



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

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### Specialty section:

This article was submitted to  
Waste Management in  
Agroecosystems,  
a section of the journal  
Frontiers in Sustainable Food Systems

Received: 23 December 2020

Accepted: 24 February 2021

Published: 25 March 2021

### Citation:

Khadka RB and Miller SA (2021)  
Synergy of Anaerobic Soil  
Disinfestation and *Trichoderma* spp. in  
Rhizoctonia Root Rot Suppression.  
*Front. Sustain. Food Syst.* 5:645736.  
doi: 10.3389/fsufs.2021.645736

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

## 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 *Plasmiodiophora 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 Erlenmeyer 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<sup>5</sup> conidia ml<sup>-1</sup> 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<sup>-1</sup> soil equivalent to ≈100 g fresh biomass kg<sup>-1</sup> soil, and chicken manure, wheat bran and molasses were applied at 10 g kg<sup>-1</sup> 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<sup>6</sup> conidia ml<sup>-1</sup> of the isolate was pipetted into each pot. Rootshield WP (6% v/v suspension) was inoculated at 1 ml kg<sup>-1</sup> 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.

## 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:

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

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 for 1 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<sup>-2</sup> suspension was serially diluted to 10<sup>-4</sup> and 200 μl suspensions from the 10<sup>-3</sup> and 10<sup>-4</sup> 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<sup>-1</sup> soil were calculated (Foght and Aislabie, 2005).

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

Carbon source <sup>z</sup>	Rate (g kg <sup>-1</sup> 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

<sup>z</sup>Mustard greens were used on a dry matter basis equivalent to 100 g fresh weight.

## 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<sup>-1</sup> 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<sup>-1</sup> to 7.4 fg µl<sup>-1</sup>) 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>).

Number copies (molecules)

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

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: [ $\Sigma$ (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* isolates
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.

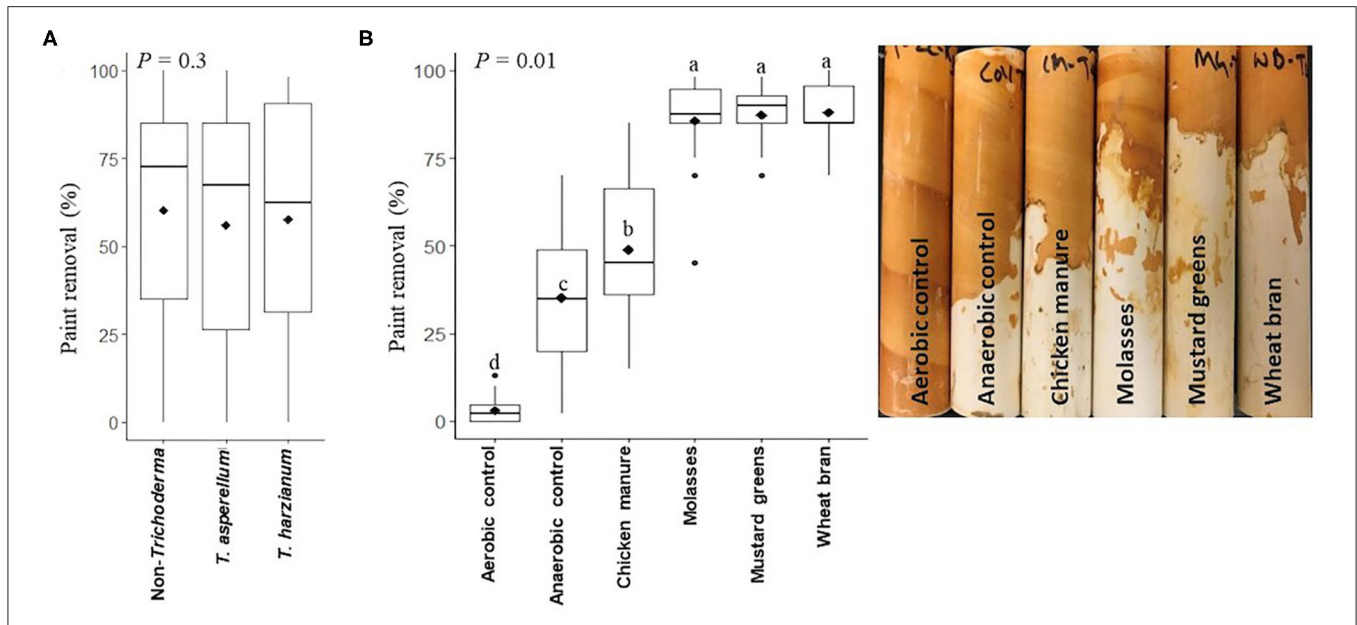
**TABLE 2 |** Gravitational moisture percentage of soil after anaerobic soil disinfestation (ASD) with different carbon source amendments and with or without *Trichoderma* spp.

Isolate	Soil gravitational moisture (%) <sup>xyz</sup>
Non-inoculated control	25.0 ± 3.8
<i>T. harzianum</i> T-22	24.8 ± 2.3
<i>T. harzianum</i> NT25	24.2 ± 4.8
<i>P-value</i>	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
<i>P-value</i>	0.004

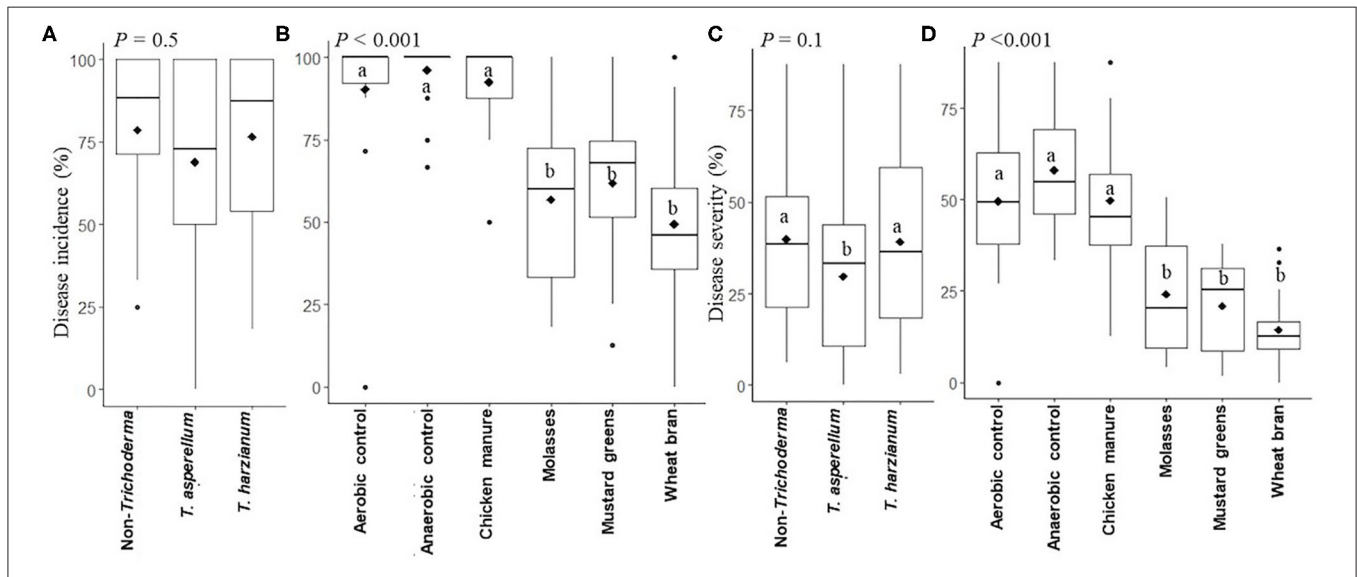
<sup>x</sup>Values in a column followed by different letters are significantly different at  $P \leq 0.05$  according to Fisher's LSD test after square root transformation. The values after ± indicate standard error of the mean.

<sup>y</sup>Average of two experiments each with three replications per treatment.

<sup>z</sup>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 <sup>x</sup> (observed) <sup>u</sup>	F <sub>UA1</sub> <sup>v</sup>	F <sub>UA2</sub> <sup>w</sup>	Expected disease control (E) (F <sub>UA</sub> ) <sup>x</sup>	O-E <sup>y</sup>	Remarks <sup>z</sup>
Non- <i>Trichoderma</i> control	Anaerobic control	67.28	0					
<i>T. harzianum</i>	Anaerobic control	55.63	0.17	0.83				
<i>T. asperellum</i>	Anaerobic control	51.30	0.24	0.76				
Non- <i>Trichoderma</i> control	Chicken manure	49.27	0.27		0.73			
Non- <i>Trichoderma</i> control	Molasses	29.17	0.57		0.43			
Non- <i>Trichoderma</i> control	Mustard greens	21.57	0.68		0.32			
Non- <i>Trichoderma</i> control	Wheat bran	20.87	0.69		0.31			
<i>T. asperellum</i>	Chicken manure	35.13	0.48			0.44	0.04	Additive
<i>T. asperellum</i>	Molasses	27.88	0.59			0.67	0.08	Additive
<i>T. asperellum</i>	Mustard greens	18.98	0.72			0.76	-0.04	Additive
<i>T. asperellum</i>	Wheat bran	8.52	0.87			0.76	0.11	Synergistic
<i>T. harzianum</i>	Chicken manure	61.67	0.08			0.39	-0.31	Antagonistic
<i>T. harzianum</i>	Molasses	15.32	0.77			0.64	0.13	Synergistic
<i>T. harzianum</i>	Mustard greens	22.42	0.67			0.73	-0.07	Additive
<i>T. harzianum</i>	Wheat bran	13.72	0.80			0.74	0.05	Additive

<sup>u</sup>Percent reduction in *Rhizoctonia* root rot severity in radish compared to non-*Trichoderma* inoculated anaerobic control.

<sup>v</sup>F<sub>UA1</sub> = 1 - Percent reduction in *Rhizoctonia* root rot severity for *Trichoderma* isolate under anaerobic control conditions compared to non-*Trichoderma* inoculated anaerobic control.

<sup>w</sup>F<sub>UA2</sub> = 1 - Percent reduction in *Rhizoctonia* root rot severity for ASD carbon source under non-*Trichoderma* inoculated conditions compared to non-*Trichoderma* inoculated anaerobic control.

<sup>x</sup>Expected percent disease control (F<sub>UA</sub>) = 1 - (F<sub>UA1</sub> for *Trichoderma* isolate under anaerobic conditions × F<sub>UA2</sub> for ASD carbon source under non-*Trichoderma* inoculated conditions).

<sup>y</sup>O - observed percent reduction in *Rhizoctonia* root rot severity, E - expected percent disease control (F<sub>UA</sub>).

<sup>z</sup>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.

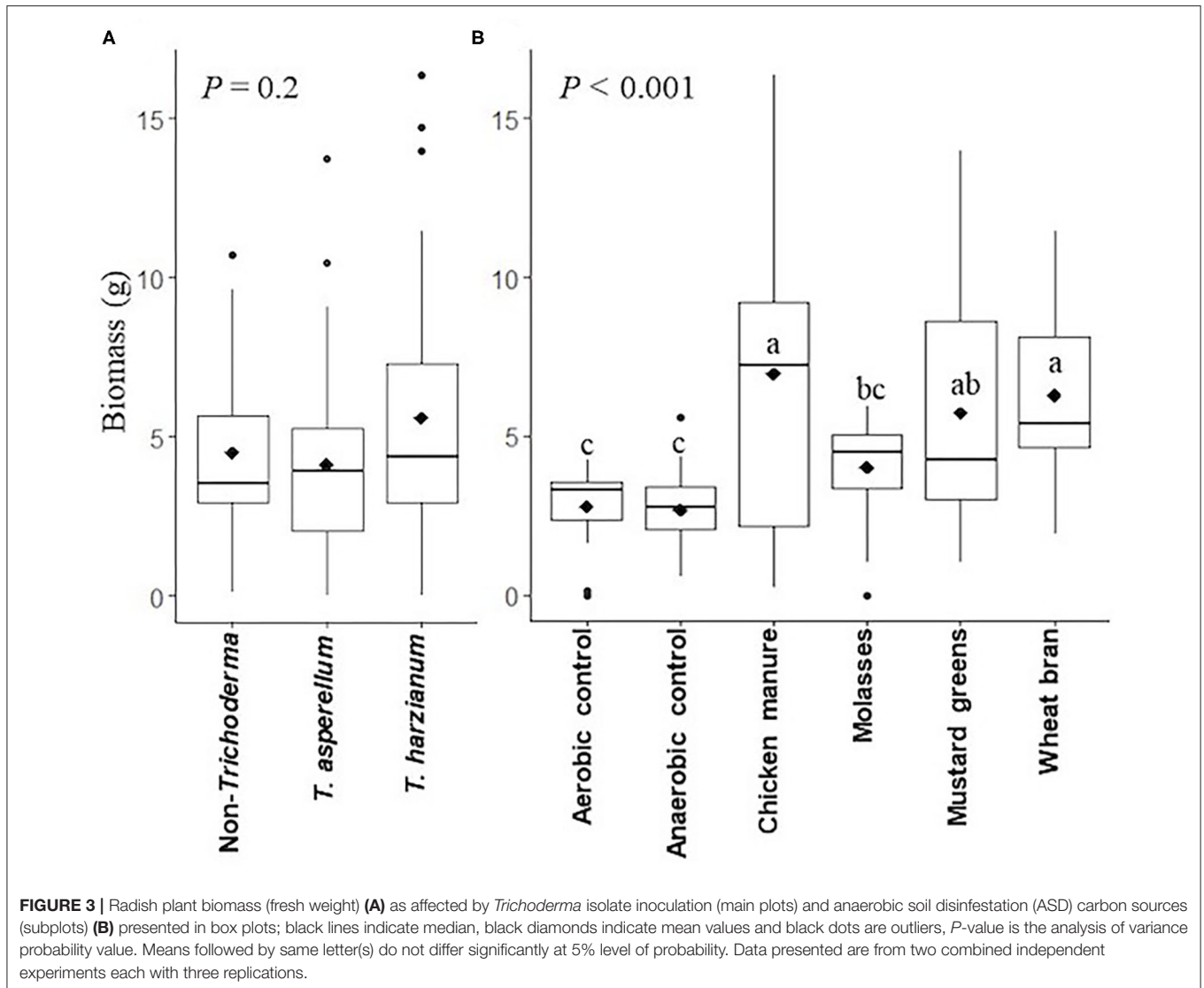
### *Trichoderma* spp. Populations in Soil After ASD Treatment

*Trichoderma* spp. populations were highest ( $P < 0.001$ ) in *T. asperellum*-inoculated (log 4.3 CFU g<sup>-1</sup>) soils after ASD compared to *T. harzianum*-inoculated (log 3.1 CFU g<sup>-1</sup>) and non-inoculated (log 2.0 CFU g<sup>-1</sup>) 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<sup>-1</sup>) in soil compared to the aerobic control (log 3.9 CFU g<sup>-1</sup>), and mustard greens-amended ASD (log 2.5 CFU g<sup>-1</sup>) 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<sup>-1</sup> 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

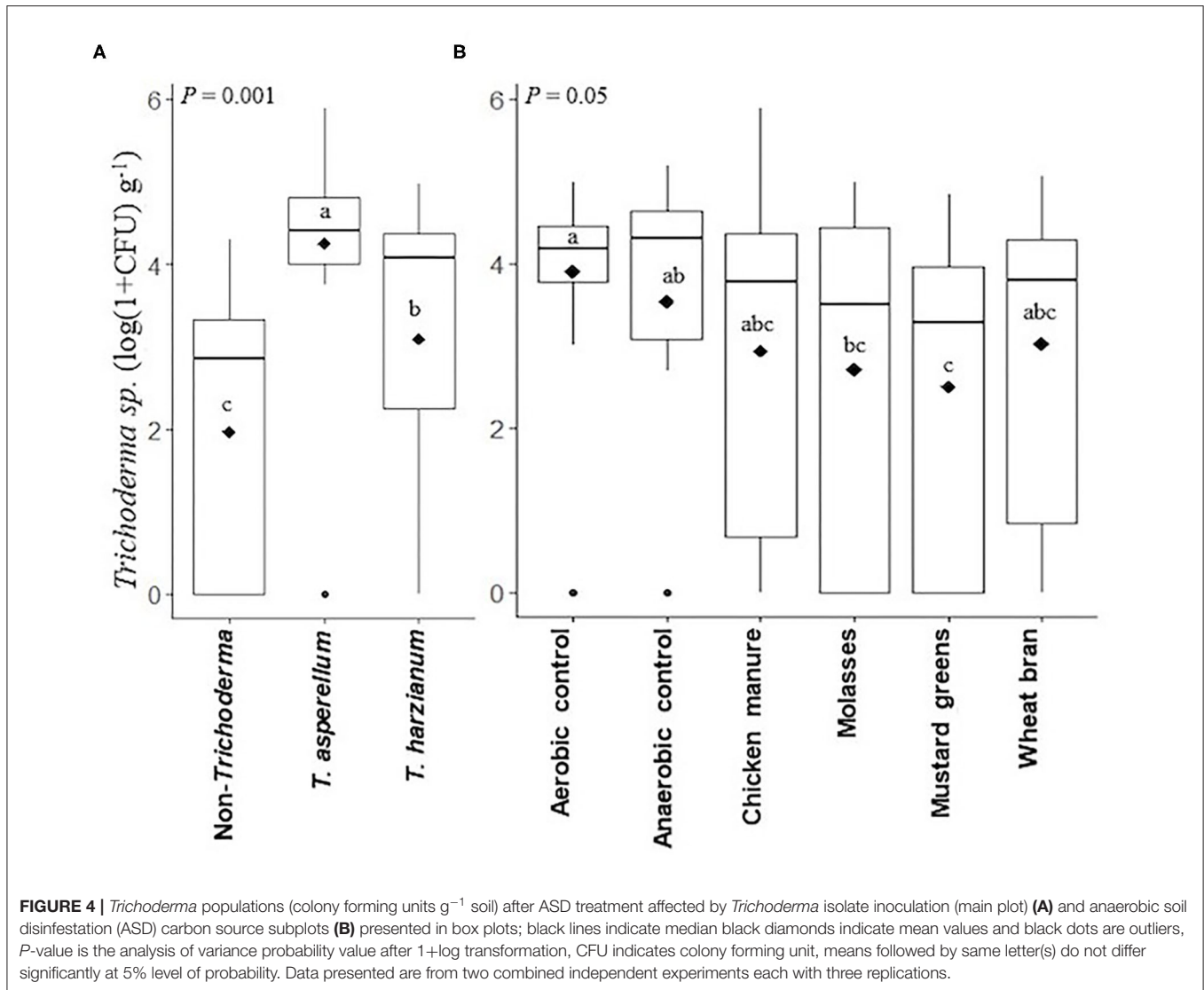
Diseases caused by *R. solani* are challenging to manage once the pathogen is established in soil because of its wide host range and production of environmentally resilient sclerotia (Cook et al., 2002). There are limited management options for these diseases. Anaerobic soil disinfestation and biological control are promising management tools that have no known negative environmental or health impacts (Harman, 2000; Roskopf 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



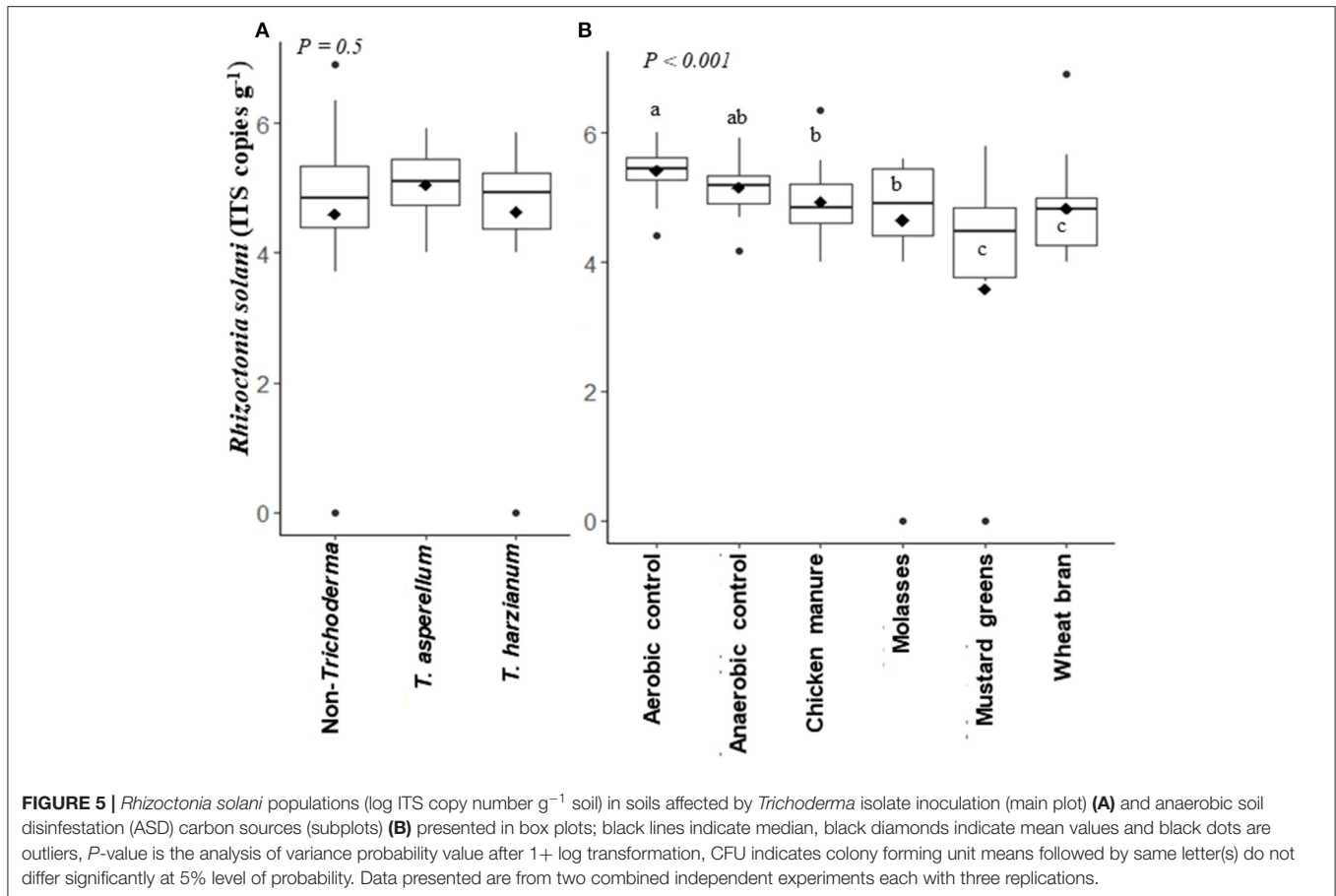


that *Trichoderma* can survive under conditions generated during ASD. Previous reports have also demonstrated the survival of *Trichoderma* spp. during ASD (Lamers et al., 2010; Momma et al., 2013; Shrestha et al., 2019). Chovanec et al. (2005) reported that *Trichoderma* could survive under hypoxic 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



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.

## FUNDING

This study was supported by the United States Agency for International Development under Cooperative Agreement No. AID-OAA-L-15-00001 to Virginia Tech and the Feed the Future Innovation Lab for Integrated Pest Management, and state and federal funds appropriated to The Ohio State University College of Food, Agricultural and Environmental Sciences.

## ACKNOWLEDGMENTS

We thank Pierce A. Paul for guidance on data analysis and Francesca Rotondo for guidance on qPCR analysis. We also thank Michelle Jones, Maria-Soledad Benitez Ponce, Kaylee South, and Laura J. Chapin for comments and suggestions on the early drafts of the manuscript.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2021.645736/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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