma inoculated anaerobic control.*

[w] $F_{UA1} = 1$ *– Percent reduction in Rhizoctonia root rot severity for ASD carbon source under non-Trichoderma inoculated conditions compared to non-Trichoderma inoculated anaerobic control.*

[x] *Expected percent disease control* (F_{UA}) $=1 - (F_{UA1 for Trichoderma isolate under anaerobic conditions} \times F_{UA2 for ASD carbon source under non-Trichoderma inoculated conditions})$.

[y] *O – observed percent reduction in Rhizoctonia root rot severity, E – expected percent disease control* (F_{UA}).

[z] *Remarks: O > E: synergistic, O < E: antagonistic, otherwise: additive.*

the *Trichoderma* inoculated and non-inoculated main plots (**Figure 3B**, **Supplementary Table 1**). The biomass of radish plants grown in ASD-treated soils amended with molasses was not different from that of radish grown in aerobic and anaerobic control soils and mustard greens-amended ASD-treated soils. Fresh radish plant biomass was increased by 156.4, 110.8, and 133.0% in ASD-treated soils amended with chicken manure, mustard greens and wheat bran, respectively, compared to the aerobic control across the *Trichoderma* inoculated and non-inoculated treatments (**Supplementary Table 1**). Fresh biomass was 173.9% higher in radish plants grown in non-*Trichoderma* inoculated, ASD-treated soil amended with chicken manure than in the non-*Trichoderma* inoculated, aerobic control (**Supplementary Table 2**). Fresh plant biomass was increased by 258.5, 215.5, and 246.1% in *T. harzianum*-inoculated soils amended with chicken manure, mustard greens, or wheat bran, respectively, then subjected to ASD, relative to the non-*Trichoderma* inoculated, aerobic control.

Trichoderma spp. Populations in Soil After ASD Treatment

Trichoderma spp. populations were highest ($P < 0.001$) in *T. asperellum*-inoculated (log 4.3 CFU g^{-1}) soils after ASD compared to *T. harzianum*-inoculated (log 3.1 CFU g^{-1}) and non-inoculated (log 2.0 CFU g^{-1}) soils across the ASD-carbon source subplots (**Figure 4A**). *Trichoderma* spp. populations were not affected by flooding; populations in the aerobic and anaerobic controls were statistically similar across all *Trichoderma* inoculated and non-inoculated treatments (**Figure 4B**). Anaerobic soil disinfestation (ASD) with chicken manure, or wheat bran amendment did not reduce total *Trichoderma* spp. populations compared to the aerobic and anaerobic controls. However, molasses-amended ASD significantly ($P = 0.05$) reduced total *Trichoderma* spp. (log 2.7 CFU g^{-1}) in soil compared to the aerobic control (log 3.9 CFU g^{-1}), and mustard greens-amended ASD (log 2.5 CFU g^{-1}) reduced total *Trichoderma* spp. populations compared to both controls.

Rhizoctonia solani Populations in Soil After ASD and *Trichoderma* Treatment

Rhizoctonia solani ITS gene copy numbers g^{-1} soil after ASD treatment were not significantly different in soils inoculated with *T. harzianum* or *T. asperellum*, or not inoculated, across the ASD carbon sources subplots ($P = 0.5$; **Figure 5A**). However, ASD treatment of soils amended with wheat bran or mustard greens significantly ($P < 0.0001$) reduced *R. solani* populations compared to the aerobic and anaerobic controls and the chicken manure- and molasses-amended ASD treatments across the *Trichoderma* main plots (**Figure 5B**). Populations of *R. solani* were similar in aerobic and anaerobic control soils, while populations in chicken manure- or molasses-amended, ASD-treated soils were significantly reduced compared to the aerobic control only.

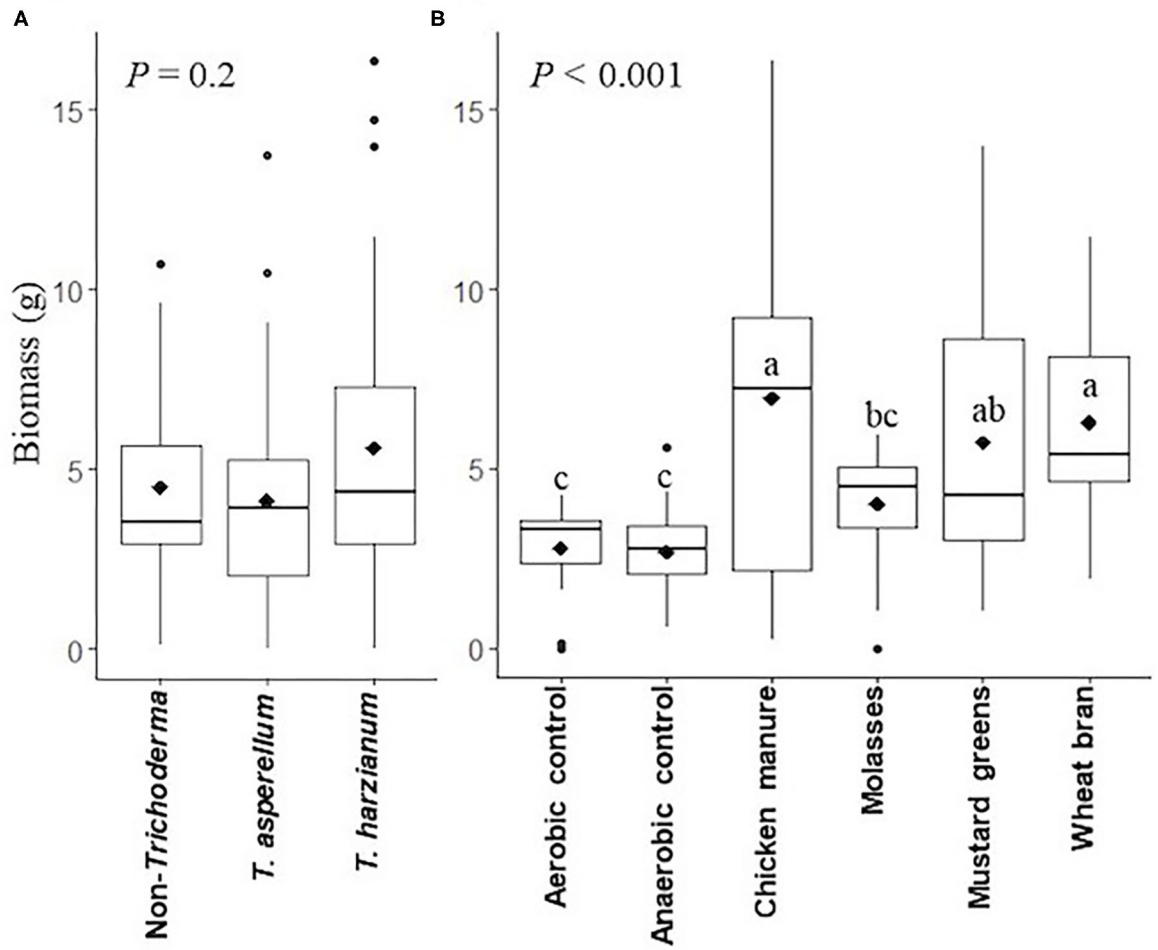

FIGURE 3 | Radish plant biomass (fresh weight) **(A)** as affected by *Trichoderma* isolate inoculation (main plots) and anaerobic soil disinfestation (ASD) carbon sources (subplots) **(B)** presented in box plots; black lines indicate median, black diamonds indicate mean values and black dots are outliers, *P*-value is the analysis of variance probability value. Means followed by same letter(s) do not differ significantly at 5% level of probability. Data presented are from two combined independent experiments each with three replications.

DISCUSSION

Diseases caused by *R. solani* are challenging to manage once the pathogen is established in soil because of its wide host range and production of environmentally resilient sclerotia (Cook et al., 2002). There are limited management options for these diseases. Anaerobic soil disinfestation and biological control are promising management tools that have no known negative environmental or health impacts (Harman, 2000; Rosskopf et al., 2015). No single method is entirely sufficient to control these diseases, therefore a combination of methods might be an effective strategy. The soil environment created by ASD is inhospitable for plant pathogens as a result of anaerobicity and the generation of toxic volatile and non-volatile fatty acids by soil microbial populations (Momma, 2015; Sanabria-Velazquez et al., 2020). It also improves crop growth by addition of soil nutrients (Paudel et al., 2018) and increases disease suppressiveness of the soil (Liu et al., 2019). *Trichoderma* spp. utilize several mechanisms to suppress soilborne diseases and to survive under a wide range of environmental conditions (Chovanec et al., 2005). If *Trichoderma* biocontrol agents can survive the toxic environment generated by ASD, the two tactics might be integrated to synergistically improve disease management in vegetable and other high value crop production systems.

The choice of carbon source plays a critical role in the efficacy of ASD. For instance, rice bran-amended ASD was comparatively less effective in suppressing root-knot severity in okra and eggplant than mustard cake- and molasses-amended ASD in Nepal (Khadka et al., 2019). The ASD carbon sources included in this study were selected based on their demonstrated efficacy in previous research (Butler et al., 2012; McCarty et al., 2014; Testen and Miller, 2018, 2019).

In this study, the *Trichoderma* populations were not reduced in ASD-treated soils when chicken manure, molasses, or wheat bran were used as carbon sources compared to the aerobic and/or anaerobic controls. This result provides strong evidence

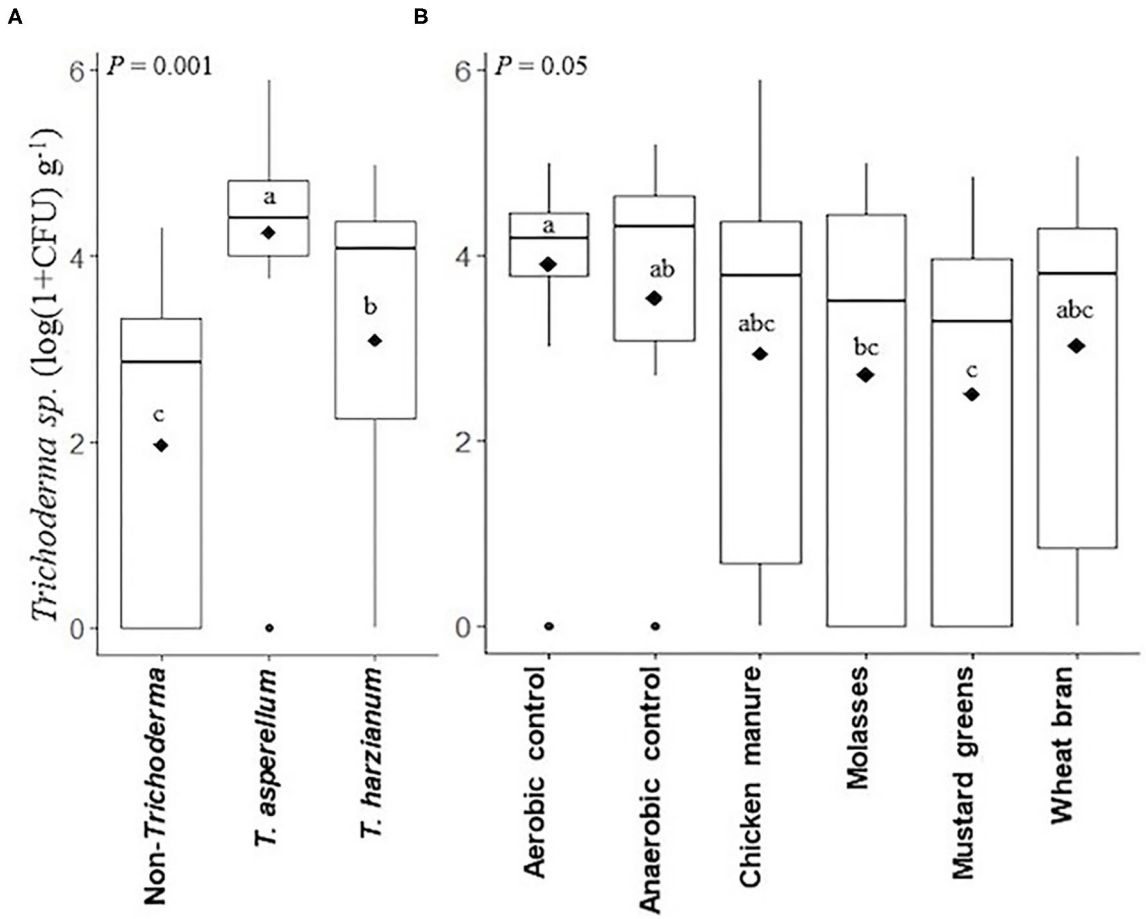

FIGURE 4 | *Trichoderma* populations (colony forming units g^{-1} soil) after ASD treatment affected by *Trichoderma* isolate inoculation (main plot) **(A)** and anaerobic soil disinfestation (ASD) carbon source subplots **(B)** presented in box plots; black lines indicate median black diamonds indicate mean values and black dots are outliers, *P*-value is the analysis of variance probability value after 1+log transformation, CFU indicates colony forming unit, means followed by same letter(s) do not differ significantly at 5% level of probability. Data presented are from two combined independent experiments each with three replications.

that *Trichoderma* can survive under conditions generated during ASD. Previous reports have also demonstrated the survival of *Trichoderma* spp. during ASD (Lamers et al., 2010; Momma et al., 2013; Shrestha et al., 2019). Chovanec et al. (2005) reported that *Trichoderma* could survive under hypoxic conditions by utilizing fermentative metabolic oxygen. Similarly, Pedreschi et al. (1997) and Poosapati et al. (2014) showed that some *Trichoderma* isolates tolerated a wide range of pH and high temperatures either by using diversified secondary metabolic pathways or producing stress protectant sugars.

The lower population of *Trichoderma* spp. we observed in mustard greens-amended ASD-treated soil may be due to biocidal effects of isothiocyanates produced by most *Brassica* spp. when glucosinolates present in these plants are hydrolyzed (Sarwar and Kirkegaard, 1998). Isothiocyanates are chemically similar to methyl isothiocyanate, which is widely used for chemical fumigation (O'Malley, 2010). However, several reports indicate that *Trichoderma* spp. are tolerant of Brassica-based biofumigation of soil (Galletti et al., 2008; Berlanas et al., 2018). Isothiocyanates exist in a gaseous state (Clapp et al., 1959, p. 1) in soil and may be trapped when the soil is covered with plastic, increasing toxicity to soil microbes such as *Trichoderma* spp. and ensuring sufficient moisture for hydrolysis of glucosinolates compared to biofumigation, which is not covered.

The lower disease severity and incidence in radish plants grown in wheat bran-, mustard greens-, and molasses-amended ASD-treated soils compared to those grown in chicken manure-amended ASD-treated and anaerobic and aerobic control soils are also supported by lower *R. solani* populations and higher soil reducing conditions in these treatments. In our studies, anaerobic conditions were increased by addition of carbon sources rather than soil moisture because gravitational soil moisture was not significantly different among covered and flooded (anaerobic) control soil and any carbon source-amended ASD-treated soil. However, reducing conditions were higher in all carbon source-amended ASD-treated soils than in the

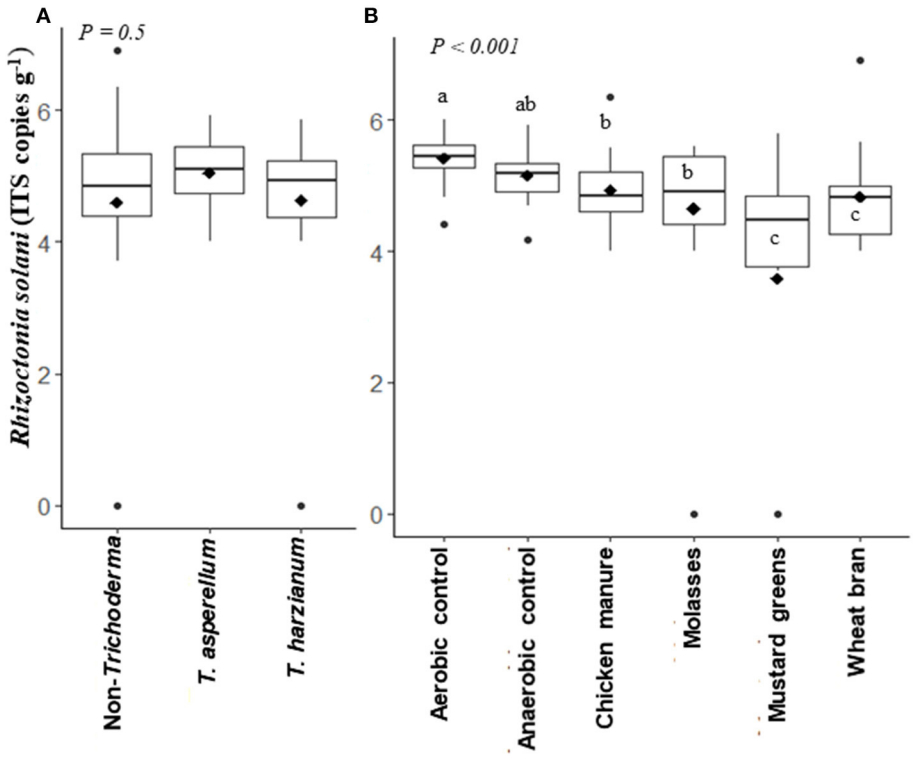

FIGURE 5 | *Rhizoctonia solani* populations (log ITS copy number g^{-1} soil) in soils affected by *Trichoderma* isolate inoculation (main plot) **(A)** and anaerobic soil disinfestation (ASD) carbon sources (subplots) **(B)** presented in box plots; black lines indicate median, black diamonds indicate mean values and black dots are outliers, *P*-value is the analysis of variance probability value after 1+ log transformation, CFU indicates colony forming unit means followed by same letter(s) do not differ significantly at 5% level of probability. Data presented are from two combined independent experiments each with three replications.

anaerobic control. Redox reactions under ASD conditions produce poorly oxidized compounds such as methane and ethylene gases, alcohol, and organic acids that are toxic to plant pathogens (Demirel and Yenigün, 2002; Merlin Christy et al., 2014). Plant pathogens including *R. solani* are aerobic microbes that require oxygen for survival and growth. Thus, hypoxic conditions may reduce the growth and multiplication of *R. solani* leading to reduced soil populations. Furthermore, ASD changes the soil microbial community composition, which leads to the domination of anaerobic microbes (Mazzola et al., 2018; Testen and Miller, 2018). Additionally, the reduced *R. solani* populations observed in this study might be due to low compatibility and poor competitiveness with anaerobic microbes under anaerobic conditions (Liu et al., 2019).

Rhizoctonia solani populations were not affected by *Trichoderma* inoculation of soils prior to ASD, but disease severity was lower in radish plants grown in *T. asperellum*-inoculated soil than in non-inoculated or *T. harzianum*-inoculated soil across all ASD treatments and the controls. *Trichoderma* spp. suppress Rhizoctonia diseases through different mechanisms, either directly killing pathogen propagules through hyperparasitism (Benhamou, 1993) or production of antibiotics (Ghisalberti and Sivasithamparam, 1991; Lorito, 1993; Tseng et al., 2008), and/or indirect mechanisms such as inducing systemic resistance in plants (Mayo et al., 2015) or competing for plant rhizosphere niches (Sivan and Harman, 1991). Reduced *R. solani* populations in *Trichoderma*-inoculated soils were not observed in this study, which indicates that indirect mechanisms may be responsible for the reduced disease severity in our *T. asperellum*-inoculated treatments.

Rhizoctonia root rot suppression was higher in radish plants grown in *T. asperellum*-inoculated soil compared to *T. harzianum*- or non-inoculated soil, this could be due to the presence of higher numbers of viable *Trichoderma* spores in *T. asperellum*-inoculated soil compared to *T. harzianum* and non-inoculated soil. In addition, *Trichoderma* isolates are known to vary in ability to suppress plant disease. For instance, Worasatit et al. (1994) tested fifty-four single spores isolates of Trichoderma koningii against *R. solani* in *in-vitro* and six isolates in *in-planta* and reported that only six isolates showed strong inhibition of pathogen growth in agar plate assays and only three isolates significantly suppressed Rhizoctonia root rot in wheat.

Few studies have been reported to date on the potential benefits of combining *Trichoderma* inoculation with ASD on soilborne disease suppression. Huang et al. (2016) reported a significant reduction in cucumber damping-off caused by *R. solani* in alfalfa-amended ASD plus *T. harzianum* T37 compared to alfalfa-amended ASD alone in the second season of

cultivation after ASD treatment when T37 was inoculated after ASD treatment. In contrast, Shrestha et al. (2019) reported no additional benefits of *Trichoderma* spp. inoculation during ASD with a dry molasses/corn starch mixture as the carbon source on sclerotial mortality of *S. rolfsi* over ASD alone. We found that the interaction between ASD and *Trichoderma* in suppression of Rhizoctonia root rot was both *Trichoderma* isolate- and ASD carbon source-dependent. Most interactions in our study were additive, indicating neither beneficial nor detrimental effects of the combinations. However, the combinations of *T. asperellum* with ASD-wheat bran and *T. harzianum* with ASD-molasses were synergistic, resulting in greater disease suppression than with either alone. The combination of *T. harzianum* with ASD-chicken manure was antagonistic, resulting in less root rot suppression than either alone. These results point to the need to optimize ASD carbon source and *Trichoderma* isolate choices in different pathosystems.

No effect of *Trichoderma* inoculation was observed on radish biomass; however differential impacts of ASD carbon sources on biomass were observed. Hewavitharana and Mazzola (2016) and Testen and Miller (2018) also reported differential impacts of ASD carbon sources on fresh plant biomass. In this study the chicken manure-amended ASD treatment resulted in higher plant biomass but not suppression of Rhizoctonia root rot, whereas ASD-molasses reduced disease severity but did not increase radish biomass. Only mustard greens- and wheat bran-amended ASD treatments both increased radish biomass and reduced root rot. Our results are supported by the observations of Testen and Miller (2018) that wheat bran-amended ASD reduced root rot severity and increased biomass in tomato, but ethanol and molasses amendments in ASD treatments reduced root rot severity but did not increase tomato biomass. Anaerobic soil disinfestation with wheat bran reduced Rhizoctonia root rot incidence and severity in radish and *R. solani* populations in soil, increased radish biomass and did not affect *Trichoderma* populations. Furthermore, *T. asperellum* NT25 interacted synergistically with ASD-wheat bran to reduce Rhizoctonia root rot severity. This study confirms the suitability of wheat bran as a preferred carbon source in ASD and suggests that ASD efficacy can be improved by addition of suitable *Trichoderma* isolates during treatment.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

RK conducted the experiments and wrote the original draft. SM supervised RK for experimental design and revised the manuscript. Both authors contributed to the article and approved the submitted version.

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Factors Influencing the Persistence of *Salmonella* Infantis in Broiler Litter During Composting and Stabilization Processes and Following Soil Incorporation

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Broiler litter (BL), a by-product of broiler meat production, is frequently contaminated with *Salmonella* and other zoonotic pathogens. To ensure the safety of crop production chains and limit pathogen spread in the environment, a pre-treatment is desired before further agricultural utilization. The objective of this study was to characterize the effect of physico-chemical properties on *Salmonella* persistence in BL during composting and stabilization and following soil incorporation, toward optimization of the inactivation process. Thirty-six combinations of temperature (30, 40, 50, and 60°C), water content (40, 55, and 70%; w/w), and initial pH (6, 7, and 8.5) were employed in static lab vessels to study the persistence of *Salmonella enterica serovar* Infantis (*S.* Infantis; a multidrug-resistant strain) during incubation of artificially-inoculated BL. The effect of aeration was investigated in a composting simulator, with controlled heating and flow conditions. Temperature was found to be the main factor significantly influencing *Salmonella* decay rates, while water content and initial pH had a secondary level of influence with significant effects mainly at 30 and 40°C. Controlled simulations showed faster decay of *Salmonella* under anaerobic conditions at mesophilic temperatures (<45°C) and no effect of NH_3 emissions. Re-wetting the BL at mesophilic temperatures resulted in *Salmonella* burst, and led to a higher tolerance of the pathogen at increased temperatures. Based on the decay rates measured under all temperature, water content, and pH conditions, it was estimated that the time required to achieve a 7 $\log_{10}$ reduction in *Salmonella* concentration, ranges between 13.7–27.2, 6.5–15.6, 1.2–4.7, and 1.3–1.5 days for 30, 40, 50, and 60°C, respectively. Inactivation of BL indigenous microbial population by autoclaving or addition of antibiotics to which the *S.* Infantis is resistant, resulted in augmentation of *Salmonella* multiplication. This suggests the presence of microbial antagonists in the BL, which inhibit the growth of the pathogen. Finally,

Salmonella persisted over 90 days at 30°C in a Vertisol soil amended with inoculated BL, presumably due to reduced antagonistic activity compared to the BL alone. These findings are valuable for risk assessments and the formulation of guidelines for safe utilization of BL in agriculture.

Keywords: poultry litter, poultry manure, antagonistic microorganisms, thermal inactivation, pathogen elimination, zoonotic microorganisms, fresh produce contamination, field crops contamination

INTRODUCTION

The poultry sector is amongst the fastest growing agriculture-based meat production industries worldwide, due to the increasing demand for meat and egg products (Bolan et al., 2010). Broiler litter (BL) is a by-product of broiler meat production. It consists of a mixture of bedding (usually sawdust or shavings, rice hulls or straw), manure, and wasted feeds and feathers. BL can become a source of zoonotic pathogens such as *Salmonella* that are pathogenic to humans (Chinivasagam et al., 2010; Wilkinson et al., 2011; Gould et al., 2013). Often, *Salmonella* that are excreted from infected chickens, contaminate the litter and eventually, the poultry house environment and the entire flock (Jones et al., 1991; Bryan and Doyle, 1995; Corrier et al., 1999; Trampel et al., 2000). Thus, besides the immediate risk to public health through the consumption of contaminated eggs and broiler meat products, *Salmonella* can be transferred to the agricultural environment by contaminated litter.

BL is a valuable alternative fertilizer and soil additive that is used in conventional and organic farming. It has been proven in numerous studies to be an effective fertilizer for row crops, including corn, cotton, and soybean (Tewolde et al., 2013) and in some cases, it was shown to be more effective and valuable than synthetic fertilizers (Tewolde et al., 2011). A meta-analysis based on 116 studies showed positive effects of poultry litter compared to inorganic fertilizers regarding P and K plant uptake and other indicators of soil fertility (Lin et al., 2016). Yet, although BL may be contaminated by a variety of zoonotic pathogens, most farmers use it without processing or after partial stabilization by stockpiling (Ogejo and Collins, 2009; Wilkinson et al., 2011; Wiedemann, 2015). These common practices may facilitate pathogen spread in agricultural fields, which in turn may lead to crop contamination. Indeed, there are numerous reports on the contamination of fresh produce in the field due to soil contamination (Beuchat, 2002; Bell et al., 2015; Gu et al., 2018; Jechalke et al., 2019). In many such cases Salmonellosis outbreaks have been associated with consuming contaminated fresh produce, like tomatoes, cantaloupe, and leafy vegetables (Fatica and Schneider, 2011; Herman et al., 2015; Chaves et al., 2016). *Salmonella enterica serovar* Typhimurium (*S.* Typhimurium) was detected in soil up to 231 days after application of poultry and cattle manure composts that were artificially inoculated with the pathogen (Islam et al., 2004a,b). In these studies, *Salmonella* was also detected in vegetable crops grown in the tested soils, 203 and 84 days after seeding of carrot and radish, respectively (Islam et al., 2004a), or 231 and 63 days after seeding parsley and lettuce, respectively (Islam et al., 2004b). In spite of strict control measures and regulations, *Salmonella* outbreaks due to consumption of contaminated fresh produce are still a threat to public health.

Thermal processing is considered as a practical and effective approach for inactivating pathogens in BL or BL-based organic fertilizers prior to land application (Williams and Benson, 1978; Macklin et al., 2008; Wilkinson et al., 2011). Although thermal inactivation may occur partially by stockpiling the litter for some time before spreading, it cannot be effective as compared to controlled thermophilic composting. The first active stage of composting is an exothermic process governed by aerobic decomposition reactions. Typically, the efficiency of the composting process depends on proper aeration of the material (Sánchez et al., 2017; Alkoaik, 2019; Oazana et al., 2020), under which the degradation rates can be maximized. These conditions in turn yield high rates of heat emission and eventually lead to thermal inactivation of pathogens. Based on international regulations (USA and Canada; also adopted in Israel), to ensure effective pathogen elimination, all compost particles need to be exposed to a minimum temperature of 55°C for a period of at least 3 consecutive days (United States Environmental Protection Agency, 2003; Wichuk and McCartney, 2007). Yet, composting piles are often not extensively controlled, such that thermal inactivation is not efficient throughout the entire pile even after several turnings (i.e., temperatures do not reach a minimum of 55°C throughout the pile; Wilkinson et al., 2011; Avidov et al., 2017). Although thermophilic temperatures are reported in numerous composting studies, only average values are usually presented, while the spatial variability of the temperature in the pile is practically unknown. The situation is even more critical in static piles, where a minimal management regime is commonly applied (Avidov et al., 2019). Pathogens surviving the composting process or any phase of stabilization, may regrow during storage or following land application. Indeed, several studies have demonstrated the persistence of zoonotic pathogens in the finished compost at different levels of maturity and in compost-amended soils (Chen and Jiang, 2014; Reynnells et al., 2014; Hruby et al., 2018).

The combination of temperature with other environmental factors may have variable effects on *Salmonella* persistence in BL. Several studies have examined the effect of temperature and water content, generally showing that thermal susceptibility of the pathogen increases with increasing water content. At low water contents, desiccation may play a major role in pathogen inactivation. Yet, desiccation-adapted *Salmonella* spp. persisted longer in aged chicken litter compared to non-adapted cells (Chen et al., 2013). The increased persistence

and temperature-tolerance of *Salmonella* at low water content was evident in a study by Liu et al. (1969), who showed that *Salmonella* Senftenberg strain 775 W in meat and bone meal of 5% water content remained relatively stable at temperatures as high as 50°C. Heat inactivation of *Salmonella* spp. in fresh poultry compost was faster at 50% water content compared to 40%, both at 50 and 55°C (Singh et al., 2012). Yet, an opposite trend was shown by Wilkinson et al. (2011) who found that at lower temperatures (35 and 45°C) more effective reduction of *S.* Typhimurium in poultry litter occurred at 30% than at 65% water content. Other co-factors may also play a role in bacterial inactivation, such as the combined and intensified effect of drying and NH_3 emissions, shown by Himathongkham and Riemann (1999). Biological mechanisms, such as competition between indigenous microorganisms and pathogens (Wichuk and McCartney, 2007), and microbial antagonism (Millner et al., 1987; Erickson et al., 2010; Gurtler et al., 2018) may also affect pathogen inactivation.

Overall, beyond thermal inactivation, which is considered as the primary mechanism responsible for the inactivation of human pathogens in BL and other livestock manures, there is a lack of knowledge about the combined effect of different physico-chemical and biological factors. This study aimed at investigating various key factors and the interactions among them that influence the persistence of *Salmonella* in BL during composting and stabilization processes and following soil incorporation.

MATERIALS AND METHODS

Broiler Litter (BL) and Soil

BL

Selected physical and chemical properties of the BL and the soil used in this study are presented in **Tables 1A**,**B**. *Un-stabilized (fresh)* BL was collected throughout the experimental period from tunnel-ventilated broiler houses of several farms located at the Jezreel Valley, northern Israel: Moshav Barak, Moshav Beit She'arim, Moshav Balfouria, and Kibbutz Yifat. The farms use raising protocols of the main poultry cooperatives in Israel, while some of the variability shown in **Table 1** may represent spatial variation within any given poultry house and between different growing cycles at the same poultry house. In all cases, the BL was collected at the end of 6-weeks growing period. *Stabilized BL* was obtained from a static pile, representing a common practice in Israel. About 35 m^3 of BL from the poultry farm of Moshav Balfouria were stockpiled in the open yard without any further treatment for a period of 8 months. This pile was closely monitored within the first 2 months, showing that 27 and 23% of the stockpile volume were below 45°C during the first and second weeks, respectively. Later, about 30% of the stockpile volume remained below that temperature (based on *ca.* 53 sampling points monitored on a weekly basis in the first month and then biweekly in the second month). *Composted BL* was prepared using a polyethylene sleeve with forced aeration (Avidov et al., 2017, 2018). For that, about 35 m^3 of BL was pre-wetted to achieve a water content of *ca.* 50–55% (51.1 ± 1.71%), which is within the optimal range for composting (Christian et al., 2009; Zakarya et al., 2018), and then packed in a polyethylene sleeve that was sealed manually (Avidov et al., 2019). Controlled composting was processed for 56 days with blower settings of 2 min on and 30 min off. The entire compost material within the sleeve maintained thermophilic temperatures (>45°C) up to 69 and 66°C during the first and second weeks, respectively. Only *ca.* 2 and 4% of the volume was estimated to be below 45°C during the third and fourth weeks, respectively (based on *ca.* 48 sampling points monitored weekly in the first month and biweekly in the second month. Temperatures were measured using Type K thermocouples, constructed on 80 cm-long stainless steel rods; Elcon Ltd., Israel). The composted BL was left (stored) within the sleeve which was partly open for additional 6 months. Finally, the material of both the stockpile and the sleeve was sampled from 9 different locations each, unified and homogenized. All materials (un-stabilized, stabilized, or composted BL) were stored at 4°C until use, except for the experiments used to assess the potential of antagonistic indigenous populations against *Salmonella*, for which the un-stabilized BL was used without any storage. Before each experiment, the BL was acclimated for *ca.* 24 h at room temperature.

Soil

A Vertisol-type soil was collected from 0 to 30 cm depth at the Newe Ya'ar Research Center, Jezreel Valley; northern Israel.

Physical and Chemical Analyses

Dry based aqueous extracts (1:9 w/w) of BL or soil samples were prepared with distilled water by shaking the suspension for 1 h at 200 RPM on a reciprocal shaker. The pH was analyzed directly in the suspension (LL-Ecotrode Plus WOC; Metrohm, Herisau, Switzerland), while the electrical conductivity (EC) was determined in the supernatant after centrifugation at 6,000 RPM for 20 min at 25°C (CyberScan CON 11, Eutech Instruments, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Total C and N were determined after grinding sub-samples (mixer mill MM 400, Retsch, Haan, Germany) by FlashSmart 2000 Elemental Analyzer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

Bacterial Strain and Inoculum Preparation

We used a clinical isolate of *Salmonella enterica* serovar Infantis; a multi-drug resistant strain containing a mega plasmid (pESI) that carries several antibiotics resistance genes (Aviv et al., 2014; courtesy of Prof. Ohad Gal-Mor, Sheba Medical Center, Tel-Ha'shomer, Israel). The culture was stored in phosphate-buffered saline (PBS) containing 15% glycerol (Duchefa biochemie, Netherlands) at −80°C. All growing media were prepared according to the manufacturer's instructions. Before each experiment, about 100 μl of the stored culture (PBS with 15% glycerol) were transferred into 100 ml of nutrient broth (NB; Oxoid, Basingstokes, UK) with tetracycline (20 μg ml^{-1}) and incubated for 24–72 h at 37°C. The culture was then transferred into several sterile polypropylene (PP) tubes, and washed 3 times with 40 ml sterile PBS by centrifugation at 6,500 RPM for 10 min at 25°C. The final pellet was re-suspended in 40 ml PBS to achieve a final concentration of *ca.* 9 $\log_{10}$ colony forming units (CFU) ml^{-1}. To inoculate the BL, the suspension was added together with the amount of water needed to adjust the BL to the desired

TABLE 1A | Selected properties of broiler litter (BL) and soil.

BL type and source	Aim and experimental setup	Bulk density (kg l^{-1})	Aqueous extract (1:9)		Total (% dry-based)	
			pH	EC (dS m^{-1})	C	N
Un-stabilized BL* (Moshav Bark)	The combined effect of temperature, water content, and pH on the persistence of *S.* Infantis in BL during lab incubation (**Figures 1**, **2** and **Supplementary Figure 1** at 40, 50, and 60°C).	0.44	6.61 (±0.08)**	10.67 (±0.95)	41.80 (±0.75)	5.44 (±0.15)
	Persistence of *S.* Infantis under controlled aerobic and anaerobic conditions using lab-scale simulations (**Figures 3**, **4**).		7.37 (±0.08)			
Un-stabilized BL (Moshav Beit She'arim)	The combined effect of temperature, water content, and pH on the persistence of *S.* Infantis in BL during lab incubation (**Figures 1**, **2** and **Supplementary Figure 1** at 30°C).	0.50	6.67 (±0.1)	11.46 (±0.11)	41.11 (±1.33)	4.65 (±0.05)
	Effect of drying and re-wetting on the persistence of *S. Infantis* in BL at 30°C (**Figure 5**).					
Un-stabilized BL (Moshav Beit She'arim)	Heat inactivation of indigenous microbial populations in BL. *S.* Infantis was grown in autoclaved BL suspension at room temperature (**Figure 6A**).	0.50	6.89 (± 0.00)	7.77 (±0.11)	42.27 (±0.68)	3.69 (±0.07)
Composted BL (Moshav Balfouria)		0.51	8.03 (± 0.74)	13.13 (±3.81)	39.52 (±0.55)	4.04 (±0.05)
Un-stabilized BL (Kibbutz Yifat)	Heat inactivation of indigenous microbial populations in BL. *S.* Infantis was grown in autoclaved BL suspension at room temperature (**Figure 6A**–inset).	0.50	6.67 (± 0.10)	7.77 (±0.86)	43.31 (±0.98)	3.92 (±0.25)
Un-stabilized BL (Moshav Beit She'arim)	Heat inactivation of indigenous microbial populations in BL. Un-stabilized and stabilized BL were artificially contaminated with the pathogen and incubated under aerobic and anaerobic conditions at room temperature (**Figure 6B**).	0.50	6.89 (±0.00)	7.77 (±0.11)	42.27 (±0.66)	3.69 (±0.07)
Stabilized BL (Moshav Balfouria)		0.50	7.12 (± 0.40)	10.16 (±1.32)	39.05 (±0.62)	3.61 (±0.21)
Un-stabilized BL (Kibbutz Yifat)	Antibiotics-induced inactivation of indigenous microbial populations in BL. *S.* Infantis was grown at room temperature in BL suspension that was amended with a cocktail of antibiotics to which the pathogen is resistant (**Figure 7**).	0.50	6.67 (± 0.10)	7.77 (±0.86)	43.31 (±0.98)	3.92 (±0.25)
Un-stabilized BL (Moshav Beit She'arim)	Persistence of *S.* Infantis in mixtures of soil with un-stabilized or stabilized BL (**Figure 8**).	0.50	6.67 (±0.1)	11.46 (±0.11)	41.11 (±1.33)	4.65 (±0.05)
Stabilized BL (Moshav Balfouria)		0.50	7.12 (± 0.40)	10.16 (±1.32)	39.05 (±0.62)	3.61 (±0.21)

**All materials (un-stabilized, stabilized, or composted BL) were stored at 4°C until use, except for the experiments used to assess the potential of antagonistic indigenous populations against Salmonella, for which the un-stabilized BL was used without any storage.*

***Standard deviation of triplicate analyses.*

water content. The final concentration of *Salmonella* was *ca.* 7 $\log_{10}$ CFU g^{-1} dry matter.

Salmonella Enumeration

The initial stock prepared for each experiment was enumerated by serial dilution in sterile PBS and plating triplicate aliquots of 100 µl on XLD agar (Oxoid Basingstokes, UK) amended with tetracycline (20 µg ml^{-1}). The plates were incubated at 37°C for 48 h before counting. BL samples, before and following *Salmonella* inoculation, were analyzed by weighing 8 g of dry matter (based on a preliminary analysis of water content) and suspending it in a sterile stomacher bag after the addition of 80 ml PBS. The stomacher (STO-4, MRC, Israel) was operated at 10 pedals s^{-1} for 3 min, and then the suspension was transferred into a sterile PP tube and let to settle for *ca.* 5 min. A 1 ml sample was taken from the top suspension to prepare serial dilutions, from which 100 µl (or 200 µl in cases where we had to decrease the detection limit) were plated in triplicate plates, as described above. Typical black colonies were counted as presumptive *S.* Infantis. No black colonies were observed in un-inoculated BL samples. *The detection limit* was determined as 10–20 CFU g^{-1} dry matter. *Salmonella* enrichment was also performed to ensure complete bacterial inactivation. A 1 ml of the undiluted suspension was transferred into each of 5–10 tubes containing 9 ml of buffered peptone water (BPW, Oxoid, Basingstokes, UK) and tetracycline (20 µg ml^{-1}). The tubes were incubated for 24 h at 37°C. Each of the enrichment tubes (undiluted) was checked for positive or negative growth on XLD agar amended with tetracycline to check for the presence of typical *Salmonella* colonies. *The detection limit after enrichment* was determined as 1–2 CFU g^{-1} dry matter.

TABLE 1B | Vertisol soil.

pH	7.54 (±0.35)[a]
EC (dS m^{-1})	1.88 (±0.19)
Organic matter (%)	2.89 (±0.27)
Sand (%)	12.57 (±1.69)
Silt (%)	24.04 (±1.86)
Clay (%)	62.16 (±0.12)
$CaCO_3$ (%)	11.93 (±0.21)

[a] *Each value represents the average of 9 sub-samples taken from 0 to 30 cm depth; Newe Ya'ar Research Center, Jezreel Valley, Israel.*

Decay Rate Calculations

Exponential decay rates of *Salmonella* were calculated using Equation (1)

$$C(t) = C_0 e^{k(t)} \quad (1)$$

where *C(t)* is the concentration of *Salmonella* (CFU g^{-1} dry matter) at point in time *t* (d), C_0 is the initial concentration of *Salmonella*, and *k* is the first-order decay constant (d^{-1}). Decay rate constants were calculated using a linear curve fit between the natural log-transformed concentrations ($ln\ C/C_0$) and time. The number of data points to be included in the linear correlation of each dataset, was selected to provide the highest R^2, while excluding data points below the detection limit.

Laboratory Simulations of Temperature, Water Content, and pH Conditions

Thirty-six combinations of four temperatures (30, 40, 50, and 60°C), three water contents (40, 55, and 70%; w/w), and three initial pH (6, 7, and 8.5) were tested. For each combination, triplicate vessels were prepared with 200 g (dry based) of un-stabilized BL that was artificially inoculated with *S.* Infantis at a concentration of *ca.* 7 $\log_{10}$ CFU g^{-1} dry matter. The BL was first placed in a biohazard bag and adjusted to the desired pH by adding acid (1 M H_2SO_4) or base (1 M NaOH). The amount of required acid or base was pre-determined in preliminary titrations on parallel samples (at 55% water content). The pH-adjusted BL was thoroughly mixed by massaging the bag over several minutes and then inoculating it with *S.* Infantis, as described above, to achieve the desired initial concentration. The inoculated BL was thoroughly mixed by massaging the bag one more time and finally the bag was placed (loosely tied) in a 600 ml glass beaker. Aeration was not controlled in these experiments. The beakers were placed in an incubator (pre-verified over 4 days before each experiment) at the desired temperature (30, 40, 50, or 60°C) for 14 days, and then transferred to 30°C for additional 14 days of incubation to evaluate regrowth potential. The desired water content was adjusted during the incubation period, based on gravimetric analyses performed in a preliminary experiment on vessels with non-inoculated BL under identical conditions. Adjustments were made every sampling day during the first 2 weeks and every 3 days during the rest of the experiment. *Salmonella* enumerations were performed on sub-samples from each vessel, on days 0, 1, 3, 7, 14, and 28. A preliminary analysis showed that the percentage of inoculated *S.* Infantis that could be recovered from the BL after incubation for 24 h at 4°C was 75 and 71% on average, at 50 and 70% BL water content, respectively (data not shown).

Before each incubation series, the BL was checked for the presence of indigenous tetracycline-resistant *Salmonella* and was always found to be below the detection limit after enrichment. Triplicate non-inoculated vessels were incubated in each of the four temperatures and served as controls. In these samples, the BL was adjusted to a water content of 55% and initial pH 7 (the water content was adjusted using 10 ml of PBS plus the needed amount of deionized water and acid/base, as done for the inoculated samples). The aim of the control vessels was to negate any possible growth of indigenous *Salmonella* or cross-contamination between samples.

Controlled Simulations of Aerobic and Anaerobic Conditions

The fate of *S.* Infantis in un-stabilized BL under controlled aerobic and anaerobic conditions was evaluated using the Agricultural Research Organization Composting Simulator (ARO-CS) in which the temperature and aeration are controlled by a programmable logic controller (Oazana et al., 2018). The system includes six 9-liter reactors; each is mounted into a separate 80-liter bath and equipped with two temperature sensors: one thermocouple in each bath and one PT-100 in each reactor. The airflow was controlled by individual mass flow controllers (MFC) and transferred to the reactors through a humidifier mounted into the water bath, ensuring water-saturated inflow, and eliminating drying of the composting mixture. However, in cases of intense aerobic activity, during which it was difficult to restrain the heat evolved (Oazana et al., 2020), the air was transferred through a bypass to enable evaporative cooling.

Two simulation experiments were performed: In the first experiment the effect of aeration on the persistence of *S.* Infantis at 40, 50, and 60°C was evaluated during 38 days. Each of the six reactors was filled with 1.4 kg of un-stabilized BL (dry-based; 4.5 liters) that was adjusted to 40% water content, initial pH 7, and was artificially inoculated with *S.* Infantis at *ca.* 7 $\log_{10}$ CFU g^{-1} dry matter. Three of the reactors were maintained under aerobic conditions with a flow of 5 l min^{-1} (10 min on and 2 min off), and three reactors were kept under anaerobic conditions, without any aeration. The simulation was divided into three phases: (I) *mesophilic phase*: Temperature was maintained between 30 and 37°C for 9 days (aerobic reactors), or between 35 and 37°C for 7 days (anaerobic reactors). The humidifier bypass was used for the aerobic reactors to restrain the heat evolved during this phase. This in turn resulted in BL drying and required re-wetting, which delayed the move to phase II in 2 days. (II) *Heating phase*: Temperature was increased to 40, 50, and 60°C, under both aerobic and anaerobic conditions, one reactor for each temperature. These temperatures were maintained for 14 (aerobic) or 7 days (anaerobic). (III). *A second mesophilic phase*: The content of each reactor was transferred into a lab incubator for additional 14 days at 30°C, to explore regrowth potential.

The BL was sampled during the simulations on days 0, 3, 7, 9, 10, 11, 12, 14, 16, 23, and 37 (aerobic), and on days 0, 3, 7, 9, 10, 14, 28, and 37 (anaerobic) and analyzed for *Salmonella*, water content, and pH. Emission of NH_3 was measured on days 1, 3, 7, 9, 11, 16, and 23 (aerobic) or days 1, 3, 8, 10, and 14 (anaerobic). Air samples (*ca.* 25 liters) were collected in Nalophan^{TM} bags (polyethylene terephthalate; thickness 20 mm; Kalle GmbH, Wiesbaden, Germany) through a sampling port located on the cape of each reactor. Before sampling, the reactors were flushed for 20 min at 2.5 l min^{-1} of air (aerobic) or N_2 (99.999% purity) (anaerobic). NH_3 was analyzed in triplicates for each bag, using the spectrophotometric method of Willis et al. (1996) with slight modifications as described by Avidov et al. (2017).

In the second simulation experiment, the persistence of *Salmonella* in un-stabilized BL was evaluated during a more gradual increase of temperature under anaerobic conditions. Duplicate reactors were filled with 1.4 kg BL (dry-based; 4.7 liters) that was adjusted to 40% water content, initial pH 7, and artificially inoculated with *S.* Infantis, as described above. The reactors were heated to 45°C by increment steps of 1°C and a total heating time of 14 days. The BL was sampled on days 0, 1, 2, 3, 5, 7, 9, 11, and 14 and analyzed for *Salmonella* concentrations.

Modulation of Microbial Populations in BL

Two sets of experiments were conducted to check the possible involvement of antagonistic microorganisms in BL that inhibit the growth of *Salmonella* under mesophilic temperatures: (1) Heat inactivation of indigenous microbial populations of the BL. (2) Addition of antibiotics to which the *S.* Infantis strain is resistant. The effect of heat inactivation was explored both in liquid suspension and in the BL itself. *For BL suspension*—aliquots of 8 g (dry-based) of un-stabilized BL pre-adjusted to 60% water content, were placed in open 50 ml PP tubes and autoclaved for 1 h (Vertical pressure steam sterilizer LS-B50L-I, KWF, China; 121°C, 0.11 MPa). Autoclave performance was validated with 1 mL spore ampules (Crosstex, USA) placed within a BL sample of similar size. The autoclaved and non-autoclaved BL samples were inoculated with 100 μl of a *Salmonella* stock, and mixed by vortexing the tubes for a few seconds. The content of each tube was diluted 1:10 in PBS (mixing in a sterile stomacher bag, as described above), and 25 ml suspension were transferred into Erlenmeyer flasks containing 100 ml of sterile NB (a total volume of 125 ml). Control flasks (no BL suspension) contained 100 ml of sterile NB that was inoculated with 100 μl of the *Salmonella* stock. The initial concentration of *Salmonella* was *ca.* 2 $\log_{10}$ CFU ml^{-1} in EXP 1 (heat inactivation) and 4 log CFU ml^{-1} in EXP 2 (a second heat inactivation experiment and the addition of antibiotics). Multiplication of the pathogen was monitored over 24 h. *For the BL itself*—aliquots of 8 g (dry-based) of un-stabilized and stabilized BL were placed in 50 ml open PP tubes and autoclaved. Both autoclaved and non-autoclaved samples were inoculated with *Salmonella* as described above. The initial concentration of *Salmonella* in this experiment was *ca.* 8 $\log_{10}$ CFU g^{-1} dry matter. The tubes were placed inside a biological hood and incubated for 11 days at room temperature (20–25°C) under aerobic or anaerobic conditions. The tubes under aerobic conditions were left with untighten caps and those under anaerobic conditions were kept closed after replacing the headspace with N_2 (purging for 30 s). *Salmonella* was enumerated by the end of the incubation period.

The effect of antibiotics was explored in a liquid suspension of un-stabilized BL as described above (without autoclaving). The suspension was amended with a cocktail of antibiotics [tetracycline, 20 μg ml^{-1}; nitrofurantoin, 64 μg ml^{-1}; trimethoprim, 50 μg ml^{-1}; nalidixic acid, 20 μg ml^{-1}; sulfamethoxazole, 50 μg ml^{-1}; and rifampin, 100 μg ml^{-1} (Aviv et al., 2014); all from Sigma–Aldrich, St. Louis, MO; ≥97% purity]. Amended and un-amended NB medium were inoculated with the same *Salmonella* stock. The initial concentration of *Salmonella* in this experiment was *ca.* 4 $\log_{10}$ CFU ml^{-1} and multiplication of the pathogen was monitored over 120 h.

Persistence of *S.* Infantis in Soil-BL Mixtures

Batch of 1 kg soil was mixed with 5% (v/v) of un-stabilized or stabilized BL containing *S.* Infantis, at an initial concentration of *ca.* 6 $\log_{10}$ CFU g^{-1} dry matter of the final soil-BL mixture. The mixtures were brought to water field capacity of 30 or 70% (13.5 and 31.5% water content, respectively) and then divided into aliquots of 8 g (dry-based), which were transferred into 50 ml PP tubes and incubated at 30°C. Triplicate tubes were sacrificed for *Salmonella* counting on days 0, 4, 8, 11, 18, 31, 45, 60, 90, and 105. The content of each tube was suspended in PBS at 1:5 ratio, vortexed for 3 min, and *Salmonella* enumeration was performed as described above. Triplicate tubes containing non-inoculated soil-BL mixture at 70% water field capacity served as control on each sampling day, and were always found to be free of *Salmonella*.

Statistical Analyses

JMPIN software was used for all statistical analyses (SAS pro 14; SAS Institute Inc.). Mean values were compared by the Tukey-Kramer Honestly Significant Difference (HSD) test at $p \leq 0.05$. Analyses of CFU counts were performed using log-transformed values.

RESULTS

The Combined Effect of Temperature, Water Content, and pH on the Persistence of *S.* Infantis in BL During Static Incubation

The persistence of *S.* Infantis under the 36 combinations of temperature, water content, and pH is presented in **Figures 1A–I**. Each figure represents the results of four temperatures at one selected water content and initial pH. The BL was incubated under the selected temperature (30, 40, 50, or 60°C) for 2 weeks (phase I), during which *Salmonella* count was reduced below the detection limit after enrichment (1–2 CFU g^{-1}

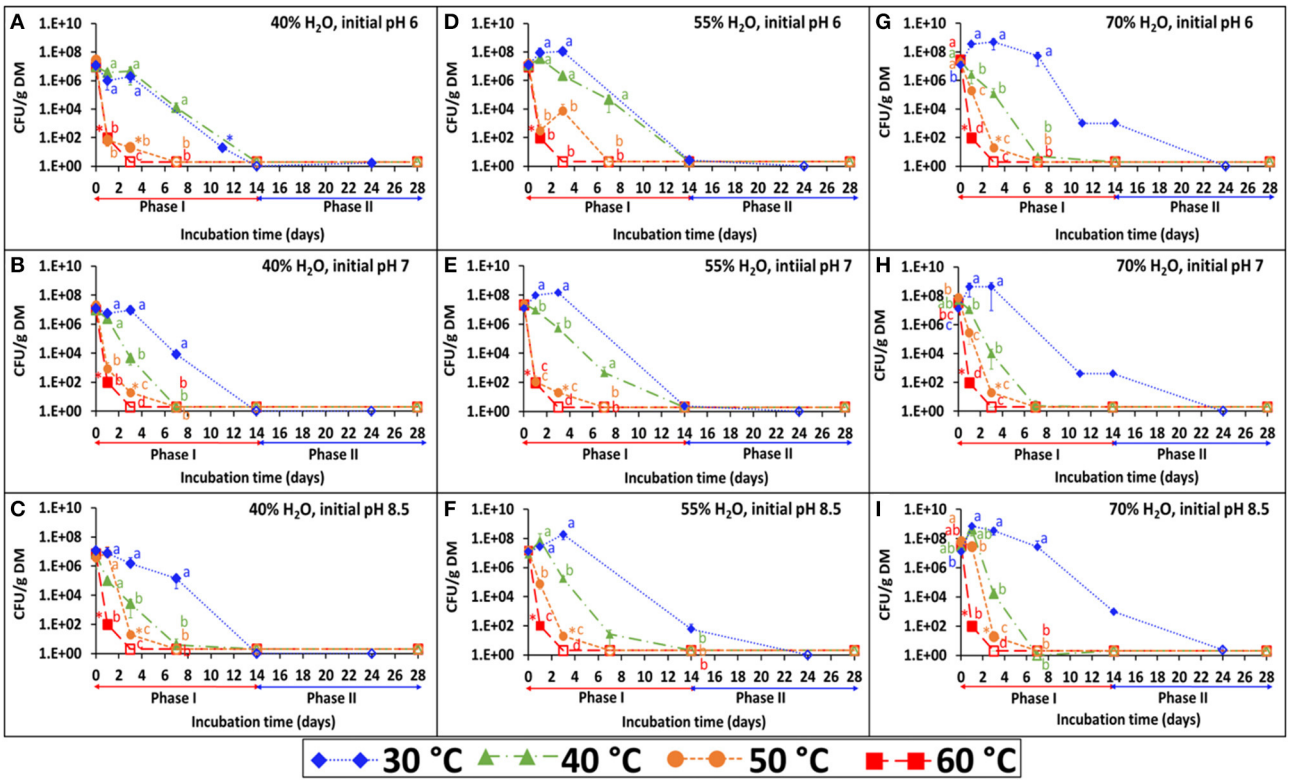

FIGURE 1 | Persistence of *S*. Infantis in broiler litter (BL) incubated at different temperatures (30, 40, 50, and 60°C), water content (40, 55, and 70%) and initial pH (6, 7, and 8.5). The different incubation temperatures were applied during phase I (weeks 1–2), while at phase II (weeks 3–4) the incubation temperature was 30°C. Error bars represent the standard deviations of triplicate vessels. Letters denote significant differences between treatments at time intervals (log-transformed CFU values; Tukey-Kramer HSD; $P \leq 0.05$). Empty symbols indicate values below the detection limit after enrichment (1–2 CFU g^{-1} dry matter); *values below the detection limit without enrichment (10–20 CFU g^{-1} dry matter). The figures are grouped according to water contents of 40% **(A–C)**, 55% **(D–F)**, and 70% **(G–I)**. DM, dry matter.

dry matter). Besides water content which was adjusted during incubation, the pH (**Figure 2**) and EC (**Supplementary Figure 1**) were dynamic and monitored in a parallel experiment of non-inoculated BL. Under lower temperatures (30 and 40°C), the pH typically increased during the first 2 weeks and stabilized at the range of 9–9.5. Different dynamics were observed under the higher temperatures (50 and 60°C), in which the pH tended to decrease during phase I and in certain cases to increase later during phase II. This effect of temperature on pH development was more evident at the lower water contents and the lower initial pH. Regarding the EC, a general increase was observed for all combinations during phase I, but it was more evident at a water content of 70% for which this increase was significantly higher in the lower (30 and 40°C) compared to the higher (50 and 60°C) temperatures.

The first-order decay constants in this experiment [$-k$ (day^{-1})], are presented in **Table 2**. Based on these values, the time needed to reduce *Salmonella* below the detection limit after enrichment (<1 CFU g^{-1} dry matter) was calculated for initial concentrations of 7 and 3 log CFU g^{-1} dry matter. Temperature was shown to be the main factor influencing *Salmonella* decay rates, while water content and initial pH were found to be of secondary level of influence with significant effects mainly at 30 and 40°C. Under all water content and pH conditions, it was estimated that the time required to achieve 7 log reduction in *Salmonella* concentration ranges between 13.7–27.2, 6.5–15.6, 1.2–4.7, and 1.3–1.5 days for 30, 40, 50, and 60°C, respectively. Thus, the effect of temperature was most evident in the transition from mesophilic to thermophilic conditions, whereas increasing the temperature from 40 to 50°C was associated with increased decay rates by a factor of 4.6. Water content had a significant effect at 30°C, with the highest decay rates at a water content of 40% at all initial pH values. In contrast, at 40°C, the highest decay rates were observed at a water content of 70% at all pH values, and also at a water content of 40% and initial pH 7 and 8.5 only. Such effects were still significant at 50°C, with the highest decay rates at water contents of 40 and 55% and initial pH 6 and 7. These effects were negligable at 60°C.

Regrowth of *Salmonella* was not observed in phase II in any of the combinations after transferring the vessels to 30°C, neither in the vessels which were initially incubated at 30°C. *Salmonella* counts in non-inoculated control samples were below the detection limit throughout the experiment, negating the possibility that indigenous *Salmonella* were present in these samples or that cross-contamination occurred between samples.

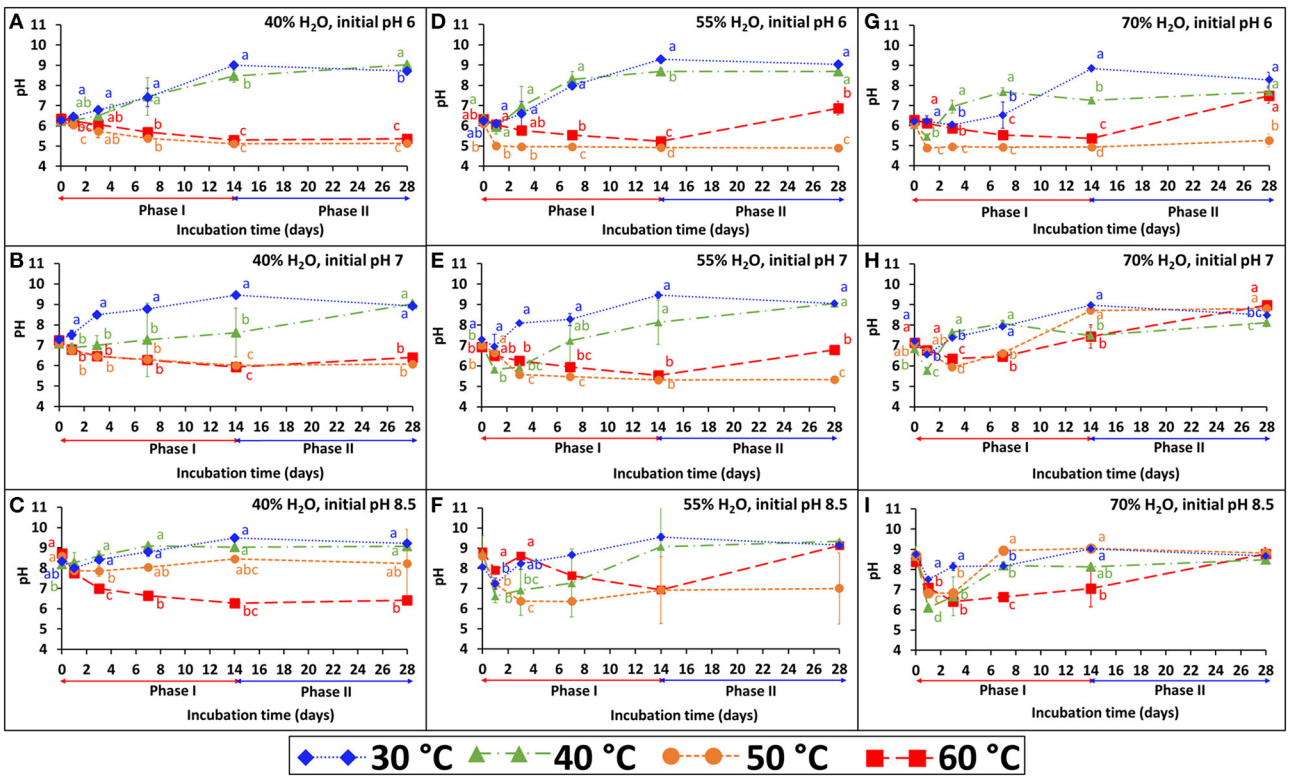

FIGURE 2 | pH dynamics during incubation of broiler litter (BL) at different temperatures (30, 40, 50, and 60°C), water content (40, 55, and 70%) and initial pH (6, 7, and 8.5). The different incubation temperatures were applied during phase I (weeks 1–2), while at phase II (weeks 3–4) the incubation temperature was 30°C. Error bars represent the standard deviations of triplicate vessels. Letters denote significant differences between treatments at time intervals (Tukey-Kramer HSD; $P \leq 0.05$). The figures are grouped according to water contents of 40% **(A–C)**, 55% **(D–F)**, and 70% **(G–I)**.

Persistence of *S.* Infantis Under Controlled Aerobic and Anaerobic Conditions using Laboratory-Scale Simulations

The persistence of *S.* Infantis under controlled aerobic and anaerobic simulations of BL, pre-adjusted to a water content of 40% and initial pH 7, is demonstrated in **Figures 3A,B**. During phase I (mesophilic), *Salmonella* concentrations decreased substantially under both conditions, with 4–5 $\log_{10}$ reduction under aerobic conditions (**Figure 3A**) and 7 $\log_{10}$ reduction (below the detection limit) under anaerobic conditions (**Figure 3B**). During this phase, the water content decreased unintentionally in the aerobic reactors from 40 to 18.7% (**Figure 3A**–inset), since aeration was provided through a bypass instead of using the humidifier (see Materials and Methods) On day 7, the water content of the three aerobic reactors was re-adjusted to 40%, which in turn resulted in a 4–5 $\log_{10}$ increase of *Salmonella* concentration in one of the reactors; the one that was set to 50°C during phase II of the simulation. When phase II started (day 9), *Salmonella* concentrations increased even more in that reactor, but dropped below the detection limit after an additional 3 days. The effect of BL drying was also recognized at 60°C, where *Salmonella* showed a higher persistence and decreased below the detection limit only after 3 days, which is longer than expected based on the static vessels experiment (**Figure 1** and **Table 2**). Moreover, following 2 days at 40°C in phase II, *Salmonella* increased by 4 $\log_{10}$ and then steadily increased, reaching a concentration similar to the initial conditions of the simulation. Under these conditions, *Salmonella* was not reduced below the detection limit, even after 2 more weeks of incubation at 30°C (Phase III). In contrast to aerobic conditions, *Salmonella* did not persist under anaerobic conditions, and no growth was observed during phases II and III of the simulation (**Figure 3B**).

NH_3 monitoring (**Figures 3C,D**) revealed a major difference between aerobic and anaerobic conditions, with no clear effect of temperature (phase II). Concentrations peaked on day 3 under aerobic conditions (*ca.* 270 mg m^{-3}) while under anaerobic conditions they were mostly below 5 mg m^{-3}. The respective pH values (**Figures 3E,F**) also showed a clear difference between aerobic and anaerobic conditions. The pH fluctuated or slightly increased during aerobic simulation, while under anaerobic conditions a distinct reduction (from 6.5–7 to 5.5) was observed in phase I.

The fate of *S.* Infantis in BL (pre-adjusted to a water content of 40% and initial pH 7) under anaerobic conditions was further investigated in another simulation, during which the mesophilic temperatures increased more gradually from 28 to 42°C (**Figure 4**). *Salmonella* concentration decreased from

TABLE 2 | First-order decay rate constants (*–k;* day^{-1}) and the calculated time needed to reduce *S.* Infantis below the detection limit after enrichment, at initial concentrations of 7 and 3 log CFU g^{-1} dry matter.

Temperature (°C)	H_2O (%)	pH	$n^{\dagger}$	$R^{2\dagger\dagger}$	Decay rate constant, *–k* (day^{-1})	Significance of differences between *–k*-values (Tukey-Kramer HSD; $P \leq 0.05$)$^{++}$		Days required to reduce *S.* Infantis below the detection limit after enrichment (<1 CFU g^{-1} dry matter)	
						Analyzed for all 36 combinations together	Analyzed for each temperature separately	Initial concentration: 7 log CFU g^{-1} dry matter	Initial concentration: 3 log CFU g^{-1} dry matter
30	40	6	5	0.96	1.174	H	a	13.72 (13.64–13.81)*	5.88 (5.84–5.92)
		7	5	0.95	1.117	H	a	14.44 (13.90–15.00)	6.19 (5.96–6.43)
		8.5	5	0.93	1.097	H	a	14.47 (13.55–16.06)	6.33 (5.81–6.88)
	55	6	5	0.85	0.770	H	b	20.95 (20.64–21.27)	8.98 (8.84–9.11)
		7	5	0.84	0.771	H	b	20.91 (20.43–21.39)	8.96 (8.76–9.17)
		8.5	5	0.89	0.717	H	bc	22.49 (21.90–23.09)	9.64 (9.39–9.90)
	70	6	7	0.77	0.636	H	cd	25.36 (25.22–25.50)	10.87 (10.81–10.93)
		7	6	0.85	0.705	H	bc	22.86 (22.73–22.99)	9.80 (9.74–9.85)
		8.5	6	0.78	0.593	H	d	27.18 (26.45–27.93)	11.65 (11.34–11.97)
40	40	6	5	0.94	1.036	H	c	15.58 (14.88–16.30)	6.68 (6.38–6.98)
		7	4	0.98	2.242	GH	a	7.20 (6.80–7.62)	3.09 (2.91–3.27)
		8.5	4	0.96	2.297	FGH	a	7.05 (6.47–7.67)	3.02 (2.77–3.29)
	55	6	5	0.91	1.055	H	c	15.31 (14.42–16.25)	6.56 (6.18–6.96)
		7	4	0.97	1.606	GC	b	10.38 (8.27–12.74)	4.45 (3.55–5.46)
		8.5	4	0.89	1.664	GC	b	9.72 (9.06–10.41)	4.16 (3.88–4.46)
	70	6	4	0.98	2.176	GC	a	7.41 (7.14–7.70)	3.18 (3.06–3.30)
		7	4	0.98	2.496	DEFGH	a	6.47 (6.09–6.87)	2.77 (2.61–2.94)
		8.5	4	0.89	2.381	EFGH	a	6.78 (6.49–7.07)	2.90 (2.78–3.03)
50	40	6	2	1.00	13.004	A	a	1.24 (1.15–1.35)	0.53 (0.49–0.58)
		7	2	1.00	12.286	AB	a	1.35 (1.09–1.65)	0.58 (0.47–0.71)
		8.5	3	0.80	3.617	CDEFG	b	4.74 (3.43–6.43)	2.03 (1.47–2.73)
	55	6	2	1.00	11.563	AB	a	1.41 (1.23–1.61)	0.60 (0.53–0.69)
		7	2	1.00	13.092	A	a	1.25 (1.09–1.42)	0.53 (0.47–0.61)
		8.5	3	0.99	4.571	CDE	b	3.53 (3.46–3.60)	1.51 (1.48–1.54)
	70	6	3	1.00	4.595	CD	b	3.51 (3.45–3.57)	1.50 (1.48–1.53)
		7	3	0.98	5.132	C	b	3.15 (3.00–3.29)	1.35 (1.29–1.41)
		8.5	3	0.87	4.486	CDEF	b	3.61 (3.34–3.89)	1.55 (1.43–1.67)
60	40	6	2	1.00	11.33	AB	bc	1.42 (1.38–1.46)	0.61 (0.59–0.63)
		7	2	1.00	11.38	AB	abc	1.42 (1.34–1.51)	0.61 (0.57–0.65)
		8.5	2	1.00	10.78	B	c	1.50 (1.37–1.64)	0.64 (0.59–0.70)
	55	6	2	1.00	11.87	AB	abc	1.36 (1.33–1.38)	0.58 (0.57–0.59)
		7	2	1.00	12.19	AB	ab	1.32 (1.27–1.38)	0.57 (0.54–0.59)
		8.5	2	1.00	11.86	AB	abc	1.36 (1.32–1.40)	0.58 (0.57–0.60)
	70	6	2	1.00	12.15	AB	ab	1.33 (1.30–1.36)	0.57 (0.56–0.58)
		7	2	1.00	12.45	AB	ab	1.29 (1.27–1.32)	0.55 (0.54–0.56)
		8.5	2	1.00	12.71	AB	a	1.27 (1.25–1.29)	0.54 (0.54–0.55)

$n^{\dagger}$, number of time points included in the linear correlation. For each set of data (including all replicate samples in each condition), the exact number of time points used for the linear correlation was selected to provide the highest R^2 (excluding data points below the detection limits).

$R^{2\dagger\dagger}$, the average linear regression coefficient of triplicate set of data (based on $n^{\dagger}$ data points).

**Minimum and maximum values calculated for triplicate –k data.*

$^{++}$Letters denote significant differences between treatments (Tukey-Kramer HSD; $P < 0.05$).

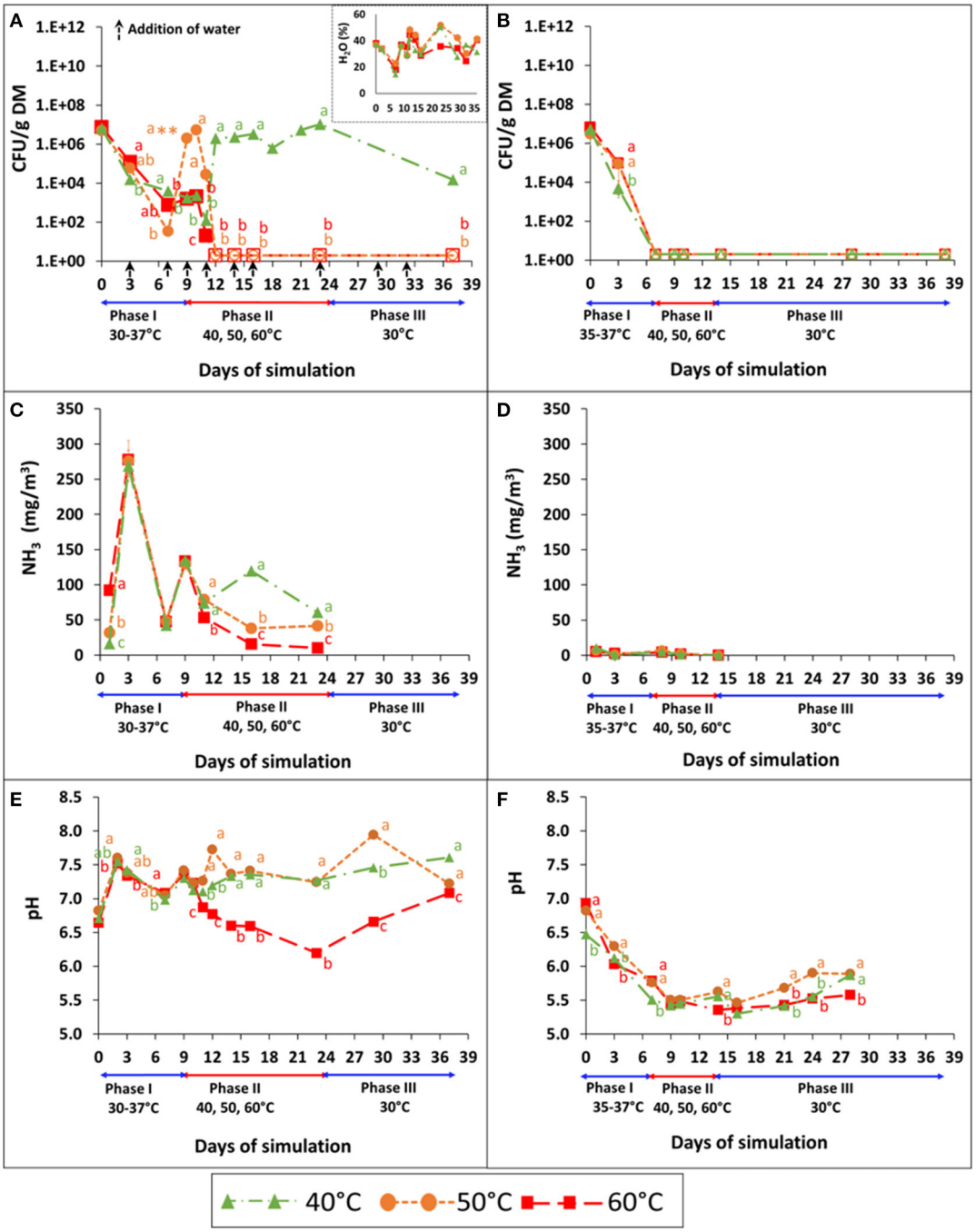

FIGURE 3 | Persistence of *S.* Infantis **(A,B)**, emissions of NH_3 **(C,D)**, and pH dynamics **(E,F)** in broiler litter (BL), during controlled aerobic and anaerobic simulations performed in six reactors (one reactor for each treatment). The water content was monitored and adjusted under aerobic conditions (**A**–inset). Mesophilic temperatures were maintained between 30 and 37°C in phase I and then increased to 40, 50, or 60°C in phase II until *Salmonella* was reduced below the detection limit. Finally, in phase III, the contents of each of the reactors were transferred to 30°C for 2 more weeks. The airflow was constant (5 l min^{-1}) during the simulations of aerobic conditions. Error bars represent the standard deviations of triplicate analyses performed on a unified sample collected from each reactor. Empty symbols indicate values below the detection limit after enrichment (1–2 CFU g^{-1} dry matter); **a value above plate countability. Letters denote significant differences between treatments at time intervals (Tukey-Kramer HSD; $P \leq 0.05$). DM, dry matter.

7.5 log_{10} to <10 CFU g^{-1} dry matter (below detection limit) within 11 days, during which the temperature increased to 38°C only. In a similar manner to the other simulations under controlled anaerobic conditions (**Figure 3F**), the pH decreased from 7.04 (±0.1) to 6.09 (±0.04) over 14 days (data not shown).

Effect of Re-wetting on the Persistence of *S.* Infantis in BL

In addition to the effect of drying and re-wetting shown in the aerobic simulations (**Figure 3**), this effect was tested at 30°C in the static vessels for all water contents at pH 7 (**Figures 1B,E,H**) and for 40% water content only, also at pH 6 and 8.5 (**Figures 1A,C**). Regardless of the initial pH, adjusting the water content to 70% in BL that was previously incubated at 40 and 55% water content, resulted in a burst of *Salmonella* growth by 5–6 $\log_{10}$ CFU g^{-1} dry matter within the next 14 days (**Figure 5**–inset). During this period, no changes in *Salmonella* concentrations were observed in the BL samples which were initially incubated at a water content of 70%.

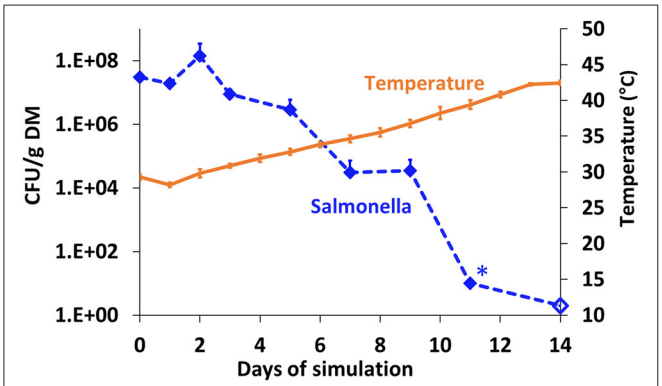

FIGURE 4 | Persistence of *S.* Infantis in broiler litter (BL) during controlled anaerobic simulation in duplicate reactors maintained at mesophilic temperatures. The BL was pre-adjusted to a water content of 40% and initial pH 7, and the temperature increased to 42°C over a period of 14 days. Error bars represent the standard deviations of two reactors. Empty symbols indicate a value below the detection limit after enrichment (1–2 CFU g^{-1} dry matter); *values below the detection limit without enrichment (10–20 CFU g^{-1} dry matter). DM, dry matter.

Potential Antagonistic Activity Against *S.* Infantis in BL

In suspensions containing autoclaved BL, *Salmonella* multiplied at a similar rate as in control medium without BL. From *ca.* 2 $\log_{10}$ CFU ml^{-1} it increased to 9.6 $\log_{10}$ CFU ml^{-1} within 20 h. On the other hand, *Salmonella* growth was completely inhibited in medium containing non-autoclaved BL (**Figure 6A**). These findings were repeated in another experiment using autoclaved BL from another farm (**Figure 6A**–inset). The effect of autoclaving was also observed in the BL itself (20–25°C; 60% water content). The concentration of *Salmonella* in the autoclaved BL increased from *ca.* 8 $\log_{10}$ to 9–10 $\log_{10}$ CFU g^{-1} dry matter within 11 days, both under aerobic and anaerobic conditions. On the other hand, *Salmonella* decreased to 3–6 log CFU g^{-1} dry matter in the non-autoclaved samples, with significantly more reduction under aerobic conditions for the un-stabilized BL (**Figure 6B**).

The effect of BL amendment with antibiotics on the persistence of *Salmonella* is presented in **Figure 7**. In the un-amended treatment (no antibiotics), *Salmonella* multiplication was inhibited within 24 h, while no such inhibition occurred in the antibiotics-amended BL suspension and in the control media with or without antibiotics (no BL). After 24 h

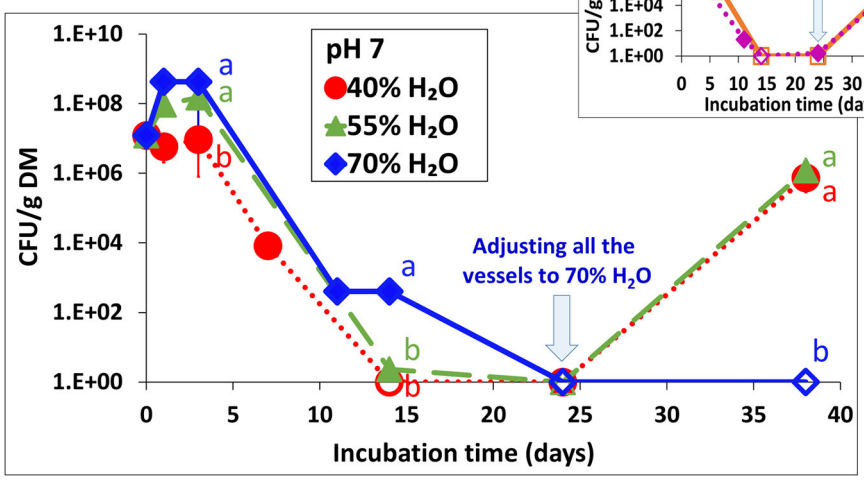

FIGURE 5 | Persistence of *S.* Infantis in broiler litter (BL) following re-wetting of samples which were incubated for 24 days at 30°C and various water contents (as shown in **Figure 1**). The vessels were incubated for additional 14 days following adjustment of water content to 70% in BL that was initially incubated at 40 and 55% water content (initial pH 7) and in the BL that was initially incubated at 40% water content also for initial pH of 6 and 8.5 (inset). Error bars represent the standard deviations of triplicate vessels. Letters denote significant differences between treatments at time intervals (log-transformed CFU values; Tukey-Kramer HSD; $P \leq 0.05$). Empty symbols indicate values below the detection limit after enrichment (1–2 CFU g^{-1} dry matter). DM, dry matter.

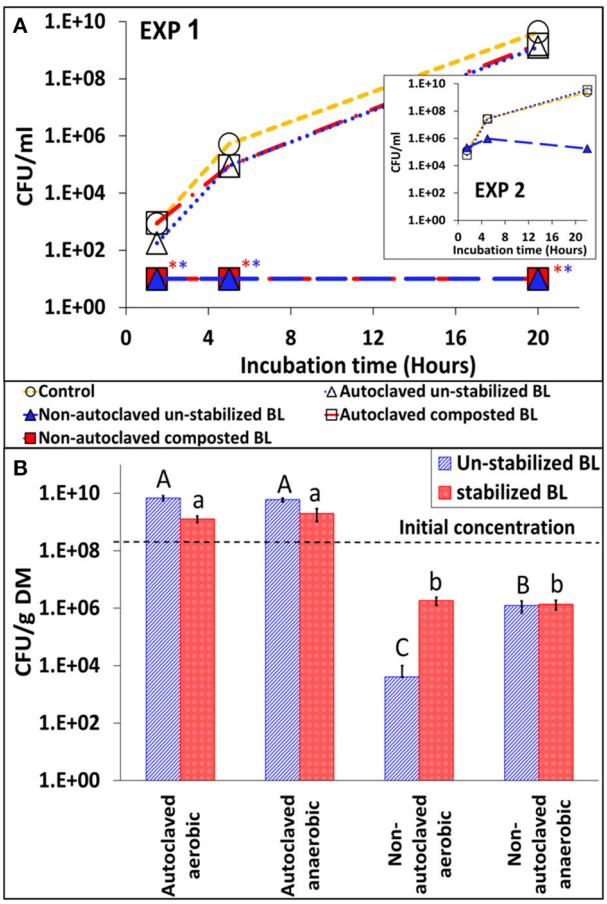

FIGURE 6 | The efect of heat inactivation of indigenous microbial populations in broiler litter (BL) on *S.* Infantis multiplication and persistance. **(A)** Growth of *Salmonella* in nutrient broth suspension of autoclaved or non-autoclaved BL (un-stabilized and composted BL; initial *Salmonella* concentration *ca.* 2 log CFU ml^{-1}). The inset figure shows the results of a similar experiment conducted with BL from another farm and at higher initial *Salmonella* concentration (*ca.* 4 log CFU ml^{-1}). *Values below the detection limit without enrichment (10 CFU ml^{-1}). **(B)** Persistence of *Salmonella* in the un-stabilized and stabilized BL itself, which were artificially contaminated with the pathogen and incubated for 11 days. Error bars represent the standard deviations of triplicate tubes. Letters denote significant differences between treatments by the end of the incubation period (log-transformed CFU values; Tukey-Kramer HSD; $P \leq 0.05$). DM, dry matter.

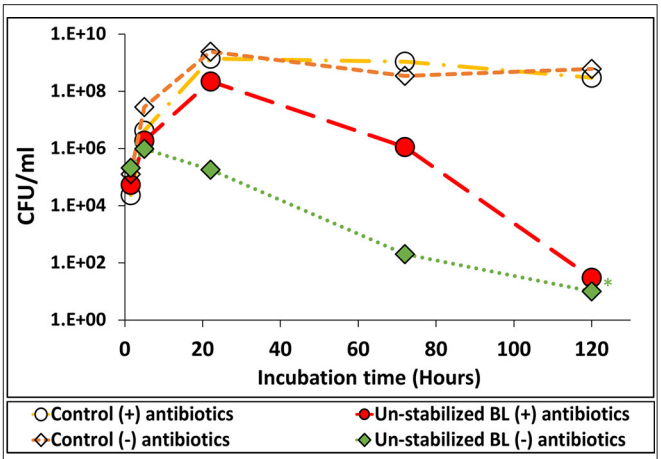

FIGURE 7 | The efect of antibiotics-induced inactivation of indigenous microbial populations in broiler litter (BL) on *S.* Infantis multiplication. Growth of *Salmonella* in nutrient broth suspension of un-stabilized BL, which was amended or not amended with a cocktail of antibiotics to which the pathogen is resistant. *Values below the detection limit without enrichment (10 CFU ml^{-1}).

Salmonella concentration declined also in the antibiotics-amended BL (compared to the maximum value reached in the controls), yet the decline rate was lower than in the un-amended treatment.

Persistence of *S.* Infantis in BL-amended Soil

Persistence of *Salmonella* in soil amended with artificially-inoculated un-stabilized or stabilized BL is shown in **Figure 8**. Within 60 days, *Salmonella* concentration reduced by 2 $\log_{10}$ in the soil with stabilized BL and by 3–4 $\log_{10}$ in the soil with un-stabilized BL. *Salmonella* reduction was substantially slower at the higher water content (70 vs. 30% of field water capacity). After 90 days, the relatively dry soil samples (either with un-stabilized or stabilized BL) were also adjusted to 70% of water field capacity, which resulted in *Salmonella* increase by 2–3 $\log_{10}$ CFU g^{-1} dry matter, 15 days later. Control samples, without addition of *S.* Infantis, were free of *Salmonella* throughout the experiment.

DISCUSSION

The persistence of *Salmonella* in poultry litter has been the subject of several studies that mainly focused on thermal inactivation (Williams and Benson, 1978; Wilkinson et al., 2011; Kim et al., 2012; Singh et al., 2012; Chen et al., 2013; Biswas et al., 2019). Most of them also demonstrated the effect of water content (Wilkinson et al., 2011; Kim et al., 2012; Singh et al., 2012; Chen et al., 2013), whereas the combined effect of multiple factors remained less understood. Beyond temperature, this study presents a more comprehensive investigation of various co-factors affecting the persistence of *S.* Infantis, an emerging pathogen associated with the poultry industry worldwide (Hindermann et al., 2017). These factors include water content, pH, drying and re-wetting, aerobic vs. anaerobic conditions, and potential antagonistic activity. The water content range was selected as being relevant for composting processes (Christian et al., 2009; Zakarya et al., 2018) and the range of initial pH was selected as being relevant for a spectrum of BL properties (Gordillo and Cabrera, 1997; Wood et al., 1999; Ekinci et al., 2000; Lopez-Mosquera et al., 2008). For practical reasons, four sources of BL were used in this comprehensive experimental scheme. Evidently, different sources may add additional factors to the already complex interactions among the co-factors examined in this study.

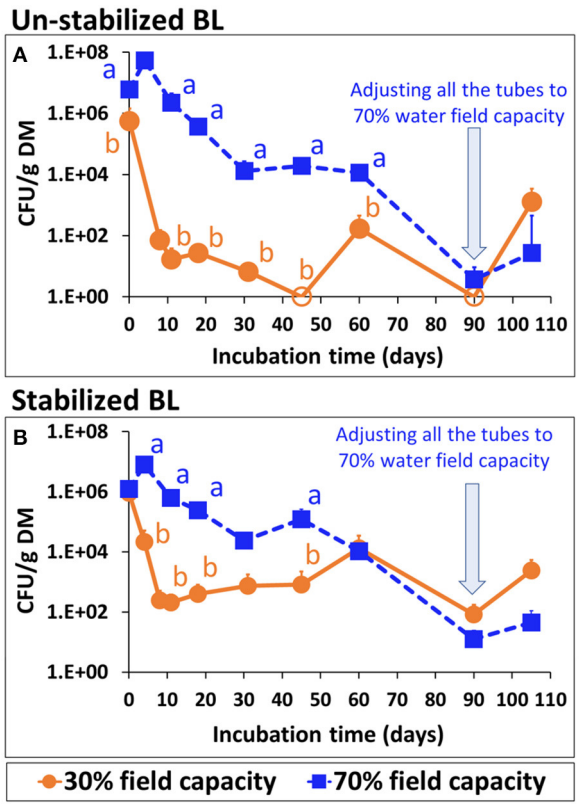

FIGURE 8 | Persistence of *S.* Infantis in a vertisol soil amended with 5% (v/v) of un-stabilized or stabilized broiler liter (BL), adjusted to 30 or 70% water field capacity. **(A)** Un-stabilized BL. **(B)** Stabilized BL. Letters denote significant differences between treatments at time intervals (log-transformed CFU values; Tukey-Kramer HSD; $P \leq 0.05$). Error bars represent the standard deviations of triplicate tubes. Empty symbols indicate values below the detection limit after enrichment (1–2 CFU g^{-1} DM). DM, dry matter.

Yet, these BL sources are from poultry growers in the same geographical region that use raising protocols of the biggest poultry cooperatives in Israel. Some of the variability shown in **Table 1** regarding the main BL properties, may be related to the spatial variability within the poultry house, as well as the time of BL storage before each of the experiments which may vary as well.

Overall, the results indicate the significant role of several co-factors under mesophilic temperatures, in which *Salmonella* may persist longer, rather than under thermophilic temperatures (above 50°C in the present study) in which thermal inactivation is the main effective mechanism. The primary role of temperature is evident from the series of BL incubation experiments, in which 36 combinations of temperatures, water contents, and pH were tested in lab vessels (**Figure 1**). A 7 $\log_{10}$ reduction of *Salmonella* was achieved within 6.5–27.2 days at 30–40°C and within 1.2–4.7 days at 50–60°C (**Table 2**). Although the bags inside the vessels were loosely tied, we could not ensure aerobic conditions throughout the entire material. However, this setup is highly relevant for real scenarios since it is practically impossible to avoid anaerobic pockets during composting, especially in the core of windrow piles (Poulsen, 2011; Stegenta et al., 2019) and it is certainly relevant for static uncontrolled litter piles. Under thermophilic temperatures, the results of Singh et al. (2012) on poultry litter-based compost, are generally in agreement with the range of decay rates found in the present study. Thermal inactivation is anticipated to remain the primary factor affecting the persistence of *Salmonella* during stabilization and composting of BL. Yet, since most of the BL worldwide is still applied without controlled processing (Ogejo and Collins, 2009; Wilkinson et al., 2011; Wiedemann, 2015), the fate of *Salmonella* under mesophilic temperatures has more practical implications. Moreover, even composting may not ensure thermophilic conditions throughout the pile. In their study on poultry litter composting, Wilkinson et al. (2011) showed that only *ca.* 35–40% of the pile's cross-sectional area was exposed to temperatures higher than 55°C during the first 2 weeks. Similarly, in their study on composting of municipal sewage sludge in open piles, Stegenta et al. (2019) estimated that 69% of the pile volume did not reach a temperature of 60°C within 7–8 weeks. Also, Isobaev et al. (2014) showed that 24% of the temperature probes that were introduced randomly into a covered aerated static pile of biosolids, did not meet sanitation conditions within 4 weeks (55°C for 3 days). Such studies emphasize that composting treatment may not ensure exposure of all particles to thermophilic temperatures and durations that are needed for pathogen elimination.

A longer persistence, yet consistent decay of *Salmonella* in BL under mesophilic temperatures (a non-thermal inactivation), has been more scarcely reported. Previous studies suggested that thermal sensitivity of microorganisms increases with increasing water content (de Bertoldi, 1988; Wilkinson et al., 2011), but at a lower temperature, desiccation may play a greater role in pathogen inactivation (Wilkinson et al., 2011). After 24 h under mesophilic temperature (35°C), Wilkinson et al. (2011) found higher persistence of *S.* Typhimurium in poultry litter at a water content of 65% compared to 30%. The present study indicates that under mesophilic temperatures and favoring water contents, *Salmonella* may initially multiply in BL following inoculation (**Figures 1**, **5**), as well as in BL-amended soil mixtures (**Figure 8**; 70% field water capacity), and then decay under all conditions. While the role of water content and initial pH was evident, there was no consistent effect with regards to these two factors. At 30°C, *Salmonella* decay rate was significantly higher at a lower water content. Likewise, *Salmonella* decayed substantially faster at 30 vs. 70% of water field capacity in soil-BL mixtures incubated at 30°C (**Figure 8**). In contrast, at 40°C, the effects of water content as well as initial pH were less consistent.

The varying effect of water content and pH and the dynamics of pH and EC during incubation (**Figure 2** and **Supplementary Figure 1**), further emphasize the complication of predicting the role of co-factors that may synergistically affect *Salmonella* persistence. It may affect *Salmonella* directly by modifying the physico-chemical conditions favored by the pathogens, or indirectly by stimulating or inhibiting the

activity of antagonistic populations. For example, high water content (70% in this study) may be associated with reduced air-filled porosity and the presence of anaerobic conditions. This, in turn, may affect the composition and density of antagonistic populations on one hand (biotic factor) and NH_3 emissions on the other hand (abiotic factor). Nevertheless, although the release of NH_3 was proposed as a factor that may inactivate *Salmonella*, the controlled simulations of the present study do not support this hypothesis. *Salmonella* decayed faster under anaerobic conditions (**Figures 3A,B**), during which NH_3 emissions were negligible compared to those obtained under aerobic conditions (**Figures 3C,D**). The lower NH_3 emissions under anaerobic conditions are expected due to the production of organic acids (Beffa et al., 1996; Naikwade et al., 2011), as evident also from the pH dynamics (**Figures 3E,F**). Thus, based on our findings, although aerobic processing of BL resulted in high levels of NH_3, it was not a major factor in *Salmonella* inactivation under mesophilic temperatures. Notably, NH_3 was shown to be effective as a process treatment designed to reduce enteric pathogens in livestock manure (Gurtler et al., 2018). Yet, this practice is based on the addition of high concentrations of liquid ammonia (Himathongkham and Riemann, 1999; Ottoson et al., 2008; Bolton et al., 2012) and not relied on the naturally associated NH_3 emissions during manure stockpiling. Other studies also suggested the involvement of NH_3 emission as a factor in the inactivation process (Kim et al., 2012; Chen et al., 2013, 2015). In these studies, inactivation was faster in more active samples (like fresh vs. aged manure), which also emitted more NH_3; however, the factor of ammonia emission was not isolated.

The effect of BL drying and re-wetting on *Salmonella* persistence was evident from three different experiments. First, during composting simulations under aerobic conditions, the unintentional drying (from 40 to 14–23% water content) and the following re-wetting, resulted in rapid multiplication of *Salmonella* with 4 and 5 $\log_{10}$ increase in bacterial counts, at 40 and 50°C, respectively. This burst of growth was associated with increased thermal tolerance of the pathogen and a longer persistence. At 40°C *Salmonella* persisted more than 30 days following re-wetting (**Figure 3A**) compared to only 6–16 days in the static vessels experiment under equivalent temperature and water content (**Figure 1** and **Table 2**). A second observation was obtained from the static vessels incubated at 30°C and water contents of 40 and 55%. *Salmonella* was reduced below the detection limit after enrichment; yet, re-wetting the BL to 70% water content, resulted in *Salmonella* increase by 5–6 $\log_{10}$ CFU within 14 days, irrespective to the initial pH (**Figure 5**). In the case of 55% water content, which is considered a suitable environment for the pathogen, the effect of water addition on *Salmonella* multiplication may suggest that the pathogen was dehydrated locally during the first phase of incubation. Finally, a third observation was obtained in soil-BL mixtures, in which the increase of field water capacity from 30 to 70% resulted in *Salmonella* multiplication within the following 15 days (**Figure 8**).

Our findings regarding the increased tolerance of *Salmonella* to high temperatures (50–60°C), are concurrent with previous studies. Increased tolerance of *Salmonella* to stress due to desiccation was investigated in pure culture (Gruzdev et al., 2011, 2012) as well as in poultry litter (Chen et al., 2013). In the study of Chen et al. (2013), a 5 $\log_{10}$ CFU reduction of desiccation-adapted cells in aged chicken litter of 20% moisture, required >6, >6, 4–5, and 3–4 h, compared to 1.5–2, 1–1.5, 0.5–1, and <0.5 h, for the non-adapted cells, at 70, 75, 80, and 85°C, respectively. Gruzdev et al. (2011) found that desiccated cells demonstrated high tolerance to a 1-h exposure to dry heat, with no substantial change in their viable counts at 60°C compared to their initial pre-challenge count and 1.5- and 3.1 $\log_{10}$ reductions at 80 and 100°C, respectively. In contrast, non-desiccated cells were highly susceptible to heat, with as much as a 3 $\log_{10}$ CFU reduction at 60°C and an 8 $\log_{10}$ reduction (below the detection limit) following 1-h incubation at 80 and 100°C. A reasonable mechanism to the increased tolerance of desiccated bacteria to high temperatures, is that very low water content in bacterial cells can inhibit or diminish the protein denaturation induced by high-temperature heating through vibration of water molecules to break S-S and hydrogen bonds of intracellular proteins. Thus, desiccation prevents the bacteria from denaturation of the membrane proteins and preserving their integrity even during exposure to a very high temperature (Earnshaw et al., 1995; Archer et al., 1998; Hiramatsu et al., 2005).

The increased thermal tolerance following re-wetting as observed in the present study has important implications. BL may dry before soil application and then be re-wetted by rain or field irrigation. The BL may also dry before composting, or any short phase of thermal stabilization, which may increase the chance of ineffective thermal inactivation. The increased stress-tolerance of desiccated cells may also be meaningful with regards to salinity. Due to organic matter oxidation (volatilization) and increased ash content, the salinity of BL increases during composting as shown in **Supplementary Figure 1**. Gruzdev et al. (2011) showed that desiccated *Salmonella* cells were able to maintain their viability in 1 to 5% bile salts and 0.1–0.5 M NaCl, while the number of the non-desiccated cells continuously declined in a dose-dependent manner. The increased tolerance to pH changes may also play a role, as the pH dynamics can vary substantially during BL processing (**Figure 2**). In this case, Gruzdev et al. (2012) showed that the survival of dehydrated *Salmonella* was maximal at pH 8.0 and decreased at lower or higher pH values, while that of the non-dehydrated cells was higher at the various pH values.

Out of the co-factors investigated in this study, the involvement of *Salmonella* antagonistic microorganisms (SAM) seems to play a major role in *Salmonella* inactivation by non-thermal mechanisms under mesophilic temperatures. Three different experiments provided indirect evidence that SAM activity was the primary mechanism for non-thermal inactivation of *S.* Infantis. Heat inactivation of the indigenous microbial populations of BL (**Figure 6**) or the addition of antibiotics to which the *S.* Infantis is resistant (**Figure 7**) resulted in augmentation of *Salmonella* multiplication, suggesting that raw BL contains microbial antagonists, susceptible to heat and

antibiotics, which inhibit the growth of *Salmonella*. In the case of antibiotics addition, this effect was lessened in the following days, presumably due to degradation of the antibiotics. Notably, the potential effect of SAM inactivation was shown both in liquid suspensions of BL (**Figures 6A**, **7**) and under aerobic and anaerobic conditions in the BL itself (**Figure 6B**), using different BL sources and degree of stabilization. Under anaerobic conditions, *Salmonella* may be inactivated also by the presence of volatile fatty acids (VFAs) that are produced during anaerobic digestion (Kunte et al., 1998; Salsali et al., 2006; Jiang et al., 2018). Yet, as shown in **Figure 6B**, *Salmonella* persisted longer in the non-autoclaved un-stabilized BL under anaerobic than under aerobic conditions, such that the effect of VFAs in this case seems unlikely. This varying magnitude of the potential effect of SAM can be related to the different experimental systems tested along this study but also due to the use of different BL sources and storage time. As suggested by Bucher et al. (2020), multiple factors and particularly physico-chemical variables are associated with litter microbiome succession. Yet, the authors emphasized the critical role of litter moisture and pH on bacterial diversity, which were pre-adjusted in all experiments of the present study. Moreover, heat inactivation by means of autoclaving was shown to facilitate the later proliferation of some heat-resistant bacterial populations in soil (Baker et al., 2020) and compost (Kim et al., 2011); yet the diversity of microbial populations after autoclaving is expected to be much lower before reaching a new equilibrium (Baker et al., 2020). Thus, although the water content and initial pH were pre-adjusted before each experiment, we cannot assess potential differences in *Salmonella* antagonistic populations. Moreover, we could not obtain specific information from the broiler growers about the possible use of antibiotics during the growing period, and we cannot assess the long-term impact of such use on BL microbiome.

The effect of antagonistic microorganisms on the fate of pathogens during composting of livestock manure and biosolids was suggested previously, based on a small number of observations (Millner et al., 1987; Sidhu et al., 2001; Jiang et al., 2002; Szala and Paluszak, 2008; Wilkinson et al., 2011; Weinberg et al., 2014). Similarly to the present study, Sidhu et al. (2001) showed the effect of sterilization of biosolids compost, in which *S.* Typhimurium multiplied rapidly as compared to non-sterilized compost in which *Salmonella* was suppressed. Likewise, Jiang et al. (2002) showed a longer persistence of *E. coli* in manure-soil mixtures, in which the soil was sterilized, compared to a mixture of non-sterilized soil. Following *Salmonella* decay in the present study, either by physico-chemical or presumably biological mechanisms, no regrowth was observed under mesophilic temperatures, besides those cases of BL re-wetting. These findings support the possibility that re-wetting is a major process inducing *Salmonella* regrowth. Then, if a *Salmonella*-contaminated BL or any BL-based organic fertilizer is applied, the pathogen may survive in the soil for several months, presumably due to a reduced antagonistic activity in the soil environment. Furthermore, multiplication of *Salmonella* may also be augmented upon soil wetting. Evidently, the fate of *Salmonella* will be further governed by horizontal and vertical transport mechanisms related to various soil properties, crop root systems, agricultural practice, and rainfall (Mawdsley et al., 1995; Islam et al., 2004a), as well as to the dynamics of SAM due to the same factors. Finally, the initial *Salmonella* concentrations used along this study (*ca.* 7 $\log_{10}$ g^{-1} dry mater) and the calculations regarding the number of days required to reduce *S.* Infantis below the detection limit, are relevant to real scenarios. *Salmonella* concentrations in the range of 3–5 $\log_{10}$ g^{-1} litter have been reported in the literature (Chinivasagam et al., 2009, 2010; Brooks et al., 2010); moreover, as shown in the present study, *Salmonella* may multiply in the litter or following soil application, and increase by several orders of magnitude.

CONCLUSIONS

Thermal inactivation is the primary mechanism of *S.* Infantis elimination in BL. Thus, composting or any thermal processing is expected to minimize the risk of *Salmonella* contamination upon soil application. In contrast, under mesophilic temperatures, other co-factors may play a significant role, including water content, pH, drying and re-wetting, aerobic *vs.* anaerobic conditions, and the presence of SAM. Although *Salmonella* persistence may be reduced at lower BL water content under mesophilic conditions, desiccation and re-wetting is critical, and increases the risk of *Salmonella* transfer from the poultry environment to soil and crops. Following soil application, *Salmonella* may persist for several months due to reduced antagonistic activity compared to the BL alone and retain its ability to multiply upon soil re-wetting. Desiccation and re-wetting of BL not only lead to augmentation of *Salmonella* multiplication, but may also increase its tolerance to thermophilic temperatures. The role of SAM under mesophilic temperatures is meaningful both under aerobic and anaerobic conditions with no clear effect of NH_3 emissions. These effects, at varying magnitudes, are expected to appear in BL from different sources, both un-stabilized, as well as stabilized or composted BL in which *Salmonella* persisted after treatment. Future studies are needed to elucidate the mode of action of SAM in BL and soil. Such studies may ultimately assist with selecting the BL-processing conditions under which SAM activity is most effective or by modulation of the BL/soil microbiome toward augmentation of SAM activity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

RA: conceptualization, methodology, data analysis, and writing original draft. VV and AL: conceptualization and methodology. IS: methodology and resources. AH: methodology. SS: conceptualization and reviewing. YC: supervision and reviewing. YL: supervision, conceptualization, writing, reviewing, and

editing. All authors: contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.645721/full#supplementary-material

Supplementary Figure 1 | Electrical conductivity (EC) dynamics during incubation of broiler litter (BL) at different temperatures (30, 40, 50, and 60°C), water content (40, 55, and 70%) and initial pH (6, 7, and 8.5). The different incubation temperatures were applied during phase I (weeks 1–2), while at phase II (weeks 3–4) the incubation temperature was 30°C. Error bars represent the standard deviations of triplicate vessels. Letters denote significant differences between treatments at time intervals (Tukey-Kramer HSD; $P \leq 0.05$). The figures are grouped according to water contents of 40% **(A–C)**, 55% **(D–F)**, and 70% **(G–I)**.

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Evaluation of Agricultural Byproducts and Cover Crops as Anaerobic Soil Disinfestation Carbon Sources for Managing a Soilborne Disease Complex in High Tunnel Tomatoes

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Anaerobic soil disinfestation (ASD) is a viable option for disease management in tomato production and reduces damage due to a soilborne disease complex consisting of *Pyrenochaeta lycopersici*, *Colletotrichum coccodes*, *Verticillium dahliae*, and *Meloidogyne* spp. There are plentiful options for ASD carbon sources using byproducts of Midwestern United States agriculture or cover crops, yet these carbon sources have not been evaluated for use in Midwestern settings. Low (10.1 Mg/ha) and high (20.2 Mg/ha) rates of corn gluten meal, distillers dried grains, soybean meal, wheat bran, and dry sweet whey were evaluated as ASD carbon sources in growth chamber and greenhouse bioassays. Cover crops including buckwheat, cowpea, crimson clover, mustard, oilseed radish, sorghum-sudangrass, white clover, and winter rye were evaluated in similar bioassays with one amendment rate (20.2 Mg/ha). Reducing conditions developed in soils regardless of carbon source or rate. Use of high rates of corn gluten meal, distillers dried grains, soybean meal, and wheat bran led to the lowest levels of root rot severity compared to non-treated controls. The higher rate of any byproduct carbon source was always more effective than the lower rate in reducing root rot severity. Use of both rates of soybean meal or corn gluten meal and the high rate of distillers dried grains or dry sweet whey led to significant increases in dry root and shoot biomass compared to controls. For cover crops, ASD with crimson clover, sorghum-sudangrass, white clover, or winter rye amendments reduced root rot severity relative to the aerobic control, but not relative to the anaerobic control. Use of cover crops did not significantly impact plant biomass. A subset of three ASD carbon sources [distillers dried grains, soybean meal, and wheat middlings (midds), all 20.2 Mg/ha] were evaluated in five on-farm ASD trials in high tunnels. Soil temperatures were low during the application period, limiting treatment efficacy. Reducing conditions developed in all soils during ASD treatment, and a moderate but significant reduction in root rot severity

was observed following ASD with the soybean meal or wheat midds compared to ASD with distillers dried grains. Tomato yield was not significantly affected by ASD treatment.

Keywords: *Pyrenochaeta lycopersici*, *Colletotrichum coccodes*, *Meloidogyne*, soil amendment, disease management, *Verticillium dahliae*

INTRODUCTION

Protected culture tomato production in Ohio is constrained by a soilborne disease complex consisting of corky root rot (*Pyrenochaeta lycopersici*), black dot root rot (*Colletotrichum coccodes*), Verticillium wilt (*Verticillium dahliae*), and root-knot nematodes (*Meloidogyne hapla* and *Meloidogyne incognita*) (Vrisman et al., 2017; Testen and Miller, 2018; Testen et al., 2020). Based on a state-wide survey, members of this complex are prevalent in Ohio high tunnels with *P. lycopersici* present on 50% of farms, *C. coccodes* present on 97% of farms, *V. dahliae* present on 75% of farms and root-knot nematodes present on 56% of farms (Testen et al., 2020). *Fusarium*, *Rhizoctonia,* and *Pythium* spp. likely also damage tomatoes but occur less frequently than the four core pathogens. The recommended approach for managing this soilborne disease complex relies on anaerobic soil disinfestation (ASD), usually applied in autumn, and grafting susceptible cultivars onto disease-resistant rootstocks (Testen and Miller, 2018; Testen et al., 2020).

Anaerobic soil disinfestation is a soilborne disease management strategy mediated by native soil microbial populations (Blok et al., 2000; Momma et al., 2013). The efficacy of ASD against the soilborne disease complex depends on carbon source (Testen and Miller, 2018), as soilborne fungi vary in their sensitivity to ASD with wheat bran, molasses, or ethanol. Root-knot nematodes are highly sensitive to ASD regardless of carbon sources examined to date (Katase et al., 2009; Butler et al., 2012b; Testen and Miller, 2018, 2019). While the efficacy of wheat bran and molasses as ASD carbon sources has been demonstrated in Midwestern production systems (Testen and Miller, 2018, 2019; Testen et al., 2020), there are more potential ASD carbon sources, including cover crops, yet to be examined for efficacy against the tomato soilborne disease complex.

Carbon sources for ASD should be readily available, inexpensive, and easily broken down by soil microbial populations. Agricultural byproducts, usually those sold as animal feed, meet these criteria. The availability and cost of these byproducts varies by region within the United States, depending on local agricultural industries. Agricultural byproducts commonly studied as ASD carbon sources include brans (Yossen et al., 2008; Momma et al., 2010; Shennan et al., 2018; Testen and Miller, 2019), seed meals (Shennan et al., 2018), molasses and molasses products (Butler et al., 2012a; McCarty et al., 2014), ethanol (Momma et al., 2010; Hewavitharana et al., 2014; Testen and Miller, 2018), crop residues (Blok et al., 2000; Messiha et al., 2007), pomaces (Domínguez et al., 2014; Achmon et al., 2016; Serrano-Pérez et al., 2017), poultry litter (Butler et al., 2012a), manures (Núñez-Zofío et al., 2011; López-Robles et al., 2013; Hewavitharana et al., 2014; Khadka et al., 2020), and high protein, fermented products (Ludeking et al., 2011; van Overbeek et al., 2014). Cover crops have potential as ASD carbon sources because they can be produced *in situ* and can be used to supplement agricultural byproducts as ASD amendments. Various grass, Brassicaceous, and legume cover crops have been used in ASD studies (Blok et al., 2000; Goud et al., 2004; Lamers et al., 2010; Núñez-Zofío et al., 2011; Butler et al., 2012b; Hewavitharana et al., 2014; Korthals et al., 2014; McCarty et al., 2014; Vecchia et al., 2020). As soils, soilborne pathogens, soil microbial communities, and cropping systems differ across regions, it is essential to test the efficacy of various carbon sources when ASD is introduced to a new region (Strauss and Kluepfel, 2015).

The objective of this study was to determine which alternative ASD carbon sources, including cover crops, could effectively reduce damage from the tomato soilborne disease complex, in order to provide Midwestern growers with a range of amendment options. Carbon sources were selected so that they would be appropriate for use by Midwestern growers, meaning they should be readily available at feed mills and relatively inexpensive, or can be produced on-farm. These carbon sources, including cover crops, were assessed in growth chamber and greenhouse bioassays and a subset of these carbon sources were assessed in on-farm trials.

MATERIALS AND METHODS

Agricultural Byproduct Carbon Source Screening

Corn gluten meal (CG), distillers dried grain (DG), soybean meal (SM), wheat bran (WB), and dry sweet whey (WY) were tested for efficacy as ASD carbon sources at high (H: 20.2 Mg/ha) and low (L: 10.1 Mg/ha) rates. Carbon sources were obtained from The Ohio State University Feedstock Processing Research Facility in Wooster, OH. Wheat bran was included as a standard carbon source known to be effective in ASD against the tomato soilborne disease complex (Testen and Miller, 2018). Soils from tomato high tunnels in three Ohio counties (Wayne, Erie, and Highland) with a known history of soilborne diseases were used in these experiments. Experiments were laid in a randomized complete block design (RCBD) with five replications. One replication consisted of one cup containing one tomato plant. Each experiment was conducted twice.

Soils were placed in 266 mL plastic cups (Hefty, Reynolds Consumer Products, USA), amended with a carbon source, flooded to saturation with sterile distilled water, covered with black polyethylene mulch (1.5 mm super strength embossed mulch, blend of LDPE and LLDPE, PolyExpert, Quebec, Canada), and sealed with rubber bands and electrical tape. Two controls were used in these experiments, a non-amended, flooded, covered control (anaerobic control) and a non-amended,

flooded, uncovered control (aerobic control). An iron oxide-painted, 7.62 by 0.635 cm diameter PVC IRIS (Indicator of Reduction in Soils) rod (Rabenhorst and Burch, 2006; Rabenhorst, 2008) (Professional Plastics, Fullerton, CA) was inserted fully into the saturated soil in each cup prior to sealing. Cups were placed in a growth chamber at 25°C in the dark.

After 4 weeks, cups were removed from the growth chamber, plastic mulch removed, IRIS rods were removed, five holes were punched into the bottom of the cups using a nail, and cups were returned to the growth chamber for 6 days to dry. Iron oxide paint loss was visually assessed after rinsing the IRIS rods in tap water. After drying, soils were placed in a plastic bag and homogenized with a rubber mallet. Homogenized soils were returned to cups and one 2-week-old tomato "Moneymaker" seedling (seed source: Everwilde Farms, Bloomer, WI) was placed in each pot exactly 1 week after the end of ASD treatments. Plants were fertilized once weekly with a 20–20–20 N–P–K fertilizer solution. Tomatoes were grown in the greenhouse in the same RCBD arrangement for 9 weeks, at which time plants were harvested and roots were washed in tap water.

Roots were evaluated for both root rot severity (percent of roots rotted or discolored) and taproot rot severity using a 1 to 5 scale [1: no taproot rot, 2: 1 to 2 small lesions on the taproot, 3: multiple lesions covering <50% of the taproot, 4: multiple lesions covering more than 50% of the taproot, 5: taproot completely rotten or missing (Testen and Miller, 2018)]. Following root rating, a random subsample of roots was taken from each plant and surface disinfested in 0.6% sodium hypochlorite for 15 s, followed by a sterile water rinse. Five, 2–3 mm long root pieces from each random subsample were plated onto each of three plates of half strength potato dextrose agar (½APDA, IBI Scientific, Dubuque, IA) for a total of 15 root pieces plated per plant. After 2 weeks, fungi growing on the medium were identified morphologically. Following plating, all aboveground tissue (harvested at the soil line) and roots were dried in a 65°C oven for 48 h to obtain dry shoot and root biomass.

Cover Crops Carbon Source Trial

Eight cover crops were assessed for efficacy as ASD carbon sources: two grasses (Sorghum-sudangrass *Sorghum* × *drummondii* "Piper" and winter rye *Secale cereale*), three legumes (cowpea *Vigna unguiculata* "Iron and Clay," crimson clover *Trifolium incarnatum*, and white clover *Trifolium repens*), two Brassicas (mustard *Brassica juncea* "Mighty Mustard Pacific Gold" and oilseed radish *Raphanus sativus*), and buckwheat (*Fagopyrum esculentum*). All seeds were obtained from Johnny's Selected Seeds (Fairfield, ME). Cover crops were direct seeded (5–7 seeds per pot) in a topsoil blend in Deepots (D40H, Steuwe and Sons, Tangent, Oregon) and were fertilized weekly as described above. After seven weeks, the aboveground portion of the cover crop was harvested and cut by hand into 0.25–0.75 cm pieces. Portions of the taproot were included in the radish cut pieces. Cover crops were mixed at a rate of 20.2 Mg/ha fresh biomass with soil obtained from a high tunnel in Highland County, OH and placed into 266 mL cups. Experiments were laid as a randomized complete block design with five replications. One replication consisted of one cup containing one tomato plant. Each experiment was conducted twice. Wheat middlings (midds) were included as a separate treatment, as a known effective ASD carbon source. Wheat midds are nutritionally similar to but less costly than wheat bran. Wheat bran is the outer seed covering, while midds are the wheat remnants following flour production. IRIS rods were placed into cups, soils were irrigated to saturation with sterile distilled water, and cups were sealed and placed into a growth chamber as described above. The ASD treatment, planting, root rot assessment, and root plating were conducted as described above for the agricultural byproduct carbon source screening. Only 10 root pieces per plant (two plates with five root pieces each) were plated for the cover crops experiments. Following plating, all aboveground tissue (harvested at the soil line) and roots were dried in a 65°C oven for 48 h to obtain shoot and root biomass.

On-Farm ASD Trials

Anaerobic soil disinfestation trials were established in high tunnels on five farms in Wayne (one trial), Holmes (one), Morrow (one), and Knox (two) Ohio counties and a randomized complete block design with four replications was established in each high tunnel. Plots were one m wide and ranged in length from 3 to 9.1 m, depending on the size of the high tunnel. Plots were amended with either wheat midds (ASD-WM), soybean meal (ASD-SM), or distillers dried grains (ASD-DG) at a rate of 20.2 Mg/ha. Carbon sources were obtained from Gerber Feed Services (Dalton, OH, USA). Carbon sources were spread over the treated area and incorporated to a depth of 10–15 cm using a walk behind rototiller. Beds were formed by hand and two lines of drip tape were laid on top of each bed. Three, 30-cm-long IRIS tubes (1.27 cm diameter PVC pipes) were placed in the center of each plot. A HOBO temperature pendant data logger (Onset Computer Corporation, Bourne, MA, USA) was placed in one plot of each treatment per trial. Black plastic mulch (1.5 mm embossed, 1.2 m wide, PolyExpert Inc., Laval, QC, Canada) was laid over each bed, and the sides of the mulch were covered with soil to prevent air exchange. Non-amended, covered plots served as anaerobic controls. Drip irrigation was applied to all plots until soils were saturated to a depth of 20 cm. Plots remained covered for 6 weeks. Trials were initiated in October 2018. Iron oxide paint loss was assessed on IRIS tubes using the visual grid method (Rabenhorst, 2012).

Soils were collected from each on-farm trial immediately following the end of ASD to determine the impacts of the treatments on root rot severity in a post-ASD bioassay. Post-ASD bioassays were laid in a randomized complete block design with four subsamples per sampled plot. One subsample consisted of one cup containing one tomato plant. Soils were placed in Deepots (D16H) and tomato "Moneymaker" seeds were directly sown into the pots and thinned to one plant per pot. Plants were grown for 9 weeks in the greenhouse (12 h day/night cycle, temperature range: 24–30°C) and then assessed for root rot severity, taproot rot severity, root-knot nematode galling (number of galls per root system) as described above, and dry root and shoot biomasses. Following root rating, a random subsample of roots was surface disinfested and plated onto ½APDA as described above. Ten root pieces were plated per plant (two plates

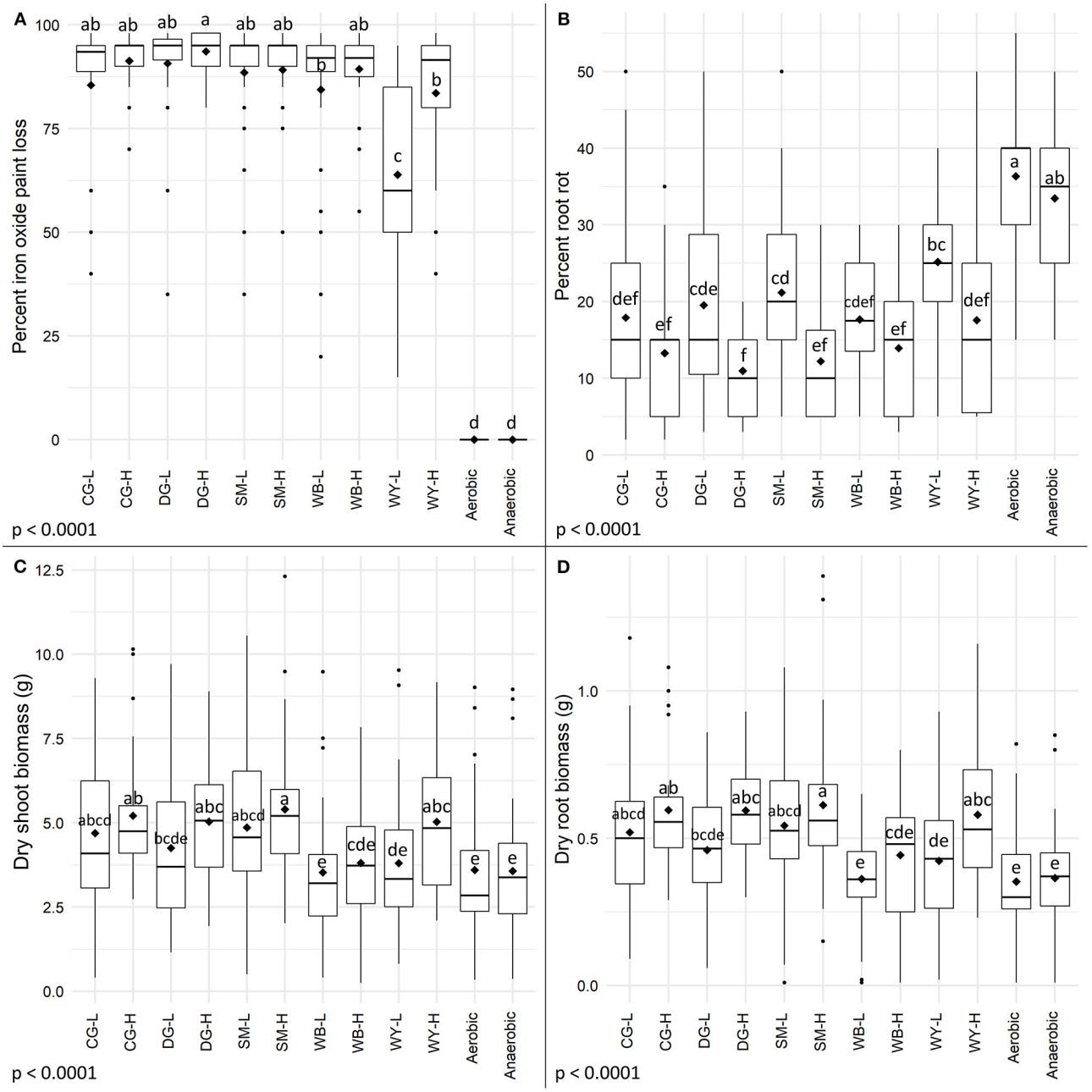
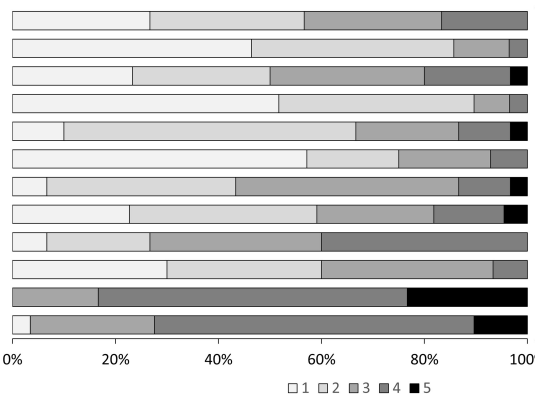


FIGURE 1 | Effects of anaerobic soil disinfestation (ASD) carbon sources and amendment rates on **(A)** soil reducing conditions as indicated by iron oxide paint loss on IRIS rods, **(B)** root rot severity, **(C)** dry shoot biomass, and **(D)** dry root biomass. Corn gluten meal (CG), distillers dried grains (DG), soybean meal (SM), wheat bran (WB), and dry sweet whey (WY) were evaluated as ASD carbon sources in bioassays at low (L, 10.1 Mg/ha) and high (H, 20.2 Mg/ha) amendment rates and performance was compared to aerobic and anaerobic controls. Treatment means are indicated by diamonds and means that do not share the same letters differ significantly based on Tukey's HSD with a 95% family-wise confidence level.

of five root pieces each), and plates were observed 2 weeks later to identify fungi morphologically.

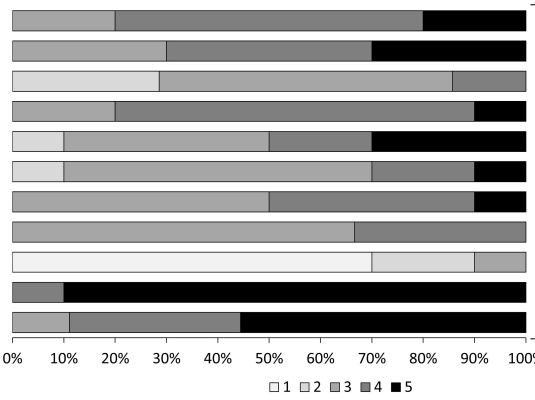
Tomatoes were planted in on-farm trials in March to April 2019. Farmers grew their preferred tomato varieties (Knox 1 and Knox 2: "Mountain Fresh," Morrow: "Red Deuce," Holmes: "Bigdena," Wayne: none: high tunnel structural failure), and trials were managed according to farmers' normal production practices. Participating farmers recorded yield data from three plants in the center of each plot during the growing season (Wayne and Knox 1 trials excepted). In August 2019, roots were collected from these three plants and assessed for root rot severity (Wayne and Knox 2 trials excepted).

A



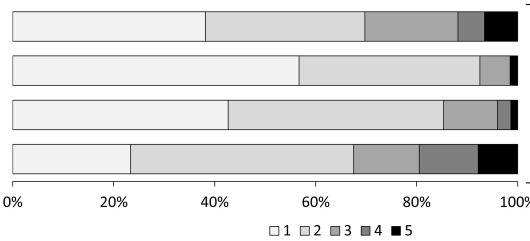
	Vs Anaerobic control	Vs Aerobic control
Corn gluten-Low	0.060 (0.022-0.16)*	0.029 (0.01-0.083)*
Corn gluten-High	0.018 (0.006-0.053)*	0.009 (0.003-0.027)*
Distillers dried grains-Low	0.079 (0.029-0.22)*	0.038 (0.013-0.11)*
Distillers dried grains-High	0.015 (0.005-0.043)*	0.007 (0.002-0.022)*
Soybean meal-Low	0.064 (0.023-0.18)*	0.031 (0.011-0.089)*
Soybean meal-High	0.016 (0.006-0.047)*	0.008 (0.003-0.024)*
Wheat bran-Low	0.11 (0.039-0.29)*	0.051 (0.018-0.15)*
Wheat bran-High	0.049 (0.018-0.14)*	0.024 (0.008-0.069)*
Whey-Low	0.24 (0.09-0.64)*	0.12 (0.041-0.32)*
Whey-High	0.046 (0.016-0.13)*	0.022 (0.007-0.064)*
Aerobic control	2.1 (0.75-5.8)	N.A.
Anaerobic control	N.A.	0.48 (0.17-1.3)

B



	Vs Anaerobic control	Vs Aerobic control	Vs Wheat midds
Buckwheat	0.29 (0.051-1.7)	0.038 (0.003-0.42)*	>1000 (>101.9->1000)*
Cowpea	0.29 (0.051-1.7)	0.038 (0.003-0.042)*	>1000 (>102.5->1000)*
Crimson clover	0.011 (0.001-0.10)*	0.001 (<0.001-0.023)*	60.4 (4.3-857.3)*
Mustard	0.23 (0.04-1.3)	0.03 (0.003-0.34)*	>1000 (81.8->1000)*
Oilseed radish	0.13 (0.023-0.78)*	0.017 (0.002-0.2)*	740.0 (48.1->1000)*
Sorghum-sudangrass	0.042 (0.006-0.28)*	0.006 (0.001-0.068)*	234.4 (15.9->1000)*
White clover	0.10 (0.017-0.62)*	0.014 (0.001-0.16)*	576.8 (37.8->1000)*
Winter rye	0.054 (0.008-0.36)*	0.007 (<0.001-0.088)*	299.9 (19.5->1000)*
Wheat midds	<0.001 (<0.001-0.003)*	<0.001 (<0.001-<0.001)*	N.A.
Aerobic control	7.6 (0.67-87.1)	N.A.	>1000 (>1000->1000)*
Anaerobic control	N.A.	0.13 (0.011-1.5)	>1000 (322.7->1000)*

C



	Vs Control
Distillers dried grains	0.62 (0.35-1.1)
Soybean meal	0.22 (0.12-0.42)*
Wheat midds	0.39 (0.21-0.70)*
Non-amended control	N.A.

FIGURE 2 | Taproot rot severity of bioassay plants grown in soils subjected to anaerobic soil disinfestation (ASD) using **(A)** various agricultural byproduct carbon sources, **(B)** cover crop carbon sources, or **(C)** a subset of ASD carbon sources evaluated in on-farm trials. Roots were rated on a 1–5 scale with 1: taproot healthy, 2: one to two small lesions or slight discoloration on taproot, 3: multiple lesions covering <50% of the taproot, 4: multiple lesions covering more than 50% of the taproot, 5: taproot completely rotten or missing. Proportional odds ratios are shown, along with confidence intervals, with a star indicating significance at $P < 0.05$. Odds ratios of <1 indicate that an ASD treatment was associated with lower taproot rot ratings compared to a control, while odds ratios of >1 indicate that an ASD treatment was associated with higher taproot rot ratings compared to a control.

Statistics

Bioassay and field trial data were combined for analysis to assess overall impacts of carbon sources across environments and soil or location (environments) were used as random blocking factors. Data were analyzed using mixed effects general linear models in Minitab 17 (Minitab Inc. State College, PA) with treatment

as a fixed factor and soil (or location), experimental run, and block as random factors. Pairwise comparisons were made using Tukey's HSD with a 5% familywise error rate. Percentages were subjected to the arcsine transformation prior to data analysis. A proportional ordinal logistic regression model using the LOGISTIC procedure of SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC (Allison, 1999; Derr, 2013) (Allison, 1999; Derr, 2013) was used to calculate odds ratios (OR) for taproot rot ratings from post-ASD bioassays. Odds ratios of <1 indicated that a treatment was associated with lower taproot rot ratings compared to a control, while odds ratios of >1 indicate that a treatment was associated with higher taproot rot ratings compared to a control.

RESULTS

Impacts of Alternative ASD Carbon Sources and Rates on Root Rot Severity in Tomato

Reducing conditions developed in all ASD-treated soils, regardless of carbon source and rate, and the amount of iron oxide paint loss from IRIS tubes was significantly more (**Figure 1A**, $p < 0.0001$) for all amended ASD-treated soils than for the aerobic and anaerobic controls. Significantly more iron oxide paint was lost from IRIS tubes in ASD-treated soils amended with DG-H than in soils amended with WB-L or either dry sweet whey amendment rate. The correlation between percentage of iron oxide paint loss and root rot severity was −0.61 ($p < 0.0001$).

Anaerobic soil disinfestation with either rate of any carbon source significantly reduced tomato root rot severity compared to the aerobic control (**Figure 1B**, $p < 0.0001$), and with the exception of WY-L, compared to the anaerobic control. Root rot severity was significantly lower for tomato plants grown in ASD-treated soil amended with the high rate of DG, SM, and WY than the low rate of each corresponding amendment, but there was no significant effect of rate when CG and WB were used as carbon sources. Both rates of all carbon sources significantly lowered the odds of having a higher taproot rot rating relative to the aerobic and anaerobic controls (**Figure 2A**, $p < 0.0001$). The anaerobic and aerobic controls did not differ significantly for proportional odds ratios associated with taproot rot severity. The incidence of *C. coccodes* recovered from roots was not significantly affected by ASD soil treatment (**Table 1**, $p = 0.17$). *Fusarium* spp. recovery was significantly lower from roots of plants grown in ASD-treated soils regardless of carbon source or rate, with the exception of WY-L, compared to the aerobic control. Only the ASD treatment with CG-H significantly reduced *Fusarium* spp. recovery compared to the anaerobic control. Significantly less *P. lycopersici* root colonization (**Table 1**, $p = 0.007$) was observed for plants grown in ASD-treated soils amended with DG-H and CG-H compared to the aerobic control.

Root rot severity for plants grown in soils subjected to ASD at low amendment rates trended higher than at high amendment rates, so data were subset by individual carbon sources to examine the impact of amendment rate. General linear models were run on subset data to assess rate effects for individual carbon sources. Root rot severity differed significantly by rate for each individual carbon source (p-value range: 0.001–0.045) indicating that use of a higher amendment rate consistently led to lower levels of root rot severity compared to use of a low amendment rate.

TABLE 1 | Average incidence of root piece infection by various fungi for plants grown in soils treated with anaerobic soil disinfestation with various agricultural byproduct carbon sources at low (10.1 Mg/ha) or high (20.2 Mg/ha) amendment rates.

	***Fusarium* spp.**	***Colletotrichum coccodes***	***Pyrenochaeta lycopersici***
Corn gluten-low	6.9 bcd[x,y]	38.0	4.4ab
Corn gluten-high	1.4d	28.3	1.4b
Distiller's dried grains-low	2.4cd	34.2	4.7ab
Distiller's dried grains-high	1.8cd	40.5	2.5b
Soybean meal-low	4.6bcd	35.3	5.1ab
Soybean meal-high	4.5bcd	27.4	4.5ab
Dry sweet whey-low	8.4ab	26.2	5.8ab
Dry sweet whey-high	4.2bcd	29.8	5.8ab
Wheat bran-low	4.7bcd	25.6	5.8ab
Wheat bran-high	3.0cd	26.2	4.4ab
Aerobic	13.8a	24.7	10.7a
Anaerobic	7.4bc	34.7	5.3ab
P-value[z]	<0.0001	0.173	0.007

[z] *Analysis of variance p-value for treatment effect.*
[y] *Percentage of root pieces per plant from which each fungus was recovered.*
[x] *Means that do not share the same letters differ significantly based on Tukey's HSD with a 95% family-wise confidence level.*

Anaerobic soil disinfestation with agricultural byproducts significantly affected both dry shoot (**Figure 1C**, $p < 0.0001$) and dry root biomass (**Figure 1D**, $p < 0.0001$). Shoot and root biomass of plants grown in soils amended with either wheat bran rate, DG-L or WY-L did not differ significantly from the shoot and root biomass of control plants. Significantly higher shoot and root biomass was observed in plants grown in soils amended with either rate of soybean or corn gluten meal, DG-H or WY-H and subjected to ASD compared to control plants.

Impacts of Cover Crops as ASD Carbon Sources on Root Rot Severity in Tomato

Reducing conditions developed in soils following ASD with cover crops, and the percentage of iron oxide paint removal with any cover crop amendment was significantly higher (**Figure 3A**, $p <$ 0.0001) than in either control treatment. The highest amount of iron oxide paint removal was observed in wheat midds-amended soils (98%) and this was significantly higher than the amount of iron oxide paint removal in any cover crop-amended soil. Among the cover crop-amended, ASD-treated soils, the highest amounts of iron oxide paint removal were observed for soils amended with sorghum-sudangrass (63.5%), crimson clover (60%), white clover (45.5%), and winter rye (41.1%). The lowest levels of iron oxide paint removal were observed in soil amended with cowpea (38.5%), buckwheat (15.2%), oilseed radish (13.7%), and mustard

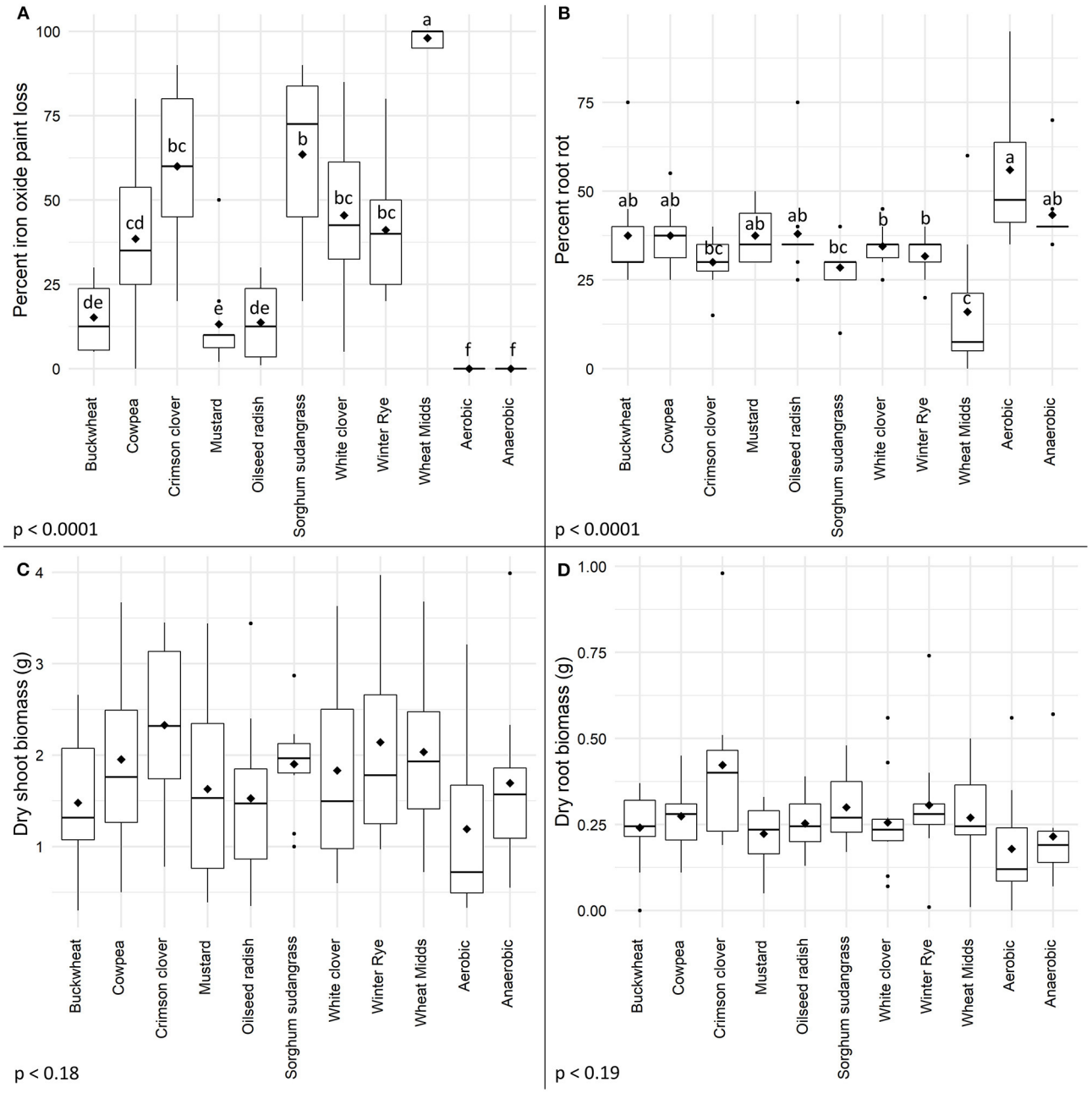


FIGURE 3 | Effects of cover crops as anaerobic soil disinfestation carbon sources on **(A)** soil reducing conditions as indicated by iron oxide paint loss on IRIS rods, **(B)** root rot severity, **(C)** dry shoot biomass and **(D)** dry root biomass. Cover crop ASD treatment (20.2 Mg/ha) amendment rates were compared to aerobic and anaerobic controls and a standard ASD treatment with wheat midds amendment. Treatments means are indicated by diamonds and means that do not share the same letters differ significantly based on Tukey's HSD with a 95% family-wise confidence level.

(13.2%). The correlation between the percentage of iron oxide paint loss and root rot severity was −0.48 ($p < 0.0001$).

Root rot severity was significantly impacted by ASD treatment (**Figure 3B**, $p < 0.0001$). A significant reduction in root rot severity was observed in plants grown in soils amended with wheat midds compared to plants grown in either control soil. No cover crop amendment significantly reduced root rot severity relative to the anaerobic control, but ASD with crimson clover, sorghum-sudangrass, white clover, or winter rye significantly reduced root rot severity relative to the aerobic control. All cover crops assessed led to significantly lower odds of increased taproot severity relative to the aerobic control (**Figure 2B**, $p < 0.0001$). Use of buckwheat, cowpea, and mustard did not significantly impact odds of higher taproot rot ratings relative to the anaerobic

control, while amendment with all other cover crops significantly reduced odds relative to the anaerobic control. Plants grown in cover crop-amended ASD-treated soils or either control soil had significantly higher odds of increased taproot rot ratings relative to those grown in soils amended with wheat midds and subjected to ASD. No ASD treatment significantly reduced the recovery of *P. lycopersici* (**Table 2**, $p = 0.063$) or *C. coccodes* ($p = 0.49$) from roots. The incidence of *Fusarium* spp. recovery from roots was reduced significantly (**Table 2**, $p = 0.003$) for plants grown in wheat midds-amended soils compared to plants grown in aerobic control soils or soils amended with oilseed radish. Anaerobic soil disinfestation with cover crops as carbon sources did not significantly affect either dry shoot (**Figure 3C**, $p = 0.18$) or root (**Figure 3D**, $p = 0.19$) biomass.

Soil Temperatures and Soil Reducing Conditions in On-Farm ASD Trials

Average soil temperatures over the treatment period were low, ranging from 12.0 to 17.3°C (**Table 3**).

TABLE 2 | Average incidence of root piece infection by various fungi for plants grown in soils treated with anaerobic soil disinfestation with various cover crop carbon sources and a wheat midds standard (20.2 Mg/ha).

	Fusarium spp.	*Colletotrichum coccodes*	*Pyrenochaeta lycopersici*
Buckwheat	32.0ab[x,y]	0.0	10.0
Cowpea	28.0ab	0.0	13.0
Crimson clover	11.4ab	2.9	21.4
Mustard	26.0ab	1.0	9.0
Oilseed radish	33.0a	3.0	11.0
Sorghum sudangrass	18.0ab	3.0	12.0
White clover	12.0ab	3.0	15.0
Winter rye	17.8ab	1.1	12.2
Wheat midds	5.0b	0.0	3.0
Aerobic	34.0a	1.0	23.0
Anaerobic	25.6ab	2.2	15.6
P-value[z]	0.003	0.49	0.063

[z] *Analysis of variance p-value for treatment effect.*
[y] *Percentage of root pieces per plant for which each fungus was recovered.*
[x] *Means that do not share the same letters differ significantly based on Tukey's HSD with a 95% family-wise confidence level.*

Reducing conditions developed in all ASD-treated plots (**Table 4**, $p < 0.0001$). The highest levels of iron oxide paint removal were observed in plots amended with soybean meal (37.4%), followed by plots amended with distillers dried grains (32.7%) and wheat midds (28.7%). Iron oxide paint loss was significantly higher in plots amended with soybean meal than in plots amended with wheat midds. All ASD treatments led to significantly higher amounts of iron oxide paint loss compared to control soils.

Impacts of ASD on Yield and Root Rot in On-Farm Trials

In post-ASD bioassays, root rot severity was significantly affected by treatment (**Table 5**, $p = 0.002$) as were dry shoot biomass ($p < 0.0001$) and dry root biomass ($p = 0.008$). Root rot severity in plants grown in soils collected from ASD-SM-treated plots (13.7%) was significantly lower than root rot severity in plants grown in soils collected from anaerobic control plots (18.4%). The odds of higher taproot rot ratings were significantly reduced (**Figure 2C**, $p < 0.0001$) for plants grown in ASD-treated soils amended with either wheat midds or soybean meal relative to plants grown in soils collected from anaerobic control plots (**Figure 2C**, $p < 0.0001$). Anaerobic soil disinfestation did not significantly reduce the incidence of *Fusarium* spp. (**Table 6**, $p = 0.15$) or *C. coccodes* ($p = 0.68$) recovered from plants grown in treated vs. control soils. The incidence of *P. lycopersici* recovered from plants grown in soils treated with ASD-WM (10.8% of root pieces, $p = 0.004$) was significantly lower than incidence in plants grown in soils collected from ASD-DG (19.9%) or anaerobic control (18.8%) plots. Dry shoot biomass was significantly higher in plants grown in ASD-treated soils with any amendment compared to plants grown in soils collected from control plots (**Table 5**). Dry root biomass was significantly higher for plants grown in soils collected from plots treated with either ASD-WM or ASD-DG compared to plants grown in soils collected from control plots. Root-knot nematodes were present in the Wayne trial post-ASD bioassays. Root-knot nematode galling was significantly reduced ($p = 0.003$) in plants grown in soils from any ASD treatment (ASD-SM: 0.9 galls per root system, ASD-DG: 1.7, ASD-WM: 1.9) compared to plants grown in soils collected from control plots (8.2 galls per root system).

The Wayne trial was lost due to a high tunnel collapse in the winter of 2019, but post-ASD bioassays were completed for this trial. Due to extenuating circumstances, yield data were not

TABLE 3 | Average soil temperatures (°C) during anaerobic soil disinfestation treatment in on-farm trials assessing distiller's dried grains, soybean meal, and wheat midds as carbon sources (20.2 Mg/ha).

Temps	Holmes	Knox 1	Knox 2	Morrow	Wayne
Distiller's dried grains	14.9	17.3	ND[z]	14.6	12.9
Soybean meal	14.9	17.3	17.0	ND	12.8
Wheat midds	14.8	17.1	16.4	14.7	13.0
Anaerobic control	14.4	16.4	17.0	ND	12.0

[z] *No data due to probe failure.*

TABLE 4 | Mean soil reducing conditions, root rot severity, and yield from on-farm trials conducted to assess three anaerobic soil disinfestation carbon sources (20.2 Mg/ha).

	Percent iron oxide paint loss[y]	Root rot[w]	Yield[v]
Distiller's dried grains	32.7ab[x]	42.3a	5.1
Soybean meal	37.4a	28.0b	6.1
Wheat midds	28.7b	28.5b	6.1
Anaerobic control	0.8c	39.6ab	5.1
P-value[z]	<0.0001	0.005	0.14

[z] *Analysis of variance p-value for treatment effect.*
[y] *Percent of iron oxide paint lost from Indicator of reduction in soils (IRIS) tubes (Data from six trials).*
[x] *Means that do not share the same letters differ significantly based on Tukey's HSD with a 95% family-wise confidence level.*
[w] *Percent roots rotted or discolored from three trials (Wayne and Knox 1 excluded).*
[v] *Yield per plant in kilograms from three trials (Wayne and Knox 2 excluded).*

TABLE 5 | Impacts of anaerobic soil disinfestation (ASD) on root rot severity, root biomass, and shoot biomass in post-ASD bioassays using soils collected from plots treated in on-farm trials.

	Root rot[y]	Shoot biomass[w]	Root biomass[w]
Distiller's dried grains	17.1a	2.9a	0.45a
Soybean meal	13.7b	2.8a	0.42ab
Wheat midds	15.3ab	2.9a	0.44a
Anaerobic control	18.4a	2.0b	0.36b
P-value[z]	0.002	<0.0001	0.008

[z] *Analysis of variance p-value for treatment effect.*
[y] *Percent root rotted and discolored.*
[x] *Means that do not share the same letters differ significantly based on Tukey's HSD with a 95% family-wise confidence level.*
[w] *Dry shoot and dry root biomass in grams.*

obtained from the Knox 2 trial and root rot severity data were not obtained from the Knox 1 trial.

Anaerobic soil disinfestation did not significantly impact average yield per plant in on-farm trials (**Table 4**, $p = 0.14$). Root rot severity in high tunnel-grown plants was significantly impacted by ASD treatment in on-farm trials ($p = 0.005$). Root rot severity was significantly lower for plants grown in plots treated with ASD-SM or ASD-WM compared to plants grown in ASD-DG soils. No treatment differed significantly from the anaerobic control, but ASD-SM and ASD-WM root rot severity trended lower.

DISCUSSION

Anaerobic soil disinfestation is effective for management of the tomato soilborne disease complex (Testen and Miller, 2018; Testen et al., 2020). To improve this management strategy for Midwestern vegetable growers, we assessed alternative ASD carbon sources, including cover crops, to determine their impact on root rot severity due caused by *P. lycopersici* and *C. coccodes*. Carbon sources were evaluated in growth chamber and greenhouse bioassays, and several amendments resulted in reduced root rot severity following ASD treatment. While a subset of carbon sources was evaluated in on-farm trials, their full efficacy was likely not realized due to unusually low soil temperatures during the treatment period.

TABLE 6 | Average incidence of root piece infection by various fungi for plants grown in post-ASD bioassays using soils treated on-farm with anaerobic soil disinfestation with various carbon sources (20.2 Mg/ha).

	Fusarium spp.	*Colletotrichum coccodes*	*Pyrenochaeta lycopersici*
Distiller's dried grains	13.8[y]	16.6	19.9a[x]
Soybean meal	14.3	13.7	12.5ab
Wheat midds	19.2	15.5	10.8b
Anaerobic control	14.6	16.6	18.8a
P-value[z]	0.15	0.68	0.004

[z] *Analysis of variance p-value for treatment effect.*
[y] *Percentage of root pieces per plant for which each fungus was recovered.*
[x] *Means that do not share the same letters differ significantly based on Tukey's HSD with a 95% family-wise confidence level.*

Agricultural byproducts are easy to procure and relatively inexpensive as ASD carbon sources. Based on 2020 pricing at a feed mill in Northeastern Ohio, the least expensive carbon sources were wheat midds, distillers dried grains and corn gluten feed [all $0.30 (USD) per kilogram] while soybean meal and dry sweet whey cost $0.43 and $1.31 per kilogram, respectively. Wheat midds are nutritionally equivalent to but less costly than wheat bran, while corn gluten feed is a corn processing byproduct with a lower protein (22% protein) content than corn gluten meal (Feedipedia, 2020). No carbon source outperformed wheat bran, a proven ASD standard, for reducing root rot severity. Two carbon sources are not realistic options for Midwestern growers. Whey is not realistic due to high cost and a lack of efficacy at low amendment rates. Corn gluten meal was not evaluated in field trials due to potential phytotoxicity as corn gluten meal is a known bioherbicide (Bingaman and Christians, 1995). The bioherbicide effects of corn gluten meal survived the ASD process. When tomato seeds were directly sown into soils treated after ASD with corn gluten meal, reduced germination and phytotoxicity were observed (data not shown), in line with the pre-emergent herbicide characteristics of corn gluten meal (McDade and Christians, 2000). This led to the need to transplant 2-week-old tomato seedlings in bioassays. Corn gluten meal may be a viable option as transplants are used for tomato production, but field trials are needed to confirm this, and corn gluten feed may be a less phytotoxic and less expensive alternative to corn gluten meal.

Lowering ASD carbon source amendment rates while maintaining treatment efficacy would greatly reduce input costs. While low amendment rates for soybean meal, distiller's grains and wheat midds led to significant reductions in root rot severity after ASD compared to controls in this study, the reductions were less than those observed when high amendment rates were used. Therefore, high amendment rates are needed for effective

control of the tomato soilborne disease complex. High carbon source rates are especially needed for autumn applications of ASD when pathogen populations are at their highest following a cropping cycle. Further research is needed to determine the lowest amendment rate that is as effective as the 20.2 Mg/ha amendment rate. One key limitation to amendment rate studies is a lack of pathogen population thresholds associated with yield losses. This information would help to determine whether an ASD carbon source and rate is sufficient to manage root rot. In a meta-analysis of ASD studies, higher carbon source amendment rates tended to lead to greater reductions in soilborne disease damage (Shrestha et al., 2016), so it is likely that most amendment recommendations will trend toward use of higher rates.

When working with disease complexes, it can be difficult to determine which pathogens are most affected by the imposed treatment as the impacts of ASD occur in a carbon source dependent manner (Hewavitharana et al., 2014; Testen and Miller, 2018). Assessment of root rot severity is a general measure of the impacts of ASD on root rotting fungi, while assessment of taproot severity allows us to infer effects on *P. lycopersici*. While all agricultural byproducts, with the exception of WY-L, reduced recovery of *Fusarium* spp. compared to the aerobic control, only high rates of corn gluten and distillers dried grains similarly reduced *P. lycopersici* recovery. No cover crop significantly reduced recovery of any soilborne fungus from roots after ASD, but use of wheat midds in that trial reduced incidence of *Fusarium* spp. compared to the aerobic control. Use of wheat midds in on-farm trials significantly reduced incidence of *P. lycopersici* in roots. We did not observe a carbon source that reduced the incidence of *C. coccodes* recovery in any study presented here. Testen and Miller (2018) demonstrated that use of wheat bran reduced the recovery frequency of *P. lycopersici* but not *C. coccodes* following ASD. Most carbon sources examined led to lower taproot rot severity compared to controls, which indicates that *P. lycopersici* damage is reduced during ASD. Root-knot nematode damage was reduced significantly following ASD with any carbon source in the Wayne trial. This suggests that root-knot nematodes are highly sensitive to ASD and pathogens vary in their sensitivity to ASD applications at low temperatures. Further studies are needed to clarify the efficacy of carbon sources against specific members of the soilborne disease complex and the differential sensitivity of pathogens to ASD carbon sources suggests the need for carbon source mixtures.

Anaerobic soil disinfestation efficacy depends not just on carbon source but also soil temperatures (Shennan et al., 2018), and pathogen populations are more greatly reduced at warmer soil temperatures (Shrestha et al., 2016). In Ohio, autumn applications of ASD fit into tomato cropping cycles used by most farmers, particularly in high tunnels. These autumn ASD applications are made when soilborne pathogen populations are at their highest. Another difficulty of autumn ASD application is that soil temperatures may become too low for effective treatments, especially if applications are made too late in the season or temperatures drop earlier than anticipated. The average soil temperatures in all on-farm trials were lower (average temperatures ranged from 12.0 to 17.3°C) than in other ASD studies conducted in Ohio in which average soil temperatures ranged from 16.3 to 27.8°C for September and October high tunnel applications (Testen et al., 2020) or 23.6 to 30.8°C for summer applications on muck soils (Testen and Miller, 2019). Despite these lower soil temperatures, soil reducing conditions as indicated by iron oxide paint loss were only slightly lower in most trials compared to ASD trials conducted with warmer soil temperatures (Testen and Miller, 2019; Testen et al., 2020). Soil reducing conditions still develop at cool soil temperatures, even if ASD does not effectively reduce soilborne pathogens (Shennan et al., 2018). Average soil temperatures for effective management of the tomato soilborne disease complex likely range from 20 to 25°C, but further growth chamber assays are needed to identify minimum average temperatures at which ASD is effective.

Cover crops had not been examined for control of the tomato soilborne disease complex prior to this study, but they did not prove to be as effective as carbon sources derived from agricultural byproducts. Cover crop amendments led to soil reducing conditions during ASD that were lower than those obtained from use of agricultural byproducts. The grass and clover amendments used in this study reduced root rot relative to the aerobic control after ASD; crimson clover and sorghum sudangrass reduced root rot severity equivalent to wheat midds. No cover crop reduced taproot rot severity in a manner equivalent to wheat midds, but taproot rot severity was, in general, reduced compared to controls. Cover crop amendments did not increase biomass, unlike biomass increases observed with some agricultural byproducts. This may be due to less overall nitrogen provided by cover crops compared to high protein agricultural byproducts. Nutritional content of the cover crops used in this study was not assessed but cover crops can have protein contents in the same range as some effective agricultural byproduct carbon sources, such as wheat midds (15.5–17.3% protein). Crude protein content of cover crops can range from 10 to 24% (Heins and Paulson, 2018), but this is very dependent on growing conditions. Additionally, the carbon within cover crops may be less available to soil microbes than the readily liable carbon in ag byproducts, but this would need to be assessed in future studies. Cover crops may be an option for more frequent ASD applications or as a supplement to agricultural byproduct carbon sources. Cover crops vary in their efficacy as ASD carbon sources. They have been shown to be slightly more effective in reducing *Rhizoctonia* populations than molasses as ASD carbon sources (McCarty et al., 2014) or as effective as molasses for reducing *Fusarium oxysporum* but not *Sclerotium rolfsii* (Butler et al., 2012b).

This study demonstrated the efficacy of multiple carbon sources used in ASD against the tomato soilborne disease complex. While no carbon source significantly reduced root rot significantly compared to controls in on-farm trials due to low treatment soil temperatures, wheat bran and midds, soybean meal, distillers dried grains, and corn gluten meal consistently reduced disease in bioassays for which soil temperatures were higher. Future studies to assess the efficacy of carbon sources against specific pathogens at a range of temperatures and timings would allow farmers to fine-tune their carbon source selection for soil pathogens

present on their farms and current weather conditions. Future studies should also examine combinations of carbon sources or use of cover crops to supplement carbon sources to design ASD amendment mixtures to target a wider range of soilborne pathogens.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AT and SM designed the study. AT, FR, MH, and MM conducted field, greenhouse, and lab work. AT conducted statistical analyses and wrote the first draft of the manuscript. All authors edited the manuscript and approved submission.

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Biodisinfestation With Agricultural By-Products Developed Long-Term Suppressive Soils Against *Meloidogyne incognita* in Lettuce Crop

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Soil biodisinfestation is the process generated after the incorporation of organic amendments followed by a plastic cover to control soilborne diseases. Among organic amendments, the use of agricultural by-products could be an interesting alternative as it promotes circular economy. In this study, beer bagasse and defatted rapeseed cake together with fresh cow manure were incorporated into the soil (1.5, 0.5, and 20 kg/m^2, fresh weight, respectively) to assess their capacity to reduce disease incidence caused by the root-knot nematode *Meloidogyne incognita* in protected lettuce crops and develop suppressive soils. The trial was conducted in a commercial greenhouse for 7 weeks during which temperature was continuously recorded at three different soil depths (15, 30, and 45 cm). Short- and long-term effects were assessed: before treatment, after treatment, after first crop post-treatment and one year post-treatment. Disease incidence and changes in nematode community structure were analyzed along with microbiological properties and general physicochemical parameters. After biodisinfestation, microbiological activity significantly increased in the treated soils and changes in the nematode community structure were detected in detriment of *M. incognita* and other plant-parasitic nematodes. These effects were more apparent after the first crop post-treatment than right after biodisinfestation. In the first crop after biodisinfestation, lettuce yield increased in the treated plots and root galling indices were significantly lower. One year after treatment, differences between treatments could be observed in the incidence of the damage caused by *M. incognita* that remained lower in the treated plots. In this trial, the addition of beer bagasse and rapeseed cake along with fresh manure in biodisinfestation treatment demonstrated nematicidal effects against *M. incognita*. Moreover, we suggest that the compounds released during the degradation of these by-products and the sub-lethal temperatures achieved in this trial during biodisinfestation (<42°C) were the key to develop suppressive soils in the long-term.

Keywords: rapeseed cake, beer bagasse, organic amendment, solarization, root-knot nematode, nematode guilds, sub-lethal temperatures

INTRODUCTION

Conventional crop management includes pathogen control and the use of chemical fumigants is the most common practice, but many of them are harmful and cause environmental damages. After the Montreal Protocol (1989), methyl bromide and other fumigants were banned or subjected to restriction in use (Gareau, 2010). The negative attributes of soil chemical fumigants are of increasing concern, and therefore more attention is given to non-chemical approaches for soilborne pest control (Klein et al., 2012; Mocali et al., 2015). Among them, soil biodisinfestation, which includes the use of organic amendments, is widespread in pest management as an effective and healthy alternative for soil treatments (Rosskopf et al., 2020). Soil biodisinfestation is a general term for the different approaches developed to control soilborne diseases using organic matter as main driver, but different terms can be found to define each approach. Biosolarization is based on the incorporation of organic amendment followed by mulching with transparent polyethylene plastic film during hot seasons in order to achieve high soil temperatures. These conditions promote certain physical, microbiological and biochemical processes that contribute to disease reduction and even a decrease in soil pathogen populations (Katan, 2014; Katan and Gamliel, 2014). In climate regions with low solar radiation, labile C-sources are used to boost anaerobic conditions and this strategy is commonly called anaerobic soil disinfestation (ASD) (Shennan et al., 2018; Rosskopf et al., 2020). Another practice is the incorporation of plants of the Brassicaceae family, which is known as biofumigation (Kirkegaard et al., 1993). This approach is based on the release of isothiocyanates (ITCs) produced by the hydrolysis of the glucosinolates (GLs) in Brassicaceous amendments (Matthiessen and Kirkegaard, 2006). These ITCs along with other biocidal compounds produced during decomposition of plant tissue, like volatile fatty acids (VFA) and NH3, have demonstrated biocidal activity (Oka et al., 2007; Dutta et al., 2019).

These biodisinfestation practices have shown positive results against different soilborne pathogens (Ntalli et al., 2020; Rosskopf et al., 2020), plant-parasitic nematodes (PPN) among them, which are important pests of a wide range of crops (Avato et al., 2013; Fourie et al., 2016; Dutta et al., 2019; Talavera et al., 2019). PPNs are very diverse and can cause different damage to host plants. Regarding yield loss, the most common are root-knot (*Meloidogyne* spp.), cyst (*Heterodera* spp. and *Globodera* spp.) and root lesion (*Pratylenchus* spp.) nematodes (Jones et al., 2013). Root-knot nematodes (RKN) of the genus *Meloidogyne* are the major plant pathogen with a wide geographical and climatic distribution affecting growth and yield of different agricultural crops (Sasser, 1980; Ijoyah and Koutatouka, 2009; Klein et al., 2012; Jones et al., 2013). Mature females induce gall formation on the root tissue when laying their eggs. This root galling is the most important symptom for RKN affecting root system growth and nutrient uptake capacity that leads to important yield loss (Jones et al., 2013). In particular, *Meloidogyne incognita* (Kofoid and White, 1919; Chitwood, 1949) is recognized as one of the most destructive species with wide host ranges (Avato et al., 2013; Jones et al., 2013). Despite the scarce related literature, lettuce (*Lactuca sativa*) is one of the host species with important yield losses recorded in different regions (Koenning et al., 1999; Correia et al., 2019).

Soil nematodes are the most abundant of the Metazoa, most are free-living species with different food sources, and occupy key positions in soil food web (Bongers, 1990; Bongers and Ferris, 1999; Ferris et al., 2001). Thus, the interpretation of the nematode community structure as a whole could provide relevant information to assess disturbances and stress, and could be useful for monitoring changes in the structure and functioning of the soil food web (Bongers and Ferris, 1999; Cesarz et al., 2015). Considering that, several authors developed different indices based on the presence and abundance of the different nematode families (Bongers, 1990; Yeates et al., 1993; Bongers and Bongers, 1998; Bongers and Ferris, 1999; Ferris et al., 2001; Ferris, 2010). However, it is important to consider that intensive agricultural soils are subjected to disturbance and stress conditions due to crop management practices (e.g., tillage, fertilization, and fumigation) in order to choose the proper indices. Despite the scarce information about the effect of biodisinfestation treatments on nematode assemblage, in this trial, together with *M. incognita,* the effects on presence of the different guilds was assessed. Moreover, analysis of soil nematode community structure could also contribute to the assessment of soil suppressiveness along with other soil parameters (Carrascosa et al., 2014).

The incorporation of organic amendments into the soil in combination with any of the mentioned practices can develop suppressive soils although there is great variability in results of previous studies (Janvier et al., 2007; Bonanomi et al., 2010). Soil suppressiveness consists in disease control, in spite of the presence of soilborne pathogens, which is attributed to an increase of antagonistic microorganisms (Baker and Cook, 1974). The incorporation of organic matter into the soil boosts microbiological activity due to degradation processes, which are enhance by the conditions generated during biodisinfestation treatments (e.g., high temperatures, moisture). This shift in microbiological community can promote antagonistic relationships in detriment of soilborne pathogens (Alabouvette et al., 1996; Janvier et al., 2007). In this trial, beer bagasse was selected to enhance microbial degradation and anaerobic conditions with the consequent release of VFAs. This is the residue obtained in beer production and becomes a source of labile carbon after the fermentation processes. Besides, it has little commercial interest and few studies have been done for agricultural use or other fields. Considering previous studies, the incorporation of crop residues and animal manures and Brassicacea-based management strategies are most common treatments used against PPN (Brennan et al., 2020; Rosskopf et al., 2020). Rapeseed (*Brassica napus* "Canola") cake is the by-product obtained after the oil extraction and is often marketed in livestock feed due to its high protein content and low glucosinolates concentration (<15 μmol/g). Unlike other *Brassica* spp., the main nematicidal effect of *Brassica napus* "Canola" is attributed to the high nitrogen content which promotes microbial activity and the release of other nematicidal

compounds (i.e., NH3, VFAs) (Mazzola et al., 2001; Dutta et al., 2019).

In this assay, beer bagasse and rapeseed cake together with fresh cow manure were incorporated into the soil as organic amendment in biodisinfestation treatment against RKN *M. incognita* in a commercial lettuce greenhouse with significant yield losses. The main objectives were (i) to assess the potential ability of these by-products to reduce the damage caused by *M. incognita* in lettuce crop and (ii) to evaluate suppressive effects, as well as (iii) the impact of biodisinfestation on the soil nematode community in both short- and long-term (before treatment, after treatment, first crop after treatment and one year after treatment). For that, root galling index and crop yield were assessed for disease incidence. The suppressive effects were evaluated through soil microbiological activity (soil respiration rate), the physiological profiles at community-level of heterotrophic bacteria and through the nematode community structure. Nematode community related variables can provide useful information in soil quality assessment, but scarce information can be found in soil biodisinfestation studies. In this case, the relative presence of *M. incognita* nematode and nematode functional guilds together with plant-parasitic index and the enrichment footprint were calculated. Similar happens with long-term effects after biodisinfestation treatments. Generally, the results right after biodisinfestation treatments are assessed, but few studies evaluated the effects in long-term.

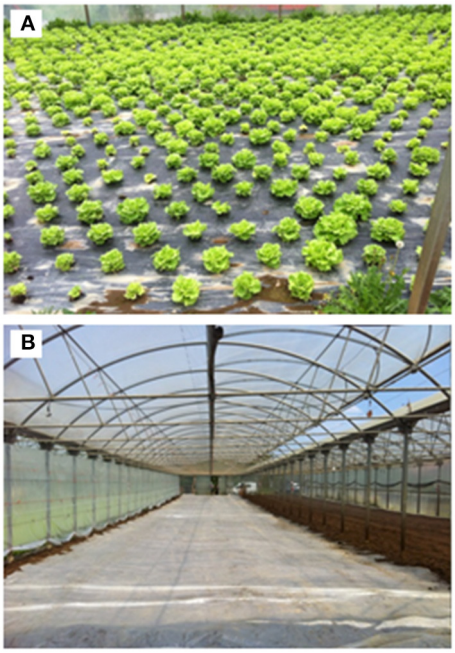

FIGURE 1 | (A) Lettuce crop damaged by *M. incognita* nematode in the treated greenhouse. **(B)** View of biodisinfested and controls plots.

MATERIALS AND METHODS

Study Site and Soil

The study was conducted in a commercial greenhouse located in the Basque Country, northern Spain (43°17′00″N; 2°45′00″W), a humid temperate region. The greenhouse selected for the study was a poly-tunnel consisting of four tunnels (8 × 50 m each tunnel), with lettuce monoculture. The lettuce varietal "Batavia," commonly grown by farmers in this region, has been continuously cultivated in the same tunnels for the last 20 years. The plants were grown over plastic cover (25 × 30 cm pattern) with a total yield of about 4,500 lettuces per tunnel (12 plants/m^2). The soil was loamy (33% sand, 49% silt, 18% clay, with 5 > 6% organic matter) and was naturally infested by *Meloidogyne incognita* which caused more than 45% yield loss in the last harvest before treatment, even with previous chemical fumigations. In fact, right before biodisinfestation (July), the farmer had to discard the entire lettuce crop grown in the treated tunnels, due to weak growth caused by *M. incognita* nematode (**Figure 1A**).

Organic Amendments

The organic amendments selected for soil biodisinfestation were fresh cow manure (FCM), beer bagasse (BB) and rapeseed cake (RC). The addition of fresh cow manure was included in farmers' crop management as a traditional practice in this region. It was obtained from a nearby farm, mixed on about 20% with straw, and applied at a dose of 20 kg/m^2 (80% moisture). Beer bagasse is the residue obtained in beer production and it was provided by *BOGA Basque Craft Beer* as residue and incorporated into the soil at a dose of 15 kg/m^2 (75% moisture). Finally, pellets of rapeseed (*Brassica napus* "Canola") cake were added at a dose of 0.5 kg/m^2 (6% moisture) and was supplied by an experimental sheep farm in Alava (Basque Country). These doses were based on previous researches with these byproducts (Gamliel et al., 2000; Mazzola et al., 2001; Guerrero-Díaz et al., 2014; Guerrero et al., 2019). Physicochemical characterizations of the organic amendments were used to adjust the final mixture (**Table 1**). The doses applied of FCM, BB, and RC represented a ratio of 78:10:12 (dry matter), respectively.

Experimental Design

The experimental design was structured as a combination of soil treatment and time. For that, two treatments were assessed: (i) non-treated control (C) and (ii) biodisinfested (BD) at different times: before treatment (T1), after treatment (T2), after the first crop post-treatment (T3), and one year after treatment (T4). Depending on the variable, different moments were assessed: soil microbiological properties at T2, T3, and T4; galling index and crop yield at T1, T3, and T4; and the structure of nematode functional guilds and *M. incognita* relative presence at T1, T2, T3, and T4. Soil treatments were arranged in a complete randomized design (CRD) with four replicate plots per treatment. Four tunnels of a poly-tunnel severely affected by *M. incognita* were virtually divided lengthwise into two parts in order to obtain four replicates per treatment with a plot size of 4 × 50 m^2 each.

Biodisinfestation was carried out for 7 weeks at the end of summer, from 1st August to 19th September. In the treated plots, BB + RC + FCM were incorporated into the soil and mixed with the rotary tiller. Soil was then spray irrigated for

TABLE 1 | Physicochemical values [dry matter (DM), organic matter (OM), total N, organic C, C/N ratio, pH, EC, some available nutrients and presence of heavy metals] of the amendments used for the biodisinfestation treatment: RC (rapeseed cake); BB (beer bagasse); FCM (fresh cow manure).

Parameters	Amendments			Soil				
	RC	BB	FCM	T1	C-T2	BD-T2	C-T4	BD-T4
DM (%)	93.80	25.17	14.75	76.75	72.70	74.41	78.84	78.58
OM (%)	17.72	5.50	65.36	5.82	5.90	7.55	5.38	5.51
N tot (%)	1.36	0.24	1.48	0.32	0.31	0.37	0.31	0.35
C org (%)	10.30	3.20	37.96	3.38	3.42	4.38	3.16	3.61
C/N	7.60	13.50	25.65	10.62	11.08	11.93	10.19	10.41
pH			8.89	7.47	7.49	7.41	7.34	7.49
CE (dS/m)			8.19	0.74	0.57	2.43	0.88	1.04
P (ppm)				229	260	207	195	174
Fe (ppm)			<0.2	63	58	250	64	160
Mn (ppm)			126	12	12	195	29	31
Cu (ppm)			14	9	8	14	9	10
Zn (ppm)			75	15	14	18	14	11
B (ppm)			17	1	1	1	1	1
Cd (ppm)			<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Pb (ppm)			7	20	17	15	25	22
Hg (ppm)			<0.5	<0.5	<0.5	<0.5	<0.50	<0.5
Ni (ppm)			3	14	12	11	16	16
Cr (ppm)			5	17	14	12	24	23

Soil physicochemical values of biodisinfested (BD) and control (C) soils at three different times: T1 (pre-treatment), T2 (post-treatment) and T4 (1 year post-treatment). All parameters are expressed in dry weight.

15 min (30 l/h/m^2) and manually covered with a 200 gauges transparent polyethylene plastic film (provided by RAISAFILM). The edges of the plastic mulch were buried 10–15 cm deep in the soil avoiding air pockets formation (**Figure 1B**). In the control plots, FCM was incorporated at the same dose as in the treated ones but the soil was not covered with plastic film. After 7 weeks of biodisinfestation treatment, plastic cover was removed from the treated tunnels and soil was aerated during 1 week before the transplanting of the next lettuce crop. One year after biodisinfestation, both biodisinfested and control soils supported four crop growing cycles without additional fertilization.

Sampling Procedure and Data Collection

Composed soil samples were randomly collected from each plot with four samples per treatment. For this purpose, topsoil was collected from 0 to 25 cm depth at ten different points in each plot using a core soil sampler (2.5 cm diameter), the margins were excluded to avoid the edge effect. Sample collection was carried out at four moments: in July 2018 before biodisinfestation treatment (T1), in September 2018 after treatment (T2), in November 2018 after harvesting the first crop post-treatment (T3) and in August 2019 one year after biodisinfestation (T4). Soil samples were air-dried at room temperature and sieved at $\leq$2 mm for physicochemical and heavy metals presence analyses. Conversely, measurements of biological parameters were done on fresh soils samples sieved at $\leq$2 mm and stored at 4°C for a maximum of 1 month until analysis. For nematode extraction, fresh non-processed soil samples (600 g) were stored at 4°C and analyzed within 1 week after sampling.

Twenty lettuce roots were randomly collected from each plot in order to assess the GI before biodisinfestation (T1), after the first crop post-treatment (T3) and one year after biodisinfestation (T4). Crop yield (plants/m^2) was also assessed at the same sampling times.

Temperature was recorded inside the treated tunnels (ambient temperature) and in the treated soil at three different depths during biodisinfestation treatment as described below.

Analyzed Variables

Organic Amendments and Soil Physicochemical Characterization

A single composed soil sample from each plot was used to determine the following parameters according to standard methods (MAPA, 1994) for all the organic amendments: dry matter (DM), pH, EC, organic matter (OM), N Kjeldhal, P Olsen and organic C. In the case of fresh manure, presence of fecal coliforms *Escherichia coli* and *Salmonella* spp. was also assessed following standard methods ISO-7251:2005 and ISO 6579-1:2017, respectively, as well as certain heavy metals and other metals of agronomical importance (USEPA, 2007) determined by ICP analyses.

The same parameters were analyzed for soil characterization, as well as pH and EC (dS/m) (**Table 2**). These parameters were analyzed only before (T1), after treatment (T2) and one year after biodisinfestation (T4). For that, soil samples were air-dried at room temperature and sieved through a 2 mm mesh before analysis. The physicochemical properties of this soil were also

TABLE 2 | Number of hours accumulated during 7 weeks of biodisinfestation treatment at different temperatures and at the different soil depths (15, 30, and 45 cm) in the treated plots.

°C	15 cm	30 cm	45 cm
24		5	5
25	2	5	12
26	2	19	17
27	6	2	7
28	12	9	18
29	46	28	25
30	116	162	178
31	186	140	618
32	173	655	287
33	166	131	4
34	126	15	
35	110		
36	80		
37	61		
38	40		
39	29		
40	10		
41	6		

determined according to standard methods (MAPA, 1994), as well as heavy metals (USEPA, 2007).

Temperature

During biodisinfestation, soil temperatures were monitored at 15, 30, and 45 cm depth, as well as the ambient temperature inside the greenhouse. Soil temperatures were recorded in one treated plot every 15 min with specific probes connected to a Hobbo Datalogger (Weather Station, OCC, US, H8-4 32K). Average values per hour at different depths were calculated in order to know the number of hours at the recorded temperatures in each depth along the treatment. The total hours at each temperature were summed to obtain the accumulated hours at each temperature.

Soil Microbiological Properties

Immediately after soil sample collection, fresh samples were stored at 4°C and sieved to <2 mm prior microbiological analyses. Microbial activity was assessed by measuring soil respiration, which indicates total belowground activity. The procedure was an adaptation of ISO standard 16072:2002 by which CO_2 emission rate of the soil was measured in a hermetic jar incubated for 3 days at 30°C and quantified by titration with NaOH. In addition, changes in bacterial community structure were determined with Biolog Ecoplates™ through the physiological profiles of the heterotrophic bacteria at community-level. This technique rapidly allows the detection of changes in microbiological structure by measuring the metabolic activity in 31 different C-sources (Garland and Mills, 1991; Insam, 1997). For that, soil water extracts were incubated in Biolog Ecoplates™ (96-well microplates with 31 different substrates) at 30°C and absorbance (590 nm) was measured every 12 h during 10 days. Then, average well color development (AWCD) and the number of utilized substrates (NUS) were calculated at half the incubation time (240 h). Composed samples from each plot were used in duplicate to work with two sub-samples in both microbiological analyses.

Root Galling Index and Yield

The root galling index (GI) of lettuce plants was estimated according to the scale of Bridge and Page (1980) where 0 represents root without galling (healthy root system) and 10 is the maximum degree of galling (root functioning is loose and decaying). For this purpose, 20 plants were randomly collected of each plot and visually evaluated.

In addition, yield data were obtained considering the commercial plants selected by the farmer. These data were adapted in order to obtain the number of commercial plants per square meter that enabled the assessment of disease incidence and yield.

Identification of Root-Knot Nematode

Identification of root-knot nematode species was done by perineal pattern characterization (Hartman and Sasser, 1985). In summary, affected roots were washed under tap water and single galls were selected for the extraction of females. Under magnifying binoculars NIKON SMZ800 (6.3X), selected galls were open and the females were extracted and immersed in lactophenol. The body was then transversally cut to obtain the perineal area. This fragment was dyed and fixed in a glass slide for visualization under optical microscope.

Soil Nematode Community and Presence of *M. incognita*

Soil nematode extraction was performed following the sieving and Baermann-Funnel technique (Barker, 1985). For that, fresh and non-processed soil samples (600 g) were divided into three sub-replicates of 200 g each. After extraction, nematodes were visually identified at family level with an optical microscope LEIKA DM6000B (200X) and counted on 100 µl of extraction but calculated in 100 g soil (dry weight). The relative presence of the pathogen species *M. incognita* and the relative presence of the different guilds found in soil samples were calculated to assess the biodisinfestation effects on the pathogen and on the nematode community structure. Bongers and Bongers (1998) proposed the term "guild" as a tool to improve the analysis of soil nematode community that classified the different nematode taxa considering the feeding group (bacterivores: Ba; fungivores: Fu; plant-parasitic: PP; predators: Pr; omnivores: Om) and the life strategy with values between 1 and 5, low values for colonizers and high values for persister (cp value). In addition, plant-parasitic index (PPI) and enrichment footprint (EF) were also calculated. The PPI is an indicator of the global plant-parasitic nematode assemblage and not only a single pathogen specie. The EF considers opportunistic nematode groups and reflects an increase in resources due to an implementation of organic amendments and the resulting increase in microbiological activity (Ferris et al., 2001; Ferris,

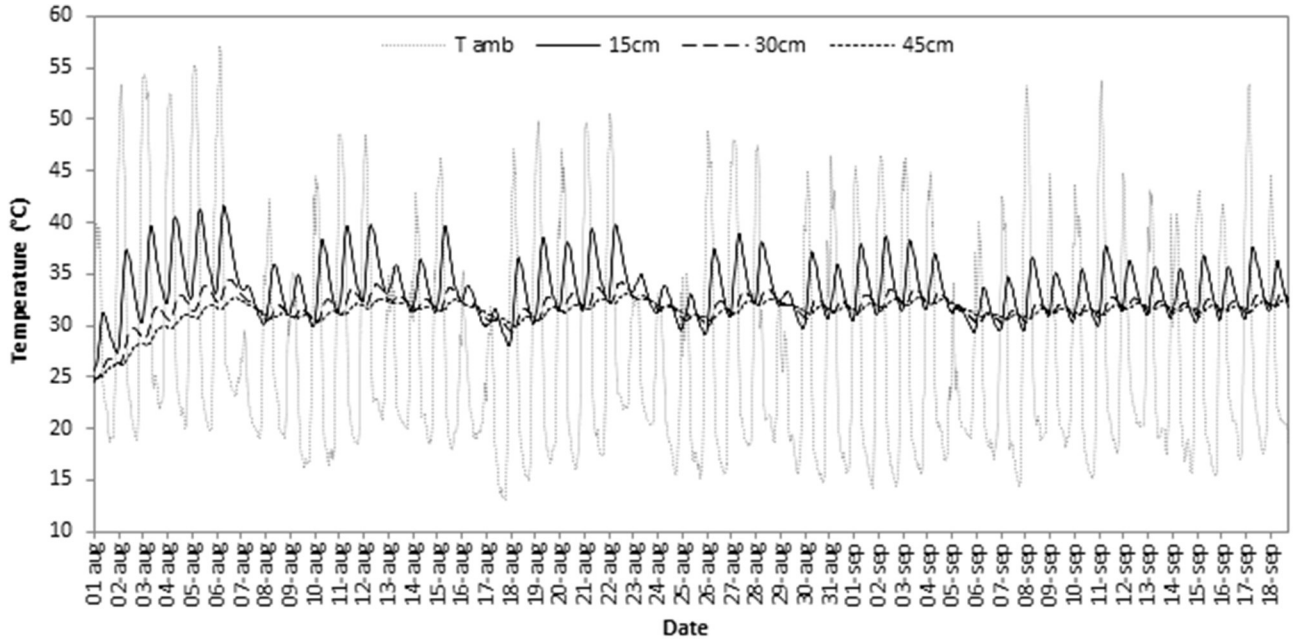

FIGURE 2 | Mean temperatures per hour for each day during seven weeks of biodisinfestation treatment at ambient temperature inside the greenhouse (T amb) and at three different soil depths (15, 30, and 45 cm) in the treated soils.

2010). Both PPI and EF values were obtained thanks to the free NINJA tool developed by Sieriebriennikov et al. (2014) and can provide information about the effects of soil biodisinfestation on other PPN and opportunistic nematodes which may contribute to develop suppressiveness.

Statistical Analysis

Analyses were performed with a one-way factor (soil treatment) analysis of variance (ANOVA) with the linear mixed model procedure (proc MIXED) of the statistical software SAS 9.4, which contained both fixed- and random-effects. Each soil treatment and time combination was considered as a fixed factor, and each replicate plot a random factor nested in each combination of soil treatment and time. Means of significant variables were separated with the multiple range Tukey HSD test with adjustment for the *P*-values against an overall experiment-wise type I error rate of $\alpha = 0.05$. Nematode related variables were squared root transformed to stabilize the variances.

RESULTS

Organic Amendments and Soil Physicochemical Characterization

Among the organic amendments, FCM presented the highest amount of organic matter (65.36%) but similar N total content as RC (1.48 and 1.36, respectively). FCM showed also a high content of organic C (37.96%) followed by RC (10.3%) (**Table 1**). The final mix of the different by-products presented a C/N ratio of 23 and OM content of 53.52% considering the application ratio (FCM:BB:RC 78:10:12).

The soil before treatment (T1) had an initial OM content of 5.82% and after biodisinfestation (T2) the controls (C) remained around this value, whereas in treated soil (BD) this value reached 7.55%. Even though N total content slightly increased in the treated soils, organic C content and EC increased after biodisinfestation compared to the controls. Likewise, Fe and Mn content increased significantly in the treated soils after treatment (249 and 195 ppm, respectively). One year after biodisinfestation (T4), all parameters recovered the initial values before treatment except for Fe, which remained high (159 ppm) regarding to the control (63 ppm). In all cases, no appreciable differences were observed and the heavy metals remained below the toxicity levels (**Table 1**).

Temperature

During the 7 weeks of biodisinfestation treatment, the ambient temperature range recorded inside the treated tunnels was 13.2–57.2°C. Soil temperature ranges were 25.5–41.6°C at 15 cm depth, 24.5–34.5°C at 30 cm and 24.8–33°C at 45 cm in the soil of the treated tunnels (BD). The highest temperatures were registered in the first week of BD from 2nd to 6th August (**Figure 2**). The soil reached the highest temperatures at 15 cm depth accumulating 29, 10, and 6 h at 39, 40, and 41°C, respectively. At 30 and 45 cm soil depth the temperature remained nearly constant between 31 and 32°C accumulating 655 h at 32°C at 30 cm and 618 h at 31°C at 45 cm (**Table 2**).

Microbiological Soil Properties

Soil respiration rate was significantly higher ($P < 0.0001$) in treated soils (4.31 mgC/g/h) than in controls (1.50 mgC/g/) after biodisinfestation and after the first crop (1.95 and 0.53

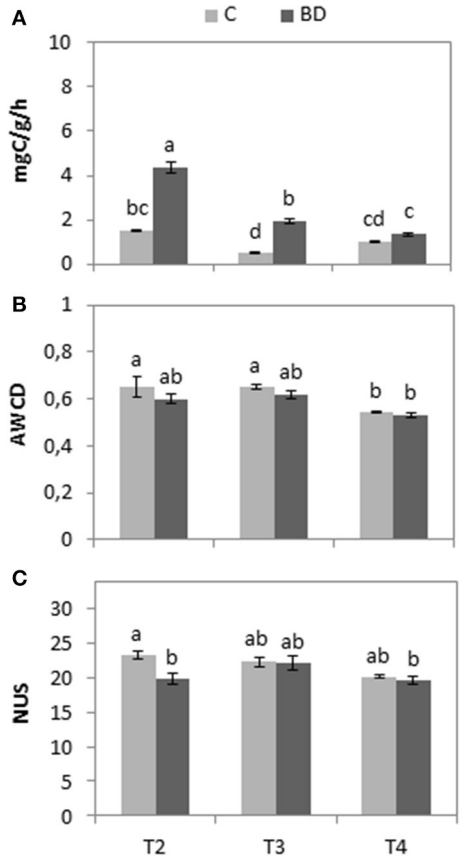

FIGURE 3 | (A) Mean values and standard errors of soil respiration rate (mgC/g/h) in dry matter after 72 h incubation time at 30°C. **(B)** Mean values and standard errors of average well color development (AWCD). **(C)** Number of used substrates (NUS) in Biolog Ecoplates™ at half of the incubation time. All values of control (C) and biodisinfested (BD) soils measured at three different times: T2 (post-treatment), T3 (post-crop after treatment) and T4 (1 year after treatment). Values with the same letter are not significantly different among time and treatment combinations according to Tukey HSD test ($P < 0.05$).

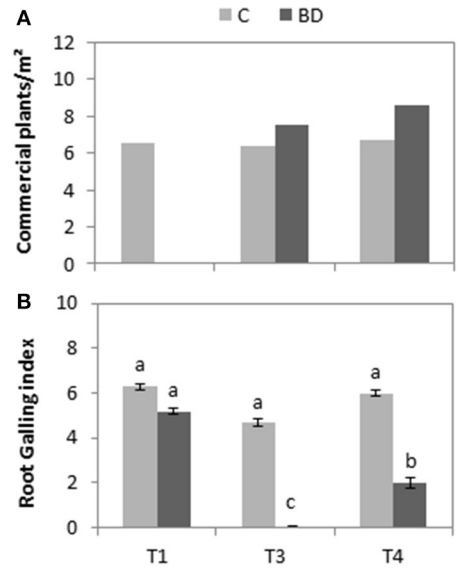

FIGURE 4 | (A) Average and standard errors for root galling index according to root-knot nematode rating chart by Bridge and Page (1980) of lettuce plants. **(B)** Number of commercial lettuces collected. For both, lettuce were collected from the biodisinfested (BD) and control (C) plots at three different times: T1 (pre-treatment), T3 (post-crop after treatment) and T4 (1 year after treatment). Values with the same letter are not significantly different among time and treatment combinations according to Tukey HSD test ($P < 0.05$).

mgC/g/h, respectively) but it gradually decreased in T4 showing values closer to those of the control (1.34 and 1.02 mgC/g/h, respectively) (**Figure 3A**). Regarding Biolog EcoPlates™, no significant differences ($P = 0.1077$ at T2; $P = 0.3166$ at T3; $P = 0.6779$ at T4) were observed between treatments in AWCD (**Figure 3B**) at half of the incubation time. Similar happened with the NUS (**Figure 3C**) but, in this case, a statistically significant decrease ($P = 0.003$) occurred in the treated tunnels in T2.

Root Galling Index and Yield Data

Lettuce plants collected before treatment (T1) in the control plots showed a GI between 5 and 6, similar values to those in the treated plots. In the first harvest after treatment (T3), considerable difference ($P < 0.0001$) was observed between the plants collected in treated and control tunnels. While in the controls the GI remained close to 5, hardly any galls were detected on the roots sampled in the treated soils. One year after BD (T4), the lettuces in the control plots still showed significant root galling and crop damage ($P < 0.0001$). The samples from the treated soil began to show root galling with index values of 1–3 (**Figure 4A**) but without crop losses caused by *M. incognita*.

Regarding yield data, the maximum number of lettuce that could be harvested were 12 plants/m^2. Before the trial, any lettuce was harvested for in the plots that were treated later due to severe damage caused by *M. incognita*. After biodisinfestation, the first crop grown faster in the treated soils and an increase of yield was observed (7.5 plants/m^2). Moreover, lettuce yield improved one year after biodisinfestation (8.6 plants/m^2) when 70% of total yield was harvested (**Figure 4B**). In contrast, in the control plots yield remained constant during the assessment period with around 55% (6.5 plants/m^2) in the three sampling moments (T1, T3, and T4).

M. incognita Presence and Nematode Community Analyses

The relative presence detected of *M. incognita* was around 50% in all plots before treatment and it decreased below 30% in all of them at T2 with no significant differences between treatments ($P = 0.4757$). Although no significant difference ($P = 0.1368$) could be observed at T3, *M. incognita* was not detected in the biodisinfested soils. One year after treatment, no statistically significant differences ($P = 0.1368$) were observed between treatments with low presence of *M. incognita* in the control (<10%) but the absence of the pathogen continued in the biodisinfested soils (**Figure 5A**).

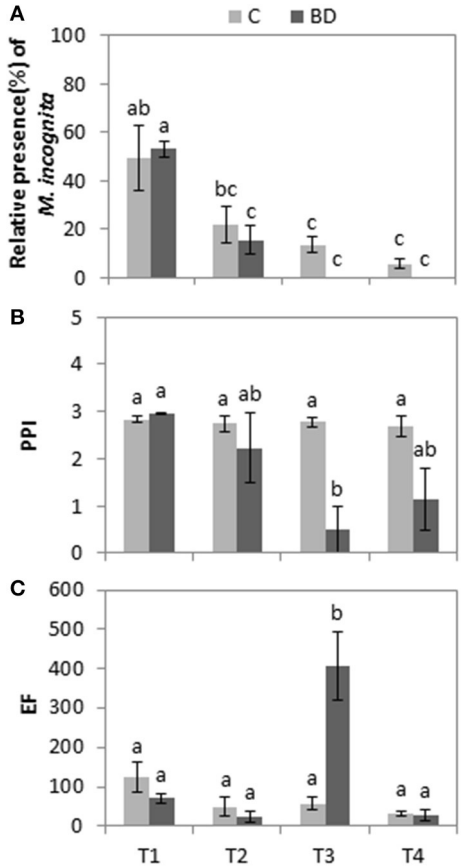

FIGURE 5 | (A) Relative presence (%) of *Meloidogyne incognita*. **(B)** Plan parasitic index (PPI). **(C)** Enrichment footprint (EF). Values at T1 (pre-treatment), T2 (post-treatment), T3 (post-crop after treatment) and T4 (1 year after treatment) in the biodisinfested (BD) and control (C) soils. Values with the same letter are not significantly different among time and treatment combinations according to Tukey HSD test ($P < 0.05$).

Regarding the nematode community indexes, in the control soils PPI remained stable throughout the study, while in the biodisinfested soil this index decreased after the treatment (T2), especially after first crop post-treatment (T3) ($P = 0.006$), and remained at low values one year later with significant difference ($P = 0.0136$) regarding to the control (**Figure 5B**). The EF stayed stable over time with low values in both biodisinfested and control soils. Only at T3 this index increased significantly ($P < 0.0001$) in the treated tunnels but shown initial levels one year later (**Figure 5C**).

According to the relative presence of the different guilds (**Figure 6**), PP3 dominated at T1 in all soils (about 50%) due to the presence of *M. incognita*, which is classified in this guild. After biodisinfestation, bacterivores became the dominant group in both soils (>48%) but at T3 this feeding group displaced the other groups in the biodisinfested soils (98.10% of presence) and stayed one year later (87.94%). While the presence of PPN in the control soils barely changed at T2, in the treated soils no presence was detected at T3 and only 2.38% one year later without detection of *M. incognita*.

DISCUSSION

The application of agricultural by-products followed by solarization has demonstrated to be effective against RKN *M. incognita* in lettuce crop. In this study, FCM + BB + RB resulted to be effective against *M. incognita* after biosolarization in a humid temperate area. The low solar radiation in these regions limits biodisinfestation processes related with high temperatures. However, positive results against *M. incognita* were obtained in similar studies carried out in northern areas (Núñez-Zofío et al., 2011; Ojinaga et al., 2020). During the assay, the maximum temperature achieved in the upper layer, at 15 cm (41.6°C), was below the maximum average temperature (45–55°C) registered in biosolarization performed in Mediterranean regions where this technique is included in crop management (Katan, 2017). However, comparable temperature values were obtained in similar trials carried out in summer in warmer geographical areas (Kaşkavalci, 2007; Núñez-Zofío et al., 2013) where maximum soil temperature averages registered were <42°C. The solar radiation is critical to achieve high temperatures in the soil but this effect is primarily achieved in the upper soil layers (Katan, 2017). Temperature data registered during this trial (**Figure 2**) showed how the temperatures in the upper soil layers increased as ambient temperature increased whereas the lower layers remained more stable at lower temperatures. Sub-lethal temperatures (30–35°C) were achieved in the lower layers during the most part of the assay which might render nematodes more vulnerable to biocidal compounds or to antagonistic microorganisms (Oka, 2010). In the upper layer (15 cm), 16 h accumulated between 40 and 41°C (**Table 2**) were enough to reduce population of *M. incognita* and GI, in agreement with the conclusion obtained by other authors in similar studies (Wang and McSorley, 2008; Guerrero-Díaz et al., 2014).

In this study, the effects of biodisinfestation were expected just after treatment, but only some physicochemical variables (**Table 1**) and soil respiration (**Figure 3**) showed differences between treatments at this time. On the contrary, nematode related variables showed significant differences after harvesting the first lettuce crop after biodisinfestation, at T3 (**Figures 5**, **6**). More complex soil microorganisms like nematodes required more time to establish after any disturbance such as tillage and organic amendment incorporation than bacteria and fungi, which reacted faster after changes in environmental conditions (Bongers, 1999; Treonis et al., 2010). This shift in microbial population was reflected in the respiration rate after biodisinfestation in the treated soils, primarily due to the incorporation of organic amendments. The incorporation of FCM + BB + RB into the soil incremented the organic matter content more than FCM alone (**Table 1**) which contributed to increase the microbiological activity boosted by the high temperatures achieved under plastic film (Katan, 2017; Waisen et al., 2020). However, while significant differences were observed in soil respiration rate (**Figure 3A**), the physiological profile

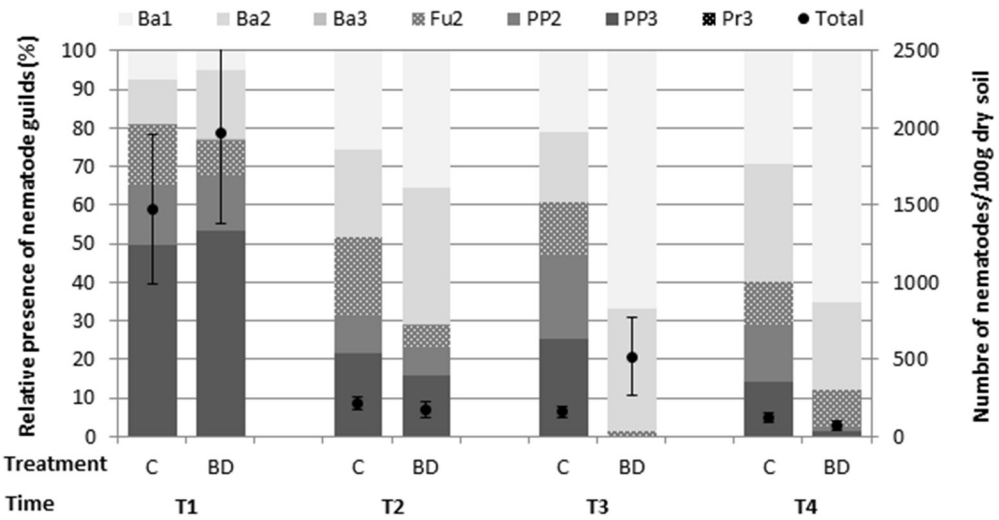

FIGURE 6 | Percentage of relative presence of different nematode guilds are represented by bar chart: Ba1 (bacterivores cp-1), Ba2 (bacterivores cp-2), Ba3 (bacterivores cp-3), Fu2 (fungivores cp-2), PP2 (plant-parasitic cp-2), PP3 (plant-parasitic cp-3) and Pr3 (predators cp-3). Total number of nematodes presented in 100 g of soil (dry matter) is represented by dot chart with the standard errors. Values of soil samples collected in the biodisinfested (BD) and control (C) soils at four different times: T1 (pre-treatment), T2 (post-treatment), T3 (post-crop after treatment) and T4 (1 year after treatment).

of heterotrophic bacterial community barely varied between treatments (**Figures 3B,C**). This might be due to changes in the soil physicochemical properties generated by biodisinfestation that favored certain indigenous microbiological communities. Previous studies have demonstrated that populations of antagonistic microorganisms like *Trichoderma*, *Bacillus,* and *Pseudomonas* spp. were enhanced after biosolarization resulting, in many cases, in suppressive soils (Stapleton, 2000; Moosavi, 2020; Rosskopf et al., 2020).

This suppressive effect was observed in the nematode related variables, which showed clearer differences after first harvest post-treatment and one year later than right after biodisinfestation. The reduction of *M. incognita* population was significant after first crop post-treatment with regard to the control (**Figure 5A**) and the same happened with PPN, both represented by PPI (**Figure 5B**). Although no statistical difference was observed in PPI one year after biodisinfestation, PPN represented <5% of total population identified in the treated soils (**Figure 6**). In concordance with previous studies, biodisinfestation effects on PPN remained longer periods by favoring antagonist microorganisms on detrimental of PPN (Ntalli et al., 2020). Conversely, bacterivorous nematodes were favored by the new conditions via enhancing the food source. In fact, after first crop post-treatment this group represented >95% of the whole community with higher number of individuals per 100 g/dry soil, manly represented by *Rhabditidae* taxa. Nematodes of this family have a short life-cycle and high colonization ability under high microbial activity (Bongers, 1990; Bongers and Bongers, 1998). This fact can be observed on EF (**Figure 5C**), which showed very high values after the first harvest after biodisinfestation, in the treated soils regarding to the other samplings. This result might also explain the increase of crop yield and even the early harvest of the first crop after biodisinfestation in the treated soils as a consequence of nutrients availability by higher microbiological activity. Thus, the incorporation of organic amendments in biodisinfestation practices might enhance the degradation rate increasing crop yield. One year after treatment, bacterivorous nematodes remained at high proportion (>85%) due to the prevalence of opportunistic bacterivores families (Ba1), nevertheless, nematode community tended toward equilibrium with the presence of fungivorous and an emerging population of PPN. The population of PPN remained lower in the treated soils than in the controls (2.38 and 29.04%, respectively) one year after treatment, after supporting four susceptible crop cycles (**Figures 5B**, **6**). Among PPN families identified in the soil samples one year post-treatment, presence of *Meloidogyne* was not detected in the samples but root galling was observed. According to Tzortzakakis (2010), GI and presence of *M. incognita* might not be necessarily correlated. Furthermore, some eggs might detach from the root and survived in the lower soil layers waiting for hutching under favorable conditions (Curtis et al., 2009). That might explain the beginning of galling one year after biodisinfestation in plants grown in the treated soils, but with low GI (1.99) that enables plant growth without disease development (**Figure 4**).

Among the soil processes generated by biodisinfestation practices, there is still no general consensus on the main cause of soil pest reduction. According to these results, we suggested that first effects were generated by the biocidal compounds, mainly VFAs and ammonia, released by the degradation of RC and BB together with FCM amendments followed by shift in antagonist microbial community, as it was suggested by several authors (Mazzola et al., 2001; Cohen et al., 2005; Mocali et al., 2015). This increase in the resident antagonist microorganisms generated beneficial effects in the following crops, even one year after biodisinfestation, after four susceptible crop cycles.

This shift in the microbiological community might have an impact on PPN reproduction rate as well. This change promoted colonization by opportunistic bacterivores nematodes at the expense of other groups. Although nematode community tended to equilibrium one year after treatment, the significant decrease of PPN after the first harvest post-treatment and one year later, along with yield increase, suggested that the proliferation of antagonistic organisms via biodisinfestation might be the main factor in *M. incognita* population decrease in long-term, resulting in suppressive soils (Moosavi, 2020). Moreover, *M. incognita* suppression might also be induced by fungivores or other nematodes when competing for same ecological niches (Oka, 2010).

The combination of organic amendments might have an important role and further research is needed on proper mixtures to be incorporated in these practices. In this study, positive results were obtained with BB + RC + FCM. The effects of fresh manures have been widely demonstrated (Rosskopf et al., 2020) but, in this case, the aim was to enhance effectiveness through implementation of certain by-products. According to previous studies, rapeseed cake (*Brassica napus* "canola") has demonstrated to be effective against soilborne pathogens despite the low glucosinolates content (Mazzola et al., 2001). In this case, the main effect was attributed to the high nitrogen concentration that led to release of NH3 via microbial degradation (Oka, 2010). On the other hand, the incorporation of BB as labile C-source could enhance the microbial degradation and the consequent release of VFAs (e.g., acetic, butyric, formic, and propionic acids) which have nematicidal effect (Oka, 2010). Besides, the increase in Fe and Mn in the treated plots (**Table 1**), which have also demonstrated biocidal effects, indicated that reductive conditions were enhanced by these amendments (Fernandez-Bayo et al., 2018). Regarding the properties of these by-products, could be interesting to consider them when choosing organic amendments in biodisinfestation treatments against *Meloidogyne* species.

In this case, the combination of these agro-industrial by-products with fresh manure demonstrated to be effective in short- and long-term against *M. incognita* and the whole PPN assemblage. The assessment of nematode functional guilds and EF provided information about the impact of biodisinfestation treatments on nematodes and their role in soil suppressiveness. The evaluation of nematode community offers useful information about the status of the soil food web that can help to better understand the development of suppressive soils due to biodisinfestation. Regarding the microbiological analysis, soil respiration rate has proven to be effective to measure microbial activity. While other analysis should be done, or combined with Biolog EcoPlates^{TM}, to understand the changes in the bacterial community structure. Metagenomics or other "-omics" techniques could be interesting alternatives to better understand these processes.

Our assay proved the effectiveness of biodisinfestation treatments in geographical areas with low solar radiation. This suggests, that high temperatures (>42°) are not necessarily required to achieve positive effects in soil biodisinfestation and soil supressiveness. Moreover, we considered that the sub-lethal temperatures were one of the main effects of soil suppressiveness. In some cases, biosolarization in Mediterranean areas can achieve high temperatures (>50°C) that lead to pasteurization with the consequent damage of possible antagonists. Sub-lethal temperatures can be enough to promote biodisinfestation processes, as well as long-term suppressiveness. The study of sub-lethal temperatures in indigenous microflora and nematode community would be interesting to understand the processes in soil suppressiveness after biodisinfestation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MG, JL-P, and SL contributed to conception and design of the study. MG, BJ, and LA conducted the assay and samplings. MG and JL-P performed the laboratory analyses. SL and MG organized the database and performed the statistical analysis. MG wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Soil Application of Almond Residue Biomass Following Black Soldier Fly Larvae Cultivation

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Insect farming has the potential to transform abundant residual biomass into feed that is compatible with non-ruminant animal production systems. However, insect cultivation generates its own by-products. There is a need to find valuable and sustainable applications for this material to enable commercial-scale insect farming. Soil application of by-products, which may be either basic broadcasting incorporation or part of a sustainable soil borne pest management practice, such as biosolarization, could offer an agricultural outlet. The objective of this study was to assess the potential of applying black soldier fly larvae (BSFL)-digested substrate as soil amendment for soil biosolarization and evaluate its impact on soil health. Sandy loam (SL) and sandy clay loam (CL) soils amended with BSFL-digested almond processing residues, i.e., spent pollinator hulls (SPH), at 2% dry weight (dw) were incubated under aerobic and anaerobic conditions for 15 days under a daily fluctuating temperature-interval (30–50°C). The microbial respiration, pH, electrical conductivity, volatile fatty acids, macronutrients, and germination index using radish seeds (*Raphanus sativus* L.) were quantified to assess the soil health after amendment application. Incubation showed a statistically significant ($p < 0.05$) increase in electrical conductivity related to amendment addition and a decrease potentially linked to microbiological activity, i.e., sequestering of ions. Under aerobic conditions, SPH addition increased the CO_2-accumulation by a factor of 5–6 compared to the non-amended soils in SL and CL, respectively. This increase further suggests a higher microbiological activity and that SPH behaves like a partially stabilized organic material. Under anaerobic conditions, CO_2-development remained unchanged. BSFL-digested residues significantly increased the carbon, nitrogen, C/N, phosphate, ammonium, and potassium in the two soil types, replenishing soils with essential macronutrients. However, greenhouse trials with lettuce seeds (*Lactuca sativa*) lasting 14 days resulted in a decrease of the biomass by 44.6 ± 35.4 and 35.2 ± 25.3% for SL and CL, respectively, compared to their respective non-amended soil samples. This reduction of the biomass resulted from residual phytotoxic compounds, indicating

that BSFL-digested SPH have the potential to be used for biosolarization and as soil amendments, depending on the concentration and mitigation strategies. Application and environmental conditions must be carefully selected to minimize the persistence of soil phytotoxicity.

Keywords: black soldier fly, almond residue, biosolarization, phytotoxcitiy, circular economy, sustainability

INTRODUCTION

In one decade, the production of almond kernels in the U.S. has increased from 640,000 t to 1.2 million t (USDA, 2020)[1]. This growth may partially be attributed to more health (Willett et al., 1995) and environmental (Pimentel and Pimentel, 2003) awareness in consumers, making them transition from animal to plant-based food and cosmetic products. Furthermore, targeted global marketing programs have effectively been implemented, resulting in a higher demand for almonds (ABC, 2019)[2]. Along with the increased production of almond kernels, the amount of hulls and shells, which by weight make up roughly 50 and 25%, respectively, of the almond fruit (Yousef et al., 2017), has surged as well. In the crop year 2018/2019 roughly 2.8 million t (ABC, 2019) of hulls and shells were generated. While hulls are mainly used for feedstock, shells are used for animal bedding due to the high lignin and cellulose content, making them unsuitable for animal feed (Velasco and Schoner, 1965). In contrast to the rising appetite for almonds, livestock production has been stable during the past 10 years, lowering the demand and thus the price for almond biomass side-streams (CDFA, 2019)[3]. Consequently, alternative uses for hulls and shells have been investigated during the last years. Many promising applications include insect farming (Palma et al., 2018), orchard soil amending before and after torrefaction (Pedrefio et al., 1996; Chiou et al., 2016), ethanol production (Offeman et al., 2014), and as plastic additives (Essabir et al., 2013).

Insect farming, using low-value organic residues, has the great advantage of generating a high-quality protein source that could be used for fish and poultry feeding or as a substitute for other commercially available protein sources (Leiber et al., 2015; Al-qazzaz et al., 2016; Gold et al., 2018). This additional and sustainable protein source could help address the major challenge of filling the predicted gap between available nourishment and effective population size by 2050 (Godfray et al., 2010; Smetana et al., 2016). Many different studies revealed that black soldier fly (*Hermetia illucens)* larvae (BSFL) grow on various waste and side streams, such as manure (Sheppard et al., 1995), food waste (Nguyen et al., 2015), or municipal organic waste (Diener and Studt, 2011). Palma et al. showed that BSF larvae could be successfully cultivated on almond hulls, whereby aeration and moisture content had significant effects on larvae growth (Palma et al., 2018). The usage of pollinator hulls showed the greatest increase in specific larvae growth compared to non-pareil hulls, monterey hulls, or mixed shells (Palma et al., 2019, 2020). Insect cultivation however leads to side-streams of leftover and non-consumed hulls, termed spent hulls, which are possibly not stable enough for direct soil applications, due to the short BSF larvae cultivation process (<20 days) relative to other organic waste stabilization systems. It has been reported that the direct soil application of amendments may have a negative impact on crop growth in the initial months or even years due to residual phytotoxicity from the amendments or limited nitrogen availability (Stamatiadis et al., 1999; Griffin and Hutchinson, 2007; Thomsen et al., 2018). In order to stabilize the organic matter in a short time-frame, achieve zero-waste goals[2] and close life cycles in a sustainable way, spent hull residues must be carefully managed to minimize negative impacts on the environment.

Soil biosolarization (SBS) is a chemical-free alternative to soil fumigation that relies on solar heating and decomposition of organic material amended into soil to achieve pathogen and weed seed inactivation (Blok et al., 2000; Ros et al., 2008). Soil amendments help overcome the limitations that arise from only solarization methods, such as long treatment times, survival of pathogens in deeper soil layers, and weather dependency regarding heating, by creating a reductive environment and producing biocidals through microbial activity under low oxygen conditions (Blok et al., 2000; Ros et al., 2008; Patel et al., 2014). This biocidal activity can be traced back to the degradation of organic material and the formation of ammonium, nitrate, nitrite, organic acids, hydrogen sulfide, aldehydes, and ketones (Angus et al., 1994; Sarwar et al., 1998; Domínguez et al., 2014; Shea et al., 2021). Several studies positively correlated the potential efficacy of SBS with the stability degree of the amendments originating from wheat bran, green waste compost, or tomato processing residues (Simmons et al., 2013; Achmon et al., 2017; Fernández-Bayo et al., 2017, 2018). In addition to a decreased mortality in crops, such as strawberry (Domínguez et al., 2014), SBS was shown to positively impact crop yields and soil health. Particularly, SBS with manure, olive pomace, or beet vinasse as organic amendment showed a significant increase of strawberry and tomato yields (Domínguez et al., 2014; Díaz-Hernández et al., 2017). Furthermore, SBS with energy crops and digestates increased phosphate, potassium, and inorganic nitrogen amounts (Pognani et al., 2009; Fernández-Bayo et al., 2017).

The main objective was to assess the potential of using spent hulls from BSFL cultivation as an amendment for SBS in two different soil types and to investigate its implications on soil

[1]USDA's National Agricultural Statistic's Service (Released July 7th, 2020): https://www.nass.usda.gov/Statistics_by_State/California.

[2]Almond Board of California's annual reports (2019): https://www.almonds.com/about-us/annual-publications.

[3]CDFA Agricultural Statistics Review: https://www.cdfa.ca.gov/Statistics/.

health. Its feasibility was characterized by monitoring metrics related to SBS efficacy (pH, volatile fatty acids, and microbial respiration) (Achmon et al., 2017), and parameters related to soil quality (conductivity, phytotoxicity, macronutrients, and crop yield). Experiments were conducted in the lab with fluctuating temperature under anaerobic (mimicking SBS) and aerobic (simulating broadcast soil application) conditions. To the best of our knowledge, there is only one study investigating the potential effects of soil application of spent hulls from BSFL (Palma et al., 2020).

MATERIALS AND METHODS

Spent Hulls and Soil Samples

Spent pollinator hulls were made available downstream of BSFL cultivation (Palma et al., 2018), originating from the VanderGheynst Lab, Department of Biological and Agricultural Engineering, UC Davis, CA, USA. As is described in Palma et al. (2018), pollinator hulls were obtained from a processor in 2017 from Chico, CA, USA from the prior years' harvest (2016). The hulls were reported to have a pH 4.74, 6 g kg^{-1} total nitrogen and 434 g kg^{-1} total carbon and particle size ($d = 6.35$ mm). The larvae cultivation conditions are described in Palma et al. (2018). Briefly, the initial C/N was set to 25 by adding urea. The hulls were inoculated with 1.3 larvae g^{-1} hulls BSFL (wet basis). Prior to inoculation onto hulls, larvae were reared for 5–10 days on chicken feed (Purina Premium Poultry Feed Layena Crumbles, Purina Animal Nutrition LLC, Shoreview, MN, USA). During the larvae cultivation studies on hulls, the aeration rate, temperature and moisture content during growing conditions were 160 mL min^{-1}, 26–28°C, and 82% of fiber saturation point (wet basis), respectively. The feeding rate was 300 g wet weight (ww) on day 0 and 610 g ww added after 5 days of incubation. After 13 days of incubation, larvae were recovered from substrate and spent pollinator hulls (SPH) were stored at 25°C. Prior to the current study, the SPH were oven-dried for 24 h at 55°C.

Almond trees are most productive in loam-textured soils due to their permeability, water retention, and root zone aeration (Micke, 1996). Soil texture is known to affect soil aeration, which may impact anaerobic conditions during biosolarization and the capacity of the soil to remediate phytotoxicity after treatment. Therefore, two relevant soil types for almond production with different textures were selected, a sandy loam (SL) and a sandy clay loam (CL). SL (41, 37, and 22% sand, silt and clay, respectively) soil was collected from the 0–15 cm depth range at the University of California Kearney Agricultural Research and Extension Center (KARE) in Parlier, CA, USA. Similarly, CL (47, 27, and 26% sand, silt and clay, respectively) soil was obtained from the 0–15 cm depth range at the UC Davis Plant Pathology Research Fields. Both soil types were air-dried, sieved through a 2 mm screen, and stored at room temperature.

Biosolarization Simulation Assay on a Lab-Scale

Four different treatments were prepared: Non-amended sandy loam (SPH: 0% dw, SL), sandy loam mixed with spent pollinator hulls (SPH: 2% dw, SL + SPH), non-amended sandy clay loam (SPH: 0% dw, CL), and sandy clay loam mixed with spent pollinator hulls (SPH: 2% dw, CL + SPH). The selected amendment rate of 2% dw is equivalent to 33 t ha^{-1} (assuming the bulk density of SL and CL is around 1.65 g cm^{-3} and that the amendment is incorporated down to 10 cm), which is within the recommended application rates for compost (Donn et al., 2014). Prior to the experiments, distilled water was added to each sample to reach 80% of the field capacity (FC) and they were left to equilibrate overnight at 4°C.

For each treatment (SL, SL + SPH, CL, CL + SPH), six replicate 250 mL flasks were filled with 100 g of sample. The flasks were placed in an incubator with a fluctuating temperature range from 30 to 50°C in 12 h intervals (Thermo Fischer Scientific, Waltham). Tubes from each reactor were connected to a Micro-Oxymax Respirometer (Columbus instruments, OH, USA). For each treatment, three flasks experienced anaerobic conditions by closing the flask with a sealed lid connected to a gas sampling tube with a check valve to prevent air going in. Three samples were placed in an aerobic environment by having a lid connected to the gas sampling tube and an air supplying tube at an aeration rate of 20 mL min^{-1} (Achmon et al., 2016). Samples were incubated for 15 days where the CO_2 that evolved was measured as an indicator for microbial respiration. In parallel, to monitor pH, electrical conductivity (EC, 25°C), volatile fatty acids (VFAs), and phytotoxicity changes during anaerobic experiments, 10 g of each sample ($n = 3$) was added to polypropylene tubes (15 mL, Fisherbrand Pittsburgh, PA, USA). These tubes were closed to promote anaerobic conditions and incubated in the same incubators for different incubation times. After 0, 1, 5, 10, and 15 days (t_0, t_1, t_5, t_{10}, t_{15}), three replicates of each treatment were taken for analysis.

Soil Analysis of Incubated Samples

At each incubation point, samples incubated in 15 mL tubes were diluted with distilled water at a dilution ratio 1:1 (mass of sample dw/mass water) and shaken for 30 min. For each extract, the pH, EC, phytotoxicity and volatile fatty acid (VFA) content were monitored using the method described in the following. The pH (SevenCompact S220, Mettler Toledo, OH, USA) and the conductivity (SevenCompact S230, Mettler Toledo, OH, USA) were measured following vendor guidelines. Then, the samples were centrifuged for 20 min at 4,000 *g*. An aliquot of the supernatant was filtered through a 0.2 µm filter (Titan3, 17 mm, PTFE membrane, Thermo Fisher Scientific, MA, USA) to measure the volatile fatty acid (VFA) content, i.e., lactic acid, formic acid, acetic acid, propionic acid, isobutyric acid, butyric acid, using HPLC-UFLC-10Ai (Shimadzu, Columbia, MD, USA) equipped with an Aminex® HPX-87H (300 × 7.8 mm) column (Life Science Research, Education, Process Separations, Food Science, Hercules, CA, USA) and a SPD-M20A diode array detector set at 210 nm. The HPLC conditions are described elsewhere (Simmons et al., 2016).

To test the phytotoxicity on radish seeds (*Raphanus sativus* L., Ferry-Morse, KY, USA), a filter paper (Dia.: 9.0 cm, P5, Cat. No.: 09-801B, Thermo Fisher Scientific, MA, USA) was sterilized with UV light on both sides for 20 min each (Biosafety cabinet, 1300 Series A2, Thermo Fisher Scientific, MA, USA) and placed

in a petri dish (Falcon, NY, USA), on which 10 seeds were placed and soaked with 5 mL of the previously mentioned supernatant. After 3 days of incubation in the dark at 25°C, the root length and number of germinated seeds were determined. As a control, 10 seeds ($n = 3$) were soaked with 5 mL of distilled water. The phytotoxicity was quantified by the germination index G_i (Equation 1) (Tiquia et al., 1996; Ko et al., 2008; Mitelut et al., 2011). G_i is defined as the product of the germination ratio and the length ratio:

$$G_i = \frac{G}{G_0} \cdot \frac{L}{L_0} \quad (1)$$

where G represents the number of seed germinated per plate, L the root lengths (mm) of germinated seed, G_0 the number of germinations of the control, and L_0 (mm) the root lengths of the control. Only seeds with root lengths >0.5 mm were considered germinated.

Analysis of Macronutrients in Soils

Samples at time t_0 and time t_{15} under anaerobic and aerobic conditions were oven-dried for 48 h at 55°C and sent to the analytical lab at the University of California Davis (Davis, CA, USA) to measure total carbon and total nitrogen (combustion method, AOAC Official Method 972.43, 1997), NO_3-N and ammonium-nitrogen content (flow injection analyzer method, Rhoades, 1982), extractable phosphorus (Olsen method, Olsen and Sommers, 1982), and exchangeable potassium (cation exchange capacity, ICP-AES, Thomas, 1982).

Lettuce Growth in Greenhouse

To measure potential fertility or residual phytotoxicity of the spent hulls, the growth of lettuce (*Lactuca sativa*) was monitored in a greenhouse at 22°C and 80% relative humidity. Prior to transplanting the lettuce, seeds were germinated in petri dishes during 3 days. Germinated seeds of similar sizes were placed into pots (180 cm^3) filled with sandy loam, SL, or sandy clay loam, CL, with fresh (2% dw) or without spent pollinator hull (SPH) amendments ($n = 10$). Coir-Lite Mix (0.75 yd^3 Coir, 0.25 yd^3 Perlite, UC Davis) was used as the control soil. After 14 days in the greenhouse, the seedlings were harvested, oven-dried (24 h, 55°C), and weighed to measure the lettuce biomass.

Data Analysis

For the statistical analysis IBM SPSS Statistics® (Version 23.0. IBM Corp., Armonk NY, USA) was used. A multiway ANOVA, followed by a one-way ANOVA with a Tukey's HSD *post-hoc* test or a paired-sample *T*-test was conducted with a confidence level of 95%. To correlate the pH, conductivity, and volatile fatty acids with the phytotoxicity and to correlate macronutrients with the lettuce biomass, Pearson's r was calculated in a two-tailed bivariate correlation test. Acetic acid concentrations were fitted with a 1st order decay model using the damped least-squares method (OriginPro 2019, OriginLab Corporation, Northampton, MA, USA) (Equation 2):

$$y = Ce^{-kt} + y_0 \quad (2)$$

where C represents the initial acetic acid concentration, k the decay constant, t the time constant, y_0 the offset.

RESULTS AND DISCUSSION

Assessment of the Potential of Spent Hulls for Soil Biosolarization

Microbial Respiration of Amended Samples

CO_2, CH_4, and H_2 were monitored, however, no quantifiable amounts of CH_4 and H_2 were measured, confirming that these gases were not produced or below the detection limit of the measurement system. To determine the degree of stability of the spent pollinator hulls (SPH) in the soils, the microbial activity was assessed by monitoring the CO_2 evolution during incubation under anaerobic (AN) and aerobic (AE) conditions. After 15 days under AE conditions, SPH addition increased the CO_2 accumulation significantly (**Supplementary Table 1**, $p < 0.05$) by a factor of 5 and 6 compared to the non-amended soils in SL and CL, respectively. Compared to AN conditions, AE non-amended soil samples (SL, AE and CL, AE) released 26 and nine times more CO_2, respectively. When spent hulls were present, 150 and 68 times more CO_2 was released for SL + SPH and CL + SPH, respectively. In the AN environment, added hulls and soil type were irrelevant (**Figure 1**). When comparing these respiration rates to other samples with slightly higher amendment rates of non-treated organic wastes, such as white wine grape pomace (5%), red wine grape pomace (5%), or tomato pomace (5%), a lower amount of CO_2 was released by SPH (Achmon et al., 2016). Compared to respiration rates of partially composted amended soils at 2.5% similar amounts of CO_2 were observed (i.e., around 200 mL/100 g dw), suggesting that digested SPH were only partially stabilized by BSFL digestion (Fernandez-Bayo et al., 2018). It has been shown that *Actinomyces* spp., *Dysgonomonas* spp., and *Enterococcus* spp. play an important role in the degradation process of organic matter in the BSFL gut, resulting in a reduced respiration rate in the soil compared with non-treated organic waste (Klammsteiner et al., 2020).

Analyses of pH, Electrical Conductivity, and VFAs Related to Biosolarization

After 15 days of incubation under AN conditions, the pH of SL and SL + SPH was at 7.29 ± 0.08 and 7.40 ± 0.14, respectively (**Figure 2A**). In the case of CL and CL + SPH, the pH reached values of 7.56 ± 0.12 and 8.02 ± 0.03, respectively (**Figure 2B**). Soil pH for all anaerobic samples was significantly affected by the soil type, the presence of spent hulls, time, interactions of soil and spent hulls, soil and time, spent hulls and time (**Supplementary Table 2**, $p < 0.05$). When comparing initial (t_0) and final (t_{15}) conditions of the same soil sample, only CL + SPH had a significantly higher pH than after incubation ($p < 0.05$). This suggests that the type of soil affected the evolution of the pH when spent hulls were incorporated. Moreover, by adjusting the water content to 80% of the field capacity, CL resulted in a higher volumetric soil moisture content. The pH of the soil tends to increase more as the ratio of water to soil increases (Thomas, 1996). Various mechanisms, such as decrease in exchangeable and soluble aluminum, consumption of

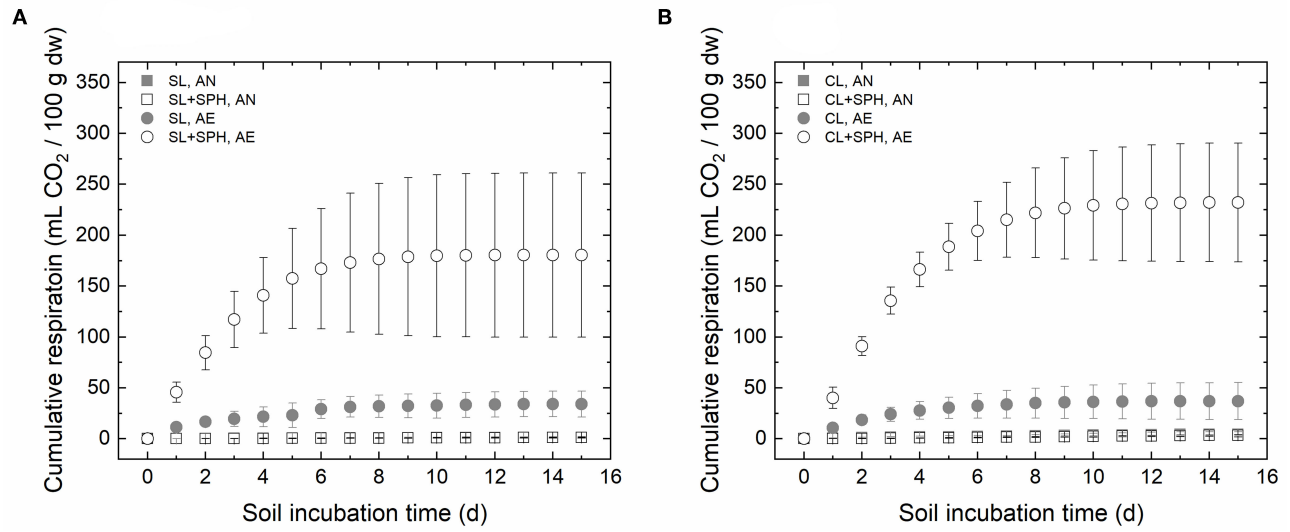

FIGURE 1 | CO_2 accumulation during 15 days in **(A)** sandy loam (SL) and **(B)** sandy clay loam (CL), with (2% dw) and without spent pollinator hulls (SPH) under anaerobic and aerobic conditions. AN, anaerobic; AE, aerobic. Bars represent standard deviations ($n = 3$).

protons by carboxylic, phenolic, and enolic groups, and liming effect of residues, have been proposed to explain an increasing pH after amendment addition (Wong et al., 1998; Haynes and Mokolobate, 2001; Naramabuye and Haynes, 2006). It appears that in CL, a sufficient amount of moisture was present, and enough time was given for soil chemical reactions to take place, thus resulting in a significantly higher pH compared with the initial state of CL + SPH.

The electrical conductivity (EC) at 25°C in AN samples was significantly affected by the soil type, the presence of spent hulls, time, interactions of soil and spent hulls, spent hulls and time (**Supplementary Table 3**, $p < 0.05$). Significant differences of pH and EC between soil types are shown in **Supplementary Figure 1**. A one-way ANOVA for SL (**Figure 2C**) and CL (**Figure 2D**) followed by a Tukey's HSD *post-hoc* test showed that the EC increased ($p < 0.05$) in non-amended soil samples of SL, however, decreased after 15 days ($p < 0.05$) when spent hulls were added. Before incubation, both SL and CL showed significantly ($p < 0.05$) higher EC when spent hulls were added. After 15 days of incubation, SL + SPH still had a significantly higher EC than the non-amended soil ($p < 0.05$), while in CL + SPH it decreased back to the original EC as was found in CL. Similarly, to the significantly increased pH in CL + SPH, the higher soil moisture content resulted in a larger soil activity. Overall, the amendments seemed to introduce salts in form of nutrients to the soil, however, they thereby increased microbial activity and decreased the EC over time. This suggests that the ions were sequestered in the microbial biomass (Stamatiadis et al., 1999; Piotrowska et al., 2011). The increased microbial activity in the soil might be a result of microbial community shifts introduced by SPH addition, originating from the BSFL gut, i.e., *Actinomyces* spp., *Dysgonomonas* spp., and *Enterococcus* spp. (Klammsteiner et al., 2020). Identifying microorganisms before and after SPH addition to the soil in the near future would allow further insights into the reasons for this increased activity.

No volatile fatty acids (VFAs) were detected in non-amended soils. Of the tested VFAs, only acetic acid was observed in samples with SPH under anaerobic conditions on a lab-scale. Acetic acid concentrations dropped to around 5 mg kg^{-1} within 10 days for SL + SPH and CL + SPH (**Figure 2E**). Upon fitting a 1st order decay model to the 5 measured points ($R^2 = 0.97$ for CL and $R^2 = 0.63$ for SL), results suggested acetic acid may degrade slightly slower in SL soil ($k = 0.25$) compared to CL soil ($k = 2$).

Based on the VFA development during 15 days, BSFL-digested pollinator hulls seem to be an appropriate organic material to be implemented in SBS approaches. The presence of acetic acid at the beginning of the incubation suggested that BSFL digestion did not manage to break down all phytotoxic organic acids in pollinator hulls. Furthermore, an increased microbial activity was attributed to the high respiration rates, indicating available sources of organic carbon that can be converted in biopesticides under the anaerobic conditions of SBS, decreasing the soil treatment time compared to traditional solarization of 4–6 weeks (Stapleton and DeVay, 1986). The pH change and detected acetic acid concentrations in SPH amended soil correspond to values, for which successful pest inactivation levels were achieved (Fernández-Bayo et al., 2018).

Phytotoxicity of Spent Hulls-Amended Soil

Spent Hulls Preliminary Assessment

The germination index (G_i) was used to determine the impact of spent almond hulls and incubation on the growth of radish seeds as the indicator of phytotoxicity. The lower the index, the higher the phytotoxicity. Preliminary analyses on water extracts from spent pollinator hulls at a dilution rate of 1:10 (g dw/g water) and at 1:100 had a G_i of 13 ± 7 and 42 ± 4%, respectively. The extract from the same SPH mixed

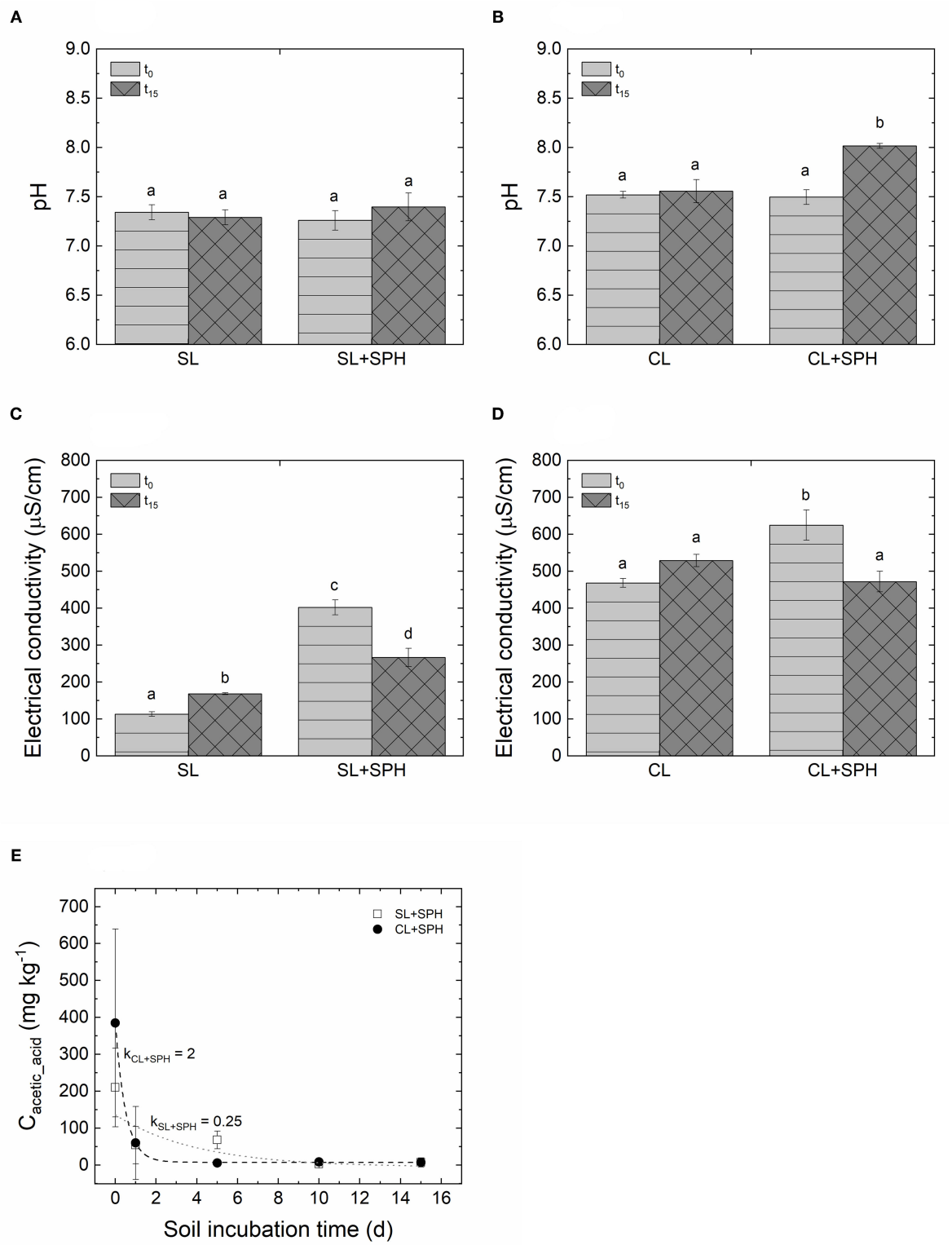

FIGURE 2 | (A) pH for sandy loam (SL), **(B)** pH for sandy clay loam (CL), **(C)** electrical conductivity (25°C) for SL, **(D)** electrical conductivity (25°C) for CL with (2% dw) and without amended spent pollinator hulls (SPH) under anaerobic conditions for initial (0 days, t_0) and final time (15 days, t_{15}). Different letters indicate significant differences ($p < 0.05$) in a one-way ANOVA followed by a Tukey's HSD *post-hoc* test. **(E)** Acetic acid concentration after anaerobic incubation measured for the time-points t_0, t_1, t_5, t_{10}, and t_{15} and fitted with a 1st order decay model (dotted line: SL + SPH; dashed line: CL + SPH). Parameter k represents the decay constant. Bars represent standard deviations ($n = 3$).

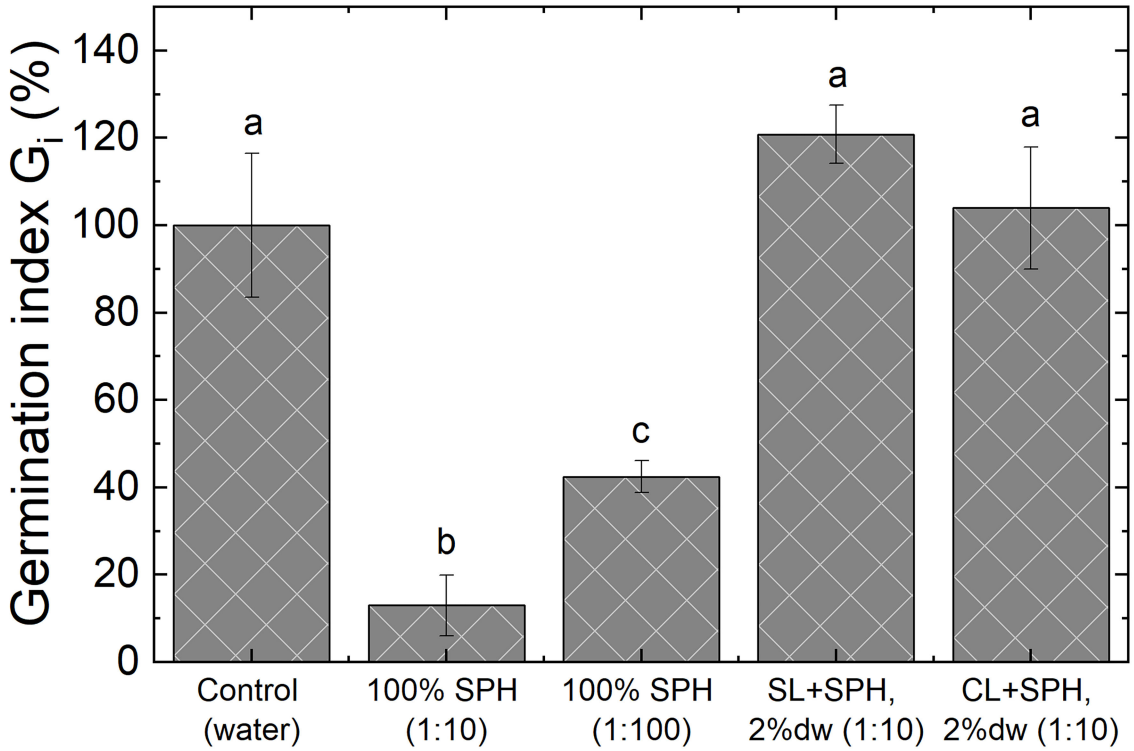

FIGURE 3 | Phytotoxicity of water extracts of spent pollinator hulls (SPH) for different concentrations (1:10 and 1:100) and the control (water) compared to 2% dw spent pollinator hulls in sandy loam (SL + SPH) and sandy clay loam (CL + SPH) at same concentrations (1:10) for radish seeds. Different letters indicate significant differences in a one-way ANOVA followed by a Tukey's HSD *post-hoc* test. Bars represent standard deviations (10 seeds, $n = 3$).

(2% dw) with SL and CL at a 1:10 (g dw/g water) dilution of non-incubated samples showed a G_i of 120 ± 7 and 104 ± 14%, respectively (**Figure 3**). Given the toxicity found in the extracted almond hulls at 1:100 dilution, the buffer effect in the phytotoxicity can be explained by the effect of mixing the spent hulls with the soils and the potential adsorption/degradation to the soil particles of the compounds responsible for phytotoxicity. The reduction in phytotoxicity over time was observed in biosolarization trials with compost (Simmons et al., 2013), tomato (Achmon et al., 2016), and wine processing solid wastes (Achmon et al., 2016).

Phytotoxicity Evolution in the Amended Soil

To assess if incubation under anaerobic (AN) or aerobic (AE) conditions of SPH in the soil can develop some phytotoxicity, a dilution ratio of 1:1 (g of sample dw/g water) was used in soils amended with SPH before and after incubation in AN and AE conditions for 15 days. The G_i of water extract from the incubated samples in AN conditions showed a significant effect ($p < 0.05$) of the interaction between SPH and soil type and between SPH and incubation type, i.e., non-incubated, incubated in AE, or incubated in AN conditions (**Supplementary Table 4**). Even though AE samples showed a slight improvement, when similar samples were compared under AN and AE conditions, there was no significant improvement of the germination index. Only SL + SPH under AN conditions showed a significantly higher G_i after 15 days compared to SL under AN conditions and CL + SPH before incubation (**Figure 4**). Furthermore, a paired-sample *T*-test (**Supplementary Table 5**) for same soil samples under the same conditions, i.e., AN and AE with and without adding SPH, differed significantly for AN SL and AN SL + SPH ($t = -7.792$, $p = 0.016$), and AE SL and AE SL + SPH ($t = -4.794$, $p = 0.041$).

Incubation of SL under AN conditions promoted some phytotoxicity in the soil. This may have been due to either changes in the soil or production of phytotoxic metabolites. This increase of the background phytotoxicity in the non-amended soil was not observed in the amended SL soil after incubation, suggesting that the amendments may have buffered the phytotoxicity or facilitated decomposition of phytotoxic metabolites. Furthermore, the drop in acetic acid (**Figure 2E**) observed in this study may have also contributed to the lower phytotoxicity observed in the SPH-amended soils undergoing incubation. It needs to be highlighted that both soils displayed a slight natural intrinsic phytotoxic effect on the model seed ($G_i \sim 80\%$).

When correlating the pH, EC, VFA concentration to the phytotoxicity levels, no significant correlations were observed. Phytotoxic compounds seemed to be present, however, non-detected VFAs and organic matter might further contribute to the evaluated phytotoxicity (Shea et al., 2021).

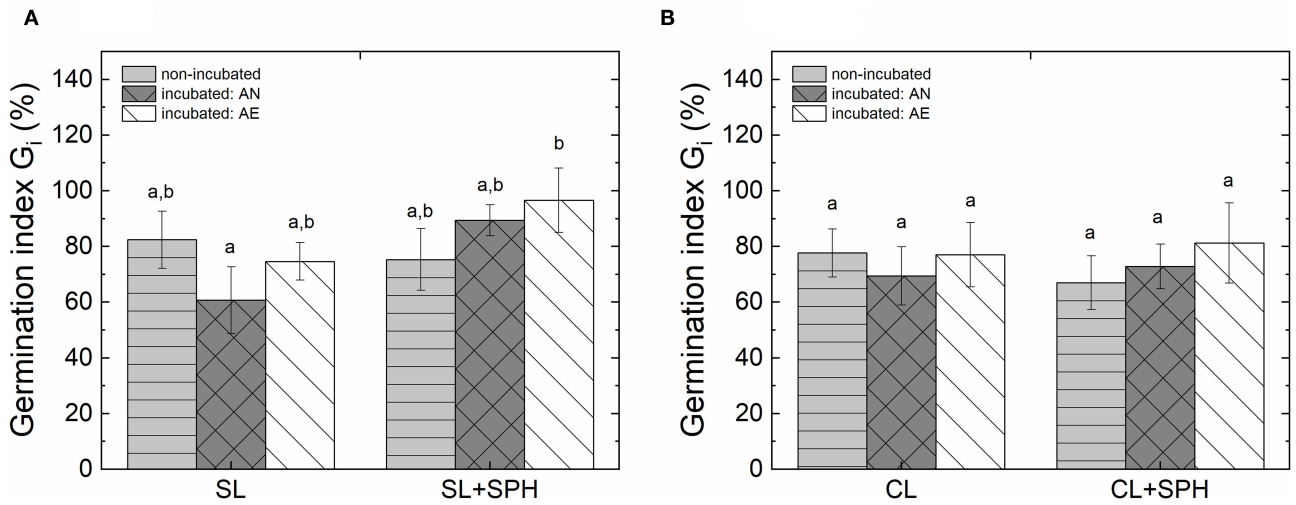

FIGURE 4 | Phytotoxicity of water extracts (Dilution rate: 1:1 g dw/g water) for non-incubated and incubated (15 days) samples for two different soil types for radish seeds: **(A)** sandy loam, SL and **(B)** sandy clay loam, CL with (SPH, 2% dw) and without amended spent pollinator hulls under anaerobic (AN) and aerobic (AE) conditions. Different letters indicate significant differences in a one-way ANOVA followed by a Tukey's HSD *post-hoc* test. Bars represent standard deviations (10 seeds, $n = 3$).

Impact of Spent Hulls on Lettuce Growth

To further elucidate positive or negative effects of SPH on soil quality, the growth of fertigated lettuce was assessed in greenhouse trials. A multiway ANOVA showed a significant effect of the soil type and SPH addition on the soil phytotoxicity (**Supplementary Table 6**). For both soils, a significant reduction of the dried biomass (shoot and root system) of lettuce seedlings was observed in the samples amended with spent hulls after 14 days post-transplantation ($p < 0.005$, **Figure 5** and **Supplementary Figure 3**) in comparison to the non-amended soils. Phytotoxic effects seemed to hinder the growth of the shoot system in particular, which was not quantified in the germination studies with radish seeds. Differing plant sensitivities to SPH phytotoxicity or differences in phytotoxin dilution in the varying growing formats could furthermore be attributed to the observation of an increased phytotoxic effect.

Impact of Spent Hulls on Soil Health

Significant effects of the incubation environment (AN, AE), soil type (SL, CL), SPH addition, and their interactions were observed ($p < 0.05$) on the macronutrient concentrations, i.e., carbon, nitrogen, nitrate, phosphate, ammonium, and potassium (**Supplementary Tables 7–13**). Spent almond hulls significantly ($p < 0.05$) increased the amount of carbon, nitrogen, C/N, phosphate, ammonium, and potassium content in SL + SPH, while nitrate did not significantly increase at the initial time t_0 (**Tables 1**, **2**, **Supplementary Figure 4**). Initial C/N values increased by about a factor of 2 with SPH amendment and remained significantly higher even after AN and AE incubation. A C/N ratio of stable and mature compost was defined by by Chefetz et al. (1996) between 10 and 15 and by Fang et al. (1999) between 17 and 19. A lower pH, a lower G_i index and a higher ammonium level furthermore indicate a lower stability (Palma et al., 2020). Alongside the rather low values of detected VFAs and rapid decrease (**Figure 2E**), this further suggests that SPH is a stable amendment. After 15 days under anaerobic conditions, nitrate in SL + SPH was reduced to 0.2 $\pm$ 0.0 mg kg^{-1}, while ammonium increased significantly to 20.9 $\pm$ 1.5 mg kg^{-1}. This effect must be considered for soil health and phytotoxicity in lower depths of the soil if amendments are distributed there. Ammonium levels were significantly higher in CL (+SPH) compared to SL (+SPH). This might potentially explain the slightly higher intrinsic phytotoxicity in CL, as ammonium can display a phytotoxic effect in sufficient quantities (Wong, 1985). Additionally, when comparing ammonium levels at t_{15} (with and without hulls), they decreased under aerobic conditions (**Tables 1**, **2**, **Supplementary Figure 4**). Ammonium is expected to oxidize to nitrate under aerated conditions. Due to the texture of the soil, anaerobic conditions are better kept in CL compared to SL, favoring turning nitrate into ammonium. For biosolarization this is beneficial, as it can be one of the mechanisms associated with pathogen inactivation (Stapleton, 2000).

The total amount of carbon that is retained in the soil is proportionally small to the additional CO_2 that is released due to the amendments under AE conditions. For SL + SPH (AE) and CL + SPH (AE) the carbon content dropped by 0.064 and 0.235% dw, respectively (**Supplementary Figure 4**). However, in a paired-sample *T*-test, both SL + SPH ($t = 0.212$, $p = 0.852$) and CL + SPH ($t = 0.953$, $p = 0.441$) were not significantly different.

When correlating these soil nutrients to the phytotoxicity in SL, SL + SPH, CL, CL + SPH at t_0 and t_{15}, no macronutrient was significant at the 95% confidence interval. Ammonium had a Pearson's coefficient (r) of 0.208 ($N = 10$), for nitrate r = 0.299 ($N = 10$) and the soil had a coefficient equal to 0.309 ($N = 10$), further suggesting an intrinsic phytotoxicity,

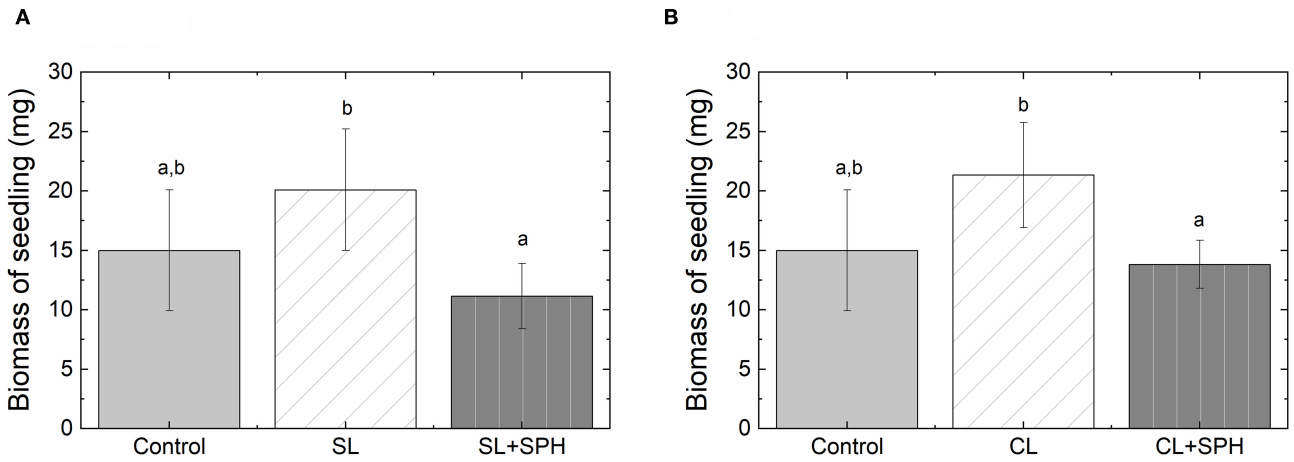

FIGURE 5 | Comparison of dried lettuce (*Lactuca sativa*) biomass in different soil types: **(A)** sandy loam, SL and **(B)** sandy clay loam, CL with (2% dw) and without spent hulls after 14 days. The control soil: Coir-Lite Mix, 0.75 yd^3 Coir, 0.25 yd^3 Perlite, no SPH. Different letters indicate significant differences in a one-way ANOVA followed by a Tukey's HSD *post-hoc* test. Bars represent standard deviations ($n = 10$).

TABLE 1 | Carbon, nitrogen, C/N ratio, nitrate, phosphate, ammonium, and potassium concentrations ($n = 3$) in sandy loam (SL) after 15 days (t_{15}) under different conditions (AN, anaerobic; AE, aerobic), with and without spent pollinator hulls (SPH) and at initial time (t_0).

Source	Carbon (%)	Nitrogen (%)	C/N	Nitrate ($N\text{-}NO_3$) (mg kg^{-1})	Phosphate ($P\text{-}PO_4$) (mg kg^{-1})	Ammonium ($N\text{-}NH_4$) (mg kg^{-1})	Potassium (K) (mg kg^{-1})
SL, t_0	0.40 ± 0.00^{a}	0.04 ± 0.00^{a}	10.7 ± 0.3^{a}	$2.2 \pm 0.6^{a,b}$	13.7 ± 0.1^{a}	3.2 ± 0.1^{a}	77.3 ± 1.5^{a}
SL, AN, t_{15}	0.38 ± 0.02^{a}	0.04 ± 0.00^{a}	10.1 ± 0.8^{a}	12.1 ± 3.3^{c}	$15.3 \pm 0.4^{b,c}$	$7.9 \pm 1.0^{b,c}$	75.0 ± 3.6^{a}
SL, AE, t_{15}	0.39 ± 0.02^{a}	0.04 ± 0.00^{a}	10.8 ± 0.7^{a}	$7.9 \pm 4.0^{b,c}$	$14.3 \pm 1.0^{b,c}$	$5.7 \pm 1.0^{a,b}$	76.3 ± 2.5^{a}
SL + SPH, t_0	1.51 ± 0.21^{b}	0.08 ± 0.01^{b}	19.8 ± 0.9^{b}	$2.3 \pm 0.7^{a,b}$	18.4 ± 1.1^{c}	11.2 ± 2.5^{c}	612.3 ± 119.3^{b}
SL + SPH, AN, t_{15}	1.12 ± 0.09^{b}	0.07 ± 0.01^{b}	16.7 ± 1.7^{b}	0.2 ± 0.0^{a}	19.8 ± 0.6^{c}	20.9 ± 1.5^{d}	517.3 ± 20.8^{b}
SL + SPH, AE, t_{15}	1.45 ± 0.36^{b}	0.08 ± 0.01^{b}	18.7 ± 2.1^{b}	$2.1 \pm 1.2^{a,b}$	16.0 ± 0.1^{b}	$8.6 \pm 1.3^{b,c}$	486.0 ± 35.9^{b}

Different letters indicate significant differences in a one-way ANOVA followed by a Tukey's HSD post-hoc test.

TABLE 2 | Carbon, nitrogen, C/N ratio, nitrate, phosphate, ammonium, and potassium concentrations ($n = 3$) in sandy clay loam (CL) after 15 days (t_{15}) under different conditions (AN, anaerobic; AE, aerobic), with and without spent pollinator hulls (SPH) and at initial time (t_0).

Source	Carbon (%)	Nitrogen (%)	C/N	Nitrate ($N\text{-}NO_3$) (mg kg^{-1})	Phosphate ($P\text{-}PO_4$) (mg kg^{-1})	Ammonium ($N\text{-}NH_4$) (mg kg^{-1})	Potassium (K) (mg kg^{-1})
CL, t_0	0.92 ± 0.03^{a}	0.10 ± 0.00^{a}	9.3 ± 0.3^{a}	52.4 ± 1.2^{b}	30.7 ± 0.3^{a}	8.5 ± 0.4^{a}	370.7 ± 5.0^{a}
CL, AN, t_{15}	0.85 ± 0.03^{a}	0.09 ± 0.00^{a}	9.2 ± 0.3^{a}	32.8 ± 14.7^{b}	34.4 ± 1.3^{c}	32.9 ± 4.5^{d}	377.3 ± 10.0^{a}
CL, AE, t_{15}	0.89 ± 0.03^{a}	0.10 ± 0.00^{a}	9.1 ± 0.2^{a}	50.3 ± 1.3^{b}	31.2 ± 0.3^{a}	$15.3 \pm 1.6^{b,c}$	370.3 ± 6.4^{a}
CL + SPH, t_0	1.75 ± 0.13^{b}	0.13 ± 0.01^{b}	13.6 ± 0.7^{b}	51.5 ± 0.7^{b}	$33.1 \pm 0.4^{b,c}$	18.6 ± 0.7^{c}	743.7 ± 6.1^{c}
CL + SPH, AN, t_{15}	1.57 ± 0.19^{b}	0.12 ± 0.01^{b}	13.0 ± 0.9^{b}	0.5 ± 0.0^{a}	37.5 ± 0.5^{d}	34.7 ± 1.1^{d}	696.3 ± 29.3^{b}
CL + SPH, AE, t_{15}	1.64 ± 0.10^{b}	0.12 ± 0.00^{b}	13.6 ± 1.2^{b}	34.4 ± 17.0^{b}	$31.3 \pm 0.6^{a,b}$	$10.0 \pm 1.8^{a,b}$	699.7 ± 16.3^{b}

Different letters indicate significant differences in a one-way ANOVA followed by a Tukey's HSD post-hoc test.

originating from undetected VFAs, ketones, aldehydes, or other phytotoxic molecules present in almond residue, such as cyanide (Chaouali et al., 2013). Cyanide was shown to reduce lettuce seed germination (Taylorson and Hendricks, 1973) and lettuce root length inhibition by more than half (Alström and Burns, 1989).

CONCLUSIONS

The addition of spent hulls from black soldier fly larvae cultivation in soils had a significant impact on the properties of the studied SL and CL soils. SPH addition resulted in a significant increase of carbon, nitrogen, C/N, phosphate,

ammonium, and potassium in SL and CL. Respiration was significantly higher in both soils when SPH were incubated under AE conditions, mimicking broadcast soil application. The electrical conductivity after 15 days decreased significantly in the amended soil, suggesting that salts introduced by the amendments were sequestered in the microbial biomass following an increased microbial activity. The results indicated that phytotoxic compounds are present in SPH and SBS may be limited due to a higher stability of the SPH compared to another non-degraded biomass. The phytotoxic effect was further quantified in greenhouse trials where there was a significant reduction of the lettuce seedling biomass after 14 days when plants were grown in soil amended with SPH relative to non-amended soil. Further research is needed to determine the responsible compounds of the phytotoxicity and the role of microbial communities in the soil after SPH addition. Possible mitigation strategies could include implementing a buffer period between amendment and planting to allow phytotoxins to degrade or using transplanting methods where more mature plants can tolerate phytotoxins better, while profiting from the added nutrients. Although lab-scale experiments suggested that BSFL-digested almond hulls have the potential to be used as amendments in various types of soil, longer-term experiments and field studies with crop production will allow more conclusive statements on soil health, biomass yield, and the ability to control soilborne diseases.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CS, JF-B, and RA contributed toward the conception of the work, designed the experiments, drafted the work, and critically revised it for intellectual content. RA conducted the experiments. RA and JF-B acquired, analyzed, and interpreted the data. RA, CS, LP, JV, and JF-B contributed the final approval of the completed revision and ensured accountability for all aspects of the work in ensuring that questions related to accuracy and integrity of the work were appropriately managed. CS and JV provided the financial support. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.664635/full#supplementary-material

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Biofumigation With Pellets of Defatted Seed Meal of *Brassica carinata*: Factors Affecting Performance Against *Phytophthora nicotianae* in Pepper Crops

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Phytophthora nicotianae is the pathogen that causes root and crown rot disease in open field paprika pepper crops of Extremadura (central-western Spain). A field experiment was established during spring, a period compatible with the pepper crop cycle in this region, to evaluate the effects of biofumigation with pellets of *Brassica carinata* defatted seed meal, at the rate recommended by the manufacturer (3 tons ha^{-1}), on the survival and infectivity of *P. nicotianae* chlamydospores inoculum. Furthermore, three biofumigant rates (3, 6, and 20 tons ha^{-1}) were assayed in laboratory experiments with natural soil inoculated with chlamydospores. In the field trial, the incorporation of pellets at 3 tons ha^{-1} did not produce remarkable changes in soil enzyme activity or soil properties. In both the field and laboratory tests, survival and infectivity in the biofumigated treatment at 3 tons ha^{-1} did not differ from those in the untreated control. On the contrary, the same rate added to autoclaved soil completely suppressed the inoculum, suggesting that the soil microbiota degrades *B. carinata* pellets before being effective against *P. nicotianae*. Increasing the recommended rate to 6 tons ha^{-1} decreased inoculum survival in the laboratory test and 20 tons ha^{-1} completely inhibited the inoculum, although the economic value of such a high rate application is a factor to be assessed. In phytotoxicity tests on radish and white mustard seeds with several concentrations (100, 50, 25, 10, 5, and 0%) of *B. carinata* pellets solution, no phytotoxic effect was observed with the two lowest concentrations, and no symptoms of phytotoxicity were found in the bioassays of pepper plants.

Keywords: biodisinfestation, Biofence, *Brassica carinata*, soil disinfestation, *Phytophthora*, phytotoxicity

INTRODUCTION

Phytophthora nicotianae Breda de Haan (=*P. parasitica* Dastur) is a very damaging soil-borne plant pathogen (Erwin and Ribeiro, 1996) that causes significant losses in a large number of host plants (Panabières et al., 2016). In Extremadura region (central-western Spain), where paprika peppers are grown in open field, *P. nicotianae* survives mainly as chlamydospores and is the principal cause

of root and crown rot in this crop (Rodríguez-Molina et al., 2010; Serrano-Pérez et al., 2017a). The banning of methyl bromide as a soil fumigant and restrictions on the use of other chemicals have made it necessary to develop sustainable alternatives for the management of this pathosystem.

In field trials in Extremadura, solarization in summer was effective in the inactivation of chlamydospores of *P. nicotianae* (Rodríguez-Molina et al., 2016), and several authors have described decreases in the density of this pathogen after soil solarization (Coelho et al., 1999; Lacasa et al., 2015). However, pepper for paprika is grown in open field in Extremadura during summer. Thus, solarization or biofumigation during the summertime combined with solarization (biosolarization) is not compatible with this crop cycle. Anaerobic soil disinfestation (ASD), i.e., incorporating high amounts of organic carbon under saturated soil conditions and sealing it with a plastic sheet, which leads to oxygen exhaustion by facultative anaerobes, has been shown to be an effective approach to control this pathogen (Serrano-Pérez et al., 2017b). Biofumigation is a term that refers to the suppression of soil pests and pathogens by volatile toxic compounds, mostly isothiocyanates (ITCs), which are released when the glucosinolates (GSLs) that *Brassicaceae* species contain in their tissues are hydrolyzed by the enzyme myrosinase (Kirkegaard et al., 1993; Angus et al., 1994). The products resulting from the hydrolysis, especially the ITCs, have shown extensive biocidal action, although they could have phytotoxic effects (Brown and Morra, 1997). The meaning of the term biofumigation is actually broader and it is associated with the release of biocidal compounds in the decomposition processes of plant tissues or any kind of organic matter (Bello et al., 2000; Gimsing and Kirkegaard, 2009). There is a considerable amount of literature on the success of this strategy, see descriptions in recent works (Brennan et al., 2020; Hanschen and Winkelmann, 2020; Santos et al., 2021). In a meta-analysis including information from 934 biofumigation tests with brassica residues, Morris et al. (2020) reported a decrease in disease incidence and about 30% increase in crop yields using this technique.

GSLs can be incorporated into the soil as fresh plant material (green manure), dried plant material, or seed meals (Matthiessen and Kirkegaard, 2006; Gimsing and Kirkegaard, 2009). The residual meal after oil extraction, the defatted seed meal (DSM), constitutes the major by-product (around 60% of the seed) of the extraction process (Santana-Méridas et al., 2012). Using DSM as a biofumigant is a sustainable way to upcycle this organic waste. Seed brassica meals have several advantages over green manures, such as their availability all year round and stable storage, with no change in their GLS profile, due to their low moisture content. In this study, commercial pellets (Biofence®, Triumph Italia SPA) produced from seed meal of *Brassica carinata* A. Braun selection ISCI 7 (Lazzeri et al., 2008; Furlan et al., 2010) were tested to control *P. nicotianae*. Seed meal is previously deffated to extract the oil, which increases its GSLs content. This formulation enables the application of doses that would be impossible to achieve with green amendments (Lazzeri et al., 2008). *B. carinata* is a well-studied species that provides a very suitable formulation for biofumigation (Porras and Dubey, 2011). Sinigrin is the principal glucosinolate in the *B. carinata* pellets and releases toxic allyl isothiocyanate (AITC) upon hydration (Galletti et al., 2008; Lazzeri et al., 2008).

In a previous paper, Serrano-Pérez et al. (2017a) showed that *B. carinata* pellets at the recommended commercial rate (3 tons ha^{-1}) and the double of this (6 tons ha^{-1}) decreased populations of *P.nicotianae* under the threshold of bioassay detection (<2 CFU g^{-1} of soil) and controlled the disease on pepper using autoclaved soil for the experiments. In some cases this product is not as effective when applied to natural soils (Guerrero et al., 2010; Núñez-Zofio et al., 2010; Núñez-Zofío et al., 2011). In a previous field experiment, the application of 3 tons ha^{-1} of *B. carinata* pellets in spring had no significant effect on the survival of *P. nicotianae* chlamydospores, regardless of the combination with *Brassica* cover crops (Rodríguez-Molina et al., 2016). In that study, it was suggested that the infectivity of the introduced inoculum was unexpectedly low, possibly by factors related to microbiological or chemical properties of the soil, decreases in density and weakening of inoculum, or even losses of pathogenicity of the *P. nicotianae* isolate used due to continuous culturing. Besides, the amount of water applied in that study after the pellets were incorporated may have been insufficient for optimizing the release of ITCs, according to Hanschen and Winkelmann (2020).

Several factors can influence the effectiveness of biofumigation with *Brassica* amendments in natural soils (Gimsing and Kirkegaard, 2009). In field conditions, GSLs can leach easily from the soil, or may be degraded and mineralized before forming ITCs (Gimsing et al., 2006, 2007; Laegdsmand et al., 2007). Also, ITCs are generally short-lived in natural soil (Gimsing and Kirkegaard, 2009). Sorption of ITCs by the organic matter in the soil or their volatilization, are other mechanisms that reduce the effect of *Brassica* amendments in soils (Kirkegaard et al., 1998; Gimsing and Kirkegaard, 2009).

Recent work proposes to apply this *B. carinata* pellets at higher rates than that recommended by the manufacturer (3 tons ha^{-1}) (Garibaldi et al., 2010; Gilardi et al., 2016, 2020). Gilardi et al. (2016) reported a reduction in the severity of *F. oxysporum* f. sp. *lactucae*, with a significant increase in *Pseudomonas* soil population, after the addition of 2.5 g L^{-1} of *B. carinata* pellets to non-autoclaved blonde sphagnum peat mixed with soil.

The objectives of this work were (i) to evaluate the effect of *B. carinata* pellets at the recommended rate by the manufacturer on survival and infectivity of *P. nicotianae* in a field experiment and to know its effect on chemical and biological soil properties, (ii) to test increasing doses of pellets incorporated into an inoculated natural soil under controlled laboratory conditions, and (iii) to discard the possible phytotoxic effect of the pellets.

MATERIALS AND METHODS

Inoculum Production

The isolate P-23 of *P. nicotianae*, isolated from a symptomatic pepper plant and previously evaluated for pathogenicity (Rodríguez-Molina et al., 2010), was used for the experiments.

Chlamydospores production followed the methodology described by Rodríguez-Molina et al. (2016), based on the

culture of *P. cinnamomi* isolate in 150 ml of V8 juice broth in 20-cm Petri plates and incubation at 25°C in the dark for 10 days. Subsequently, the V8 juice broth was removed, replaced with distilled water and plates were incubated again in the dark at 18°C for 7 days. Then, the mycelium clumps were collected, washed with sterile distilled water, homogenized in 50 ml of sterile distilled water (homogenizer MICCRA D-1, ART-moderne Labortechnik, Germany) and sonicated (HD 2070, Sonoplus, Bandelin, Germany) on a 60-s cycle (active interval: 0.9 s, passive interval: 0.1 s). After centrifugation (2 min; 1,760 rpm), the resulting pellet was resuspended in distilled water. The concentration of chlamydospores was assessed with a Neubauer counting chamber and their viability was estimated by staining with Rose Bengal solution (Tsao, 1971).

For field and laboratory experiments, inoculum bags were prepared using field disinfected soil (autoclaved twice 1 h at 120°C) which subsequently was infested with chlamydospores of *P. nicotianae,* according to the procedure described by Rodríguez-Molina et al. (2016). The soil was placed inside the Agryl cloth bags which were closed with string. Bags with 5 g of soil and 500 chlamydospores g^{-1} were prepared for the laboratory trials, and bags with 100 g of soil and 50 chlamydospores g^{-1} for the field trial.

Field Experiment

The trial was performed in a field with sandy loam soil (pH = 6.5; organic matter = 0.55%) at the Agricultural Research Institute Finca La Orden-Valdesequera (Extremadura, central-western Spain). The experimental design was a randomized complete block design with 4 replicates. The experimental plot size was 1.5 × 1.5 m, and each plot was artificially infested with 4 inoculum bags, with 100 g of soil and 50 chlamydospores per gram of dry soil each, which were buried at 20-cm depth, as used by Serrano-Pérez et al. (2017b).

The trial was conducted using commercial pellets of defatted *B. carinata* seed meal (BioFence®, organic N 6%, P 2.2%, K 2%, organic C 52%; Triumph Italia SPA). The concentration of sinigrin, the predominant glucosinolate, was 84.31 micromol g^{-1} dry weight (Rodríguez-Molina et al., 2021) and the pellets also contained active myrosinase (Galletti et al., 2008). The pellets were added to the soil at 3 tons ha^{-1}, according to the manufacturer's recommendations, and incorporated 20-cm depth using a motorized tiller. The plots were covered with transparent polyethylene film (0.05 mm thick). Two control treatments were included: control non-amended (CP) and control non-amended and without plastic cover (C). The soil was irrigated with 50 mm of water at the beginning of the experiment and again after 14 days with the same dose to maintain it near saturation [volumetric water content (VWC) at 0.21 m^3 m^{-3}] in the first 24-cm depth. The treatment was carried out for 4 weeks. Plastic covers were removed after that time and inoculum bags were dug up for determining inoculum survival and infectivity. A composite soil sample was collected at 0 to 25-cm depth from each plot to analyze soil physicochemical properties and microbial activity.

Soil Temperature, Moisture, and pH During the Field Experiment

As was performed in Serrano-Pérez et al. (2017b), soil temperature was continuously monitored at 20-cm depth, using soil probes and an automatic data logging system (HOBO Weather Logger, Pocasset, MA, USA) as well as moisture (volumetric water content, VWC) using 10HS sensors (ECH_2O, Decagon Devices Inc., Pullman, WA, USA), during the 4 weeks. Soil samples were collected before the beginning of the treatments and every 7 days during the trial to determine the evolution of soil pH. A pH electrode (HI12963, Hanna Instruments Inc., Woonsocket, RI, USA), in a 1:1 v/v slurry of soil and deionized water, was used for pH determination.

Weed Biomass and Native *Fusarium* spp. Population Density

To evaluate the possible herbicidal effect of the biofumigation treatment, all vegetation grown during the treatments within the quadrat of each experimental plot was cut, weighed and dried in the oven (102°C) and the dry weight m^{-2} was calculated. Total *Fusarium* density (CFU g^{-1}) in each plot was estimated on two occasions: before and after application of the treatments. Soil samples were air-dried, crushed and sieved (0.2 mm mesh size), and *Fusarium* density was evaluated by the soil-plate method (Warcup, 1950) using Komada's medium modified (Tello et al., 1991). Four replicates were prepared per soil sample.

Soil Physicochemical Properties

Soil samples were randomly collected from each field plot at two times: (i) before treatments application (0 weeks) and (ii) at the end of the experiment (4 weeks). Before analysis, soils were air-dried and ground to pass through a 2-mm sieve. Organic matter (OM) was determined by dichromate oxidation (Walkley and Black, 1934). The electrical conductivity (EC) was measured in water at a soil:extractant ratio of 1:5. Ammonium (NH_4^+) content was determined after extraction with 2 M KCl (Mulvaney, 1996). Available phosphorus (P) was measured using the molybdate reactive method (Murphy and Riley, 1962) after bicarbonate extraction (Olsen et al., 1954).

Soil Enzyme Activity

Enzyme activities were determined on the same soil samples collected for physicochemical characterization. The methodology used for the determination of each of the enzymes analyzed can be found in Serrano-Pérez et al. (2017b). Briefly, the β-glucosidase activity was determined as the amount of p–nitrophenol (PNP) formed from p–nitrophenyl-β-D-Glucoside (PGN) (Eivazi and Tabatabai, 1988). Acid phosphatase activity was determined at pH 6.3, using 16mM p-nitrophenyl phosphate (EC 206.353.9) as substrate (Tabatabai and Bremner, 1969). For both enzyme activities, the concentration of PNP was determined photometrically at 400 nm. Dehydrogenase activity was determined by measuring the amount of triphenylformazan (TPF) released after incubating the soil with 2,3,5-triphenyl-tetrazolium chloride. TPF was extracted with methanol (Trevors et al., 1982) and determined by reading at 490 nm. Urease activity was assayed by the method modified by Nannipieri et al.

(1980). The N-NH_4^+ was measured colorimetrically at 667 nm (Mulvaney, 1996).

Laboratory Experiments

The soil used in the laboratory experiment was taken from the field experiment plot at the Agricultural Research Institute Finca La Orden-Valdesequera. Before use, the soil was sieved (2 mm sieve).

The experimental set up to evaluate the effectiveness of different rates of pellets is practically the same as the one proposed by Serrano-Pérez et al. (2017b), in which closed controlled-temperature system was established to emulate the physical, chemical and microbial changes that take place in the field during the treatment. In this system, chlamydospores were in contact with all compounds released into the soil solution during the biofumigation process or only exposed to the volatile compounds generated during the treatments. The controlled system consisted of 1-liter airtight glass containers (14-cm height and 10-cm diam) filled with 600 g of soil mixed with the pellets and inoculated with a suspension of chlamydospores. The inoculum concentration was 50 chlamydospores g^{-1} dry soil. In addition, small bags of soil (5 g) inoculated with 100 chlamydospores g-1 were prepared and hung in the headspace of the container, avoiding contact with the soil placed at the bottom of the container. Ninety ml of tap water was added to saturate the bottom soil and the containers were hermetically sealed for 4 weeks. They were placed in a programmable incubator with a complete randomized design with 4 replicates per treatment. The temperature regime in the incubator was: 17.5°C for 5 h/day; 22.5°C for 5 h/day; 27.5°C for 4 h/day; 32.5°C for 2 h/day; 27.5°C for 3 h/day; 22.5°C for 5 h/day. These temperatures were selected as they had been recorded during a spring solarization field trial in Extremadura (Rodríguez-Molina et al., 2016).

Three different pellets rates were assayed: 3 tons ha^{-1} (BF3 = biofumigant commercial rate), 6 tons ha^{-1} (BF6), and 20 tons ha^{-1} (BF20). For the conversion between weight and surface area units, the bulk density of the soil (1.163 g cm^{-3}) and a product incorporation depth of 20-cm were considered. Control non-amended and inoculated (CPhy+) and non-amended and non-inoculated (CPhy–) were included in the experiment. Besides, to verify the importance of soil microbiota in the process, a treatment with autoclaved soil (1 h at 120°C twice in 2 consecutive days) mixed with the pellets at the commercial field rate (3 tons ha^{-1}) was included (BF3-AS).

When finished the incubation period, the containers were opened, and the soil from the inoculum bags was analyzed to estimate the number of chlamydospores of *P. nicotianae* surviving after the volatile exposure as described by Serrano-Pérez et al. (2017b) as follows: "the 5 g of soil from the bags were added to 45 ml of 0.25% water-agar (1:10, v:v) and stirred for 2 min; five 1-ml aliquots from this slurry were spread evenly over each of five Petri plates containing 12 ml of NARPH medium (Romero et al., 2007) giving the detection threshold of 2 colony-forming units (CFU) g^{-1} of soil. After 48 h of incubation in the dark at 25°C, the soil overlay was removed by gently washing the agar surface with tap water. Macroscopically visible colonies of *P. nicotianae* were counted and reported as CFU g^{-1} dry soil."

In the treated soil at the bottom of the containers, inoculum survival and infectivity were analyzed.

Survival and Infectivity of Inoculum

For the assessment of inoculum survival, 2 g soil samples (10 samples per container in laboratory experiments and 3 samples per inoculum bag in the field experiment) were analyzed for the presence of *P. nicotianae*. The 2 g soil samples were placed in 9-cm Petri plates and flooded with distilled water. Immature carnation petals floating on the water were used as baits for the detection of *P. nicotianae* (Tello et al., 1991). Results of survival are expressed as the percentage of soil samples in which *P. nicotianae* was detected.

The soil remaining in the containers (580 g per container) and in the bags (94 g per bag) after sampling for survival assessment was used for chlamydospores infectivity bioassays on pepper plants. The soil was put into 250-cm^3 plastic pots (5 pots per container, with 116 g soil each, and 1 pot per inoculum bag). The soil in each pot was mixed with a previously disinfected (autoclaved for 1 h at 120°C) substrate of peat and vermiculite (1:3, v:v). The ratio soil:substrate (v:v) was 1:2 in pots from container and 1:2.5 in pots from bags. One pepper seedling (*Capsicum annuum* L. cv. *Jaranda*) at the 2 to 4-true-leaf stage was transplanted into each pot and the pots were placed in a growth chamber with a 16 h light at 28°C/8 h dark at 24°C cycle. Disease symptoms were assessed weekly for 2 months, when the bioassay was concluded. To confirm plant death by *P. nicotianae*, as the plants died, fragments of the crown and tap water-washed roots were plated on potato dextrose agar and NARPH medium. At the end of the bioassay, roots of all plants were inspected for the presence of disease symptoms and analyzed. Infectivity results are expressed as the percentage of diseased plants. As the blocks were kept separate, there were 4 replicates per treatment.

Phytotoxicity Test of *B. carinata* Pellets

Radish (*Raphanus sativus* L.) and white mustard (*Sinapis alba* L.) seeds were used in these experiments as sensitive species to phytotoxic metabolites. The germination tests were conducted according to methods described previously (Zucconi et al., 1981; Carballo et al., 2009) with slight modifications. A pellet solution was prepared using sterile distilled water at 1:5 ratio (w:v), stirred vigorously for 30 min, and then centrifuged 1 min at 2,500 rpm. Dilutions from the solution were prepared using sterile distilled water to give the following concentrations: 100, 50, 25, 10, 5, and 0%. A sterile filter paper was placed inside each 55-mm diameter Petri plate, and 10 disinfected seeds were placed on the filter paper. One milliliter of each dilution was used to moisten the paper, and the plates were closed. Ten replicates per dilution and species were prepared. Seeds were incubated at 25°C in darkness, and germination and root length were recorded after 3 days. If the primary root was higher than 1 mm, seed germination was considered positive. The germination index (GI) was calculated for each dilution and species with the mean from 10 replicates according to the following formula: $GI = G/G_{(control)} \times L/L_{(control)}$, where G and $G_{(control)}$ are the mean percentage of germination with *B. carinata* pellets and with the

control, respectively, and L and $L_{(control)}$ are the mean root lengths with *B. carinata* pellets and with the control, respectively.

Data Analysis

Data of survival and infectivity of inoculum were analyzed by a Generalized Linear Model (GLM) with a binomial error distribution and logit link (binary dependent variable: detection/non-detection of *P. nicotianae* for Survival, and disease/non-disease for Infectivity). The treatment was included as fixed independent factor with three levels in the field experiment and five levels in the laboratory experiment and *post-hoc* comparisons of means were performed by Tukey's tests. The program R Core Team (2020) was used for these analyses.

Data of *P. nicotianae* chlamydospores survival in laboratory experiments, expressed as CFU g^{-1} dry soil, were subjected to one-way ANOVA on log-transformed data [log(x+1)] followed by Tukey's multiple range test. Field pH was analyzed with two-way ANOVA for repeated measures (rmANOVA), including statistical significance for the effects of treatment (between-subjects factor) and sampling time (within-subjects factor), as well as the interactions between them. The degrees of freedom were corrected using Greenhouse-Geisser estimates of sphericity. Data on soil enzyme activity and soil properties were also subjected to two-way rmANOVA, studying the effects of treatments (between-subjects factor) and sampling time (within-subjects factor) and their interactions. Post-rmANOVA means comparisons were carried out with Bonferroni's correction. Data of *Fusarium* spp. population density was analyzed using Kruskal-Wallis non-parametric test. Phytotoxicity data were subjected to two-way ANOVA with dose and species as factors followed by Tukey's multiple range tests. Effects and differences were considered significant at $P < 0.05$. All analyses were performed with the software package SPSS version 20.0 (SPSS Inc., Chicago, Illinois, USA).

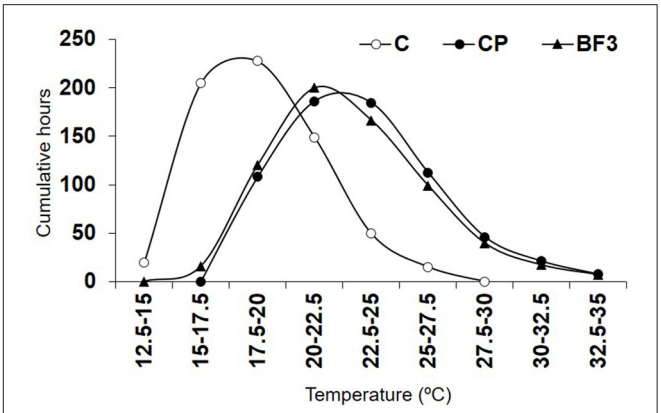

FIGURE 1 | Mean soil temperature at 20-cm depth during the biofumigation field experiment, expressed as cumulative hours at different temperature ranks. Control non-amended without plastic cover (C); control non-amended (CP); *Brassica carinata* pellets 3 tons ha^{-1} (BF3). Values represent means of data collected from two sensors.

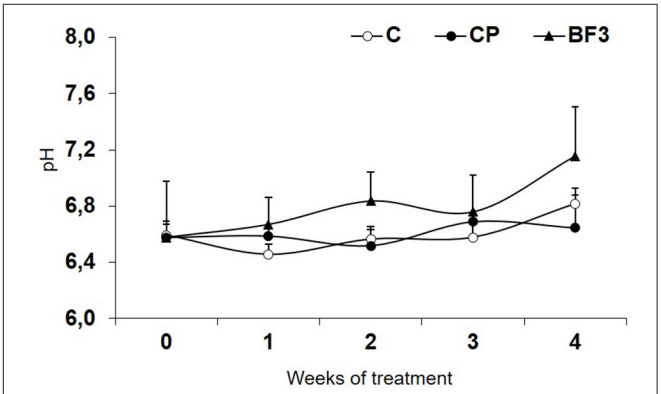

FIGURE 2 | Evolution of soil pH during the biofumigation field experiment. Control non-amended without plastic cover (C); control non-amended (CP); *Brassica carinata* pellets 3 tons ha^{-1} (BF3). Values are means ± SD ($n = 4$).

RESULTS

Soil Temperature, Moisture, and pH During the Field Experiment

Soil temperatures at 20-cm in plastic-covered treatments (CP and BF3) fluctuated between 15 and 35°C, and a similar pattern in soil temperature was observed in these treatments. In covered plots, the greatest number of cumulative hours registered (∼200 h) were between 20 and 25°C and only around 150 h above 25°C (**Figure 1**). Soil temperatures in the non-covered treatment fluctuated between 12 and 27.5°C, with the highest number of accumulated hours (217 h) around 17.5°C and only 15 h above 25°C.

Soil moisture in covered plots was maintained around field capacity (20 to 22 m^3 m^{-3} of VWC) throughout the experiment, while in control without plastic cover soil moisture decreased, inconstantly due to occasional rains, to values of 12 m^3 m^{-3} of VWC.

Soil pH did not significantly change in time, either in the control treatments (C and CP) or in BF3 treatment (**Figure 2**). Soil pH was kept between 6.5 and 7.2, and no differences were found in pH values among treatments throughout the experiment.

Effect of Commercial Rate of *B. carinata* Pellets on Survival and Infectivity of Chlamydospores on Pepper in the Field Experiment

There was no noticeable effect of the biofumigation with *B. carinata* pellets on the survival of *P. nicotianae* in soil from recovered field inoculum bags. The estimated survival was very high for all treatments, and no statistical differences were found in any case ($Chi^2 = 1.363$; $P = 0.506$). The percentages of positive baits detected were 88 ± 14% (mean ± sd; $n = 4$) in CP and 94 ± 13% in BF3, achieving 100 ± 0% of positive baits when the plastic cover was not used (C). The use of a plant disease bioassay confirmed the pathogenicity of the surviving population of *P. nicotianae*. There was no significant effect of treatment on

infectivity ($Chi^2 = 8.6\ e^{-6}$; $P = 1.00$), with trends very similar to that observed related to survival. Both covered treatments (CP and BF3) showed the same infectivity results (88 ± 14% of diseased pepper plants), while all plants showed root and crown rot disease in control without plastic cover (C).

Effects of Commercial Rate of *B. carinata* Pellets on Weed Biomass and Native *Fusarium* spp. Population Density in the Field Experiment

Weed biomass was not affected by treatment [$F_{(2)} = 1.869$, $P = 0.210$]. Biofumigated plots (BF3) had the highest level of weeds (31.5 ± 39 g of dry weight m^{-1}; $n = 4$), with *Cyperus rotundus* and *Setellaria media* being the predominant species, although these results were inconsistent due to differences between blocks and did not show significant differences compared with CP (23.8 ± 5.5 g of dry weight m^{-1}). Weed populations were lower in the non-covered control (C) (1.43 ± 1.9 g of dry weight m^{-1}), probably due to less favorable temperature and moisture conditions for seed germination in these plots.

Four *Fusarium* species were isolated from the soil before and after the treatments: *F. oxysporum*, *F. solani*, *F. roseum* and *F. moniliforme*. The total *Fusarium* spp. population density was over 3,982 ± 1,795 CFU g^{-1} of soil ($n = 48$) in all plots at the end of the experiment. The Kruskal-Wallis test conducted did not show differences among treatments [$H(2) = 1.69$, $P = 0.428$].

Effects of Commercial Rate of *B. carinata* Pellets on Soil Physicochemical Properties and Soil Enzyme Activity in the Field Experiment

Physicochemical soil properties before and after field experiment are presented in **Table 1**. Before pellet incorporation (0 weeks), no significant differences were found between plots for any of the soil properties studied, indicating the homogeneity of the plots before the setting up of the trial. At the end of the field experiment, there were significant increases in EC. No significant increase was observed in OM in BF3 treatment. The increases in NH_4^+ were significant in all cases, but no differences were found between treatments at the end of the experiment. The increases in available P were significant only in BF3, although there were no differences among treatments at the end of the experiment.

Soil enzymatic activities before and after field experiment are presented in **Table 2**. Just as with the physicochemical properties, there were no differences among plots at the beginning of the experiments. The treatment with pellets (BF3) did not enhance the soil enzymatic activities, and the results showed no significant differences compared with the controls (C and CP) for any enzyme studied.

Effects of Volatiles Released From *B. carinata* Pellets on Chlamydospores in the Laboratory Experiments

Volatile compounds released into the headspace of containers from the biofumigant rate BF3 did not suppress the chlamydospore germination of *P. nicotianae* (**Table 3**). Similarly, doubling the recommended commercial rate (BF6) was also ineffective on chlamydospore inactivation. However, when the pellets rate was increasing (BF20) or the rate BF3 was added into autoclaved soil (BF3-AS) no germination of chlamydospores was observed in any plate.

Effects of *B. carinata* Pellets on Survival and Infectivity of Chlamydospores on Pepper in the Laboratory Experiments

All rates of pellets assayed decreased the infectivity of the pathogen with respect to the control without pellets and the effect of treatment was significant on infectivity ($Chi^2 = 10.17$; $P = 0.037$). No significant differences were detected between BF3 and BF6 biofumigant rates and the control treatment (**Figure 3**). This lack of significance may be due to low infectivity reported in the control treatment (25% of diseased plants). Marginally significant differences ($P = 0.056$) were detected between the BF20 and BF3-AS treatments and the control treatment. The effect of the *B. carinata* pellets was more noticeable on the survival of *P. nicotianae* inoculum than on infectivity on pepper ($Chi^2 = 66.23$; $P < 0.001$). The doubled commercial rate (BF6) reduced the inoculum survival significantly, but no differences were found between the commercial rate BF3 and the control without pellets. On the contrary, when the rate BF3 was added into the autoclaved soil (BF3-AS) or the rate was increased (BF20), the infectivity on pepper and the inoculum survival were drastically reduced (**Figure 3**).

Phytotoxicity Test of *B. carinata* Pellets

The germination indices (GI) of radish and mustard with increasing *B. carinata* pellet dilutions found in the plate experiment are shown in **Figure 4**. The ANOVA indicated that the dilution had a significant effect [$F_{(4)} = 69.46$; $P < 0.001$], while the interaction between the two factors (dilution × species) was not significant [$F_{(4)} = 1.697$; $P = 0.158$). The GI was gradually decreasing for rising concentrations higher than 10%, and no germination was observed either with 100% concentration in radish seeds or with 50 and 100% concentrations in mustard. Mustard seeds were significantly more sensitive [$F_{(1)} = 13.358$; $P < 0.001$] than radish seeds, with lower GI for all dilutions assayed.

DISCUSSION

The ability of *B. carinata* commercial pellets (Biofence ®) against *P. nicotianae* has been previously demonstrated to inhibit the mycelial growth and the germination of chlamydospores and reduce the survival and infectivity of the inoculum on pepper plants in laboratory conditions (Serrano-Pérez et al., 2017a). However, it is noteworthy that the experiment was carried out with autoclaved soil, without any soil microorganism interference in biofumigation effectiveness.

The present work was carried out to elucidate the effect of *B. carinata* pellets at the recommended rate by the manufacturer (3 tons ha^{-1}) on the survival and infectivity of *P. nicotianae* in a

TABLE 1 | Soil properties before *Brassica carinata* pellets incorporation (0 weeks) and at the end of the treatments in the field experiment (4 weeks).

	EC	OM	NH_4^+	P
	dS/m	%	mg/kg soil	mg/kg soil
0 weeks[z]				
	0.042 ± 0.002	0.54 ± 0.02	0.20 ± 0.03	31.83 ± 1.23
4 weeks[x]				
C	0.069 ± 0.005 ab*	0.53 ± 0.07 a	7.72 ± 0.42 a*	33.61 ± 1.90 a
CP	0.059 ± 0.005 b*	0.52 ± 0.08 a	8.19 ± 0.76 a*	34.93 ± 5.15 a
BF3	0.088 ± 0.005 a*	0.56 ± 0.15 a	17.66 ± 8.07 a*	37.90 ± 2.08 a*

Control non-amended without plastic cover (C); control non-amended (CP); B. carinata pellets 3 tons ha^{-1} (BF3).
[z]No significant difference between plots. Values for each variable are means of all plots ± SD (n=20).
[x]Means ± SD (n = 4). For each variable, means with a different letter are statistically different [rmANOVA (P < 0.05) followed by multiple comparison tests with Bonferroni's corrections (P < 0.05)].
**Means are statistically different from the mean at 0 weeks for each variable.*

TABLE 2 | Soil enzyme activities before *Brassica carinata* pellets incorporation (0 weeks) and at the end of the treatments in the field experiment (4 weeks).

	Dehydrogenase	Phosphatase	Urease	β-Glucosidase
	mg TPF kg^{-1} soil h	mg PNP kg^{-1} soil h	mg N-NH_4^+ kg^{-1} soil h	mg PNP kg^{-1} soil h
0 weeks[z]				
	1.02 ± 0.11	148.38 ± 8.02	2.57 ± 0.21	55.69 ± 3.92
4 weeks[x]				
C	0.99 ± 0.29 a	165.77 ± 15.80 a	1.93 ± 0.26 a	84.43 ± 6.11 a*
CP	0.77 ± 0.16 a	148.01 ± 9.24 a	1.28 ± 0.24 a	52.67 ± 18.83 a
BF3	1.24 ± 0.36 a	145.13 ± 11.23 a	1.94 ± 0.77 a	68.24 ± 7.41 a

Control non-amended without plastic cover (C); control non-amended (CP); B. carinata pellets 3 tons ha^{-1} (BF3).
[z]No significant difference between plots. Values for each variable are means of all plots ± SD (n = 20).
[x]Means ± SD (n = 4). For each variable, means with a different letter are statistically different [rmANOVA (P < 0.05) followed by multiple comparison tests with Bonferroni's corrections (P < 0.05)].
**Means are statistically different from the mean at 0 weeks for each variable.*

TABLE 3 | Number of chlamydospores of *Phytophthora nicotianae* surviving in the inoculum bags after the exposure to the volatile compounds generated during the biofumigation process in the laboratory experiments.

	Treatments[z]	CFU g^{-1} dry-soil[y]
B. carinata pellets	BF3-AS[x]	0 ± 0 b
	BF3	377 ± 109 a
	BF6	383 ± 62 a
	BF20	0 ± 0b
Control	CPhy+	376 ± 81 a

[z]Brassica carinata pellets at 3 tons ha^{-1} (BF3); B. carinata pellets at 6 tons ha^{-1} (BF6); B. carinata pellets at 20 tons ha^{-1} (BF20); control non-amended and inoculated (CPhy+).
[y]Values are means ± SD (n = 5) of CFU g^{-1} dry soil. Means for each treatment followed by a different letter are statistically different [ANOVA (P < 0.05) followed by Tukey's multiple range test (P < 0.05)].
[x]Autoclaved 1 h, 121°C twice in 2 consecutive days.

field experiment. Previous works that applied pellets at similar rates, combined or not with plastic cover and fresh *Brassica* amendments during the spring, did not show the expected results to replace chemical treatments for the control of *P. nicotianae* in Extremadura (Lacasa et al., 2015; Rodríguez-Molina et al., 2016).

Sealing the soil following biofumigant incorporation retains fumigants and creates an anaerobic environment if enough water is applied. The treatments under these conditions have been found effective against soil-borne pathogens through different mechanisms, including the production of organic acids via anaerobic decomposition of the added organic matter, production of volatiles (Momma et al., 2006; Mazzola and Hewavitharana, 2014), and biocontrol activity of fungal and bacterial communities that grow during the process (Momma et al., 2010; Mowlick et al., 2012, 2013; Butler et al., 2014; Rosskopf et al., 2015). In the present study, to ensure that an anaerobic soil environment was achieved in the biofumigation process, the soil was abundantly watered, according to Serrano-Pérez et al. (2017b). The results of the field experiment of the present study are consistent with previous works (Lacasa et al., 2015; Rodríguez-Molina et al., 2016), although initially it was considered that the lack of pathogenicity of the *P. nicotianae* isolate used, or the insufficient water applied after pellet incorporation into the soil (Hanschen and Winkelmann, 2020) could have been a contributing factor in the results obtained by Rodríguez-Molina et al. (2016). Furthermore, the *P. nicotianae* isolate (P-23) infectivity used in the current work was higher than the infectivity of the

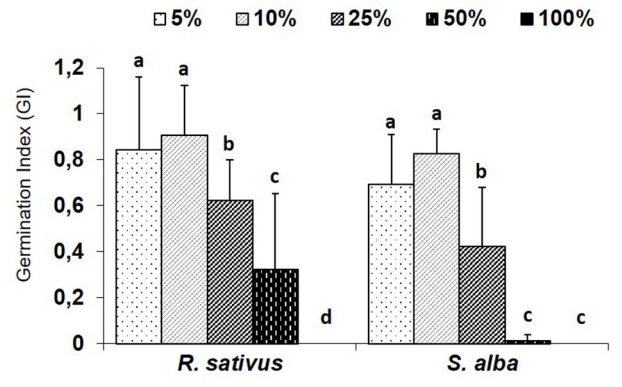

FIGURE 3 | Survival and infectivity of *Phytophthora nicotianae* after laboratory experiments. *Brassica carinata* pellets at 3 tons ha^{-1} (BF3); *B. carinata* pellets at 6 tons ha^{-1} (BF6); control non-amended and inoculated (CPhy+). Treatment followed by AS was prepared with autoclaved soil. Survival was determined by detecting *P. nicotianae* with carnation petals as baits and was expressed as % of positive baits ($n = 10$). Infectivity was determined by bioassays with pepper plants and expressed as % of diseased plants ($n = 5$). Values are means ± SD ($n = 4$). For survival, different letters in bars indicate significant differences [Chi$^2 = 66.23$; $P < 0.001$, followed by Tukey's multiple range test ($P < 0.05$)]. For infectivity, different letters in bars indicate marginally significant differences (Chi$^2 = 10.17$; $P = 0.037$), followed by Tukey's multiple range test ($P < 0.056$).

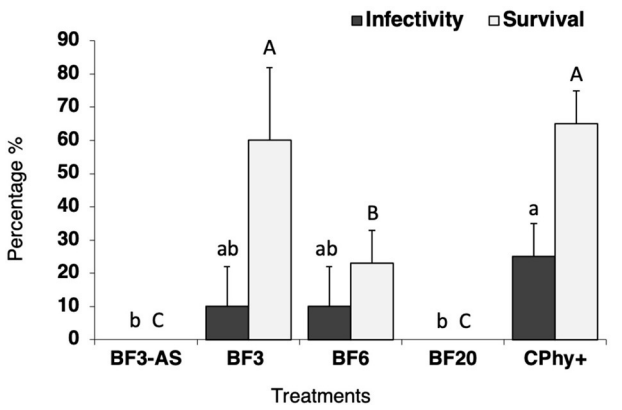

FIGURE 4 | Phytotoxic effect of dilution ratios of *Brassica carinata* pellets extract on the germination index of radish (*Raphanus sativus*) and mustard (*Sinapis alba*). Values are means ± SD ($n = 10$). For each species, bars with a different letter are significantly different [ANOVA ($P < 0.05$) followed by Tukey's multiple range test ($P < 0.05$)].

isolate used previously (P-13) in Rodríguez-Molina et al. (2016), evidencing the inefficacy of *B. carinata* pellets at that rate (3 tons ha^{-1}) to inactivate the chlamydospores in natural soils.

When similar treatments were combined with solarization in summer, both survival and infectivity of chlamydospores of *P. nicotianae* and oospores of *P. capsici* were significantly reduced, but the high soil temperature registered was the main factor to inactivate the inoculum (Guerrero et al., 2010; Rodríguez-Molina et al., 2016). In a strawberry field trial, the combination of solarization with 2 tons ha^{-1} of *B. carinata* pellets significantly increased the percentage of plant survival with respect to the untreated control (Domínguez et al., 2014).

The present work aimed to identify changes in chemical and biological soil properties with the incorporation of the pellets into natural soils. The incorporation of organic matter can enhance microbial biomass and enzyme activities due to increased organic C content in the soil (Haynes, 1999). In our field experiment, dehydrogenase levels in the soil before treatments were like those reported in other studies (Leirós et al., 2000; Trasar-Cepeda et al., 2000; Quilchano and Marañón, 2002), and the incorporation of pellets at 3 tons ha^{-1} did not lead to a significant increase in enzyme activity of the soil. However, dehydrogenase activity increased slightly in biofumigated soil. Similar results have been reported in soils treated with the same pellets rate at the end of the summer season (Núñez-Zofío et al., 2011). The incorporation of pellets into the soil did not produce remarkable changes in the soil properties. Soil pH was minimally affected, suggesting that the buffer capacity of the soil maintained stable pH values. Higher concentrations of NH_4^+ were measured in biofumigated plots, but the results did not differ significantly.

Although some authors have reported the allelopathic effect of *Brassicas* on weeds (Boydston and Hang, 1995; Eberlein et al., 1998; Krishnan et al., 1998; Mattner et al., 2008), this effect was not observed in our field experiment. The biofumigation with 3 tons ha^{-1} of pellets had not either significant effect on total *Fusarium* spp. density in soil. However, repeated applications of biosolarization using manure amendments have shown similar or even more significant reductions on *Fusarium* population than the effect of methyl bromide (Martínez et al., 2011).

We intended to understand the lack of success of the commercial pellets in field conditions through the standardized laboratory experiments that were set up to emulate the biofumigation process in field with different pellets rates. The toxicity of the *B. carinata* pellets added to the soil against chlamydospores of *P. nicotianae* was verified in this study, although it depended on the rate and on the presence of soil microbiota. The commercial rate (BF3; 3 tons ha^{-1}) reduced populations of *P. nicotianae* below the limits of detection of our assay (<2 CFU g^{-1} of soil) and controlled disease on pepper, but only when the pellets were added to autoclaved soil. The effects observed in the present study against *P. nicotianae* agreed with previous results when vermiculite-inoculum was used under controlled conditions (Serrano-Pérez et al., 2015). However, the control efficacy was drastically reduced when the same rate of pellets was incorporated into non-autoclaved soil. Some authors reported that the biological activity of pure 2-propenil isothiocyanate (=AITC), as well as *Brassica* tissues, was reduced when it was incorporated into soils (Matthiessen and Shackleton, 2005). A laboratory study showed an efficacy of only 27% on *Verticillium dahliae* microsclerotia using 1.6 g kg^{-1} of *B. carinata* pellets (equivalent to BF3 dose) in naturally infested soil (Wei et al., 2016). On the contrary, a study demonstrated that incorporating this commercial product into pasteurized compost at the same rate as the BF3 treatment significantly reduced the carpogenic germination of *Sclerotinia sclerotiorum* sclerotia

comparing with the untreated control (Warmington and Clarkson, 2016).

Some authors associate the failure of biofumigation with *B. carinata* pellets (3 tons ha^{-1}) to the high organic carbon content of the soil (5 to 10% of humus) (Neubauer et al., 2014). However, this factor does not seem to be the leading cause of the inefficacy of the lower pellets rates in our experiments, given the low organic carbon content in the soil.

In this study, it was proved that the biofumigation with commercial *B. carinata* pellets at 3 ton ha^{-1} was ineffective in reducing infectivity and survival of chlamydospores when applied with the spring soil temperatures of western Spain. The results suggest that microbial degradation of the ITCs is the main factor involved in the unsatisfactory efficacy of the *B. carinata* pellets in our experiments. Numerous authors have reported the degradation of ITCs by soil microorganisms (Borek et al., 1995; Rumberger and Marschner, 2003; Warton et al., 2003; Price et al., 2005; Gimsing and Kirkegaard, 2009; Hanschen et al., 2015). Three times more AITC was found in autoclaved soil compared with non-autoclaved soil (Price et al., 2005), and phenylethyl-isothiocyanate added to the soil was degraded within 96 h by soil microorganism (Rumberger and Marschner, 2003). Pellets are rich in organic carbon and nitrogen and are chemically and physically readily available for soil microbial degradation (Hu et al., 2011).

The volatile compounds released by the lower doses (BF3 and BF6) in non-autoclaved soil did not inhibit the germination of the chlamydospores in the bags hung in the containers' headspace. For soil disinfestation via biofumigation, high isothiocyanate levels are needed (Hanschen and Winkelmann, 2020). Isothiocyanate levels in soils after biofumigation can range widely from 1 to 100 nmol isothiocyanate g^{-1} soil (Gimsing and Kirkegaard, 2009). Mazzola and Brown (2010) and Wang and Mazzola (2019) reported that the Brassicaceae seed meals optimized for biofumigation have a glucosinolate range from 170 μmol up to 303 μmol g^{-1} seed meal. According to Rodríguez-Molina et al. (2021), *B. carinata* pellets (Biofence ®) used in the present study has a concentration of 84.31 μmol g^{-1}, which is below this range.

There was a decrease in the survival of the chlamydospores treated with the BF6 rate suggesting that other factors than GSL hydrolysis may be involved in suppressing *P. nicotianae* (Mazzola et al., 2001; Weerakoon et al., 2012; Hanschen et al., 2015). Besides ITCs, numerous other sulfur-containing products have been identified as secondary products of glucosinolates in *Brassica* tissues (Gamliel and Stapleton, 1993; Wang et al., 2009), which may have influenced the effectiveness of the rate BF6 of pellets in the laboratory experiments. The *B. carinata* pellets added to non-autoclaved soil could provide food for the resident communities involved in natural disease suppressiveness by regulating soil bacterial community structure (Mazzola et al., 2001). Furthermore, according to Rosskopf et al. (2015), the effects due to volatile fatty acids generated by anaerobiosis should not be excluded when adding a high dose of labile carbon.

The review of previous works using *B. carinata* pellets, the promising results of the double commercial rate to decrease survival of *P. nicotianae,* and the drastic reduction of propagules of *P. nicotianae* when the maximum rate of 20 tons ha^{-1} was added in our experiments suggest that an increase in the amount of amendment incorporated into natural soils would be necessary to suppress soil-borne pathogens. Gilardi et al. (2014, 2015) did not obtain reasonable control of *P. nicotianae* on tomato or *P. capsici* on zucchini by adding 2.5 g L^{-1} of *B. carinata* pellets. Previously, some authors had shown that these pellets at 2 g L^{-1} or 4 g L^{-1} doses were ineffective in controlling Fusarium wilt of lettuce and basil (Garibaldi et al., 2010). Moreover, unsatisfactory results on mortality of *Verticillium dahliae* microsclerotia using 4 tons ha^{-1} of *B. juncea* seed meal have been found (Neubauer et al., 2015).

The laboratory experiments revealed the drastic reduction of propagules of *P. nicotianae* when the maximum rate (20 tons ha^{-1}) was added. The values are barely distinguishable from Gilardi et al. (2020), who used 15 tons ha^{-1} to control *Rhizoctonia solani* on lettuce. This rate was included in our experiments according to the suggestion of Butler et al. (2014) for ASD when soil temperatures were low (15–25°C) (Serrano-Pérez et al., 2017b). However, the tested application rate was more than 6 times greater than the suggested for the Biofence® product for biofumigation treatment. This rate is so high that it is likely to be neither sustainable nor applicable under field conditions. The economic value of such a high-rate application should be evaluated (Gilardi et al., 2020), as well as the associated environmental risks.

GI is a complete indicator to describe the potential phytotoxicity (Zucconi et al., 1981; Emino and Warman, 2004; Tiquia, 2010). A GI lower than 0.5 indicates strong phytotoxicity (Zucconi et al., 1981). According to this criterion, the pellets showed high phytotoxicity when were applied at high concentrations. No phytotoxicity symptoms have been observed in the pepper plants used in the bioassays. Therefore, the phytotoxicity of Biofence® at the tested doses is considered unlikely. Given that this finding is based on a limited number of plants, the results from such observation should therefore be treated with considerable caution. In Wang and Mazzola (2019) research, *Brassica juncea* and *S. alba* seed meal showed phytotoxicity and tree mortality when it was applied at 6.6 tons ha^{-1}.

The soil microbiota degrades the rate of *B. carinata* pellets recommended by the manufacturer (3 tons ha^{-1}) before being effective against *P. nicotianae* in the field and laboratory conditions studied. Increasing the recommended rate to 6 tons ha^{-1} decreases inoculum survival in the laboratory test and has no phytotoxic effects. Although pellets at 20 tons ha^{-1} completely inhibited the inoculum, the cost of such a high rate of the product could be the limiting factor for its application. Phytotoxic effects of Biofence® at the tested doses are considered unlikely.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available on request from the authors.

AUTHOR CONTRIBUTIONS

The study conception and design were performed by RM-MC and PS-P. The first draft of the manuscript was written by PS-P. All authors contributed to material preparation, data collection, analysis, commented on previous versions of the manuscript, read, and approved the final manuscript.

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Low Temperature Biodisinfection Effectiveness for *Phytophthora capsici* Control of Protected Sweet Pepper Crops in the Southeast of Spain

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Biodisinfection using fresh sheep manure in August is effective in controlling *Phytophthora* root rot in greenhouses in southeast Spain, but this is not the case after the beginning of September. The effectiveness of biodisinfection of new amendments has been evaluated beginning in October in sweet pepper greenhouses to control *Phytophthora capsici*. The amendments used were: T1: wheat husk + fresh sheep manure (FSM), 3.5 kg m^{-2}; T2: sunflower pellets 3.5 kg m^{-2}; T3: FSM: 3.5 kg m^{-2}; T4: Control. Temperatures above 40°C were obtained in some amendments; anoxic conditions were found in all amendments, and also a reduction of the viability of *P. capsici* oospores with respect to the control, as well as a higher yield. The contribution of fresh sheep manure to the amendments did not lead to an improvement in their effectiveness. Autumn biodisinfection under low temperature conditions using fresh organic amendments that enhance soil anaerobic conditions may be a promising strategy for the control of *P. capsici* in pepper greenhouses in southeastern Spain.

Keywords: soil disinfestation, *Capsicum annuum*, organic amendment, *Phytophthora*, oospores, greenhouse

INTRODUCTION

Greenhouses of pepper (*Capsicum annuum* L.) in southeast Spain suffer from serious economic losses caused by the oomycetes *Phytophthora capsici* and *P. nicotianae*, which contaminate most of the region's soils (Lacasa et al., 2013). Both of these oomycetes limit the crop, leading to important losses in plants and harvests if preventive measures are not adopted before planting (Lacasa et al., 2010; Guerrero et al., 2013). Nowadays, *P. nicotianae* is the most prevalent (Lacasa et al., 2013; Blaya et al., 2014), favored by the climatic conditions in the greenhouses and farming practices. Chlamydospores and/or oospores may survive for up to 6 years (Erwin and Ribeiro, 1996). The main soil-borne pathogens of protected peppers are *Phytophthora* spp. and *Meloidogyne incognita* in addition to soil fatigue issues which are specific of this crop. The previous soil phytopathological problems are both caused by the repeated growing of pepper crops in the same soil for over 30 years (Guerrero et al., 2014).

Between 1982 and 2005, soil disinfestation with methyl bromide was the way to palliate the effects of *Phytophthora* root rot in more than 95% of the greenhouses (Lacasa et al., 2010). The mix

of chloropicrin and 1,3-dichloropropene has replaced methyl bromide in over 70% of the surface area. Biodisinfection is used in 20% of the surface area, combining solarization with biofumigation (Lacasa et al., 2010; Guerrero et al., 2013).

The combination of solarization with biofumigation shows that the two methods have synergic effects (Katan, 2005). Sealing the soil with plastic produces the gas retention that occurs during organic matter decomposition (Bonanomi et al., 2007; Oka, 2010). If saturation levels are reached when the soil is wetted, then the effects of anaerobiosis are also added and phytoparasites and pathogens are affected (Blok et al., 2000).

If water saturation levels are not reached, but organic amendments containing a high carbon content are used anaerobiosis occurs under the plastic during biodecomposition (anaerobic soil disinfestation, ASD) which affects the pathogens (Shennan et al., 2014; Rosskopf et al., 2015). During the degradation process of the organic matter, changes occur in the composition of the microbiota that confer a suppressive character against some pathogens (Mazzola, 2011; Núñez-Zofío et al., 2011; Mazzola and Manici, 2012; Mazzola et al., 2012). The suppressive effects and the anaerobiosis depend on the soil characteristics, the organic amendment used, the soil temperature, the characteristics of the microbiota and the disinfectant effectiveness or the pathogens susceptibility (Butler et al., 2012, 2014). The choice of the organic matter used in the biodisinfection is one of the determining elements for its efficacy. Bonanomi et al. (2007) suggested that the application of specific organic matters: crop residues and organic wastes, compost and peat could be useful to increase soil suppressiveness. Núñez-Zofio et al. (2013), found an effective biofumigant in beetroot stillage. Guerrero et al. (2013), checked the effect of *Brassica carinata* pellets on *Meloidogyne incognita* in pepper greenhouses in southeast Spain.

European Union restrictions on the use of chemical products, as well as the increase in the surface area dedicated to ecological agriculture, require the use of soil organic amendments for soilborne disease management. Biodisinfection using fresh sheep manure in August gives good results in controlling *Phytophthora* in greenhouses in southeast Spain, although this is not the case when the process is performed after the start of September (Guerrero et al., 2010). The majority of the greenhouses soils in the region are clay loam (Haplocalcids), basic pH, with an organic matter content between 1.5 and 2.5%. The climate is Mediterranean with marine influence, hot dry summers, and mild winters. The greenhouse pepper crop is planted in December-January, and finishes in August-September.

In order to fit the start of the biodisinfection to the end of the cropping season it is necessary to commence in October. Using the amendments that are typically used in August (fresh sheep manure), the biodisinfection does not reduce the levels of pathogens, and are thus unviable (Guerrero et al., 2010, 2013; Lacasa et al., 2010), so it is necessary to improve the biodisinfection effects by adding amendments that strengthen the anaerobic effect or increase soil temperature, both effects by the organic matter decomposition.

Moreover, the southeast of Spain is in a vulnerable zone due to nitrates (Directive 91/676/CEE, of 12 December, relating to the protection of waters against contamination produced by agricultural origin nitrates) which means that there are restrictions on the contribution of nitrogen. This premise severely limits the use of organic matter in the zone.

The objective of this study was to obtain biodisinfection amendments that are effective in controlling *Phythophthora* in pepper greenhouses with low temperatures whilst also reducing the amount of fresh organic amendments.

MATERIALS AND METHODS

Experimental Design and Treatments

The field experiment was carried out over two crop seasons in an experimental sweet pepper greenhouse of 1,000 m^2, at the experimental station of the IMIDA, located in the Campo de Cartagena, Region of Murcia (southeast Spain), where pepper has been grown periodically for over 20 years.

The clay loam soil was free from pathogens and was not previously disinfested.

The biodisinfection was performed in October for both seasons, starting on 2 October 2019 and 9 October 2020, respectively. Treatments were arranged in a randomized complete block design with four replicates and were repeated in the same plots in each of the two seasons. Each experimental unit consisted of a 60 m^2 plot. The treatments evaluated were: T1: wheat husk (Alimer SCoop)+fresh sheep manure (WH+FSM) 3.5 (2+1.5) kg m^{-2}; T2: sunflower pellets (Alimer SCoop) (S) 3.5 kg m^{-2}; T3: (FSM): 3.5 kg m^{-2}; T4: Control (non-amended and non-covered with plastic). Amendments and the non-amended control soil treatment were analyzed in the 2 years (**Table 1**).

The amendments were applied and plots were rototilled to a depth of 25–30 cm. The soil was irrigated using a drip irrigation system using 3 L h^{-1} emitters spaced 0.40 × 0.60 m for 4 h on 2 consecutive days. Amended soil was covered with a 0.05 mm-thick transparent polyethylene film. The plastic was maintained for 6 weeks. The "Beniel" (Syngenta Seeds) pepper cultivar was planted in December at the habitual density of the zone: 1 m separation between rows and 0.4 m between plants in the same row (2.5 plants m^{-2}).

Inoculum Production

The inoculum was obtained from a *P. capsici* isolate from pepper plants that presented symptoms and was conserved in the IMIDA mycology collection in PDA medium. When this isolate was inoculated into healthy pepper plants, it showed to be aggressive and maintained its pathogenicity. The isolate grew in controlled conditions at 25°C for 3 weeks until it reached the edge of a 9 mm diameter Petri dish. A micellar solution with a concentration of 1×10^3 CFU mL^{-1} was obtained by grinding the content of one dish in 100 mL of distilled sterile water and subsequent adjustment by dilution and direct count using a Neubauer counting chamber. A volume of 100 mL of soil, previously autoclaved at 120°C for 1 h on 2 consecutive days, was inoculated with 1 mL of micellar solution, and was placed inside muslin forming a bag. In three different points of each treatment

TABLE 1 | Composition of organic amendments and non-amended control soil treatment.

	Wheat husk	Sunflower pellets	Fresh sheep manures	Control
Total OM %	91.3 ± 22.1	93.8 ± 23.0	52.23 ± 3.92	2.82 ± 0.65
Total N g kg^{-1}	22.8 ± 1.5	48.5 ± 2.3	16.5 ± 0.10	0.7 ± 0.39
C/N	20.4 ± 14.9	11.5 ± 14.1	17.9 ± 1.38	9.54 ± 0.81
P_2O_5 g kg^{-1}	25.2 ± 01.1	14.4 ± 3.2	0.95 ± 0.03	0,11 ± 0.04
K_2O g kg^{-1}	20.1 ± 01.5	17.4 ± 1.2	3.98 ± 0.11	2.18 ± 0.13
pH	6.54 ± 0.5	6.47 ± 2.2	8.50 ± 0.05	7.71 ± 0.23
Electrical conductivity (25°C) dS m^{-1}	2.59 ± 0.86	2.89 ± 0.41	8.62 ± 0.07	3.99 ± 0.54
Moisture%	8.12 ± 0.15	5.60 ± 0.12	51.53 ± 1.25	42,45 ± 1.07

Values are the mean of two replicates corresponding to the 2 years ± standard errors.

replicate plot, soil inoculated bags were buried at 15 and 30 cm soil depth.

Oospores of *P. capsici* were produced *in vitro* by pairing isolates of different mating types on soft pea agar following Núñez-Zofio (2011). Oospores embedded in nylon mesh were buried at 15 and 30 cm soil depth in three different points of each treatment replicate plot.

Variables Measured

- Ambient greenhouse temperature and soil temperatures in one replicate plot of each treatment at 15 and 30 cm depth were registered with 12-bit S-TMB-M017 temperature Hobo probes (accuracy < 0.2°C) connected to an H21-002 Hobo datalogger. Readings were taken every 30 min throughout the biodisinfection treatment.
- Soil oxygen percentage content in one replicate plot of each treatment at 15 cm depth was registered with SO-200 galvanic cell type oxygen Apogee-Instruments probes (accuracy < 0.02% O_2) buried at a depth of 15 cm connected to an H22-001 Hobo datalogger. Readings were taken every 30 min throughout the biodisinfection treatment.
- Viability of *P. capsici* oospores. First season. After 6 weeks of biodisinfection treatment, *P. capsici* oospore viability was determined using the plasmolysis method (Jiang and Erwin, 1990), adapted by Núñez-Zofio et al. (2013).
- Infectivity of *P. capsici* inoculum soil buried. Second season. After biodisinfection the bag with inoculated soil were removed, and the soil was placed in 150 mL pots into which "Lamuyo" cultivar (Clause Seeds) sensitive pepper plants were transplanted when they had four true leaves. The potted plants (three points for each treatment in each of the three replicate plots) were kept for 12 weeks in a chamber at 25°C and a relative humidity of 60–70% with 14:10 h light: darkness of photoperiod and were kept. Notes were taken on the presence of yellowing, wilting or death once per week and those that presented symptoms were analyzed in PDA medium. As plants died, damaged tissue was washed with tap water and fragments of roots and crown were placed on potato dextrose agar and on PARPH (Jeffers and Martin, 1986) medium and incubated at 25°C for 4–6 days. After that, the fungus was identified using microscopic observation. At the end of the bioassay, the plant roots were examined to detect symptoms of the disease. The results of infectivity were expressed as a percentage of diseased plants (Coelho et al., 2000; Lacasa et al., 2015).
- Marketable yield. Each 15 days, from April through to August in each season, the production of the plants was harvested and weighed separately for each treatment replicate plot. This variable was expressed in kg m^{-2}. Each replicate plot consisted of two rows and 45 plants were harvested from each row.

Statistical Analysis

The effects of treatments were studied using two way analysis of variance (ANOVA) with the Software Statgraphics Centurion 16. In order to fulfill the assumptions of analysis of variance (homocedasticity and normality), the infectivity bioassay data were transformed using arcsine ($\sqrt{x/n}$), where x = total number of dead plants and n = total number of plants. Oospores viability data were transformed using arcsine ($\sqrt{x/n}$), where x=total number of oospores survival and n = total number of oospores. Data on crop yield were transformed using log transformation log (x+1), where x = total yield. Means for significant main effects (biodisinfection treatment, soil depth) and all pairwise differences among least squares means within the biodisinfection treatment × soil depth interaction were separated by Fisher's LSD test ($p < 0.05$).

RESULTS

Soil Temperatures

Temperatures exceeding 38°C were reached in the amendments consisting of wheat husk+fresh sheep manure, in the sunflower pellets, and fresh sheep manure, at 15 cm in the first season. In the second season, although the largest number of hours was accumulated in the order of 33°C, the soil was subjected to temperatures of up to 37°C for certain periods of time at both soil depths, 15 and 30 cm. In the second season, the lowest number of accumulated hours above 33°C was found in the non-treated control. There were no temperatures exceeding 42°C in any of the treatments (**Table 2**). The mean ambient greenhouse temperature ranged from 13.6 to 31.8°C in the first season, and from 15.9 to 44.3°C in the second.

There was a period of anoxia during the biodisinfection process in both seasons in all the biodisinfection treatments (**Figure 1**). Variations were found among the treatments and

TABLE 2 | Number of cumulative hours in each crop cycle at 15 and 30 cm soil depth within different temperature ranges.

		First season		Second season	
Treatment	**Depth (cm)**	**34–37°C**	**38–41°C**	**34–37°C**	**38–41°C**
Wheat husk + Fresh sheep manures	15	48	51	120	0
	30	0	0	39	0
Sunflower pellets	15	66	57	40	0
	30	0	0	18	0
Fresh sheep manures	15	60	52	223	0
	30	0	0	147	0
Control	15	0	0	30	0
	30	0	0	0	0

First and second season.

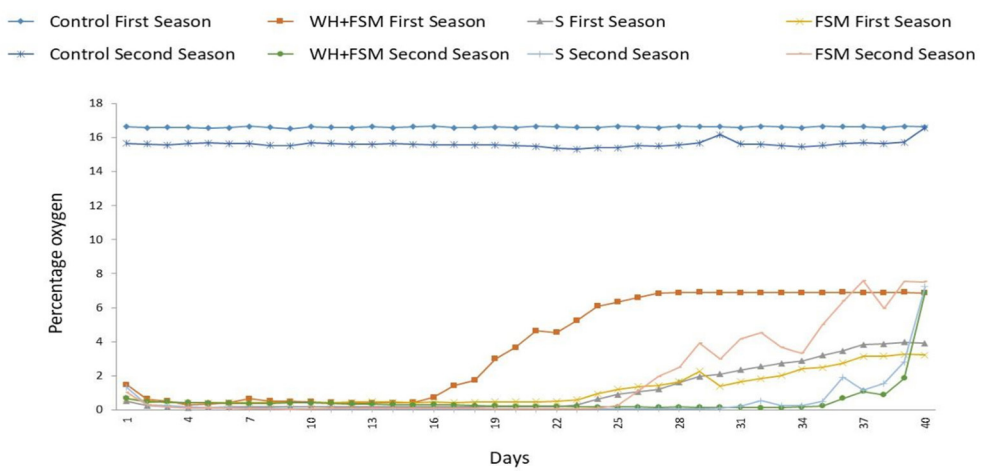

FIGURE 1 | Evolution of the percentage of oxygen in each treatment during the biodisinfestation process started in October at 15 cm depth. Abscisa axis: days from the start of the biodisinfestation. First and second seasons.

between years for the same treatment. In WH+FSM, at least 35.5% of the disinfestation time the O_2 level was below 2% in the first year, whilst in the second year this was the case 84.4% of the time. In FSM at least 64.4% of the time the oxygen levels were below 2%, whilst in the second year this occurred 57.7% of the time. In the first year the time that the oxygen level was below 2% in S and FSM was similar (at least 64.4%) whilst in WH+FSM the figure was, of at least 35.5%. In the second year, the oxygen level remained below 2% for 84.4% of the biodisinfection time in both S and WH+FSM, whilst in FSM the proportion of biodisinfection time with oxygen levels below 2% was at least 57.7%.

Effect of Treatments on *P. capsici* Oospore Viability

First Season

Table 3 shows the viability of the oospores in each treatment and at each depth. The depth had an influence on oospore viability. At 15 cm the oospore viability was reduced in all the treatments, except for the control, while at 30 cm the reduction in viability was greater in the WH+FSM and S treatments than in the FSM. Biodisinfection reduced the viability of the oospores buried in the amendments with FSM as well as in those that did not contain it [$F_{(3,11)=}$ 5.69; $p = 0.0028$ at 15 cm; $F_{(3,11)} = 2.92$; $p = 0.004$ at 30 cm].

Effect of Treatments on Infectivity of Introduced Soil Inoculum of *P. capsici*

Second Season

The results obtained in the bioassay were different at 15 cm and 30 cm soil depth (**Table 4**). There were differences between the percentage of dead pepper plants in each treatment and the control at 15 cm (**Table 4**). The percentage of dead plants in the bioassay at 30 cm differed between the FSM and the control, although this was not the case for the other two amendments [$F_{(3,11)} = 1.07$; $p = 0.040$ at 15 cm; $F_{(3,11)} = 3.30$; $p = 0.047$ at 30 cm].

Effect of Treatments on Marketable Yield

Yields were significantly higher in the biodisinfested plots than in the control. The effect of biodisinfection treatments on marketable yield was significant in the first season [$F_{(3,15)} = 4.16$; $p = 0.009$], and in the second season [$F_{(3,15)} = 0.65$; $p =$

TABLE 3 | Effect of biodisinfection treatments on *P. capsici* oospore viability percentage at two different soil depths (15 and 30 cm) after 6 weeks of exposition.

Biodisinfection treatment	15 cm	30 cm
Wheat husk + Fresh sheep manure	2.89 ± 3.40bA	3.34 ± 2.23bA
Sunflower pellets	4.46 ± 4.93bA	4.66 ± 4.5bA
Fresh sheep manure	3.29 ± 2.91bB	8.88 ± 3.98aA
Control	22.8 ± 6.71aA	5.11 ± 2.85abB

x = mean percentage of viable oospores. Mean values (n = 12) ± standard deviation. Data were transformed using arcsine (√x+0.5) where x= mean percentage of viable oospores in each biodisinfection treatment at each soil depth.
The significant differences among biodisinfection treatments and each soil depth at 6 weeks were tested by two-way ANOVA.
Values in the same column followed by a different lower-case letter indicate significant differences between biodisinfection treatments within a given soil depth and upper-case letters compare soil depths for a given biodisinfection treatment based on the Fisher's LSD test ($p < 0.05$) within the interaction of biodisinfection and soil depth treatments. When differences were non-significant, letters were omitted.

TABLE 4 | Infectivity of introduced soil inoculum of *Phytophthora capsici* at 15 and 30 cm depth in bioassays with sweet pepper variety "Lamuyo" plants expressed as percentage of dead plants during bioassays in each treatment.

Treatment	15 cm	30 cm
Wheat husk + Fresh sheep manures	77.7± 12 a	77.7± 12 ab
Sunflower pellets	77.7± 19 a	88.9±19 ab
Fresh sheep manures	77.7± 18 a	100.0± 0 a
Control	44.4± 19 b	66.6± 3 b

Mean values (n = 12) ± standard deviation. Data were transformed using arcsine (√x+0.5) where x = mean percentage of lived plants at each soil depth.
Values followed by the same letter are not significantly different according to Fisher's LSD ($p < 0.05$).

TABLE 5 | Marketable yield (kg m^{-2}) in each crop cycle.

Treatment	1st season	2nd season
Wheat husk + Fresh sheep manures	9.43 ± 1.06 a	6.86 ± 1.30 a
Sunflower pellets	9.72 ± 1.79 a	6.96 ± 0.80 a
Fresh sheep manures	9.21 ± 1.17 a	6.30 ± 1.20 a
Control	8.23 ± 0.44 b	5.00 ± 0.39 b

Mean values (n = 4) ± standard deviation. Data were transformed using log (x+1), where x = total yield. Values followed by the same letter are not significantly different according to Fisher's LSD ($p < 0.05$).

0.6028] (**Table 5**). The non-contribution of FSM had no negative influence on yield in the amendment composed of sunflower pellets. This fact increases the interest of the amendment, due to the situation of greenhouses in the zone subjected to restrictions in the N application with origin on livestock manures due to Nitrates Directive implementation.

DISCUSSION

In our trial, the temperatures obtained during the biodisinfection carried out in October with the amendments of wheat husk+fresh sheep manure, sunflower pellets, and fresh sheep manure exceeded 38°C in the first season, varying between 51 and 57 h, but no in the second. At both seasons soil temperatures accumulated in October during the biodisinfection in the greenhouse were considerably lower than those obtained for the month of August in the same zone (Guerrero et al., 2019). Etxeberria et al. (2011) found that for 1,680 h at 15–35°C, the survival of the oospores ranged between 88 and 36%. The oospore viability in the two seasons was reduced by the presence of minimum temperatures above those reported by Etxeberria et al. (2011). The results also concur with those obtained by Lacasa et al. (2015) in which biodisinfection in spring with *Sinapis alba* fresh green manure did reduce the infectivity of *Phytophthora nicotianae* chlamydospores introduced inoculum (Gandariasbeitia et al., 2019). The infectivity of the surviving inoculum corresponded to an insufficient reduction of the inoculum buried in the bags. Coelho et al. (2000) considered that inoculum levels in excess of 1 propagule per gram of soil are necessary in order to detect the surviving inoculum using plant indicators in bioassays.

The biodisinfection with the assayed amendments induce anoxia in the soil for several weeks, which is one of the factors indicated for Gamliel et al. (2000) as cause of the decrease in the pathogen populations and of the efficacy of the disinfestation. In the first season, the level of oxygen in the soil was below 2% for more than 64% of the disinfestation time for S and FSM treatments, and over 35% of the time in WH + FSM. In the second season, the corresponding time was more than 57% in FSM and over 84% in S and for WH + FMS. The production of sporangia and the mycelial growth of several species of *Phytophthora* (including *P. capsici*) was considerably lower when the oxygen level was at 1% of oxygen in the crop solution. Below 2.5% of oxygen, the formation of sporangia is reduced and also the mycelial development of *P. cinnamomi*, affecting the viability of the inoculum (Davison and Tay, 1987).

The efficacy of the biodisinfection when temperatures are low improves with the contribution of amendments that are rich in organic matter or by increasing the amount of carbon that is applied to the soil. The contribution of 4 mg C g^{-1} of soil has been recommended to improve the efficacy of ASD at low temperatures (Butler et al., 2014; Rosskopf et al., 2015). It is recommended that the carbon of the organic amendment should be labile, so that it can easily and rapidly be degraded by the soil microorganisms and create conditions for persistent anaerobiosis. Additionally, an adequate ratio of C/N must be considered so that, on the one hand, the N that is applied is enough so that N is not immobilized by the soil microorganisms in the degradation/mineralization of the organic amendments and, at the same time, there is not a potentially leachable excess of N. In our trials, the amendments presented a balanced composition, and ratios of C/N within the limits that are considered as adequate for biodisinfection and the generation of hypoxia.

The efficacy or failure of soil biodisinfection may be due to the soil characteristics, the degree of moistening, the electrical conductivity, the characteristics of the plastic, and the period of solarization (Chellemi, 2002). The temperate-humid climate has obstructed the application of biosolarization, very extended in

Mediterranean horticultural areas such as South-eastern Spain. However, previous experimentation with different amendments, doses and application times compatible with crop cycles showed the applicability of these practices in the humid temperate climate region of Northern Spain (Ojinaga et al., 2020).

In the north of Spain, the survival of oospores of *P. capsici* was reduced at 15 and 30 cm when the biodisinfection was applied in August with both fresh and semi-composted manure (Núñez-Zofío et al., 2012). Using rice bran, rapeseed cake, or grape pomace as the carbon source, Serrano-Pérez et al. (2017) obtained significant reductions in chlamydospores viability and inoculum infectivity of pepper plants by *P. nicotianae*, when biodisinfection was carried out in low temperature open air conditions of the spring season in south western Spain.

Despite the low disinfectant efficacy assessed by the reduction in the survival of *P. capsici* oospores in sub-optimal conditions for solarization (absence of thermal inactivation), improvements were observed in the suppressiveness and in the chemical, physical and biological soil properties when the organic amendments were applied in biodisinfection, which caused a reduction of crop diseases and a yield increase (Núñez-Zofío et al., 2012; Rosskopf et al., 2015; Gandariasbeitia et al., 2019). It was concluded that repeated biodisinfection for the control of *Phytophthora* root and crown rot in protected pepper crops located in temperate climate regions can improve soil quality and suppressiveness, as well as enabling reduced doses of the organic amendment needed for biodisinfection. However, this disinfestant efficacy worsened when the biodisinfection was performed in March (Arriaga et al., 2011), and also when it was carried out in September (Núñez-Zofío et al., 2011).

One strategy employed when biodisinfection fails due to the climate or crop cycles is to turn to anaerobic disinfection. This strategy has been applied in limiting solarization conditions in Japan (Shinmura, 2000; Momma et al., 2005, 2006, 2010, 2011; Katase et al., 2009) and also in the Netherlands (Blok et al., 2000; Agtmaal et al., 2015).

The effect of the application of organic matter produced a yield increase in both seasons. Although there was no naturally occurring presence of pathogens, the increased yield in the biodisinfested plots might be explained by the reduction of soil fatigue in the crop. The fatigue accumulated in greenhouses used for pepper monocropping is highly specific toward pepper (Guerrero et al., 2014). The greenhouse assayed in this study fits the conditions of pepper monocropping. Biodisinfection with fresh sheep manure of greenhouses used to grow pepper crops, improved the physical and chemical characteristics of the soil (Fernández et al., 2005). The application of wood waste and sunflower husk biochar improved soil porosity and the structural stability of soil aggregates (Sokołowska et al., 2020). Improvement of soil water infiltration, soil drainage, and roots aeration have been associated with a better control of crop root rot caused by soilborne pathogenic species of *Phytophthora* (Ristaino, 1991).

The principal cause of this fatigue is considered to be biotic and related with the extent to which the soils are contaminated with *Fusarium* sp. (Martínez et al., 2009, 2011). It is additionally known that the fatigue accumulated in greenhouses used for pepper monocropping is highly specific toward pepper (Guerrero et al., 2014). In soils contaminated by *Fusarium* spp. and *Phytophthora* spp., Song et al. (2020a,b) obtained a significant reduction in the incidence of *Fusarium* and *Phytophthora* in strawberry crops in conditions of biodisinfection with chicken manure or with maltose as carbon sources at 28°C and covering the soil with total impermeable film. They also observed a yield improvement related with the increase of soil available nutrients after the biodisinfection treatment.

In those plots where FSM was not used in this study, the yields were similar to those in which it had been used. This fact is of great interest since the cropping zone of Southeast Spain is subject to organic matter restrictions. Moreover, it is known that the incorporation of organic correctives improves soil properties and fertility, as reported by Bonanomi et al. (2007).

The results obtained with the carbon-rich and balanced amendments and in time periods that are compatible with the pepper crop cycle in greenhouses in southeast Spain, showed a yield improvement. This increased yield may also be due to the effect highlighted by Núñez-Zofío et al. (2011) that repeated biodisinfection treatments in temperate climate areas improve soil suppressiveness and quality. This would enable growers to extend the crop cycle and thereby the corresponding economic benefit.

In conclusion, using the specific organic amendments, significant reductions were obtained in the viability of the inoculum resting structures of *P. capsici* as well as increases in the marketable yield, when biodisinfection is performed on dates that are compatible with the pepper growing cycle in the Campo de Cartagena. This way of soil disinfestation is recommendable in the context of the strategies of sustainable integrated production.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MG designed the experiment and wrote the original draft. CL, VM, MM, and AM supervised the experiment and contributed to the data. SL revised the manuscript. All authors approved the submitted version.

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Gases Released During Soil Biodisinfestation of Pepper Greenhouses Reduce Survival of *Phytophthora capsici* Oospores in Northern Spain

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Phytophthora capsici is one of the oomycetes that affects protected pepper crops in different agroclimatic areas of Spain. Currently, environmentally friendly strategies such as biodisinfestation for plant disease control have become increasingly popular. In this study, the effect of released gases during biodisinfestation with a fresh manures mixture amendment on *P. capsici* oospore viability was determined. A biodisinfestation trial was performed in a greenhouse located in northern Spain (Biscay), with a mixture of fresh sheep (2 kg m^{-2}) and dry poultry manures (0.5 kg m^{-2}) followed by soil sealing with a transparent polyethylene plastic film for 21 days (onset June 15th). Gases were sampled from the aerial cavity of biodisinfested plots at different days after soil sealing (0–1–2–3–4–7–9–11, and 14 days). Vacutainer tubes were incubated at 20°C with oospores of *P. capsici* that were previously placed under vacuum and refilled with extracted gases. Treatments assayed were gases from different sampling times (0–1–2–3–4–7–9–11–14 days, and succession of days 1–2–3–4–7–9–11–14) combined with different exposure times (7–14–21 days) at 20°C in the laboratory. Control treatments were included: air-tubes and vacuum-tubes. An additional reference treatment under real field conditions was also considered: buried oospores at 15 cm depth in the biodisinfested plots. Oospore viability was determined with the plasmolysis method. The most effective treatment was the succession of gases collected during all sampling days. The significant but slight reduction in oospore viability by exposure to the different gas treatments was consistent with the low dose of applied amendment and the low soil temperature registered at 15 cm depth during soil biodisinfestation (>25°C−100% time, >35°C−23%, >40°C−3%). The above circumstances might have generated a small quantity of gases with low impact on oospore viability. The biodisinfested soil at 15 cm depth reference treatment showed the lowest oospore viability in all the exposure times assayed. The overlap of thermal and higher biofumigation effects in this treatment could likely be responsible for its greater efficacy. A disinfectant effect purely attributable to

released gases throughout biodisinfestation has been demonstrated. We believe that our research will serve as a base for future application in agro-environments with reduced thermal inactivation effects.

Keywords: capsicum annuum, pepper, *Phytophthora capsici*, oospores, biodisinfestation, biosolarization, biofumigation, animal manure

INTRODUCTION

Phytophthora root rot is a soilborne plant disease of pepper plants (*Capsicum annuum* L.) that imposes important economic losses for pepper crops worldwide, including the Mediterranean area (De-Cara-García et al., 2018), and areas with a humid temperate climate, such as the Basque Country (Larregla et al., 2015) in Spain. In the Basque Country (northern Spain), the main causal agents of this disease are the oomycetes *Phytophthora capsici* Leonian and *P. cryptogea* Pethybridge & Lafferty. The existence of the two complementary mating types of *P. capsici* in the area allows sexual reproduction, formation of survival spores (oospores) and its adaptability to the environment due to potential genetic variation resulting from meiosis (Etxeberria et al., 2011a). *P. capsici* is able to survive in soil for extended periods of time due to its oospores that act as the main source of initial inoculum that causes primary infection in the next crop cycle (Erwin and Ribeiro, 1996).

Strategies recommended for management of *Phytophthora* root rot involved integrated approaches that focus on cultural practices. For many years, plant disease control was broadly based in pre-plant chemical fumigation. However, the use of chemical fumigants involves human health risks, environmental regulations, and economic restraints (Rosskopf et al., 2020). Thus, at the present time, there is a growing pursuit for the use of secure, healthy, and environmentally friendly strategies.

Several strategies based on the use of organic amendments for soilborne disease management have been studied to improve its efficacy and to elucidate the soil disease suppression action mechanisms that are directly or indirectly involved in their application (Bonanomi et al., 2010, 2018; Gamliel and Stapleton, 2017; Rosskopf et al., 2020). One of these approaches combines soil solarization with organic amendments for an improved control of soilborne pests, named "biosolarization" (Katan, 2005; Ros et al., 2008) or "biodisinfestation" (de la Fuente et al., 2009). The term soil solarization refers to a soil disinfestation method which uses passive solar heating of moist soil mulched with transparent plastic sheeting for the control of pathogens, mainly through a direct thermal inactivation mechanism (Katan et al., 1976; Stapleton, 2000). Furthermore, the term biofumigation was first coined to define the application of brassicaceous plant material in soil with the aim of controlling soilborne pathogens (Kirkegaard et al., 1993). Since then, it has adopted a broader meaning that includes the use of other organic materials and reflects the mode of action of active volatile compounds that are generated in the soil after incorporation and decomposition of organic amendments (Stapleton, 2000; Stapleton and Bañuelos, 2009). In this study, the term biodisinfestation will be applied for the combined use of an organic amendment and soil plastic tarping without implying that soil heating is the most important mechanism.

Numerous studies have demonstrated the benefits of combining organic amendments with solarization (Gamliel and Stapleton, 1993a,b; Gamliel et al., 2000; Stapleton, 2000). Early on, it was observed that a series of chemical and microbial processes in the soil is activated when the soil is heated by covering it with a transparent plastic film and proper organic material is added as amendments. This includes its vapor and liquid phases as well as processes such as the degradation of organic matter, the release of various volatile and soluble compounds, and changes in the microbial balance of the soil. Most notably, studies suggested these chain reactions produce better control of soilborne pests, including some types that were not well-controlled by either method separately (Gamliel and Stapleton, 2017).

Previous reports have related the release of nitrogenous compounds from animal manures combined with soil solarization to the lethal effect on certain soil microbiota (Oka, 2010; Arriaga et al., 2011; Núñez-Zofío et al., 2011, 2012) and also to the increased crop growth response after treatment (Gamliel and Stapleton, 1993a; Stapleton, 2000). Ammonia (NH_3) has been widely reported to negatively affect the survival or germination of certain soilborne oomycetes (Tsao and Oster, 1981; Riga et al., 2000; Arriaga et al., 2011; Núñez-Zofío et al., 2011, 2012), and soilborne fungi and nematodes (Tenuta and Lazarovits, 2002; Oka, 2010; Gandariasbeitia et al., 2021).

In southeastern Spain, biosolarization with fresh sheep manure showed its efficacy in the reduction of *Phytophthora* spp. (*P. capsici* and *P. nicotianae*) soil inoculum and disease control when it was carried out in August or at the beginning of September (Núñez-Zofío et al., 2013; Lacasa et al., 2015) as well as if it was repeated for two or three consecutive years (Guerrero et al., 2005, 2006) under greenhouse conditions. In the humid subtropical climate of south-eastern United States, increased efficacy of soil solarization by cabbage amendment on the reduction of populations of *P. capsici* and *P. nicotianae* was observed, but these reductions were not as effective as the chemical fumigant methyl bromide in eliminating the pathogens at a depth of 25 cm under open field conditions. Soil temperature under solarization treatments reached a maximum of 47°C at a 10 cm depth, but only 41°C at 25 cm (Chellemi et al., 1997; Coelho et al., 1999). In the temperate humid climate of northern Spain, biodisinfestation under greenhouse conditions with fresh animal manure in spring (March-April) significantly reduced the *P. capsici* inoculum survival rate (30.6%) when compared with the non-treated control (61.1%), and the solarization treatment (94.4%) (Núñez-Zofío et al., 2010; Arriaga et al., 2011). Similarly, biodisinfestation in autumn (September-October) with fresh and

semicomposted animal manures reduced initial oospore viability by 40%. However, oospores continued to be infective when they were tested in a bioassay with pepper plants (Núñez-Zofío et al., 2011). Greenhouse soil temperature at 15 cm depth did not exceed 33°C in the biodisinfestation treatment with fresh manure in spring or autumn. Repeated biodisinfestation with fresh and semicomposted animal manures during three consecutive crop seasons improved soil quality and provided effective control of pepper *Phytophthora* root rot in protected crops located in the temperate humid climate of northern Spain, a region where solarization has been proven non-effective. The repeated manure applications allowed for a reduction in the dose of organic amendment needed for an effective disease control (Núñez-Zofío et al., 2010, 2011, 2012; Arriaga et al., 2011).

One advantage of biodisinfestation is that it can be applied in organic production as well as conventional farming (Guerrero et al., 2013; Gamliel and Stapleton, 2017). Furthermore, the use of these strategies can not only be observed from a crop and soil health point of view, but also from a circular bioeconomy or a life cycle assessment point of view (Oldfield et al., 2017). This is due to the upcycling of the extremely heterogeneous organic materials, such as green and animal manures along with agro-industrial by-products that are used as amendments for soil biodisinfestation.

In situations where there is not solar radiation enough, biosolarization effectiveness is reduced and the biofumigation effect acquires more importance. This is the case of the Basque Country (northern Spain), where the greenhouse pepper crop season (March-September) prevents the practice of biodisinfestation in the months of maximum solar radiation (June–August) (Gandariasbeitia et al., 2019; Ojinaga et al., 2020). To better understand the mechanisms involved in the reduction of *P. capsici* soil inoculum survival previously observed after biodisinfestation with fresh animal manure in spring (Arriaga et al., 2011) or autumn (Núñez-Zofío et al., 2011) in northern Spain, the main objective in the present study was to assess the effect only attributable to gases sampled at different time intervals throughout biodisinfestation on *P. capsici* oospore viability. This study was carried out in early summer (June) with the aim of increasing the effectiveness of biofumigation component in biodisinfestation. The experiment was carried out at an innocuous controlled temperature for the pathogen (20°C) in order to ensure the absence of oospores inactivation by thermal effects. Oospore viability change rates over exposure time to the different gases sampled throughout the biodisinfestation treatment were also integrated in the study.

MATERIALS AND METHODS

Field Trial Characteristics

A field trial was established in an experimental greenhouse located at NEIKER Research Station (Derio, 43°18'20" N-−3°53'0" W; Biscay, northern Spain) in spring-summer 2009. The greenhouse had 1,200 m^2. Soil type texture was clay loam (46.3% clay, 40.0% silt, and 13.7% sand), with a pH of 6.8, an organic matter content of 55 g kg^{-1}, a total nitrogen content of 2.2 g kg^{-1}, a C/N ratio of 14.2, a phosphorous content of 99.0 mg kg^{-1} and an electrical conductivity of 1.42 dS m^{-1}. The region has a temperate humid climate with an annual mean temperature of 12°C (maximum mean temperature in summer 25°C) and rainfall of 1,200 mm $year^{-1}$.

A mixture of fresh sheep manure and dry poultry manure (70%: 30% on a dry weight basis) was added at a dose (fresh weight) of 25,630 kg ha^{-1} (equivalent to 9,920 kg ha^{-1} dry weight) and incorporated in the soil until a depth of 20 cm using a rototiller. The applied amendment mixture had the following characteristics: pH 8.3 and EC 10.1 dS m^{-1}, organic matter: 587 g kg^{-1}, total N: 29.4 g kg^{-1}, total P: 16.1 g kg^{-1}, total K: 30.9 g kg^{-1} and a relation C/N = 11.6. The applied amendment represented a total nitrogen fertilization dose of 292 kg N ha^{-1} which was consistent with the advised rates for green pepper greenhouse fertilization in the region (CBPA, 2011) for an expected pepper fruit yield of 57,600 kg ha^{-1}. The organic nitrogen mineralization rates for the sheep and poultry manures in the first year after the amendment mixture application were estimated at 45 and 75% respectively, which provided a mineral nitrogen dose of 170 kg ha^{-1}. This amount was equivalent to the advised mineral nitrogen fertilization dose based on the green pepper crop yield in the region (3 kg N t^{-1} yield x yield 57.6 t ha^{-1} = 173 kg N ha^{-1}). A drip irrigation system (comprised of 2 L h^{-1} emitters spaced 0.40 m apart in the same row, with 0.50 m between drip rows) was placed over the amended soil. Amended soil was then sealed with a transparent polyethylene plastic film 0.05 mm thick and was moistened the first day for 4.5 h, and for another 4.5 h on the second day, equivalent to a total volume of water of 90 L m^{-2}. Amended and moistened soil remained sealed for approximately 3 weeks (from June 15th to July 6th).

Throughout the experiment, ambient and soil temperature (15 cm depth) were registered using sensors connected to a data-logger (HOBO H21-002 Micro Station, Onset Computer Corporation, USA).

Production of Oospores

Oospores of *P. capsici* were produced *in vitro* by pairing isolates of different mating types on soft pea agar (filtered cooking broth of 200 g L^{-1} of peas in distilled water for 30 min was supplemented with 7.5 g of agar and autoclaved at 121°C for 20 min) in order to assist oospore extraction according to the method proposed by Pittis and Shattock (1994). The agar was supplemented with 0.1 g L^{-1} β-sitosterol to increase oospore formation. Spanish isolates from pepper (00/004, 02/206 and 06-13-03) of the A1 genetic compatibility type were mated with A2 isolates (CBS 554.88 and CBS 370.72) from the Dutch Type Culture Collection (Centraalbureau voor Schimmelcultures, CBS). After 4 weeks of incubation in the dark at 20°C, oospores were extracted from the agar by blending in sterile distilled water (10 mL $plate^{-1}$). The oospore suspension was filtered through a 100 μm nylon mesh and oospores were then placed in 1 x 1 cm^2 25 μm nylon meshes (Sefar Nitex 03 25/19, SEFAR, Switzerland) by vacuum filtration according to the method proposed by Lumsden (1980). At least 500 oospores were placed in each mesh piece, to ensure that a minimum number of 100 oospores were available for counting and observation. The oospores initial viability was determined before treatments and was equal to 41.2

± 1.5% (mean ± standard error). Oospores viability percentage values were relativized to the initial viability value which was equal to 100 ± 3.6%.

Sampling of Buried Oospores From the Greenhouse Biodisinfestation Trial

In the case of the biodisinfested soil control treatment at 15 cm depth (SOIL; see treatment abbreviations in epigraph 2.6), embedded oospores in 25 μm nylon meshes were placed within 5 × 5 cm bags made from permeable 1,550 μm nylon mesh (Sefar Nitex 06-1550/60). A total of three bags were buried at 15 cm soil depth in each greenhouse experimental plot in one location (1 location × 1 depth × 3 exposure times) before the biodisinfestation treatment onset. One bag from each greenhouse biodisinfested plot and location at 15 cm depth was removed after 7, 14, and 21 days from the treatment onset.

Sampling of Gases From the Greenhouse Biodisinfestation Trial

Several gas-tight silicone plugs were inserted in the sealing plastic film of each greenhouse biodisinfested plot and were placed two meter away from the edge of the plastic film in each plot. Gases produced during soil biodisinfestation were sampled at different time intervals in the aerial cavity between the amended soil surface and the plastic film. The samples were taken with a plastic syringe (50 mL volume) that was punctured into the silicone plugs of the plastic film.

Oospores Incubation in Laboratory Controlled Conditions With Gases From the Greenhouse Biodisinfestation Trial

Embedded oospores in 25 μm nylon meshes were placed in 10 mL volume Vacutainer® (BD, Plymouth, England) glass tubes, with each tube containing one nylon mesh. The vacutainer tubes containing oospores were closed with a hermetic silicone septum and maintained at 20°C in a precision stove in the laboratory until they were used for collecting gas samples in the greenhouse biodisinfestation trial. A vacuum was created in each vacutainer tube containing oospores by means of a syringe. Next, gases sampled from the aerial cavity of the greenhouse biodisinfested plots were injected into the tubes. Once the tubes were filled with the gases, they were transported back to the laboratory and incubated in the stove at 20°C. The duration of the journey was <2 h and the tubes with oospores were transported in a portable fridge to maintain temperatures of 20–25°C, a range considered to be innocuous for *P. capsici* oospores viability, as reported by Etxeberria et al. (2011b). Vacutainer tubes containing oospores and gases samples collected in the greenhouse were incubated at 20°C in a precision stove in the dark with an exposure time of 7, 14, and 21 days for each Biodisinfestation gas, as well as the two control treatments C_va and C_ai (see treatment abbreviations in epigraph 2.6).

Treatments and Experimental Design

The samples of biodisinfestation gases were obtained from three points located in the center (two meters from the end of the plastic) of each of three biodisinfested plots located in the central zone of the greenhouse. The plot size was 24 m x 4 m. The samples of gases released in each biodisinfested replicate plot were taken at different time intervals from the onset of biodisinfestation (days after setting sealing plastic film): 0, 1, 2, 3, 4, 7, 9, 11, 14, and succession of sampling days 1–2–3–4–7–9–11–14.

The experiment was factorial with a treatment structure of two crossed factors: (i) Biodisinfestation gas (gases sampled at different time intervals from biodisinfestation onset) with thirteen levels that included three control treatments: day 0 (G_00), day 1 (G_01), day 2 (G_02), day 3 (G_03), day 4 (G_04), day 7 (G_07), day 9 (G_09), day 11 (G_11), day 14 (G_14), every sampling day (1-2-3-4-7-9-11-14) (G_ev), Control 1 of vacuum. (C_va), Control 2 of air (C_ai), and Control 3 of biodisinfested soil at 15 cm depth (SOIL); and (ii) Exposure time with three levels: 7, 14, and 21 days. These exposure times were selected for incubation of oospores and gases because it has been shown that the formation and release of biotoxic volatile compounds are predominantly found during the first 3 weeks from the biodisinfestation onset (Gamliel and Stapleton, 2017). Thus, the total number of combined treatments for the two crossed factors was 39. The vacuum-tube control treatment (C_va) consisted of vacuum-tubes. The air-tube control treatment (C_ai) consisted of an air sample that was taken outside the greenhouse experiment at the onset of biodisinfestatión. The biodisinfested soil control treatment at 15 cm depth (SOIL) consisted of embedded oospores in 25 μm nylon meshes which were buried at 15 cm soil depth in each of the three replicate plots (see epigraph 2.3).

The experimental unit was the nylon mesh inside each vacutainer tube, where at least 100 oospores were counted and observed for each combined factorial treatment (Biodisinfestation gas x Exposure time) and replicate plot. The factorial treatments were arranged in a complete randomized design of three replicate plots.

Determination of Oospores Survival

At the end of incubation period, oospore survival was determined using the plasmolysis method (Jiang and Erwin, 1990), effective on *P. capsici* oospores according to Etxeberria et al. (2011a). For each mesh, 100 oospores were counted and observed microscopically and the number of oospores that plasmolyzed was considered viable.

Statistical Analysis

Discrete percent data of oospore viability (number of oospores that plasmolyzed out of the number observed) in each experimental unit (mesh of oospores) was the response variable, for which a binomial distribution was used. The response variable was analyzed using the generalized linear mixed models (GLMM) procedure (proc GLIMMIX) of SAS 9.4 software with a repeated measures mixed model ANOVA of one factor (Biodisinfestation gas) over time (Exposure time). Biodisinfestation gas and Exposure time were considered fixed factors and each replicate plot was a random factor. Each factorial treatment was considered to be nested in each replicate plot. The Laplace direct likelihood approximation method was used to model goodness of fit. A first order autoregressive covariance structure provided

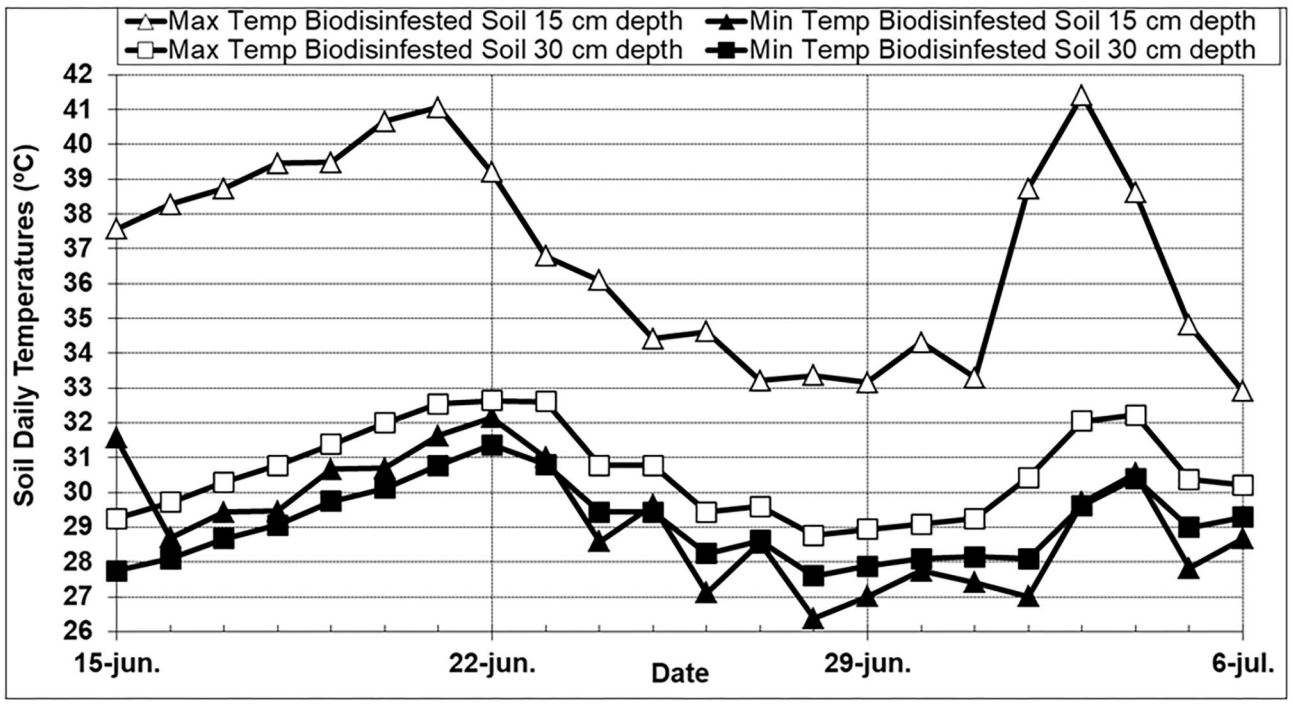

FIGURE 1 | Maximum and minimum daily soil temperatures (15 and 30 cm depth) registered for the 21-day duration (June 15–July 6th) of the biodisinfestation treatment in the greenhouse field trial located in northern Spain (Derio; Biscay). Biodesinfestation amendment was a mixture of fresh sheep and poultry manures (2 + 0.5 kg.m^{-2}). Soil was tarped with a 0.05 mm-thick transparent low density polyethylene plastic film.

the best relation of data over time (lower AICC statistics). The default residual pseudo-likelihood estimation method was used to continue the analysis. All pairwise differences among least squares means within the interaction of Biodisinfestation gas × Exposure time were adjusted for *P*-values with the Tukey-Kramer test to maintain an overall experiment-wise type I error rate of $\alpha = 0.05$. For significant interactions, tests of simple effects (Schabenberger and Pierce, 2002) were performed to detect differences. A linear regression model was adjusted for the proportion of viable oospores over exposure time with separate slopes and intercepts for each level of the Biodisinfestation gas factor treatment and to test differences among them according to Stroup (2018). Linear contrasts of different combinations of the Biodisinfestation gas factor levels were tested for significant differences among slopes and intercepts.

RESULTS

Daily Air and Soil Temperatures in the Greenhouse Biodisinfestation Trial

In the greenhouse biodisinfestation trial, the average daily temperatures for ambient air, biodisinfested soil at 15 cm, and 30 cm depth were 25.3, 32.5, and 29.8°C respectively. Maximum daily air temperatures varied from 29.8 to 54.0°C with an average value of 42.1°C and minimum daily air temperatures varied from 11.5 to 19.2°C with an average value of 15.6°C. In the biodisinfested soil at 15 cm depth, maximum daily soil temperatures ranged from 32.9 to 41.4°C with an average value of 36.8°C and minimum daily soil temperatures ranged from 26.4 to 32.2°C with an average value of 29.2°C. In the biodisinfested soil at 30 cm soil depth, maximum daily soil temperatures ranged from 28.8 to 32.6°C with an average value of 30.6°C and minimum daily soil temperatures ranged from 27.6 to 31.4°C with an average value of 29.1°C (**Figure 1**). In the biodisinfested soil at 15 cm depth, the number of cumulative hours above 30, 35, 37.5, and 40°C were 367, that represented percentages of the biodisinfestation treatment duration of 73, 23, 12, and 3% respectively. In the biodisinfested soil at 30 cm depth, the number of cumulative hours above 30°C was 204 h (40% treatment duration) and no temperature exceeded 32.5°C. The averaged hourly soil temperatures calculated for the total duration of the biodisinfestation treatment showed a temperature variation in each 24-h cycle that ranged from 7°C (36.1–29.1) to 1.2°C (30.4–29.2) at 15 and 30 cm depth respectively.

Effects of Treatments on Oospore Viability

Gases produced during biodisinfestation and exposure time both affected pathogen survival. Viability of *P. capsici* oospores was significantly affected by the factors Biodisinfestation gas ($P < 0.0001$), Exposure time ($P < 0.0001$) and the interaction Biodisinfestation gas x Exposure time ($P < 0.0001$) (**Table 1**). The statistical generalized linear mixed model selected for the binomial data was valid as indicated by the "Generalized Chi-Square/DF" fit statistics which was close to 1 (Generalized Chi-Square/DF = 1.03) (**Table 1**). Viable oospores were detected

TABLE 1 | Effect of Biodisinfestation gas and Exposure time on *Phytophthora capsici* oospores viability. Results of analysis of variance, mean differences among main factors levels, and analysis of simple effects of the significant interaction.

One-Factor (Biodisinfestation gas) repeated measures ANOVA over time (Exposure time)[a]		
Biodisinfestation gas[b]	$F_{12,32.83}$ [c]	18.60
	P-value	<0.0001
Exposure time[b]	$F_{2,53.25}$	16.66
	P-value	<0.0001
Biodisinf. gas × Exposure time	$F_{24,51.53}$	4.54
	P-value	<0.0001
CPE[d]	$AR(1)_{Biodisinfestationgas \times Plot}$	0.0128
CPE[e]	Variance $_{Biodisinfestationgas \times Plot}$	0.0282
Model Fit Statistics	Generalized Chi-Square/DF	1.03

Mean differences among main factors levels based on the Tukey-Kramer test ($P < 0.05$)

Biodisinfestation gas	**Exposure time**	**Viabr (%)[f]**
C_va		50.7 ± 2.3 (b)[g]
C_ai		46.9 ± 3.6 (b)
G_00		50.7 ± 3.6 (b)
G_01		46.7 ± 2.2 (b)
G_02		42.9 ± 2.3 (bc)
G_03		45.8 ± 2.5 (bc)
G_04		50.2 ± 2.5 (b)
G_07		43.1 ± 1.7 (bc)
G_09		42.3 ± 2.8 (bc)
G_11		41.5 ± 2.6 (bc)
G_14		62.3 ± 6.4 (a)
G_ev		35.9 ± 1.6 (c)
SOIL		23.5 ± 2.4 (d)
	07 days	49.3 ± 2.3 (A)[h]
	14 days	43.6 ± 1.8 (B)
	21 days	41.6 ± 1.6 (B)

Analysis of Simple Effects of the Interaction Biodisinfestation gas × Exposure Time

Factor	**Num DF[i]**	**Den DF[i]**	***F* value**	***P*-value**
Biodisinfestation Gas				
C_va	2	49.13	3.25	0.0473
C_ai	2	50.86	8.70	0.0006
G_00	2	50.13	7.17	0.0018
G_01	2	49.02	0.82	0.4465
G_02	2	50.68	2.93	0.0627
G_03	2	50.04	2.92	0.0634
G_04	2	49.38	4.60	0.0148
G_07	2	50.01	0.22	0.8051
G_09	2	50.85	2.06	0.1382
G_11	2	50.52	0.81	0.4507
G_14	2	61.76	32.47	<0.0001
G_ev	2	54.26	0.14	0.8695
SOIL	2	77.02	3.51	0.0349
Exposure Time				
07 days	12	74.93	12.86	<0.0001

(Continued)

TABLE 1 | Continued

Analysis of Simple Effects of the Interaction Biodisinfestation gas × Exposure Time

Factor	Num DF[i]	Den DF[i]	*F* value	*P*-value
14 days	12	73.83	7.43	<0.0001
21 days	12	74.61	6.39	<0.0001

Three replicate plots with one location per plot and one count of 100 oospores per location were used per treatment. Mean values (n = 3) ± standard errors.
[a]Values of significance probability (P-values), parameter estimates ($F_{12,32.83}$, CPE), and model fit statistics in one-factor repeated measures ANOVA over time.
[b]Factorial experiment with a treatments structure of two crossed factors: (i) Biodisinfestation gas with 13 levels (C_va, C_ai, G_00, G_01, G_02, G_03, G_04, G_07, G_09, G_11, G_14, G_ev, SOIL). C_va: Control 1 of vacuum. C_ai: Control 2 of air. G_00, G_01, …, G_14: Gases sampled on day 00, 01, …, 14. G_ev: Gases sampled on every sampling day. SOIL: Control 3 of biodisinfested soil at 15 cm depth.
(ii) Exposure time with three levels (7, 14, 21 days).
[c]F statistic with numerator and denominator degrees of freedom used in its calculation.
[d]CPE: Covariance parameter estimate for a first-order autoregressive [AR(1)] covariance structure type of the statistical model subject "Biodisinfestation gas×Plot".
[e]CPE: Covariance parameter estimate of the variance of the statistical model defined by the random unit effect "Biodisinfestation gas×Plot".
[f]Viabr (%): oospores viability percentage values relativized to the initial viability which was Viabr0 = 100 ± 3.6%.
[g]Values in the same column followed by the same lower-case letter in brackets indicate non-significant differences between levels of the main factor Biodisinfestation gas based on the Tukey-Kramer test (P < 0.05).
[h]Values in the same column followed by the same upper-case letter in brackets indicate non-significant differences between levels of the main factor Exposure time based on the Tukey-Kramer test (P < 0.05).
[i]Num DF, Den DF: Numerator and denominator degrees of freedom, respectively.
When differences were non-significant, letters were omitted.

under all treatments combinations of gas sampling times and exposure times (**Figure 2**).

On average, the most effective treatments ordered from higher to lower efficacy, for the main factor Biodisinfestation gas, were: SOIL, G_ev, G_11, G_09, G_07, G_03, and G_02, and for the main factor Exposure time, were: 21 and 14 days (**Table 1**).

The analysis of simple effects of the interaction Biodisinfestation gas x Exposure time was significant in six of the thirteen levels of the first factor and in all the levels of the second factor respectively, indicating that oospore viability was differently affected by Exposure time within six levels (C_va, C_ai, G_00, G_04, G_14, SOIL) of the Biodisinfestation gas and was also differently affected by Biodisinfestation gas within the three levels of Exposure time (7, 14, and 21 days) (**Table 1**).

For the shortest exposure time (7 days), the gases sampled at day 9, day 11, and every day (G_09, G_11, G_ev) showed a significant lower viability than the air and vacuum untreated controls (C_ai, C_va) with reduction rate percentages of 34, 34, and 36% when compared with the vacuum control (C_va), respectively (**Figure 2**). Conversely, for exposure time of 14 days, none of the biodisinfestation gases differ significantly from the untreated controls (**Figure 3**), with reduction rates comprised between 16 and 21% for gases sampled in days 2, 3, 7, and 9 (G_02, G_03, G_07, G_09), and 28% for every day (G_ev), when compared with the vacuum control (C_va), respectively. For the longer exposure time of 21 days, differences among biodisinfestation gases were reduced and none of them differ significantly from the untreated controls, with highest reduction rates of 15, 9, 13, and 23% for gases sampled in days 2, 11, 14, and every day (G_02, G_11, G_14, G_ev), when compared with the vacuum control (C_va), respectively (**Figure 2**).

Gases sampled at day 14 (G_14) produced a significant higher viability than the untreated controls (C_ai, C_va) for exposure times of 7 and 14 days and showed increase rates of 38% and 38% when compared with the vacuum control (C_va), respectively. On the contrary, for the longer exposure time of 21 days, non-significant differences were shown and a decrease rate of 13% was observed when compared with the vacuum control (**Figure 2**).

The gases sampled every day (G_ev) was the second most effective biodisinfestation gas treatment and was significantly different from untreated controls (C_va, C_ai) for the shorter exposure time (7 days) but was not different for the longer exposure times (14 and 21 days) with oospore viability reduction percentages of 36, 28, and 23% when compared with the vacuum control (C_va) for exposure times of 7, 14, and 21 days, respectively (**Figure 2**).

In contrast, the biodisinfested soil control treatment at 15 cm depth (SOIL) was always the most effective treatment, significantly different from the untreated controls (C_va, C_ai), and with reduction percentage rates of 45, 47, and 55% when compared with the vacuum control (C_va) for exposure times of 7, 14, and 21 days, respectively (**Figure 2**).

The graphical analysis of the proportion of viable oospores over exposure time for each biodisinfestation gas treatment appeared to have a different pattern of change (**Figure 3**). With this in mind, a linear regression model was fitted for the proportion of viable oospores over exposure time with separate slopes for each Biodisinfestation gas type. A first model included a term for lack of fit, in order to test for non-linear trends (**Table 2A**). The result for the term Biodisinfestation gas * Exposure time ($F_{13,35.35} = 1.57$; $P = 0.1425$) indicated that there was no evidence of lack of fit from linear regression over Exposure time (**Table 2A**). This allowed us to move to the next step: drop the lack-of-fit term (Biodisinfestation gas * Exposure time) from the model and focus on the estimated slopes for linear regression of the proportion of viable oospores over Exposure time and whether there was statistical evidence that they differ by Biodisinfestation gas.

A second model of linear regression was fitted and statistically significant evidence was obtained for the model terms

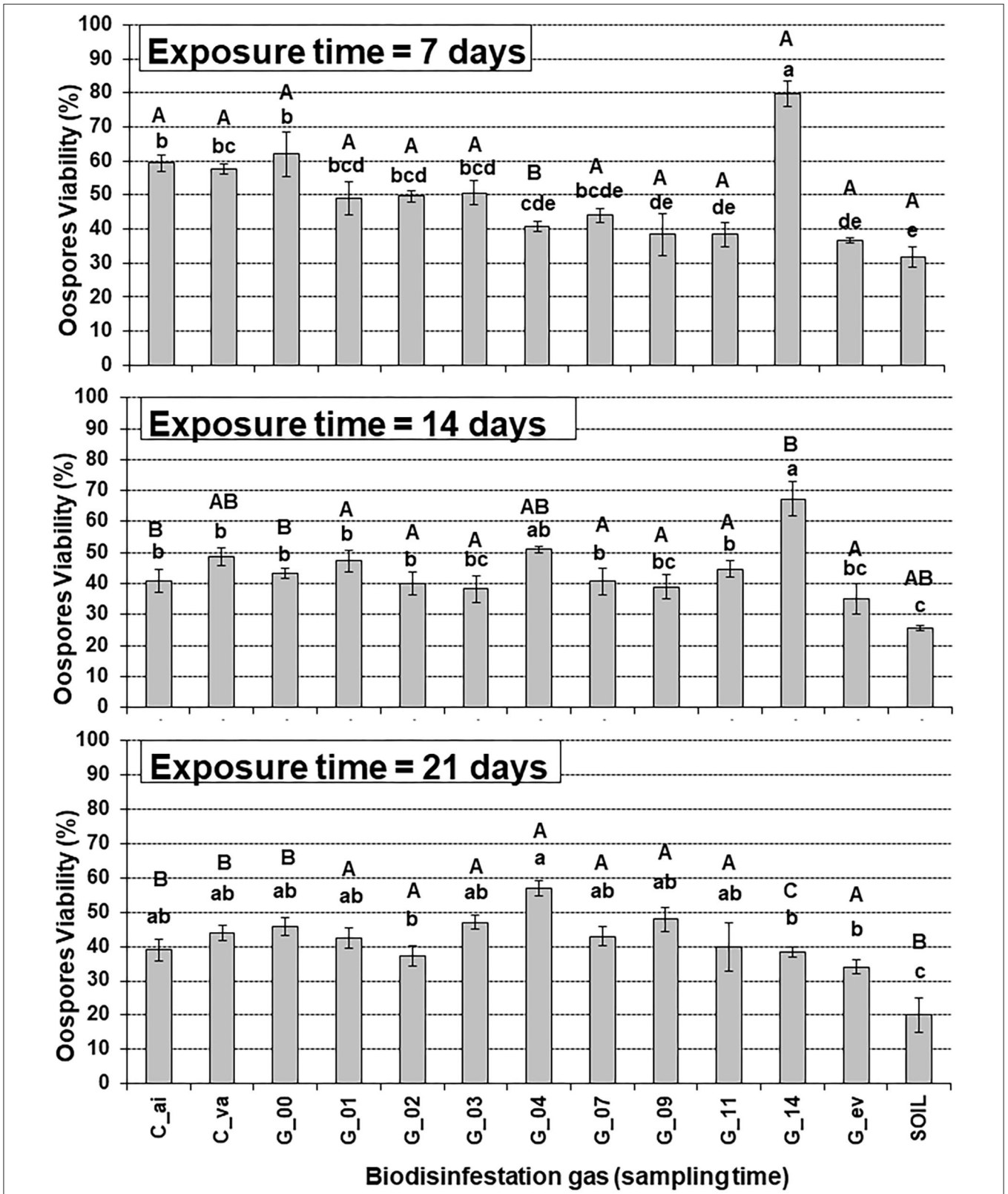

FIGURE 2 | Analysis of the interaction of Biodisinfestation gas and Exposure time on *Phytophthora capsici* oospores viability. Error bars represent standard error of the mean ($n = 3$) from three replicate counts of 100 oospores each. Values with different lower-case letter indicate significant differences between Biodisinfestation gas within a given exposure time and upper-case letters compare exposure times for a given Biodisinfestation gas based on the Tukey-Kramer test ($P < 0.05$). C_va: Control 1 of vacuum. C_ai: Control 2 of air. G_00, G_01, …, G_14: Gases sampled on day 00, 01, …, 14. G_ev: Gases sampled on every sampling day. SOIL: Control 3 of biodisinfested soil at 15 cm depth.

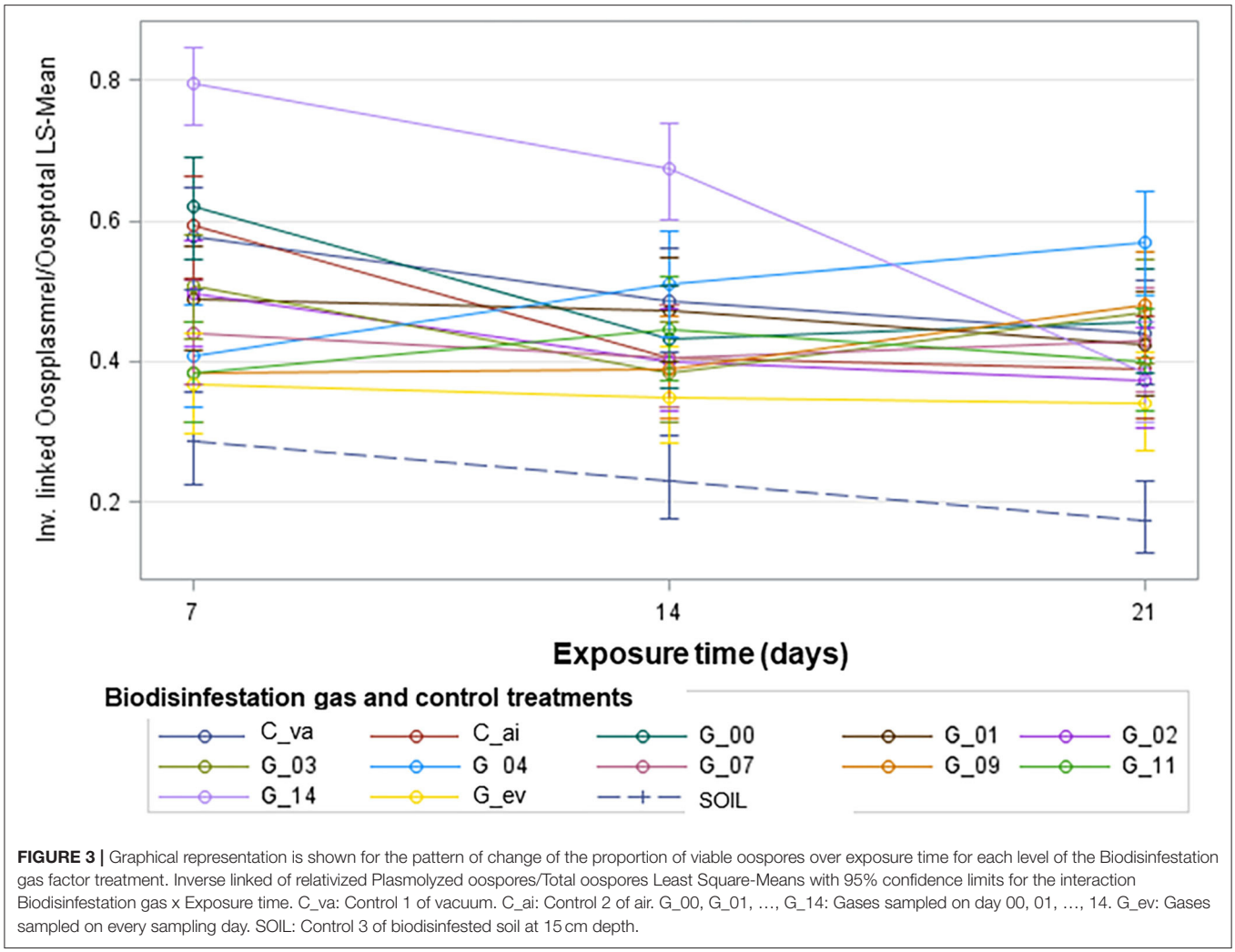

FIGURE 3 | Graphical representation is shown for the pattern of change of the proportion of viable oospores over exposure time for each level of the Biodisinfestation gas factor treatment. Inverse linked of relativized Plasmolyzed oospores/Total oospores Least Square-Means with 95% confidence limits for the interaction Biodisinfestation gas x Exposure time. C_va: Control 1 of vacuum. C_ai: Control 2 of air. G_00, G_01, …, G_14: Gases sampled on day 00, 01, …, 14. G_ev: Gases sampled on every sampling day. SOIL: Control 3 of biodisinfested soil at 15 cm depth.

TABLE 2A | Results of type I tests of fixed effects.

Effect	Num DF	Den DF	F value	Pr > F
Biodisinfestation gas	12	32.83	18.56	<0.0001
E* Biodisinfestation gas	13	75.1	9.04	<0.0001
Biodisinfestation gas*Exposure time	13	35.35	1.57	0.1425

*This first model includes the lack-of-fit term (Biodisinfestation gas * Exposure time) in order to test significance for non-linear trends.*

(Biodisinfestation Gas (*intercept*), $F_{13,71.83} = 10.28$, $P < 0.0001$; E * Biodisinfestation Gas (*slope*), $F_{13,77.57} = 8.73$, $P < 0.0001$) (**Table 2B**). Separate slopes and intercepts for each level of the Biodisinfestation gas factor treatment (**Supplementary Table 1**) were estimated for the linear regression of the proportion of viable oospores over exposure time. The linear regression model equation was:

$$\frac{\text{Viable oospores}}{\text{Total oospores}} = \frac{1}{(1 + e\,(\,-\,(\text{intercept} + \text{slope E})))}$$

where E = Exposure time (days).

Significant differences among intercepts and slopes of the linear regression equations of the proportion of viable oospores over exposure time were tested with linear contrast of different combinations of the Biodisinfestation gas factor levels (**Supplementary Table 2**). Linear contrast showed that there was evidence of statistically significant difference for slopes and intercepts between the average of vacuum and air untreated controls (C_va, C_ai) vs. the average of biodisinfestation gases ($P_{\text{intercept}} = 0.0054$; $P_{\text{slope}} = 0.0139$), the average of untreated controls vs. gases sampled every day ($P_{\text{intercept}} = 0.0004$; $P_{\text{slope}} = 0.0439$), the average of untreated controls vs. gases sampled in day 14 ($P_{\text{intercept}} < 0.0001$; $P_{\text{slope}} = 0.0001$), the average of

TABLE 2B | Results of type III tests of fixed effects.

Effect	Num DF	Den DF	*F* value	Pr > *F*
Biodisinfestation Gas (*intercept*)	13	71.83	10.28	<0.0001
E * Biodisinfestation Gas (*slope*)	13	77.57	8.73	<0.0001

This second model is a linear regression of the proportion of viable oospores over exposure time for each level of the Biodisinfestation gas factor treatment. This model includes two terms in order to detect if each Biodisinfestation gas (intercept) shows different patterns of change over time (slope).
E: Exposure time (days).

untreated controls vs. the average of gases sampled in days 1–2–3–4 ($P_{intercept} = 0.0010$; $P_{slope} = 0.0021$), the average of untreated controls vs. the average of gases sampled in days 7–9–11 ($P_{intercept} < 0.0001$; $P_{slope} = 0.0001$), the average of gases sampled in days 7–9–11 vs. the gases sampled in day 14 ($P_{intercept} < 0.0001$; $P_{slope} < 0.0001$), and the average of gases sampled in days 1–2–3–4 vs. the gases sampled in day 14 ($P_{intercept} < 0.0001$; $P_{slope} < 0.0001$). Significant differences were also found for slopes of the biodisinfested soil vs. the average of gases sampled in days 1–2–3–4–7–9–11 ($P_{slope} = 0.0124$), the biodisinfested soil vs. the average of gases sampled in days 1–2–3–4 ($P_{slope} = 0.0357$), and the biodisinfested soil vs. the average of gases sampled in days 7–9–11 ($P_{slope} = 0.0061$). Lastly, significant differences were found for intercepts of the gases sampled every day vs. the average of gases sampled in days 1–2–3–4–7–9–11–14 ($P_{intercept} = 0.0328$), and the biodisinfested soil vs. the average of gases sampled in days 1–2–3–4–7–9–11–14-every day ($P_{intercept} = 0.0336$).

DISCUSSION

Although none of the biodisinfestation gases completely eliminated the *P. capsici* ooospore viability, a significant effect purely attributable to the gases released at different time intervals from the onset of biodisinfestation was observed in the reduction of inoculum, in agreement with previous studies with other soilborne pathogens and biotoxic volatile compounds generated in solarized organic-amended soil (Gamliel and Stapleton, 1993a,b; Stapleton, 2000; Zhang et al., 2021).

The small effect on oospore viability of sampled biodisinfestation gases in our experiment was consistent with the low-medium dose of organic amendment applied (25,630 kg ha^{-1}) for an intensive greenhouse crop, and with the low-medium temperature registered at 15 cm depth in the biodisinfested soil (only 115, 61, and 13 cumulative hours above 35, 37.5, and 40°C respectively). Both factors could have ultimately resulted in the generation of a small quantity of biotoxic volatile compounds and therefore, in a low impact on the survival of *P. capsici* oospores, as previously reported with various *Phytophthora* spp. survival spores (Guerrero et al., 2010; Larregla et al., 2014; Lacasa et al., 2015; Gandariasbeitia et al., 2019) or with other soilborne fungal pathogens survival structures (Gamliel et al., 2000; Stapleton, 2000, Gamliel and Stapleton, 2017).

The low disinfectant efficacy of biodisinfestation gases on oospore viability observed in this study in northern Spain (Biscay) contrasted with the higher efficacy of a previous experiment carried out with the same methodology in southeastern Spain (Murcia) (Larregla et al., 2014). The differences in efficacy between both studies seemed to be closely related to the different applied doses of organic amendment (70,000 kg ha^{-1} of fresh sheep manure in southeastern Spain vs. 25,630 kg ha^{-1} in northern Spain) and the 15 cm depth biodisinfested soil temperatures (average daily minimum-maximum values of 37.7–42.1°C in southeastern Spain vs. 29.2–36.8°C in northern Spain). In the trial of this study in northern Spain, the assayed conditions might have been insufficient to achieve the critical threshold (Gamliel and Stapleton, 1993a) which is required for the generation of a sufficient amount of biotoxic gases in the soil atmosphere to affect the oospore survival.

Although no analyses were made for identification of the released gases sampled in the field biodisinfestation trial of this study, ammonia volatilisation, among other volatile compounds, might have contributed to the reduction of oospore survival during biodisinfestation. In a previous experiment carried out in early spring (17th March to 21st April) in the same soil and with the same amendment mixture type applied at a quadruple dose (fresh weight) of 10,000 kg ha^{-1} (equivalent to 1,360 kg N ha^{-1}), mean ammonia concentration measured in the manure amended soil atmosphere under plastic sheets was 14.8 mg NH_3 m^{-3} and it decreased 45% after 35 days of biodisinfestation (Arriaga et al., 2011). Ammonia volatilisation is regulated by NH_{4+}-N concentration in the soil solution and is modeled by factors such as pH and temperature (Beutier and Renon, 1978). A low soil organic carbon content is also another critical factor in the accumulation of ammonia, while a high level prevents its generation (Tenuta and Lazarovits, 2002). The differences in NH_3 concentration between both experiments could be attributed to the different NH_{4+}-N content, which would be determined by the rate of N organic content (N_{org}) mineralisation. We could hypothesize similar or even slightly higher NH_3 concentration in our study when compared with the previous experiment of Arriaga et al. (2011). Indeed, higher NH_{4+}-N availability would be expected by higher rates of N_{org} mineralisation which are favored by temperature differences during soil biodisinfestation. Average daily temperatures of air and 15 cm depth biodisinfested soil were 25.3 and 32.5°C in the present experiment versus 18.3 and 21.2°C in the previous experiment of Arriaga et al. (2011).

Our results with oomycetes share similarities with the findings obtained in previous studies with other soilborne pathogens such as fungi and nematodes. Laboratory and field tests showed that fresh chicken manure was an effective non-chemical soil fumigant that effectively prevented soil-borne

pathogens, reduced *Fusarium oxysporum* and *Phytophthora* spp. soil inoculum, improved soil condition, and increased strawberry yield (Zhang et al., 2021). Stapleton et al. (1991) observed that composted chicken manure alone at 5,381 kg ha^{-1} (equivalent to 304 kg ha^{-1} of total N and 17.6 kg ha^{-1} of NH_{4+}-N) significantly reduced *Pythium ultimum*, and when combined with a diurnal heating regime (42°C high 8 h; 18°C low) and an incubation time of 4 weeks, the *Pythium* population was eradicated. Although increasing NH_{4+}-N concentrations gave better control of *P. ultimum* and *Meloidogyne incognita* in incubator fertilizer/solarization simulation laboratory experiments, field results showed that the addition of nitrogen sources to the solarization process did not increased the control efficacy against *P. ultimum* and *V. dahliae* (Stapleton et al., 1991). In contrast, the high levels of ammonia detected in soil atmospheres 3 and 7 days after the incorporation of *Vicia villosa* as green manure were related to the reduction of chlamydospores viability of *Thielaviopsis basicola* and ammonia was responsible for the observed suppressiveness in field (Candole and Rothrock, 1997).

In our study, the most effective treatment for all the gases sampled at different times was the succession of gases sampled every day (G_ev). This treatment showed a viability reduction rate of 41% compared with the untreated control for an exposure time of 21 days. Similarly, the higher reduction (72% for an exposure time of 34 days) was also obtained for the same treatment in the previous study in southeastern Spain (Larregla et al., 2014). The following more effective biodisinfestation gas treatments were similar in both studies, since they were detected between days 4 and 11 in the present study in northern Spain (reduction of 24–34%) and between days 3 and 9 (reduction of 17-28%) in the previous study in southeastern Spain. This behavior would be in line with studies which have shown that the formation and release of biotoxic volatile compounds are predominantly found during the first 3 weeks of solarization of the soil amended with organic materials. The concentrations of volatile compounds drop to low levels after this time (Gamliel and Stapleton, 2017).

In our experiment, longer exposure times (14 and 21 days) were not more effective than the shorter time (7 days) for all the gases sampled at different times and even the differences among them were no longer observed when exposure time increased. These results were not expected and differ considerably from earlier studies (Ebben et al., 1983; Katan and Gamliel, 2010), which showed that *in vitro* toxicity of a chemical fumigant to soilborne phytopathogenic fungi was higher when the fumigant concentration x time product was increased. However, it is likely that the explanation for this is that the sampled gas volume in the vacutainer tubes was small (10 mL) and with the increase of exposure time, the escape of gases could have caused a decrease in their concentration and the absence of toxic effect on the pathogen spores. This explanation would be consistent with the result that the biodisinfested soil control (SOIL) was the only treatment that improved disinfectant efficacy when exposure time increased. It also confirms our previous findings where the succession of gases sampled every day (G_ev) was the most effective treatment (Larregla et al., 2014). In order to avoid gas exhaustion and negligible effects on survival by low gas concentrations, the use of methodological devices (Klein et al., 2007) which allow the exposition of fungal spores to larger volumes of gas samples are recommended in future experiments. In that way, the methodological underestimation of the biofumigant effect of gases on actual field conditions could be minimized.

The lowest oospore viability was shown in the biodisinfested soil control treatment (SOIL) for all the assayed exposure times. The overlap of thermal, higher biofumigation effects and microbial activity effects in a synergistic mode of action could well be responsible for these results (Stapleton et al., 1991; Hoitink and Boehm, 1999; Gamliel et al., 2000). Therefore, this could explain that oospore survival in the field biodisinfested soil control treatment (SOIL) would be lower than in the remaining laboratory treatments with only biofumigation effects caused by gases.

Oospores in the moist biodisinfested soil control treatment (SOIL) received a "heat dosage" (13 cumulative hours above 40°C) that was below the threshold (112 cumulative hours at 40°C) required for direct thermal inactivation (Etxeberria et al., 2011b). However, oospores in the biodisinfested soil received a considerable amount of "sub-lethal" heat (115 and 61 cumulative hours above 35 and 37.5°C respectively), that could make them less pathogenic and more susceptible to stress factors such as chemical toxicants or microbial antagonists (Chellemi et al., 1994; Stapleton and DeVay, 1995; Tjamos and Fravel, 1995; Oka, 2010). Although thermal sensitivity of the target pathogen(s) varies widely among species, in general terms, sublethal heat can be defined as a temperature range to weaken target pathogen(s) and, in general, is established for soil temperatures below 38-40°C (Stapleton, 2000; Oka, 2010).

The linear regression of the proportion of viable oospores over exposure time for each level of the biodisinfestation gas factor treatment evidenced significant differences in quantity (intercept) and change rate (slope). Significant differences were found between the succession of biodisinfestation gases sampled every day (G_ev) and the untreated controls (C_va, C_ai). This is in agreement with *in vitro* biofumigation experiments using different volatility isothiocyanates and the growth-rate response of soilborne fungal pathogens (Sarwar et al., 1998; Kirkegaard, 2009). In our experiment, a significant higher change rate (slope) on survival of oospores was detected in the biodisinfested soil control treatment (SOIL) with regard to the average of gases sampled in days 1–2–3–4–7–9–11 (G_11). This is consistent with Gamliel and Stapleton (1993b), where they obtained an interactive effect of heating soil amended at 38°C with composted chicken manure on the reduction of inoculum densities of fungal pathogens.

A methodology to evaluate a high number of organic amendments for their greater biofumigant efficacy has been obtained in this work. This methodology could be applied for an initial screening of a high number of

organic amendments with the final aim of choosing the most suitable amendments for further evaluation with the use of more expensive, time-consuming, and long-lasting field tests that would include the pathosystem to be controlled.

In our study, an effect purely attributable to the released gases through biodisinfestation has been observed in the reduction of long term survival propagules (oospores) of the oomycete plant pathogen *P. capsici*. Thus, our results suggest the significant biofumigant effect of fresh animal manures in the reduction of the inoculum of a soilborne pathogen in suboptimal conditions for solarization.

In conclusion, the biodisinfestation practice could also be effective in agro-environments with reduced thermal inactivation effects. In a circular bioeconomy context, it appears necessary to continue further the assessment of varied proximity byproducts for their use as organic amendments including a good biofumigant effect and also adapted to the different crops requirements.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SL carried out the conception and design of the research. MG, MO, SM, MMG, AO-B, and SL participated in the other stages of the work, including the performing of experiments, data analyzing, revision of the intellectual content and the drafting of the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.663915/full#supplementary-material

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Amendment Properties Affect Crop Performance, Leaf Tissue Nitrogen, and Soil Nitrogen Availability Following Soil Treatment by Anaerobic Soil Disinfestation

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Efficacy of anaerobic soil disinfestation (ASD) for soilborne plant pathogen suppression is strongly influenced by soil environment and organic amendment attributes. At the same time, these factors influence soil nutrient availability, crop nutrition, and crop performance, but published information on ASD amendment property effects, including carbon to nitrogen (C:N) ratio and C substrate bioavailability, on crop performance and soil nutrient availability is limited. We evaluated ASD amendment effects on soil N availability, crop N status, and solanaceous crop performance in a series of trials: (1) greenhouse/growth chamber study of amendments (primarily molasses/soybean hulls and wheat bran) formulated at 10:1, 20:1, 30:1 and 40:1 C:N ratios (4 mg C g^{-1} soil), (2) field study with molasses/soybean hull-based amendments at equivalent C:N ratios/C rates (3) on-farm study with molasses/soybean hull-based amendments (4 mg C g^{-1} soil) compared to grower-standard control, and (4) field study of labile to recalcitrant amendment substrates at 30:1 C:N ratio (~3.4 mg C g^{-1} soil). ASD amendment C:N ratio strongly influenced soil inorganic N and the lowest (10:1) ratio was associated with highest soil inorganic N at ASD treatment termination in both trials 1 and 2, which often persisted into the cropping phase. Accordingly, the lowest amendment C:N ratio was also associated with the highest biomass (trail 1), leaf tissue N (trial 2), and crop yield (trials 1, 2) among treatments, even with application of recommended fertigation rates to all treatments in the field study. In trial 3, ASD treatment induced higher soil inorganic N and crop yield than the control, but no differences were observed in plant tissue N. In trial 4, more decomposable ASD substrates reduced soil inorganic N at ASD treatment termination, with the highest soil inorganic N associated with the most recalcitrant amendment, but there was no effect on crop yield. ASD amendment C:N ratio, and to a lesser extent, amendment decomposability, exert a strong influence soil inorganic N and crop performance. Optimization of ASD treatments for disease management will require simultaneous optimization of crop nutrition practices to facilitate more holistic, less confounded assessment of crop performance and to facilitate recommendations for grower adoption.

Keywords: biological soil disinfestation, organic soil amendments, agricultural by-products, solanaceous crops, soil fertility

INTRODUCTION

Anaerobic, or biological, or reductive soil disinfestation has emerged as a biologically-based alternative to soil fumigation for suppression of soil borne pests (Shrestha et al., 2016). Twenty years ago, the technique of using anaerobic decomposition of organic soil amendments to control soil borne pathogens was developed separately in Japan and the Netherlands (Shennan et al., 2014). More recently, research studies on ASD have been conducted in multiple regions and cropping systems of USA. Comprehensive studies and reviews of ASD (Rosskopf et al., 2005; Shennan et al., 2014; Strauss and Kluepfel, 2015) have shown that ASD is a versatile technique that can be adapted regionally by using varying types of locally-available organic amendments to control various soil borne pathogens and plant-parasitic nematodes, and with effects on weed pests. ASD has also shown promising effects on yields of horticultural fruit and vegetable crops when compared to non-treated systems and fumigated systems (McCarty et al., 2014; Shrestha et al., 2016; Guo et al., 2017; Paudel et al., 2018; Gilardi et al., 2020). Varying organic amendments can be used as carbon sources for ASD treatment depending on cost effectiveness, availability, and ease of application. Further, application of organic amendments can improve soil physical and chemical properties of soil leading to ASD treatment impacts on vegetable and fruit yield (Butler et al., 2014a). However, limited studies have evaluated ASD impacts on soil nutrients, crop nutrition, or crop performance. Further, existing published work tends to be limited in scope because it was generally not designed to specifically evaluate mechanistic impacts of varying ASD amendment properties on soil/crop nutrients and crop performance.

In this study we evaluate the importance of ASD amendment properties, including C:N ratio, C rate and substrate decomposability on soil/crop nitrogen and crop performance under environmental conditions typical to the southeastern USA. While ASD implementation relies on relatively simple techniques of amendment incorporation, irrigation to saturate topsoil and covering the treated plot to create anaerobic conditions for a few weeks, optimization of ASD techniques, including amendment characteristics, is essential to optimize effectiveness against pests and simultaneously maintain or improve crop performance (Shrestha et al., 2016). The ASD process relies on the bioavailability of organic matter in ASD amendments, which increases soil microbial respiration leading to strongly anaerobic soil conditions that facilitate shifts to anaerobic microbial decomposition and the formation of anaerobic decomposition metabolites (Shennan et al., 2014). Amendment C:N ratio, rate, and decomposability all potentially affect these microbial decomposition dynamics (Sinsabaugh et al., 2013; Spohn, 2015; Truong and Marschner, 2018), potentially affecting ASD treatment effectiveness for pathogen control (Shrestha et al., 2018a, 2020a). At the same time, soil amendments and their biochemical composition potentially impact a range of soil chemical, physical, and biological properties (Inglett et al., 2005; Butler et al., 2014a) and of these, changes affecting soil nutrient availability are especially important in affecting crop performance post-ASD treatment (Butler et al., 2014a; Di Gioia et al., 2017).

The overall goal of our study was to evaluate ASD amendment composition effects on soil N availability, crop N status, and solanaceous crop performance across a series of four greenhouse and field trials. Our hypotheses were that (1) ASD treatments will increase plant biomass and crop yield compared to anaerobic (unamended, saturated and plastic covered; Trials 1, 2) or grower standard (compost only; Trial 3) controls and have similar yield to fumigated controls (Trial 2), (2) soil inorganic N, plant tissue N and plant biomass will increase at lower (< 20:1) ASD amendment C:N ratios compared to higher ASD amendment C:N ratios and controls (Trials 1, 2), and (3) ASD treatments with labile amendments will increase yield compared to recalcitrant ASD amendments at a similar C:N ratio and a control, with similar soil N and leaf tissue N among treatments (Trial 4).

MATERIALS AND METHODS

Trial 1. Growth Chamber/Greenhouse Trial of Amendment Type and C:N Ratio

A pot trial with two amendment mixtures (dry molasses/soy hull-based, or wheat bran-based) at four C:N ratios was conducted to evaluate C:N ratio effects on soil inorganic N, plant tissue N and tomato *(Solanum lycopersicum L.)* fruit and plant biomass following ASD treatment. The experimental design was a factorial completely randomized design with four replicates, which was repeated. The ASD treatment was conducted in an environmental growth chamber (ECG, Chagrin Falls, OH, USA) at 25°C for 14 h and 15°C for 10 h to simulate soil temperature regimes in relevant production regions during spring in Tennessee and similar warm-temperate to subtropical production regions. Top soil [Dewey silt loam (fine, kaolinitic, thermic, typic Paleudult)] from the Organic Crops Unit at the University of Tennessee, Knoxville, TN, USA was collected, sieved (<10 mm) and mixed in equal proportion with sand (w/w). Treatment factors included dry molasses/soy hull-based and wheat bran-based amendments (low-cost livestock feed supplements) mixed with either soybean meal (high nitrogen) or corn starch (low nitrogen) amendments to formulate four amendment C:N ratios (10:1, 20:1, 30:1, and 40:1; **Table 1**) at 4 mg C g^{-1} soil (**Table 1**; also described in Shrestha et al., 2021). Total C and N content of amendments was determined by combustion (Flash EA 1112 NC Soil Analyzer, CE Elantech, Lakewood, NJ, USA) and other nutrient analysis of amendments determined following digestion (**Table 1**). Amendments were mixed with soil and placed in polyethylene pots (2.6-L volume, 12-cm diameter by 23-cm height). Pots were saturated with tap water (∼375 mL kg^{-1} soil), covered with black polyethylene mulch (0.032 mm) and secured with heavy-duty rubberbands. Oxidation-reduction electrodes (ORE, Sensorex Corp., Garden Grove, CA, USA) were inserted in each pot to measure redox potential and assess accumulated anaerobic conditions as described in Shrestha

et al. (2020b). For a control treatment, pots were non-amended, irrigated and covered (i.e., anaerobic control). All treatment pots were incubated for 3 weeks during the ASD treatment period.

After 3-week incubation, soil samples were collected and pots were transferred to a greenhouse (average temperature 25–30°C), and 3-week-old seedlings of dwarf tomato (cv. Florida Lanai) were planted in each pot to evaluate plant growth characteristics. Dried blood meal (5 g/plant) was applied to each pot at 2 weeks after transplanting. Cropping phase soil samples were collected after 3 weeks and fruit weight and number of fruits per plant were recorded at 8 weeks after transplanting. Plants were removed, cleaned and oven dried at 65°C for 48 h and dry biomass of shoots and roots recorded.

Soil pH, Soil Inorganic N

Subsamples of soil collected were air-dried and sieved (<2 mm) prior to determining soil pH and inorganic N. Soil pH was recorded by inserting a pH electrode (Orion 3-Star Plus pH Benchtop Meter, Thermo Scientific, Waltham, MA, USA) in suspension of 5-g soil and 0.01 M $CaCl_2$ (1:2). The value was reported as equivalent soil pH determined in deionized water by adding 0.6 (Kissel et al., 2009). For inorganic soil N and total soil N and C, 5-g of sieved (<2 mm) soil was extracted with 1-M KCl for 30 min, centrifuged, and filtered (Whatman 42) prior to colorimetric analyses for NH_4-N and NO_2-N + NO_3-N using a microplate spectrophotometer (Powerwave XS, Biotek, Winooski, VT, USA) as described by (Sims et al., 1995).

Leaf/Shoot Tissue N Analysis

Whole shoot biomass of tomato plants was collected for tissue N analysis 8 weeks after transplanting. Samples were dried at 65°C for 48 h, then ground and analyzed for total N content by combustion (Flash EA 1112 NC Soil Analyzer, CE Elantech, Lakewood, NJ, USA).

Trial 2. Field Trial of ASD Amendment C:N Ratio Effects on Soil N and Bell Pepper Performance

A 2-year field study was conducted at the experimental farm located in Plateau Research and Education Center, University of Tennessee, Crossville, TN, USA to evaluate the effect of C:N ratio of ASD amendment on soil inorganic N, plant tissue N, and bell pepper (*Capsicum annum* L.) crop yield compared to anaerobic and fumigated controls. The soil type according USDA classification system is in the Lily series (fine-loamy, siliceous, semiactive, mesic Typic Hapludult). Treatments included an ASD amendment mixture (molasses/soy hulls) mixed with soybean meal or corn starch to maintain four C:N ratios of 10:1, 20:1, 30:1, and 40:1 at 4 mg C g^{-1} soil, a low C amendment treatment (2 mg C g^{-1} soil at C:N ratio of 30:1), a non-amended anaerobic control, and a methyl bromide (MeBr) fumigated control (67:33 mixture with chloropicrin, 224 kg/ha) control (**Table 1**; also described in Shrestha et al., 2021). The experimental design was a randomized complete block with four replications and the experiment was repeated in different sites in 2 years. Soil amendments for ASD treatment were applied in each plot (7.6 × 1.8 m) using a drop fertilizer spreader and were thoroughly incorporated with a rotovator. Raised beds (∼5-cm) were formed, mulched with standard black polyethylene (0.025-mm, Berry Global, IN, USA) and then drip irrigated (5 cm total irrigation applied over 9 h) to fill soil pore space to a ∼20-cm depth. To assess anaerobic conditions, IRIS (IRIS = indicator of reduction in soils), iron oxihydroxide coated PVC (Castenson and Rabenhorst, 2006) tubes were inserted in each plot at 0 to15-cm depth and were retrieved after the ASD termination. The removal of iron coating was assessed as described by Rabenhorst (2012). Oxidation-reduction electrodes were limited to only one trial within two blocks.

At ASD treatment termination, and 3 weeks after ASD termination, soil samples were collected to determine soil pH and soil inorganic N as described in trial 1. Bell pepper transplants (cv. Aristotle F1) were planted at 30-cm between and within a double row per bed (28 to 30 plants per bed) to assess crop performance and plant nutrition. The pepper crop was drip fertigated according to standard grower practice for the southeastern USA (Kemble et al., 2013) beginning the week of transplanting. In total, 148 kg N/ha, 36 kg P/ha and 138 kg K/ha were applied to the crop throughout the growing season, with the final fertigation 2 weeks prior to the last harvest. Pepper fruits were harvested based on size, dark green color, and firmness, and graded according to the standard USDA fruit grading system in fancy, number 1, number 2 or cull categories (USDA-AMS, 2005). Culled fruits included small, diseased, deformed and sunscalded fruits. Fruits were harvested from each plot (24 to 26 plants), except plants at the end of each row. Plants were harvested once per week from early August to late September in both years. Fruits were counted and weighed in each grade class and summed for each harvest time, then data extrapolated to a per ha basis based on bed length harvested. For leaf tissue N, recently matured, clean pepper leaf tissue (∼20 leaves per plot) was sampled from 5 randomly selected plants in each plot at 7 weeks after transplanting and leaf N analyzed as described in trial 1.

Trial 3. On-Farm, High Tunnel Trial of Tomato Crop Performance

In spring 2016 and 2017 an on-farm evaluation of ASD with molasses and soybean hull-based amendments plus compost amendment compared to a grower-standard control (compost only) was conducted on a privately-owned certified organic vegetable farm, Loudon County, TN, USA to evaluate impacts on soil pH and soil N changes over time, tomato leaf tissue N, and tomato yield. The soil type was Litz silt loam (mixed, active, mesic Ruptic-Ultic Dystrudept). The trial began in mid-February and concluded at the end of the tomato growing season in mid-July. The trial was conducted within a 9.1 m by 15.2 m high tunnel in a randomized complete block design with six replicates in each year. Each plot was 0.9 × 7.6 m long with 0.6 m alleys between plots. The study was identically repeated on the same site in 2017. The two treatments were (i) ASD treatment with dry molasses/soyhull + soybean meal

TABLE 1 | Amendment nutrient content and application rates in Trials 1, 2, 3, and 4.

	Crop	Amendments	C:N ratio	Rate of application	Nutrients applied								
					C	N	P	K	Ca	Mg	S	Fe	Mn
				g kg soil^{-1}	mg kg^{-1} soil								
Trial 1:	Tomato	DM† + SM	10	6.4 + 3.6	4009	401.4	31.5	341.0	73.0	42.8	63.0	5.2	3.9
Growth chamber/		DM + SM	20	9.4 + 0.9	4019	201.8	15.7	402.0	87.6	46.7	74.3	6.7	4.9
greenhouse study		DM + CS	30	10.3 + 0.1	4023	133.9	9.7	416.0	91.0	47.1	76.8	7.1	5.1
		DM + CS	40	7.7 + 2.6	4024	100.6	7.7	313.0	68.6	35.6	58.3	5.5	4.2
		WB + SM	10	7.8 + 1.8	4020	403.3	112.0	140.0	16.0	57.0	21.8	0.9	0.8
		WB + CS	20	6.4 + 3.3	3998	201.0	81.2	81.0	6.5	41.1	12.2	0.6	0.8
		WB + CS	30	4.2 + 5.6	4006	132.6	53.8	53.7	4.5	27.1	8.7	0.6	1.0
		WB + CS	40	3.2 + 6.7	4032	101.5	41.4	41.4	3.6	20.8	7.1	0.5	1.1
Trial 2:	Pepper	DM + SM	10	6.4 + 3.6	3992	398.3	31.2	337	72.1	42.4	62.4	3.0	0.0
Field study		DM + SM	20	9.4 + 0.9	4001	200.6	11.9	277	60.2	32.7	51.3	2.2	0.0
		DM + CS	30	10.3 + 0.1	3999	133.0	9.7	413	90.7	46.8	76.5	3.7	0.0
		DM + CS	40	7.7 + 2.6	4007	100.3	7.4	309	67.6	34.9	57.2	3.0	0.0
		LC (DM + SM)	30	5.1 + 0.04	2002	70.6	4.5	207	45.3	23.0	37.9	1.5	0.0
Trial 3:	Tomato	DM* + SM + Compost	12	9.5 + 0.9 + 9.8	7248	620.8	71.1	414	234.7	118.2	75.5	164.1	6.0
On farm high		Compost/Control	14	9.8	3204	230.8	58.8	132	173.6	85.3	23.5	161.8	5.9
tunnel study													
Trial 4:	Pepper/ Eggplant	Suc + FM	34	7.2 + 0.7	3365	98.5	1.4	0.7	3.2	0.3	9.0	0.1	0.0
High tunnel study		CS* + FM	30	7.6 + 0.7	3415	114.0	2.6	1.1	3.8	0.5	11.3	0.2	0.0
		PS + FM	27	6.2 + 0.7	3287	120.9	15.2	6.0	35.4	3.0	99.0	1.1	0.1
		FM/Control	3	0.3	161	54.2	0.8	0.3	1.7	0.1	4.9	0.1	0.0

† DM, dry molasses/soy-hull product (Westway Feed Products, New Orleans, LA, USA); WB, wheat bran (Siemer Milling Company, Hopkinsville, KY, USA); SM, soybean meal (Hi Pro, Fiona, TX, USA); CS, corn starch (Tate and Lyle,. Decatur, IL, USA); DM, dry molasses/soy-hull product (Sweetix, Mankato, MN, USA); Suc, sucrose (Michigan sugar, Bay City, MI, USA); CS*, corn starch (Ingerdion Inc, Westchester, IL, USA), PS, pine shavings (America's Choice, Columbia, MD, USA); FM, feather meal (Mason City By-Products, Mason City, IA, USA).*

amendment (20:1 C:N ratio, 4 mg C g^{-1} soil) + compost, and ii) a standard practice treatment (compost amendment only at 1.31 kg dry matter m^{-2}) (**Table 1**). The ASD amendments and C:N ratio were chosen based on crop performance and inoculated pathogen mortality assessments in previous trials (Shrestha et al., 2018a, 2021). Prior to treatment, five 0–15 cm soil cores were collected from each plot. Amendment and compost samples were composited and analyzed for nutrient content as for ASD amendments in Trial 1 (**Table 1**). Amendments and compost were incorporated into plots using a rotovator to ∼15-cm depth. After incorporating amendments, plots were mulched with black polyethylene (0.032 mm) and drip irrigation installed. Plots were irrigated with 5-cm of water to ensure saturation to ∼20-cm depth. All plots were equipped with ORE and combination soil temperature/moisture sensors (5TM Soil Moisture and Temperature Probe, Decagon Devices, Pullman, WA, USA). Five 0–15 cm soil cores from each plot were collected at 7, 14, 21, and 32 days from treatment initiation. The core samples from each plot were composited and then used to evaluate soil pH and soil inorganic N as described in trial 1.

After 4 weeks of treatment incubation, three tomato cultivars (cvs. Sungold, Cherokee Purple and Valencia) were transplanted (16 plants per plot; 45-cm spacing) with each block planted to a single cultivar (two blocks per cultivar). Crop performance and leaf N were assessed as described in Trial 2. Total marketable tomato fruit yield data was collected 2–3 times each week from mid-June to mid-July.

Trial 4. ASD Amendment Substrate Bioavailability Effects on Soil Nutrients and Bell Pepper and Eggplant Performance

Experiments were established in two separate high tunnels at the University of Tennessee, Organic Crops Unit in Knoxville, TN, USA in spring 2016 to evaluate ASD amendment bioavailability effects on soil inorganic N, bell pepper and eggplant (*Solanum melongena* L.) leaf tissue N and crop yield. The experimental details are provided in Shrestha et al. (2020a) and ASD treatments included a range of amendments based on substrate bioavailability (sucrose, corn starch, pine shavings, each with feather meal added to bring to a ∼30:1 C:N amendment C:N ratio and ∼3.4 mg C g^{-1} soil) which were compared to an anaerobic control with feather meal amendment only (**Table 1**). The design of the experiment was a split plot randomized complete block design with six replicates. Crop (bell pepper or eggplant) was assigned as the whole plot and soil treatments (ASD treatments or control) as the split plot. Each high tunnel had six beds of length 12.2 × 1.22 m each, which were divided into four

plots receiving split-plot treatments at 3 × 0.6 m. The soil type according to the USDA classification system was a Dewey silt loam (fine, kaolinitic, thermic, Typic Paleudult). Treatments were established as described in other trials with irrigation supplied over 12 h through drip irrigation to fill soil pore space to an approximate 20-cm depth. IRIS tubes and ORE were inserted in each treatment on three beds of each high tunnel to determine anaerobic conditions, and soil samples collected from each plot as described previously.

Bell pepper (cv. Sweet Sunrise) and eggplant (cv. Traviata) were randomly assigned to whole plots and transplanted after 3 weeks of ASD treatment. Pepper transplants were planted double row per bed at 30-cm spacing. Eggplant transplants were planted in a single row with 45-cm spacing. Plants were harvested five times from mid-July to mid-August for bell pepper (at mature, yellow color) and seven times from late-June to mid-August for eggplant. End of row plants in each treatment were excluded and harvests were graded using standard USDA fruit grading as described previously. Pepper and eggplant leaf tissue from high tunnels were collected as described previously to evaluate leaf N for both crops.

Statistical Analysis

A mixed model analysis of variance was conducted with SAS (9.3 SAS Institute, Cary, NC, USA) for each trial; data were checked for normality and homogeneity of variances and transformed as needed (log or rank transformation). For trial 1, amendment type, amendment C:N ratio were considered fixed effects and trial was treated as a random effect, and a two-way factorial analysis between C amendment and C:N ratio performed. Data were also analyzed separately by C amendment to compare treatments with the anaerobic control and also by C:N ratios to compare with the anaerobic control. For field and high tunnel studies (trials 2, 3, 4) soil treatment was considered the fixed effect and block and year (or tunnel in trial 4) were considered random effects. Soil pH in trial 3 was analyzed separately by treatment and time points. Least squares means were compared with Fisher's P-LSD at 5% significance level and untransformed means and standard errors are reported. Relationships of crop yield (or biomass for trial 1) with soil inorganic N and leaf N were assessed for each trial with Pearson or Spearman correlation analysis at $P < 0.05$.

RESULTS

Soil Characteristics

Data for soil pH and soil anaerobic conditions for trials 1, 2 and 4 have been published previously (Shrestha et al., 2018a, 2020a). Here we summarize the overall results briefly to provide an overview of the environmental conditions during soil treatments (see **Supplementary Table 1**). There was no significant effect of ASD treatments on soil pH measured after ASD termination and/or during cropping phase for all studies except trial 1 (pot study) where the lowest pH of 5.1 was observed at ASD amendment C:N ratio of 10:1 for both amendment types, whereas for all other treatments soil pH ranged from 5.3 to 5.4. In trial 2 (field study) soil pH at ASD termination ranged from 5.8 to 6.0, and later during the cropping period increased by 0.1 to 0.3 pH units. Both high tunnel study (trials 3, 4) sites had higher soil pH than other studies, ranging from 6.9 to 7.3 (data not shown), which can be typical in protected culture systems in the region, largely due to lack of leaching rainfall under plastic cover and different amendment and irrigation rates and strategies than in open-field production systems (Knewtson et al., 2012). Soil pH taken in a series of sampling time points in trial 3 showed increases in soil pH during ASD incubation, which then trended downwards during the cropping phase regardless of treatment (**Figure 1**).

Accumulated soil anaerobic conditions were higher in all ASD treatments compared to their respective controls in all trials. For trial 1 (growth chamber/greenhouse studies) the more anaerobic condition was generated in dry molasses/soy hull amended treatments (190 to 234 V h) and wheat bran amended treatment (166 to 194 V h) compared to anaerobic controls (106 V h). In trial 2, although accumulated anaerobic conditions (173–201 V h) trended higher in all ASD treatments amended at 4 mg C g^{-1} soil when compared to ASD at 2 mg C g^{-1} soil and the anaerobic control, this comparison was not statistically significant due to a limited number of replicates for this measure. However, there were significant treatment effects on the percentage of iron oxyhydroxide reduction observed. The percentage of oxyhydroxide paint removal was higher in all ASD treatments at 4 mg C g^{-1} soil amendment rates (31 to 35% Fe solubilization) than in the reduced amendment rate treatment (17.5% Fe solubilization) and anaerobic control (8.6% Fe solubilization). Similar results were observed in trial 3 with higher accumulated anaerobic conditions observed for ASD (136 V h) than the compost-only control (15 V h) and for trial 4, ASD treatment with more bioavailable substrates (sucrose, corn starch) had more anaerobic soil conditions (127 and 67 V h, respectively) than less bioavailable substrate (pine shavings, 18 V h) or the feather meal-only control (7 V h). Similar differences among treatments were observed for the percentage of oxyhydroxide paint removal (**Supplementary Table 1**).

Effect of ASD Treatment on Soil and Plant N

Soil Inorganic N

Soil inorganic N (NH_4-N, NO_2-N + NO_3-N) measured at the termination of ASD treatment was significantly affected by ASD amendment and amendment C:N ratio in trial 1 (**Table 2**), amendment C:N ratio and amendment C rate in trial 2, by soil treatment in trial 3, and by soil treatment in trial 4 (**Table 3**). Soil inorganic N during the cropping phase was also significantly affected by treatments for all studies where evaluated (trials 1,2, and 3).

For trial 1, no significant interaction effect of ASD amendment and amendment C:N ratio was observed on total soil inorganic N at termination of ASD treatment (**Table 2**). Soil inorganic N at ASD termination was primarily NO_2-N + NO_3-N (72 to 92% of total inorganic N) at ASD termination, with lower NH_4-N (8 to 28% of total inorganic N), indicating sufficient soil oxidation allowing for nitrification of mineralized N by the termination of ASD treatments. Among C:N ratios, the highest mean soil

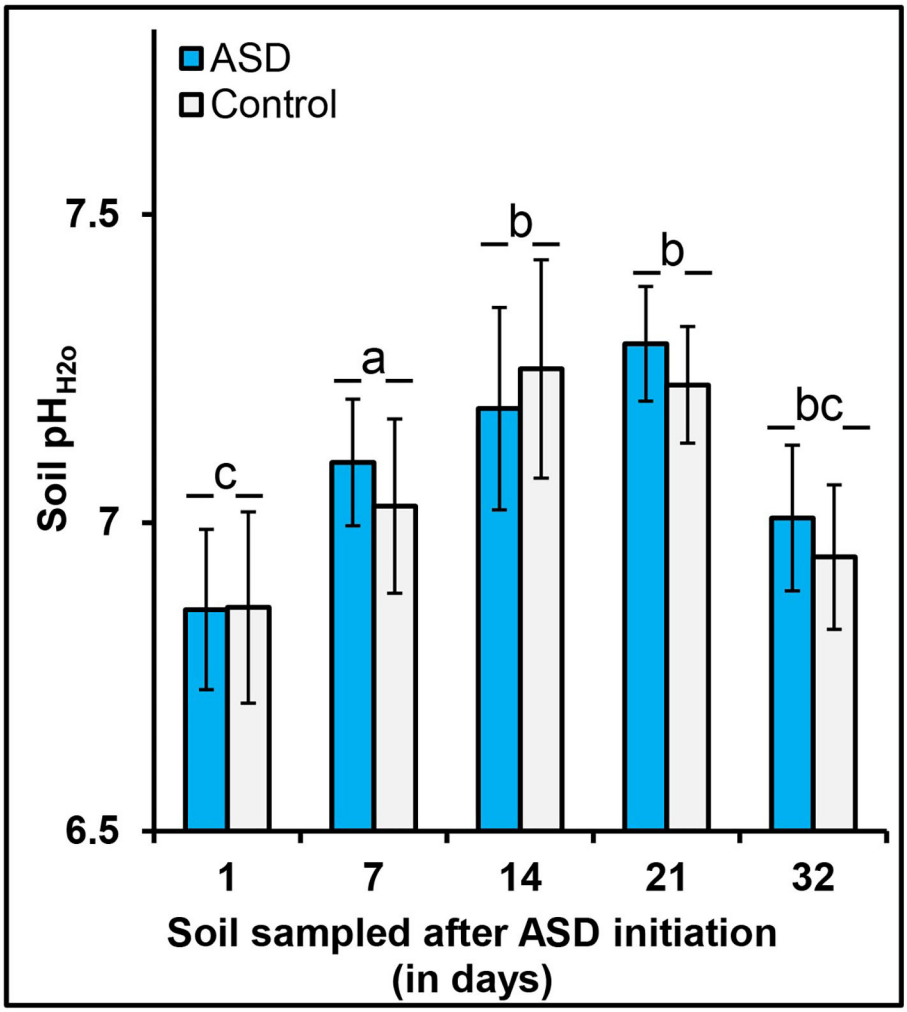

FIGURE 1 | Effect of ASD treatment and sampling time on soil pH, high tunnel, trial 3. Bars indicated by similar letters for sampling time are not significantly different at $P < 0.05$ according to Fisher's protected LSD test. Error bars indicate standard error with 12 total replicates (6 replicates × 2 experiments).

TABLE 2 | Main effect of ASD amendment and analysis of variance for soil nitrogen response variables after ASD and/or during cropping phase as affected by soil treatment in trial 1.

	Post ASD			Cropping phase		
	NH_4-N	**$NO_2 + NO_3$-N**	**Total inorganic N**	**NH_4-N**	**$NO_2 + NO_3$-N**	**Total inorganic N**
			mg N kg^{-1} soil			
Dry molasses	9.1 ± 1.8 b	74.7 ± 13.2 a	83.8 ± 14.2 a	18.1 ± 3.7 a	64.8 ± 9.6 a	82.8 ± 12.0 a
Wheat bran	15.4 ± 2.6 a	77.0 ± 14.3 a	95.6 ± 16.9 a	9.5 ± 1.0 a	51.7 ± 5.8 a	61.2 ± 6.2 a
Control	3.7 ± 0.4 c	24.5 ± 3.2 b	28.2 ± 3.4 b	12.9 ± 4.6 a	33.1 ± 8.0 b	46 ± 12.4 b
			P-value			
Amendment	<0.001	0.04	0.02	NS[†]	0.01	0.01
C:N ratio	<0.001	<0.001	<0.001	NS	0.01	0.02
Amendment × C:N ratio	NS	NS	NS	NS	NS	NS

[†] *NS, not significant; P > 0.05.*

Values represent means and standard errors of 8 total replicates (4 replicates × 2 experiments).

TABLE 3 | Analysis of variance for soil nitrogen and leaf tissue nitrogen response variables after ASD and/or during cropping phase as affected by soil treatment in trials 2, 3 and 4.

	Post ASD			Cropping phase			
	NH_4-N	NO_2+NO_3-N	Total inorganic N	NH_4-N	NO_2+NO_3-N	Total inorganic N	Leaf tissue N
				P-value			
Trial 2: Field study	<0.001	<0.001	<0.001	0.04	<0.001	<0.001	<0.001
Trial 3: On farm high tunnel study	0.002	NS†	NS	NS	0.003	0.003	NS
Trial 4: High tunnel study	NS	<0.001	<0.001	N/A‡	N/A	N/A	N/A

†*NS, not significant; P > 0.05;* ‡*N/A, not applicable, was not evaluated in trial 4.*

inorganic N was observed at C:N ratio of 10:1 (212 mg N kg^{-1} soil; **Figure 2A**), and among amendments the highest total soil inorganic N was observed for wheat bran-based (241.0 mg N kg^{-1} soil for C:N 10:1, 9 times higher than control) followed by dry molasses/soy hull-based (181.9 mg N kg^{-1} soil for C:N 10:1, 6 times higher than the control). The anaerobic control had the least amount of total inorganic N at the time of ASD treatment termination (28.2 mg N kg^{-1} soil; **Figure 2A**). During the cropping phase, soil NO_2-N + NO_3-N was highest at C:N ratios of 10:1 and 20:1 (>60 mg NO_2-N + NO_3-N kg^{-1} soil) and total soil inorganic N was similarly higher than the control only at C:N ratios of 10:1 and 20:1 when compared to the control (**Figure 2B**). There was no significant effect of ASD amendment or amendment C:N ratio on soil NH_4-N during the cropping phase (**Table 2**).

Prior to ASD treatment in trial 2, total soil inorganic N ranged from 5.7 to 7 mg N/kg of soil (3 to 4.7 mg NH_4-N kg^{-1} soil and 1.9 to 2.8 mg NO_2-N + NO_3-N kg^{-1} soil). As in trial 1, dry molasses/soy hull-based amendments at the 10:1 C:N ratio significantly increased soil total inorganic N (63.3 mg N kg^{-1} soil), soil NO_2-N + NO_3-N (27.7 mg NO_2-N + NO_3-N kg^{-1} soil) and NH_4-N (35.4 mg NH_4-N kg^{-1} soil) at the termination of ASD treatment (**Figure 3A**). The fumigated treatment and the ASD treatment at the 20:1 amendment C:N ratio had intermediate total inorganic N (17 mg N kg^{-1} soil) and the total inorganic N was similar at all other ASD/C:N ratio treatments and the anaerobic control (9.1 mg N kg^{-1} soil; **Figure 3A**). However, during the cropping phase with equal fertigation management, higher soil inorganic N was observed at all ASD treatments except for the reduced amendment rate when compared to control and fumigated treatments (**Figure 3B**). This was especially the case at the 10:1 amendment C:N ratio, where total inorganic N was five-fold higher than the fumigated or anaerobic controls, whereas at 20:1 to 40:1 C:N ratios total inorganic N was only two-fold higher than the controls.

In trial 3, ASD treatment increased total soil inorganic N as the incubation period progressed and was significantly higher than the compost-only control at 2 to 3 weeks post treatment termination (**Figure 4**). During the ASD incubation period, soil NH_4-N was significantly higher in the ASD treatment at 2 weeks (4.8 vs. 3.5 mg NH_4-N kg^{-1} soil) and 3 weeks (6.3 vs. 4.9 mg NH_4-N kg^{-1} soil, **Table 3**) compared to the compost-only control (**Figure 4**). There was no significant difference among treatments in soil inorganic N during the 1st week of treatment incubation (**Table 3**). At the end of ASD treatment incubation in trial 4, ASD treatments amended with pine shavings + feather meal had the highest total inorganic N (116.0 mg N kg^{-1} soil; primarily NO_2-N + NO_3-N), the feather meal-only control was intermediate (83.0 mg N kg^{-1} soil), and total soil inorganic N was lowest from the ASD treatments amended with sucrose + feather meal or corn starch + feather meal (44.5–55.7 mg N kg^{-1} soil; **Figure 5**).

Leaf/Shoot Tissue N and Correlation With Soil N

In trial 1, there was no significant correlation between plant tissue N and soil inorganic N during ASD termination or the cropping phase (**Table 4**). Tomato shoot tissue N was significantly affected by the main effect of ASD amendment, but not amendment C:N ratio or the interaction between the ASD amendment and ASD amendment C:N ratio (**Table 5**). Wheat bran-based ASD amendments, averaged across C:N ratios, had the highest shoot tissue N (36.9 mg N g^{-1}) compared to dry molasses/soyhull-based amendments and the control (27.8–28.7 mg N g^{-1}; **Table 5**).

In trial 2, bell pepper leaf tissue N was positively correlated with soil NH_4-N and total soil inorganic N (0.3, $P < 0.05$) and significantly affected by soil treatments ($P < 0.001$, **Table 4**). Among treatments, there was a higher leaf tissue N concentration from treatments with low C:N ratio ASD amendments (e.g., 10:1, 57.1 mg N g^{-1}) than high C:N ratio ASD amendments (e.g., 40:1, 50.5 mg N g^{-1}; **Figure 6A**). The lowest leaf tissue N was observed in the low carbon rate amendment treatment (30:1 C:N ratio, 48.7 mg N kg^{-1}). Leaf tissue N in the fumigated treatment and anaerobic control treatment was intermediate (51.6–54.9 mg N kg^{-1}), and the fumigated treatment did not differ from any ASD treatment at the 4 mg C g^{-1} soil amendment rate (**Figure 6A**).

In the on-farm high tunnel trial (trial 3), tomato leaf tissue N did not differ in the ASD treatment (53.5 mg N g^{-1}) compared to the compost-only control (51.5 mg N g^{-1}; **Figure 6B**). Similarly, in the research farm high tunnel trial (trial 4) ASD amendments of sucrose + feather meal (36.8 mg N g^{-1}) and pine shavings + feather meal (38.2 mg N g^{-1}) had similar leaf tissue N compared to corn starch + feather meal and the feather meal only control (40.2–42.0 mg N g^{-1}; **Figure 6C**). For eggplant leaf tissue N, no differences were observed among amendments with leaf tissue concentrations ranging from 44.2 to 46.8 mg N g^{-1} for all treatments (**Figure 6D**). A significant, but moderate positive relationship of leaf tissue N with post-ASD soil NH_4-N was observed in trial 3 with tomato (0.4, $P < 0.05$) and in

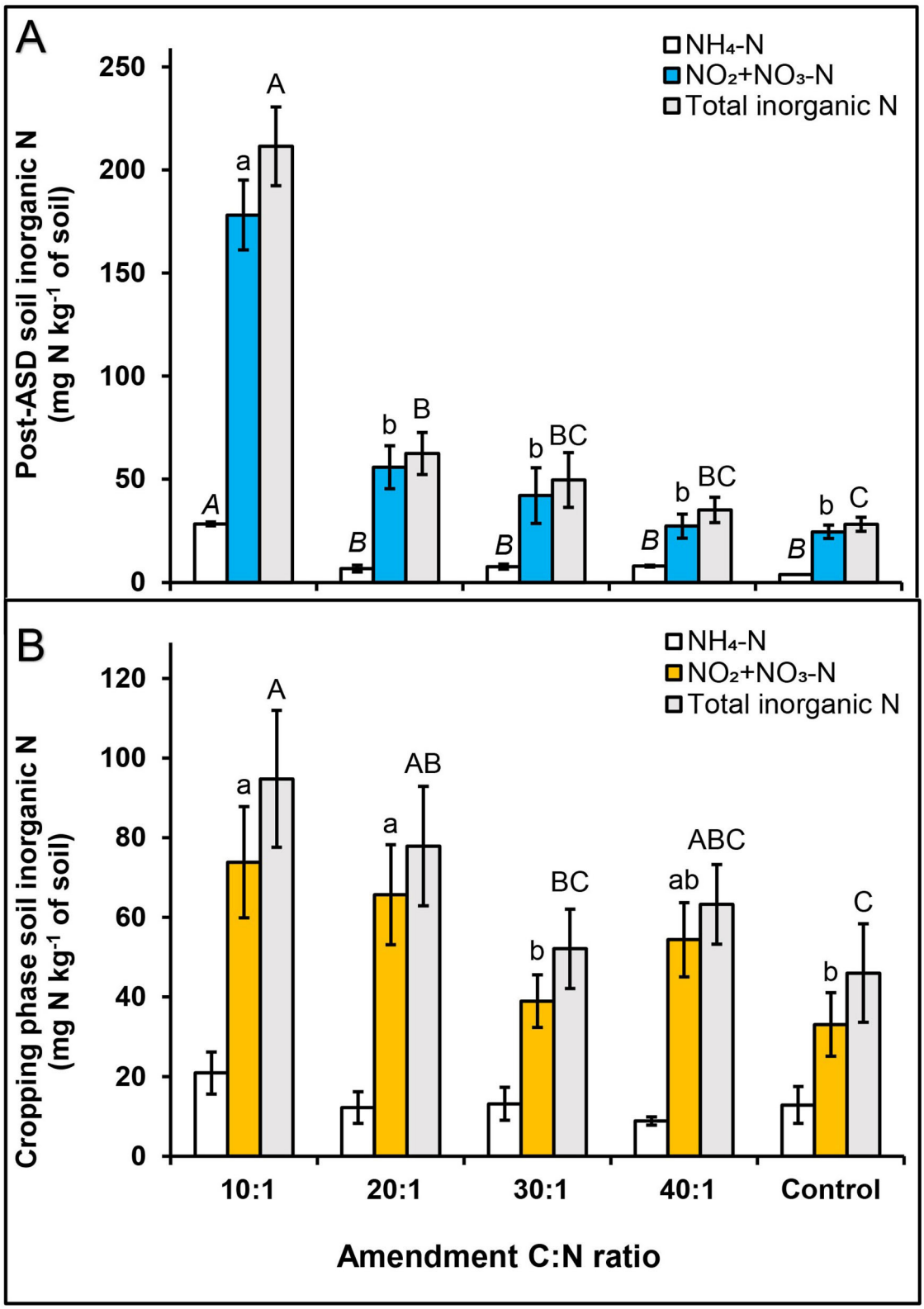

FIGURE 2 | Effect of ASD amendment C:N ratio across amendment types (dry molasses/soybean hull-based and wheat bran-based) on soil inorganic N at **(A)** post-ASD treatment and **(B)** during cropping phase, trial 1. Bars indicated by similar letters for each category are not significantly different at $P < 0.05$ according to Fisher's protected LSD test. Error bars indicate standard error with eight total replicates (4 replicates × 2 experiments). Control = anaerobic, non-amended control.

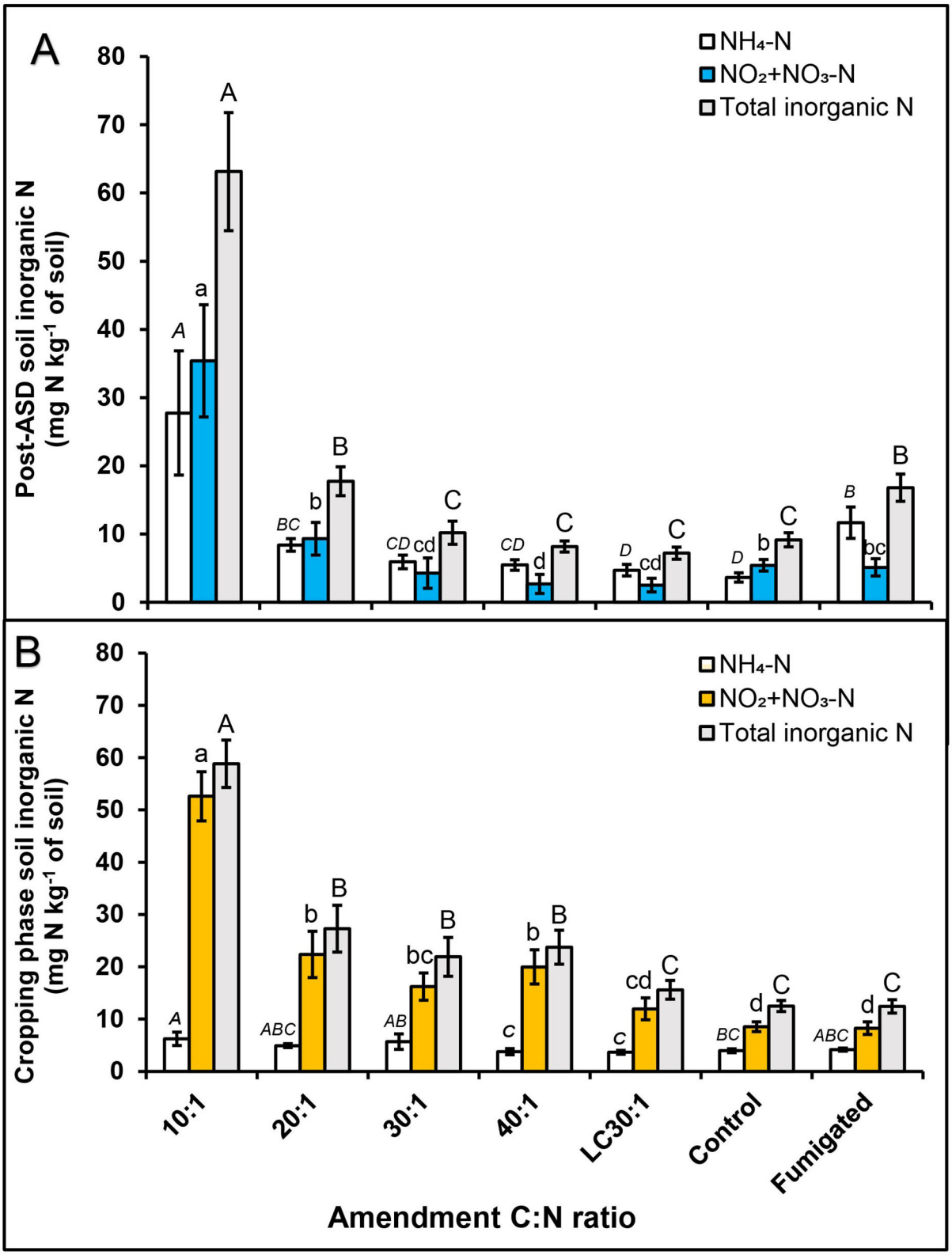

FIGURE 3 | Effect of dry molasses/soybean hull-based ASD amendment C:N ratio on soil inorganic N, **(A)** post-ASD treatment and **(B)** during cropping phase, trial 2. Bars indicated by similar letters for each category are not significantly different at $P < 0.05$ according to Fisher's protected LSD test. Error bars indicate standard error with eight total replicates (4 replicates × 2 experiments). LC = Low carbon, Control = anaerobic, non-amended control, Fumigated = methyl bromide (MeBr) fumigated control (67:33 mixture with chloropicrin, 224 kg ha^{-1}).

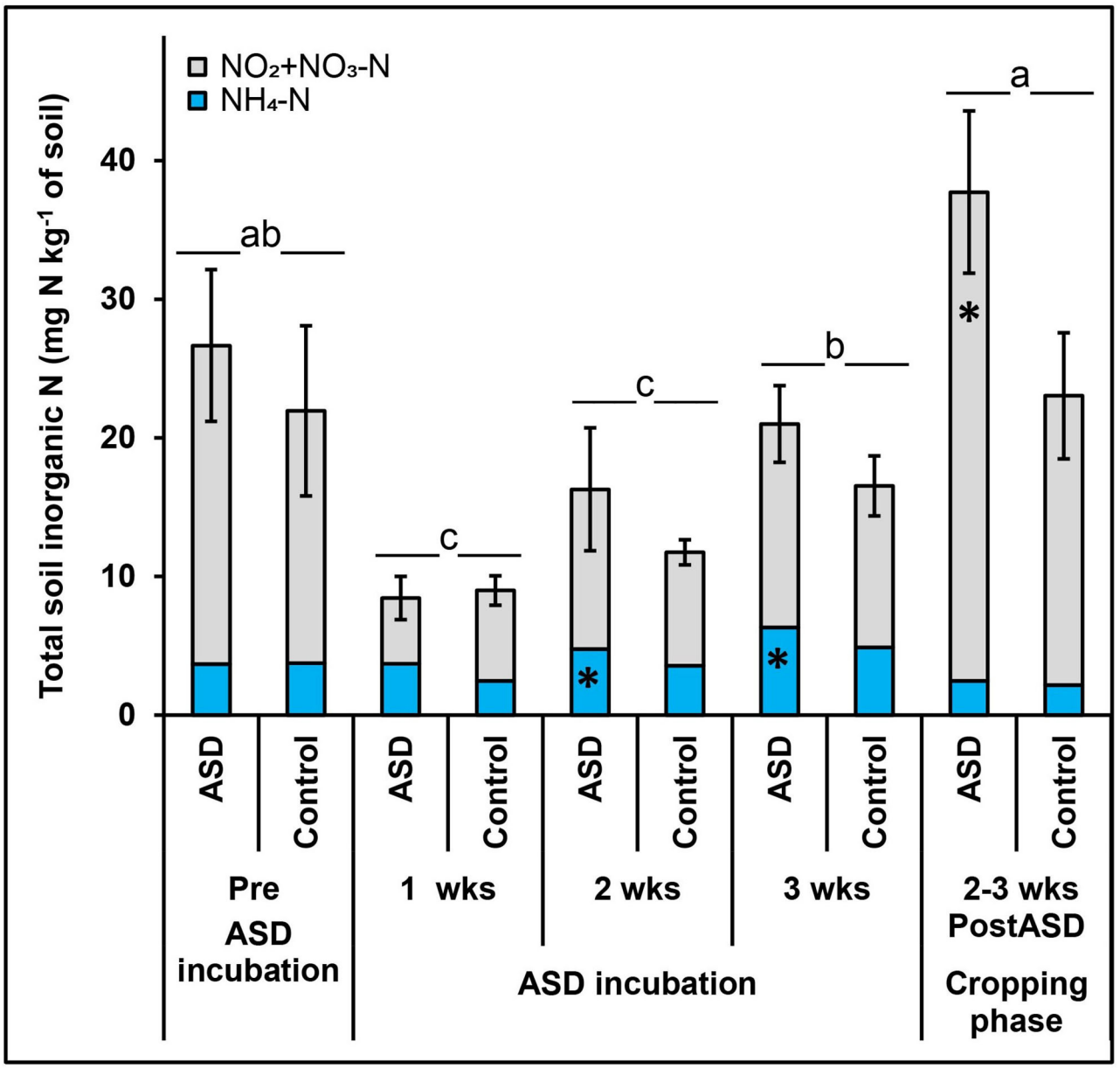

FIGURE 4 | Effect of ASD on soil inorganic N post-ASD treatment high tunnel study, trial 3. Bars indicated by similar letters for each category are not significantly different at $P < 0.05$ according to Fisher's protected LSD test and means indicated by * are significantly different from the control. Error bars indicate standard error with twelve total replicates (6 replicates × 2 experiments). Control = anaerobic, compost-amended control.

trial 4 with bell pepper (0.5, $P < 0.01$). Eggplant leaf tissue N in trial 4 correlated moderately and positively with post-ASD soil NO_2-N + NO_3-N and total soil inorganic N (0.4, $P < 0.05$, **Table 4**).

Crop Performance and Correlation to Soil and Leaf Tissue N

Tomato plant growth in trial 1 was significantly affected by ASD amendment and amendment C:N ratio without any interaction (**Table 5**). Fruit yield and dry shoot biomass was higher in treatments with dry molasses/soy hull-based ASD amendments (57 g fruit plant^{-1} and 51 g dry biomass plant^{-1}, respectively) compared to the wheat bran-based ASD amendments (37 and 42 g plant^{-1}, respectively) and the non-amended control (20 and 38 g plant^{-1}, respectively; **Table 5**). Among C:N ratios, the highest mean fruit weight, root and shoot biomass were observed at C:N ratio of 10:1 (**Table 5**).

In trial 2, the total number of bell pepper fruit per plant was significantly higher in ASD treatments with lower C:N ratios (10 and 20:1), compared to fumigated and anaerobic control treatments. However, this difference was only observed in the

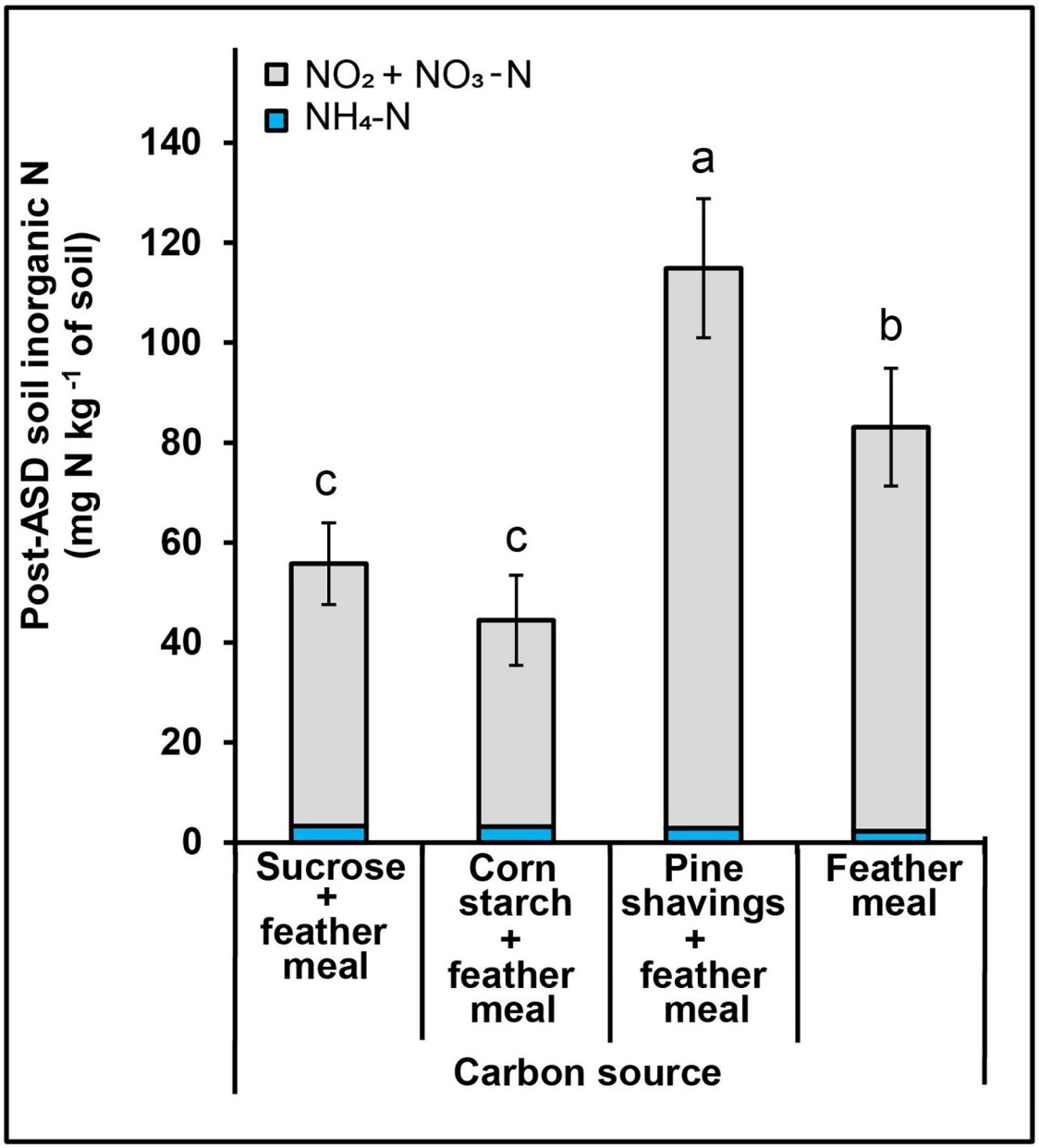

FIGURE 5 | Effect of ASD amendment (various C substrate) on soil inorganic N post-ASD treatment high tunnel study, trial 4. Bars indicated by similar letters are not significantly different at $P < 0.05$ according to Fisher's protected LSD test. Error bars indicate standard error with twelve replicates.

2014 season (data not shown). Marketable yield (30 to 35 Mg ha^{-1}) and fancy-grade yield (11.3 to 15.7 Mg ha^{-1}) was higher across both years in all ASD treatments amended at 4 mg C g^{-1} soil, and lowest in fumigated and anaerobic control treatments (marketable, 20 to 24 Mg ha^{-1} and fancy-grade, 4.4 Mg ha^{-1}; **Figure 6A**). Similarly, total fruit yield (marketable + culled fruits) was highest in ASD treatments at 10, 20 and 30:1 ASD amendment C:N ratios (at 4 mg C g^{-1} soil; 33.9 to 39.2 Mg ha^{-1}), and lowest in fumigated (25.2 Mg ha^{-1}) and anaerobic control treatments (29.3 Mg ha^{-1}).

In trial 3, the number of tomato fruit per meter of row was increased by 21% in ASD treatment (8.1 kg m^{-1}) compared to the compost-amended control treatment (6.7 kg fruit m^{-1}; **Figure 6B**). In trial 4, the highest fancy-grade bell pepper yield among treatments was observed for corn starch + feather meal ASD treatment (6 kg m^{-1}), however, this was not significantly higher than the feather meal only control (**Figure 6C**). Similarly, eggplant yield did not differ significantly among treatments and ranged from 7.3 kg m^{-1} in the sucrose + feather meal ASD treatment to a low of 6.2 kg m^{-1} in the feather meal only control

TABLE 4 | Correlation analysis of crop performance variables and soil and leaf tissue N in each trial.

	Post-ASD soil			Cropping phase soil			Plant N
	NH_4-N	NO_2+NO_3-N	Total inorganic N	NH_4-N	NO_2+NO_3-N	Total inorganic N	
Trial 1: Greenhouse study[†]				Correlation coefficient			
Fruit weight	0.4*[†]	0.37*	0.42**	0.09	−0.14	0.003	−0.27
Dry shoot biomass	0.04	0.28	0.04	0.1	0.06	0.1	−0.47**
Dry root biomass	−0.01	0.08	−0.06	0.45**	0.64***	0.52***	0.19
Trial 2: Field study							
Leaf tissue N	0.33*	0.16	0.28*	−0.03	0.14	0.13	–
Fancy yield	0.11	0.31*	0.28*	−0.003	0.47***	0.45**	0.34*
Total marketable yield	0.11	0.11	0.14	−0.08	0.18	0.17	0.56***
Total yield	0.11	0.1	0.13	−0.1	0.14	0.12	0.6***
Trial 3: On farm high tunnel study							
Leaf tissue N	0.42*	0.12	−0.08	0.24	0.13	0.12	–
Tomato total yield	0.73***	0.06	0.37	0.73***	0.65***	0.67***	0.51*
Trial 4: High tunnel study							
Eggplant leaf tissue N	0.16	0.44*	0.43*	–	–	–	–
Pepper leaf tissue N	0.52**	−0.08	−0.05	–	–	–	–
Eggplant total yield	−0.33	−0.22	−0.24	–	–	–	−0.17
Pepper total yield	−0.43*	−0.14	−0.15	–	–	–	−0.43*

[†] *Each row within each variable represents Pearson or Spearman correlation coefficients. Correlation is significant at *P < 0.05, **P < 0.01, and ***P < 0.001. Correlation coefficients without * are non-significant at P > 0.05.*

TABLE 5 | Mean values by main effects and analysis of variance for growth characteristics tomato plant 8-week post termination of ASD treatment as affected by carbon amendment, C:N ratio and the interaction in trial 1.

	Shoot N (mg N g^{-1})	Fruit weight (g $plant^{-1}$)	Fruit number (fruit $plant^{-1}$)	Shoot height (cm)	Dry shoot biomass (g $plant^{-1}$)	Dry root biomass (g $plant^{-1}$)
Amendment						
Dry molasses	27.8 ± 0.9 b	57.1 ± 5.2 a	6.4 ± 0.5 a	36.9 ± 1.1 a	50.6 ± 2.1 a	0.9 ± 0.1 a
Wheat bran	35.7 ± 1.2 a	36.9 ± 4.8 b	5.4 ± 0.6 a	31.6 ± 1.1 b	41.7 ± 2.6 b	0.8 ± 0 a
Control	28.7 ± 1.1 b	19.7 ± 4.5 c	3.4 ± 0.4 b	33.7 ± 1.1 ab	37.5 ± 1.3 b	0.8 ± 0.1 a
C:N ratio						
10	30.4 ± 1.2	70.0 ± 6 a	7.1 ± 0.9 a	36.3 ± 1.6	53.5 ± 3.7 a	1.0 ± 0.1 a
20	30.7 ± 1.4	48.7 ± 8.7 b	6.3 ± 0.6 a	34.6 ± 1.6	45.9 ± 4 ab	0.8 ± 0.1 b
30	30.7 ± 2.0	41.0 ± 6.6 bc	6.6 ± 0.8 a	34.3 ± 1.7	45.5 ± 2.9 ab	0.8 ± 0.1 b
40	35.3 ± 2.1	28.4 ± 4.1 cd	3.6 ± 0.4 b	31.8 ± 1.6	39.7 ± 2.8 b	0.7 ± 0.1 b
Control	28.7 ± 1.1	19.7 ± 4.5 d	3.4 ± 0.4 b	33.7 ± 1.1	37.5 ± 1.3 b	0.8 ± 0.1 b
			P-value			
Amendment	<0.001	<0.001	0.004	0.002	0.001	NS
C:N ratio	NS	<0.001	<0.001	NS	0.004	0.028
Amendment × C:N ratio	NS	NS	NS	NS	NS	NS

Values represent means and standard errors of 8 total replicates (4 replicates × 2 experiments).

(**Figure 6D**). The only significant difference was observed in culled fruit mass where the highest culled fruit mass was recorded for the sucrose + feather meal ASD treatment for both pepper and eggplant (**Figures 6C,D**).

There was significant moderate positive relationship between tomato fruit weight with post-ASD total soil inorganic N (0.4, $P < 0.05$), tomato root biomass with cropping phase soil inorganic N (0.4 to 0.6, $P < 0.01$) and a moderate negative correlation between plant N and dry shoot biomass (−0.5, $P < 0.01$) in trial 1 (**Table 4**). For trial 2, total fancy-grade bell pepper yield had a positive moderate relationship with cropping phase soil NO_2-N+NO_3-N and total soil inorganic N (0.5, $P < 0.01$), but neither correlated significantly to total yield or total marketable yield. Bell pepper leaf N in trial 2 had a

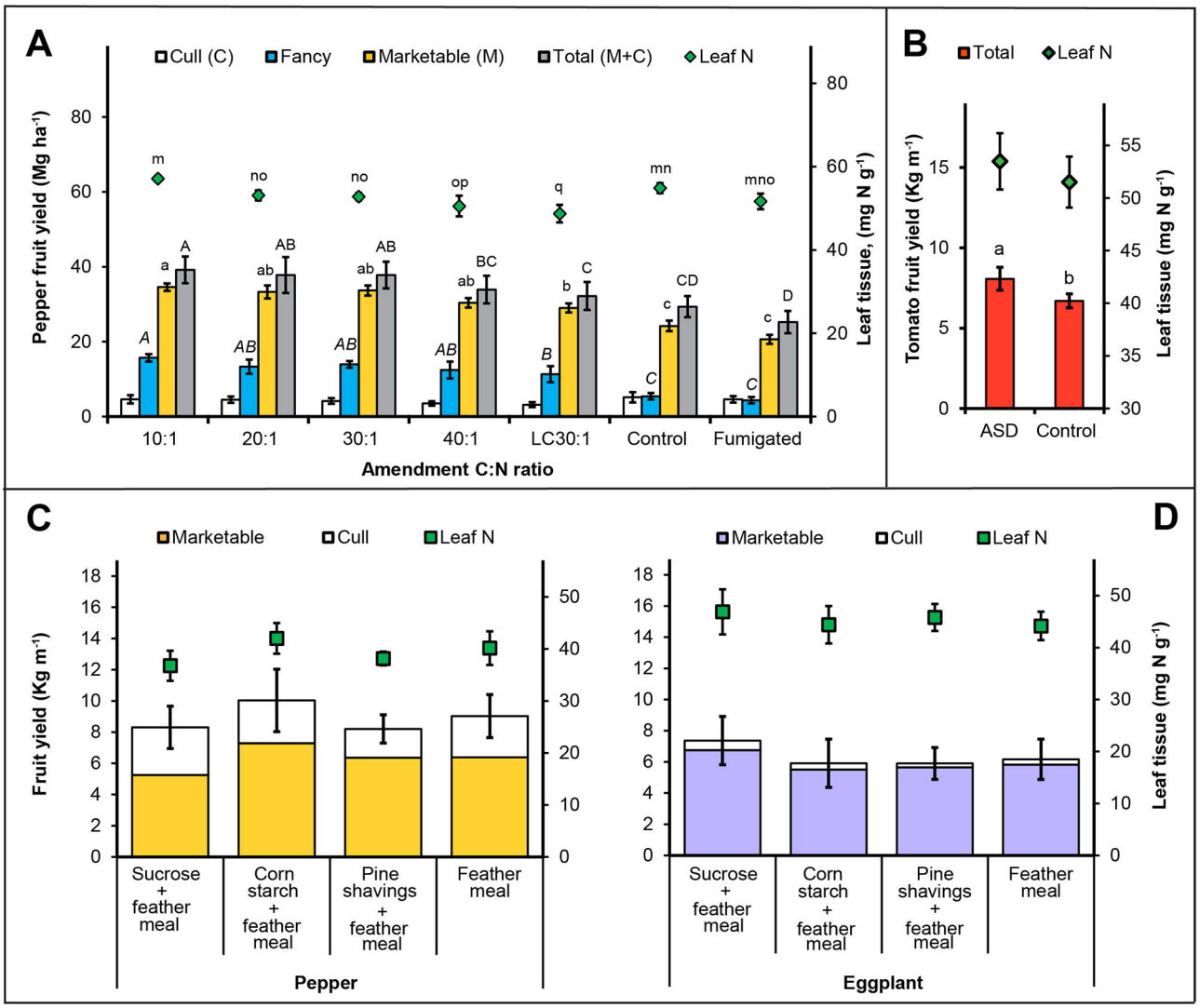

FIGURE 6 | Crop yield and leaf tissue N in response to ASD amendment **(A)** C:N ratio field study, trial 2, **(B)** on-farm study, trial 3 and **(C)** ASD substrate study, trial 4. Bars indicated by similar letters are not significantly different at P<0.05 according to Fisher's protected LSD test. Error bars indicate standard error with eight **(A)**, 12 **(B)**, six **(C)** and six **(D)** replicates.

moderate positive relationship with fancy and total marketable yield (0.6, $P < 0.001$). Similarly, total yield in trial 3 was strongly positively correlated with post-ASD soil NH_4-N and cropping phase soil inorganic N (0.7, $P < 0.001$). Leaf tissue N content was moderately correlated to total yield. We did not observe any significant positive correlation of fancy-grade or total marketable yield, leaf N or post-ASD soil inorganic N for eggplant or pepper in trial 4 (**Table 4**).

Discussion

Soil treatment by ASD has been proven to be an effective method to control various pests, especially fungal pathogens and plant parasitic nematodes (e.g., Shrestha et al. 2016). The effect of ASD treatments on horticultural crop yield is not as comprehensively described in the literature, as much research and developmental work in ASD systems has focused on evaluating mechanisms of plant pathogen control, or on applied research optimizing ASD treatment systems to specific local environmental factors and available amendments to control important diseases of regional cropping systems. The relationship of ASD treatment to crop yield is also complex (Butler et al., 2014a), not unlike the mechanisms that increase crop yield post soil fumigation or solarization (Stapleton et al., 1985; Chen et al., 1991; Stapleton, 2000) or with crop rotation (Bennett et al., 2012) or the addition of organic amendments in aerobic soil environments (Gamliel et al., 2000; Wortman et al., 2017; Chen et al., 2018; Barzee et al., 2019; Bonanomi et al., 2020). First, and perhaps most importantly considering the motivation of ASD treatment or other soil disinfestation practices, is the potential yield benefit of controlling plant diseases as compared to systems without soil disinfestation or soil fumigation treatment. In research studies, this would require sufficient plant disease that negatively impacts

yield, which is not always predictable given the environmental conditions important to disease occurrence and the difficulty in controlling such conditions in field research. At the same time, ASD systems may also increase crop yield through organic-amendment induced changes in soil chemical, physical and biological properties (Butler et al., 2014a; Rosskopf et al., 2015). These changes include beneficial changes to the soil environment post ASD treatment such as increased soil nutrient availability (Butler et al., 2014a; McCarty et al., 2014), increased water or nutrient holding capacity especially in sandy soils (Chen et al., 2018; Minasny and McBratney, 2018), or increased populations and crop-associations of plant growth promoting fungi or bacteria (e.g., Mazzola et al., 2018; Poret-Peterson et al., 2019; Shrestha et al., 2020b).

In a meta-analysis of work published prior to 2016, Shrestha et al. (2016) reported that of 68 published comparisons of an ASD treatment to a non-amended control treatment, ASD treatments had 30% higher yield than control treatments on average but this was not statistically different. In 55 published comparisons of an ASD treatment to a fumigated treatment, yields were essentially equivalent in ASD and fumigated treatments (ASD yields numerically 6% higher). Results from this meta-analysis also suggest that yield response can vary due to effects of amendment properties and rates of application, although the limited number of published studies limits inferences that can be made (Shrestha et al., 2016). This is congruent with our understanding of the mechanisms of ASD effects on crop yield, as we would expect both effects on soilborne plant pathogens and soil physical, chemical and biological properties to be affected by amendment properties and application rates (Butler et al., 2014b; Shrestha et al., 2016, 2018a; Mazzola et al., 2018).

In the present study, we show that ASD amendment types and properties such as C:N ratio have important effects on solanaceous crop biomass and yield, even under recommended crop fertilization regimes, and in the relative absence of yield limiting crop disease. To our knowledge, this is the first study to specifically evaluate ASD amendment C:N ratio effects on soil inorganic N, crop N status and crop yield. At the same time, our results are consistent with other reports of high soil inorganic N following soil disinfestation with ASD amendments with relatively low C:N ratios (Butler et al., 2014a; McCarty et al., 2014; Di Gioia et al., 2017; Shrestha et al., 2018b). Soil microbial stoichiometry is fairly constrained within a C:N ratio of ∼5:1 to 7:1, which along with microbial energetic needs causes substrate C:N ratios above ∼20:1 to lead to N limitations on microbial decomposition and reduced available soil inorganic N (Sinsabaugh et al., 2013; Spohn, 2015). In the absence of data to optimize ASD treatments to both control important soilborne plant pathogens and to promote crop yield, crop managers may find utility in using ASD amendment mixtures with a C:N ratio near 20:1 (and amendment rates near 4 mg C g^{-1} of soil), where both N mineralization or N immobilization caused by the added amendment will be limited. This will allow for minimal changes to existing solanaceous crop fertilization practices, while still potentially improving crop yield compared to grower standard treatments, as we observed in trials 2 and 3. As our trials were conducted across relatively similar soil and environmental conditions typical to many warm-temperate to tropical production conditions for solanaceous crops, our results may be less applicable to environmental conditions typical to production of cooler climate crops or with vastly different soil types (such as coarsely textured soils).

While we expect that ASD amendment substrate decomposability has important effects on soilborne plant pathogens (e.g., Shrestha et al., 2016, 2021), initial effects on soil inorganic N in our study did not lead to substantial differences in crop performance, likely because C:N ratios were relatively standardized (∼30:1) across substrates with feather meal, a relatively easily available form of organic N. This suggests that crop performance benefits of ASD treatments (other than soilborne disease suppression) may result from a range of substrate types, if ASD amendment C:N ratios are relatively low. Highly recalcitrant substrates, such as the pine shavings in trial 4, likely do not induce microbial growth at rates sufficient to significantly induce N immobilization during the ASD incubation or during the post-ASD cropping phase. This suggests that there are limits to use of C:N ratio in guiding crop fertility decisions with ASD treatment (e.g., Bengtsson et al., 2003). While highly recalcitrant substrates are not typically suitable for ASD treatment due to low decomposability and thus low production of anaerobic decomposition metabolites during treatment incubation, amendment mixtures can contain relatively recalcitrant forms of C (such as lignified components of cover crop biomass or crop residues, or highly processed C compounds in composted materials), and these components may exert less influence on post-treatment N availability than would be evident from C:N ratio alone. At the same time, amendment mixtures often do not mineralize in a purely additive manner, as antagonistic and synergistic (i.e., priming) effects of amendment mixture components can alter decomposition rates of mixed residues (e.g., Bending and Turner, 1999; Maisto et al., 2011; Truong and Marschner, 2018). Given there are many well-developed simulation models to describe and estimate nitrogen transformation dynamics in anaerobic conditions (e.g., DNDC; Li, 1996) and decomposition dynamics and N mineralization from organic residues (e.g., CERES–N; Quemada and Cabrera, 1995) based on environmental conditions and biochemical composition, future work to model these dynamics using similar tools for ASD treatment systems would likely have high utility for crop managers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

US co-designed all trials and conducted trials 1, 2 and 4, assisted in trial 3, performed related lab work, completed

all statistical analyses, and wrote the initial draft of the manuscript. KS conducted trial 3, assisted in trial 4. DB co-designed all trials, obtained funding for all trials, supervised trial completion in his lab group, and provided critical revisions to the manuscript draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2021.694820/full#supplementary-material

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Mechanisms of Anaerobic Soil Disinfestation: Volatile Fatty Acids Reduce Viability of *Athelia* (*Sclerotium*) *rolfsii* Sclerotia in Acidic Soil Conditions and Have Limited Effects on Endemic *Trichoderma* spp.

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Volatile fatty acids (VFAs), such as acetic and *n*-butyric acid, released during anaerobic decomposition of organic soil amendments during anaerobic soil disinfestation (ASD) likely play a role in soilborne plant pathogen inoculum suppression. However, research is limited on the direct effects of soil VFA exposure on fungal plant pathogen inoculum, effects on pathogen antagonists such as *Trichoderma* spp., and the role of soil microbial VFA metabolism on reducing exposure effects. The present study addresses these limitations through a series of studies evaluating the effects of VFA (acetic or *n*-butyric acid), VFA concentration (4, 8, or 16 mmol/kg soil), soil sterilization by autoclaving, and soil amendment on the viability of *Athelia rolfsii* (*Sclerotium rolfsii*) sclerotia post VFA exposure, and soil populations of *Trichoderma* spp. HCl and water-only controls were included. After 4-days exposure in an acidic, anaerobic environment, sclerotial viability, and colonization by culturable fungi or bacteria were assessed with standard procedures. Greenhouse experiments were similarly conducted to evaluate endemic soil populations of *Trichoderma* spp. following soil exposure to VFAs and *Trichoderma* spp. populations assessed with standard soil dilution plating onto semi-selective medium. Sclerotial germination was generally reduced by soil exposure to acetic (35.1% germination) or *n*-butyric (21.9% germination) acids compared to water (74.3% germination) and HCl (62.7% germination). Germination was reduced as VFA concentration increased from 4 to 8 and 16 mmol/kg (39.5, 29.1, and 16.9%, respectively). In amended soils, there was no difference in sclerotial germination compared to non-amended soils, but in the greenhouse experiment there was a *Trichoderma* spp. population increase of over 300% in amended soil [3.4×10^6 colony forming units (CFU)/g soil] compared to the non-amended soil (9.6×10^5 CFU/g soil). Soil autoclaving had no effect on sclerotial germination at low VFA concentrations, but sclerotial germination was reduced at higher VFA concentrations compared to non-autoclaved soil. Our results suggest that VFAs

contribute to sclerotial mortality in strongly acidic soil environments, and mortality is influenced by VFA components and environment. Antifungal activity is less for acetic acid than for *n*-butyric, and less in non-sterile soil environments more typical of field conditions than in sterile laboratory conditions.

Keywords: biological soil disinfestation, organic acids, organic amendment, biological control, soilborne fungal plant pathogens, southern blight

INTRODUCTION

Biological or anaerobic soil disinfestation (ASD) is a feasible alternative to soil fumigation for soilborne plant pathogen inoculum control in several environments and cropping systems (Butler et al., 2014; Shennan et al., 2014; Rosskopf et al., 2015; Shrestha et al., 2016), but specific control mechanisms are not well-described across environments and pathosystems. Numerous changes to soil chemical, physical, and biological properties likely contribute to ASD treatment effects (Runia et al., 2014; Hewavitharana et al., 2015; Rosskopf et al., 2015; Hewavitharana and Mazzola, 2016; Shrestha et al., 2021). Volatile fatty acids (VFAs), including acetic acid, *n*-butyric acid, isobutyric acid, valeric acid, and isovaleric acid, are present in varying rates in biologically active moist soils, but are formed at high rates during the anaerobic decomposition of labile organic amendments during ASD and can create strongly acidic soil conditions for a brief time (Blok et al., 2000; Momma et al., 2006; Runia et al., 2014). The VFAs are then readily metabolized by aerobic microbes during soil oxidation that would follow ASD treatment (Adeleke et al., 2017). There are multiple reports that ASD treatment induces relatively high soil concentrations of acetic and *n*-butyric acid (Momma et al., 2006; Runia et al., 2014; Huang et al., 2015; Shrestha et al., 2020a). These compounds are likely an important factor in control of inoculum of some soilborne fungal plant pathogens, including *Fusarium oxysporum* and *Verticillium dahliae*, and plant parasitic nematodes such as *Pratylenchus penetrans* and *Pyrenochaeta terrestris* (Blok et al., 2000; Shinmura, 2004; Browning et al., 2006; Momma et al., 2006; Oka, 2010; Runia et al., 2014; Huang et al., 2015), but it is unclear how VFAs and carbon amendments affect viability of large fungal sclerotia (like those of *Athelia rolfsii*) or potential mycoparasites of sclerotia-forming plant pathogens, such as *Trichoderma* or *Mucor* spp., during ASD treatment in field (i.e., non-autoclaved) soils.

Studies by Tenuta et al. (2002) and Abbasi et al. (2009) indicated that VFAs reduced numbers of microsclerotia of *V. dahliae* after several days, and the effect was more pronounced at lower soil pH. Similarly, Swilling et al. (2021) reported that soil pH and soil texture affected the antifungal activity of VFAs against *A. rolfsii* in autoclaved soils under laboratory conditions. The pKa values for both acetic and *n*-butyric acids are near 4.8 so in an acidic soil, a larger ratio of the VFAs would be in a non-dissociated state. Non-dissociated forms are generally more toxic to soilborne plant pathogens because in the undissociated state the compounds likely more able to readily diffuse across cell membranes, thereby leading to cytoplasm acidification (Browning et al., 2006; Runia et al., 2014). It follows that because VFAs suppress soilborne plant pathogens (such as *A. rolfsii*), that VFAs may also affect other organisms present in the soil such as the fungal mycoparasite, *Trichoderma*.

Athelia rolfsii (Sclerotium rolfsii) is an economically-damaging fungal plant pathogen that causes southern blight in a wide range of host plants including tomato, pepper, and many other vegetable and legume crops (Mullen, 2001). Southern blight disease symptoms typically form initially at the base of the plant and spread toward the roots, but the pathogen can also infect leaves and fruits in contact with soil. *Athelia rolfsii* occurs in tropical to subtropical regions globally, preferring warm moist soils and temperatures over 25°C, however, it can grow at temperatures as low as 8°C (Punja, 1985; Mullen, 2001). Long term survival structures (sclerotia) are formed when mycelium condenses to form a protective, melanized rind. Sclerotia can remain dormant in soil for multiple years and are easily dispersed with soil movement (Punja, 1985; Xu, 2008).

The genus, *Trichoderma,* contains numerous species commonly found in soils, especially those rich in root systems. Selected isolates of *Trichoderma*, particularly *T. harzianum*, are effective biocontrol agents for soilborne plant pathogens including *Fusarium*, *Phytophthora*, and *A. rolfsii* (Mishra et al., 2011). *Trichoderma* species have also been linked to promotion of plant growth and drought resistance in plants (Duffy et al., 1997; Yedidia et al., 2001; Benítez et al., 2004). As a biocontrol, *Trichoderma* spp. use several mechanisms to slow down or inhibit the growth of plant pathogens. *Trichoderma* spp. can directly parasitize and lyse the mycelia of other fungi and nematode integuments through enzymes (Chet et al., 1981; Sharon et al., 2001) and produces antibiotics that negatively affect other soilborne organisms (Mishra et al., 2011). *Trichoderma* species typically grow rapidly and can displace other fungi in the soil environment (Benítez et al., 2004). Bulluck and Ristaino (2002) observed that *Trichoderma* suppressed *A. rolfsii* and that *Trichoderma* populations were enhanced by addition of organic amendments. Shrestha et al. (2018) similarly observed *Trichoderma* parasitizing *A. rolfsii* post ASD treatment, at percentages higher than observed for non-amended controls. It follows that populations of *Trichoderma* spp. may be an important consideration in evaluation of non-chemical and biological techniques of soil disinfestation. Similarly, *Mucor* spp. have been observed colonizing sclerotia post ASD treatment (Shrestha et al., 2018) and *Mucor* spp. have been reported as probable mycoparasites of sclerotia of *Sclerotinia sclerotiorum* (Merriman, 1976; Adams and Ayers, 1979; Harvey et al., 1995). Increased mechanistic understanding

of ASD treatment and effects on these potential mycoparasites colonizing sclerotia will facilitate development of treatment recommendations for effective use of biologically-based ASD treatment.

Based on preliminary data and previous studies, our objectives were as follows: (1) evaluate effect of ASD amendment and soil autoclaving on sclerotial colonization and VFA-induced suppression of germination of *A. rolfsii* sclerotia during ASD, and (2) evaluate impact of acetic and *n*-butyric acids and organic soil amendment during ASD on *Trichoderma* spp. population dynamics post ASD treatment. We hypothesized that: (1) VFA exposure will reduce *A. rolfsii* germination in a VFA concentration dependent manner, and that soil microbial activity in non-autoclaved soil and organic amendments used during ASD will alter the effect of VFAs on *A. rolfsii* germination, and (2) acetic and *n*-butyric acids will increase soil *Trichoderma* spp. populations as a function of VFA concentration and organic amendment.

MATERIALS AND METHODS

Experiment 1, Role of Soil Autoclaving and ASD Amendment on VFA-Induced Suppression of *A. rolfsii*

To evaluate the effect of endemic soil microbial activity and ASD amendment on VFA-induced suppression of *A. rolfsii* germination, the activity of VFAs in field soils that were either autoclaved (45 min at 121°C and 103 kPa, twice, 24 h apart) or not autoclaved, and amended with an organic amendment or not amended were evaluated. Based on the work of Shrestha et al. (2018), the organic amendment was a mixture of dry molasses on a soy hull carrier and corn starch at a rate of 4 mg C/g soil, with a carbon to nitrogen ratio of 30:1. The study was a completely randomized factorial design with two levels of soil autoclaving (autoclaved, non-autoclaved), two ASD organic amendment treatments (amended, non-amended), two VFAs (acetic, *n*-butyric acids), and three VFA concentrations (4, 8, and 16 mmol/kg soil at a single soil pH of 5.0). HCl was used as an acid control at a soil pH of 5.0 and 16 mmol/kg soil concentration, and sterile water was used as a baseline control. There were four replicates in each of two repeated trials of the study. The soil was air-dried field soil from the surface horizon at the University of Tennessee Organic Crops Unit, Knoxville, TN, USA (Dewey silt loam, fine, kaolinitic, and thermic typic Paleudult) mixed in equal parts with sand (particle size range from 0.0625 to 1.5 mm) by volume, this resulted in a sandy loam soil texture with a clay content of 10%.

Concentrations of VFA were selected based on previous studies (4, 8, and 16 mmol/kg soil) to represent a typical range of the VFA concentrations present in soil during the ASD process (e.g., Shrestha et al., 2020a). Working solutions were created by combining reagent-grade concentrated VFA with autoclaved double deionized water to achieve concentrations of 0.027, 0.053, and 0.107 M. These concentrations were equivalent to final soil concentrations of 4, 8, and 16 mmol/kg dry soil given treatment application rates. Stock solution pH was determined using a pH electrode (Orion Star A221, Thermo Scientific, Waltham, MA, USA). To determine soil solution pH, 750 μL of VFA solution was added to 5 g soil. After a 10 min equilibration, soil pH was measured using a pH electrode in soil mixed with 10 mL of 0.01M $CaCl_2$ (Kissel et al., 2009). To achieve the desired pH value, based on soil buffering, $Ca(OH)_2$ or 1.2 M HCl solution was added to VFA solutions in small quantities while pH was monitored with a submerged pH electrode. Final soil pH of the VFA and soil mixture measured in 0.01M $CaCl_2$ was 4.5, and was 5.0 when measured in water (as expected when comparing pH measurements in water vs. 0.01 M $CaCl_2$) (Kissel et al., 2009).

Sclerotia of *A. rolfsii* were cultured from an isolate originally isolated from hybrid field tomatoes at the East Tennessee Research and Education Center, Knoxville, TN, USA cultured at room temperature (21°C) on pepper (*Capsicum annuum*, cv. Felicity F1) leaf and stem tissue. Briefly, frozen pepper tissue was chopped (<2.5 cm) and placed into 1-L Erlenmeyer flasks corked with Poly-fil and cheesecloth and covered with aluminum foil. Plant tissue was autoclaved twice with 24-h between sterilizations. After the second autoclave cycle, flasks were cooled to room temperature and ten sclerotia of *A. rolfsii* were added to each flask. Flasks were incubated for 3–4 weeks until large numbers of sclerotia were produced. Sclerotia were harvested under a biosafety cabinet and left in an open Petri dish in the cabinet to dry overnight. Sclerotia were stored at 8°C in Petri dishes sealed with Parafilm until needed. Using this method, with 300-cm^3 pepper tissue (autoclaved volume), ∼1,000 sclerotia were produced per flask.

To evaluate sclerotial germination after exposure to VFAs, 10 g of soil and 10 sclerotia each were added to autoclaved 20-mL glass scintillation vials and mixed with light shaking. Treatments were randomly assigned, and 1.5 mL of solution (acetic acid, *n*-butyric acid, HCl, or water) was added to each vial of soil to bring the 10 g of soil to the water-holding capacity of the soil mixture (without standing water in the vials). Vials were then lightly shaken to ensure that all areas of the soil were thoroughly moistened by the solution. Lidded vials were placed into a controlled atmosphere chamber (Model 855-AC, PLAS LABS, Lansing, MI, USA) with an atmosphere of 90% N_2, 5% CO_2, and 5% H_2 and lids were removed for 5 min while the palladium molecular sieve of the anaerobic chamber removed existing oxygen from the chamber and vials. Vials were then re-lidded and then incubated in the anaerobic chamber at room temperature for 4 days. A 4-day period was selected based on the cycles of VFA concentrations seen in previous ASD field experiments (Shrestha et al., unpublished data). After the 4-day period, vials were removed from the chamber, and sclerotia plated individually into 24-well-plates with 32 g/L PDA with 6.9 mg/L fenpropathrin (Sigma-Aldrich, St. Louis, MO, USA) and incubated at room temperature. Germination of sclerotia (yes, no) was observed and recorded over the 1-week period after plating, and colonization of sclerotia (yes, no) by *Trichoderma* spp., *Mucor* spp., *Bacillus* spp., or other uncharacterized fungi or bacteria was recorded at 3 days after plating. Percentage germination or colonization of the 10 sclerotia in each vial was calculated for each replicate vial.

Experiment 2, Response of Endemic Soil Populations of *Trichoderma* spp. to VFA, VFA Concentration, and ASD Soil Amendment

To evaluate *Trichoderma spp*. population post ASD treatment, 20-cm plastic pots were filled with 1.3 kg sandy loam soil (50:50 mixture of sand and field soil from the University of Tennessee Organic Crops Unit, Knoxville, TN, USA). The experiment was established as a randomized complete block design with four replicates per trial, which was repeated. Treatments included a factorial combination of two VFAs (acetic, *n*-butyric), three VFA concentrations (4, 8, and 16 mmol/kg soil), and two ASD organic amendment treatments (amended, non-amended). The organic amendment was a mixture of dry molasses, soyhulls, and corn starch at a rate of 4 mg C/g soil and a carbon to nitrogen ratio of 30:1 as in experiment 1. Control treatments included sterile water and HCl at 16 mmol/kg soil as described in experiment 1, factorially combined with both amended and unamended treatments. Following organic amendment incorporation into the soil mixture, 300 mL of VFA (or water or HCl for controls) solution was applied to each pot and the pot surface was sealed using polyethylene mulch secured on each pot with heavy-duty rubber bands. The pots were incubated for 3 weeks in a greenhouse (13–18°C at night and 21–27°C during the day). After 3 weeks, the polyethylene mulch was removed and soil samples (30 g) were taken from the center of the pot at a depth of 0–3 cm and stored at 4°C until analysis.

To determine the population of *Trichoderma* spp. in each sample, 1 g of soil was added to 9 mL of sterile double deionized water, and serial dilutions from 10^{-1} to 10^{-3} were prepared. From each dilution, 0.1 mL was spread onto *Trichoderma* selective medium (TSM) containing 39 g/L PDA amended with 0.02 g/L rose bengal, 0.3 g/L chloramphenicol, 0.02 g/L streptomycin sulfate, prepared at pH 6 (Gil et al., 2009). The plates were covered and incubated in dark storage boxes for 3 days, after which colony forming units (CFUs) of *Trichoderma* spp. were counted.

Statistical Analysis

Data were subjected to mixed models analysis of variance using PROC GLIMMix in SAS 9.4 (SAS Institute, Cary, NC, USA). Experiment 1 was a factorial completely randomized design with four factors ($2 \times 2 \times 2 \times 3$). The main effects and interactions of soil autoclaving (autoclaved, non-autoclaved), ASD organic amendment (amended, non-amended), VFA (acetic, *n*-butyric), and VFA concentration (4, 8, and 16 mmol/kg soil) were treated as fixed effects, and repeated trials were treated as random effects. Experiment 2 was a factorial randomized complete block design with three factors ($2 \times 3 \times 2$). The main effects and interactions of VFA (acetic, *n*-butyric), VFA concentration (4, 8, and 16 mmol/kg soil), and ASD amendment (amended, non-amended) were treated as fixed effects and trial and block treated as random effects. Differences between means were determined with an F-protected LSD at $P \leq 0.05$. Rank (*Trichoderma* spp. populations) or arcsine square root (percentage sclerotia germination and colonization) transformations of data were used to satisfy the non-normal distribution and unequal variances of residual error. Untransformed means and standard error of the mean are reported. Given that controls could not be included in the factorial analysis described previously, controls were analyzed with a separate statistical analysis by comparing to each experimental treatment in the factorial design.

RESULTS

Experiment 1

Significant main effects on sclerotial germination were observed for VFA, VFA concentration, and soil autoclaving, but not ASD soil amendment in experiment 1 (**Table 1**). A significant interaction effect was observed between VFA and soil autoclaving, VFA concentration and soil autoclaving, and ASD soil amendment and soil autoclaving (**Table 1**).

For the main effect of VFA, in acetic acid treatments, percentage germination of sclerotia averaged 35.1% compared to 21.9% for *n*-butyric acid treatments (**Table 2**). For comparison, sclerotia in the HCl controls had an average germination of 62.7%, and in water controls germination averaged 74.3% (averaged across both autoclaved and non-autoclaved soil treatments). For the main effect of VFA concentration, germination of sclerotia in the 4 mmol/kg treatment averaged 39.5% compared to the 29.1% at the 8 mmol/kg soil treatment and 16.9% at the 16 mmol/kg soil treatment, which all differed significantly. In non-control treatments, sclerotial germination averaged 18.1% for the autoclaved soil which was significantly less than that for the non-autoclaved soil (38.9% germination; **Table 2**).

A significant interaction effect between VFA concentration and soil autoclaving was observed, such that at 4 mmol/kg soil VFA concentration, sclerotial germination did not significantly differ between the autoclaved soil treatments (36.9%) and the non-autoclaved soil treatments (42.2%) when averaged across VFAs (**Figure 1**). At 8 mmol/kg soil, autoclaved soil had a lower sclerotial germination (16.6%) compared with that of the non-autoclaved soil (41.6%). At 16 mmol/kg soil concentration, a similar trend was observed with lower sclerotial germination (0.9%) in autoclaved soil treatments compared to non-autoclaved soil treatment (32.8%; **Figure 1**). Germination of sclerotia in the HCl and water controls was 50.4 and 64.4%, respectively, in non-autoclaved soil and 75.0 and 84.4%, respectively, in autoclaved soil, an inverse relationship to effects of soil autoclaving in VFA treatments (**Figure 1**). The interaction of VFA with soil autoclaving resulted in the highest germination observed from acetic acid treatments in non-autoclaved soil (49.8%), intermediate with acetic acid in autoclaved soil (20.4%) or *n*-butyric acid in non-autoclaved soil (27.9%) and the lowest from *n*-butyric acid treatments in autoclaved soil (15.8% germination; data not shown). Irrespective of ASD soil amendment, sclerotial germination in VFA-amended treatments was higher in non-autoclaved soil (36.4–41.5%) than in autoclaved soil with (13.5% germination) or without (22.7%) ASD soil amendment (data not shown).

Colonization of sclerotia by *Trichoderma*, *Mucor*, *Bacillus*, and other unidentified bacteria was significantly affected by both

TABLE 1 | Analysis of variance of the response variables percentage germination of *Athelia rolfsii* sclerotia and percentages colonization of sclerotia by soil fungi and bacteria in experiment 1 as affected by the main effects of volatile fatty acid (VFA) type, VFA concentration, ASD soil amendment, soil autoclaving, and their interactions.

		Sclerotia colonization				
	Sclerotial germination	*Trichoderma*	*Mucor*	*Bacillus*	Other fungi	Other bacteria
			P-value			
VFA	<0.001	<0.001	<0.001	0.008	NS	0.01
VFA concentration	<0.001	0.01	<0.001	NS	NS	<0.001
VFA × VFA concentration	NS	0.02	NS	0.03	NS	NS
Soil amendment	NS	<0.001	<0.001	NS	NS	NS
VFA × soil amendment	NS	0.02	0.05	NS	NS	NS
VFA concentration × soil amendment	NS	NS	NS	NS	NS	NS
VFA × VFA concentration × soil amendment	NS	NS	NS	NS	NS	NS
Soil autoclaving	<0.001	<0.001	<0.001	0.003	<0.001	<0.001
VFA × soil autoclaving	0.02	<0.001	NS	0.003	NS	NS
VFA concentration × soil autoclaving	0.004	0.02	0.03	0.01	NS	<0.001
VFA × VFA concentration × soil autoclaving	NS	0.05	<0.001	NS	NS	NS
Soil amendment × soil autoclaving	0.04	<0.001	NS	NS	NS	NS
VFA × soil amendment × soil autoclaving	NS	<0.001	NS	NS	NS	NS
VFA concentration × soil amendment × soil autoclaving	NS	NS	NS	NS	NS	NS
VFA × concentration × soil amendment × soil autoclaving	NS	NS	0.05	NS	NS	NS

NS, not significant, P > 0.05.

TABLE 2 | Mean percentage germination of *Athelia rolfsii* sclerotia and percentages colonization of sclerotia in experiment 1 as affected by main effects of volatile fatty acid (VFA) type, VFA concentration, ASD soil amendment, and soil autoclaving.

		Sclerotial colonization				
	Sclerotial germination	*Trichoderma*	*Mucor*	*Bacillus*	Other fungi	Other bacteria
			Percentage (%)			
VFA						
Acetic	35.1 (±3.3)[a]	11.9 (±2.5)[a]	38.2 (±3.9)[a]	4.0 (±1.3)[a]	7.8 (±1.2)	7.9 (±1.7)[a]
n-butyric	21.9 (±2.8)[b]	0.9 (±0.8)[b]	18.0 (±3.1)[b]	0.6 (±0.3)[b]	6.8 (±1.6)	4.2 (±1.2)[b]
VFA concentration						
4 mmol/kg soil	39.5 (±3.8)[a]	8.4 (±2.7)[a]	35.6 (±4.7)[a]	3.3 (±1.5)	8.3 (±1.6)	11.7 (±2.6)[a]
8 mmol/kg soil	29.1 (±3.5)[b]	8.8 (±2.8)[a]	32.7 (±4.9)[a]	2.5 (±1.3)	9.1 (±2.2)	4.2 (±1.4)[b]
16 mmol/kg soil	16.9 (±3.5)[c]	2.0 (±0.9)[b]	16.1 (±3.4)[b]	1.1 (±0.6)	4.5 (±1.2)	2.2 (±0.9)[b]
Soil amendment						
Amended	27.5 (±3.0)	9.5 (±2.3)[a]	36.6 (±3.9)[a]	2.5 (±1.0)	8.3 (±1.6)	4.7 (±1.1)
Non-amended	29.5 (±3.2)	3.3 (±1.3)[b]	19.7 (±3.3)[b]	2.1 (±0.9)	6.3 (±1.2)	7.4 (±1.8)
Soil autoclaving						
Autoclaved	18.1 (±2.5)[b]	12.3 (±2.6)[a]	7.1 (±2.0)[b]	4.1 (±1.3)[a]	2.4 (±0.7)[b]	0.1 (±0.1)[b]
Non-autoclaved	38.9 (±3.3)[a]	0.5 (±0.3)[b]	49.2 (±3.7)[a]	0.5 (±0.3)[b]	12.2 (±1.7)[a]	12.0 (±2.0)[a]

Within main effects, means represented by the same letter or no letters are not significantly different according to an F-protected LSD, P ≤ 0.05. Values in parentheses represent standard error of the mean.

VFA and VFA concentration; colonization by *Trichoderma* and *Mucor* was significantly affected by ASD soil amendment; and colonization by all (*Trichoderma*, *Mucor*, *Bacillus*, other fungi and other bacteria) was significantly affected by soil autoclaving (**Table 1**). There were also numerous significant interaction effects on sclerotial colonization by *Trichoderma* spp. (**Table 1**).

Colonization was higher in acetic acid treatments than in *n*-butyric acid treatments for *Trichoderma* spp. (11.9 vs. 0.9% colonization, respectively), *Mucor* spp. (38.2 vs. 18.0%), *Bacillus* spp. (4.0 vs. 0.6%), and other bacteria (7.9 vs. 4.2%; **Table 2**). The main effect of VFA concentration showed reduced percentage colonization as VFA concentration increased, with the lowest colonization of *Trichoderma* spp., *Mucor* spp., and other bacteria observed at 16 mmol/kg soil concentration (**Table 2**). ASD soil amendment increased colonization by *Trichoderma* spp. (9.5% colonization vs. 3.3% in non-amended treatments) and *Mucor* spp. (36.6 vs. 19.7%; **Table 2**). Soil autoclaving effects on colonization varied with organisms; autoclaving reduced

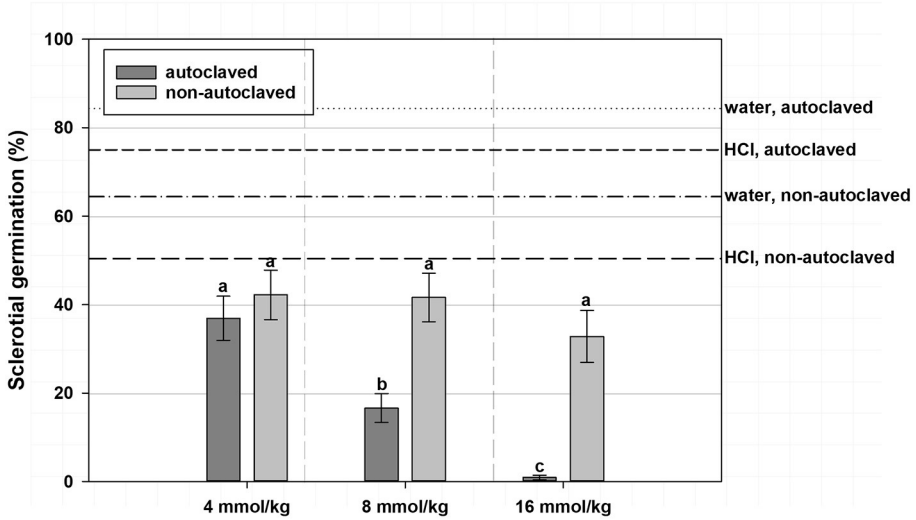

FIGURE 1 | Mean percentage germination of *A. rolfsii* sclerotia in experiment 1 as affected by volatile fatty acid (VFA) concentration, and soil autoclaving. Bars indicated by the same letter are not significantly different according to an F-protected LSD at $P \leq 0.05$. Error bars represent standard error of the mean.

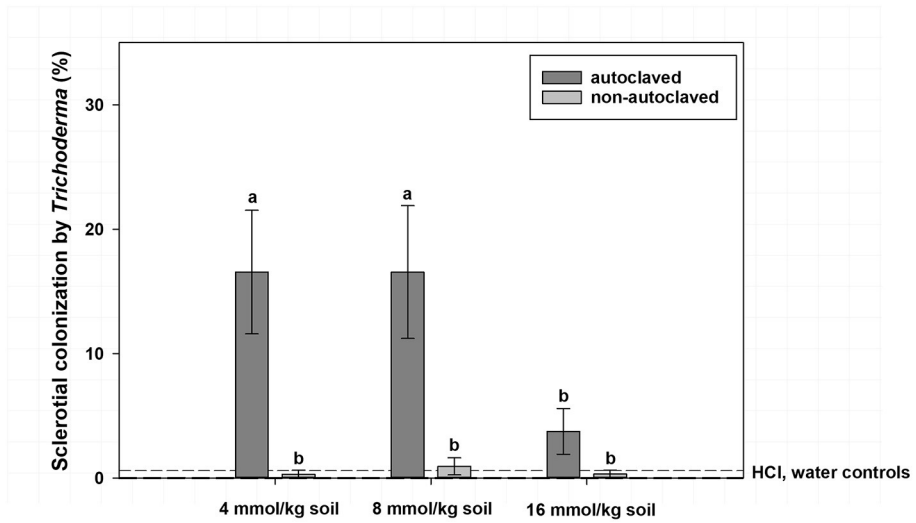

FIGURE 2 | Mean percentage colonization of *A. rolfsii* sclerotia by *Trichoderma* spp. in experiment 1 as affected by volatile fatty acid (VFA) concentration and soil autoclaving. Bars indicated by the same letter are not significantly different according to an F-protected LSD at $P \leq 0.05$. Error bars represent standard error of the mean.

colonization by *Mucor* spp. (7.1 vs. 49.2%), other fungi (2.4 vs. 12.2%), and other bacteria (0.1 vs. 12.0%), but increased colonization by *Trichoderma* spp. (12.3 vs. 0.5%) and *Bacillus* spp. (4.1 vs. 0.5%; **Table 2**).

Mean sclerotial colonization by *Trichoderma* spp. was low (<1% colonization) regardless of VFA concentration when soil was not autoclaved and statistically similar to autoclaved soil with the 16 mmol/kg soil VFA concentration (4% colonization; **Figure 2**). This compares to 17% colonization by *Trichoderma* spp. for the 4 or 8 mmol VFA/kg soil treatments in autoclaved soil. Autoclaved treatments with acetic acid were characterized by the highest colonization by *Trichoderma* spp., especially at 4 or 8 mmol VFA/kg soil (28 and 33% mean colonization, respectively; **Figure 3**) or autoclaved and ASD soil amendment treatments (34% colonization, **Figure 4**).

Experiment 2

In experiment 2, there was a significant main effect of ASD soil amendment ($P = 0.02$), but not VFA (acetic or *n*-butyric) or VFA concentration on populations of *Trichoderma* (**Table 3**). For VFA treatments, soil with ASD amendment had a population of 3.4 $\times$ 10^6 CFU/g soil whereas soil without ASD amendment had a lower population of 9.6 $\times$ 10^5 CFU/g soil. When compared to control treatments, the population of the non-amended HCl control group was the lowest at 1.6 $\times$ 10^4 CFU/g soil, while the controls with no VFA or HCl had a population similar to

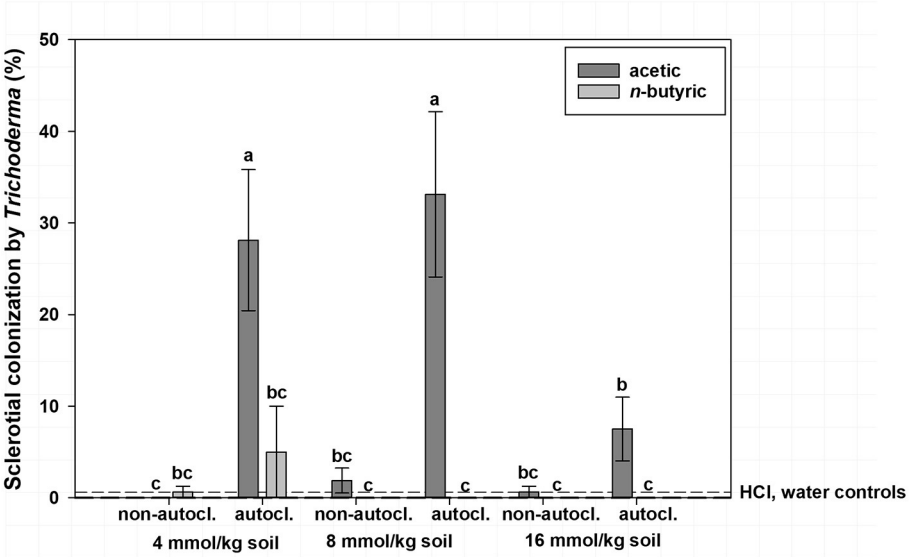

FIGURE 3 | Mean percentage colonization of *A. rolfsii* sclerotia by *Trichoderma* spp. in experiment 1 as affected by volatile fatty acid (VFA), VFA concentration and soil autoclaving. Bars indicated by the same letter are not significantly different according to an F-protected LSD at $P \leq 0.05$. Error bars represent standard error of the mean.

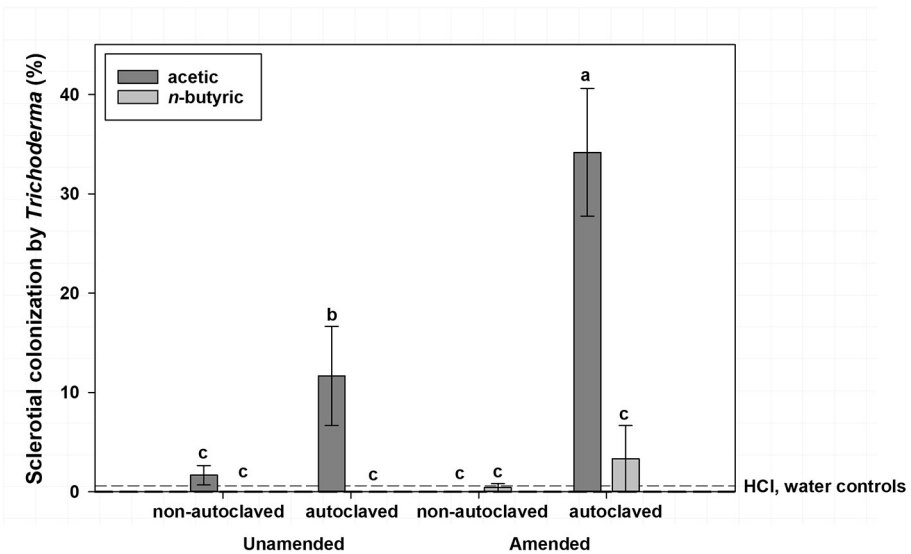

FIGURE 4 | Mean percentage colonization of *A. rolfsii* sclerotia by *Trichoderma* spp. in experiment 1 as affected by volatile fatty acid (VFA), soil autoclaving, and soil amendment. Bars indicated by the same letter are not significantly different according to an F-protected LSD at $P \leq 0.05$. Error bars represent standard error of the mean.

treatments with VFAs (1.3 to 1.6 $\times$ 10^5 CFU/g soil) regardless of soil amendment.

DISCUSSION

Our hypotheses were generally supported as related to the effects of VFAs on sclerotial viability as defined by germination post-exposure. While acetic and *n*-butyric acid were significantly different in terms of sclerotial germination, both VFAs significantly reduced sclerotial viability over that of the water and HCl control treatment groups; the effects increased with increasing VFA concentration in autoclaved soil. Our hypothesis that endemic soil microbial activity would reduce VFA-induced suppression was also supported in that an average of 38.9% of sclerotia incubated with VFAs in non-autoclaved soil germinated, compared with only 18.1% germination when incubated in soil that had been sterilized by autoclaving. This suggests that there are processes in biologically active soil that reduce

TABLE 3 | Analysis of variance for response variables of *Trichoderma* spp. populations in experiment 2 as affected by the main effects of volatile fatty acid (VFA), VFA concentration, ASD soil amendment, and their interactions.

	***P*-value**
VFA	NS
VFA concentration	NS
VFA × VFA concentration	0.05
Amendment	0.02
VFA × amendment	NS
VFA concentration × amendment	NS
VFA × VFA concentration × amendment	NS

NS, not significant, P > 0.05.

effectiveness of VFAs against *A. rolfsii*. This is likely because these short-chain organic acids are readily metabolized by aerobic and anaerobic soil microorganisms such as *Bacillus* and *Clostridium* (Massie et al., 1985; Coates et al., 1998; Chauhan and Ogram, 2006a,b). Interestingly, although there was a trend of lower sclerotia germination in non-autoclaved soil as VFA concentration increased, percentage germination at 16 mmol/kg soil was not significantly different than that at 4 mmol/kg soil. It may be expected that increased soil VFA concentrations could reduce germination percentages in non-autoclaved soils, as in field conditions, but more work is needed to evaluate VFA effects in these conditions.

There was no significant effect of VFA or VFA concentration on soil populations of endemic *Trichoderma* spp., and treatments with VFAs did not have higher *Trichoderma* spp. populations than treatments amended with water alone, which did not support our hypothesis. This is also in contrast with that reported by Rosskopf et al. (2014), who reported increased soil *Trichoderma* spp. when soils in Florida, USA were treated with a blend of organic acids. As the authors did not report the composition of the proprietary mixture used in their study, it is unclear what accounts for the difference between studies, although differences in acid types, concentrations, and soil type could all be expected to play a role. In the present study, the addition of labile ASD amendment to field soil increased *Trichoderma* spp. populations by more than 300% over that of the non-amended soil. This population increase under ASD conditions is consistent with other studies involving organic soil amendments (Bulluck and Ristaino, 2002; Kurakov et al., 2008, 2011) and ASD treatment (Shrestha et al., 2018, 2020b). *Trichoderma* spp. can grow at a reduced rate under anaerobic conditions they are not eliminated by the process and can utilize metabolites produced, such as sugars and alcohols (Bulluck and Ristaino (2002) and Kurakov et al. (2008, 2011). This, in turn, can give *Trichoderma* spp. an advantage over other soilborne fungi when soil conditions return to aerobic, post ASD treatment.

Acetic acid consistently promoted higher fungal colonization than *n*-butyric acid at both the 8 and 16 mmol/kg soil concentrations, and was less suppressive to sclerotia of *A. rolfsii* than *n*-butyric acid. Small difference in dissociation of acetic (pKa = 4.75) and butyric acids (pKa = 4.82) in the pH 5.0 soil mixtures may have contributed to these findings, but this is unlikely to be the major factor in differences between the VFAs. As a shorter carbon chain fatty acid, acetic acid (a two C compound) is both more readily broken down by microorganisms and is more volatile than butyric acid (a four C compound) and so does not persist as long in soil compared to *n*-butyric acid (Massie et al., 1985; Coates et al., 1998; Chauhan and Ogram, 2006a,b). This indicates that endemic soil microbes may metabolize higher rates of acetic acid more readily than *n*-butyric acid.

This study provides documentation of the direct effects of soil VFA exposure on *A. rolfsii*. This work confirms that the VFAs and the transient strongly acidic soil environment induced by those VFAs characteristic of anaerobic decomposition of organic amendments during soil treatment with ASD contribute to loss of sclerotial viability. Antifungal activity of VFAs is less for acetic compared to *n*-butyric acid and increases in a concentration-dependent manner. The importance of evaluating these mechanisms under realistic field environmental conditions is emphasized by the differences in antifungal VFA activity and by differences in sclerotial colonization in non-sterile soil environments more typical of field conditions than in sterile laboratory conditions. Continued mechanistic evaluations of ASD under realistic field environmental conditions will help to maximize the efficacy of this cultural control against soilborne plant pathogens.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

KS co-designed and conducted all trials, performed related lab work, and wrote the initial draft of the manuscript as a chapter of an M.S. thesis project. US assisted with trial implementation and data collection, assisted with statistical analysis, and critically reviewed the manuscript. BO co-designed all trials, supervised fungal assessments, assisted with data analysis, and critically reviewed the manuscript. KG assisted with data analysis and interpretation and critically reviewed the manuscript. DB co-designed all trials, obtained funding for all trials, supervised trial completion in his lab group, and provided critical revisions to the manuscript draft. All authors contributed to the article and approved the submitted version.

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A Combined Field–Lab Approach for Assessing *Salmonella* Infantis Persistence in Broiler Litter in a Stockpile and Composting Sleeve

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Broiler litter (BL) is often contaminated by a variety of zoonotic pathogens. This study attempts to assess the persistence of *Salmonella enterica* serovar Infantis (*S.* Infantis) in BL based on spatial and temporal variation of physicochemical properties in a stockpile and composting sleeve. A single trial of two pilot-scale setups, ∼35 m^3 each, included an open static pile (stockpile) and composting in a polyethylene sleeve with forced aeration. The initial water content was adjusted only for the sleeve (∼50% w/w) as in a common composting practice. Both systems were monitored weekly and then biweekly during 2 months in 47–53 sampling points each on every campaign. Measurements included temperature, water content, pH, electrical conductivity (EC), gas-phase oxygen, and ammonia, and the collected data were used to construct multiple contour grid maps. Of the stockpile volume, 83, 71, and 62% did not reach the commonly required minimum temperature of 55°C for three consecutive days during the first, second, and third weeks, respectively. Oxygen levels showed a strong gradient across the stockpile, while anaerobic conditions prevailed in the core. Variation was also recorded within the sleeve, but due to the water content adjustment and active aeration, the conditions favored more intense degradation and higher temperatures. Combining the grid maps drawn in this study with decay rate constants recently published for S. Infantis in BL under 36 combinations of temperature, water content, and pH, we assessed the spatial persistence of S. Infantis in the stockpile and the sleeve. Temperature was shown as a major factor, while water content and pH had only a small effect, in the stockpile only. Co-correlations between temperature, water content, EC, and oxygen suggest that selected physicochemical properties may be sufficient for such assessments. Up to 3 weeks would be recommended to achieve 7–8 $\log_{10}$ reduction in *Salmonella* in a stockpile, while this would be fully achieved within 1 week in a sleeve. This approach of combining high-resolution spatial field sampling along with decay rates of pathogens under controlled lab conditions may improve quantitative microbial risk assessments and future regulations of manure utilization.

Keywords: poultry manure, pathogens, grid maps, contour maps, decay constants, quantitative microbial risk assessment, *Salmonella* survival/die-off

INTRODUCTION

The poultry sector is one of the largest and among the fastest growing agriculture-based meat production industries worldwide, due to the growing demand for both meat and egg products (Bolan et al., 2010). Broiler litter (BL) is a byproduct of broiler meat production, consisting of a mixture of fecal droppings, bedding materials (usually sawdust or shavings, rice hulls, or straw), feathers, and wasted feed (Wilkinson, 2007; Kim et al., 2012). Often, pathogens excreted from infected chickens contaminate the litter, which, in turn, becomes a source of zoonotic pathogens such as *Salmonella*, limiting the safe use of BL (Chinivasagam et al., 2010; Wilkinson et al., 2011; Gould et al., 2013). At the same time, due to the relatively high concentrations of nitrogen and other plant nutrients along with low water content, BL is considered a valuable fertilizer and soil additive in conventional and organic farming (Wilkinson, 1979; Stephenson et al., 1990; Kingery et al., 1994; Chaudhry et al., 1998; Marshall et al., 1998; Mitchell and Tu, 2006; Cassity-Duffey et al., 2015). The extensive global application of BL as a plant nutrient is expected to continue, considering the increasing demand for broiler production; yet, although BL may be contaminated by a variety of zoonotic pathogens, most farmers use it either without composting or after partial stabilization, by temporarily stockpiling the material in the field, covered or uncovered under uncontrolled conditions (Bush et al., 2007; Wilkinson, 2007; Ogejo and Collins, 2009; Wilkinson et al., 2011). In Israel, about 250 million broilers were grown in 2020 (Poultry Council Chicken Health Labs, 2020), generating nearly 290 thousand tons of BL per annum (Cnaan, N., Director of the Growth Division of "Off Tov Group", Israel; personal communication) of which the majority is used directly in agriculture (Grinhut et al., 2015).

Salmonella outbreaks due to consumption of contaminated fresh produce are still a threat to public health (Beuchat, 2002; Islam et al., 2004a,b; Fatica and Schneider, 2011; Bell et al., 2015; Herman et al., 2015; Chaves et al., 2016; Gu et al., 2018; Jechalke et al., 2019). To minimize such outbreaks, pathogen inactivation prior to land application is commonly sought, which is the main drive for thermophilic composting of manures (Williams and Benson, 1978; Vinnerås et al., 2003; Wichuk and McCartney, 2007; Macklin et al., 2008; Wilkinson et al., 2011). Thermal inactivation may be partially achieved also by stockpiling the litter for some time before spreading, but it cannot be effective compared with controlled thermophilic composting. To ensure effective pathogen elimination during composting, international guidelines require a minimum of 55°C for three consecutive days under in-vessel or aerated static pile methods. In open windrows, this minimal temperature should be maintained for at least 15 consecutive days with a minimum of five turnings during the thermophilic phase (USEPA, 2003; Wichuk and McCartney, 2007). These guidelines correspond with the work of Isobaev et al. (2014) who presented a statistical modeling based on temperature measurements in a covered aerated static pile of biosolids and wood chips. They estimated that following five turnings, the likelihood of every particle to be exposed to the required time and temperature conditions (≥55°C for three consecutive days) was 98%. Multiple studies, however, reported a variety of composting settings in which these temperature requirements have not been fully met in the entire volume of the composting material (Pereira-Neto et al., 1986; Fernandes et al., 1994; Erickson et al., 2010; Wilkinson et al., 2011; Avidov et al., 2017). Although thermophilic temperatures are reported in multiple composting studies, only average or min and max values are often reported, while the spatial distributions remain unknown (e.g., Déportes et al., 1998; Tiquia et al., 1998; Raviv et al., 1999; Larney et al., 2003; Van Herk et al., 2004; Aviani et al., 2010). Clearly, average temperatures or a single measurement point cannot indicate the achievement of pathogen lethal conditions. It should be noted that pathogens surviving the composting process or any phase of stabilization may regrow during storage under favorable conditions or following land application (Yeager and Ward, 1981; Zaleski et al., 2005; Chen and Jiang, 2014; Reynnells et al., 2014; Avidov et al., 2017, 2021a; Hruby et al., 2018). In our previous study (Avidov et al., 2021a), soil-BL mixtures were inoculated with *S.* Infantis and incubated at 30°C for a period of 90 days. *Salmonella* decreased by 4–6 $\log_{10}$ but then increased again within 2 weeks by 2–3 $\log_{10}$, in response to the increase in water content from 30 to 70% water holding capacity.

Variable oxygen concentrations during composting was also demonstrated in several studies (Haga et al., 1998; Erickson et al., 2010; Poulsen, 2011; Stegenta et al., 2019). Anaerobic zones within the pile were reported during the first week of composting at the bottom of the core in a static pile of dairy cattle feces (Haga et al., 1998). Similarly, Stegenta et al. (2019) recorded the lowest oxygen concentrations at the core in windrow piles of sewage sludge, and Poulsen (2011) reported the lowest oxygen concentrations at the bottom of the core in a turned windrow pile, which contained sewage sludge, yard and park waste, and screening residue from processing of finished compost. In six unturned static piles of chicken litter and peanut hulls, Erickson et al. (2010) showed oxygen concentrations above 18% at the pile surface and below 10% at about 30-cm depth, during the first week of composting. Aerobic–anaerobic gradients may have an effect on pathogen persistence. Avidov et al. (2021a) observed higher decay rates of *S.* Infantis in BL at mesophilic temperatures under anaerobic compared with aerobic conditions and postulated that it might be related to the different composition and density of antagonistic microbial populations. Such variability of multiple physicochemical properties has not been used in quantitative assessments of pathogen persistence in stockpiles or composting systems. The combination of temperature with other environmental factors may have varying effects on *Salmonella* persistence in BL. Several studies have examined the effect of temperature and water content, generally showing that thermal susceptibility of *Salmonella* spp. increases with increasing water content (Liu et al., 1969; Wilkinson et al., 2011; Singh et al., 2012; Chen et al., 2013). At low water content, desiccation may play a major role in pathogen inactivation. Yet, desiccation-adapted *Salmonella* spp. persisted longer in aged chicken litter compared with non-adapted cells (Chen et al., 2013). Other mixed physiochemical properties may also play a role in bacterial inactivation, such as the combined and intensified effect of drying and NH_3 emissions, as shown by Himathongkham and Riemann (1999). Biological mechanisms,

such as competition between indigenous microorganisms and pathogens (Wichuk and McCartney, 2007), and microbial antagonism (Millner et al., 1987; Erickson et al., 2010; Gurtler et al., 2018) may also affect pathogen inactivation. Recently, Avidov et al. (2021a) explored the combined effect of temperature (30, 40, 50, and 60°C), water content (40, 55, and 70%; w/w), and initial pH (6, 7, and 8.5) on the persistence of *S.* Infantis in BL under laboratory controlled conditions. The authors showed that temperature was the main factor influencing *Salmonella* decay rates, while water content and initial pH were of secondary level of influence with significant effects mainly at 30 and 40°C.

This study presents a combined field-lab approach for assessing pathogen persistence in BL before land application. It is based on high-resolution spatial field sampling of BL during temporary storage or composting, coupled with lab-scale evaluations of the pathogen persistence under controlled conditions. The first objective was to evaluate in detail the spatial–temporal distribution of key physicochemical properties that can affect the persistence of pathogens in BL. Two systems were examined: 1. A static stockpile (without any special treatment), which represents the common practice in Israel and 2. composting in a closed polyethylene sleeve with forced aeration as a cheap alternative of enclosed composting (Avidov et al., 2017, 2018). The second objective was to combine such field data with recently published decay rate constants of *S.* Infantis under controlled lab conditions (Avidov et al., 2021a) and use the data in assessing the persistence of the pathogen in real scenarios. Besides being a unique systematic database of decay constants, both the present and the previous study used BL from tunnel-ventilated broiler houses of farms in northern Israel that use raising protocols of the main national poultry cooperatives.

MATERIALS AND METHODS

Experimental Setup

The study was conducted at the Newe Ya'ar Research Center, Israel, between August and September 2018 during which the ambient min and max temperatures ranged between 18.9–22.8°C and 33.4–36.7°C, respectively (Israel Meteorological Service; data station No. 186; https://ims.data.gov.il). A single trial of two pilot-scale setups, *ca.* 35-m^3 BL each, included one open static pile (stockpile) and one closed polyethylene sleeve with forced aeration (Avidov et al., 2017, 2018). The BL was brought from two broiler houses located in a poultry farm at Moshav Balfouria (northern Israel) at the end of an extensive indoor rearing period of 6 weeks. The stockpile was prepared directly by unloading a truck on a concrete floor located outdoor at the Newe Ya'ar campus. This pile consisted of three subpiles; each was monitored at 15 points (three sampling locations at depths of 10, 30, 50, 70, and 85 cm) plus 8 points at the lower edges of the pile for temperature measurements, or 2 points for other measurements. A total of 47 or 53 sampling points (**Figures 1A,B**) were employed on the entire stockpile. For composting in a sleeve, the BL was initially brought to a water content of *ca.* 50% (w/w), considered to be within the optimal range for composting (Bernal et al., 2009; Christian et al., 2009). For that, water was added to the pile manually using a hose and a water flow meter. Then the BL was mixed several times by means of a front-end and backhoe loader. The amount of added water was predetermined based on the initial water content of the BL (drying at 70°C for 24 h). The sleeve was constructed manually, using a polyethylene sheet of 25-m long × 8.5-m wide and 1,500 μm thick (A. A. Politiv Ltd., Kibbutz Einat, Israel). The sheet was placed on the ground, a solid 2.5-inch diameter PVC flexible pipe was connected to a blower on one side (centrifugal Model PB 50-3, Shevah Blowers Ltd., Ashdod, Israel), while the other side was connected to a 3.5-inch diameter perforated PVC flexible pipe (hole area *ca.* 10 mm^2; the pattern included six holes in circumferential rings along the pipe at 1.7-cm intervals). The perforated pipe was placed on a plastic sheet in the form of a longitudinal ring. Another piece of solid pipe was placed for exhaust on the opposite side of the sleeve (**Figure 1C**). The BL was piled on the sheet (on top of the perforated pipe), and the sleeve was sealed using a manual impulse hand sealer (hpl ISZ, HAWO, Germany). Finally, the sheet and the pipe were taped together on both sides using a heavy-duty masking tape (Avidov et al., 2019). This manual procedure was developed in the present study as an alternative to the use of a dedicated machine [like Euro-bagging CM 1.5 CCS (Avidov et al., 2017, 2018)]. The principles of the technology remain similar, although the specific pipe geometry can affect airflow paths. The blower was operated by a programmable logic controller (PLC; Vision1040™, Unitronics, Israel) for 2 min ON and 30 min OFF with flowrates of *ca.* 150 $m^3\ h^{-1}$. The sleeve was monitored in four cross-sections, at 1.5-, 6-, 10- to 12-, and 16-m distance from the blower (sections I–IV, respectively). Each cross-section was monitored in 12 sampling points, a total of 48 points (**Figure 1D**). To represent the results of the whole sleeve, we calculated the weighted average of the four cross-sections, considering the relative volume of each section. All sampling campaigns, both in the stockpile and the sleeve, were conducted during daytime, usually between 8 a.m. and 4 p.m.

Spatial–Temporal Mapping of Physicochemical Properties of the Stockpile and the Sleeve

Temperature, water content, pH, electrical conductivity (EC), as well as gas-phase oxygen and ammonia, were measured weekly during the first month and biweekly during the second month of the experimental period. Temperatures were measured by a mobile type K thermocouple that was mounted on an 80-cm-long stainless steel rod and connected to a 305 thermometer (Elcon Ltd., Israel). BL samples were collected from the designated depths using a soil auger (Edelman head, 3.5-cm core diameter; Eijkelkamp Soil and Water; Giesbeek, The Netherlands). Water content (w/w) was determined on *ca.* 5- to 6-g samples by oven drying at 70°C for 48 h. The pH and EC were measured in aqueous extracts at 1:9 dry weight:deionized water. The extracts were prepared by using a reciprocal shaker at 200 rpm for 1 h. The pH was analyzed directly in the suspension (LL-Ecotrode Plus WOC; Metrohm, Herisau, Switzerland), while the EC was determined in the supernatant after centrifugation at 6,000 rpm

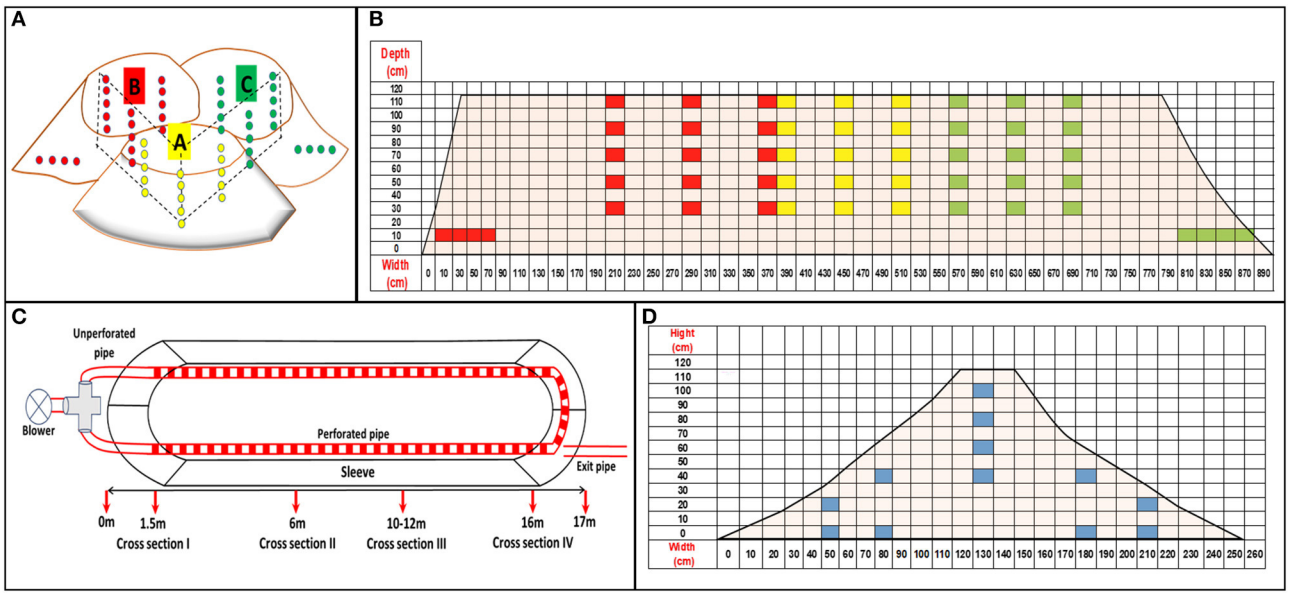

FIGURE 1 | Setup geometry and the location of sampling points in the stockpile **(A,B)** and the sleeve **(C,D)**. The cross-section of the stockpile connects the centers of three subpiles. The sleeve was divided into four sections, each was represented by a cross-section at 1.5-, 6-, 10- to 12-, and 16-m distance from the blower (denoted as CS-I – CS-IV).

for 20 min at 25°C (CyberScan CON 11, Eutech Instruments, Thermo Fisher Scientific Inc., Waltham, MA, USA). Gas-phase oxygen and ammonia were monitored in the same designated points (as used for temperature measurements and BL sampling) by pumping the headspace of each point using two pocket pumps (SKC 210 and SKC 220-1000TC, 84, PA, USA). The air was pumped through a Teflon tube (1/4″ inner diameter) that was connected in parallel to an acid trap (for ammonia analyses) and an oxygen sensor (SO-210, Apogee Instruments, Inc., Logan, UT, USA) (**Supplementary Figure 1**). The oxygen sensor was calibrated, and its readings were validated at the beginning of each sampling day against zero (pure N_2; 99.999%) and ambient air (21%). Ammonia (trapped in 0.1 M sulfuric acid) was analyzed using a spectrophotometric method with slight modifications after that of Willis et al. (1996) and similar to that of Avidov et al. (2021b). The method was validated against a standard of 50-ppm ammonia (Balance N_2 58L standard gas; Calgaz, Staffordshire UK), showing a bias of <4%. On each sampling campaign, the headspace was withdrawn at a single time in each point of the stockpile, while for the sleeve, it was done at intervals during aeration-off periods. In that case, sampling was done for a period of about 30 min following aeration-on periods of 2 or 7 min. Each spatial dataset was used to construct a grid map, which was then converted into a kriged contour map using the Surfer 7 software (Golden software, CO, USA). The initial average height of the stockpile was 113 (±5.8) cm and that of the sleeve was 120 (±0) cm. For simplicity, both the stockpile and the sleeve were normalized to the same initial height of 120 cm, as well as throughout the experiment, during which we observed height loss (and volume) due to natural compaction and organic matter degradation. Due to normalization of the sampling depths, the relative position of the sampling points in all maps overlapped each other.

BL Analyses

Selected properties of the fresh and processed BL are presented in **Table 1**. The fresh BL was sampled from nine random locations right after constructing the stockpile and the sleeve (time 0). A composite sample of each setup was then divided into triplicate subsamples. After 2 months, samples were collected from the stockpile and the sleeve from all designated sampling points and combined into composite samples (one for the stockpile and four for each cross-section of the sleeve). Ash content was determined after incinerating samples at 550°C for 4 h. Total C and N were determined after grinding the samples (mixer mill MM 400, Retsch, Haan, Germany) and analyzing them by FlashSmart 2000 Elemental Analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Total P was determined after digestion with H_2SO_4 and H_2O_2 using the ascorbic acid method (SM 4500 P-E; APHA, 2005). Analyses of water content, pH, and EC followed the same procedures as described above for the spatial–temporal mapping.

Assessing the Persistence of *S.* Infantis in Broiler Litter at the Stockpile and Composting Sleeve

Using exponential decay constants recently published for *S.* Infantis (Avidov et al., 2021a), along with the contour maps drawn in the present study, we assessed the persistence of the pathogen in the stockpile and the sleeve. The work of Avidov et al. (2021a) was based on controlled static lab vessels, covering 36 combinations of four temperatures (30, 40, 50, and 60°C),

TABLE 1 | Selected properties of the broiler litter (BL) at time 0 and after 60 days in a static stockpile and a closed sleeve with forced aeration.

Property	Stockpile		Sleeve†					
	Day 0	Day 60	Day 0	Day 60 CS-I	Day 60 CS-II	Day 60 CS-III	Day 60 CS-IV	Day 60 Weighted average
Height (cm)	113 (±6)	77 (±6)	120	65	65	65	70	66 (±3)
Water content (w/w; %)±	31.2 (±0.72)	20.1 (±7.8)	51.1 (±1.7)	42.7 (±6.3)	41.5 (±8.0)	42.0 (±15.2)	42. 2 (±10.5)	42.1 (±21.1)
pH±	7.0 (±0.0)	7.1 (±0.4)	7.0 (±0.00)	8.4 (±0.3)	8.3 (±0.4)	7.7 (±0.4)	7.9 (±0.4)	8.0 (±0.7)
EC (dS m^{-1})±	9.9 (±0.00)	10.3 (±1.2)	11.8 (±0.0)	13.6 (±1.7)	13.7 (±2.4)	10.9 (±1.5)	15.3 (±1.9)	13.2 (±3.8)
Ash (%)#	14.9** (±0.7)	16.2 (±0.8)	15.6 (±0.9)	18.5 (±0.5)	20.0 (±0.9)	17.1 (±0.2)	18.6 (±0.7)	18.4 (±0.5)
C_{total} (%)#	40.4 (±0.2)	39.1 (±0.6)	40.9 (±0.4)	38.8 (±0.5)	39.0 (±0.2)	40.0 (±0.9)	40.2 (±0.3)	39.5 (±0.5)
N_{total} (%)#	3.85 (±0.2)	3.61 (±0.2)	5.03 (±0.1)	4.2 (±0.06)	3.7 (±0.07)	4.1 (±0.1)	4.4 (±0.06)	4.04 (±0.0)
C/N	10.5	10.8	8.1	9.3	10.6	9.9	9.2	9.8
P_{total} (%)#	0.8 (±0.01)	0.9 (±0.06)	0.7 (±0.08)	1.0 (±0.08)	0.99 (±0.03)	0.91 (±0.11)	0.93 (±0.11)	0.95 (±0.08)

† *Measured in four cross-sections (CS-I – CS-IV). The weighted average of the whole sleeve is based on the relative volume fraction represented by each cross-section.*

± *On day 0, the values represent the average and standard deviations of triplicate subsamples obtained from a composite sample from nine random locations right after the construction of the stockpile and the sleeve. On day 60, the average and standard deviations are calculated for all grid cells, and for the sleeve weighted average, it is calculated for all grid cells in the four cross sections.*

On day 0, the values represent the average and standard deviations of triplicate subsamples obtained from a composite sample from nine random locations right after the construction of the stockpile and the sleeve. On day 60, the values represent the average and standard deviations of triplicate subsamples obtained from a composite sample from all designated sampling points used to characterize the physicochemical properties.

three water contents (40, 55, and 70%), and three initial pH (6, 7, and 8.5). Thus, according to the contour maps drawn for temperature, water content, and pH, the stockpile and the sleeve were divided into volume fractions of specific combinations. To link between the discrete values used by Avidov et al. (2021a) and the ranges applied in this study, we assumed the following matching: Temperatures of 30, 40, 50, and 60°C were considered as ≤35°C, 35–45°C, 45–55°C, and >55°C; water contents of 40, 55, and 70% were considered as <47.5%, 47.5–62.5%, and >62.5%; and pH of 6, 7, and 8.5 were considered as ≤6.5, 6.5–7.75, and >7.75. Based on the specific combination, each grid cell was assigned with a respective k value from Avidov et al. (2021a), representing the first-order decay constant according to Equation (1).

$$C(t) = C_0 e^{k(t)} \quad (1)$$

where $C(t)$ is the concentration of *Salmonella* (CFU g^{-1} dry matter) at point in time t (days), C_0 is the initial concentration of *Salmonella*, and k is the first-order decay constant ($days^{-1}$). After assigning k values, the $\log_{10}$ reduction of *Salmonella* ($C(t)/C_0$) during the first week (t =7 days) was calculated for each cell. Then the $\log_{10}$ reduction after 2 weeks (t = 14 days) in each cell was calculated as the sum of $\log_{10}$ reductions in week 1 and week 2. Likewise, the $\log_{10}$ reduction can be calculated for longer times based on the temporal physicochemical grid maps constructed for each week.

Statistical Analyses

JMP Pro 15 (SAS Institute Inc.) was used for all statistical analyses. The comparisons between kriged contour maps (performed using the Surfer 7 software; see above) were made on selected number of grid cells (n value) that is comparable to the number of physical sampling points measured in the sleeve and the stockpile. This limited number of cells were evenly distributed throughout the cross-section (50 points for the single cross-section of the stockpile and 12 points for each of the four cross-sections of the sleeve) to represent the overall spatial distribution of physicochemical properties. Significant differences were determined using Tukey's honestly significant difference (HSD) test at $p \leq 0.05$. Pearson's r correlations were determined among the physicochemical variables determined for all grid cells in the stockpile and the sleeve.

RESULTS AND DISCUSSION

Spatial and Temporal Variations of Key Physicochemical Properties in the Stockpile and the Sleeve

Temperature

Stockpile

A clear trend was evident since the first week, showing higher temperatures in the center of the stockpile and relatively cool temperatures at the margins and top surface (**Figure 2A**). The temperature gradient ranged from 61°C at the core, down to

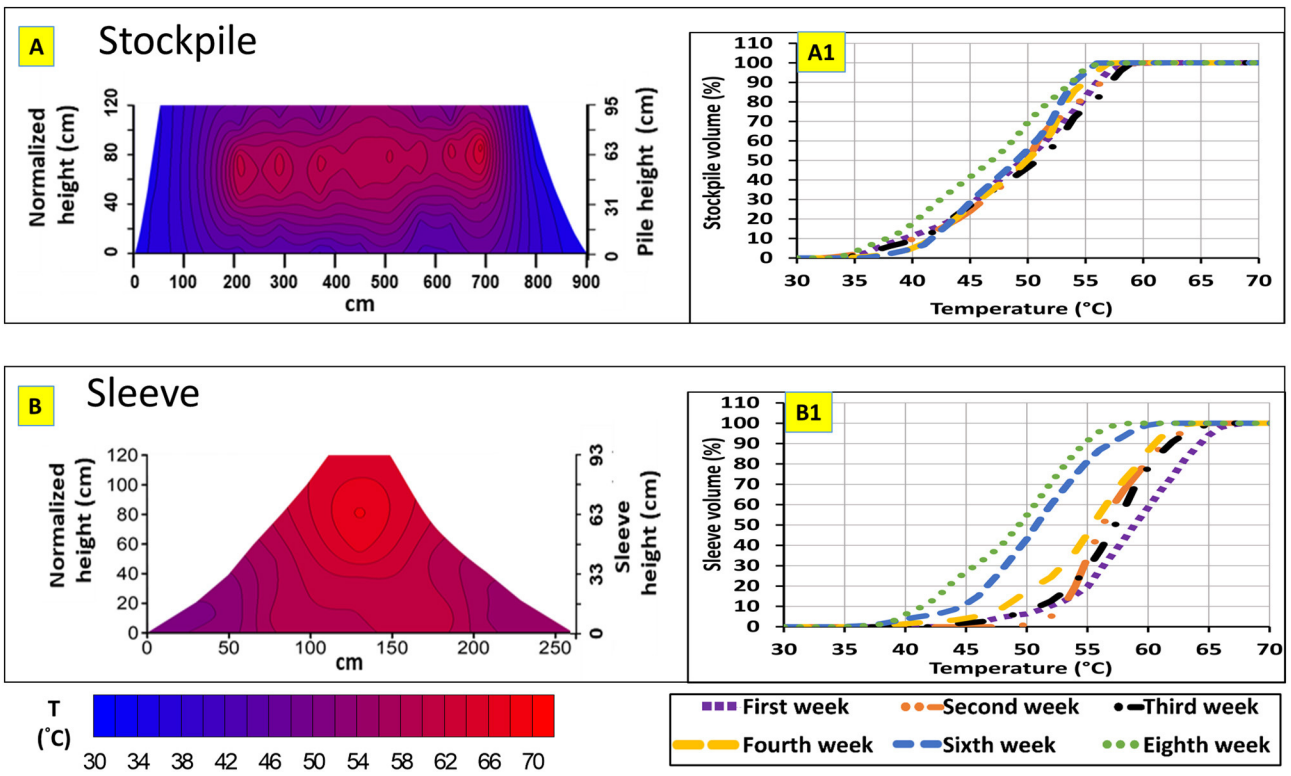

FIGURE 2 | Spatial–temporal variation in temperature (°C). Contour maps (week 1) and the cumulative volumetric percentage of temperature in the stockpile **(A,A1)** and the sleeve (**B**; cross-section II, **B1**; weighted average of cross-sections I–IV).

33–34°C toward the surface. Within this range, 83% of the total cross-section (considered here as the total pile volume) was below (≤) 55°C. Moreover, 27% of the entire pile volume, within the margins and top surface, did not reach thermophilic conditions (≤45°C). This trend did not change significantly during a period of 2 months (**Supplementary Figure 2-1** and **Supplementary Table 1B**), although this gradient slightly weakened over time, and the hot regions were reduced. A plot of cumulative volume fractions (**Figure 2A1**) shows a slight cool down on weeks 6 and 8, such that 50% of the pile volume was below 49–50°C, 49–50°C, 50–51°C, 49–50°C, 49–50°C, and 46–47°C, on weeks 1, 2, 3, 4, 6, and 8, respectively. This trend is also expressed by the average temperature of all grid cells, which was 48.8, 49.0, 49.5, 48.8, 48.3, and 46.1°C, on weeks 1, 2, 3, 4, 6, and 8, respectively.

Sleeve

Temperatures on weeks 1–4 and 6 were significantly higher than in the stockpile (**Supplementary Table 1A**), while the core and the top of the sleeve were still warmer than the lower sides (**Figure 2B**; CS-II). During the first week, the weighted average gradient of the four cross-sections ranged from 68 to 69°C in the core, down to 45–46°C at the lower sides. Within this range, 20% of the volume was below 55°C. The proximity to the blower seemed to have a chilling effect only in the first cross-section (CS-I; **Supplementary Figure 3-1**, significantly lower than other cross-sections only on weeks 2 and 4; **Supplementary Table 1C**); during the first week, 32, 10, 25, and 11% of the volume exhibited temperatures below 55°C in CS-I, CS-II, CS-III, and CS-IV, respectively. This trend did not change substantially during the entire period of 2 months (**Supplementary Figure 3-1**), although the gradient weakened over time and the upper part cooled down gradually, such that it finally became less spatially heterogeneous. A plot of the cumulative volume fractions (**Figure 2B1**) shows a more substantial cool down trend compared with the stockpile, such that 50% of the volume was below 58–59°C, 56–57°C, 57–58°C, 55–56°C, 50–51°C, and 49–50°C, on weeks 1, 2, 3, 4, 6, and 8, respectively. This cool down is also expressed by the weighted average temperature of all grid cells, which was 58.5, 56.7, 56.7, 54.8, 50.6, and 48.6°C, on weeks 1, 2, 3, 4, 6, and 8, respectively. No clear effect of the proximity to the blower was shown on these average temperatures.

Sleeve geometry and the setting of the perforated aeration pipe as well as the selection of blower type (**Figure 1C**) could all have an effect on temperature distribution and the relatively cool low edges. In our previous studies on biosolids and green waste (Avidov et al., 2017, 2018), the perforated pipe was placed in the center of the sleeve and showed lower temperatures along the centerline in the proximity to the aeration pipe. These studies also showed extensive heat accumulation within the middle and top parts of the sleeve. The sleeve clearly provided better conditions for thermal inactivation of pathogens: Compared with 83, 71, and

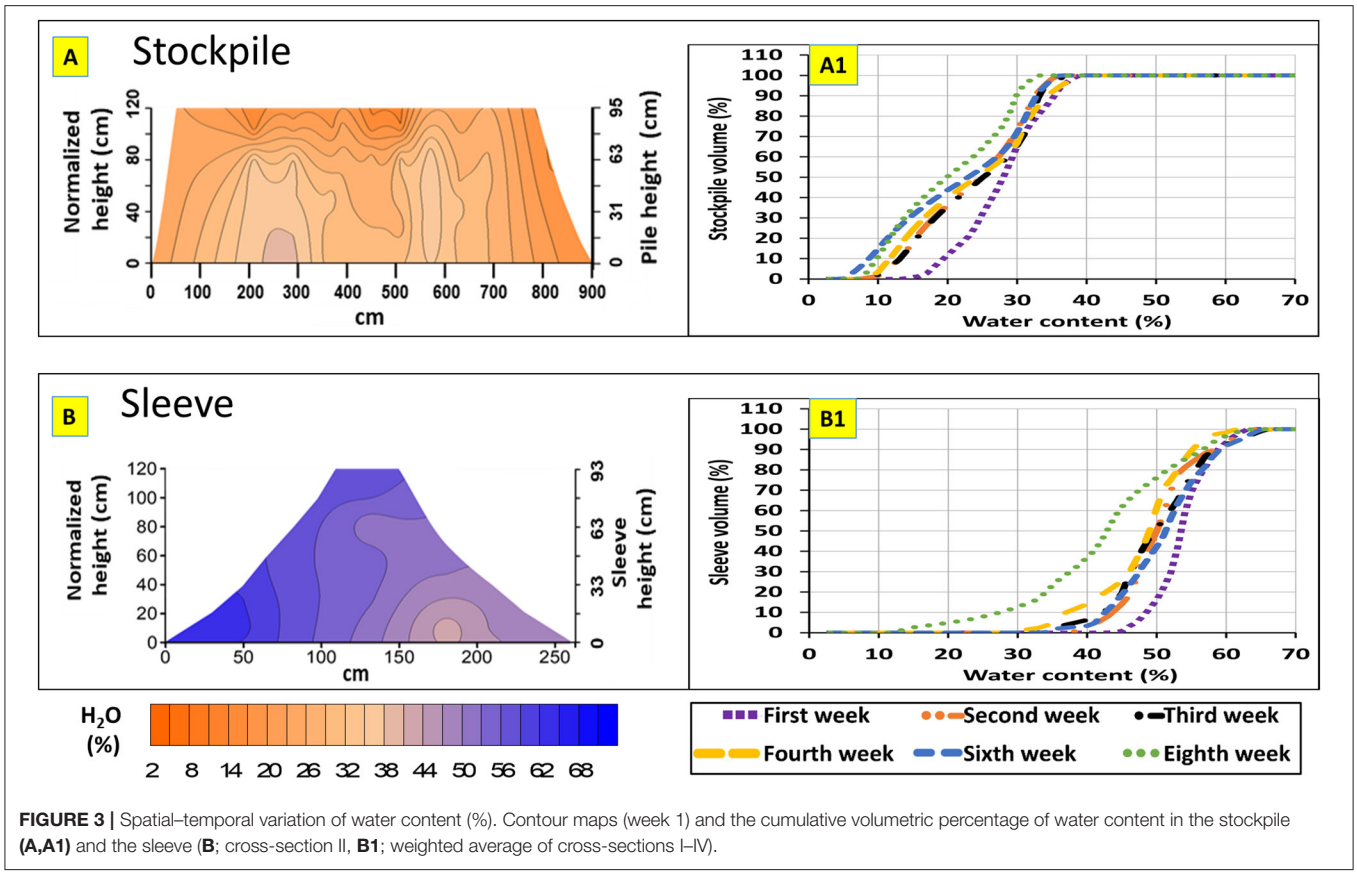

FIGURE 3 | Spatial–temporal variation of water content (%). Contour maps (week 1) and the cumulative volumetric percentage of water content in the stockpile **(A,A1)** and the sleeve (**B**; cross-section II, **B1**; weighted average of cross-sections I–IV).

62%, of the stockpile volume, only 20, 14, and 8% of the sleeve volume did not reach the commonly required minimum of 55°C for three consecutive days (USEPA, 2003) by the end of the first, second, and third weeks, respectively.

Water Content

Stockpile

The initial water content of the BL was relatively low (31.2 ± 0.72%; **Table 1**), and it was not adjusted prior to stockpiling as in a common practice. The gradient ranged from 14% at the stockpile edges and up to 39% at some locations in the center (**Figure 3A**). Within this range, none of the locations were at optimal conditions for biological activity (50–60%; Bernal et al., 2009, 45–65%; Christian et al., 2009). The water content gradient slightly decreased during the 2 months (**Supplementary Figure 2-2**, significantly lower on weeks 6 and 8; **Supplementary Table 1B**); a plot of the cumulative volume fractions (**Figure 3A1**) shows a gradual drying over time, such that 50% of the material was below 28–29%, 24–25%, 25–26%, 24–25%, 22–23%, and 19–20%, on weeks 1, 2, 3, 4, 6, and 8, respectively. This gradual drying is also expressed by the average water content of all grid cells, which was 27.7%, 23.6%, 24.1%, 23.5%, 21.7%, and 20.1% on weeks 1, 2, 3, 4, 6, and 8, respectively.

Sleeve

In contrast to the stockpile, the water content of the BL in the sleeve was pre-adjusted before composting to 51.1 ± 1.7 (**Table 1**). During the first week, the weighted average gradient of all cross-sections ranged between 43 and 64%. Within this range, only 16% of the sleeve volume was below 50% water content and 7% above 60% (**Figure 3B**; CS-II). The BL significantly dried out over time (**Supplementary Table 1B**), without a clear spatial trend. However, slightly drier conditions were observed at the sleeve's base next to the location of the perforated aeration pipe, while the proximity to the blower did not show a consistently significant effect (**Supplementary Figure 3-2**). A plot of the cumulative volume fractions (**Figure 3B1**) shows a gradual drying, such that 50% of the material was below water contents of 53–54%, 50%, 49–50%, 49%, 51–52%, and 42–43%, on weeks 1, 2, 3, 4, 6, and 8, respectively. This gradual drying is also expressed by the weighted average water content of all grid cells, which was 53.6, 50.2, 50.1, 47.8, 50.9, and 42.1%, on weeks 1, 2, 3, 4, 6, and 8, respectively. The range of water content that is ideal for composting is not definite (e.g., suggested as 50–60% by Bernal et al., 2009, or 45–65% by Christian et al., 2009). Water contents at the upper range (above 60%) increase water film thickness that fill small pores between particles and, in turn, limit oxygen diffusion throughout the composting material (Richard et al., 2002; Richard, 2004).

The different initial water content of the stockpile vs. the sleeve reflects the standard practice of farmers in which the relatively dry BL is stockpiled (Ogejo and Collins, 2009), while water content is adjusted before composting to optimize biological

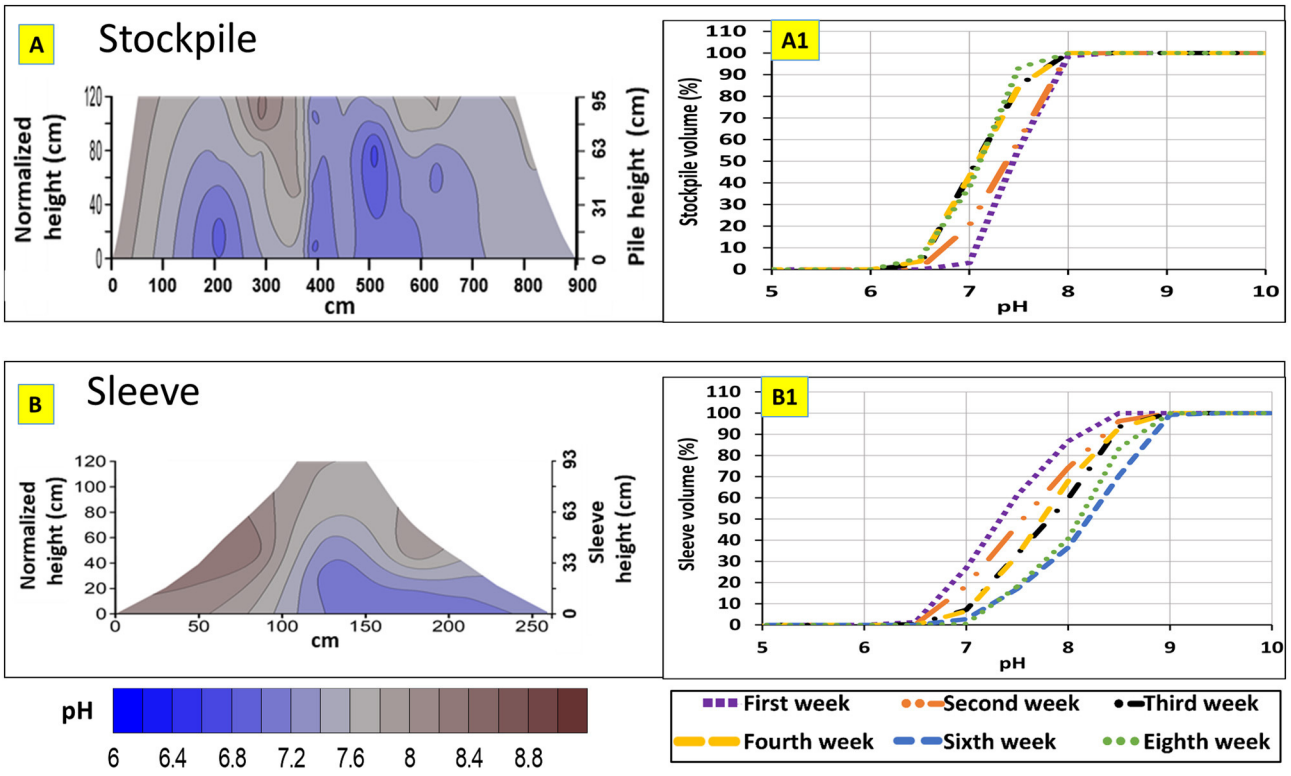

FIGURE 4 | Spatial–temporal variation of pH. Contour maps (week 1) and the cumulative volumetric percentage of pH in the stockpile **(A,A1)** and the sleeve (**B**; cross-section II, **B1**; weighted average of cross-sections I–IV).

activity (Walker, 2004). The relatively dry and poorly aerated static stockpile of this study resulted in low biological activity and, in turn, less heat accumulation compared with the moist and forced aerated sleeve setup. Further drying of the stockpile surface was eased by the dry summer weather (a total of 1.7-mm rain) and warm temperatures (max day temperatures of 33.4–36.7°C) during the experimental period.

pH

Stockpile

The initial pH of the BL was 7.0 (**Table 1**). During the first week (**Figure 4A**), the pH gradient ranged between *ca.* 7 at the core and 8.5 at the surface. A similar trend was observed during the whole period (**Supplementary Figure 2-3**). The average pH was significantly higher in weeks 1–2 than in all other weeks (**Supplementary Table 1B**). A plot of the cumulative volume fractions (**Figure 4A1**) does not show a substantial trend over time, such that 50% of the material was below 7–7.5 during the entire period.

Sleeve

The initial pH of the BL was 7.0 (**Table 1**). During the first week, the weighted average gradient of all cross-sections ranged between 6.5 and 8.5 with a slightly lower pH in the core (**Figure 4B**; CS-II). Except week 1, the average pH values were significantly higher in the sleeve compared with the stockpile (**Supplementary Table 1A**). A similar spatial pattern was observed in all cross-sections during the 2 months, while it was more noticeable in CS-I and CS-II (**Supplementary Figure 3-3**). A plot of the cumulative volume fractions (**Figure 4B1**) shows a gradual increase in pH, such that 50% of the material was below 7–7.5 on week 1, 7.5–8 on weeks 2–4, and 8–8.5 on weeks 6 and 8. This increase in pH, which was more apparent in the sleeve, is also expressed by the weighted average pH values of all grid cells during the entire period, which was 7.4, 7.6, 7.8, 7.8, 8.1, and 8.0, on weeks 1, 2, 3, 4, 6, and 8, respectively. No consistently significant effect of the proximity to the blower was observed.

In general, the higher pH values in the sleeve can be expected due to the more intense aerobic activity, which, in turn, results in the degradation of organic acids and the release of ammonia during mineralization of proteins, peptides, and amino acid (Gigliotti et al., 2012). On the other hand, poor aeration would lead to the production of acidic compounds, which might explain the slightly lower pH in the core of the stockpile and in some locations within the sleeve. Avidov et al. (2021a) showed a pH decrease from 6.5–7 to 5.5 and an increase from 6.5–6.8 to 7.5–7.6 under anaerobic vs. aerobic degradation of BL, respectively.

Electrical Conductivity (EC)

Stockpile

The initial EC of the BL was 9.9 dS m^{-1} (**Table 1**). During the first week (**Figure 5A**) the EC gradient ranged between 7 and 8 dS m^{-1} at the surface and 13 dS m^{-1} at the core and the bottom

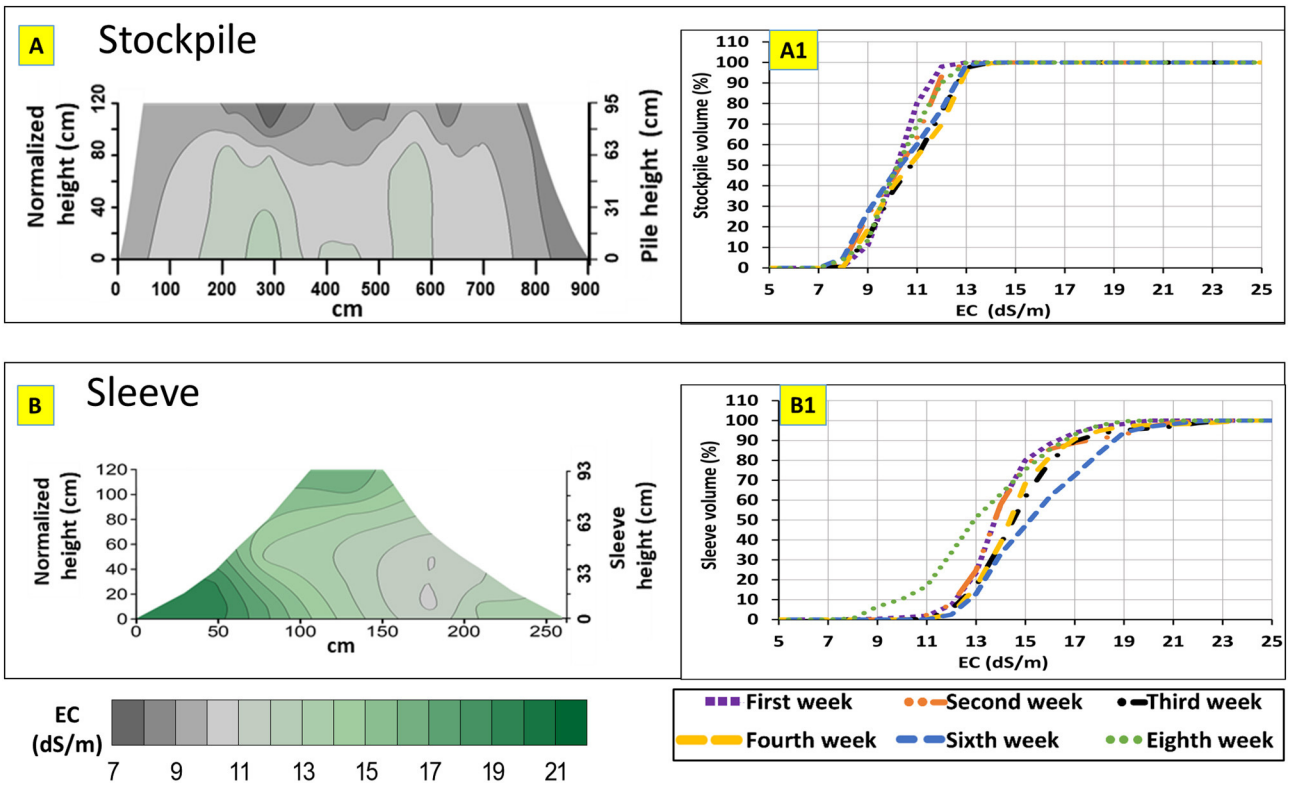

FIGURE 5 | Spatial–temporal variation of EC (dS m^{-1}). Contour maps (week 1) and the cumulative volumetric percentage of EC in the stockpile **(A,A1)** and the sleeve (**B**; cross-section II, **B1**; weighted average of cross-sections I–IV).

of the stockpile. A similar trend was observed during the whole period (**Supplementary Figure 2-4**). A plot of the cumulative volume fractions (**Figure 5A1**) does not show a trend over time, such that 50% of the material was below 10–11 dS m^{-1} during the entire period. The average EC values of all grid cells were also similar during the 2 months.

Sleeve

The initial EC of the BL was 11.8 dS m^{-1} (**Table 1**). In general, significantly higher EC values were measured in the sleeve compared with the pile (**Supplementary Table 1A**). During the first week, the average gradient of all cross-sections ranged between 8 and 20 dS m^{-1}, with the lower values appearing on one side (**Figure 5B**; CS-II). Compared with the stockpile in which the maximum value on week 1 was 13 dS m^{-1}, between 63 and 88% of the sleeve cross-sections was above that value. No clear spatial trend was observed during the whole period, while a more noticeable gradient was observed in CS-II and CS-IV (**Supplementary Figure 3-4**). A plot of the cumulative volume fractions (**Figure 5B1**) shows some EC increase and then another decrease, such that 50% of the material exhibited values below 13–14 dS m^{-1} on weeks 1–2, 14–15 dS m^{-1} on weeks 3–4, 15–16 dS m^{-1} on week 6, and finally again below 12–13 dS m^{-1} on week 8. However, no clear spatial trend was observed for the weighted average EC values of all grid cells during the whole period; neither any consistently significant effect could be related to the proximity to the blower.

On the average, the higher EC values in the sleeve can be the result of salt dissolution under higher water content compared with the stockpile. It can also reflect the more intensive aerobic degradation, which in turn results in the reduction of organic matter and increase in mineral (ash) content. Such increase in EC values during composting was reported in multiple studies (Zaha et al., 2013; Avidov et al., 2017; Karanja et al., 2019), although the opposite trend has been reported as well and was attributed to the release of volatile organic sulfur compounds, precipitation of mineral salts, microbial consumption of salts, and leaching of compost piles (Gondek et al., 2020).

Gas-Phase Oxygen

Stockpile

During the first week (**Figure 6A**), a strong gradient was observed, ranging from 21% at the surface, down to 0–1% at the core. A similar spatial trend was observed during the whole period, whereas the anaerobic fraction was lower on weeks 1 and 8. Of the pile volume, 14, 35, 32, 31, 37, and 19% was below 5% of oxygen on weeks 1, 2, 3, 4, 6, and 8, respectively (**Supplementary Figure 2-5**). A plot of the cumulative volume fractions (**Figure 6A1**) is another indication for this trend, such that 50% of the material was below 12–13%, 9–10%, 9–10%, 10–11%, 9–10%, and 12–13% oxygen, on weeks 1, 2, 3, 4, 6,

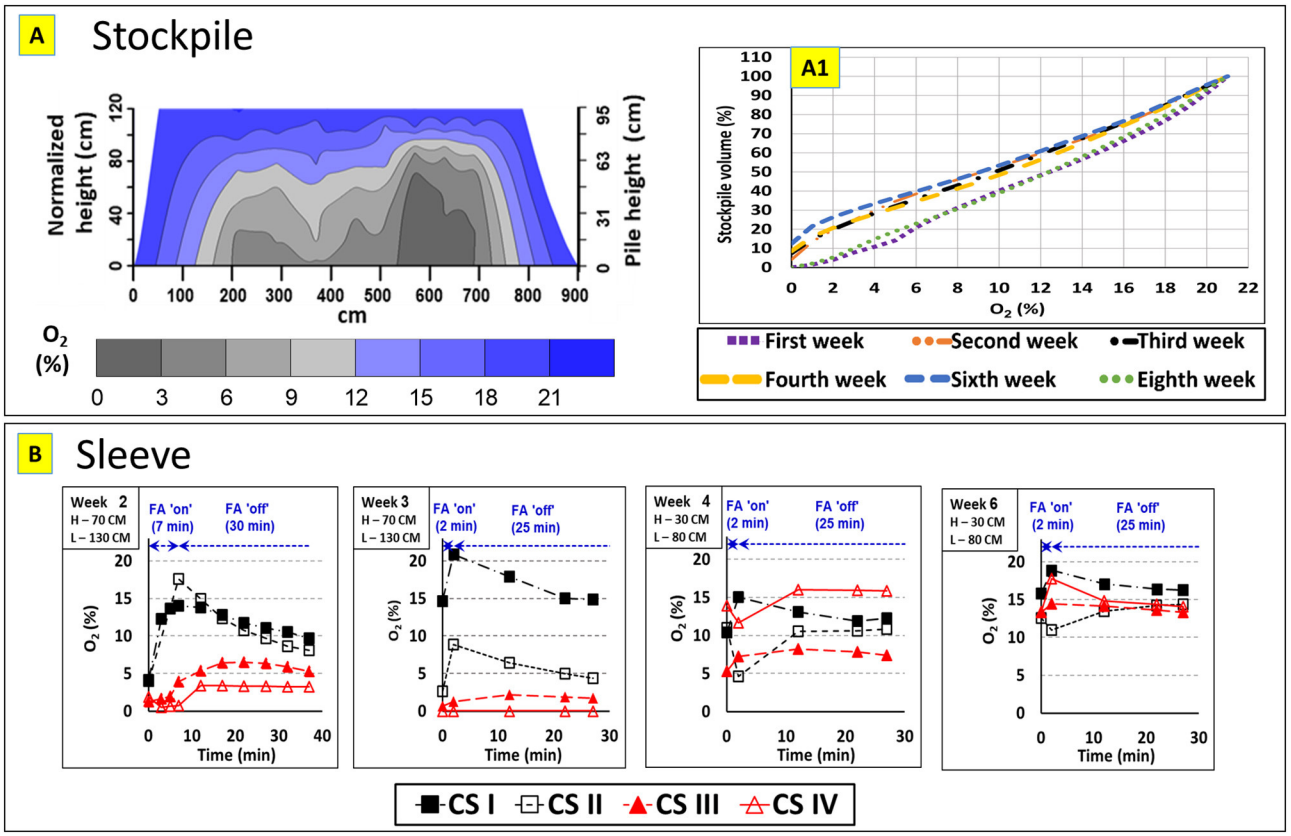

FIGURE 6 | Spatial–temporal variation of gas-phase oxygen (%). A contour map (week 1) and the cumulative volumetric percentage of oxygen are presented for the stockpile **(A,A1)**. Gas-phase oxygen in the sleeve is presented at time windows during which the blower was operated for 2–7 min, and the air was sampled at time intervals up to 30 min thereafter **(B)**.

and 8, respectively. Similarly, the average oxygen values of all grid cells were 12.1, 9.5, 9.7, 9.9, 9.1, and 11.8%, on weeks 1, 2, 3, 4, 6, and 8, respectively, although these differences were not significant.

Sleeve

The levels of oxygen are expected to fluctuate according to aeration on/off episodes. Thus, air sampling was conducted during off-periods following operation of the blower for 2–7 min (**Figure 6B**). On weeks 2 and 3, it was evident that aeration was more effective in proximity to the blower (CS-I and CS-II), whereas CS-III was mainly below 5% oxygen and CS-IV was around 0% on week 3. This effect decreased on weeks 4 and 6, during which no clear advantage was shown for the proximity to the blower. Moreover, as the composting process progressed, a lower rate of oxygen decline was observed following aeration episodes.

There is a basic difference between gas sampling of the stockpile and the sleeve: Oxygen levels measured in the static stockpile represent the headspace around the sampling point, while those measured in the sleeve following aeration episodes represent an unknown mix of headspace volumes depending on the dynamics of airways, and thus, a full spatial–temporal picture is hardly resolved. Without active turning, the stockpile was poorly aerated, whereas the extensive heat built up at the core did not trigger efficient air convection (the "chimney effect;" Stegenta et al., 2019). Since the water content in the stockpile was low, this poor passive aeration cannot be attributed to reduced air transport due to water-filled pores. Notably, poor aeration was also reported in turned windrow piles with high oxygen variability (Poulsen, 2011; Stegenta et al., 2019).

Regarding the sleeve, the inefficient aeration at the cross-sections away from the blower has not been reported in our earlier studies in which we monitored oxygen levels at limited sampling points (Avidov et al., 2017, 2018). Evidently, the specific configuration of the perforated pipe would affect the flow paths within the sleeve and, in turn, the magnitude of this effect. Compaction of the BL during composting may also reduce aeration efficiency (Avidov et al., 2017), although the present study shows that aeration was improved with time in sections III and IV, presumably due to the gradual drying of the composting material (**Figure 3**B1). In any case, as long as the holes of the perforated pipe are uniform in size and density (a shelf product), the loss of pressure along the sleeve would reduce aeration efficiency at increased distances from the blower. Practically, longer "on" periods may be applied during sleeve operation to improve aerobic conditions. Alternatively, a stronger blower may be used.

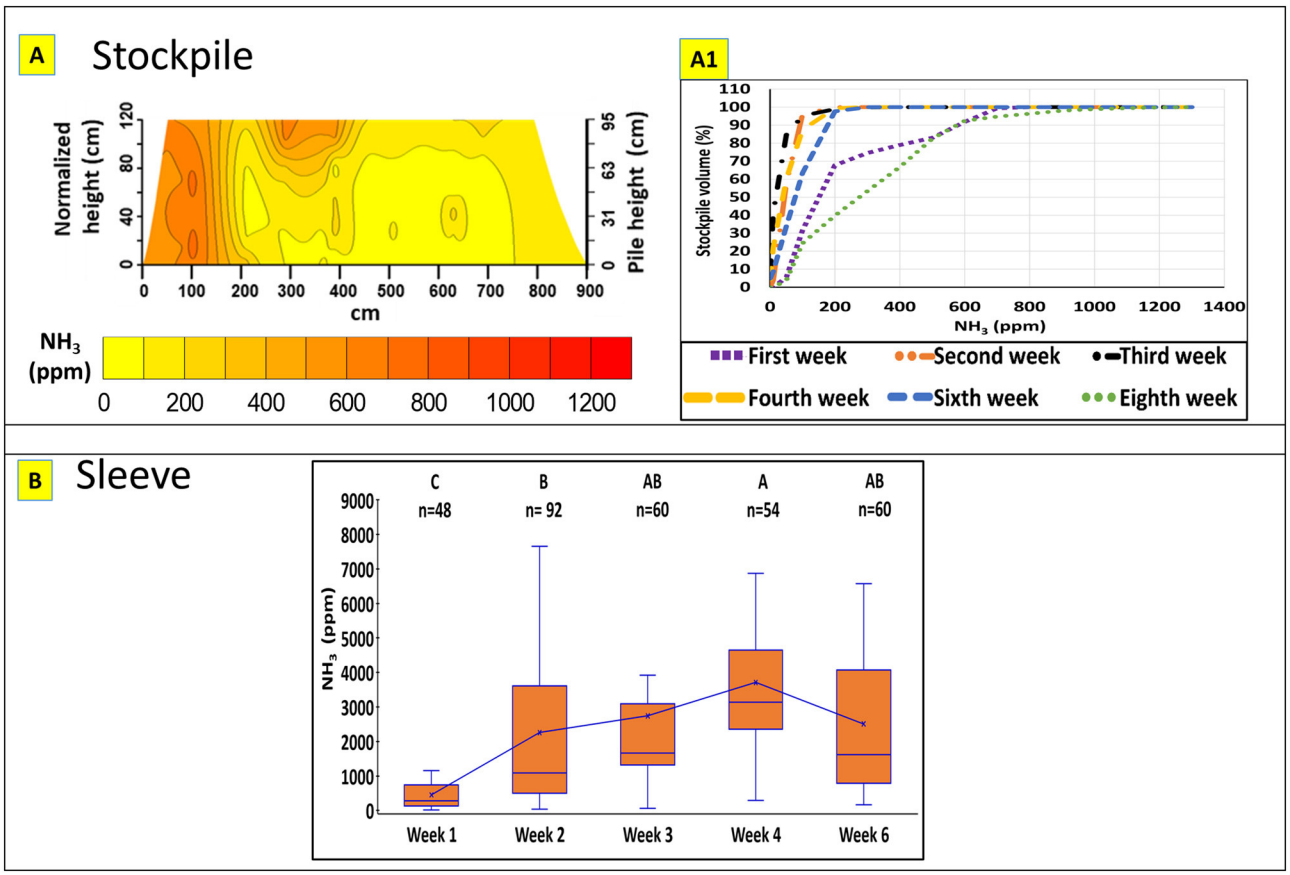

FIGURE 7 | Spatial–temporal variation of gas-phase ammonia (ppm). A contour map (week 1) and the cumulative volumetric percentage of ammonia are presented for the stockpile **(A,A1)**. Gas-phase ammonia in the sleeve is presented in a boxplot of the collective results obtained from all cross-sections on each sampling week **(B)**.

Gas-Phase Ammonia

Stockpile

During the first week (**Figure 7A**) and later on (**Supplementary Figure 2-6**), no clear spatial trends were observed. A large variability of ammonia concentrations of 50–700 ppm was recorded throughout the pile, with significantly higher values on week 8 (extreme values over 1,000 ppm). A plot of the cumulative volume fractions (**Figure 7A1**) shows fluctuations over time, such that 50% of the material was below 100–200, 10–50, 10–50, 10–50, 50–100, and 200–300 ppm on weeks 1, 2, 3, 4, 6, and 8, respectively. These fluctuations are also expressed by the average ammonia concentrations of all grid cells, which were 227, 46.1, 27.7, 47.6, 80.2, and 305 ppm on weeks 1, 2, 3, 4, 6, and 8, respectively.

Sleeve

Like oxygen, gas-phase ammonia concentrations were clearly related to the blower operation. However, in contrast to oxygen, no clear dynamics was found during aeration episodes; neither any effect of the proximity to the blower has been observed. Thus, the collective results of all cross-sections are presented for each week on a boxplot (**Figure 7B**). Generally, as composting proceeded, ammonia emissions increased, but no significant trend was observed between weeks 2, 3, and 6. Overall, ammonia concentrations during aeration episodes were an order of magnitude higher than those measured at the static pile, with average values of 451, 2,258, 2,742, 3,711, and 2,507 ppm, on weeks 1, 2, 3, 4, and 6, respectively.

As noted for oxygen, measurements of gas-phase ammonia in the stockpile represent the headspace around the sampling point, while following aeration episodes of the sleeve, it represents an unknown mix of headspace volumes. Higher ammonia emissions in the sleeve compared with the stockpile are expected, whereas the overall microbial activity is enhanced under aerobic conditions and due to the initially adjusted water content. Kirchmann and Witter (1989) reported that during composting of poultry manure, <1% of the nitrogen was volatilized as NH_3 during anaerobic decomposing due to the low pH values.

Mass Degradation and Element Losses in the Stockpile and the Sleeve

The average height of the stockpile was reduced during an 8-week process by 32%, from 113 to 77 cm, and that of the sleeve was reduced by 45%, from 120 to 66 cm. About 50% of this decline occurred during the first week in both setups, indicating

the effect of material compaction after BL piling (**Table 1** and **Supplementary Figure 4**). The four cross-sections of the sleeve had a similar height at the beginning, then slightly differentiated, and finally became similar (**Supplementary Figure 4B**). Other properties measured at the beginning and after 2 months are summarized in **Table 1**. Ash content slightly increased in both setups, while the ratios between the initial and the final contents were used to estimate the losses of dry matter (Larney and Buckley, 2007; Avidov et al., 2018), yielding 8.4 and 15.5% losses in the stockpile and the sleeve, respectively. The gap between height reductions and dry matter loss estimations is another indication of the co-effect of material compaction and biodegradation. Total nitrogen concentrations decreased by 6.2% in the stockpile and by 19.6% in the sleeve. Based on dry matter losses, these values are equivalent to 14.1 and 32.0% nitrogen losses in the stockpile and the sleeve, respectively. Likewise, the general increase in total P concentrations (**Table 1**) reflects organic matter degradation and an increase in mineral concentrations (Sommer and Dahl, 1999; Osada et al., 2001). Presumably, dry matter losses are mainly attributed to the degradation of the broiler droppings, and much less to the sawdust that is used for bedding, which is expected to be fairly recalcitrant due to its high contents of lignin and cellulose (Leconte et al., 2009). Nevertheless, the sawdust in Israel is estimated to comprise only about 7% of the total BL on a dry matter basis (N. Cnaan, Director of the Growth Division at "Off Tov Group", Israel; personal communication). The lower N losses in the stockpile (also expressed by lower ammonia emissions; **Figure 7**) can be attributed to the anaerobic conditions prevailing in extensive zones of the stockpile (**Figure 6**). In contrast, the larger amounts of N losses in the sleeve correspond with the study of Avidov et al. (2017), who reported 38–45% losses during 6 months of municipal biosolid composting in sleeves. Ignoring sanitary issues that trigger the motivation for thermal treatment through composting, ammonia volatilization is a negative consequence, being a major mechanism of N losses. From the point of view of plant nutrition, stabilizing BL in a stockpile would be preferred, as less N is lost through ammonia volatilization.

Co-correlations Between Physicochemical Properties in the Stockpile and the Sleeve

Co-correlations between the measured physicochemical properties are visually apparent from the contour maps. Pearson's r linear correlations were determined for every pair of variables, including the values of all grid cells in the constructed contour maps (**Table 2** and **Supplementary Table 2**). Due to the large dataset (all grid cells), the significance of the correlations is always very high (low *p*-value) and, therefore, not indicated. Generally, higher correlations, either positive or negative, were obtained for the stockpile compared with the sleeve. Considering the contour maps constructed for all weeks in one dataset (**Table 2A**), high positive correlations ($r = 0.74–0.90$) were obtained in the stockpile for the pairs of temperature/water content, temperature/EC, water/EC, and pH/oxygen, while high negative correlations ($r = -0.60$ to -0.87) were observed for the pairs of temperature/pH, temperature/oxygen, water/pH, water/oxygen, pH/EC, and EC/oxygen. The highest correlations were obtained for EC/water ($r = 0.90$), EC/oxygen ($r = -0.87$), and water/oxygen ($r = -0.85$). Ammonia was the least correlated with any of the other properties. The same trends (positive or negative), but less strong correlations, were obtained for the sleeve (**Table 2B**). These correlations do not include ammonia and oxygen due to the different methodologies used to characterize the gas phase (during "off" aeration, following active aeration episodes). The pair of water/pH did not show a correlation in the sleeve, although it was negatively correlated in the pile. Also, the pair of temperature/EC showed a positive correlation only in CS-III and CS-IV (away from the blower). The dynamics of these correlations was not consistent as composting progressed, except for the sleeve on the first week, during which temperature and water positively correlated with pH, while the opposite trend was observed in the stockpile and sometimes in the sleeve (**Supplementary Table 2**).

These correlations reflect the co-dynamics of key properties during composting, while the static *vs.* dynamic settings of the stockpile and the sleeve affected such interactions differently. Under static conditions, passive aeration is inefficient, which is reflected by a strong oxygen gradient. Oxidation reactions within the core of the stockpile still result in heat accumulation (less than in the sleeve), while temperature correlates with the water content, which is required for biological activity, on one hand, and is a byproduct of aerobic degradation, on the other hand. Also, biological activity at the stockpile margins and the top surface may respond to the cooling and drying effect of the ambient atmosphere. Intense aerobic degradation also correlated with EC, thus, reflecting, on one hand, possible salt dissolution at higher water content, and it is also related to the process of increases in ash content. The same trends, which were found in the sleeve, yet of less strong correlations, probably reflect highly dynamic spatial–temporal distribution of oxygen and related physicochemical properties, as resulting from active aeration. This interpretation is supported by the slightly stronger correlations observed for CS-III and CS-IV that were aerated less efficiently and, thus, presented similar environments to the static stockpile.

Assessing the Persistence of *S.* Infantis in the Stockpile and the Sleeve

Assessment of the spatial persistence of *S.* Infantis in the stockpile is presented in **Figure 8**, based on the exponential decay constants reported by Avidov et al. (2021a) and the grid maps constructed in this study. As shown in **Supplementary Table 3**, the measured conditions of temperature, water content, and pH, are matched with 10 (stockpile) and 17 (sleeve) out of the 36 combinations studied by Avidov et al. (2021a), whereas combinations including temperatures of $\leq$35°C prevailed only in the stockpile. The spatial persistence of *Salmonella* is expressed by $\log_{10}$ reduction after 1 and 2 weeks (**Figure 8**). For example, 68.3% of the stockpile volume reached the conditions under which at least 8 $\log_{10}$ reduction is expected after 1 week. Thus, assuming an initial concentration of 7–8 $\log_{10}$ CFU g^{-1} BL, we

TABLE 2 | Pearson's r linear correlation coefficients among the physicochemical properties measured in the stockpile and the sleeve during 2 months.

A. Stockpile

Stockpile—all weeks	Temperature	Water	pH	EC	Ammonia	Oxygen
Temperature						
Water	**0.77**					
pH	**–0.64**	**–0.60**				
EC	**0.74**	**0.90**	**–0.77**			
Ammonia	–0.29	–0.16	0.23	–0.23		
Oxygen	**–0.79**	**–0.85**	**0.79**	**–0.87**	0.32	

B. Sleeve

Sleeve—all weeks (Sections I + II)	Temperature	Water	pH	EC
Temperature				
Water	0.32			
pH	–0.44	0.03		
EC	0.16	**0.69**	0.16	
Sleeve—all weeks (Sections III + IV)				
Temperature				
Water	**0.67**			
pH	–0.39	–0.05		
EC	0.42	**0.61**	–0.02	
Sleeve—all weeks (all sections)				
Temperature				
Water	**0.54**			
pH	–0.41	–0.01		
EC	0.31	**0.64**	0.07	

Positive correlations are marked in green. Negative correlations are marked in red. All correlation coefficients above 0.3 are underlined, and those above 0.5 are also bolded. Due to the large dataset (all grid cells of contour maps), the significance of the correlation is always very high and, therefore, not indicated.

expect that 25.3% of the stockpile volume (6–7 $\log_{10}$ reduction) plus 1.4% (3–4 $\log_{10}$ reduction) will still be contaminated with the pathogen after 1 week. Similarly, a volume fraction of 1.3% will still be contaminated by the end of 2 weeks. If the initial concentration of the pathogen would be lower, e.g., 2–3 $\log_{10}$ CFU g^{-1} BL, then it will be eliminated from the entire stockpile volume within 1 week only. The zones that are most prone to incomplete inactivation of the pathogen would be located at the pile margins, which were generally cooler than the core. Moreover, the physicochemical properties at the pile margins are affected by the environmental condition, like temperature, wind, precipitations, shading, or direct sun exposure (the potential role of UV radiation is considered irrelevant since the polyethylene sheet becomes heavily dirty). A secondary effect of both water content and pH, is shown at the top of the stockpile, which was also relatively cool but matched with a different combination of temperature, water content, and pH, for which Avidov et al. (2021a) reported higher *Salmonella* persistence.

Since *Salmonella* concentrations in poultry litter are typically in the range of 3–5 $\log_{10}$ CFU g^{-1} litter (Chinivasagam et al., 2009, 2010; Brooks et al., 2010), the assessments shown in **Figure 8** suggest that 2 weeks should be sufficient for *S.* Infantis elimination in a stockpile. Yet, under certain conditions, such as drying and re-wetting, *Salmonella* can multiply in the BL and increase by several orders of magnitude (Avidov et al., 2021a). Thus, for minimizing risks, a total of 3 weeks may be recommended before land application of BL stockpiles. In contrast, the same analysis for the sleeve yields over 8 $\log_{10}$ reduction of *S.* Infantis in the entire volume after 1 week only. This result is a direct expression of the significantly higher temperatures prevailing in the sleeve, while the relatively cool margins were still warm enough to be represented by a relatively low persistence of the pathogen. A secondary effect of water content and pH was noticeable only in the relatively cool stockpile, in which mesophilic temperatures were recorded in the first few weeks. Avidov et al. (2021a) showed that temperature was the main factor influencing *Salmonella* decay rates, while water content and pH mainly had an influence at 30 and 40°C.

Overall, this approach that combines high-resolution spatial field data along with decay rates of pathogens under controlled lab conditions may improve quantitative microbial risk assessments of manure utilization. Such assessments can hardly be validated in the field, since BL stockpiles or other field-scale setups cannot be artificially inoculated with any specific pathogen. Moreover, homogeneous inoculation of such volumes is technically impossible and would not be allowed for sanitary

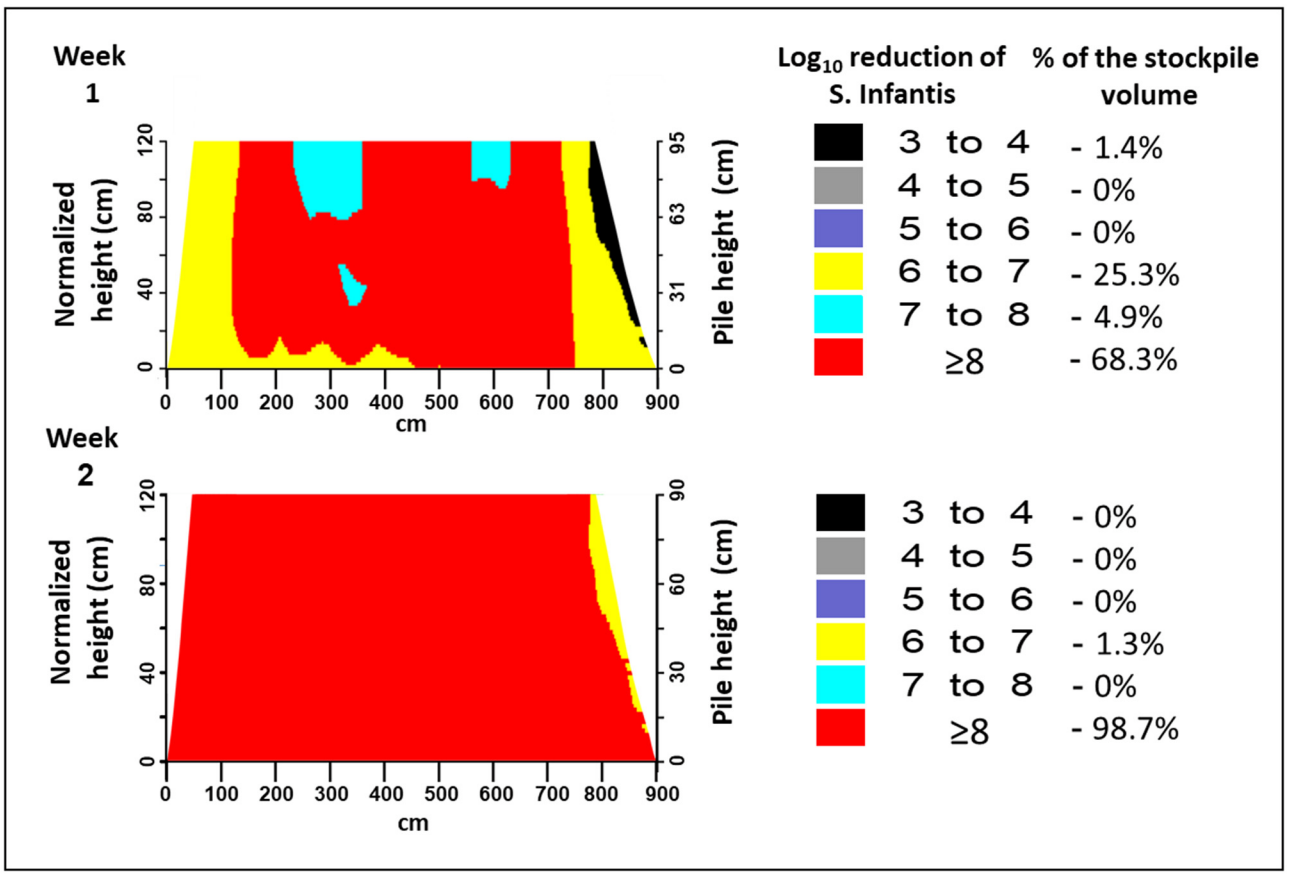

FIGURE 8 | Assessments of the spatial persistence of *S.* Infantis in the stockpile, based on the exponential decay constants from Avidov et al. (2021a) and the grid maps constructed in this study for temperature, water content, and pH.

reasons. Yet, the approach presented in this study can assist with regulations for any non- or forced-aerated static setups. It is much more informative than common regulations, e.g., the requirement for a minimum of 55°C for three consecutive days. As stated, although thermophilic temperatures are reported in multiple composting studies, only average or min and max values are often presented, while the spatial distribution remains unknown. In such cases, a pseudo-compliance with regulations may be shown just because of misrepresentation of the litter. As shown in this study, 83, 71, and 62% of the stockpile volume did not reach the commonly required minimum temperature of 55°C for three consecutive days during the first, second, and third weeks, respectively. Using the combined field-lab assessments, we would recommend a period of 3 weeks before safe land application. The assessment made in this study are based on temperature, water content, and pH only. Yet, the co-correlations found between temperature, water content, EC, and oxygen suggest that selected physicochemical properties may be sufficient for such assessments. Moreover, upon mapping the spatial distribution of key physical properties of relevant manure processing setups, this approach can be used to assess the spatial persistence of any pathogen that is tested under lab-controlled physicochemical conditions.

CONCLUSIONS

Static BL stockpiles are expected to develop a highly spatially non-homogeneous environment. The spatial–temporal distribution of physicochemical properties, combined with decay models, can be used to determine the persistence of zoonotic pathogens residing within livestock manure. Out of the measured properties, temperature is expected to remain a major factor, although the distribution of other properties should be considered, based on lab-scale controlled experiments. Co-effects of other physicochemical properties besides temperature are most relevant under mesophilic conditions and, thus, are applicable for manure stockpiles, but not for enclosed sleeves with forced aeration or any enclosed setups that ensure high thermophilic temperatures in the entire volume of the BL. Although only a single trial was conducted due to the intense labor demand in such a study, we assume that similar trends will be observed in future trials of such representing setups. Up to three weeks would be recommended to achieve 7–8 $\log_{10}$ reduction of *S.* Infantis in BL stockpile, while this would be fully achieved within 1 week in composting sleeves. Ammonia volatilization during composting, on one hand, and the cost and labor associated with the sleeves, on the other

hand, imply that composting in such setups may be longer than the time needed for pathogen inactivation but is still relatively short, sufficient enough to achieve other goals like reduction of odors and vector attraction. An approach that combines high-resolution field data, along with decay rates of pathogens under controlled lab conditions, may improve quantitative microbial risk assessments of manure utilization. It can be recommended as a universal approach in assessing the spatial persistence of other pathogens tested under controlled physicochemical conditions and analyzed against field-based detailed grid maps.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

RA: conceptualization, methodology, data analysis, and writing original draft. VV: conceptualization and methodology. IS: methodology and resources. OK: methodology. YC: supervision and reviewing. YL: supervision, conceptualization, writing, and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2022.811530/full#supplementary-material

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Integrated Soil Health Management for Plant Health and One Health: Lessons From Histories of Soil-borne Disease Management in California Strawberries and Arthropod Pest Management

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Many soil health assessment methods are being developed. However, they often lack assessment of soil-borne diseases. To better address management strategies for soil-borne disease and overall soil and plant health, the concept of Integrated Soil Health Management (ISHM) is explored. Applying the concept of Integrated Pest Management and an agroecological transdisciplinary approach, ISHM offers a framework under which a structure for developing and implementing biointensive soil health management strategies for a particular agroecosystem is defined. As a case study, a history of soil-borne disease management in California strawberries is reviewed and contrasted with a history of arthropod pest management to illustrate challenges associated with soil-borne disease management and the future directions of soil health research and soil-borne disease management. ISHM system consists of comprehensive soil health diagnostics, farmers' location-specific knowledge and adaptability, a suite of soil health management practices, and decision support tools. As we better understand plant-soil-microorganism interactions, including the mechanisms of soil suppressiveness, a range of diagnostic methodologies and indicators and their action thresholds may be developed. These knowledge-intensive and location-specific management systems require transdisciplinary approaches and social learning to be co-developed with stakeholders. The ISHM framework supports research into the broader implications of soil health such as the "One health" concept, which connects soil health to the health of plants, animals, humans, and ecosystems and research on microbiome and nutrient cycling that may better explain these interdependencies.

Keywords: soil health assessment, soil-borne disease management, integrated pest management, non-fumigant alternatives, soil suppressiveness, agroecology, soil-plant-microbe interactions, organic farming

INTRODUCTION

The concept of soil health recognizes soil as a living ecosystem with one of the greatest diversities on the earth. These organisms interact with each other, plants, and the complex abiotic environment (Wall et al., 2012; Orgiazzi et al., 2016; USDA-NRCS, 2021). Healthy soil can provide multiple ecosystem services such as food and fiber production, water quality and supply, pest and disease suppression, atmospheric composition, and climate regulation, and biodiversity conservation (Kibblewhite et al., 2008; Lehman et al., 2015; Bünemann et al., 2018).

Many laboratory-based soil health assessment methods and indicators have also been proposed and developed (Andrews et al., 2004; Moebius-Clune et al., 2016; Stott, 2019; Norris et al., 2020). These typically analyze chemical (pH, electrical conductivity, available nutrients contents, soil organic carbon, labile carbon, potentially mineralizable nitrogen, protein nitrogen, etc.), physical (water-stable aggregates, slake test, bulk density, etc.), and biological (various enzyme activities, respiration, microbial biomass, phospholipid fatty acid, etc.) properties. Yet, they often lack the assessment of soil-borne diseases. According to the Web of Science database, 3,120 papers were published on the topic "soil health" between 2000 and 2020. Among these, only 4.7% included topics of "soil-borne (or soilborne) pathogen," "soil-borne (or soilborne) disease," "suppressive," "suppressiveness," "suppressive soil," or "plant health."

Soil-borne diseases by fungal or bacterial pathogens and nematodes cause severe damage in agricultural production worldwide (Strange and Scott, 2005) and soil health assessment without assessing soil-borne diseases can be misleading. Healthy soil, defined using common soil health indicators, can produce unhealthy low-yield crops due to soil-borne diseases (Lazicki and Geisseler, 2021). To ensure healthy crop production, the inclusion of a soil-borne disease management perspective in soil health assessments is critical (van Bruggen and Semenov, 2000; Janvier et al., 2007; Larkin, 2015; Hodson and Lewis, 2016; van Bruggen and Finckh, 2016). However, many pathogens are plant-specific and effective management requires development of crop-, agroecosystem-, or location-specific soil health assessment and management strategies (Miner et al., 2020). While fumigants are widely used to control soil-borne diseases, the negative environmental and human health impacts are spurring development of non-fumigant alternatives for cropping systems worldwide (Labarada, 2008; Porter et al., 2010; López-Aranda et al., 2016; Daugovish et al., 2021).

Agroecology is the integrative study of the food system, encompassing ecological, economic, and social dimensions (Francis et al., 2003; Center for Agroecology, 2021). To create ecologically sound, economically viable, and socially just food systems, agroecology embraces science, practices, and social movements (Gliessman, 2018; Wezel et al., 2020) using transdisciplinary participatory approaches (Méndez et al., 2013). Transdisciplinary approaches value different types of knowledge systems including western scientific, indigenous, and farmer-generated practical knowledge on specific locations (Mendez et al., 2016:5) and co-production of knowledge by stakeholders and experts to realize more just food systems (Anderson et al., 2021).

Though first proposed to connect health between animals, humans, and the environment (Karesh et al., 2012; Wolf, 2015), a novel concept of "One Health" connects soil, plant, animal, human, and ecosystem health through the cycling of diverse microbiomes (Keith et al., 2016; van Bruggen et al., 2019; Altier and Abreo, 2020).

The concept of Integrated Soil Health Management (ISHM) can address management strategies for soil-borne disease and overall soil health. Melakeberhan (2010) used the term "agro-biologically, economically, and ecologically ISHM" that ties nematology and cross-disciplinary gaps for developing agrobiologically sustainable soil health management practices. Manter et al. (2018) argued the importance of underlying soil biology for soil conservation and regeneration. They have proposed a 5-step ISHM approach (knowledge, initial assessment, threshold for action, management, and reassessment) based on the adaptive management framework. However, there has been no examination of ISHM in the context of soil-borne disease management.

Applying the concepts of Integrated Pest Management (IPM) (Cook, 2000) and agroecological transdisciplinary and participatory approaches (Mendez et al., 2016; Anderson et al., 2021), we argue that ISHM and its four components, including farmer's location-specific knowledge and adaptability (**Figure 1**), offer a framework for developing and implementing a comprehensive site-specific biointensive soil health and soil-borne disease management strategy.

We begin with a brief case study review of the history of soil-borne disease management in California strawberries. Then, we contrast this with a history of arthropod pest management to illustrate the unique challenges associated with soil-borne disease management and future directions of soil health research. Lastly, we discuss the ISHM system and its relationship with One Health.

CASE STUDY: HISTORY OF SOIL-BORNE DISEASE MANAGEMENT IN CALIFORNIA STRAWBERRY

California produces ∼90% of strawberries in the US. In 2019, 1.0 million tons of fruits, valued at 2.2 billion dollars, were produced from 14,326 hectares of strawberry fields in the state (California Department of Food and Agriculture, 2021). The large-scale monocultural production of this lucrative crop has evolved dependent on the core technology of pre-plant soil fumigation (Guthman, 2019). Since the 1960s, chemical fumigation using methyl bromide mixed with chloropicrin, was the primary tool to control soil-borne diseases and weeds in California strawberries (Wilhelm et al., 1961; Holmes et al., 2020). Later, methyl bromide was identified as a significant stratospheric ozone-depleting compound by the Montreal Protocol (Ozone Secretariat Team, UNEP, 2020) and was phased out for strawberry production in 2016.

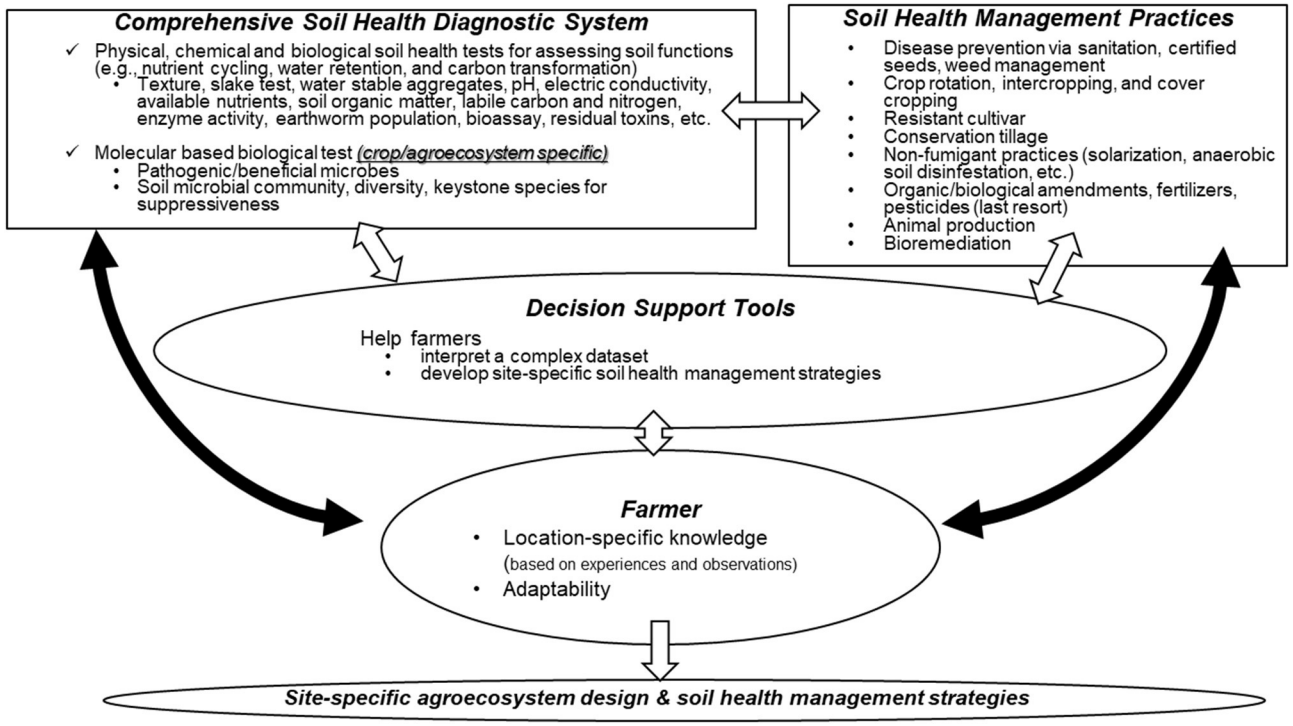

FIGURE 1 | The structure of the integrated soil health management (ISHM) system consists of farmers location-specific knowledge and adaptability, comprehensive soil health diagnostics, a suite of soil health management practices, and decision support tools (optional). Studies on relationships between varying soil health management practices and disease/pest suppression, nutrient uptake, and plant growth will help improve the diagnostic methods and develop actionable thresholds in diagnostics. Rectangular shapes and oval shapes indicate hardware/physical elements and software/intellectual elements of the system, respectively. Roles of decision support tools can be substituted or enhanced by agricultural professionals, consultants, and/or farmer-to-farmer networks (social learning).

In response, growers increased the use of alternative fumigants, such as chloropicrin and 1,3-dichloropropene, but they lacked effectiveness over the methyl bromide/chloropicrin mixture (Holmes et al., 2020).

The use of fumigants is highly regulated due to their toxicity and high application rates (California Department of Pesticide Regulation (CDPR), 2020) and negative impacts of fumigants on soil health (Dangi et al., 2017) and human health (Gemmill et al., 2013) have been reported. CDPR has documented hundreds of acute illnesses due to accidental exposure for both agricultural workers and populations adjacent to fumigated fields since 2003 (California Department of Pesticide Regulation (CDPR), 2013).

The California Strawberry Commission (CSC) initiated the "Farming without Fumigants" initiative in 2007 (Shennan et al., 2008). Non-fumigant approaches such as anaerobic soil disinfestation (ASD) (Shennan et al., 2018; Muramoto et al., 2020; Rosskopf et al., 2020), crop rotation with disease suppressive crops (Subbarao et al., 2007), use of host plant resistance (Guthman, 2019; Holmes et al., 2020), integration of these techniques (Shennan et al., 2020; Zavatta et al., 2021), substrate production (Thomas et al., 2014), and steaming with a mobile machine (Fennimore and Goodhue, 2016; Xu et al., 2017) have been examined. Overall, however, the adoption of non-fumigant approaches at conventional strawberry fields is yet limited.

Organic strawberry production may have the highest levels of adoption of fumigant alternatives. The acreage of organic strawberries has been gradually increasing since the 1980s (Gliessman and Muramoto, 2010) reaching 1,982 hectares, 13% of total strawberry acreage in California in 2021(California Department of Food and Agriculture (CDFA), 2021). Although typical organic yield is about 60% of the conventional counterpart (Bolda et al., 2016, 2019) disease suppressive strategies such as crop rotation with broccoli, host plant resistance, and ASD, alone or in combination, have supported the growth in organic strawberry acreage.

The recent development of rapid and accurate molecular diagnostic techniques is gradually making "scouting" of soil-borne pathogens a reality. For major lethal soil-borne pathogens in California strawberries, molecular approaches for *Verticillium dahliae* in plants (Dan et al., 2001) and soil (Bilodeau et al., 2012), *Fusarium oxysporum* f. sp. *fragariae* in plants (Burkhardt et al., 2019), and *Macrophomina phaseolina* in plant and soil (Burkhardt et al., 2018) have been established.

Recently, Lazcano et al. (2021) found that the rhizosphere microbiome plays a role in the resistance to soil-borne pathogens. Strong genotype by environment interactions observed suggests that soil health may also play a role in establishing beneficial plant-microbial interactions.

LESSONS FROM A HISTORY OF ARTHROPOD PEST MANAGEMENT

A history of arthropod pest management may offer some lessons for the future of soil-borne disease and soil health management. Between the 1940s and 1960s, broad-spectrum, highly toxic insecticides were widely used in arthropod pest management (Carson, 1962) following the motto, "the only good bug is a dead bug." (Warner, 2007: 141). In the late 1960s to early 1970s, due to "(insecticide) resistance, resurgence of primary pests, upsurges of secondary pests, and overall environmental contamination (Kogan, 1998: 245)," the concept of IPM was developed (Council on Environmental Quality, 1972) recognizing "there are good bugs (beneficial) as well as bad bugs (pests)." In the IPM system, transitioning to biointensive (National Research Council, 1996) or prevention-based IPM (Jacobsen, 1997) as well as redesigning of cropping systems (Hill, 1998) aimed at fostering plant and insect community and population dynamics that self-regulated pest presence and damage. More recently, the extinction of some arthropod species (Kiritani, 2000) and the decline of honeybee colonies (vanEngelsdorp et al., 2009; Ratnieks and Carreck, 2010) has raised awareness of the benefits of arthropod biodiversity and pollinators leading to the realization that "without bugs, we might all be dead." (Worrall, 2017). In biological control, social learning among farmers, rather than top-down extension, became more critical to implementing and disseminating knowledge-intensive approaches (Fakih et al., 2003; Warner, 2007).

In contrast, for soil-borne disease management in California strawberries, relatively broad-spectrum fumigants are still in use, and the IPM approach (Katan, 2014) is just beginning. The slow transition is partially due to the unique challenges associated with soil-borne disease management. For example, compared to arthropod pests, soil-borne pathogens are microscopic and require specific processes for identification that are still in the nascent stages of development and utilization. Identification and scouting are typically the first step of the IPM approach (Kogan, 1998). Unlike arthropod pest management, there are effectively no post-symptomatic treatments for soil-borne diseases. Instead, currently available treatments are all pre-plant treatments and the availability of non-fumigant alternatives is limited. The complexity and heterogeneity of soil ecosystems, the diversity of soil organisms, and the lack of basic understanding of plant-soil-microbiome interactions have limited a quicker transition to non-fumigant-based IPM approaches (Bardgett and van der Putten, 2014; Mazzola and Freilich, 2017; Thomashow et al., 2019). Further, risks due to the substantial financial investment required in wholesale marketing of high-value horticultural crops hinder the adoption of less proven non-fumigant soil-borne disease management approaches (Chellemi and Porter, 2001; Guthman, 2020).

However, advances in molecular techniques, computational power, and statistics over the last 20 years have rapidly increased our knowledge of soil-plant microbiomes and their functions. Similar to the "discovery" of "good bugs" in arthropod management, we are now understanding the importance of beneficial (Mendes et al., 2013), commensal (Teixeira et al., 2019), and core microbes (Banerjee et al., 2018; Toju et al., 2018). Mechanisms of suppressive soil conditions are a highly active area of research (Schlatter et al., 2017; Duran et al., 2021; Samaddar et al., 2021). To understand plant-soil microbe interactions as a part of the plant defense system, concepts of soil (Lapsansky et al., 2016) and plant memory (Kong et al., 2019), and plant (Han, 2019; Teixeira et al., 2019) and rhizosphere immunity (Wei et al., 2020) have been proposed. As we better understand the soil biome's life cycles, structures, and functions and their relationships with plant health, indicators and thresholds of beneficial soil microbes and soil microbial communities may be developed for specific crops or agroecosystems (Blundell et al., 2020).

European Union (EU) has one of the world's most stringent fumigant regulations and is leading in the development of the IPM approach for soil-borne disease management. They developed "Soil Health Strategy Actions" (The Agricultural European Innovation Partnership (EIP-AGRI) Focus Group, 2015) consisting of prevention (certified seed, sanitation, and weed control), monitoring (soil sampling, bioassay), crop rotation (frequency, sequence, green manure, resistant varieties), and additional measures (grafting, biological control agents, biofumigation, ASD, organic amendments, solarization, etc.).

INTEGRATED SOIL HEALTH MANAGEMENT SYSTEM, AGROECOSYSTEM HEALTH AND ONE HEALTH

We propose that ISHM, as a science and practice, with social movement advocacy for non-toxic agriculture, may evolve similarly to IPM for arthropod pest management; toward biointensive management, increasing prioritization of the role of beneficial organisms, and redesigning cropping systems and cultural practices that prevent soil-borne diseases and induce sustained soil and plant health. At the same time, ISHM is more than a simple application of integrated "soil-borne disease" management, it also encompasses soil's many other functions by improving overall soil health using transdicisplinary participatory approaches.

The proposed ISHM system in this context consists of 4 components (**Figure 1**). First, a comprehensive soil health diagnostic system created by integrating molecular approaches for quantifying pathogens, beneficials, and soil microbial indicators and their thresholds, developed with an existing soil health measurement system measuring physical, chemical, and biological soil properties for assessing soils' other functions such as nutrient cycling, water retention, and carbon transformation (Andrews et al., 2004; Moebius-Clune et al., 2016; Norris et al., 2020). The diagnostic system will determine the disease potential both from the pathogens density in the soil relative to their economic thresholds and the disease suppressiveness of the soil toward target pathogens evaluated by its biotic and abiotic properties (Postma et al., 2014; Schlatter et al., 2017). To ensure healthy crop production, monitoring of plant health indicators (e.g., nutrients and chlorophyll contents, mycorrhiza and endophyte colonization rates, pathogen infection rates,

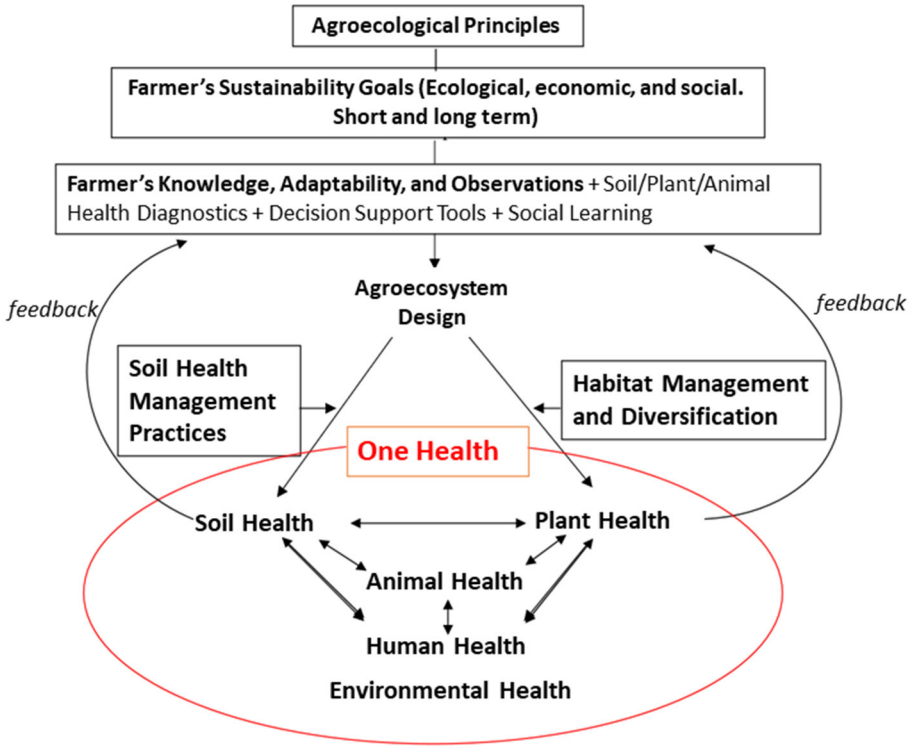

FIGURE 2 | Agroecosystem management and the concept of One Health. Integrated soil health management is embedded in this system. Agroecological principles include recycling, input reduction, soil health, animal health, biodiversity, synergy, economic diversification, co-creation of knowledge, social values and diets, fairness, connectivity, land and natural resource governance, and participation, according to a recent review by Wenzel et al. (2020). Habitat management and diversification have spatial (farm and landscape levels) and temporal dimensions. Environmental health includes the health of air and water. See **Figure 1** for soil health diagnostics, decision support tools, and soil health management practices. Modified from Altieri and Nicholls (2003).

etc.) will complement the soil health assessment during the cropping season.

EU (Clarkson et al., 2015) and Australia lead molecular plant-pathogen diagnostics services. PREDICTA® by the South Australian Research and Development Institute (Stirling et al., 2016; Government of South Australia, 2021), for example, is a fee-based public service for cereals, potatoes, and research, in which more than 10 pathogens and some beneficial microbes are quantified. The cost of quantifying soil microorganisms may hinder accessibility and affordability among diverse stakeholders. Development of portable, accurate, and easy to operate sequencers (Baldi and La Porta, 2020; Cunha et al., 2020) may allow farmers to determine soil and plant biomes in the field as "point-of-care" and may reduce the costs of diagnostics and empower farmers (Clarkson et al., 2015).

This information will then be integrated with farmers' location-specific knowledge and adaptability. Although often overlooked and underappreciated, farmers' location-specific knowledge gained from day-to-day fieldwork and observations and their adaptability to dynamic agroecosystems and climate change (Stockdale, 2011) is central to ISHM. Integration of scientific data obtained by diagnostics and farmers' experiential location-specific knowledge can be synergistic (Lobry de Bruyn and Andrews, 2016; Šumane et al., 2018). Dialogue between farmers and scientists centers farmers as an active player in examining, fine-tuning, and scaling-out agroecological knowledge and practices (Blundell et al., 2020; Anderson et al., 2021). Such participatory and transdisciplinary approaches mobilize knowledge for social change and engage stakeholders in research (Mendez et al., 2016).

The third component is a suite of soil health management practices (SHMPs) known to improve soil health. As seen in the EU program, various SHMPs including practices for prevention and enhancing disease suppression via general or specific suppressiveness (see **Figure 1**. e.g., applying organic amendments, cover cropping, crop rotation, using host resistance) (Abawi and Widmer, 2000; Raaijmakers et al., 2009; Hiddink et al., 2010; Larkin, 2015; Rosskopf et al., 2020) are integrated to tailor a site-specific soil-borne disease and soil health management strategy. A more intensive approach such as ASD and steaming is applied on an "as-needed" basis, depending on the soil health diagnostic result.

Lastly, decision support tools will assist growers in developing site-specific soil health management strategies based on their goals, knowledge, environmental conditions (e.g., soil type, climate, etc.), available SHMPs, results of soil health diagnostics, and other factors. **Figure 2** illustrates how ISHM is embedded in agroecosystem management and how it relates to the health

of soil, plants, animals, humans, and agroecosystems and the concept of One Health.

DISCUSSION

Although ISHM provides a framework, there are many knowledge gaps in the components parts. Primary research needs for developing ISHM include utilizing mechanistic models in plants-soil microbe functions such as soil suppressiveness, plant immunity, nutrient uptake (Liu et al., 2016; Trivedi et al., 2017), better chemical and biological characterization of organic amendments and crop residues, and their relationships with soil-borne disease suppressiveness (Bonanomi et al., 2018; Subbarao et al., 2020), increased efficacy of plant growth-promoting microbes in soil-borne disease suppression and nutrient uptake in field conditions (Rosier et al., 2018; Hestrin et al., 2019), and development of crop cultivars with ability to modify their rhizosphere microbiome for their benefits (Berg et al., 2016; Mendes et al., 2018).

ISHM is characterized as a location-specific and knowledge-intensive approach (Jacobsen, 1997), contrasted with the location-general and chemical-intensive fumigation and industrial farming approach. However, the transition to knowledge-intensive systems can present significant obstacles for farmers. As it worked in biocontrol (Warner, 2007), social learning, as seen in farmer-to-farmer networks, has facilitated the implementation and extension of knowledge-intensive soil health management (De Bruyn et al., 2017; Stockdale et al., 2019; Wick et al., 2019; Skaalsveen et al., 2020). Policies and extension activities that support such a process and the adoption of ISHM will be necessary for the greater engagement in the co-development of ISHM with and among stakeholders.

ISHM is additionally important as impacts of soil health may go beyond plant health. Indeed, our understanding of the direct and indirect effects of soil health on human health through microbiomes (Wall et al., 2015; Stegen et al., 2018; Samaddar et al., 2021) is increasing. The "One Health" concept suggests the interconnectedness of soil, plant, animal, human, and ecosystem health through microbiome cycling (van Bruggen et al., 2019, **Figure 2**). More than 70 years ago, Sir Albert Howard, an early student, and advocate of organic farming (Heckman, 2006), wrote, "The birthright of all living things is health. This law is true for soil, plant, animal, and man: the health of these four is one connected chain. Any weakness or defect in the health of any earlier link in the chain is carried on to the next succeeding links, until it reaches the last, mainly, man." (Howard, 1947). Although our understanding is yet at its infancy, future research on microbiome cycling and nutrient cycling (Altieri and Nicholls, 2003; Datnoff et al., 2007) may hold the key to better understanding the chains connecting healthy soils to plants, animals, humans, and ecosystems.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

JM: conceptualization and draft manuscript preparation. DP, JP, and DW: critically revised it, adding conceptual material and clarity. All authors contributed to the article and approved the submitted version.

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Spatial and Temporal Changes of Soil Microbial Communities in Field Tomato Production as Affected by Anaerobic Soil Disinfestation

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Anaerobic soil disinfestation (ASD) has been demonstrated as an effective alternative to pre-plant chemical soil fumigation (CSF) commonly used to control soilborne pathogens. However, the ASD effects on spatial and temporal changes in soil microbial communities remain poorly understood in production systems with low soilborne disease pressure. The objective of this study was to assess the influence of ASD treatments on soil microbial community composition at different soil depths during the spring tomato production season in Florida. Soil treatments included ASD using 6.9 m^3 ha^{-1} of molasses with 11 Mg ha^{-1} of composted poultry litter (CPL) (ASD0.5), ASD with 13.9 m^3 ha^{-1} of molasses and 22 Mg ha^{-1} CPL (ASD1.0), and chemical soil fumigation (CSF) using a mixture of 1,3-dichloropropene and chloropicrin. Soil microbial community composition was measured at soil depths of 0–15 and 15–30 cm using phospholipid fatty acid (PLFA) analysis at 0, 36, 76, and 99 days after transplanting (DAT). Fatty acid methyl esters were categorized into biomarker groups including total microbial biomass (TMB), G+ bacteria (G+), G− bacteria (G−), actinomycetes (Actino), arbuscular mycorrhizal fungi (AMF), protozoa, and general fungi (F). Soil concentrations of G+, Actino, F, AMF, and the ratio of F:bacteria (B) were significantly impacted by a soil treatment × soil depth × sampling time three-way interaction. All the microbial biomarkers were significantly affected by soil treatment × sampling depth two-way interactions except for protozoa and F:B ratio. Concentrations of TMB, Actino, AMF, F, G+, and G− bacteria were significantly increased in ASD treated soils at both 0–15 and 15–30 cm soil depths across different sampling times compared with CSF. In addition, the concentrations of G+ and G− bacteria, AMF, F, and TMB were higher at 0–15 vs. 15–30 cm soil depth under ASD treatments, whereas no soil depth differences were observed in CSF. Discriminant analysis further confirmed that soil microbial community composition was distinctly different in CSF compared with ASD treatments. The soil microbial profile was well-differentiated between the two soil depths under ASD treatments but not in CSF, while the enhancement of PLFA biomarkers by ASD decreased with increasing soil depth.

Keywords: ASD, chemical soil fumigation, discriminant analysis, FAME, microbial biomarker, PLFA, soil depth

INTRODUCTION

Tomato (*Solanum lycopersicum*) is an important high-value vegetable crop worldwide. In the United States, Florida ranks first in the production of fresh market tomato with a reported production area of 9,308 ha and production value of over $323 million [United States Department of Agriculture, National Agricultural Statistics Service (USDA-NASS), 2022]. Following the phase-out of methyl bromide for soil fumigation, alternative chemical soil fumigants have been identified for use in tomato production prior to field transplanting to aid in suppression of weeds and soilborne pathogens. However, fumigants such as 1,3-dichloropropene (Group II, halogenated hydrocarbon) are subject to stringent environmental regulations and often lack adequate efficacy (Poret-Peterson et al., 2019). Commonly used pre-and post-plant non-triazine herbicides including glyphosate, acetolactate synthase (ALS) inhibitors, and acetyl coenzyme A carboxylase (ACC) inhibitors have resulted in rapid growth of selective herbicide resistance (LeBaron and Hill, 2008). In addition, increasing public health awareness and consumer demand for sustainable food products provide a unique incentive for the development of environmentally sustainable agricultural practices. Thus, to promote the long-term sustainability of vegetable production systems, there is a need to explore non-chemical alternatives for managing soilborne pests (Rosskopf et al., 2005, 2020; Shi et al., 2019) while carefully considering economic viability, environmental impact, and social acceptability.

Anaerobic soil disinfestation (ASD) has been reported as an effective, environmentally benign alternative to pre-plant chemical soil fumigation for managing soilborne pathogens in various high-value crops across a range of production systems (Momma et al., 2013; Mazzola et al., 2018). The ASD method involves incorporation of a labile carbon (C) source, followed by irrigation to saturate soil pores and covering the soil with gas impermeable film for ∼3 weeks (Butler et al., 2014; Paudel et al., 2020). Some commonly used C sources include rice or wheat bran (Strauss and Kluepfel, 2015), liquid or dried molasses (Butler et al., 2012b; Shrestha et al., 2018), and ethanol (Momma et al., 2010). In greenhouse studies, Butler et al. (2012b) also investigated the use of some warm-season cover crops as a C source for ASD on the suppression of *Fusarium oxysporum*, yellow nutsedge (*Cyperus esculentus*) tubers, and root-knot nematode (*Meloidogyne incognita*) eggs and juveniles. In Florida, ASD has been successfully demonstrated to manage weeds, plant parasitic nematodes, and some soilborne pathogens in eggplant (*Solanum melongena*), pepper (*Capsicum annuum*), tomato, and strawberry (*Fragaria* × *ananassa*) production while improving crop yield (Butler et al., 2012a,b; Di Gioia et al., 2016, 2020; Guo et al., 2017; Paudel et al., 2020). The mechanisms of ASD are likely related to shifts in soil community composition from aerobic organisms to facultative and obligate anaerobic organisms, production of volatile organic compounds, release of organic acids, and generation of metal ions (Momma, 2008; Strauss and Kluepfel, 2015; Hewavitharana et al., 2019; Rosskopf et al., 2020). However, information regarding the response of soil microbial communities to ASD during the cropping season remains limited.

van Agtmaal et al. (2015) assessed the impact of stress-induced changes in soil microbial community composition on microbially produced volatile organic compounds (VOCs) for suppression of *Pythium intermedium* in the production of hyacinth flower bulbs using pyrosequencing of 16S ribosomal gene fragments. At 3 months after ASD treatment, an increase in the relative abundance of the phylum *Bacteroidetes* and a significant decrease of the phyla *Acidobacteria*, *Planctomycetes*, *Nitrospirae*, *Chloroflexi*, and *Chlorobi* were observed. In two separate field studies, Poret-Peterson et al. (2019) also investigated shifts in bacterial communities after ASD soil treatments with different C sources. In their study, ASD treatments using molasses, mustard seed meal, tomato pomace, and rice bran led to increases in the abundances of Bacteroidales, Clostridiales, Selenomonadales, and Enterobacteriales compared with untreated controls. It was also found that the phylogenetic and taxonomic composition of communities in ASD treated soils with different C sources did not show pronounced differences. However, the authors did not investigate in-season microbial community composition dynamics, as no crops were grown during their study. In a previous study using phospholipid fatty acid (PLFA) analysis, Guo et al. (2018) examined the influence of two ASD soil treatments [6.9 m^3 ha^{-1} molasses (M) + 11 Mg ha^{-1} composted poultry litter (CPL) and 13.9 m^3 ha^{-1} M + 22 Mg ha^{-1} CPL] in contrast with chemical soil fumigation (CSF; a mixture of 1,3-dichloropropene and chloropicrin) on dynamic changes of soil microbial communities at 0–15 cm soil depth in a tomato production system to identify possible legacy effects of ASD on soil microbial community composition during the tomato growing season. It was observed that soil microbial groups were depleted in CSF treatment compared with ASD treatments in bulk and rhizosphere soils, while the composition of soil microbial communities was similar between ASD treated soils. Additionally, greater concentrations of total microbial biomass (TMB), actinomycetes, and G− bacteria were detected in ASD treated soils as opposed to CSF at 0, 36, 76, and 99 days after tomato transplanting. However, it is unclear whether and how the impact may be altered at soil depths beyond 0–15 cm. Therefore, the objective of this follow-up study was to compare the spatial and temporal changes of soil microbial communities between 0–15 and 15–30 cm soil depths in response to ASD soil treatments during the field-tomato production season in an effort to clarify possible legacy effects on soil microbial community composition.

MATERIALS AND METHODS

Field Experiment

The field experiment was conducted at the University of Florida Plant Science Research and Education Unit in Citra, FL from August to December 2015, with the soil type as Gainesville loamy sand (Hyperthermic, coated Typic Quartzipsamments). The field had prominent levels of weed infestation (primarily nutsedge) and root-knot nematodes (Guo et al., 2018). A thorough rototilling at 15 cm below the soil line was conducted in the experimental plots at the time of field preparation. The field trial was arranged in a split plot design with four replications. The pre-plant soil treatments were included in the whole plots following a randomized complete block design

with four blocks, with herbicide treatments in the subplots. The soil treatments consisted of ASD with 6.9 m^3 ha^{-1} of molasses (Agricultural Carbon Source, TerraFeed, LLC, Plant City, FL, USA) and 11 Mg ha^{-1} of composted poultry litter (CPL) (ASD0.5), ASD with 13.9 m^3 ha^{-1} of molasses and 22 Mg ha^{-1} (ASD1.0), and chemical soil fumigation [CSF; Pic-Clor 60 (Soil Chemical Corporation, Hollister, CA, USA) applied at 224 kg ha^{-1}, containing 1,3-dichloropropene (39.0%) and chloropicrin (59.6%)]. The herbicide treatments included application of halosulfuron-containing Sandea® (Gowan Company, Yuma, AZ, USA) with the rate of 70 g ha^{-1} and the no-herbicide control.

Three raised beds (24.4 m long, 0.9 m wide, 0.30 m high, and 1.8 m between centers) were made in each of the four blocks (replications) on 26 September 2018 and randomly assigned to ASD0.5, ASD1.0, or CSF. Each bed received an application of the pre-plant compound fertilizer (10N-10P_2O_5-10K_2O) at a rate of 560 kg ha^{-1}. A 1:1 (v:v) water dilution of molasses and CPL was used to set up ASD0.5 and ASD1.0 treatments. The mixture was applied to the top of the bed and tilled at the soil depth of 15 cm using a rotary cultivator, evenly amending the soil. The 24.4 m-long bed was divided in two 12.2 m-long sections (each serving as a subplot) for each whole plot. A random assignment of the herbicide Sandea® treatment and the no-herbicide control were applied in each half of the bed plots. Following application of the herbicide, the CSF treatment was applied *via* shank injection. A 0.025 mm white (on black) VaporSafe® TIF (Raven Industries Inc., Sioux Falls, SD, USA) polyethylene mulch with an ethylene vinyl alcohol (EVOH) barrier layer was used to cover all the beds. Each bed was irrigated through two drip lines positioned about 2.5 cm beneath the soil surface under the mulch. The beds undergoing ASD were the only treatments irrigated one time applying 68.9 kPa water pressure for a 4-h period. The soil pore space in the upper 10 cm of the bed (5-cm irrigation) was saturated to promote anaerobic conditions (Butler et al., 2012a). Tomato transplanting took place 3 weeks after the soil treatments were initiated.

On 3 September, 2015, tomato cultivar 'Tribute' (Sakata Seed America, Morgan Hill, USA) was transplanted at the four-true-leaf stage. Twenty-six plants with in-row spacing of 0.45 m were planted per subplot. A timer-controlled drip irrigation system was used to water plants twice daily. Initially, irrigation time was set to 30 min and later adjusted as plants matured. A weekly injection through the drip tape of fertilizer 6N-0P_2O_5-8K_2O plus micro blend (2% Ca, 0.4% Mg, 0.02% Zn, and 0.02% B; Mayo Fertilizer Inc., Mayo, FL, USA) began 7 days after transplanting (DAT), with in-season application rates of N and K_2O by fertigation at 161 and 215 kg ha^{-1}, respectively.

Soil Sampling and Analyses

Bulk soil samples were collected four times from the soil depth of 0–15 and 15–30 cm for soil microbial analysis during the tomato season: 3 September, 2015 (0 DAT), 9 October, 2015 (36 DAT), 18 November, 2015 (76 DAT), and 11 December, 2015 (99 DAT). Six bulk soil samples were collected from each subplot using a handheld soil probe (1.75 cm internal diameter) at each sampling time. The six soil samples were then combined and homogenized and kept at −20°C until microbial community analysis.

Microbial Community Profiling

Soil microbial communities for each treatment were characterized using PLFA analysis outlined by Guo et al. (2018). All collected soil samples passed through a 2-mm sieve to remove root and fresh litter materials, and then were freeze-dried before further analysis. Fatty acid methyl esters (FAMEs) were extracted from the freeze-dried soil samples using high throughput procedures described by Buyer and Sasser (2012). After thawing to room temperature, samples were used to extract PLFAs. A Bligh-Dyer extractant (chloroform/methanol/phosphate buffer, 1:2:0.8, v/v/v, 50 mM, pH 7.4; 4.0 mL) with an internal standard 19:0 (1,2-Dimyristoyl-sn-glycero-3-phosphocholine) was used for PLFA extraction. Lipid classes were isolated by solid phase extraction (SPE) with a 96-well SPE plate containing 50 mg of silica per well (Phenomenex, Torrance, CA, USA). The FAMEs were analyzed using an Agilent 7890N gas chromatography system (Agilent Technologies, Wilmington, DE, USA), which was equipped with an autosampler and flame ionization detector, and was controlled with MIDI Sherlock® software and Agilent ChemStation (Microbial ID, Inc., Newark, DE, USA). The FAMEs were classified and placed into six biomarker groups: Gram positive (G+) bacteria, iso and anteiso saturated branched fatty acids; Gram negative (G−) bacteria, monounsaturated fatty acids, and cyclopropyl 17:0 and 19:0; actinomycetes, 10-methyl fatty acids; arbuscular mycorrhizal fungi (AMF), 16:1ω5c; general fungi, 18:2ω6c; protozoa, 20:3ω6c and 20:4ω6c. Total microbial biomass (TMB) was determined as a sum of all quantified PLFAs in each sample. The concentrations of different biomarker groups and total microbial biomass in the soil were expressed in the unit of nmol PLFAs g^{-1} soil. In addition, the ratios of fungi:bacteria (F:B) and G+ bacteria:G− bacteria (G+:G−) were calculated.

Statistical Analyses

Prior to statistical analysis, data were checked for normality and log transformed when necessary to meet assumptions of linear mixed models. All data and results demonstrated in tables and figures present non-transformed values. Data were analyzed using a linear mixed model in the GLIMMIX procedure in SAS (Version 9.3; SAS Institute, Cary, NC, USA). Soil treatment, herbicide treatment, sampling time, and soil depth were analyzed as fixed effects. Block and soil treatment, soil treatment × herbicide treatment, soil treatment × herbicide treatment × sampling time within the block were considered as random effects. Soil treatment × herbicide treatment × soil depth within the block was analyzed as random residual effects by fitting a first-order autoregressive [AR(1)] model to account for the repeat measures of sampling time, using the "slice" statement to compare the composition of soil microbial communities over time for each soil depth. Multiple comparisons for each microbial biomarker were conducted using Tukey's Honest Significant Difference (HSD) test at $\alpha = 0.05$. The influence of soil treatment and soil depth combinations on soil microbial community structure was further examined using discriminant analysis (DA; JMP V.15.0.0; SAS Institute). Specific microbial biomarkers with the greatest impact on treatment segregation were identified with DA. Canonical discriminant analysis was performed on the

TABLE 1 | Analysis of variance of the effects of soil treatment, soil depth, sampling time, and herbicide application, and their two-way and three-way interactions on microbial group concentrations.

Effect	TMB	G+	G−	Actino	AMF	Fungi	Protozoa	F:B	G+:G−
Soil treatment (S)	***	***	***	***	***	*	**	*	***
Depth (D)	***	***	***	NS	***	***	***	***	NS
Time (T)	***	***	**	***	***	***	NS	***	***
Herbicide (H)	NS	NS	NS	NS	NS	NS	NS	NS	NS
S*D	***	***	***	**	***	**	NS	NS	**
S*T	NS	***	NS	***	***	***	**	**	***
S*H	NS	NS	NS	NS	NS	NS	*	NS	NS
S*T*D	NS	**	NS	*	***	**	NS	**	NS
S*H*D	NS	NS	NS	NS	NS	NS	NS	NS	NS
S*H*T	NS	NS	NS	NS	NS	NS	NS	NS	NS

$^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$. NS, no significant difference.

TMB, total microbial biomass; G+, Gram positive bacteria; G−, Gram negative bacteria; Actino, actinomycetes; AMF, arbuscular mycorrhizal fungi; F:B, ratio of fungi to bacteria; G+:G−, ratio of Gram positive bacteria to Gram negative bacteria.

ensuing discriminant model. The quantity of elements used to differentiate treatment groups was found through the number of significant ($P \leq 0.05$) canonical discriminant functions (linear combinations of important microbial biomarkers identified in discriminant analysis).

RESULTS

Impacts of Soil Treatment, Soil Depth, and Sampling Time on G+ and G− Bacteria, Ratio of G+:G− Bacteria, Protozoa, and Actinomycetes

Herbicide application had no significant main effect on any of the soil microbial biomarkers measured, while soil treatment significantly impacted all PLFA biomarker groups (**Table 1**). Soil depth showed significant main effects on all the microbial parameters except for G+:G− bacteria ratio and actinomycetes, while sampling time showed significant effects on all microbial biomarkers except for protozoa. Each of the PLFA biomarkers were significantly affected by the soil treatment × soil depth interaction except for F:B ratio and protozoa. The soil treatment × sampling time interaction had significant effects on all the biomarkers except for TMB and G− bacteria. Only protozoa were significantly affected by the soil treatment × soil herbicide interaction (**Table 1**). Furthermore, concentrations of G+ bacteria, actinomycetes, general fungi, AMF, and F:B ratio during the tomato production season were significantly influenced by a three-way interaction of soil treatment, soil depth, and sampling date (**Tables 1**, **2**).

Within 0–15 cm soil depth, both ASD treatments had significantly higher levels of G+ bacteria compared with CSF at all DATs, whereas no significant differences were observed between ASD 0.5 and ASD 1.0 at each DAT (**Table 2**). Similarly, there were no significant differences between the two ASD treatments at each DAT within 15–30 cm soil depth, but the ASD treatments led to significantly higher levels of G+ bacteria at all DATs except for 99 DAT when compared with CSF. When comparing G+ bacteria concentrations between the two soil depths within each soil treatment, both ASD treatments showed significantly higher levels at 0–15 cm compared with 15–30 cm soil depth at all DATs, but no differences were found between depths in CSF (**Table 2**).

The main effects of soil treatment, soil depth, and sampling time were significant for the concentrations of G− bacteria, while only the soil treatment × soil depth interaction significantly affected G− bacteria (**Table 1**). Both ASD treatments showed significantly higher concentrations of G− bacteria at 0–15 cm soil depth compared with 15–30 cm soil depth, while no significant differences between soil depths were observed for CSF (**Figure 1A**). At both soil depths, both ASD treated soils showed higher concentrations of G− bacteria compared with CSF.

Regarding G+:G− bacteria ratio, at 0 DAT, it was significantly higher in CSF compared with ASD0.5, but it was similar between CSF and ASD1.0 (**Figure 2A**). However, the ratio of G+:G− bacteria were significantly greater in CSF treatment compared to both ASD treatments at the other DATs. There were no significant differences between ASD0.5 and ASD1.0 at 0, 36, and 76 DAT, whereas ASD0.5 showed significantly higher concentration of G+:G− bacteria compared with ASD1.0 at 99 DAT. Within each soil treatment, it showed significantly higher ratio of G+:G− bacteria at 0 DAT compared to the other DATs (**Figure 2A**). The ratio of G+:G− bacteria did not significantly differ between soil depths under ASD0.5, ASD1.0, and CSF, respectively (**Figure 1B**). Within both soil depths, CSF exhibited a higher ratio of G+:G− bacteria compared with ASD0.5 and ASD1.0, while the difference between CSF and ASD1.0 appeared to be greater at the soil depth of 15–30 vs. 0–15 cm.

In terms of protozoa, soil treatment and soil depth showed significant effects, while the interaction effect of soil treatment × sampling time was significant as well (**Table 1**). At 0, 36, and 99 DAT, the concentrations of protozoa in ASD0.5 and ASD1.0 were significantly higher compared with CSF (**Figure 2B**). The concentration of protozoa steadily decreased from 0 to 99 DAT under ASD0.5, resulting in a significant difference between 0 and 99 DAT. However, there were no significant differences among

TABLE 2 | Microbial biomarker concentrations (nmol g^{-1}) and ratio of fungi:bacteria in the bulk soil as affected by the three-way interaction of soil treatment, sampling date, and soil depth.

	G+		Actino		Fungi		AMF		F:B	
	0–15 cm	15–30 cm	0–15 cm	15–30 cm	0–15 cm	15–30 cm	0–15 cm	15–30 cm	0–15 cm	15–30 cm
ASD0.5										
0 DAT	9.69 Aa	4.74 Ba	1.40 Aab	1.37 Aab	1.56 Aab	0.28 Babc	0.28 Aabc	0.34 Aa	0.09 Ade	0.06 Bcd
36 DAT	3.08 Ab	2.29 Bbcde	1.47 Aab	1.19 Babc	0.90 Aab	0.37 Babc	0.39 Aa	0.27 Ba	0.15 Aabcd	0.11 Aabc
76 DAT	3.95 Ab	3.03 Bbc	1.70 Aa	1.52 Aa	0.81 Aab	0.45 Babc	0.45 Aa	0.34 Ba	0.13 Abcd	0.10 Babcd
99 DAT	2.83 Ab	2.12 Bbcdef	1.47 Aab	1.29 Aabc	0.50 Ab	0.17 Bc	0.39 Aab	0.27 Ba	0.10 Ade	0.07 Abcd
ASD1.0										
0 DAT	8.26 Aa	4.90 Ba	0.96 Acd	1.09 Acd	1.40 Aa	0.23 Bbc	0.20 Bbcd	0.27 Aa	0.11 Acde	0.05 Bd
36 DAT	3.88 Ab	2.49 Bbcd	1.19 Abc	1.08 Abc	1.08 Aab	0.50 Bab	0.46 Aa	0.22 Bab	0.15 Aabcd	0.12 Aabc
76 DAT	4.01 Ab	3.13 Bb	1.49 Aab	1.34 Aab	1.24 Aab	0.74 Ba	0.51 Aa	0.33 Ba	0.18 Aabcd	0.13 Aab
99 DAT	3.81 Ab	2.01 Bcdef	1.51 Aab	1.16 Babc	1.16 Aab	0.56 Babc	0.47 Aa	0.23 Bab	0.16 Aabcd	0.11 Babc
CSF										
0 DAT	2.89 Ab	2.70 Abcd	1.0 Ac	0.98 Acd	0.11 Ac	0.39 Ac	0.18 Acd	0.21 Aab	0.05 Ae	0.08 Abcd
36 DAT	1.63 Ac	1.60 Aef	0.63 Ae	0.69 Ae	0.93 Aab	0.55 Babc	0.11 Ae	0.13 Ac	0.27 Aa	0.17 Ba
76 DAT	1.69 Ac	1.89 Adef	0.72 Ade	0.83 Ade	0.75 Aab	0.58 Aabc	0.14 Ade	0.15 Abc	0.22 Aabc	0.14 Aab
99 DAT	1.38 Ac	1.51 Af	0.65 Ae	0.75 Ae	0.73 Aab	0.33 Babc	0.12 Ade	0.13 Ac	0.24 Aab	0.13 Babc

Within each microbial biomarker, means followed by the same uppercase letter within a row and means followed by the same lowercase letter within a column are not significantly different according to Tukey's HSD test at $P \leq 0.05$.

CSF, chemical soil fumigation control with Pic-Clor 60 at a rate of 224 kg ha^{-1}; ASD0.5, anaerobic soil disinfestation with 6.9 m^3 ha^{-1} of molasses and 11 Mg ha^{-1} of composted poultry litter; ASD1.0, anaerobic soil disinfestation with 13.9 m^3 ha^{-1} of molasses and 22 Mg ha^{-1} of composted poultry litter. G+, Gram positive bacteria; Actino, actinomycetes; AMF, arbuscular mycorrhizal fungi; F:B, ratio of fungi to bacteria; DAT, days after transplanting.

different DATs for ASD1.0 and CSF treatments (**Figure 2B**). Across soil treatments and sampling dates, the comparison between the two soil depths revealed a significantly higher concentration of protozoa within the 0–15 cm soil depth than in the deeper soil at 15–30 cm (data not shown).

With respect to the concentration of actinomycetes, within both 0–15 and 15–30 cm soil depths, ASD0.5 had significantly higher levels of actinomycetes than CSF at each DAT, while ASD1.0 showed significantly higher levels at 36, 76, and 99 DAT (**Table 2**). Only at 0 DAT, ASD0.5 showed a significantly higher level of actinomycetes compared with ASD1.0 at both soil depths. When comparing the levels of actinomycetes between the two soil depths, higher levels were found at 0–15 vs. 15–30 cm in ASD0.5 at 36 DAT and ASD1.0 at 99 DAT, but no significant differences were observed in CSF at each DAT (**Table 2**).

Impacts of Soil Treatment, Soil Depth, and Sampling Time on AMF, General Fungi, F:B Ratio, and TMB

The concentration of AMF was positively affected by both ASD soil treatments. Although no significant differences were observed at 0 DAT, the concentrations of AMF in the ASD1.0 and ASD0.5 treated soils were significantly higher compared with CSF at both soil depths at 36, 76, and 99 DAT (**Table 2**). Furthermore, at each sampling date the concentration of AMF under CSF did not differ significantly between soil depths, whereas higher concentrations of AMF were observed at the 0–15 cm soil depth compared with 15–30 cm soil depth under both ASD treatments at 36, 76, and 99 DAT. At 0 DAT, higher concentrations of AMF were observed at the 15–30 cm soil depth compared with 0–15 cm soil depth for ASD1.0.

The concentrations of general fungi significantly increased at 0 DAT in both ASD soil treatments compared with CSF at the 0–15 cm soil depth, while no differences were observed at later sampling dates. At the 15–30 cm soil depth, the concentrations of general fungi did not differ significantly among soil treatments at each DAT (**Table 2**). With respect to the comparison between soil depths, significantly higher levels of fungi were observed at 0–15 cm than at 15–30 cm under both ASD treatments at each DAT, while such a difference was only observed at 36 and 99 DAT for CSF (**Table 2**).

Under CSF, the F:B ratio significantly increased from 0 to 36 DAT and then remained relatively stable until 99 DAT at both 0–15 and 15–30 cm soil depths (**Table 2**). No significant changes were observed in the F:B ratio under the two ASD treatments despite soil depth and DAT except that a significant increase from 0 to 36 DAT was found at the 15–30 cm soil depth in ASD1.0 (**Table 2**). The ratio of F:B was significantly greater at 0–15 vs. 15–30 cm soil depth at 0, and 76 DAT for ASD0.5, while a similar trend was observed in ASD1.0 at 0 and 99 DAT, and in CSF at 36 and 99 DAT (**Table 2**).

The soil treatment × soil depth interaction had a significant impact on TMB (**Table 1**; **Figure 1C**). Across all sampling dates, both ASD treatments demonstrated significantly higher levels of TMB at the 0–15 cm soil depth in contrast to the deeper soil at 15–30 cm, whereas no difference between the soil depths was observed in CSF. The two ASD treatments

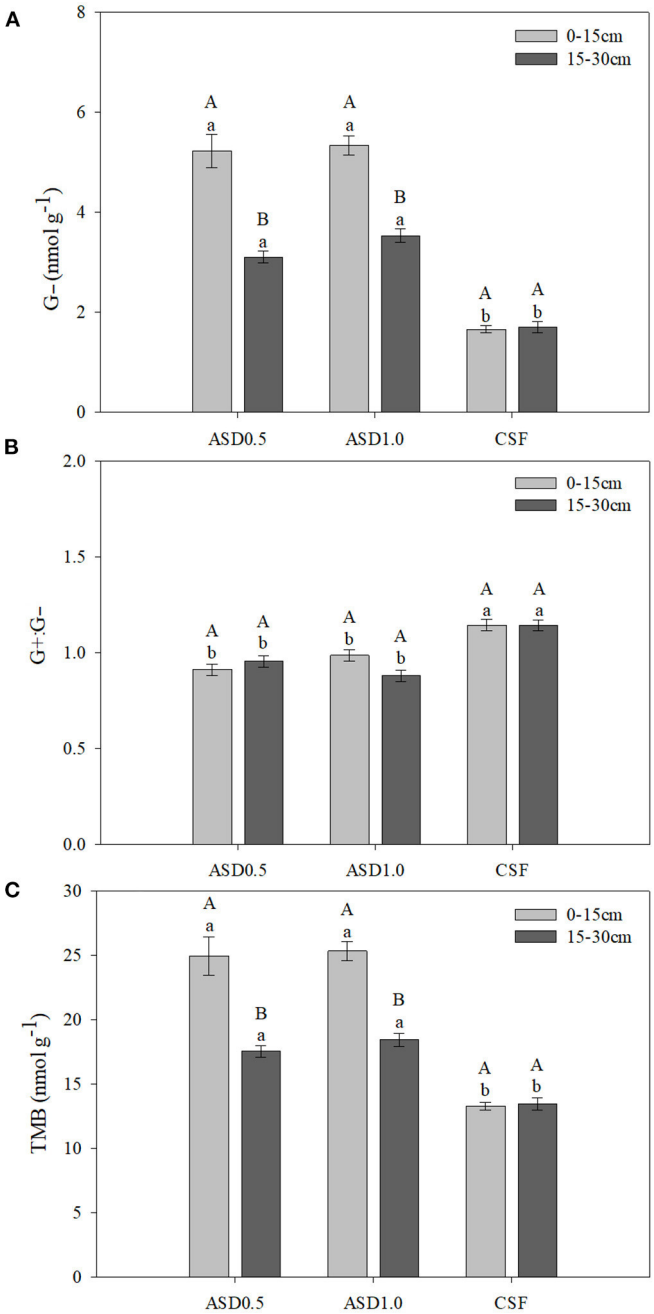

FIGURE 1 | Bulk soil PLFA concentrations of G− bacteria **(A)**, ratio of G+:G− **(B)**, and PLFA concentrations of TMB **(C)** as affected by the two-way interaction of soil treatment and soil depth across sampling dates. Error bars represent standard error. Within a soil treatment, bars sharing the same uppercase letter are not significantly different at $P \leq 0.05$ according to Tukey's HSD test. Within a sampling depth, bars sharing the same lowercase letter are not significantly different at $P \leq 0.05$ according to Tukey's HSD test. CSF, chemical soil fumigation control with Pic-Clor 60 at a rate of 224 kg ha^{-1}; ASD0.5, anaerobic soil disinfestation with 6.9 m^3 ha^{-1} of molasses and 11 Mg ha^{-1} of composted poultry litter; ASD1.0, anaerobic soil disinfestation with 13.9 m^3 ha^{-1} of molasses and 22 Mg ha^{-1} of composted poultry litter. G−, Gram negative bacteria; G+:G−, ratio of Gram positive bacteria to Gram negative bacteria; TMB, total microbial biomass.

resulted in significantly greater concentrations of TMB than CSF at both soil depths, but to a lesser extent in the deeper soil at 15–30 cm (**Figure 1C**). The main effect of sampling time also significantly impacted TMB as reflected by a significantly higher concentration of TMB at 0 DAT than that at 36, 76, and 99 DAT, and no significant differences were observed at these 3 later sampling dates (data not shown).

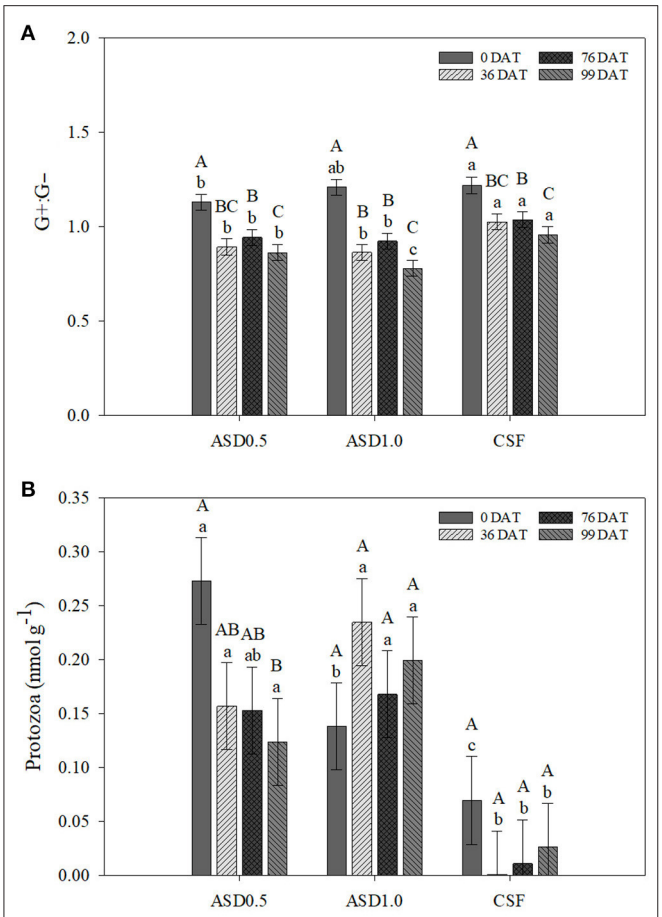

FIGURE 2 | Ratio of G^+:G^- **(A)** and PLFA concentrations of protozoa **(B)** in the bulk soil. As affected by two-way interaction of soil treatment and sampling date across soil depths. Error bars represent standard error. Within a soil treatment, bars sharing the same uppercase letter are not significantly different at $P \leq 0.05$ according to Tukey's HSD. Within a sampling date, bars sharing the same lowercase letter are not significantly different at $P \leq 0.05$ according to Tukey's HSD test. CSF, chemical soil fumigation control with Pic-Clor 60 at a rate of 224 kg ha^{-1}; ASD0.5, anaerobic soil disinfestation with 6.9 m^3 ha^{-1} of molasses and 11 Mg ha^{-1} of composted poultry litter; ASD1.0, anaerobic soil disinfestation with 13.9 m^3 ha^{-1} of molasses and 22 Mg ha^{-1} of composted poultry litter. G+:G−, ratio of Gram positive bacteria to Gram negative bacteria; DAT, days after transplanting.

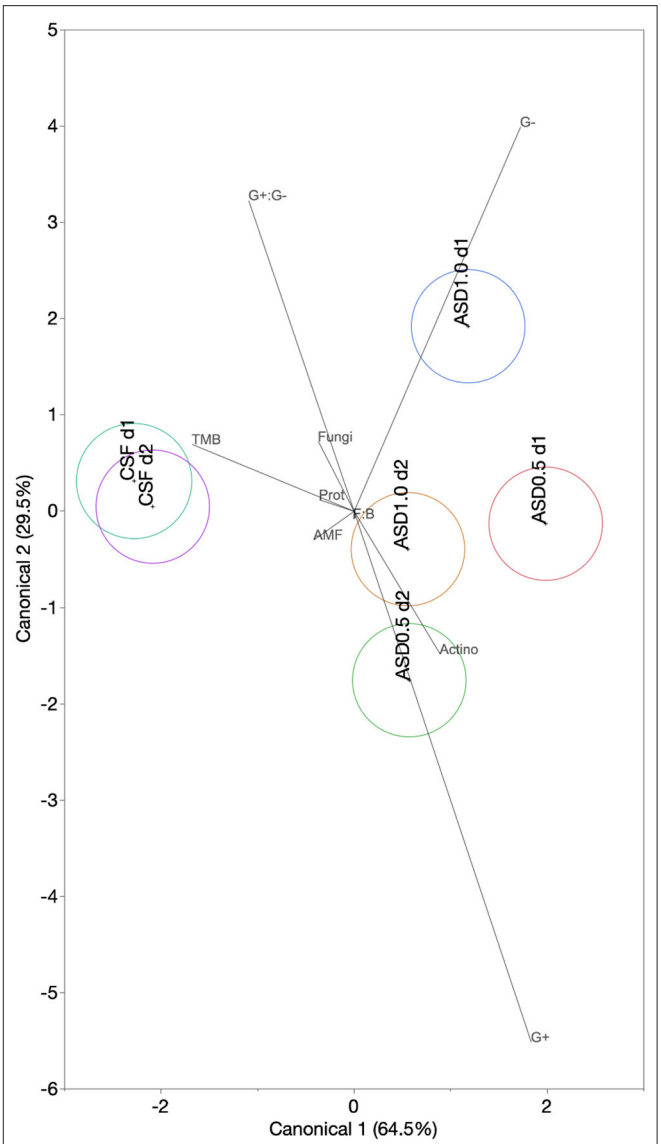

FIGURE 3 | Canonical discriminant analysis of PLFA biomarkers for the two-way interaction of soil treatment and soil depth across sampling dates. Vectors represent standardized canonical coefficients and indicate the relative contribution of each biomarker group to each canonical variate. Ellipses represent 95% confidence region of the mean. CSF, chemical soil fumigation control with Pic-Clor 60 at a rate of 224 kg ha^{-1}; ASD0.5, anaerobic soil disinfestation with 6.9 m^3 ha^{-1} of molasses and 11 Mg ha^{-1} of composted poultry litter; ASD1.0, anaerobic soil disinfestation with 13.9 m^3 ha^{-1} of molasses and 22 Mg ha^{-1} of composted poultry litter. G+:G−, ratio of Gram positive bacteria to Gram negative bacteria; F:B, ratio of fungi:bacteria; TMB, total microbial biomass; G+, Gram positive bacteria; Prot, protozoa; G−, Gram negative bacteria; Actino, actinomycetes; AMF, arbuscular mycorrhizal fungi; ASD0.5 d1, ASD0.5 at 0–15 cm soil depth; ASD0.5 d2, ASD0.5 at 15–30 cm soil depth; ASD1.0 d1, ASD1.0 at 0–15 cm soil depth; ASD1.0 d2, ASD1.0 at 15–30 cm soil depth; CSF d1, chemical soil fumigation control at 0–15 cm soil depth; CSF d2, chemical soil fumigation control at 15–30 cm soil depth.

Discriminant Analysis of Microbial Community Compositions as Affected by Soil Treatment at 0–15 and 15–30 cm Soil Depths

Different microbial biomarkers (**Table 1**) were included in the discriminant analysis to characterize changes in overall soil microbial community structure in response to the interaction of soil treatment and soil depth. Canonical discriminant analysis indicated two significant discriminant functions accounting for 94.0% of the total variance. The first canonical axis explained 64.5% of the variability, while 29.5% of the variability was explained by the second canonical axis (**Figure 3**). Microbial biomarkers positively correlated to the first canonical component included G+ bacteria, G− bacteria, and actinomycetes (in order of strongest to weakest correlation). In contrast, a negative

correlation was observed between the first canonical component and TMB, ratio of G+:G− bacteria, AMF, fungi, protozoa, and F:B (in order of strongest to weakest correlation). The second canonical component was positively correlated with G− bacteria, ratio of G+:G− bacteria, fungi, TMB, and protozoa (in order of strongest to weakest correlation), while it was negatively correlated with G+ bacteria, actinomycetes, AMF, and F:B (in order of strongest to weakest correlation).

The canonical discriminant analysis results further demonstrated that the microbial community composition characterizing the two ASD treatments were clearly different from that of CSF (**Figure 3**). Particularly, TMB, G+ bacteria, and G− bacteria were the key biomarkers differentiating CSF from ASD0.5 and ASD1.0 along canonical axis 1. CSF at both soil depths showed similar microbial biomarker characteristics according to canonical axis 1 and canonical axis 2, whereas the ASD treatments at 0–15 and 15–30 cm soil depths were well-separated along canonical axis 2. Moreover, the difference in microbial biomarkers between ASD0.5 and ASD1.0 appeared to be more pronounced at the soil depth of 0–15 cm than at 15–30 cm. The G+ bacteria played a more important role among other biomarkers in differentiating the two ASD treatments, while the G− bacteria tended to be more important in differentiating soil microbial composition between the two soil depths across the ASD treatments.

DISCUSSION

Soil fumigants are used extensively in Florida to manage soilborne pests and pathogens prior to growing strawberries, tomatoes, and other high-value crops. Although the effects of fumigants on beneficial non-target organisms at the field scale remain largely unknown (Jackson et al., 2013; Liu et al., 2015), previous studies have demonstrated fumigants including dimethyl disulfide (DMDS), Telone (1,3- dichloropropene or 1,3-D), and chloropicrin (CP) exhibit broad biocidal activity against non-target soil organisms (Dangi et al., 2015). The present study showed that shank-injected Pic-Clor 60 at a rate of 224 kg ha^{-1} to a soil depth of 30 cm (CSF) resulted in a reduction in the relative abundance of several soil microbial PLFA biomarkers. In general, concentrations of TMB, actinomycetes, AMF, G+, and G− bacteria, and protozoa were reduced at both 0–15 and 15–30 cm soil depths under CSF compared with ASD treated soils. Specifically, concentrations of protozoa under ASD0.5 and ASD1.0 increased exponentially at 0–15 cm soil depth compared with CSF, while concentrations of G− bacteria increased by more than 200% at 0–15 cm soil depth under ASD treated soils compared with CSF. At 15–30 cm soil depth, concentration of G− bacteria increased by 83% under ASD0.5 and by 108% under ASD1.0 compared with CSF. These results agree with findings from Dangi et al. (2015) who reported that microbial communities including G+ bacteria, G− bacteria, fungi, and AMF under fumigated soils were significantly lower compared with non-fumigated control plots. Other studies have also observed a decline in total microbial biomass after fumigation (Klose et al., 2006; Ge et al., 2021). However, previous studies on shifts in bacterial populations are inconsistent. While Yao et al. (2006) suggested G− bacteria may recover more rapidly following fumigation, others have reported that concentrations of G+ bacteria recover preferentially (Ibekwe et al., 2001). These inconsistencies in the literature may also be related to the availability and diversity of C-rich substrates (Hewavitharana et al., 2019).

The ratio of G+:G− bacteria was significantly greater under CSF vs. ASD treatments (except for the similar level between CSF and ASD1.0 at 0 DAT) at both soil depths, which may be primarily linked to the marked increase of G− bacteria populations in the ASD soil treatments. These results are in line with a previous study by Breulmann et al. (2014) who reported an increase in G+:G− bacteria ratio with decreasing labile C substrates along the soil profile. Gram negative bacteria generally utilize more labile, plant derived C sources, while G+ bacteria use C sources derived from soil organic matter or recalcitrant sources (Fanin et al., 2019). Thus, the structure and function of soil microbial composition are affected by substrate availability. In the present study, CSF soil treatment did not receive composted poultry litter or molasses as a source of mineral N and labile C substrate, respectively. Gram negative bacteria exhibit r-selected Monod growth kinetics, enabling rapid growth and reproduction in nutrient rich environments. It is likely that G− bacteria concentrations were promoted by the addition of molasses and CPL. In addition, some studies have reported on the influence of soil nutrient availability on soil microbial community composition. For example, Demoling et al. (2008) demonstrated that the addition of N changed microbial community composition compared with unfertilized plots. In their study, fungal growth rates were less negatively affected by fertilization compared with bacterial growth rates, while overall fungal biomass decreased more compared with bacterial biomass as a result of N fertilization. In our study, the addition of CPL and molasses contributed 372.5 and 745 kg ha^{-1} N and 4,907.5 and 9,815 kg ha^{-1} C for ASD0.5 and ASD1.0, respectively (Di Gioia et al., 2017). Thus, the observed differences in soil microbial community composition could be linked to creating an anaerobic environment during the ASD treatment period and the change in soil C and nutrient pools resulting from the incorporation of organic amendments.

The top 30 cm of soil is generally considered to be the root zone of many horticultural crops. The ability of soil microorganisms to establish and function within this zone after soil treatment is critical for maintaining productive soils. The canonical discriminant analysis revealed that for either ASD treatment, the soil microbial profile was well-differentiated between the two soil depths, whereas there was a lack of differentiation in the CSF treatment. In general, the concentrations of G+ and G− bacteria, AMF, fungi, and TMB were higher at 0–15 cm compared with 15–30 cm soil depth under ASD treatments, whereas no consistent differences were observed between 0–15 and 15–30 cm soil depth under CSF treatment. Regardless of soil treatment, the level of protozoa also decreased with increasing soil depth. One previous study that examined the effects of ASD in tree-crop nursery conditions in California reported soil microbial community changes as a

function of soil depth (down to 76.2 cm) in ASD treated soils when the C source was only incorporated to a soil depth of 15.2–20.3 cm. The reduction of soilborne plant pathogens was significantly greater at the 15.2 cm soil depth, likely due to enhanced microbial metabolic activity resulting from the higher concentration of the C source at that soil depth (Strauss et al., 2017). This might be the similar case in our study, as molasses and CPL amended to a soil depth of ∼15 cm, thus higher concentrations of several microbial biomarkers at 0–15 cm soil depth were observed.

The investigation of soil microbial community composition following ASD treated soils compared with fumigated soils at different soil depths is not well-documented in the literature. In a previous study, Guo et al. (2018) reported soil microbial community structure differed substantially between ASD and fumigated soils at 0–15 cm soil depth. Similarly, in the present study, canonical discriminant analysis of PLFA microbial biomarkers in CSF and ASD soil treatments at 0–15 and 15–30 cm soil depths clearly indicates differences in soil microbial community composition between ASD treated soils and fumigated soil. Soil microbial communities in ASD treated soils were distinctly different from fumigated plots, regardless of soil depth. The difference in soil microbial community structure between ASD and fumigated soils may be due to high toxicity of many fumigants to soil organisms (Ibekwe et al., 2001), while the addition of CPL and labile C sources in ASD soils likely promote a greater abundance of soil microbial populations (Guo et al., 2018). Mazzola et al. (2018) also observed distinctly different bacterial and fungal communities in ASD treated soils using rice bran at 20 Mg ha^{-1} or molasses at 20 Mg ha^{-1} compared with methyl bromide-chloropicrin soil fumigation. However, in their study soil samples were examined only to a depth of 0–15 cm.

CONCLUSIONS

In the present study, the concentration of selected microbial biomarkers including G+ bacteria, actinomycetes, general fungi, arbuscular mycorrhizal fungi, and fungi:bacteria ratio were impacted by the three-way interaction of soil treatment, sampling time, and soil depth while all of the microbial biomarkers were affected by the interaction of soil treatment and soil depth except protozoa and fungi:bacteria ratio. In general, ASD treatments increased the overall abundance of total microbial biomass, actinomycetes, arbuscular mycorrhizal fungi, protozoa, G+, and G− bacteria compared with CSF at both 0–15 and 15–30 cm soil depths across multiple sampling dates. Moreover, the concentrations of G+ and G− bacteria, arbuscular mycorrhizal fungi (except for at 0 DAT), general fungi, and total microbial biomass were higher at 0–15 cm compared with 15–30 cm soil depth under ASD treatments, whereas no differences were observed between 0–15 and 15–30 cm soil depths under CSF treatment. Overall, the soil microbial profile was well-differentiated between the two soil depths under ASD treatments but there is a lack of soil depth differences in CSF. These findings suggest that ASD soil treatments as an alternative to CSF may also exhibit potential for promoting soil health over the long-run in vegetable production systems, particularly within the area of the crop rhizosphere. In order to pinpoint specific players in different soil microbial functional groups, future research may use high-throughput DNA sequencing or other advanced approaches to elucidate soil microbial community composition in response to ASD application.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

This present field study was conducted by HG, XZ, FD, JH, and ER. Statistical data analyses were performed by IV, NX, BP, JC, and XZ. Lab analyses of PLFAs and FAMEs were conducted by DM. Manuscript drafting and compilation were completed by IV. NX, LA, XZ, JH, FD, and ER contributed to editing and finalizing the manuscript. All authors contributed to the article and approved the submitted version.

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