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# Anaerobic Soil Disinfestation: Meta-analysis and Optimization of Amendment Carbon Rate and C:N Ratio to Control Key Plant Pathogens and Weeds

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To the Graduate Council:

I am submitting herewith a dissertation written by Utsala Shrestha entitled "Anaerobic Soil Disinfestation: Meta-analysis and Optimization of Amendment Carbon Rate and C:N Ratio to Control Key Plant Pathogens and Weeds." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Plants, Soils, and Insects.

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# Anaerobic Soil Disinfestation: Meta-analysis and Optimization of Amendment Carbon Rate and C:N Ratio to Control Key Plant Pathogens and Weeds

**A Dissertation Presented for the**

**Doctor of Philosophy**

**Degree**

**The University of Tennessee, Knoxville**

**Utsala Shrestha**

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## DEDICATION

In the memory of

# Sheela Shrestha

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# ABSTRACT

Anaerobic soil disinfestation (ASD) is an environmentally friendly and cost effective pre-plant soil treatment technique that allows effective control of soilborne pests by creating anaerobic conditions, particularly for specialty and organic crop production under diverse environmental conditions. In spite of being a proven technique, ASD has to be optimized to fit into local production systems with specific pathogen pressure using locally available amendments for successful implementation on a commercial scale. Our meta-analysis study on soilborne pathogens, plant parasitic nematodes, and weeds validated that ASD is an effective approach to control various soilborne pathogens. This study aims to optimize the carbon rate and carbon to nitrogen ratio (C:N) of two ASD amendments namely, dry molasses and wheat bran to suppress *Fusarium oxysporum*, *Sclerotium rolfsii* and *Cyperus esculentus* tubers for a moderate soil temperature regime. Evaluation of survivability of recovered tubers, *Fusarium oxysporum* and *Sclerotium rolfsii* inocula corroborated with the finding of our meta-analysis that ASD effectively promotes tuber and pathogen propagule mortality. Evaluation of various carbon amendment rates maintained at a C:N ratio of 30:1 showed that 4 milligrams of carbon per gram of soil was the most effective to induce sclerotial mortality and parasitism. We found that maintaining an amendment C:N ratio within the range of 20:1 to 30:1, with carbon rate at 4 milligrams of carbon per gram of soil, is effective in generating favorable anaerobic conditions resulting in higher pathogen suppression and enhancement of beneficial mycoparasites.



**Keywords:** Anaerobic / biological soil disinfestation, beneficial microorganisms, *Fusarium oxysporum*, meta-analysis, mycoparasitism, *Sclerotium rolfsii*, soilborne pathogens, *Trichoderma*, yellow nutsedge

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# ACRONYMS

| | |
|---|---|
| AM | Arbuscular mycorrhizal |
| ASD | Anaerobic soil disinfestation |
| AIA | Actinomycete isolation agar |
| BSD | Biological soil disinfestation |
| BIA | *Bacillus* isolation medium |
| C | Carbon |
| C:N | Carbon to nitrogen ratio |
| CFU | Colony forming units |
| *Fol* | *Fusarium oxysporum* f. sp. *lycopersici* |
| IRIS | Indicator of reduction in soils |
| MeBr | Methyl bromide |
| $NH_4$ | Ammonium |
| $NO_2$ | Nitrite |
| $NO_3$ | Nitrate |
| OA | Organic amendment |
| ORP | Oxidation-reduction electrodes |
| PDA | Potato dextrose agar |
| TSM | *Trichoderma* selective medium |
| *Sr* | *Sclerotium rolfsii* |
| VFA | Volatile fatty acid |

# Introduction

Methyl bromide (MeBr) is a very effective pre-plant fumigant against soil pests. However, it is a serious ozone depleting fumigant; therefore, its use was banned as part of an international treaty known as the Montreal Protocol. Several chemical and non-chemical approaches have been introduced as a replacement for this effective soil fumigant. Many chemical alternatives have been registered, but these chemicals do not reach the level of broad spectrum control, with many shortcomings on effectiveness, consistency and safety. Anaerobic soil disinfestation (ASD; synonymous to biological soil disinfestation) appears to be a potential non-chemical alternative to MeBr, which has been successfully implemented or researched in Japan, the Netherlands, Florida, California (Blok et al., 2000; Butler et al., 2012b; Messiha et al., 2007; Momma et al., 2013; Shennan et al., 2007), and other parts of the world (Figure A1). Other non-chemical alternatives have shown promising results, including flooding, solarization, steam sterilization and biofumigation but these alternatives are either effective only in certain regions or cost prohibitive. For Tennessee vegetable crop production, ASD has shown some promise (McCarty et al., 2012a) but more research and commercial development is needed to make ASD technically and economically feasible for the state and the southeastern region.

ASD utilizes locally available organic amendments (OAs) as carbon (C) sources, and unlike many chemical fumigants, is safe to use near residential areas without any safety concerns. Therefore, it is potentially an economically and environmentally sustainable technique for soil disinfestation. Further, ASD has shown efficacy against many soilborne pathogens such as *Fusarium oxysporum*, *Sclerotium rolfsii*, *Verticillium dahliae*, *Pythium ultimum*, *Rhizoctonia solani*, *Ralstonia solanacearum*, and others (Butler et al., 2012b, Shennan 2014 ). Quantitative analysis of ASD studies on soilborne pests has not been reported and the meta-analytic review of previously published results on pest suppression due to ASD is useful to understand the efficacy of ASD practice.

In ASD, OAs provide labile C sources to soil microbes that create anaerobic conditions through increased microbial activity in moist, plastic mulched soils (Butler et al., 2012a; Butler et al., 2012b). The antagonistic properties and pesticidal compounds are generated in the soil by indigenous anaerobic microorganisms with the production of volatile fatty acids, which act

against plant diseases and pests (Blok et al., 2000). The ratio of C:N, as well as the rate and solubility of the C source, plays a critical role in microbial growth and metabolism (Nicolardot et al., 2001), plant growth and crop nutrition (Akhtar and Malik, 2000). Different OAs, such as grasses, wheat bran, molasses, potato haulms, cruciferous plants and cover crops have been examined as an ASD C source but no study has identified a suitable C:N ratio and carbon rates of the ASD OAs. Recent studies in Japan with ethanol (Momma et al., 2013) and in Tennessee with cover crops (McCarty, 2012a) as a C source amendment suggested on optimization of ASD for its practical application. On the other hand it is also important to note that ASD has shown a significant shift in the microbial community composition (Mazzola et al., 2012), and this is considered due to application of organic amendments (Shennan et al., 2013). *Bacillus* spp. (Momma et al., 2013) and *Trichoderma* spp. (Kredics et al., 2003) are well-known antibiotic and toxin producers in controlling mechanisms of soilborne pathogens. Studies on various biocontrol agents are in progress to replace chemical applications. Recently, actinomycetes were reported as sclerotial parasites that have a positive response toward soil amendments. However, studies on these microbes in response to ASD C sources are lacking and it is necessary to determine the effect of ASD on these beneficial organisms.

This dissertation provides a meta-analytic review of ASD and consists of experiment to optimize the ASD amendment C:N ratio and C rate to provide specialty crop growers with a pest control tools for replacing MeBr and other chemical fumigants, while maintaining high value crop production systems that are profitable and sustainable. This dissertation consists of five chapters. The first chapter is a manuscript submitted to the journal, *Frontiers in Plant Science*, summarizing the meta-analysis of efficacy of ASD on soilborne pathogens, nematodes, weeds, and crop yield. The second chapter discusses a growth chamber study that examined the C:N ratio of C amendments at 4 mg C $g^{-1}$ of soil to optimize ASD to evaluate soil anaerobic conditions and mortality of yellow nutsedge tuber. The third and fourth chapters summarize the research conducted in growth chamber and field conditions to evaluate the effectiveness of dry molasses and wheat bran maintained at different C:N ratios or C rates to suppress introduced inocula of the *Fusarium oxysporum* f. sp. *lycopersici* and sclerotia of *Sclerotium rolfsii* respectively. The fifth chapter is research study to evaluate the impact of ASD amendment maintained at 4 mg C $g^{-1}$ of soil on populations of *Trichoderma*, actinomycetes, *Bacillus* and root

colonizers. In this chapter, the impact of ASD amendment against germination and parasitism of sclerotia of *S. rolfsii* is also examined.

# Appendix

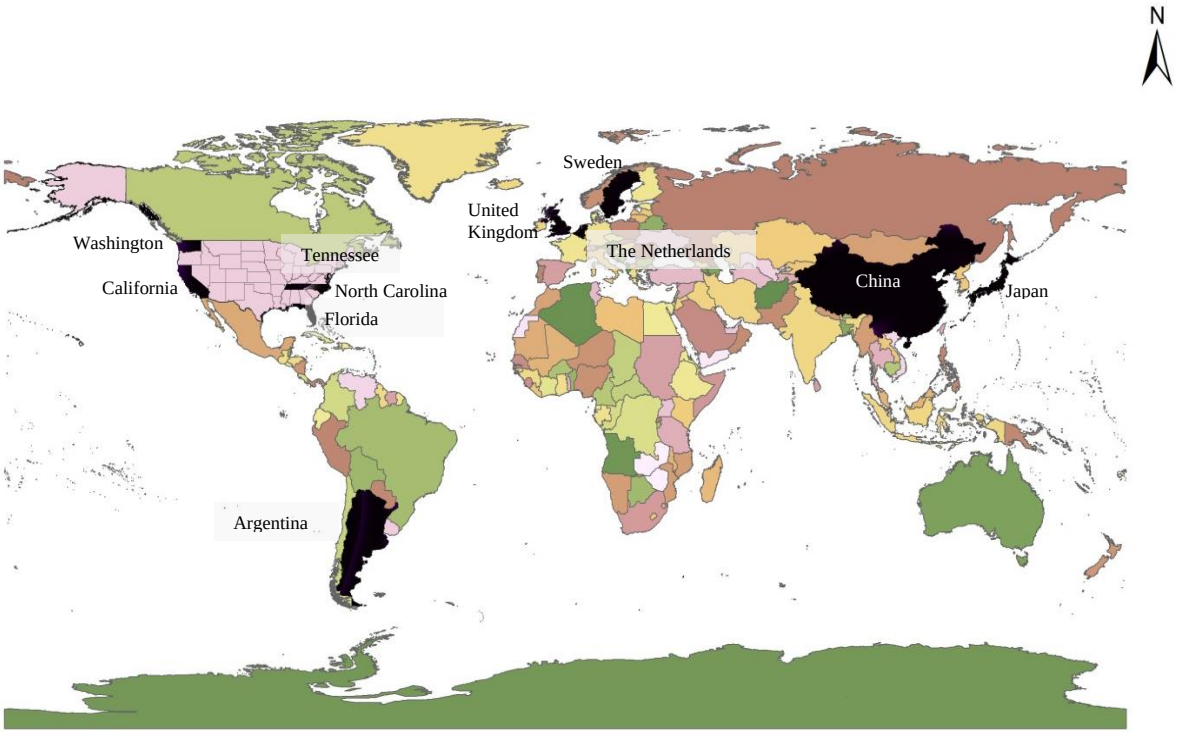


Figure A-1. Map of countries/states (in black) conducting ASD studies.

# Chapter 1

# A meta-analysis of the impact of anaerobic soil disinfestation on pest suppression and yield of horticultural crops

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My primary contributions to this manuscript include data collection and analysis, results interpretation and writing. Dr. Robert Augé contributed to the methodology and meta-analysis section along with reviewing of figures and tables.

## Abstract

Anaerobic soil disinfestation (ASD) is a proven but relatively new strategy to control soilborne pests of horticultural crops through anaerobic decomposition of organic soil amendments. The ASD technique has primarily been used to control soilborne pathogens; however, this technique has also shown potential to control plant parasitic nematodes and weeds. ASD can utilize a broad range of carbon (C) amendments and optimization may improve efficacy across environments. In this context, a meta-analysis using a random-effects model was conducted to determine effect sizes of the ASD effect on soilborne pathogens (533 studies), plant parasitic nematodes (91 studies), and weeds (88 studies) compared with unamended controls. Yield response to ASD was evaluated (123 studies) compared to unamended and fumigated controls. We also examined moderator variables for environmental conditions and amendments to explore the impact of these moderators on ASD effectiveness on pests and yield. Across all pathogen types with the exception of *Sclerotinia* spp., ASD studies show suppression of bacterial, oomycete, and fungal pathogens (59 to 94%). Pathogen suppression was effective under all environmental conditions (50 to 94%) and amendment types (53 to 97%), except when amendments were applied at rates less than 0.3 kg $m^{-2}$. The ASD effect ranged from 15 to 56% for nematode suppression and 32 to 81% for weed suppression, but these differences were not significant. Significant nematode moderators included study type, soil type, sampling depth, incubation period, and use of mixed amendments. Weed suppression due to ASD showed significant heterogeneity for all environmental conditions, confirming that these studies do not share a common effect size. Total crop yield was not reduced by ASD when compared to a fumigant control and yield was significantly higher (30%) compared to an unamended control, suggesting ASD as a feasible option to maintain yield without chemical soil fumigants. We conclude ASD is effective against soilborne pathogens and while not conclusive due to a limited number of studies, we expect the same for nematodes and weeds given observed effect sizes. Findings should assist researchers in exploring ASD efficacy in particular environmental conditions and allow for development of standard treatment protocols.



**Keywords:** Anaerobic / biological soil disinfestation, meta-analysis, soilborne pathogens, nematodes, weeds, suppression, yield

# 1. Introduction

Methyl Bromide (MeBr), a broad-spectrum soil fumigant, was completely phased out in 2005 (with the exception of critical use exemptions) due to its stratospheric ozone depleting nature. Specialty crop growers have used this fumigant to control soilborne pathogens, nematodes, and weeds since the mid-twentieth century. Due to restriction on its use, growers are seeking alternatives that will provide comparable crop yield to that of MeBr. A number of chemical fumigant alternatives have been registered as replacements to MeBr fumigation (Rosskopf et al., 2014), but growers may not be willing or able to adopt them due to geographic limitations, reduced efficacy, safety issues, and regulatory constraints of these chemicals (Csinos et al., 2002; Martin, 2003). Further, worldwide awareness of environmental degradation and reduced-pesticide agricultural concepts (Carvalho, 2006) is driving many growers to seek non-chemical techniques to control crop pests. Non-chemical techniques such as flooding, solarization, steaming, and biofumigation (with cruciferous plant residues) are some available options for disease suppression. However, these generally environmentally friendly approaches have limitations (Shennan et al., 2010; Muramoto et al., 2014), such as high use of water (Runia and Molendijk, 2010; Runia et. al., 2014a), high temperature requirements (Katan, 1981), use of costly equipment (Backstrom, 2002; Runia and Molendijk, 2010), and site-specific variability (Larkin and Griffin, 2007; Lopez-Aranda, 2014), respectively.

Another promising non-chemical option available to growers is anaerobic soil disinfestation (ASD), also known as biological soil disinfestation or anaerobically-mediated biological soil disinfestation, has been studied since 2000 in Japan (Shinmura, 2004; Momma, 2008), the Netherlands (Blok et al., 2000; Messiha et al., 2007) and the USA (Butler et al., 2012b; Rosskopf et al., 2014; Shennan et al., 2014). This technique relies on organic amendments to supply labile C to soil microbes to create anaerobic conditions in moist and plastic-covered soil. Soil microbes consume available oxygen and depletion of oxygen shifts the balance toward facultative anaerobes. Gases (such as $CO_2$, $NH_3$, $H_2S$, $CH_4$, and $N_2O$) and volatile fatty acids (VFAs) produced due to microbial decomposition of labile C during ASD lead to suppression of plant pathogens and nematodes. Among these compounds, VFAs (e.g., butyric acid and acetic acid), are particularly known to contribute to the soil disinfestation process (Momma et al., 2006).

ASD is an environmentally friendly pest control practice (Porter et al., 2010; Shennan et al., 2014; Rosskopf et al., 2015) where soil microbial growth can be enhanced, and soil fertility potentially enhanced by the addition of organic amendments. A number of active research programs across the world continue to refine ASD techniques to control plant pathogens, nematodes, and weeds, and to further elucidate mechanisms of ASD treatment success (Shennan et al., 2014). Although ASD incurs relatively low implementation costs when locally available amendments are utilized, currently, ASD application in the USA has largely been limited to a few organic crop producers and early-adopter conventional growers. ASD requires further refinement of protocols to system variables and cost benefit analysis in comparison to other chemical fumigants (Butler et al., 2012b; Shennan et al., 2014). Quantitative review of ASD literature may be useful to researchers in terms of clarifying its efficacy across environments and help to make more exacting recommendations for wide-scale adoption.

Only narrative reviews of ASD amendments and ASD comparisons in different countries have been published (Shennan et al., 2014; Rosskopf et al., 2015; Strauss and Kluepfel, 2015). However, a quantitative synthesis of the literature in reference to the efficacy of ASD on a range of soilborne pathogens, nematodes, and weeds has not been reported. Meta-analysis is a powerful tool that uses a set of statistical techniques to analyze independent studies quantitatively rather than qualitatively (Ojiambo and Scherm, 2006). The meta-analytic approach has provided useful results in medicine and psychology, and has been increasingly applied in agro-ecological systems and pest management (Madden and Paul, 2011; Ngugi et al., 2011; Poeplau and Don, 2015). The purpose of this meta-analytic review of previously published results on pest suppression due to ASD is to understand the efficacy of this non-chemical practice on a range of soilborne pathogens, nematodes, and weeds. The meta-analysis also addresses comparative data on pathogens, nematodes, and weeds using different moderator groups or explanatory variables. Likewise, ASD effectiveness on crop yield is an important study group for meta-analysis that can help growers make ASD adoption decisions. Many researchers rely on results from lab tests or pot (e.g., greenhouse, growth chamber) studies only. However, soil disinfestation using organic amendments under field conditions is a challenge for researchers as pathogen suppression is subject to numerous environmental factors such as soil temperature, soil type, pathogen type, and more (Bonanomi et al., 2010). Moderator analysis is thus important to understand how these factors influence the efficacy of treatment. In this study, we examined the overall impact of

various environmental and ASD treatment factors as moderators on ASD efficacy and effect size of pest suppression and yield.

## 2. Materials and Methods

### *2.1 Data Collection*

Literature databases were explored using the search engine Thompson Reuters Web of Science on 20 August 2015. The terms used for the initial search, "soil disinfestation" OR "soil amend*" OR "soil treat*", returned 78,019 search results. These search results were filtered to 116 articles using the search terms "anaerobic soil disinfestation" OR "biological soil disinfestation" OR "reductive soil sterilization" OR "non-chemical fumig*" OR "non-chemical alternative*". Records were retrieved from Web of Science Core Collection (70), CABI (37), BIOSIS Citation Index (6), and MEDLINE (3). Five books were excluded from 116 articles, and of the remaining 111 eligible articles, 65 were excluded because data described was presented in other original articles, full text could not be found, or did not meet one of the following inclusion criteria related to ASD-treatment: ASD treatment not applied, ASD was not compared with non-amended control, or experiment was conducted in petri dishes only (Figure 1-1). In addition to the remaining 46 articles, we identified nine additional eligible articles using 'Google scholar™' search. The meta-analysis included a total of 55 published and unpublished works (posters, theses, and conference papers) spanning 16 years from 2000 to 2015 and written in English (50), Japanese (2), Dutch (3) and Chinese languages (1).

We collected treatment means and sample sizes from each study to evaluate effectiveness of ASD for pest suppression (soilborne pathogens/diseases, nematodes and weeds) and crop yield in relation to 11 factors identified as moderator variables. If the means were reported in graphical form, we used WebPlotDigitizer (Rohatgi, 2011) to estimate their values. ASD treatment means were those that used any type of C amendment(s), soil saturation, or flooding and covering of soil (usually polyethylene mulch) during the study period, while the non-amended and covered or non-covered treatments were considered control means. Only for yield response, we also collected means of fumigated treatments to compare with ASD treatment means. Multiple treatments or pathogens from one article were treated as independent studies (sometimes referred

to as paired observations in the meta-analysis literature) and represented individual units in the meta-analyses. For example, Butler et al., (2012b) reported pathogen data for two trials for seven different C amendments, resulting in 14 studies from that article. Although designating multiple studies from one publication has the disadvantage of increasing the dependence among studies that for the purposes of meta-analysis are assumed to be independent (Gurevitch and Hedges, 1999), the greater number of studies increases statistical power (Lajeunesse and Forbes, 2003). This approach has been used commonly in plant biology meta-analyses (e.g. Holmgren et al., 2012; Veresoglou et al., 2012; Mayerhofer et al., 2013; Chandrasekaran et al., 2014). The entire data set included 900 studies from eight countries (Table 1-1).

### *2.2 Moderator variables*

Several variables affecting pest suppression and yield were categorized and employed in moderator analysis. Our first moderator of interest was the method of characterizing ASD efficacy against each pest (i.e.,'measure of efficacy moderator'), which represented studies that reported ASD effectiveness against pathogen, nematode and weed abundance in various quantifiable units (e.g., counts of pests, germination of pest propagules, ratings of disease; Table 1-2A). The different levels of 'measure of efficacy' were analyzed separately for each pest to understand the variation in effect sizes (Figure 1-3). We categorized soilborne pathogens into 3 levels: bacterial, fungal or oomycete and within each are specific pathogens (Table 1-2B). We also separated the beneficial soil organism *Trichoderma* to evaluate ASD effects. Further, realizing importance of the *Fusarium* genus that has been widely studied, we categorized *Fusarium* (*F*) spp. into 6 levels according to species and forma speciales (f. sp.) [*Fusarium* spp., *F. oxysporum* (*F. o.*), *F. o.* f. sp. *asparagi*, *F. o.* f. sp. *cubense*, *F. o.* f. sp. *spinaciae*, *F. o.* f. sp. *lycopersici*]. We also categorized available studies on nematodes and weeds according to their genus. Yield had two levels, non-amended control and fumigated control. We did not examine total vs. marketable yield as a moderator due to insufficient studies representing the total moderator level and we included total yield as a proxy for marketable yield where marketable yield was not reported. In addition, we recorded information for six categorical environmental moderators (explanatory variables) as study type, soil temperature, soil type, control type (with or without plastic mulch), depth of sampling, and incubation period for both pests and yield (summarized in Table 1-2F). These moderators are likely important determinants of the

effectiveness of ASD in response to pest control and crop yield. In addition, ASD heavily relies on amendments for C supplement and directly affect the ability of ASD to suppress pests. Accordingly, ASD amendment was categorized in four moderators: form (liquid or solid), single amendment or mixed, type, and rate (Table 1-2G). For environmental condition and amendment groups all moderator levels may or may not be present in the analysis.

### *2.3 Effect size and meta-analysis*

Our analyses followed the methodology and terminology of Borenstein et al. (2009) and were guided by the criteria suggested by Koricheva and Gurevitch (2014). We computed summary effects and associated statistics using Comprehensive Meta-Analysis Version 3 (CMA) software (Biostat, Englewood, NJ, USA; 2014). We used a random-effects model for the meta-analyses, considering that true effects are likely to have varied across studies (rather than a fixed-model, which assumes the same value or true effect for all studies).

The effect sizes were calculated as the natural log response ratio (*lnR*) of treatment mean to control mean and subjected to analysis of overall effect sizes (pest suppression and yield responses) of ASD for each moderator. *lnR* for each observation was calculated as

$$lnR = ln(X_t/X_c)$$

where $X_t$ is the ASD treatment mean and $X_c$ is the control mean (non-amended, untreated or fumigated control mean for yield). The log transformation was needed to balance positive and negative treatment effects and to maintain symmetry in the analysis (Borenstein et al., 2009). Given that approximately 80% of papers did not report a measure of dispersion, non-parametric variance was calculated as:

$$V_{lnR} = (n_t + n_c)/(n_t * n_c)$$

where, $V_{lnR}$ is the variance of the natural log of the response ratio, and $n_t$ and $n_c$ are the samples sizes of the treatment and control means, respectively. In studies in which several treatments were compared with one control group, sample size of the one control group was partitioned across treatment means. For example, for a study with one control and three treatments, each

having four replicates, the control sample size (4) was divided by three. This was done to avoid overweighting studies by incorporating the same experimental units (e.g., plot, plants) in more than one effect size. Values of zero are biologically common but mathematically not possible to incorporate into meta-analysis (ratio denominator cannot be zero; cannot calculate the natural log of 0). A common technique used in the medical literature is to add a small fixed number to any zero value (NCSS, 2015). In pathogen control research, however, this technique yields very inconsistent results, owing to the wide variety of units and the wide range of maximal pathogen growth/survival values. Further, small non-zero values result in unreasonably inflated response ratios. In order to analyze effect sizes of zero and near zero, we calculated 1% of the highest pathogen abundance value for a study and raised any other value below 1% to that level: for example, to 0.75 for 75 log CFU $g^{-1}$ of soil, and to 0.03 for 3.0-cm colony diameter. Negative values of pathogen abundance were equated to zero before applying the 1% adjustment.

Heterogeneity was assessed with the *Q* statistic, a measure of weighted squared deviations. Total heterogeneity ($Q_t$) is composed of expected or within-study variation ($Q_w$) and excess or between-study variation ($Q_b$). Heterogeneity was quantified using $I^2$, a descriptive index that estimates the ratio of true variation (heterogeneity) to total variation across studies:

$$I^2 = (Q_t - df)/Q_t * 100\%$$

where $df$ denotes the expected variation $Q_w$ and $Q_t - df$ the excess variation ($Q_b$) ($I^2$ is set to zerowhen *df* exceeds $Q_t$). A value of 0% indicates no true heterogeneity, and positive values indicate true heterogeneity in the data set with larger values reflecting a larger proportion of the observed variation due to true heterogeneity among studies. Assumptions of homogeneity were considered invalid when *p* values for the *Q* test ($P_{hetero}$) for heterogeneity were less than 0.1 (e.g., Bristow *et al*., 2013; Iacovelli *et al*., 2014). We assumed a common among-study variance across moderator subgroups.

### *2.4 Publication bias and sensitivity analysis*

Publication bias is the term applied to a body of research in the refereed literature that is systematically unrepresentative of all completed studies (Rothstein et al., 2006). Literature

reviews can be subject to publication bias, and the standard narrative review more so than quantitative meta-analysis review (Borenstein et al., 2009). The issue is raised more often with meta-analysis, likely because this method is intended to be comprehensive. The concern is the possibility that significant treatment differences are more likely to be published than non-significant findings. Direct evidence of publication bias is difficult to obtain, but it is important to check for it (Sutton, 2005; Madden and Paul, 2011; Koricheva and Gurevitch, 2014). Methods generally involve exploring the relationship between study effect size and precision. The idea is that studies with smaller sample sizes or higher variance will tend to have larger effect sizes than larger studies with greater precision. Hence, potential publication bias was assessed statistically with Begg and Mazumbar rank (Kendall) correlation and represented graphically with funnel plots of effect sizes versus their standard errors (estimated from their non-parametric variances) (Begg and Mazumdar, 1994; Borenstein, 2005; Borenstein and Cooper, 2009; Borenstein et al., 2009). The Duval and Tweedie iterative trim and fill method was used to demonstrate how the summary effect size would shift if apparent bias were to be removed (Duval and Tweedie, 2000). Sensitivity analysis was performed for the overall summary effects by removing one study and re-running the meta-analysis for every study in the analysis. This shows how much each study contributed to the summary effect, by noting how much the summary effect changes in its absence. Possible temporal changes in effect size were evaluated with meta-regression using publication year as a quantitative moderator (Koricheva & Gurevitch, 2014). Meta-regression analysis was conducted with the CMA software, with the restricted maximum likelihood and Knapp-Hartung methods (IntHout et al., 2014).

## 3. Results

We did not see evidence of publication bias. Visually, the funnel plots for each of the summary effects showed no pattern that would reflect bias toward not reporting small positive or negative effect sizes (Table 1-3). Large and small studies across the range of standard errors had the expected variability around the summary effect size. Within the Begg and Mazumdar (1994) rank correlation test, each of the summary effects had absolute Kendall tau values below 0.02, indicating no publication bias (no tendency for effect sizes to increase as study size decreases) (Table 1-3). The Duval and Tweedie (2000) trim and fill procedure imputes missing studies

needed to make the funnel plot symmetrical, removing the most extreme small studies and re-computing the effect size at each iteration until the funnel plot is symmetric on either side of the new (adjusted) summary effect. To maintain proper variance, the original studies are added back into the analysis along with a mirror image for each. The adjusted value is suggestive only, as when between-study heterogeneity exists (as was the case in our meta-analysis), trim and fill may inappropriately adjust for publication bias, where none exists, and thereby led to spurious changes in the summary effect (e.g., Terrin et al., 2003). A main concern about missing studies is that their absence in the analysis may result in an exaggerated summary effect. In our analysis, however, the summary value adjusted for potential missing studies is further from zero than the original value for the pathogen and weed summary effects (Table 1-3). The test revealed no potential missing studies and hence no adjustments for nematode control or yield assessed relative to non-amended controls or to fumigated controls. Therefore, the trim and fill analysis indicates no concern that publication bias has resulted in inflated summary effects. In fact, if the suggested adjustments are legitimate for pathogen and weed control (if there really are missing studies) then the Duval and Tweedie analysis points to an even greater impact of ASD in controlling these pests.

The stability of the overall summary effects was assessed with sensitivity analysis. One study was removed and the summary effect recalculated. This was repeated for all studies to determine how much any one study affected the summary effect size. The study with the largest influence on pathogen control was study 379 (*lnR* = -5.510, *Verticillium* treatment, Runia et. al., 2014b), whose removal changed the summary effect by 0.4% (from a 67.5 to 67.1% reduction in pathogens). The study with the largest influence on nematode control was study 720 (*lnR* = -0.401, sandy soil with solid amendment treatment, van Overbeek et al. (2014), whose removal changed the summary effect by 3.4% (from a 36.4 to a 33.0% reduction). The study with the largest influence on weed control was study 794 (*lnR* = -0.810, trial 5, McCarty et al., 2013), whose removal increased the size of the summary effect by 2.4% (from a 52.7 to a 55.1% reduction in weeds). The study with the largest influence on yield was study 871 (*lnR* = 0.205, eggplant treatment, (Butler et al., 2012b), whose removal reduced the summary effect by 5.9% (from a 28.6 to a 22.7% promotion of yield, relative to unamended controls). Koricheva and Gurevitch (2014) recommended testing whether a summary effect has changed over time, when

studies comprising the effect have been published over many years. Changes in the summary effect could potentially result from publication bias, changes in methodology, or real biological changes. Investigating chronology (year of publication), as a quantitative moderator using meta-regression, ASD control of pathogens has changed slightly over time; the yearly average change was -1.0% ($p$=0.81) over the data's 16 publication years. ASD control of nematodes has changed somewhat more over its 12 years of data, with an average decline of -1.8% per year ($p$=0.07). There was an insufficient range of publication years of articles and studies to characterize the influence of ASD on weed control or yield.

For our analysis, a natural log response ratio (*lnR*) value below zero indicates suppression of pests (i.e., soilborne pathogens, plant parasitic nematodes and weeds), a value above zero indicates an increase in pests with ASD, and a zero value signifies no effect of ASD treatments on pest suppression. The levels within moderators are considered significantly different from each other or from the overall mean when confidence intervals do not overlap. $I^2$ and $P_{hetero}$ characterized heterogeneity (the presence of underlying structure, i.e., true differences among studies) within moderators. For each pest, we grouped our results as each pest type or crop type, experimental condition and amendment used in ASD. We reported ASD yield response separately for the fumigated control and unamended control.

### *3.1 Measure of efficacy*

We detected an overall negative ASD effect on pathogen abundance in various quantifiable units (-1.18 [CI -1.56 to -0.80]). When growth of pathogens was measured in colony size, ASD effect was highest with 91% suppression and was significantly different from other units (Figure 1-2A). Such a high significance in colony size was reported as pathogen suppression indicator during ASD treatment in one article (Mazzola and Hewavitharana, 2014) with 15 studies, but realizing the importance of the study and the slight difference in the overall effect size (5%) after removal of the colony size unit, we decided to include all studies in our analysis. In the case of nematodes, all units ranged between 20 to 40% and we observed 37% overall effectiveness for nematode suppression (Figure 1-2B). Number of weeds in terms of 'count' (i.e., population or density) was highly reduced by ASD compared to germination of weed propagules (82% vs. 29%; Figure 1-2C).

### *3.2 Pathogens*

Overall ASD effect on suppression of different soilborne pathogens, which were categorized as bacterial, oomycete or fungal pathogens was -1.22 [CI -1.57 to -0.87] showing 70% suppression over 533 studies (Figure 1-3). Suppression was significantly higher for oomycete pathogens than for fungal pathogens and similar for oomycete and bacterial pathogens (Figure 1-3A). Between oomycetes, *Phytophthora* had higher suppression by ASD than *Pythium* but the difference was not statistically significant as CIs for the two summary effects overlapped (Figure 1-3B). More studies on ASD were conducted for fungal pathogens (7 soilborne genera), among which *Sclerotinia* was least suppressed by ASD (15%). ASD effect on *Sclerotinia* suppression significantly differed from *Fusarium* suppression (70%). All soilborne pathogens except *Sclerotium* were better suppressed by ASD (>63%) than non-amended controls although these pathogens did not differ significantly (Figure 1-3C). *Cylindrocarpon* was the most suppressed pathogen (86%), but with high CI values. To get an idea of the ASD effect on beneficial organisms, we also evaluated ASD effect on *Trichoderma* (n=24) and we observed a positive effect of ASD on these beneficial fungi (Figure 1-3D).

Since *Fusarium* was the most studied pathogen with 237 individual studies, it was of interest to observe the ASD effect on different host specific *Fusarium* pathogens (f. sp.) within *Fusarium* level. It also included uncharacterized *F. oxysporum* (54) and uncharacterized *Fusarium* spp. (19). Overall effect size of *Fusarium* level within pathogen was -1.05, [CI -1.55, -0.54] (representing an ASD suppression of 65% in raw terms), with significant heterogeneity $p<0.001$. True variation among studies, estimated by $I^2$, accounted for 13% of total variation. We observed a significantly higher suppression level of ASD for the spinach and tomato wilt pathogens; *F. o.* f. sp. *spinaciae* (87%) and *lycopersici* (74%), respectively. The uncharacterized *F. o.* also showed a similar effect size and was significantly higher than other levels of *Fusarium* (76%). The *F. o.* f. sp. *cubense* and other uncharacterized *Fusarium* spp. were less suppressed by ASD (Figure 1-3E). When we compared the ASD effect on sclerotial germination percentage of sclerotia-bearing pathogens, we found germination percentage was effectively lowered in *Verticillium*, *Rhizoctonia* and *Sclerotium*, but not in *Sclerotinia* (Figure 1-3F).

### *3.2.1 Experimental conditions for pathogen studies*

Experimental conditions for pathogens included meta-analysis results from only soilborne pathogens and excluding beneficial mycoparasites and non-amended treatments (e.g., flooding only). Small studies carried out in the laboratory and growth chamber conditions showed 61% pathogen suppression and large studies conducted in the field and the greenhouse showed slightly higher suppression (72%, Figure 1-4A). At high soil temperature, the pathogen reduction by ASD effect was ~10% higher than at moderate and lower soil temperatures (Figure 1-4B), however, a significance difference was not observed due to extended confidence interval of high temperature. The ASD treatment in volcanic soil from Japan showed significantly higher suppression of pathogens than sandy soil (83%). While both types of soil did not differ with clay, gray low land and loam soil. ASD effectiveness was significantly higher for 'other media,' which included greenhouse media, perlites, etc. (94%; Figure 1-4C). Pathogen suppression was not affected by whether ASD treatments involved covering (Figure 1-4D), and degree of suppression has been similar across different sampling depths (64 to 71%; Figure 1-4E). ASD incubation periods of greater than 10 weeks and 3 to 5 weeks were less effective than other periods. It is interesting to see >78% pathogen suppression for the less than a three-week period. Three weeks is by far the most used ASD incubation period for pathogen suppression (222 studies) and is among the most effective periods (64%; Figure 1-4F).

### *3.2.2 Amendment effect on pathogen suppression*

The type and amount of amendment is a crucial component of ASD to provide labile C to microbes, and so we examined amendment characteristics for influence on the efficacy of ASD on pathogen suppression. Across all pathogen studies (n=533), five amendment moderators were categorized and analyzed separately. Figure 1-5A provides results of liquid vs solid amendments (n=533) and Figure 1-5B depicts mixed vs non-mixed amendments (n=533). We found 533 studies were amended with various C sources (Figure 1-5C) and 41 studies were non-amended and were analyzed separately (Figure 1-5E). Ethanol, organic acid and other C source (glucose, sucrose and xylose) in amendment type moderator are applied as liquid amendments. Besides liquid molasses included in ag-by-product, all other amendments were solid amendments.

Amendment in liquid form was more effective than solid form, 77% vs 64% (Figure 1-5A). Mixing different amendment types did not increase the effectiveness of ASD as compared to single amendment (Figure 1-5B). Most C amendments significantly reduced pathogen measures (Figure 1-5C) and overall ASD effect on plant pathogens was -1.24, (CI [-1.56, -0.91] $p<0.001$). When ASD was conducted with ethanol, ASD effectiveness increased dramatically and was significantly different from other amendments: organic acid, combination, ag-by-product, cruciferous, grass, and legume (91%). 'Other C source', which includes glucose, sucrose and xylose showed the most pathogen suppression among amendments. Suppression of pathogens was however lower than 61% when amendments were cruciferous, legume and grass. We also examined anaerobic and flooding situations (i.e., without C amendment) to gain a sense of whether pathogen survival under these conditions was similar to ASD treatment and we found that while flooding was effective, anaerobic conditions are not as effective as ASD (28%, Figure 1-5D). Effectiveness of ASD on pathogen suppression also relies on rate of amendments. Amendment rates less than 0.3 kg $m^{-2}$ and 5 to 6 kg $m^{-2}$ did not show as much suppression as other rates (Figure 1-5E). Generally, the trend was that higher suppression was observed with higher rates of amendment but in meta-analysis of amendment rate, we could see response of pathogen suppression is not only subject to application rate.

### *3.3 Nematode suppression*

Over all studies, ASD decreased nematode abundance by 37% ($lnR$ = -0.45), with the confidence interval slightly overlapping zero ($p$ = 0.066; Figure 1-2B). The four individual efficacy measures ranged from 20 to 40%, with confidence intervals also crossing zero. Among the three most studied plant parasitic genera, ASD-induced inhibition was significant only for *Globodera*, at 56% (Figure 1-6A). The summary effect was not significant for *Pratylenchus*, *Meloidogyne* and the 3 genera grouped as 'Other'. Among the six moderators characterizing experimental conditions, most have at least one level with a significant ASD effect (Figure 1-6B-G). Unlike pathogen suppression, ASD has resulted in substantial nematode suppression in large studies (63%, $p$=0.002), with no suppression in small studies (38%, $p$=0.40) (Figure 1-6B). Suppression was greatest at moderate soil temperatures (68%, $p$=0.01) and insignificant at the higher and lower reported temperatures (Figure 1-6C). The ASD effect varied with soil type, with significant suppression of nematodes (94%) occurring only in loam soils (Figure 1-6D). The size

of the ASD-induced suppression has not differed as a function of its comparison to uncovered vs. covered controls (Figure 1-6E). Sampling depth markedly affected estimation of ASD efficacy, with nematodes reduced by 82% and 70%, respectively, in deep and shallow regions of the soil profile, while at moderate depth a near significant ASD stimulation of nematodes has been observed (Figure 1-6F). Incubation of less than 2 weeks has dramatically promoted nematode survival, while an incubation of 4 to 6 weeks has resulted in significant nematode suppression (Figure 1-6G). Amendment characteristics have had less influence on the extent to which ASD suppressed nematodes than fungal pathogens (Figure 1-6H, I, J, K). Liquid and solid forms of amendment have given similar nematode control (Figure 1-6H). Not mixing amendments has been far more efficacious than mixing them (Figure 1-6I). None of the amendment types resulted in a significant effect of ASD on nematode suppression (Figure 1-6J), although the small numbers of studies representing several of the amendment types give low statistical power for resolving differences. It was surprising that ASD showed nematode suppression at amendment rates less than 2 kg $m^{-2}$ and 3 to 4 kg $m^{-2}$, but rates at 2 to 3 kg $m^{-2}$ and 4 to 5 kg $m^{-2}$ did not show any significant effect (Figure 1-6K), but again, the relatively low number of studies which were performed under varying amendment types and soil temperatures limits interpretation.

### *3.4 Weed suppression*

Few studies have addressed the influence of ASD on weed suppression (88 studies from 5 publications) and all studies were conducted in sandy soil. Overall weed reduction was 63% when examined as both weed count and germination percentage (Figure 1-2C). Weed measures have been much more affected by ASD when assessed as weed population density (82%, $p<0.001$) than as germination of introduced propagules (29%, $p=0.189$). *Chenopodium album*, *Cyperus esculentus* (yellow nutsedge), and less frequently studied species have shown significant reductions with ASD (Figure 1-7A). *Digitaria sanguinalis* (crabgrass) has not been affected by ASD in the few studies reported, and growth of *Amaranthus retroflexus* (pigweed) has actually been substantially promoted by ASD. Large-scale application of ASD has resulted in significant weed suppression whereas small-scale application has not suppressed weeds (Figure 1-7B). The effect of ASD has been evident only when soil temperatures are high (Figure 1-7C). ASD treatments have suppressed weeds only when compared to uncovered controls; covering soils has given better weed control than ASD treatments (Figure 1-7D). Another interesting observation

for ASD was seen for sampling depth (or burial depth), with shallow depth being significantly more suppressive to weeds and moderate depth promoting weed populations (Figure 1-7E). Incubation periods of greater than 10 weeks showed far better control than a three-week period, with the latter having little effect on weed measures (Figure 1-7F).

Each of the four amendment moderators affected ASD efficacy on weed suppression (Figure 1-7G-J). The applied liquid form showed 97% weed suppression, about twice as effective as solid amendments at 44% weed reduction (Figure 1-7G). Mixed and single amendment forms of ASD have had similar, significant effects (Figure 1-7H). Among amendment types, ag by-products, manure, ethanol and the less frequently used other C sources led to substantial ASD-induced weed suppression in the range of 77 to 97% (Figure 1-7I). ASD resulted in significant weed suppression only when the rate of amendment was greater than 1 kg $m^{-2}$ (Figure 1-7J).

### *3.5 Yield*

ASD treatment promoted yields of eggplant when compared to both non-amended and fumigated controls (>130%, Figure 1-8A). Yield of bell pepper, strawberry, tomato, potato, and other crops has remained unaffected by ASD. The lack of effect on yield occurs whether ASD efficacy is viewed relative to non-amended or fumigated controls (Figure 1-8A-E). The absence of ASD influence on yield has not been affected by study type (Figure 1-8B). ASD tended to promote yield at sandy soil (33%, Figure 1-8C), higher temperatures (>54%, Figure 1-8D) and shorter incubation times (34%, Figure 1-8E). Yield response increased to 6% when ASD was compared with fumigated treatments and 30% with non-amended control (Figure 1-9A). ASD effect on yield compared to both control treatments was highest for solid amendments compared to liquid (15 to 32%, Figure 1-9B). Mixing of amendments increased yield 13 to 14% in both cases (Figure 1-9C). Similar to weed suppression, manure amendment tended to have the most positive effect on yield in both cases (>78%, Figure 1-9D). In addition, yield response increased with respect to increase in application rate of amendment (Figure 1-9E).

# 4. Discussion

### *4.1 Is ASD effective for pathogen suppression?*

Our results indicate strong evidence of pathogen suppression by ASD and that ASD plays a critical role in minimizing pathogen inoculum by inhibiting germination of inoculum or reducing the vigor of pathogens. We observed that colony size as a 'measure of efficacy' of pathogen suppression was highly sensitive to ASD. Colony size during ASD would likely be affected by the range of volatile compounds and other toxic anaerobic decomposition by-products. Along with colony size, we also observed suppression of pathogens in terms of colony forming units, germination percentage, infection percentage, and microsclerotia production. Given the various efficacy measurements, we confirmed that overwintering forms of pathogens that impact crops could potentially be effectively suppressed by ASD.

Studies have shown that ASD is effective in suppressing various soilborne pathogens (as reviewed by Shennan et al., 2014; Rosskopf et al., 2015) and our meta-analysis results were concurrent with those narrative reviews. Our meta-analysis also demonstrated the importance of statistical power in terms of study number; for example, the only two studies for *Cylindrocarpon* (infection percentage) showed no statistical difference, although disease reduction was 86%. Banana wilt by *F. o.* f. sp. *cubense* was reported by Huang et al. (2015) and Wen et al. (2015) in China and we are not surprised that these were less suppressed than all other *Fusarium* spp. as treatments where flooding of soil and use of amendments like rice and corn straw, which have an altered microbial response than that of more labile C amendments. We observed a significantly higher suppression level of ASD for the spinach wilt pathogen *F. o.* f. sp. *spinaciae* (87%) and tomato wilt pathogen *Fo* f. sp. *lycopersici* (76%). For *Sclerotinia*, which was less affected by ASD, data was reported only from species *sclerotiorum* and it was reported that sclerotial germination was highly influenced by the low amendment rate and soil temperature (Butler et al., 2014b). Further, sclerotial viability, release of biochemical compounds, and infection ability vary under different growing conditions and ineffectiveness of ASD in such cases may relate to a combination of factors. At the same time, Thaning and Gerhardson (2001) reported sclerotia of *S. cepivorum* from onion was unaffected by ASD (since data was not reported, *Sclerotium cepivorum* is not included in the meta-analysis). On the other hand, sclerotia of *Verticillium* and

*Sclerotinia* both failed to survive in the same study. Variability in sclerotial infection mechanisms (e.g., production of apothecia or mycelium; Imolehin et al., 1980) can also impact ASD effectiveness. Nevertheless, from our meta-analysis, we can grasp the degree of fungistasis (soil property preventing germination of viable propagules) being enhanced in ASD relative to size of sclerotia; specifically, compared to *Sclerotinia*, smaller sclerotia of other sclerotial pathogens are more effectively suppressed by ASD (see Figure 1-3F). *Macrophomina*, although a sclerotia producer, the size of sclerotia are too small to enumerate (100um-200um) so sclerotial germination is typically not reported. Recent studies on the bacterial pathogen *Agrobacterium tumefaciens* in tree crops was reported to be suppressed by ASD (Strauss et al., 2014), confirming ASD can be expanded to target other new plant pathogens and other crops.

Interestingly, our meta-analysis showed that ASD promoted the population of the mycoparasite *Trichoderma*. This mycoparasite along with other fungi parasitizing sclerotia of *S. rolfsii* were reported in Shrestha et al. (2013) and Thaning and Gerhardson (2001). Likewise, occurrence of the *S. sclerotiorum* sclerotial parasite *Coniothyrium minitans* was reported by Thaning and Gerhardson (2001). However, ASD effects on these beneficial organisms are not reported. Looking at the positive impact of ASD on *Trichoderma*, although nonsignificant in this study, suggests that more studies on ASD effects on beneficial microorganisms are needed. Studies have revealed that Firmicutes, Clostridia, and *Bacillus* are prominent in microbial communities during ASD (Mowlick et al., 2012). Further studies will help to further elucidate dynamics of beneficial organisms during and post-ASD treatments, which will allow for treatment adaptations to increase impact on beneficial organisms.

### *4.2 Conditions favoring ASD effectiveness on pathogen suppression*

Our analysis suggests that ASD can work as a replacement to chemical fumigants for pathogen suppression as we observed consistent pathogen suppression under various conditions (Figure 1-4). These results also suggest that ASD significantly suppresses pathogens across a range of temperatures. ASD treatments were more effective under higher soil temperature for both nurseries and field conditions. If soil temperature is relatively high (>16°C ) the incubation period can be reduced to less than 3 weeks since our analysis showed >80% of pathogen suppression is achieved when temperature ranged from 16 to 30°C  and pathogens were not

suppressed (40%) when temperature was low (data not shown). However, under low temperature (<16°C ), ASD can be effective when certain factors are modified, for e.g. *Ralstonia* and *Verticillium* under low temperature were effectively suppressed when higher amendment rates (grass) and longer incubation periods of 10 to 25 weeks were practiced.

It is not uncommon to see greater suppression of pathogens in media such as potting soil and other laboratory media other than soil, potentially due to reduced heterogeneity and reduced populations of other soil microorganisms than in field conditions. These media based studies are usually accompanied by smaller studies in a greenhouse, growth chamber, or laboratory with controlled environmental conditions. Among various types of soil, clay and sandy soils showed low suppression of pathogens in response to ASD treatment. Reasons for this observation may include low availability of C to microorganisms due to rapid loss of soluble C in sandy soil and greater adsorption and reduced water infiltration rate that affects the distribution of decomposition by-products in clay soils. Clay soils are also likely to be more buffered against changes in soil pH that may affect the accumulation of VFAs. Further, these acids are weakly adsorbed to the soil's exchange phase and have rapid turnover rate with short half-life (Jones et al., 2003) and transitory when exposed from anaerobic to aerobic condition (Lazarovits et al., 2005). Whereas volcanic ash, loam and gray lowland soil showed more suppression than clay and sandy as these soils are themselves more fertile with high mineral contents, which often enhance microbial activity.

One of the benefits of ASD is that it may be able to control pathogens under relatively short incubation periods for a biological soil treatment. Surprisingly, ASD suppressed pathogens under relatively short incubation periods. For an incubation period <3 weeks, we noticed 80% pathogen control which was directly related to study type and soil type. Most of the studies less than 3 week incubation periods were reported from small-scale studies, including lab studies and other C sources with eight amendment types in this analysis (110 studies) and only 24 studies reported from large-scale studies, which included volcanic ash and gray lowland studies. Lower percentages of pathogen suppression for 3 to 5 week and >10 week incubation periods may be attributed to few amendment types (ag-by-product, brassica, grass or protein-by-product) included in the meta-analysis. Pathogen suppression even after ASD treatment (post ASD)

duration reveals that ASD prevents resurgence of pathogens. However, post ASD treatments in this analysis included only organic acid as the C amendment and this case may not be same for the other amendments.

### *4.3 Contribution of ASD amendments to pathogen suppression*

Amendments such as ethanol, organic acids, and liquid molasses are easier to apply in the soil through drip application or by spraying. Liquid amendments are easily incorporated in soil and rapidly translocate to the soil profile, which our results suggest makes them more effective in ASD than solid amendments. In Japan, ethanol for ASD is already practiced at a relatively large scale (Momma et al., 2013) and in Florida liquid molasses is commonly used (Butler et al., 2012a; Rosskopf et al., 2014).

The categorization of amendment types in Figure 1-5C as moderator levels clearly shows differences in various C amendments for pathogen suppression. It also indicates the importance of moderator analysis as we get a clearer indication of effect sizes for various amendments. The category 'other C sources' in this analysis (glucose, xylose and sucrose) showed the highest suppression of pathogens, and studies were conducted in plastic boxes against *Fusarium* pathogens. This illustrates that ASD is highly effective in controlled environments, likely due to high anaerobic activity and confinement of VFAs and other volatile compounds (Hewavitharana et al., 2014). Recently, Daugovish et al. (2015) used diluted glycerol as liquid amendment in field soil and found that this C source was not as effective as rice bran to create long lasting anaerobic conditions, which suggests that ASD effectiveness may in some cases differ in field conditions.

From our analysis, ethanol is established as the most effective ASD amendment in controlling plant pathogens. ASD effectiveness due to ethanol is directly related to concentration and incubation period (Momma et al., 2006); a minimum incubation of 9 days is required for effective ASD treatment when 0.5% (of soil volume) of ethanol is used. In addition, almost all amendments used as C sources in the studies in this meta-analysis are considered to produce high VFAs relative to non-amended controls (Figure 1-5).

For effective disease suppression, relatively high rates of amendment incorporation are reported as necessary (Mowlick et al., 2013; Butler et al., 2014b). From our results, we confirmed higher amendment rates lead to higher suppression. However, amendment rates at 5 to 6 kg $m^{-2}$ rate showed slightly less suppression and the reason may be that represented studies utilized only grass and cruciferous plants. These amendments are less readily decomposed due to more complex C compounds in whole plant tissue than in simpler and more labile C sources such as ethanol, molasses, and glucose. Our results do suggest that ASD implementation costs could potentially be lowered by application of low amendment rates in some cases (~ 300 g $m^{-2}$) of amendment, which should be studied further.

### *4.5 ASD effect on nematode suppression*

Measure of efficacy results indicated that hatching and number of nematodes, infection incidence, and density of nematodes in roots were not significantly suppressed by ASD treatment. Only potato cyst nematode (*Globodera*) was effectively controlled by ASD and half of studies used protein-by-product amendment (Runia et al., 2014b; Streminska et al., 2014; van Overbeek et al., 2014). *Meloidogyne* and *Pratylenchus* showed some suppression, but this was non-significant. Nematode studies were approximately 7 times fewer than pathogen studies and Figure 1-6 shows how this low number of studies affected nematode suppression evaluation, with large confidence intervals due to error (Borenstein et al., 2009). We observed that nematode suppression with ASD is not as effective as pathogen suppression. However, higher suppression of nematodes by ASD treatments in field conditions, high organic content soil (e.g., loam and volcanic soil) and 2 to 6 weeks of incubation period was observed. Both liquid and solid amendments seem effective in nematode control. Besides manure and combination levels, all other amendments were applied in ASD suppressed nematodes. In our analysis, moderator levels manure and combination consist of poultry litter (7 studies each), which is known to have nematicidal activity (Riegel and Noe, 2000) and was always associated with soil solarization to increase soil temperatures. However, it was not effective enough for nematode suppression. Since the early twentieth century, studies have revealed that decomposed organic matter helps in reduction of nematodes (Linford et al., 1938). Reviews on various amendments and mechanisms of suppression against various nematodes are reported (Rodriguez-Kabana, 1986; Rodriguez-Kabana et al., 1987; Oka et al., 2007), but very few studies have been conducted to evaluate

efficacy of ASD on nematode suppression. More studies are encouraged under a range of ASD treatment factors and environmental conditions in order to better evaluate ASD impact on plant parasitic nematodes.

### *4.6 ASD effect on weed suppression*

Although there are few reports on weed suppression by ASD compared to pathogens and nematodes, our meta-analysis indicated that ASD is effective in suppressing weeds as well. Except *Amaranthus retroflexus*, all other weeds evaluated were found to be suppressed with ASD treatment (Figure 1-7B). *Amaranthus* is troublesome persistent weed with an extended germination period (Karimmojeni et al., 2014) and the study included in our meta-analysis was a pot observation thus emphasizing the need for additional research. *Digitaria* suppression likely needs some refinement in ASD while *Cyperus* tuber germination was suppressed by ASD. Although these weed suppression studies were conducted in pots, we believe that ASD can be equally effective if used in field condition as *Chenopodium album* and other weeds in field study showed high suppression when grass and other C sources were used as amendments. An ASD effect on weeds at shallow depths with almost 100% control of weeds could potentially be of large benefit; however, this represents few observations (n=9) which were reported from a single paper (Muramato et al., 2008) conducted in pots, with high temperature and without a covered control. More studies are needed with more variables for such cases to better assess suppression effects. When amendments were in liquid form, almost 99% weed control was achieved and reasons are likely similar to that discussed previously for pathogen suppression. It was not surprising to see that ethanol and manure amendments in ASD are more capable of weed suppression than other amendments as these may promote more toxicity than other C sources to control the weed propagules. But, there is a need to explore more cover crops, ethanol and manures as ASD amendments, and for an increase in the number of these studies. For effective weed suppression, rates of amendments greater than 1 kg $m^{-2}$ are likely needed.

### *4.7 ASD effect on crop yield*

We found that total fruit yield of crops was not reduced by ASD when compared to a fumigant control and yield was significantly higher when compared to a non-amended control. Our results

indicate that ASD is promising for sandy soil and high soil temperature and the result may be due to suppression of pathogens and weeds by lethal temperatures, as well as substantial beneficial effects of organic matter additions on chemical, biological, and physical properties of sandy soils (Butler et al., 2014a). Application of manures and increased amendment rate increased the yield (>50%) compared to both fumigated and non-amended controls. However, due to low number of studies, we see overlapping of confidence intervals and it is expected that if the number of studies on ASD using manures increases, we may see a significant crop yield result from meta-analysis.

Not surprisingly, a far higher number of publications on ASD are related to disease suppression than to yield response. The small numbers of published yield studies do not allow a comprehensive meta-analysis. This, and the numerous variations inherent to field studies, led to large CIs and likely insufficient power to determine with statistical confidence if yield summary effects differ from zero. Further, analysis of yield data faces several limitations. First, many papers do not report standard deviation and so use of non-parametric variance may have added additional uncertainty to our results. Second, although our mean yields include mostly marketable yield, in some instances (20 studies from McCarty et. al., 2014) we included total yield as a proxy for marketable yield where marketable yield was not reported. As concluded by (Belova et al., 2013), the lack of detail provided in many studies about field experimental protocols, horticultural practices and field management history hinders conclusive analysis. The wide confidence intervals for yield in our results likely reflect the fact that yield is affected by many environmental factors, soil factors and other cultural practices.

## 5. Conclusion

Given that pests evaluated in ASD studies differ widely in biological characteristics, it is not surprising that biologically-based ASD treatments may differentially impact survival and growth of these organisms. ASD treatment showed high reduction in bacterial (*Ralstonia*), oomycete (*Pythium* and *Phytophthora*) and fungal (except for *Sclerotinia*) pathogen inoculum. Among fungal pathogens, ASD response to pathogen supression was high for *Cylindrocarpon*, *Fusarium*, *Macrophomina*, *Rhizoctonia*, *Sclerotium*, and *Verticillium*. Among different host specific *F. oxysporum* pathogens, *F.o.* f. sp. *spinaciae* and *F.o.* f. sp. *lycopersici* were

significantly suppressed by ASD. Under most environmental conditions (i.e., a range of study types, soil temperature, soil types and incubation period), suppression of pathogen inoculum due to ASD treatment ranged from 50 to 94%. While our results indicate that ASD is effective for a suppression of a broad range of plant pathogens as compared to an non-amended control across a range of amendment types, amendment rates (>0.3 kg $m^2$), soil temperatures, soil types, and treatment incubation periods, research and demonstration studies often report variable results when compared to conventional soil fumigant controls. While this is not surprising given that ASD treatment relies on a more complex biological process that is influenced by environmental conditions and interactions with existing soil biology as compared to chemical fumigants, it does suggest that further refinement to improve ASD techniques could lead to more consistent field suppression compared to fumigants. Accordingly, ASD methods likely will need refinement based on the pests of interest and environmental conditions in a given production system. Due to a limited number of studies and variability in reported research, we cannot conclude that ASD is consistently effective in suppressing nematode or weed pests, although suppression has been achieved for some species under specific environmental and treatment conditions. Given broad-based suppression of plant pathogens under ASD treatments, future research should focus on further improving consistency of ASD treatment for soilborne plant pathogens to improve competitiveness of this biologically-based technique with conventional soil fumigants.

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# Appendix

Table 1-1. Number of studies by country and USA states and response variables examined

| S.no. | Country | Soilborne pathogens[1] | Nematodes | Weeds | Yield | Non-Amended | *Trichoderma* |
|---|---|---|---|---|---|---|---|
| 1 | Argentina | 2 | - | - | - | - | - |
| 2 | Belgium | - | 2 | - | - | - | - |
| 3 | China | 56 | - | - | 1 | 4 | - |
| 4 | United Kingdon | 4 | - | - | - | - | - |
| 5 | Japan | 84 | 2 | - | - | 7 | - |
| 6 | Netherlands | 117 | 54 | 20 | 4 | 19 | - |
| 7 | Sweden | 12 | - | - | - | 8 | - |
| 8 | USA (California) | 36 | - | 3 | 56 | - | - |
| 9 | USA (Florida) | 111 | 28 | 25 | 32 | - | 24 |
| 10 | USA (Tennessee) | 91 | - | 40 | 30 | - | - |
| 11 | USA (Washington) | 20 | 5 | - | - | 3 | - |
| | Grand Total | 533 | 91 | 88 | 123 | 41 | 24 |

[1]Does not include studies without organic amendment (i.e., anaerobic and flooding only studies; 41 studies)

Table 1-2. Levels and attributes within each categorical moderator variable tested for significance of pest suppression and yield responses

| Categorical moderator variables | Levels | Attributes |
|---|---|---|
| A. Measure of efficacy (3levels) | Pathogen | Colony size, germination (%), infection (%), colony forming units (log), microsclerotia count |
| | Nematode | Mass in root (g), hatching (%), counts, rating of disease |
| | Weed | Count, germination (%) |
| B. Soilborne pathogen genera (3 levels) | Bacterial (1) | *Ralstonia* |
| | Oomycete (2) | *Phytophthora*, *Pythium* |
| | Fungal (7) | *Cylindrocarpon*, *Fusarium*, *Macrophomina*, *Rhizoctonia*, *Sclerotium*, *Sclerotinia*, *Verticillium* |
| C. Nematodes (4 levels) | Plant parasitic | *Globodera*, *Pratylenchus*, *Meloidogyne* and others *(Heterodera*, *Pratylenchus*, *Trichodorus*, *Tylenchorhynchus*) |
| D. Weeds (5 levels) | Weed type | *Amaranthus retroflexus*, *Chenopodium album*, *Cyperus esculentus*, *Digitaria sanguinalis* and others |
| E. Yield (2 levels) | Control | Fumigated control, non-amended control |
| F. Environmental conditions | | |
| . Study type (2 levels) | Small scale | Study mostly in controlled environment using glass, bag, bucket, box, pot, growth chamber |
| | Large scale | Field / plots |
| . Soil temperature (3 levels) | Low | <16°C |
| | Moderate | 16 to 35°C |
| | High | >35°C |
| . Soil type (6 levels) | Sandy | Sandy, sandy peat, sandy loam, loamy sand, sandy clay loam, glacial sand |
| | Clay | Clay, clay loam |
| | Loam | Loam, silty loam, marine loam |
| | Gray lowland | Poorly drained soil |
| | Volcanic ash | Andosol |
| | Other media | Greenhouse soil, peat, perlite and other |
| . Control (2 levels) | Yes | Plastic sealed to create anaerobic conditions |
| | No | Uncovered treatment |
| . Depth of sampling (3 levels) | Shallow | 0 to 5-cm |
| | Moderate | 6 to 15-cm |
| | Deep | >15-cm |
| . Incubation period | Variable | Ranged from <3 weeks to>10 weeks |

Table 1-2. contd.

| Categorical moderator variables | Levels | Attributes |
|---|---|---|
| G. Amendments | | |
| . Amendments form (2 levels) | Liquid | Ethanol, organic acids, semi-solid molasses |
| | Solid | All other amendment types |
| . Amendments mixed (2 levels) | No | Single amendment only |
| | Yes | 2 or>2 different amendments mixed |
| . Amendment type (11 levels) | Agricultural by-product | Wheat bran, rice bran/straw, maize stalks/straw, molasses (solid and liquid), grape pomace, onion waste, potato residue |
| | Cruciferous | Arugula, broccoli, radish, mustard and other mustard products |
| | Combination | >2 amendments used |
| | Protein by-product | "Herbie[1]", volatiles from Herbie |
| | Legume | Cowpea, crimson clover, hairy vetch, sunn hemp, alfalfa |
| | Grass | Oats, cereal rye, perennial ryegrass, Italian ryegrass, pearl millet, sorghum-sudangrass, wheat and other grasses |
| | Manure | Poultry litter with or without solarization, composted steer manure |
| | Organic acid | Acetic acid, butyric acid, lactic acid, 'SPK' |
| | Ethanol | Ethanol, bio-ethanol (0.5%, 1%, 2%) |
| | Other C source | Glucose, sucrose, xylose, C media (other organic material) |
| . Non-amended | No amendments | Anaerobic or flooding |
| . Rate per $m^2$ | Variable | Ranged from <0.3 kg to>9kg |

[1]Proprietary blend of plant products, see Runia et al., 2014b for more information.

Table 1-3. Measures used in characterizing publication bias for each effect size (after Borenstein, 2005)

| Effect sizes | Summary effect[1] | | | | Funnel plot[2] | Kendall tau[3] | Duval & Tweedie adjusted[6] | No. impute |
|---|---|---|---|---|---|---|---|---|
| | n | *lnR* | *p* | No. var. | | | | |
| Pathogen | 533 | -1.12 | <0.001 | 0.005 | No | -0.07 | -1.29 | 66 |
| Nematode | 91 | -0.04 | 0.027 | 0.060 | No | -0.14 | -0.04 | 0 |
| Weed | 88 | -0.75 | 0.002 | 0.058 | No | -0.11 | -1.49 | 17 |
| Yield with non-amended control | 68 | 0.26 | 0.034 | 0.015 | No | 0.02 | 0.26 | 0 |
| Yield with fumigated control | 55 | 0.05 | 0.687 | 0.018 | No | -0.07 | 0.05 | 0 |

[1]Summary effect: n=number of studies, *lnR* = natural log of overall summary effect, *p*= probability that summary effect ≠ 0, No. var. = number of different variance values of studies comprising the summary effect

[2]Funnel plot appears asymmetrical

[3]Begg and Mazumdar Kendall rank correlation: tau = rank correlation coefficient (with continuity correction)

[4]Duval and Tweedie trim and fill: adjusted summary effect after imputing missing studies using an iterative trim and fill procedure, No. impute = number of studies imputed in the trim and fill exercise.

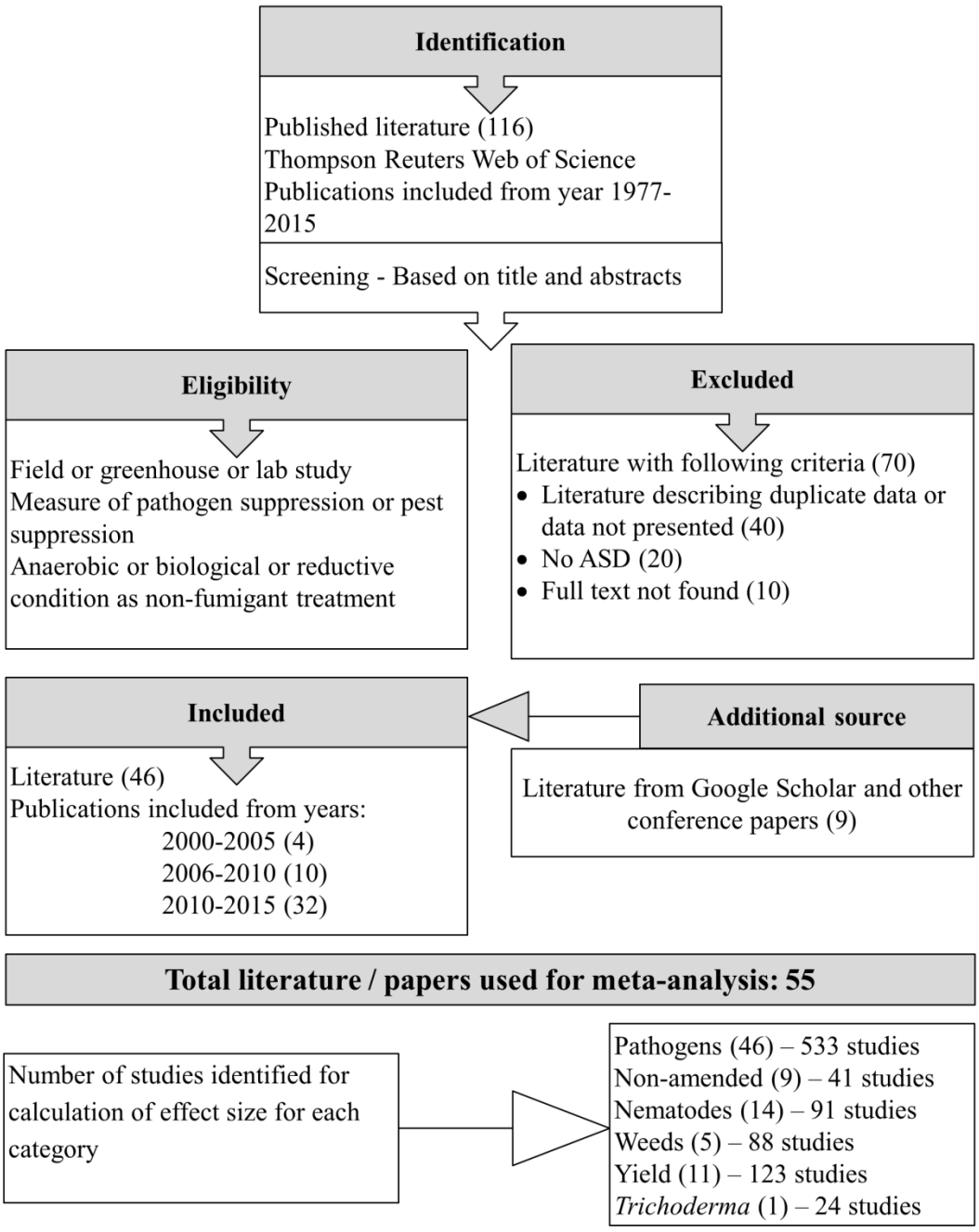


Figure 1-1. Flow diagram showing the study selection procedure

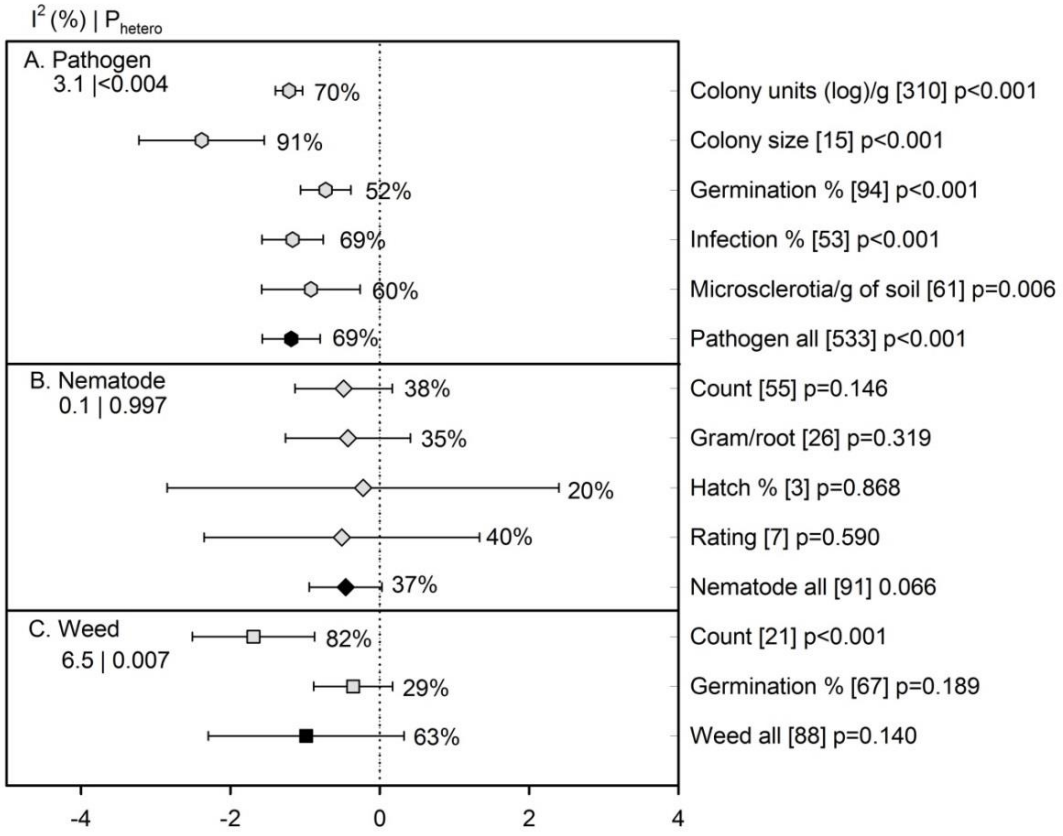


Figure 1-2. Weighted summary effect sizes (*lnR*) and 95% confidence intervals (CIs) for ASD moderator 'measure of efficacy' (various measures of pathogen growth and survival used in the literature).

Comparisons among levels of (A) Pathogen (⬢-hexagon symbols), (B) Nematode (◆-diamond symbols) and (C) Weed (■-square symbols). For each level of moderator, values to the right of the CI line with negative effective size are percent pest suppression and with positive effect size are percent of promotion. Number of studies reporting data for each level of moderator is given in brackets. The moderator level was significantly different from zero if $p$-value $\leq 0.05$. Values below panel titles to the left are $I^2$ (percentage of heterogeneity due to true variation among moderator levels) and $P_{hetero}$ (test of the null hypothesis, that all studies share a common effect size when it is greater than 0.1) for each moderator. Open symbols denote levels of each moderator (subgroups); closed symbols denote overall moderator summary effect.

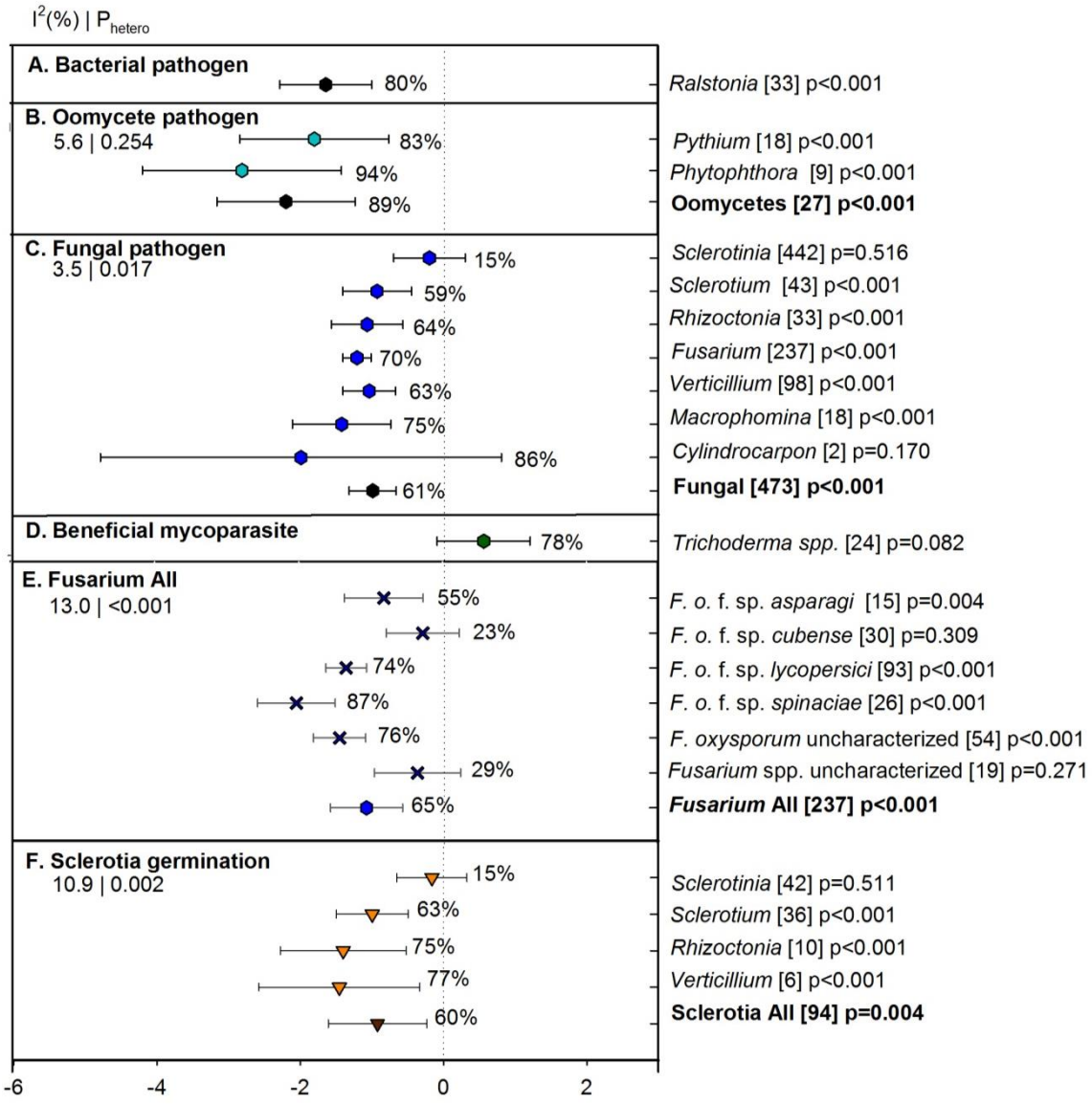


Figure 1-3. Weighted summary effect sizes (*lnR*) and 95% confidence intervals (CIs) for ASD effect on suppression of pathogens and beneficial mycoparasites.
Comparisons among levels of (A) Bacterial pathogen, (B) Oomycete pathogen, (C) Fungal pathogen, (D) Beneficial mycoparasite, (E) *Fusarium* all and (F) Sclerotial germination. For each level of moderator, values to the right of the CI line with negative effective size are percent pathogen suppression and with positive effect size are percent of promotion. Number of studies reporting data for each level of moderator is given in parentheses. The moderator level was significantly different from zero if $p$-value $\leq 0.05$. Values below panel titles to the left are $I^2$ (percentage of heterogeneity due to true variation among moderator levels) and $P_{hetero}$ (test of the null hypothesis, that all studies share a common effect size when it is greater than 0.1) for each moderator.

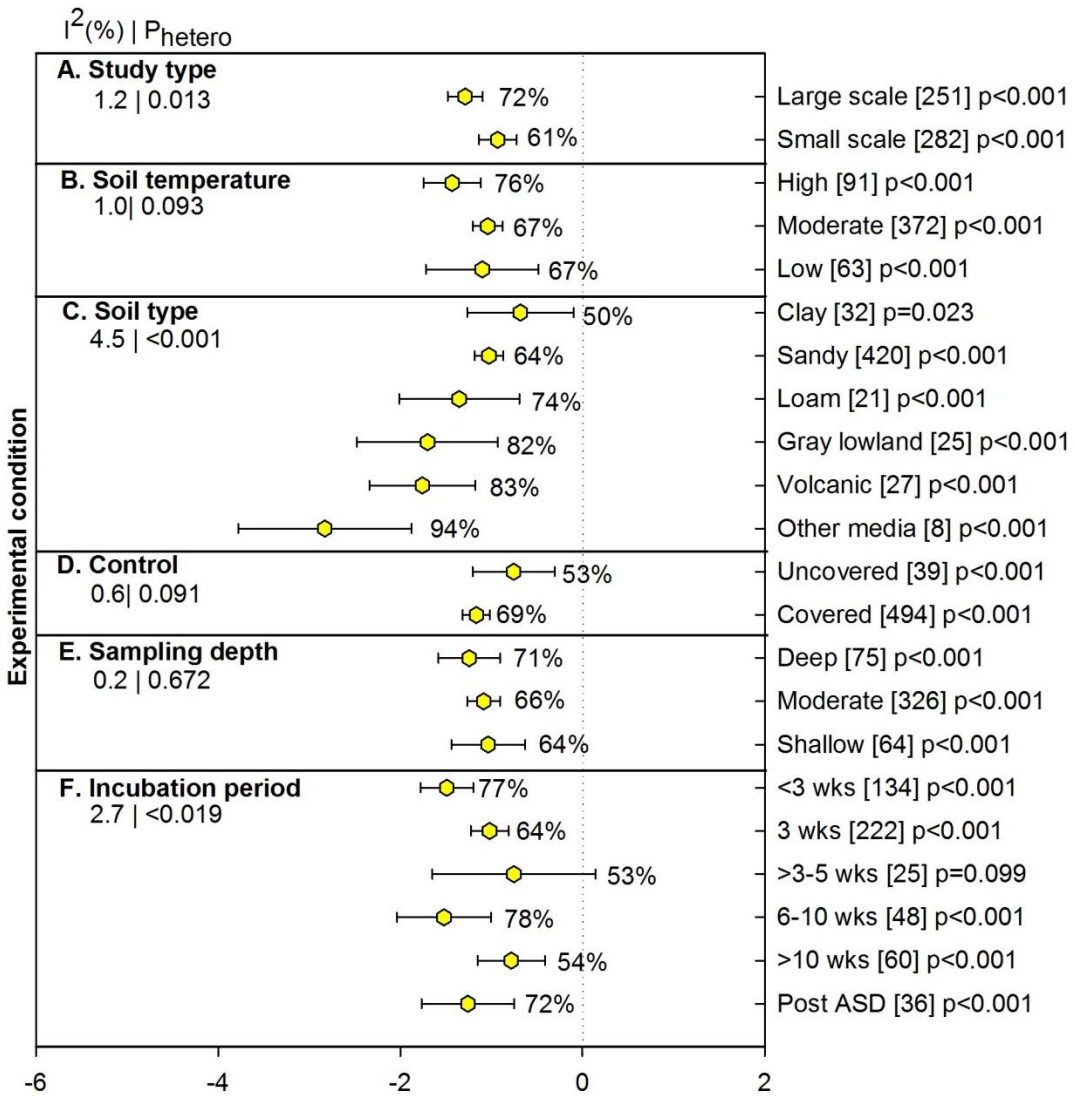


Figure 1-4. Weighted summary effect sizes (*ln*R) and 95% confidence intervals (CIs) for ASD effect on pathogen suppression under various experimental conditions. Comparisons among levels of (A) Study type, (B) Soil temperature, (C) Soil type, (D) Control, (E) Sampling depth and (F) Incubation period. For each level of moderator, values to the right of the CI line with negative effective size are percent pathogen suppression and with positive effect size are percent of promotion. Number of studies reporting data for each level of moderator is given in parentheses. The moderator level was significantly different from zero if $p$-value $\leq 0.05$. Values below panel titles to the left are $I^2$ (percentage of heterogeneity due to true variation among moderator levels) and $P_{hetero}$ (test of the null hypothesis, that all studies share a common effect size when it is greater than 0.1) for each moderator.

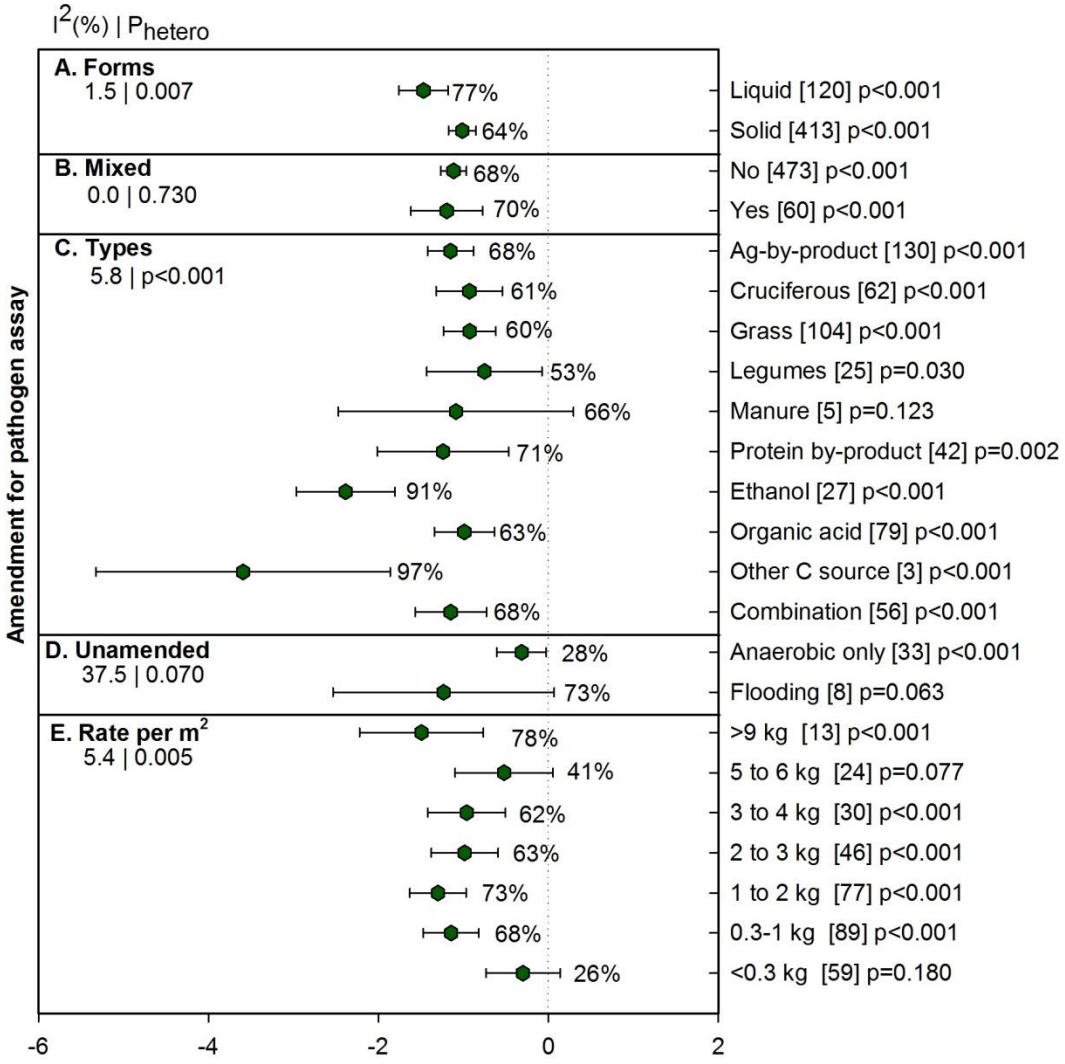


Figure 1-5. Weighted summary effect sizes (*lnR*) and 95% confidence intervals (CIs) for ASD amendment effect on pathogen suppression.
Comparisons among levels of (A) Forms, (B) Mixed, (C) Types, (D) Non-amended and (E) Rate per $m^2$. For each level of moderator, values to the right of the CI line with negative effective size are percent pathogen suppression and with positive effect size are percent of promotion. Number of studies reporting data for each level of moderator is given in parentheses. The moderator level was significantly different from zero if $p$-value $\leq 0.05$. Values below panel titles to the left are $I^2$ (percentage of heterogeneity due to true variation among moderator levels) and $P_{hetero}$ (test of the null hypothesis, that all studies share a common effect size when it is greater than 0.1) for each moderator.

Figure 1-6. Weighted summary effect sizes (*lnR*) and 95% confidence intervals (CIs) for ASD effect on nematode suppression.

Comparisons among levels of (A) Plant parasitic nematode (Other = *Heterodera*, *Trichodorus* and *Tylenchorynchus*), (B) Study type, (C) Soil temperature, (D) Soil type, (E) Control, (F) Sampling depth, (G) Incubation period, (H) Forms, (I) Mixed, (J) Types and (K) Rate per $m^2$. For each level of moderators, values to the right of the CI line indicate percent changes induced by ASD in raw terms: negative values represent suppression or reduction, positive values represent promotion. Number of studies reporting data for each level of moderator is given in parentheses. The moderator level was considered significantly different from zero if its *p*-value $\leq$ 0.05. Values below panel titles to the left are $I^2$ (percentage of heterogeneity due to true variation among moderator levels) and $P_{hetero}$ (test of the null hypothesis, that all studies share a common effect size when it is greater than 0.1) for each moderator.

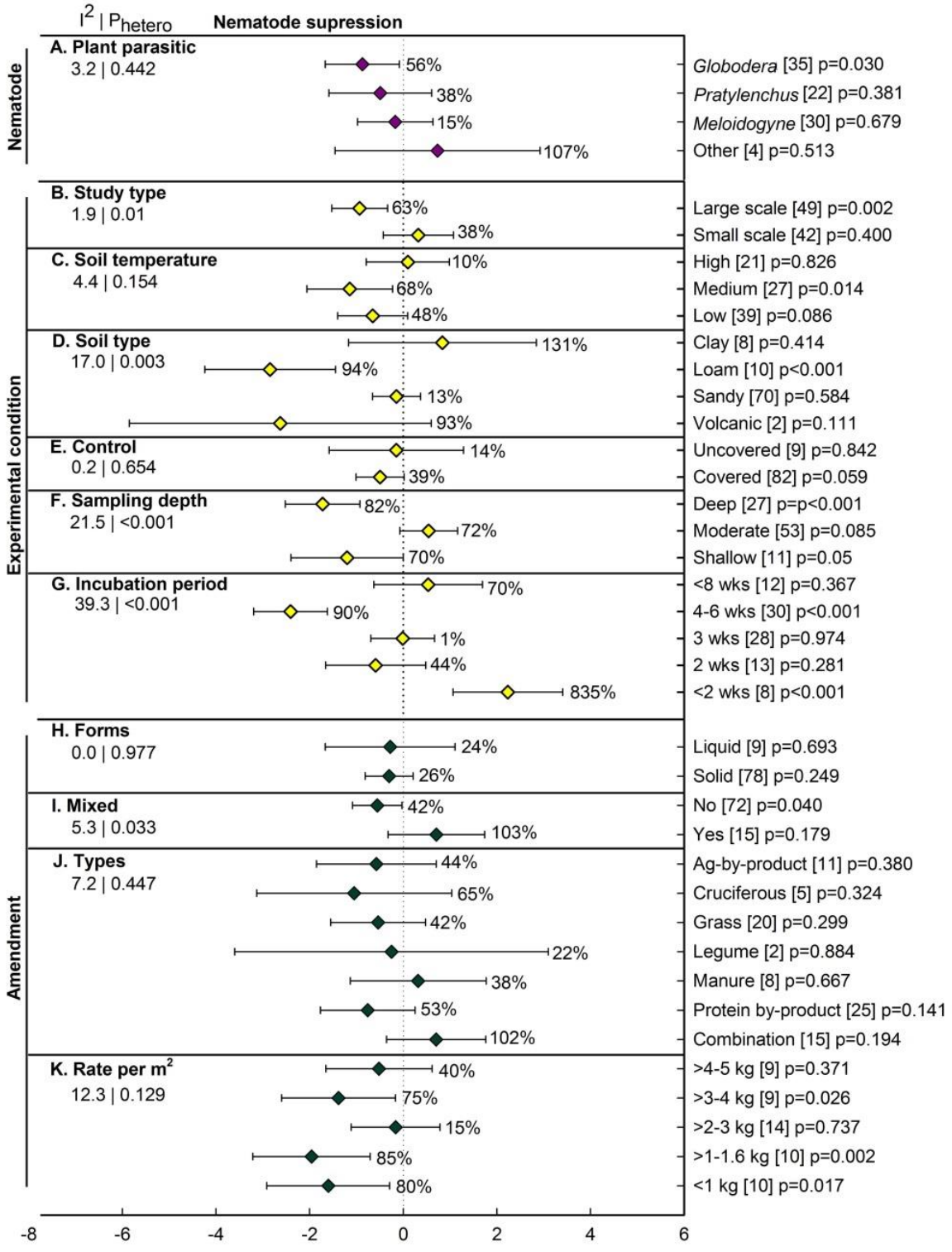


Figure 1-6. contd.

Figure 1-7. Weighted summary effect sizes (*lnR*) and 95% confidence intervals (CIs) for ASD effect on weed suppression.
Comparisons among levels of (A) Weed type, (B) Study type, (C) Soil temperature, (D) Control, (E) Sampling depth, (F) Incubation period, (G) Forms, (H) Mixed, (I) Types and (J) Rate per $m^2$. For each level of moderator, values to the right of the CI line with negative effective size are percent weed suppression and with positive effect size are percent of promotion. Number of studies reporting data for each level of moderator is given in parentheses. The moderator level was significantly different from zero if $p$-value $\leq 0.05$. Values below panel titles to the left are $I^2$ (percentage of heterogeneity due to true variation among moderator levels) and $P_{hetero}$ (test of the null hypothesis, that all studies share a common effect size when it is greater than 0.1) for each moderator.

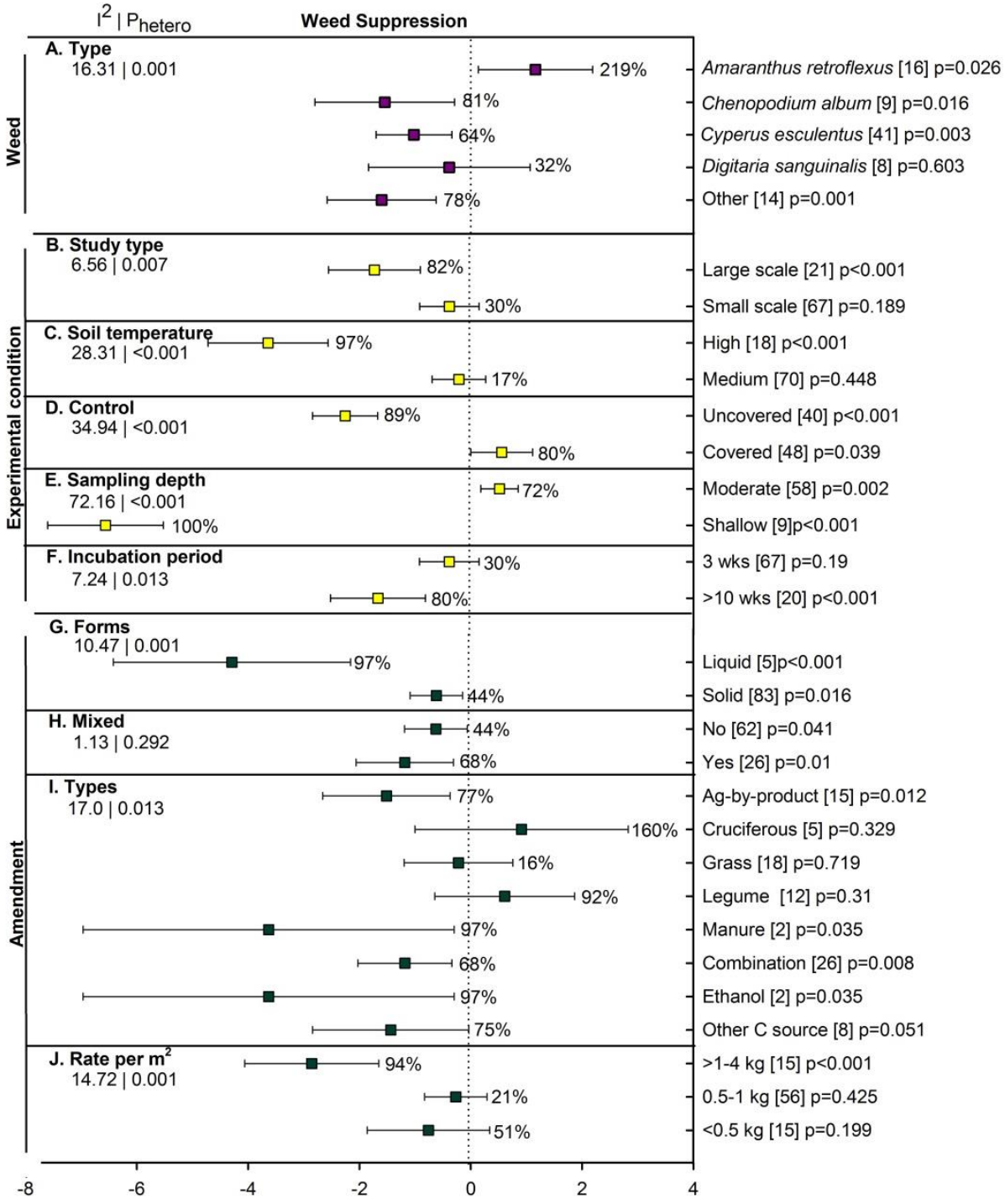


Figure 1-7. contd

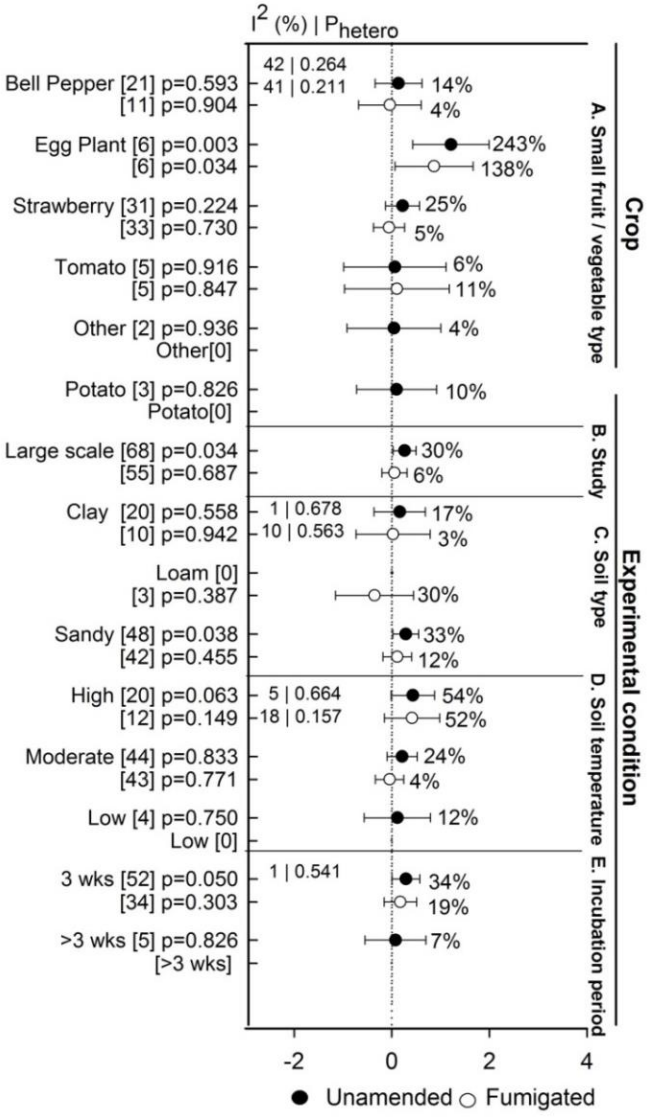


Figure 1-8. Weighted summary effect sizes (*lnR*) and 95% confidence intervals (CIs) for ASD effect on yield response.
Comparisons among levels of (A) Crop type, (B) Study type, (C) Soil type, (D) Soil temperature and (E) Incubation period. For each level of moderator, values to the right of the CI line with negative effective size are percent yield decrease and with positive effect size are percent of yield increment. Number of studies reporting data for each level of moderator is given in parentheses. The moderator level was significantly different from zero if $p$-value $\leq 0.05$. Values below panel titles to the left are $I^2$ (percentage of heterogeneity due to true variation among moderator levels) and $P_{hetero}$ (test of the null hypothesis, that all studies share a common effect size when it is greater than 0.1) for each moderator. Closed symbols (●) denote ASD compared with non-amended untreated control; open symbols denote (○) ASD compared with fumigated control.

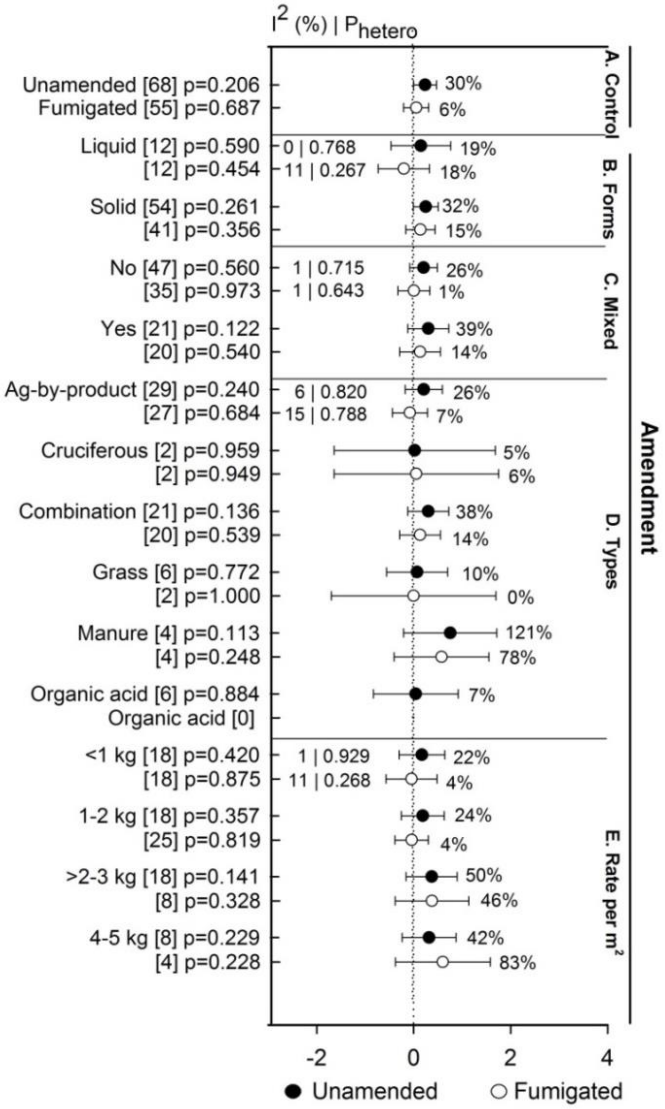


Figure 1-9. Weighted summary effect sizes (*lnR*) and 95% confidence intervals (CIs) for ASD amendment effect on yield response.
Comparisons among levels of (A) Control, (B) Forms, (C) Mixed, (D) Types and (E) Rate per $m^2$. For each level of moderator, values to the right of the CI line with negative effective size are percent yield decrease and with positive effect size are percent of yield increment. Number of studies reporting data for each level of moderator is given in parentheses. The moderator level was significantly different from zero if $p$-value $\leq 0.05$. Values below panel titles are $I^2$ (percentage of heterogeneity due to true variation among moderator levels) and $P_{hetero}$ (test of the null hypothesis, that all studies share a common effect size when it is greater than 0.1) for each moderator. Closed symbols (●) denote ASD compared with non-amended untreated control; open symbols (○) denote ASD compared with fumigated control.

# Chapter 2

## Effect of anaerobic soil disinfestation amendment C:N ratio on tuber germination, growth and reproduction of yellow nutsedge (*Cyperus esculentus*) and organic acid production

A version of this chapter is a manuscript in preparation for *Weed Science by* Utsala Shrestha, Erin N. Rosskopf and David M. Butler.

My primary contributions to this manuscript include experimental setup, data collection and analysis, results interpretation and writing. Dr. Rosskopf contributed constructive feedback to the article.

## Abstract

Anaerobic soil disinfestation (ASD) is a cultural technique to manage key pests especially soilborne pathogens in a specialty crops using organic amendments, saturating and covering the soil for at least a three-week period. ASD can be economic to growers if weed propagules survival can be decreased with suitable organic amendments. Replicated growth chamber and greenhouse studies were conducted in spring 2013 using two organic amendments, dry molasses and wheat bran adjusted to four C:N ratios 10:1, 20:1, 30:1 and 40:1 ( 4mg C $g^{-1}$ of soil) with a non-amended control to evaluate ASD treatment impact on germination and growth of introduced yellow nutsedge tubers. There was no interaction of C amendments and C:N ratios observed for cumulative anaerobic condition, pH, soil N, nutsedge growth and tuber production except for soil P. Mean cumulative anaerobic condition recorded for all treated pots was 58% higher than in the control. We did not see any difference between dry molasses and wheat bran as ASD amendments for cumulative anaerobic condition, soil pH, soil total nitrogen and carbon to nitrogen ratio. Among the C:N ratio treatments, C:N 10:1 showed significantly lowest soil pH (6.12) and soil C:N ratio (10.3) but highest total soil inorganic nitrogen (59.8 mg N $kg^{-1}$ soil) and inorganic phosphorus (28 mg P $kg^{-1}$ soil) than the control and other C:N treatments (except soil P). Regarding buried nutsedge tubers, mean non-germinated tubers recovered from pots were higher for wheat bran (86%) than dry molasses (66%) and production of tubers from germinated tubers were 44% more in dry molasses than wheat bran. Nevertheless, lowest non-germinated tubers (23%) and increased in tubers production (40-60%) were obtained from control pots. Tubers buried at 15-cm depth produced a greater number of large tubers (79%) than these buried at 5-cm depth at all treatments. The wheat bran amended treatment successfully reduced the shoot and root dry biomass compared to the control while dry molasses enhanced the shoot biomass production. Among C:N ratios, germinated tubers and the number of both large and small-sized tubers were lowest for C:N ratios 10:1, however, shoot dry biomass increased. Results from this study indicate that while weed eradication using ASD technique was not observed, both wheat bran and dry molasses used as ASD amendments resulted in higher tuber mortality with lowest new tuber production than the control. Wheat bran at a lower C:N ratio significantly reduced tuber germination and growth, hence emphasizing the importance of

amendment properties along with soil properties and nutrients for nutsedge tuber growth and production during ASD treatment.

**Keywords**: Anaerobic soil disinfestation, C:N ratio, dry molasses, organic acid, organic amendments, tuber, wheat bran, yellow nutsedge

# 1. Introduction

Yellow nutsedge is a noxious weed that competes with crops for light, soil nutrients, soil moisture (Volz, 1977;Keeley and Thullen, 1978;Keeley, 1987;Morales-Payan et al., 2003a), and can adversely affect crop plants by producing allelopathic compounds (Drost and Doll, 1980;Westendorff et al., 2013) and by serving as a host of fungal plant pathogens and nematodes (Miller and Dittmar, 2014). Yellow nutsedge can be a problem if rhizomes are present in the field (Stoller et al., 1972; Stoller and Sweet, 1987; Schippers et al., 1993), but a major concern with yellow nutsedge control is its ability to produce large numbers of tubers (Anderson, 1999). In yellow nutsedge, tubers are overwintering structures that can remain viable in the soil under extreme climatic situations (Bendixen and Nandihalli, 1987) and a single plant can produce hundreds to thousands of tubers per square meter (Tumbleson and Kommedahl, 1961) in a single season. Morales-Payan et al. (2003b) buried 25 tubers/$m^2$ in a plastic mulched tomato field, which produced 2150 tubers/$m^2$ in a single season showing potential of a single tuber to produce 86 additional tubers. Although, the number of tubers produced per unit tuber buried for the same time period decreased drastically when initial buried tuber density increased from 25 to 50 and 100 tubers per square meter, tuber production often results in high economic loss. For example, interference by these tubers during early growth stages can cause 45-50% of marketable yield loss in tomato and pepper (Morales-Payan et al., 2003b; Bangarwa et al., 2011). Further, tubers production in tilled soil can increase by 3 folds as compared to no-tilled plots (Johnson et al., 2007).

Historically, the broad-spectrum fumigant, methyl bromide in combination with chloropicrin was effective in minimizing nutsedge interference but the phase-out of the use of this fumigant has necessitated new strategies to control yellow nutsedge (Rosskopf et al., 2005). Alternative fumigant approaches to control yellow nutsedge tuber production are available, such as soil-applied herbicides (Banks, 1983), drip applied herbicides ( halosulfuron, imazosulfuron, and trifloxysulfuron) (Dittmar et al., 2012a; Monday, 2014) and many other chemicals. However, under plasticulture systems, herbicide application is inconsistent, requires higher doses and more frequent application (Dittmar et al., 2012a; Dittmar et al., 2012b)

Herbicides such as naproamide and trifluralin were inconsistent and ineffective at controlling nutsedge, while registered herbicides like fomesafen, s-metachlor, imazosulfurom failed to achieve season-long control of nutsedge when used alone (Miller and Dittmar, 2014). These herbicides also need pre-mulching and post-planting application, increasing costs and consuming more time and labor. Devkota et al. (2013) reported high rates of allyl isothiocyanate (AITC) and metam sodium reduced yellow nutsedge tuber density compared to MeBr-treated plots. However, metam sodium and 1, 3-dichloropropene + choloropicrin in tomato trials failed to improve crop yield (EPA 2011). Regarding soil fumigants, Gilreath et al. (2005) tested the efficacy of metam sodium along with 1, 3-dichloropropene, chloropicrin, and a combination of these fumigants, but these approaches also did not meet expectations for control. Along with these fumigants, many other chemical fumigants pose limitations in terms of buffer zone requirements and regulatory contraints (for example, the loss of registration of iodomethane). Further, several studies have reported crop injury due to the application of these chemicals (Dittmar et al., 2012a; Devkota et al., 2013) and are obviously inappropriate for organic or chemical-free production systems.

Fewer options are available for specialty crops in organic production systems. Cultural controls such as hand weeding, and fallow tillage often require more labor and limit the growing season, respectively. Mechanical weeding may also spread rhizomes, increasing weed distribution (Keeling et al., 1990). Solarization with or without organic materials is found to be an effective cultural practice to control nutsedge but requires temperatures lethal to tuber survival (Chellemi et al., 1997; Johnson et al., 2007). Organic mulches (Chen et al., 2013) and biological control practices (for e.g. *Bactra verutana* , *Dactylaria higginsii* and *Puccinia canaliculata*) can also be options, but the effects of these organisms on tuber growth have not been tested (Keeley et al., 1970; Charudattan, 2000; Morales-Payan et al., 2005; Riemens et al., 2008; Shabana et al., 2010) and still need in-depth studies for commercial production (Evans, 1995; Li et al., 2003).

In plasticulture systems, a biofumigation study showed 50% reduction in tuber density, but this did not reduce nutsedge competition (Bangarwa et al., 2011; 2012). Anaerobic soil disinfestation (ASD), which was initially designed to control soilborne pathogens (Blok et al., 2000; Shinmura, 2004; Shennan et al., 2014) has been also studied for effectiveness against weed propagules and yellow nutsedge tubers (Messiha et al., 2007; Muramoto et al., 2008; Butler et al., 2012b;

McCarty, 2012; Rosskopf et al., 2014a). This practice involves the incorporation of an organic or carbon (C) amendment to supply a labile C source to microbes in order to create anaerobic conditions under plastic (polyethylene) - mulched soils. Organic acids (Momma et al., 2006) and reduced forms of iron and manganese (Momma et al., 2011) have been reported as major control mechanisms in ASD. In the case of yellow nutsedge tubers, different C amendments especially cover crops with C rate ranging from 0.5-2.5 mg C $g^{-1}$ soil and C:N ratios ranging from 14:1 to 42:1 have been shown to reduce germination percentages compared to untreated controls (Butler et al., 2012a; McCarty, 2012). However, large variation in suppression of tuber germination (35 to 70%) indicates that the practice may need optimization with characterization of amendment C:N ratios to control weed germination, growth and reproduction. The appropriate C:N ratio is necessary for subsequent crop performance, not only under aerobic conditions (Rodriguez-Kabana et al., 1987) but also under anaerobic conditions of ASD (Butler et al., 2014b). Moreover, the C:N ratio is known to be important for shifts in microbial population structure and decomposition of organic matter (Akhtar, 2000; Högberg et al., 2007; Wan et al., 2015). Previous studies on ASD microbial community changes investigated bacterial populations (Momma et al., 2010; Mowlick et al., 2012; Mowlick et al., 2013a; Hong et al., 2014; Mowlick et al., 2014) and fungal communities (Mazzola et al., 2012; Runia et al., 2014; Streminska et al., 2014) showing biocidal effects on pathogens. Beneficial mycoparasites (*Trichoderma*) with antagonistic activity against pathogens have also been isolated (Shrestha et al., 2013; Rosskopf et al., 2014b) showing potential biological control effects associated with ASD treatment. However, it is unknown how microorganism response to C:N ratio could impact tuber germination at different amendment rates, or as amendment types and rates are changed, thus affecting size and structure of the soil microbiome (Wan et al., 2015). Assuming enhanced microbial activity and organic acid production during ASD could impact nutsedge tuber germination, various C:N ratios were examined for their effectiveness in decreasing nutsedge tuber survival and reproduction. For this, a growth chamber study was conducted with dry molasses and wheat bran as ASD C amendments at four C:N ratios to evaluate the impact on tuber germination and growth of yellow nutsedge and organic acid production.

# 2. Materials and Methods

### *2.1 Experimental setup*

Experiments were arranged in an environmentally-controlled growth chamber in spring 2013, in which the temperature was maintained at 25°C (14 h day) and 15°C (10 h night) to simulate soil temperature regimes that represent the relevant production regions during spring in Tennessee (McCarty et al., 2014), as well as many other production regions where ASD is under investigation (Shennan et al., 2014). Soil from the 'Ap' horizon from the University of Tennessee's Organic Crops Unit, Knoxville, TN (previously planted with maize) and fine sand were collected and sieved (<10 mm) to remove organic debris. The soil type is characterized as a Dewey silt loam (fine, kaolinitic, thermic, Typic Paleudult) and at collection had a total carbon (0.94%) and pH of 6.8. Treatment factors included two types of soil C amendments; dry molasses (molasses) or wheat bran supplemented with either high nitrogen (soybean meal) or low nitrogen (corn starch) C amendments to achieve four levels of amendment C:N ratios (10:1, 20:1, 30:1, and 40:1; Table 1). A non-amended, untreated control treatment was also included. The total rate of added C in each amendment mixture was maintained at 4 mg C $g^{-1}$ of soil. The amendment nutrient analysis was done by Agricultural and Environmental services and Laboratories, University of Georgia. The experimental design was a completely randomized design with 4 replications, and the experiment was repeated. The relative biological availability of C in amendments, soil, and sand was assessed by determining cold water extractable C and hot water extractable C with modification of the procedure described by Ghani et al. (2003). Briefly, 2 g of C amendment samples or 4 g of soil or sand samples were extracted in 40 mL of deionized water for 30 min on rotational shaker at 20°C for cold-water extraction. For hot water extraction, 40 mL of deionized water was added to sediments obtained after cold-water extraction and incubated in a water bath at 80°C for 16 h before centrifugation to obtain extract. Total organic C in water extracts was measured by acidification and sparging method to eliminate inorganic carbon using a total organic carbon analyzer (TOC-VCPH model, Shimadzu, Kyoto, Japan).

### *2.2 Experimental procedure*

An equal amount of sand and soil containing C amendments was mixed by hand and used to fill 2,600-$cm^3$ pots (12-cm diameter, 23-cm height). Six yellow nutsedge tubers were buried in each pot, three at 5-cm, and three at 15-cm. Pots were saturated with tap water to fill soil pore space. Oxidation-reduction potential (ORP) electrodes and temperature-moisture sensors (Combination ORP Electrode, Sensorex Corp., Garden Grove, CA, USA and 5TM Soil Moisture Probe, Decagon Devices, Pullman, WA, USA) were inserted at 10 to 15-cm depth and immediately covered with black polyethylene mulch (0.03175 mm), which was secured with heavy-duty rubber bands, and incubated in the growth chamber for three weeks. The first trial treatment period was 15 March to 6 April 2013 and the second trial treatment period was 8 to 29 April 2013.

### *2.3 Cumulative redox potential*

Soil temperature, redox potential, and soil moisture were continually monitored and recorded hourly during treatment using ORP electrodes and an automatic data logging system (CR1000 with AM 16/32 multiplexers, Campbell Scientific, Logan, UT) over the three-week ASD treatment. Due to a limited number of temperature probes available at the time of the study, soil temperature was monitored only in 20 randomly selected pots while ensuring each treatment was monitored in two replicate pots. Cumulative soil anaerobic activity was calculated as described in Butler et al. (2012b). The data logging system provided raw soil redox potential (RP) values on hourly basis and critical redox potential (CRP) was calculated as (CRP = 595mV – (60mV*soil pH ). The absolute value of the difference between CRP and RP value was determined for each RP. Cumulative soil anaerobic activity was then calculated by summing absolute values over the three-week ASD treatment period.

### *2.4 Soil properties*

At the end of ASD treatment, probes were removed, and a soil sample (~80 g wet wt.) was collected using a clean plastic spoon from 0 to 8-cm depth of each pot. Subsamples were oven-dried (105°C for 48 h) to determine gravimetric moisture content and the remaining sample was

air-dried and sieved (<2 mm). Soil pH was determined on air-dried samples in 0.01 M $CaCl_2$ (1:2) using a pH electrode (Orion 3-Star Plus pH Benchtop Meter, Thermo Scientific, Waltham, MA, USA) and are reported as soil pH determined in deionized water by adding 0.6. For inorganic soil N and total soil N and C, 5-g of air-dried, 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 $NO_3$-N + $NO_2$-N using a microplate spectrophotometer (Powerwave XS, Biotek, Winooski, VT, USA) as described by (Sims et al., 1995). Air-dried, sieved (<2 mm) and pulverized soil samples were analyzed for total N and total C by flash combustion (Flash EA 1112 NC Soil Analyzer, Thermo Scientific). For extractable soil P, 5-g of air-dried, sieved (<2 mm) soil was extracted with Mehlich I extractant for 5 min (Mehlich, 1953), centrifuged, filtered (Whatman 42) and extractant was determined using a malachite green microplate method (D'Angelo et al., 2001).

### *2.5 Yellow nutsedge survival assessment*

After ASD treatment, pots were incubated in the growth chamber and hand irrigated with tap water supplied regularly throughout the growing period. Each pot was fertilized with 0.2 g N (as blood meal) after ASD termination. After an 8-week period following soil treatment, yellow nutsedge tuber mortality and biomass were assessed. Nutsedge roots with tubers were washed, numbers of newly formed tubers were categorized as small (<0.5-cm ) and large (>0.5-cm) based on average dry diameter and counted. Any non-germinating nutsedge tubers were recovered from the soil and assayed for germination potential. The visual inspection of viable tubers was completed by dissecting the tubers and then examining the internal color and condition of tubers (Stoller and Wax, 1973). Ratings were assigned based on a 1 to 5 scale, 1 being firm and undecomposed and 5 being completely rotten/decomposed with outer covering only. The cream colored tissue of tubers with solid texture are viable tubers (Banks, 1983). Both root (including tubers) and shoot biomass of plants were recorded after oven drying at 65°C for 48 h.

### *2.6 Organic acid assay*

Organic acid production using carbon amendments with four C:N ratio during 7 day and 14 day time course were carried out in the growth chamber separately in 9-cm tall pots. A potting

mixture was prepared similarly as described above. Three previously soaked yellow nutsedge tubers were buried at 5-cm depth with IRIS tubes in each pot, which were irrigated until water from pot's bottom is discharged and then covered with polythene. Experiment design was completely randomized design with three replications. On the $7^{th}$ day and the $14^{th}$ day, soil from pots were mixed well and 20-30 cc of moist soil was placed in 50 ml centrifuge tubes to which 20 ml of 1 M KCl was added. Tubes were shaken for 30 min, at 180 rpm and centrifuged for 30 min, at 3500 rpm at room temperature. Supernatant (~ 10ml) was collected using 0.45 um membrane filter, stored, and refrigerated in 20ml scintillation vials until analysis.

Accumulated organic acids were analyzed using a high-performance gas chromatograph (Shimadzu GC-2010, Shimadzu, Kyoto) equipped with (Carbopack, B-DA/4% Carbowas 20M columns). The liquid carrier consisted of helium, 24 ml/min. The 0.25 ml of internal standard 50 ppms of trimethylacetic acid in 0.03 M oxalis acid with ~ 25% meta phosphoric acid was mixed with 1 ml of sample extract and 1 ul of supernatant was injected into column for analysis. We analyzed acetic, butyric, isobutyric, methyl butyric, propionic, valeric, and isovaleric acids.

### *2.7 Statistical analysis*

Data were analyzed with Mixed Model Analysis of Variance (MMAOV) macro (Saxton and Auge, 2014), and Fisher's P-LSD at $p$=0.05 using SAS version 9.3 (SAS Institute Inc., Cary, NC). Repeated trials were treated as a random factor, whereas C amendments and C:N ratios were treated as fixed factors for randomized completely block design factorial analysis. Data were analyzed separately by C amendment to compare treatments with untreated control and also by C:N ratio to compare with untreated control. Data were checked for normality and homogeneity of variances. The data were rank transformed for non-normal data and unequal variances separately and untransformed means and standard error of mean are reported.

## 3. Results and Discussion

### *3.1 Soil temperature and moisture*

Soil temperature at 15-cm depth showed overall average temperature of 25°C. The mean soil temperature generally ranged from a low of 15 to a high of 30°C during the treatment period and

was consistent in both trials. Soil moisture content was similar among carbon amended pots and non-amended pots in both trials. Average gravimetric soil moisture at the beginning of trials was 0.08 g $g^{-1}$ and at the end of ASD treatment soil moisture content increased to 0.23 g $g^{-1}$. Relative increase in moisture in all treatments (65%) and fluctuating temperature in our experiment should have significant effect on tuber germination and growth. Yellow nutsedge is reportedly found in abundance in flooded soil (Ransom et al., 2009) and presence of moisture helps to break the dormancy of buds. Usually, diurnal temperature fluctuation below 10°C creates problems under plasticulture systems as nutsedge tubers actively sprout around 15 to 23°C (Stoller and Woolley, 1983; Stoller and Sweet, 1987; Daugovish and Mochizuki, 2010) and nutsedge shoots perforate plastic mulch, negatively influencing the efficacy of any disinfestation process under plastic covering (Chase et al., 1999). During the three-week treatment period, some shoot emergence through plastic was observed in all covered treatments (31 to 36% emergence). The perforation is due to the sharp leaf tip emerging from germinated tubers placed in saturated soil (Li et al., 2001) and some air trapped inside plastic. The 13-hour photoperiod maintained in the growth chamber may have resulted in enhanced rapid rhizome differentiation (Stoller and Woolley, 1983).

### *3.2 Cumulative anaerobic activity and soil pH*

There was no interaction between C amendments and C:N ratios observed for cumulative anaerobic activity and soil pH. The cumulative anaerobic activity significantly differed between amended pots and the control pots with the lowest cumulative soil anaerobic activity observed in the control (46,777 mV h; $p=0.01$). Among C amendments and C:N ratios, generation of anaerobic conditions was statistically similar with average cumulative anaerobic activity of 110,175 mV h (Figure 2-1). Soil pH did not differ among C amendment treatments and the control at treatment termination. However, soil pH differed ($p<0.01$) among C:N ratios with the lowest numerical value in C:N ratio of 10:1 (pH 6.12; Figure 2-2).

Effectiveness of ASD for pathogen control is associated with anaerobic condition and lower pH. As soil continues to deplete oxygen in saturated covered soil due to decomposition of amendment by microbial activity, the anaerobicity is believed to be increased. In this regard high anaerobicity generated in our treated pots which was similar to several ASD studies (Butler et

al., 2012a; Shrestha et al., 2013; McCarty et al., 2014), indicatimg that microbial response to addition of amendments was higher. Treated pots could have stimulated the growth and multiplication of anaerobic bacterial species (Mowlick et al., 2013b) rather than the fungal population (Rosskopf et al., 2014b). Previous studies on ASD soil pH indicated that soil pH decreased due to the release of organic acids from the anaerobic breakdown of added carbon. All our treatments had lower soil pH than initial average soil pH (6.8) which may be due to the production of acetic acid and butyric acid (Momma et al., 2006; Huang et al., 2015) and the organic acid content may be higher for C:N 10:1, although organic acid extraction was not performed. Surprisingly, lower pH and a slightly anaerobic condition in our non-amended control suggest that some C required for microbial activity was already present in the soil. This was revealed from our water extracted C in the soil:sand mixture which showed cold and hot water extracted C rate of 2.76 mg $kg^{-1}$ and 31.49 mg $kg^{-1}$ respectively.

### *3.3 Soil nutrients and C:N ratio*

Total soil inorganic N (i.e., $NH_4$-N, $NO_3$-N, and $NO_2$-N) was significantly affected by treatment ($p$ = 0.001). Throughout this study, following ASD treatment, the lowest mean total soil inorganic N was observed from the C:N ratio of 40:1 (lower than 4 mg N $kg^{-1}$ soil). Total soil inorganic N was primarily comprised of $NO_3$ + $NO_2$-N (76 to 91% of total inorganic N; Table 2) rather than $NH_4$-N (9 to 24%). The non-amended control had the least amount of soil $NH_4$-N. There was no significant interaction between C:N ratios and amendment type for soil nitrogen content. However, there were significant differences in total soil inorganic N among C:N ratio treatments ($p$<0.01; Table 2-2). Soil C:N ratio prior to treatment was (11.7). After termination of ASD, the highest soil C:N ratio was observed for control (12.2) and least for treatment with C:N 10:1 (10.3). It was not surprising that total soil inorganic N was highest for 10:1 (60 mg) and lowest for 40:1 (3.4 mg) given that soil C:N ratio was pre-adjusted. More mineralization of amendments may have occurred at lower C:N ratio releasing high soil $NH_4$-N but the overall soil $NH_4$-N was lower than that reported by Butler et al., (2012a). Nevertheless, our $NO_3$ + $NO_2$-N was fairly higher and total N was comparable to that in a study conducted by McCarty (2014) for C:N 15:1. We assume that soil N being measured on air-dried soil, the nitrification process increased $NO_3$ + $NO_2$-N after ASD treatment assuring beneficial impact of ASD on nitrifying

bacteria (McCarty et al., 2014). Further, there may be microbial immobilization of available soil inorganic N at higher C:N ratio, thus lowering total N.

Unlike soil N, Mehlich1 extractable soil P was significantly higher in wheat bran treatments than molasses treatments and the non-amended control ($p<0.0001$). Among the C:N ratios, the Mehlich-1 extractable soil P ranged from 17 to 28 mg P $kg^{-1}$ soil and the highest values were observed for C:N ratios 10:1 and 20:1. There was a significant interaction of C amendments with C:N ratio for soil P. In treatments with wheat bran, the soil P was significantly higher at C:N ratios 10:1 and 20:1, while soil P did not differ among C:N ratios maintained for molasses which was similar to the study conducted by (Butler et al., 2012a; Butler et al., 2014a). The higher value of Mehlich1 extractable soil P for wheat bran at lower ratio is due to high P content (81 to 112 mg/kg) of amendments. The dry molasses amendment mixture had significantly higher levels of other nutrients besides P (8 to 32 mg $kg^{-1}$of amendments; Table 2-2) compared to the wheat bran mixture.

### *3.4 Tuber germination*

Several studies have confirmed that nutsedge tuber germination is not correlated to anaerobicity; however, after ASD implementation, buried tubers were controlled by the method (Muramoto et al., 2008; Butler et al., 2012a; McCarty, 2012). Moreover, buried tubers at greater depth (15-cm) showed limited germination when compared to tubers deeper in the soil (Muramoto et al., 2008) but, here two different burial depths (5 and15-cm) did not result in differences in germination. At the shallower depth of 5-cm, tuber germination did not differ significantly among C treatments but was higher in the non-amended control (77%; Figure 2-3). The germination of tubers at the 15-cm depth differed significantly among C treatments with lower percentage of germination for wheat bran (29%) and C:N ratio 10:1 resulting in the lowest percentage of germination (33.3%) and highest for the untreated control (81.3%). It was not surprising that our germination percentage for dry molasses (65%) at greater depth was not comparable to the results of (Muramoto et al., 2008; Butler et al., 2012a) as soil temperatures in those studies were higher than those used here.

Nevertheless, the percentage of non-germinated tubers was significantly higher for wheat bran ($p$<0.05; 86%) treatments compared to dry molasses and the control. Visual inspection of non-germinated tubers based on their internal state showed that tubers turned to grey to black (rating = 5) when they are totally decomposed and red, yellow, to brown when they were soft and in various states of decomposition (rating = 4-3); and white (rating = 1) when firm and undecomposed. The mortality of tubers in the wheat bran treatments is attributed to a higher number of rotted tubers (scale of 4) and slightly decomposed tubers (scale of 2) which were significantly greater than those harvested from dry molasses and control treatments ($p$<0.05; Table 4).

### *3.5 Nutsedge growth and reproduction*

Yellow nutsedge growth was significantly affected by C amendment and C:N ratio without any interaction. Dry biomass of shoots and roots was greatly reduced by wheat bran compared to the non-amended control. Between C amendments, the greater shoot and root biomass was recorded for molasses. Among C:N ratios, both shoot and root biomass was lower for C:N ratio of 20:1; however, the difference was not statistically significant for shoot biomass (Figure 2-5, 2-6). The higher root biomass in the non-amended control and C:N 40:1 is in line with the production of new tubers. Though no interaction was observed between C amendment type and C:N ratio on mean tuber production, the number of tubers per pot was significantly higher from the control treatment (33) and the lowest for wheat bran (13). Among total tubers, 79% of tubers were large (>0.5-cm) in size and 21% small (<0.5cm) in size. The number of small tubers was highest for 40:1 and large tubers were highest in the non-amended control. Among C:N ratios, the number of both large and small-sized tubers was lowest for C:N ratios 10:1 and greatest for 40:1 (Figure 2-4). The total tuber production was synchronous with the dry root biomass except C:N 10:1. It was not surprising that both dry root biomass and tuber production were lower at C:N 10:1 ratio as higher soil N content at this ratio promotes vegetative growth by enhancing basal bulb formation rather that tuber formation (Garg et al., 1967; Stoller and Sweet, 1987). Also organic acid, volatile fatty acids (VFA) and other toxic products may have led to biotransformation in the soil, directly influencing tuber production and germination (Huang et al., 2015). Though we maintained N fertility of soil after ASD treatment, only dry molasses enhanced the shoot growth as compared to wheat bran confirming shoot growth is highly dependent on nutrient content of

applied amendments (see Table 2-2) rather than supplemental application of N fertilizer (Ransom et al., 2009; McCarty et al., 2014). Production of acetic acid during amendment decomposition did not seem to impact nutsedge sprouting (Ozores-Hampton et al., 1999). As N availability becomes limited, allocation of nutrients is made to tuber production (Chellemi et al., 2013) which may account for the larger number of tubers seen produced in the higher C:N (>10:1) ratios.

### *3.6 Organic acid assay*

Although ASD is a proven technique for pathogen control, in the case of weeds, particularly with yellow nutsedge, complete eradication may be unattainable. Tubers buried in plastic-covered pots produced 17% more tubers per $m^2$ while amended pots showed only 0.4% increase in tuber production. Although, temperature may be a limiting factor with regard to complete tuber mortality, ASD treatment did significantly reduce tuber density, which can prevent crop yield loss over time. The presence of high total soil inorganic N from amendment incorporation at the lower C:N ratio (<30:1) could increase relative availability for crop uptake, thus reducing fertilizer application, but nutsedge interference with the crop due to high denitrification (Volz, 1977) after ASD treatments could possibly interfere with crop yield. However, field application of the target ratios would enhance understanding of this potential interaction.

At different C:N ratios, acetic, butyric and isobutyric were the primary organic acids observed in soil solution during treatment which coincided with past studies (Momma et al., 2006; Rosskopf et al., 2015). Total organic acids in soil at 7 and 14 days post treatment initiation were highest at an amendment C:N ratio of 40:1 (Figure 2-7). The acid production was significantly higher using dry molasses or wheat bran as ASD amendment during 7 days post treatment than in 14 days post treatment (Figure 2-8). We did not observe any tuber germination or decomposition in amended pots. We believed that our experimental period i.e. 7 days and 14 days was not enough to cause any significant impact on tuber decomposition.

# 4. Conclusion

Overall, the use of wheat bran as an ASD amendment, applied at lower C:N ratio, provided better tuber control than dry molasses. These amendments not only differ in their nutrient content, but also differ in decomposition rate i.e. dry molasses has more sucrose content making it sticky, leachable during treatment application, and results in faster decomposition (Stock, 2008) while wheat bran is more fibrous, absorbing more water and decomposes more slowly (Stevenson et al., 2012). As described earlier, in ASD, microbial decomposition of these amendments, associated with increases in *Clostridia* and *Bacilli* spp. (Mowlick 2012; 2013) plays an important role in the production of VFA and organic acids. The concentration of acids produced during ASD may not be high enough for decomposition of tubers alone, as the concentration of acetic acid needed to effectively kill 1- to 4-week old sprouted nutsedge tubers is 30% (Abouziena et al., 2009). It is more likely that multiple mechanisms are at work. Adebajo (1993) reported that yellow nutsedge tubers contain sucrose and have inhabiting microbes such as yeasts, *Pseudomonas*, and *Bacillus* spp. In the same study, tuber tissue was found to inhibit some microbial growth. Microbial community analysis of tubers is currently underway to ascertain the potential roll of biological degradation of tuber tissue. It is possible that extended ASD treatment might increase weed suppression and this will be investigated in the future.

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# Appendix

Table 2-1. Amount of C amendments for different C:N ratios at 4 mg C $g^{-1}$ of soil for each pot

| Treatments | | | | | | | |
|---|---|---|---|---|---|---|---|
| C amendment | | | | Rate of amendments | | | |
| | ratio | percentage | | g $kg^{-1}$ of soil | | | |
| | C:N | C | N | CN10 | CN20 | CN30 | CN40 |
| Dry molasses | | | | | | | |
| DM | 29.7 | 38.7 | 1.3 | 6.4 | 9.4 | 10.3 | 7.7 |
| Soybean meal | 4.8 | 42.6 | 8.8 | 3.6 | 0.9 | - | - |
| Corn starch | - | 40.3 | 0.0 | - | - | 0.1 | 2.6 |
| Wheat bran | | | | | | | |
| WB | 13.3 | 41.7 | 3.13 | 7.8 | 6.4 | 4.2 | 3.2 |
| Soybean meal | 4.8 | 42.6 | 8.84 | 1.8 | - | - | - |
| Corn starch | - | 40.3 | 0.02 | - | 3.3 | 5.6 | 6.7 |

N.B. C amendments are mixed uniformly with 3 kg of soil: sand for each pot. CN10=C:N ratio 10:1, CN20=C:N ratio 20:1, CN30=C:N ratio 30:1 and CN40=C:N ratio 40:1, Dry molasses (DM), Wheat bran (WB)

Table 2-2. Soil inorganic phosphorus, soil nitrite+nitrate-N ($NO_2$+$NO_3$-N) and ammonium-nitrogen ($NH_4$-N) and total inorganic N as affected by soil amendments and amendment C:N ratios

| Treatment | Soil nutrients * | | | | Soil C:N ratio |
|---|---|---|---|---|---|
| | Inorganic P | $NO_2$ + $NO_3$-N | $NH_4$-N | Total inorganic N | |
| Amendment | mg nutrient $kg^{-1}$soil | | | | |
| Control | 14 ± 0.8 b | 4.7 ± 0 c | 1.4 ± 0.2 b | 6 ± 1.6 b | 12.2 ± 0.1 a |
| DM | 16.2 ± 0.9 b | 19.6 ± 0 b | 2 ± 0.2 a | 21.5 ± 1.2 a | 11.1 ± 0.2 b |
| WB | 28.1 ± 2.2 a | 22.7 ± 0 a | 2.2 ± 0.2 a | 25 ± 1.2 a | 10.7 ± 0.2 b |
| | $p$<0.001 | $p$=0.0309 | $p$=0.001 | $p$<0.001 | $p$<0.001 |
| C:N ratio | | | | | |
| CN10 | 28.3 ± 3.4 a | 57.6 ± 0 a | 2.3 ± 0.2 a | 59.8 ± 1.7 a | 10.3 ± 0.4 d |
| CN20 | 24.9 ± 3 a | 19.3 ± 0 b | 1.9 ± 0.2 a | 21.2 ± 1.7 b | 11.3 ± 0.1 c |
| CN30 | 18.4 ± 1.9 b | 6.4 ± 0 c | 2.1 ± 0.2 a | 8.5 ± 1.7 c | 11.6 ± 0.1 bc |
| CN40 | 17.1 ± 1.5 bc | 1.4 ± 0 d | 2 ± 0.2 a | 3.4 ± 1.7 e | 11.9 ± 0.3 ab |
| Control | 14 ± 0.8 c | 4.7 ± 0 c | 1.4 ± 0.2 b | 6 ± 1.7 d | 12.2 ± 0.1 a |
| | $p$<0.001 | $p$<0.001 | $p$=0.009 | $p$<0.001 | $p$<0.001 |

* Within columns values (mean ± SE) followed by different letters are significantly according to Fisher's Protected LSD test at $p$<0.05.

Table 2-3. Tuber ratings of non-germinated tubers for amendment and C:N ratio treatments

| Treatments | No. of tubers buried in the experiment | No. of non-germinated tubers* | No. of tubers for each rating scale | | | | |
|---|---|---|---|---|---|---|---|
| | | | 5 | 4 | 3 | 2 | 1 |
| Control | 96 | 20 | 11 | 4 | 5 | 0 | 0 |
| | | | Dry molasses | | | | |
| 10:1 | 48 | 25 | 8 | 7 | 7 | 2 | 1 |
| 20:1 | 48 | 18 | 9 | 7 | 1 | 0 | |
| 30:1 | 48 | 7 | 3 | 4 | 0 | 0 | 0 |
| 40:1 | 48 | 13 | 7 | 3 | 3 | 0 | 0 |
| Total | 192 | 63 | 27 | 21 | 11 | 2 | 2 |
| | | | Wheat bran | | | | |
| 10:1 | 48 | 28 | 10 | 5 | 5 | 2 | 6 |
| 20:1 | 48 | 26 | 8 | 7 | 7 | 3 | 1 |
| 30:1 | 48 | 31 | 8 | 14 | 6 | 2 | 1 |
| 40:1 | 48 | 28 | 14 | 6 | 4 | 3 | 1 |
| Total | 192 | 113 | 40 | 32 | 22 | 10 | 9 |
| Total tubers | 480 | 196 | | | | | |

*Rating scale: 5- Completely rotten/decomposed with outer covering only, 4- Soft and decomposed, 3- Slightly soft and decomposed, 2- Firm and slightly decomposed and 1- Firm and undecomposed.

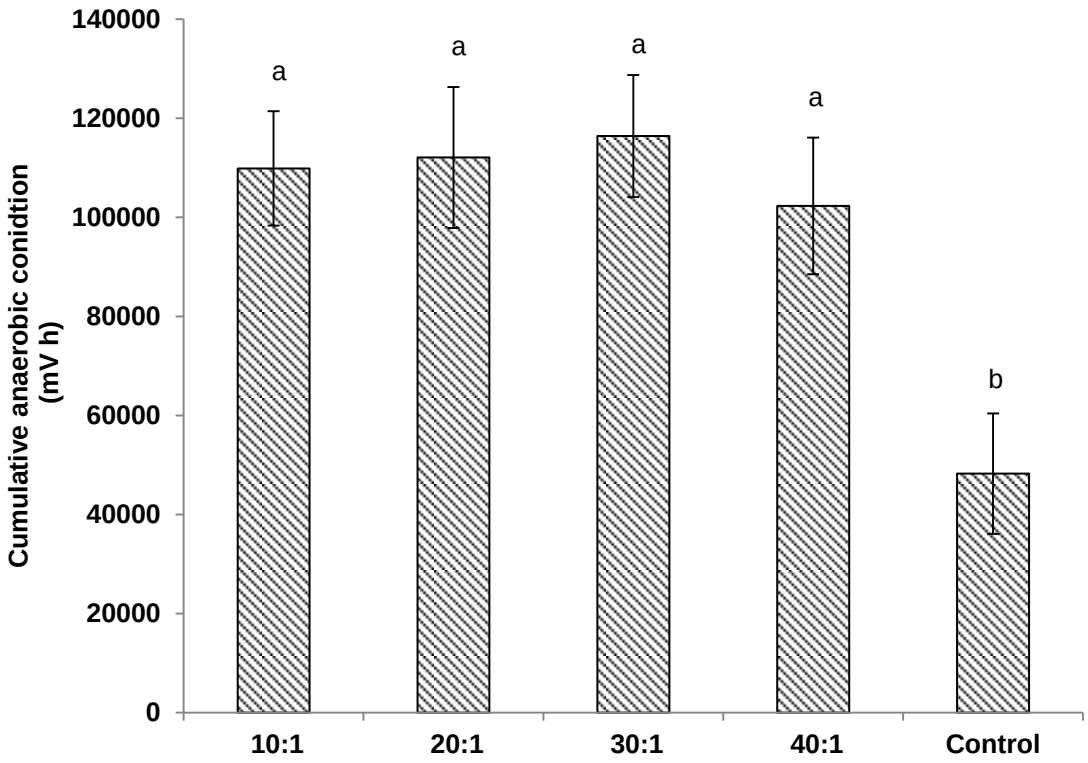


Figure 2-1. Effect of amendment C:N ratio on mean cumulative anaerobic condition during ASD.

Bars indicated by different letters are significantly different, $p<0.05$. Error bars indicate standard error with four replicates. Control=non-amended control, 10:1=C:N ratio 10:1, 20:1=C:N ratio 20:1, 30:1=C:N ratio 30:1 and 40:1=C:N ratio 40:1

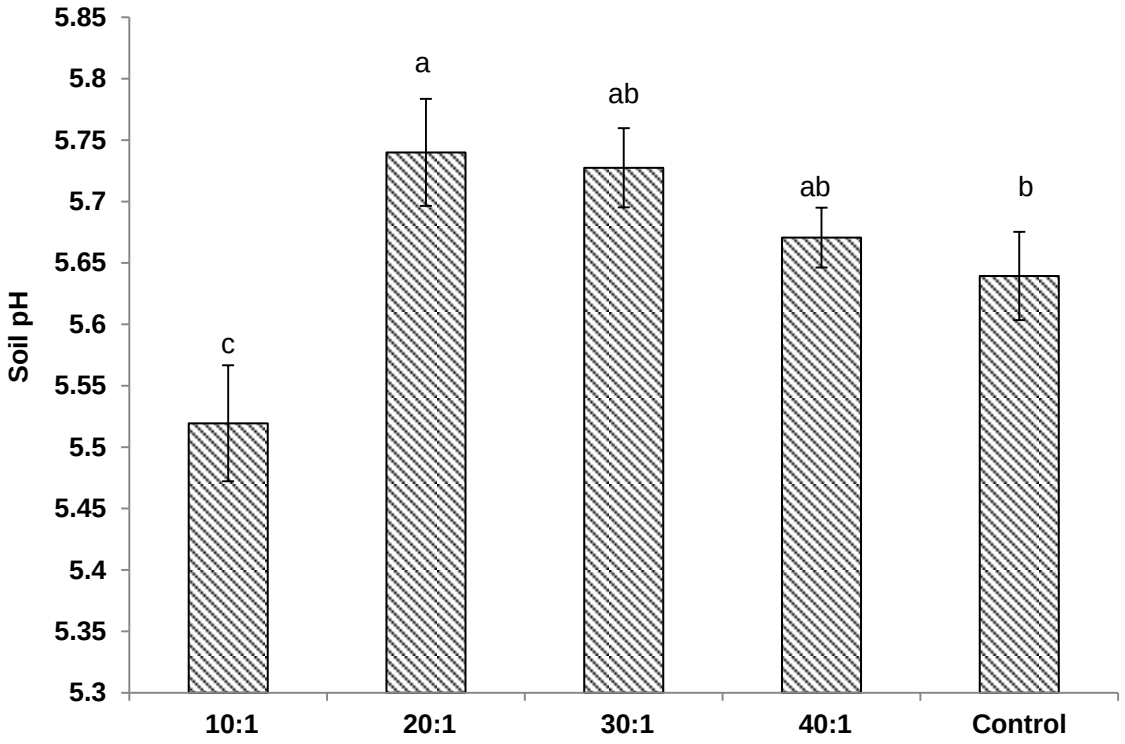


Figure 2-2. Effect of amendment C:N ratio on mean soil pH in $CaCl_2$ at treatment termination. Bars indicated by different letters are significantly different, $p<0.05$. Error bars indicate standard error with four replicates. Control=non-amended control, 10:1=C:N ratio 10:1, 20:1=C:N ratio 20:1, 30:1=C:N ratio 30:1 and 40:1=C:N ratio 40:1

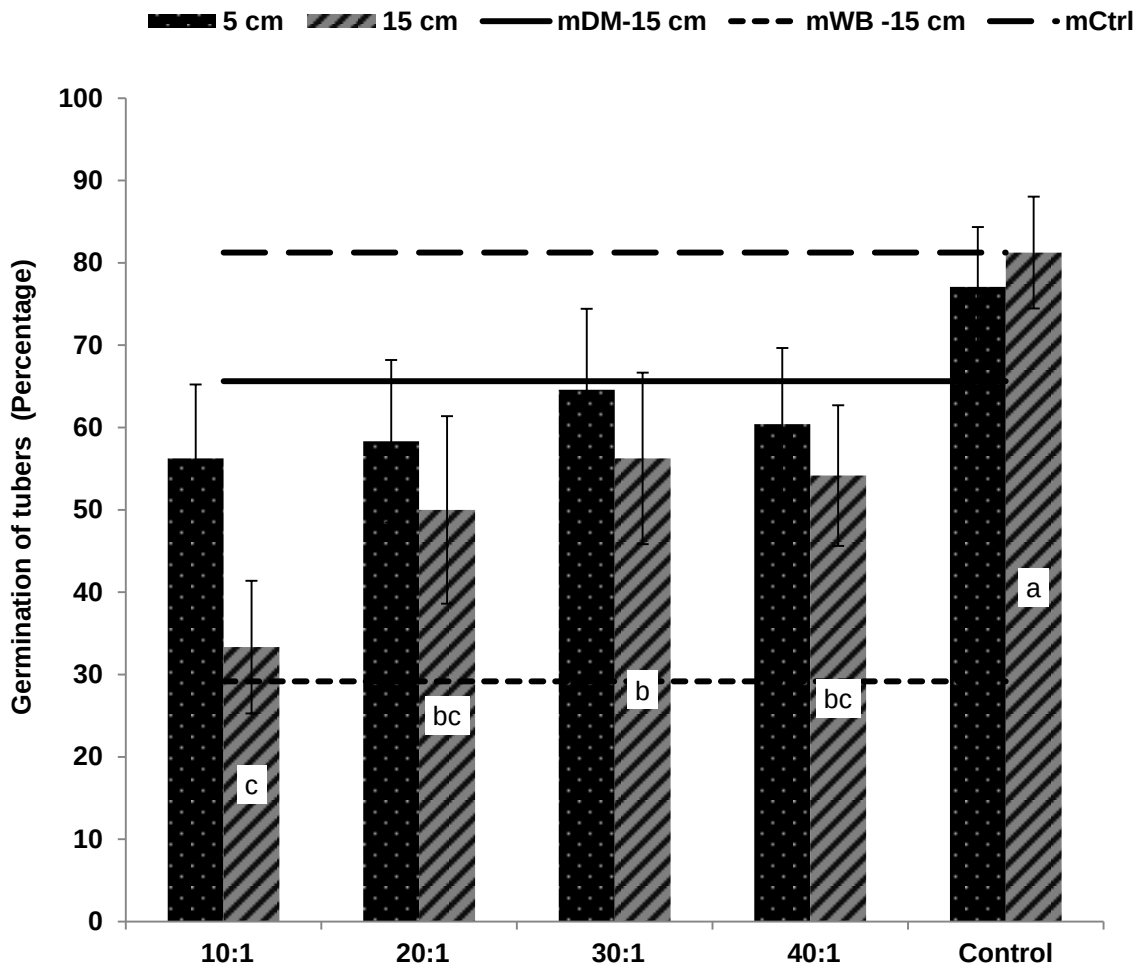


Figure 2-3. Effect of amendment and amendment C:N ratio on mean percentage of tuber germination per pot during ASD.
Bars indicated by different letters are significantly different, $p<0.05$. Error bars indicate standard error with four replicates. Control=non-amended control, 10:1=C:N ratio 10:1, 20:1=C:N ratio 20:1, 30:1=C:N ratio 30:1 and 40:1=C:N ratio 40:1,m=mean, DM=dry molasses, WB=wheat bran.

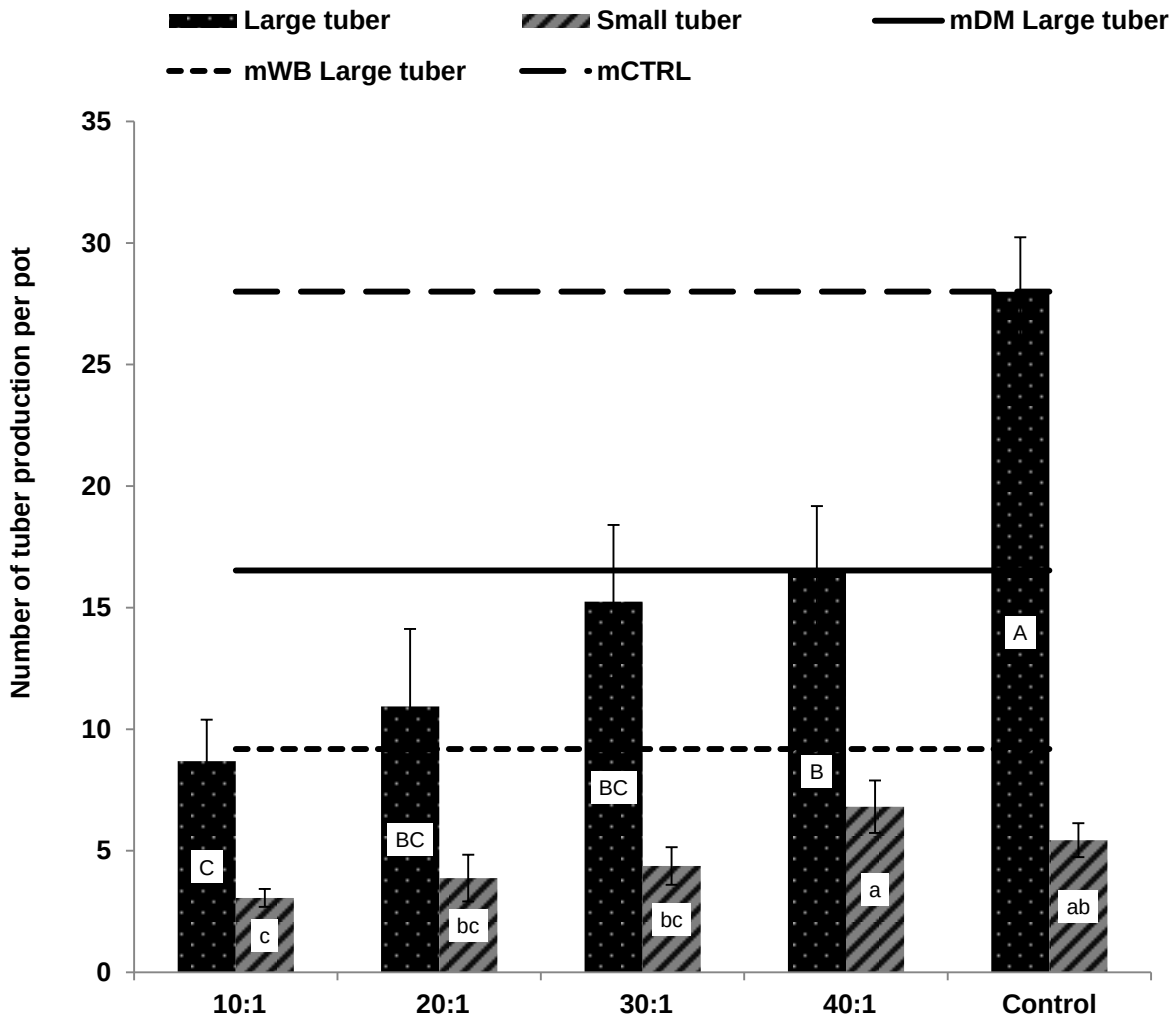


Figure 2-4. Effect of amendment and amendment C:N ratio on mean percentage of number of tuber production per pot during ASD.

Bars indicated by different letters are significantly different, $p<0.05$. Capital letters are used to compare the respective means of large tuber production and small letters are used to compare respective means small tuber production. Error bars indicate standard error with four replicates. Control=non-amended control, 10:1=C:N ratio 10:1, 20:1=C:N ratio 20:1, 30:1=C:N ratio 30:1 and 40:1=C:N ratio 40:1,m=mean, DM=dry molasses, WB=wheat bran.

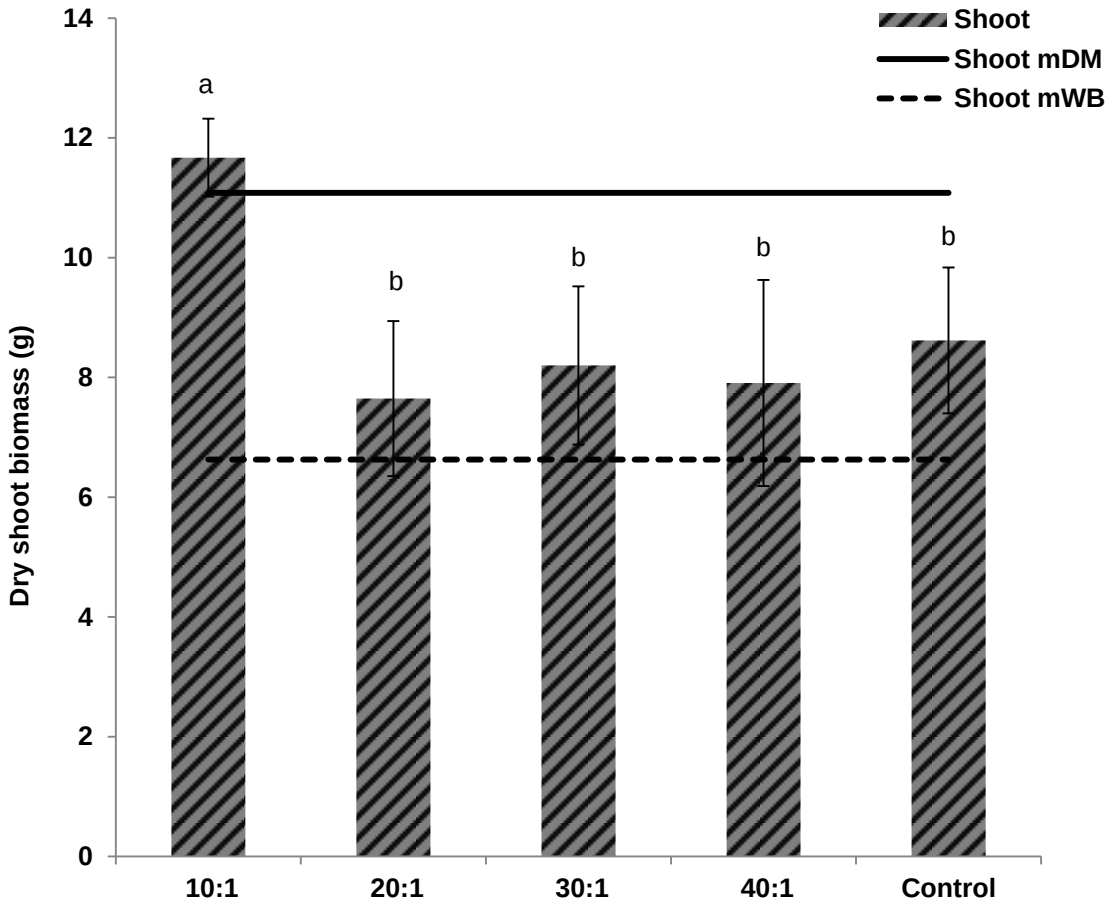


Figure 2-5. Effect of amendment and amendment C:N ratio on mean percentage of dry shoot biomass production per pot during ASD.

Bars indicated by different letters are significantly different, $p<0.05$. Error bars indicate standard error with four replicates. Control=non-amended control, 10:1=C:N ratio 10:1, 20:1=C:N ratio 20:1, 30:1=C:N ratio 30:1 and 40:1=C:N ratio 40:1, m=mean, DM=dry molasses, WB=wheat bran.

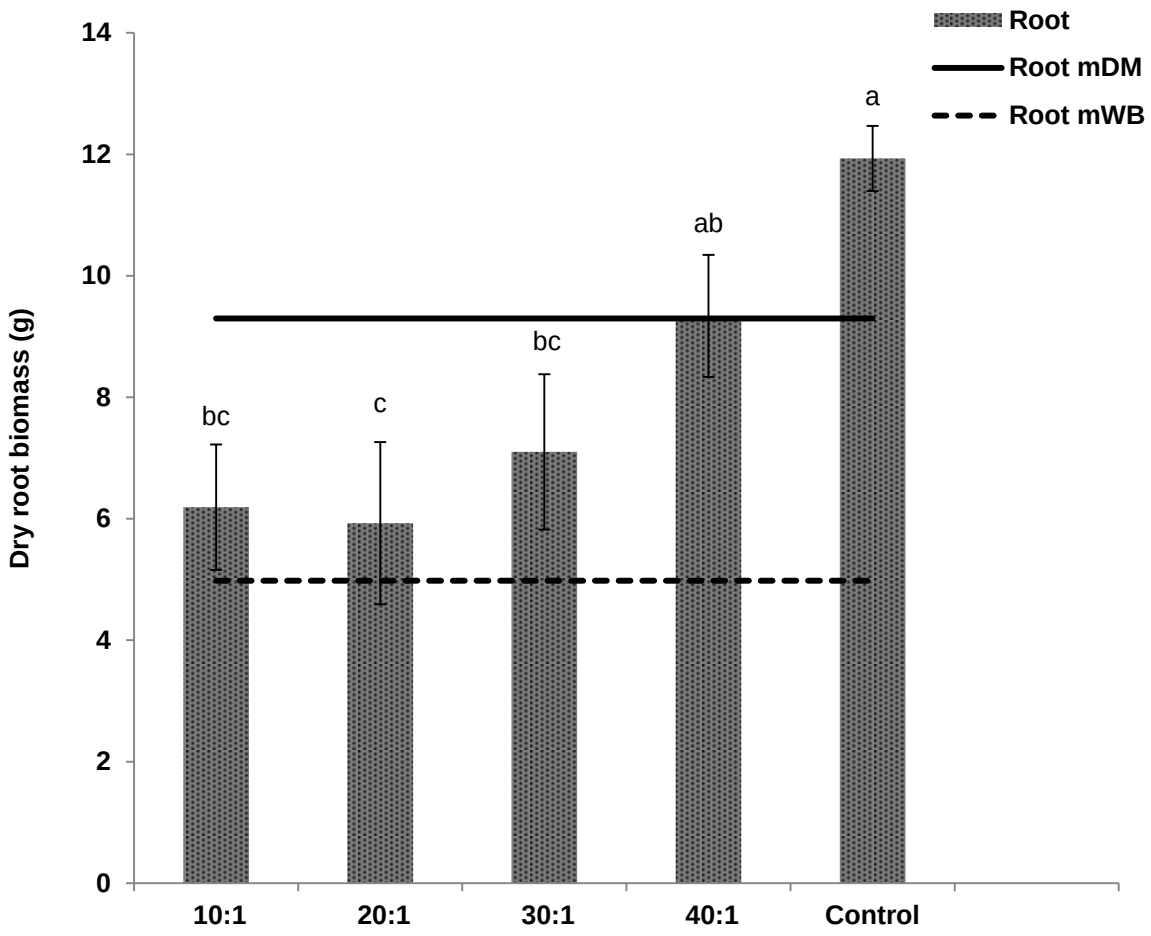


Figure 2-6. Effect of amendment and amendment C:N ratio on mean percentage of dry root biomass production per pot during ASD.

Bars indicated by different letters are significantly different, $p<0.05$. Error bars indicate standard error with four replicates. Control=non-amended control, 10:1=C:N ratio 10:1, 20:1=C:N ratio 20:1, 30:1=C:N ratio 30:1 and 40:1=C:N ratio 40:1, m=mean, DM=dry molasses, WB=wheat bran.

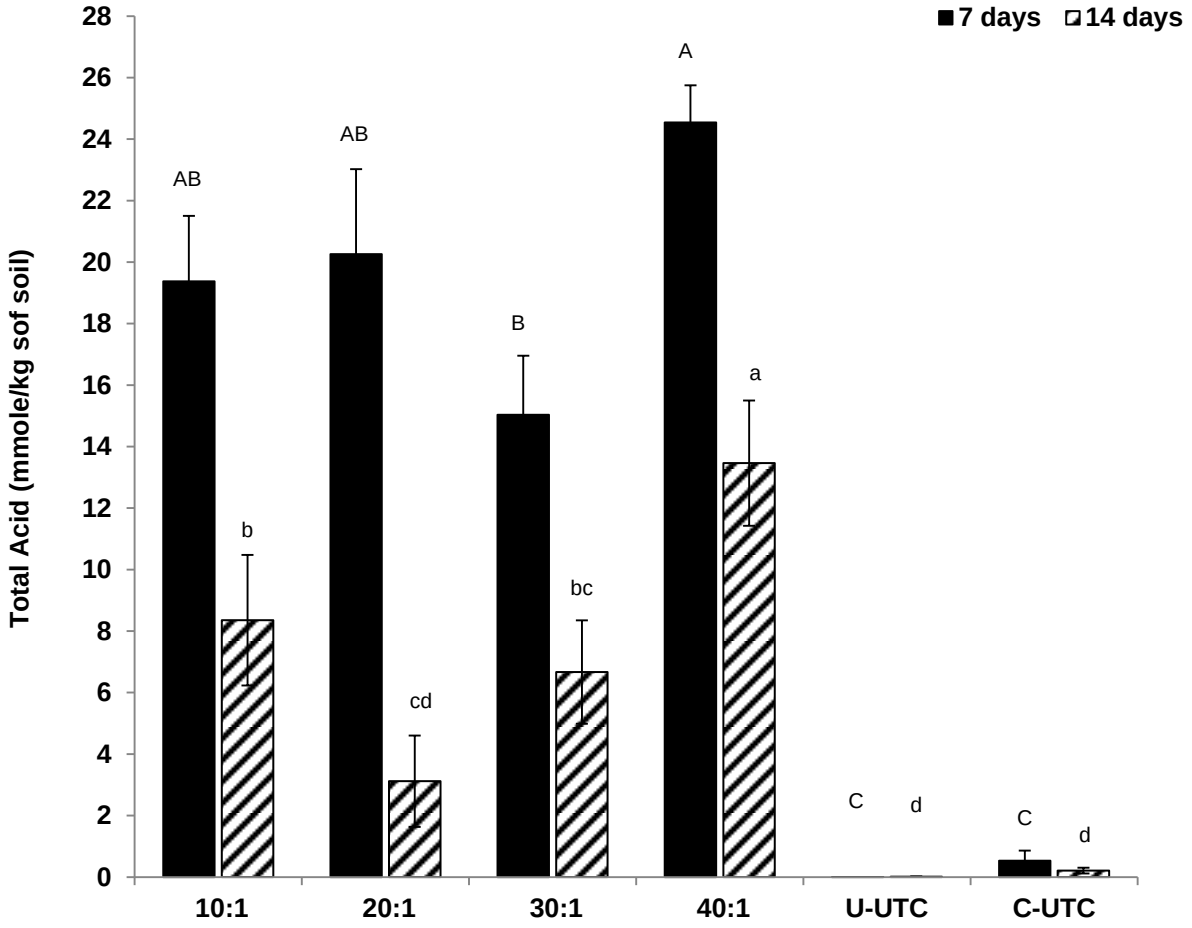


Figure 2-7. Total organic acids present in soil at 7 and 14 days post treatment initiation. Bars indicated by the same letters are not significantly different ($p>0.05$). Capital letters are used to compare the respective means of total acid production at 7 days and small letters are used to compare respective means of total acid production at 14 days. 10:1=C:N ratio 10:1, 20:1=C:N ratio 20:1, 30:1=C:N ratio 30:1, and 40:1=C:N ratio 40:1, all at C rates of 4 mg C $g^{-1}$ soil; U-UTC=uncovered and untreated (non-amended ) control, CUTC=plastic covered and untreated (non-amended ) control. Error bars represent standard error.

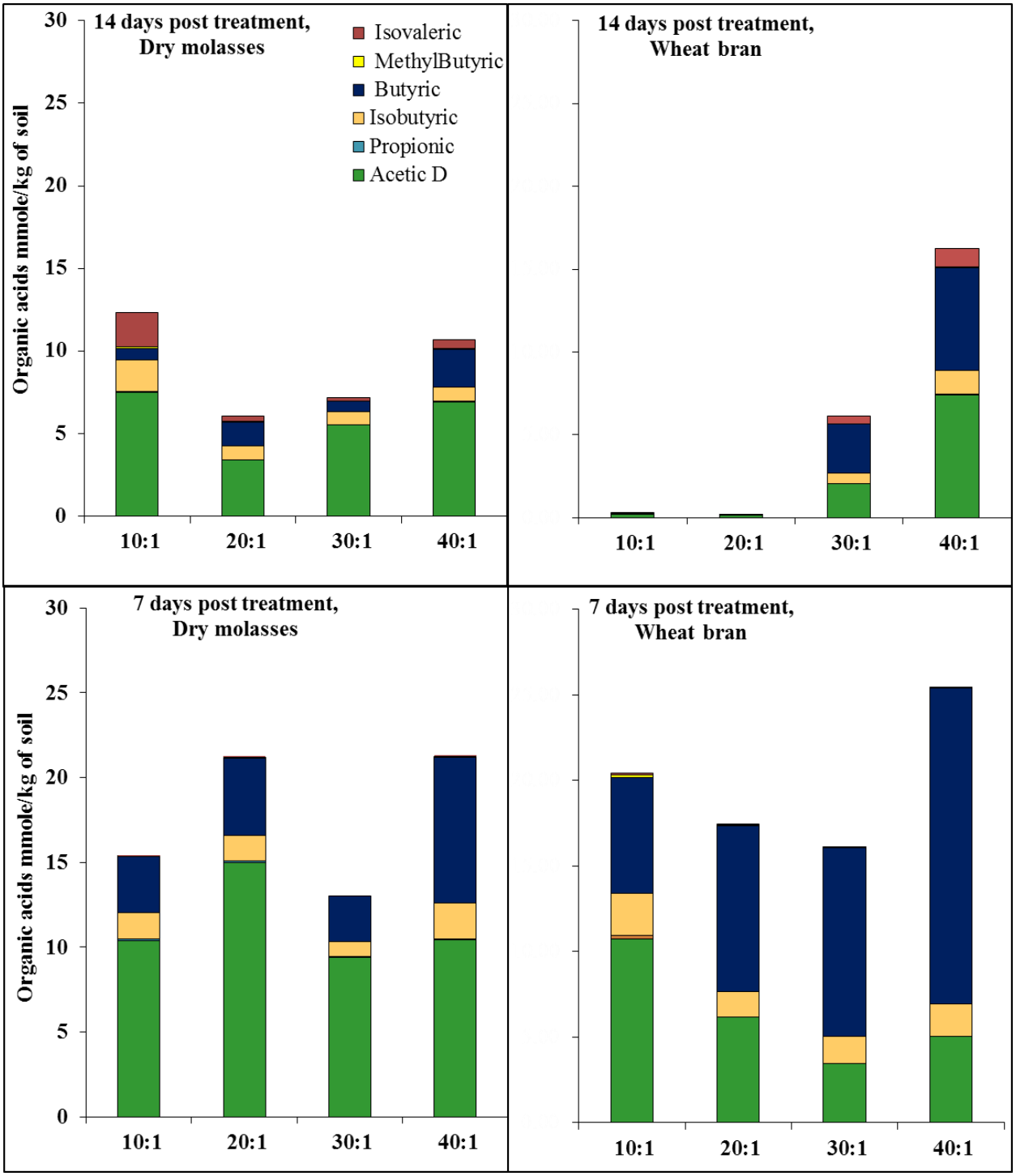


Figure 2-8. Various organic acids present in soil at 7 and 14 days post treatment initiation. 10:1=C:N ratio 10:1, 20:1=C:N ratio 20:1, 30:1=C:N ratio 30:1, and 40:1=C:N ratio 40:1, all at C rates of 4 mg C $g^{-1}$ soil.

# Chapter 3

## Organic amendment type and C:N ratio impact on *Fusarium oxysporum* f. sp. *lycopersici* following anaerobic soil disinfestation

A version of this chapter is a manuscript in preparation for *Phytopathology* by Utsala Shrestha, Alex Bruce, Bonnie H. Ownley and David M. Butler.

My primary contributions to this manuscript include experimental setup, data collection and analysis, results interpretation and writing. Alex Bruce helped in data collection of *Fusarium* population from soil.

## Abstract

Anaerobic soil disinfestation (ASD) relies on the incorporation of organic amendments to provide labile carbon (C) to stimulate microbial activity in saturated soil mulched with polyethylene. Two organic amendments, dry molasses and wheat bran, were incorporated independently in soil:sand mixture in pots with ASD treatment to evaluate effectiveness of these organic amendments at 4 mg C $g^{-1}$ soil, with varying C:N ratios of 40:1, 30:1, 20:1 and 10:1 against introduced *Fusarium oxysporum* (*Fo*) propagules. Soil pH and cumulative anaerobic condition were assessed to determine soil anaerobic condition. Similarly, a field study with dry molasses as the primary C-source amendment, with the same four C:N ratios was carried out. In addition, a C:N ratio of 30:1 at a lower C rate of 2 mg C $g^{-1}$ soil, an untreated control, and a MeBr-fumigated control were included. After three weeks of ASD treatment, *Fo* survivability was assessed by dilution plating of recovered inoculum bags from the soil on Snyder-Nash agar. Across both OAs, soil pH was least for the C:N ratio of 10:1, but there were no soil pH differences among other treatments in the pot study. We did not observed any soil pH difference before and after ASD treatment. For both OAs in the pot study and dry molasses in the field study, cumulative anaerobic condition was greater (more anaerobic) than control treatment. *Fo* colonies were fewer for dry molasses maintained at C:N ratio 20 to 30 than wheat bran in the pot study. All dry molasses treated plots in the field study significantly suppressed *Fo* than in non-amended plots. Our results suggest that application of C rates at 4 mg C $g^{-1}$ soil for ASD treatment induces more anaerobic soil conditions and greater mortality of *Fo* inoculum compared to lower C rate (2 mg C $g^{-1}$ soil), at a C:N ratio of 20:1 and 30:1.



**Keywords:** Anaerobic soil disinfestation, *Fusarium oxysporum*, organic amendments, pepper yield

# 1. Introduction

Anaerobic soil disinfestation (ASD) is an anaerobically mediated pre-plant soil treatment developed to control soilborne pathogens in high-value specialty crop production. ASD is one of the substitutes to chemical fumigation that relies on the incorporation of organic amendments to provide labile carbon (C) to stimulate microbial activity in saturated soil mulched with polyethylene (Butler et al., 2012b). Suppression of various soilborne pathogens utilizing different carbon amendments during ASD have been reported (Shennan et al., 2014) and compared (Strauss and Kluepfel, 2015) showing its viability to replace the chemical fumigants. However, results on suppression of different pathogens across diverse cropping systems are not common. Even similar types of pathogens showed variation in suppression level during ASD treatment as effectiveness of ASD is dependent upon chemical, physical and biological soil properties (Rosskopf et al., 2015). To date, disease suppression due to ASD treatment is inferred as a consequence of microbial shifts, production of organic acids (Momma et al., 2006), and other volatile compounds like alcohols, organic sulfides, esters, ketones, hydrocarbons, and isothiocyanates (Hewavitharana et al., 2014).

*Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) is a persistent soilborne pathogen that causes wilt and dry rot disease by producing pectin degrading enzymes (Jones et al., 1972) in tomato (*Solanum lycopersicum* L.), resulting in great production losses (Walker, 1971; McGovern, 2015). After phase out of the ozone depleting fumigant methyl bromide, sustainable disease management practice to control *Fol* is warranted along with environmentally friendly cultural practices that enhance soil quality and microbial activity (Ristaino and Thomas 1997). ASD has evolved as an alternative practice for methyl bromide and other chemical and non-chemical fumigants where they are restricted due to various limitations such as registration issue or economic and ecological limitations. ASD can be easily adapted to plastic culture system and utilizes a broad range of organic amendments to generate anaerobic conditions in the tarped soil. Incorporated organic amendments increase the organic matter in the soil making soil rich in nutrients and pathogen suppressive (Bonanomi et al., 2013). The wide range of amendments used in ASD have proven to control various host specific *Fusarium oxysporum* (*Fo*). More than 50% mortality of *Fo* against non-amended covered control was achieved with various agriculture by-

products (plant residue, cereal bran, mustard meal) cruciferous plant, grasses, cover crops (Blok et al., 2000; Yossen et al., 2008; Butler et al., 2012a; Butler et al., 2012b; Mowlick et al., 2012a; Mowlick et al., 2012b; Momma et al., 2013; Mowlick et al., 2013b; McCarty et al., 2014), and liquid amendments like molasses, ethanol (Momma et al., 2011; Mazzola and Hewavitharana, 2014), bioethanol (Horita and Kitamoto, 2015), and organic acids (Shennan et al., 2014; Horita and Kitamoto, 2015). The most common incubation period was 2 to 3 weeks with average soil temperature greater than 20°C while maximum incubation period of 13 weeks was reported in a study conducted by Blok et al. (2000) using rye grass, clipped grass and broccoli and a maximum temperature of greater than 35°C reported in Florida using liquid molasses and broiler litter (Butler et al., 2012a). Effective control of *Fo* f. sp. *asparagi* was observed in a study conducted by Blok et al. (2000) and similar effects were obtained in Mowlick et al. (2013a) using mustard, oat grass, radish and wheat bran to control *Fo* f. sp. *spinaciae*. ASD was also found to be effective for banana wilt caused by *Fo* f. sp. *cubense* when 1% corn stalk was used in pot study (Huang et al., 2015). So far, *Fol* is the most studied *Fusarium* pathogen with 50-99% reduction in propagules under ASD pot and field treatment conditions. However, in the field situation effectiveness is observed only when organic amendments applied were wheat bran or ethanol/ bioethanol or liquid molasses in combination with broiler litter or organic acid accompanied by high temperature (>28°C). Under controlled pot or box environments, under moderate temperature (>24°C), warm-season cover crops pearl millet, sunn hemp, sorghun-sudangrass, cowpea alone or mixed with pearl millet or sorghum-sudangrass (Butler et al., 2012b), ethanol, bioethanol, wheat bran (Momma et al., 2010; Horita and Kitamoto, 2015) effectively controlled the *Fol* pathogen.

Previous results showed that wheat bran and molasses have significant positive effects in the eradication of *Fol*. Wheat bran at the rate of 2 to 3 kg $m^{-2}$ in (Mowlick et al., 2013a; Mowlick et al., 2014) or at lower rate of 1 kg $m^{-2}$ (Yossen et al., 2008; Momma et al., 2010; Mowlick et al., 2012a) was found to reduce *Fo* inoculum in various soil types (sandy, loam and volcanic and gray lowland soil). Mortality of both fungal spores and chlamydospores of *Fol* under wheat bran is reported to occur with the production of acetic acid and butyric acid (Momma et al. 2005; Momma et al. 2006). Only liquid molasses is applied as a carbon supplement to treat soil for *Fol* disease (Momma et al., 2010; Butler et al., 2012a) and dry molasses has not been studied against

*Fol*. Considering the efficacy of wheat bran and the availability of dry molasses, we selected these as organic amendments to test effectiveness in controlling introduced *Fol* inoculum following ASD treatment. Realizing the importance of carbon to nitrogen ratio (C:N) of these amendments, we also sought to evaluate the effectiveness amendments at different C:N ratios. Amendment C:N is widely reported to influence soil microbial activity, post-treatment nutrient availability for crops following ASD treatments that ultimately affects yield of crops (Butler et al., 2014a). The overall aim of this study was to i) evaluate the cumulative anaerobic soil condition, followed by amendment application for ASD treatment and ii) evaluate the effectiveness of wheat bran and dry molasses maintained at C:N ratios of 10, 20, 30 and 40 as a carbon source amendment to suppress introduced inocula of *Fol* after ASD treatment.

## 2. Materials and Methods

### *2.1 Fusarium inoculum preparation*

*Fusarium oxysporum* f. sp. *lyscopersici* as a target pest was isolated from diseased tomato plants at the Organic Crops Unit, Knoxville, Tennessee, USA. The diseased plant parts were cleaned with tap water and then surface disinfested using 10% commercial bleach solution for 1 min. The plant was cut in 2.5-cm sections, plated on water agar medium (1.5%), and then incubated at 23 ± 2°C until fungal growth was apparent from each plant part. After 3 days, the fungal mycelium were isolated in antibiotic amended potato dextrose agar (PDA), amended with 10 mg liter$^{-1}$ rifampicin (Sigma-Aldrich, St. Louis, MO) and 3.45 mg liter$^{-1}$ fenpropathrin (Danitol 2.4EC, Valent Chemical, Walnut Creek, CA), and re-isolated after several days to obtain pure isolates. For inoculum production, 100 g of rice (Uncle Ben's® Whole Grain Brown Rice) was hydrated in double deionized water for 24 h in a 500 ml Erlenmeyer flask. Excess water from flasks was drained, autoclaved at 121°C for 55 min and shaken afterward to avoid any clumps. Ten 5- to 7-mm plugs of mycelium from the actively growing edge of fungal colonies were placed inside the autoclaved flask to inoculate the grains. Flasks were incubated at room temperature under fluorescent light for 2 weeks to allow fungal growth and colonization of rice grains. The colonized rice grains were air dried for 2 to 3 days under the fume hood on an absorbent under pad and used immediately or stored in airtight zip lock bags at 4°C for preparation of pathogen inoculum packets. About 2 g of dehydrated rice grain inoculum was sealed in 5-cm × 5-cm

packets made of apertured Delnet® polyolefin fabric (DelStar Technologies, Austin, TX). Strings were attached in each packet for easy removal for both pot and field experiments.

### *2.3 Pot experiment*

The experimental design for the pot study was completely randomized, with a 2 × 4 factorial, with organic amendments and C:N ratios as the main effects. As described above, dry molasses (C:N~29.7, Westway, New Orleans, LA) and wheat bran (C:N~13.3, Siemer milling company) were selected as carbon sources. For C:N ratios, dry molasses and wheat bran were adjusted to four ratios of 40:1, 30:1, 20:1 and 10:1 at 4 mg of C $g^{-1}$ soil using soybean meal (C:N~4.8, Hi Pro Soybean meal, Fiona, TX) and/or corn starch (C:N~0, Tate & Lyle ingredients Americas, INC. Decatur, IL). Each mixed amendment was well incorporated into sieved soil (<10 mm, Dewey silt loam, total carbon = 0.94%, pH = 6.2, and collected from University of Tennessee, Organic Crops Unit, Knoxville, TN) and fine sand (50:50). The control without organic amendment was included for both dry molasses and wheat bran and the study was repeated. The first trial started in June 12, 2013 and the second in July 8, 2013. Soil was collected from different locations each time. Initially the soil sand mixture had a C:N ratio of 11.6 and 12.4, and a mean cold water extractable C of 2 and 3.1 mg k $g^{-1}$, and a total N of 3.3 and 2 mg k $g^{-1}$ of soil in trial 1 and trial 2, respectively.

Polyethylene black pots (size 12-cm diameter and 23-cm height) were filled with the amendment soil and sand mixture and two *Fusarium* inoculum packets, were buried at 5- and 15-cm depths in each pot. Oxidation-reduction electrodes (ORP) and temperature-moisture sensors (Combination ORP Electrode, Sensorex Corp., Garden Grove, CA, USA and 5TM Soil Moisture Probe, Decagon Devices, Pullman, WA, USA) were inserted at 10- to 15-cm depth to measure cumulative redox potential and temperature of soil. Each treatment with four replicates received ORP probes, but only two replicates received temperature probes. Pots were filled with tap water until water flowed from the bottom of pots to attain complete saturation. Pots were covered with black polyethylene (0.03-mm), held in place with heavy-duty rubber band and arranged in the growth chamber, which was maintained at 25°C for 14 h and 15°C for 10 h, with 50% relative humidity for 3 weeks.

At completion of ASD, ORP were removed, and a soil sample (~80 g wet wt.) was collected using a clean plastic spoon up to 8-cm depth of each pot. Subsamples were oven-dried (105°C for 48 h) to determine gravimetric moisture content and the remaining sample was air-dried and sieved (<2 mm) to measure soil pH. Soil pH was measured in 0.01 M $CaCl_2$ (1:2 soil to solution ratio) using a pH electrode (Orion 3-Star Plus pH Benchtop Meter, Thermo Scientific, Waltham, MA, USA) and values were adjusted to soil pH determined in deionized water by adding 0.6 units. Cumulative soil anaerobic condition was calculated as described in Butler et al., (2012b) and McCarty et al., (2014).

### *2.3.1 Assessment of Tomato plant*

Three-week-old seedlings of dwarf patio tomato 'Florida Lanai' variety were planted in each pot after removal of *Fol* packets at the termination of ASD treatment to evaluate plant growth characteristics. After approximately 8 weeks, plants were removed, scored for diseases and total dry biomass determined.

### *2.4 Assessment of Fusarium population*

After ASD termination, *Fusarium* populations were assessed by standard dilution plating of recovered inoculum onto Nash-Snyder medium1(5 g Difco Peptone, 1 g $KH_2PO_4$, 0.5 g $MgSO_4$ $7H_2O$, 20 g Agar, 1 g Pentachloronitrobenzene) (Nash and Snyder, 1962). After two weeks, identification of *Fol* colonies were confirmed by microscopic examination of morphological features, and colony forming units were counted.

### *2.5 Field experiment*

### *2.5.1 Layout treatment establishment*

The field study was established at the UT Plateau Research and Education Center in Crossville, TN on May 16, 2013 and May 13, 2014 to assess the ASD effect on *Fusarium* populations. The design was a randomized complete block design with four replications. Each plot size was 7.6 m × 1.8 m. The soil type is a Lily series (Fine-loamy, siliceous, semiactive, mesic Typic Hapludult), which was tilled just to remove any debris. Soil amendments were applied in each

plot using a drop fertilizer spreader and were thoroughly incorporated with a rotovator. Raised beds (~5-cm) were formed, mulched with standard black polyethylene, and drip irrigated to fill pore space. Anaerobic soil conditions were monitored using iron oxihydroxide-coated tubes, which were installed after applying plastic mulch and before irrigation (Castenson and Rabenhorst, 2006). Dry molasses at a total C rate of 4 mg C $g^{-1}$ soil was used as the primary C source amendment at four C:N ratios of 10:1 (ASD10), 20:1 (ASD20), 30:1 (ASD30) and 40:1(ASD40) and the application rate was 0.86, 1.26, 1.38 and 1.03 kg $m^{-2}$ respectively. The C:N ratios of 10:1 and 20:1 were adjusted using soybean meal (0.48 and 0.12 kg $m^{-2}$ respectively) and C:N ratios 30:1 and 40:1 were adjusted using corn starch (0.01 and 0.35 kg $m^{-2}$ respectively). In addition, a C:N of 30:1 at a lower C rate of 2 mg C $g^{-1}$ soil (LCASD) with 0.69 kg $m^{-2}$ dry molasses and 0.01 kg $m^{-2}$ of corn starch were included. A non-amended untreated control (UTC) and a methyl bromide (MeBr) fumigated control (67:33 mixture with chloropicrin, 200 lbs $acre^{-1}$) were also included. Research was conducted in different sites each year. In 2013, soil had initial gravimetric soil moisture content of 2.5 to 2.7 g $g^{-1}$, soil C:N of 12.9, total N of 6.5 mg N $kg^{-1}$ soil and 4.9 mg P $kg^{-1}$ soil. Initial gravimetric soil moisture content in 2014 was 2.7 to 3.1 g $g^{-1}$ with soil C:N of 13.38, total N of 6.8 mg N $kg^{-1}$ soil and 9.3 mg P $kg^{-1}$ soil.

### *2.5.2 Anaerobic soil conditions and Fusarium assay*

To measure the anaerobic condition of soil two iron oxihydroxide coated IRIS tubes (polyvinyl-chloride pipe) were inserted per plot (Castenson and Rabenhorst, 2006). Reduction of iron (Fe) was assessed as described by Rabenhorst (2012). In 2014, along with IRIS tubes, the anaerobic condition was assessed inserting two ORPs in each plot of one block and soil temperature was recorded with datalogger temperature probes. Due to unavailability of datalogger temperature probes in 2013, hand held temperature probes were inserted in the soil to measure soil temperature. *Fusarium* packets were prepared as describe above for the pot study. Two packets containing propagules of *Fol* were buried at a 10- to 15-cm depth in each bed in 2013 and two additional inoculum packets were buried in 2014. Due to logistical constraints, *Fo* inoculum packets were not introduced into the MeBr fumigated plots. At the end of the ASD treatment (3 weeks), inoculum packets were retrieved and *Fol* propagule survival was assessed similarly as in the pot study. Soil samples (10 composite cores to 15-cm depth) from each plot were collected before applying amendments (preASD), at ASD termination (endASD), and 3 weeks following

ASD treatment termination (postASD) to access soil moisture content, and endemic *Fusarium* populations. Soil samples for gravimetric moisture content was assessed at 105°C immediately after collection and soil samples for *Fusarium* assay soil were kept at 4°C until analysis. *Fusarium* populations were assessed similar to *Fo* packet inoculum after soil samples were dried (24 h) and used for serial dilution.

### *2.5.3 Crop performance and root disease assessment*

On June 10, 2013, bell pepper transplants (cv. Aristotle F1) were planted 30.5-cm between and within a double row per bed (30 plants per bed) to assess crop performance, plant nutrition, and incidence of disease. Weed populations and incidence of soilborne disease were monitored throughout the growing season at least three times. Plant heights were measured each week in July beginning three weeks after transplanting. At first harvest (after 9 weeks) 20 recently mature leaves were taken from each treatment, dried at 65°C, ground and total N and C were determined by combustion (data not shown). Three root systems were taken randomly at the end of harvest to evaluate root galling and condition. Ratings of galling by root-knot nematode were based on Bridge and Page (1980), with the extent of root galling present on bell pepper plants rated as follows: 0 = no nematodes, 1 to 4 = galling of secondary roots only, 5 to 10 = galling of primary laterals and tap root. Each root system was also evaluated on a scale of 1 to 5 (1 = brown with signs of decay; 5 = white and healthy) that represented health status of the root system.

Peppers were harvested and graded according to standard USDA fruit grading; Fancy, No. 1, No. 2, and cull (USDA-AMS, 2005). Bell pepper were harvested once a week during mid-to-late August. Peppers were harvested based on size and dark green color and firmness. Fruit were harvested from the middle (~26 plants) except plants at the end of each row. Fruit were counted and weighed in each grade class. For culls, reason for culling and number of fruit culled were recorded.

### *2.6 Statistical analysis*

Mixed model analysis of variance was conducted with SAS (9.3 SAS Institute, Cary, NC); data were checked for normality and homogeneity of variances, and transformed as needed. Least

squares means were compared with Fisher's P-LSD at 5% significance level and untransformed means are reported. Relationships between cumulative anaerobic conditions and *Fusarium* colony counts were assessed using correlation analysis at $p<0.05$. Data were analyzed separately by C amendment to compare treatments with untreated control and also by C:N to compare with untreated control. *Fusarium* inoculum survival data was collected as colony forming unit per gram of soil and transformed using the formula log10(x+1) before statistical analysis.

## 3. Results

### *3.1 Pot study*

No apparent relationship between cumulative anaerobic condition, pH and *Fusarium* population was observed in trial 2 and only moderate negative correlation between cumulative anaerobic condition and *Fusarium* population was observed in trial 1 (-0.4, $p<0.02$). The weak negative relationship between soil C:N at the end of ASD treatment and *Fusarium* population was observed in trial 2 (-0.34 , $p=0.03$). At the end of treatment, the soil sand mixture irrigated and covered had 2.4 mg $kg^{-1}$ of $NH_4N$, 24.4 mg $kg^{-1}$ of $NO_3NO_2$, 5.8 mg $kg^{-1}$ of inorganic P, and10.1 C:N in trial 1. Whereas in trial 2 the mixture had 5 mg $kg^{-1}$ of $NH_4N$, 24.4 mg $kg^{-1}$ of $NO_3 + NO_2$, 6.8 mg $kg^{-1}$ of inorganic P, and 12.1 C:N.

#### *3.1.1 Gravimetric soil moisture, soil temperature, and soil pH*

Gravimetric soil moisture content before ASD treatment initiation was 0.11 g $g^{-1}$ for trial 1 and 0.18 g $g^{-1}$ for trial 2. After 3 weeks, average soil moisture content increased by 0.53% for trial 1 and 0.26% for trial 2. The gravimetric soil moisture content was higher for amended pots than control pots in both trials. However, volumetric soil content did not differ across organic amendment and C:N treatments (Table 1). Mean soil temperature across C:N ratios ranged from 23.7 to 24.7°C in trial 1 and 23.3 to 24.4°C in trial 2 and was significantly higher in organic amendment treated soil (23.9 to 24.1°C) than control (23.3°C) only in trial 2. Soil pH did not differ among organic amendments and control in the both trials, and there was no interaction of organic amendment and C:N. However, across C:N ratios, soil pH was lowest for the 10:1 (Figure 3-1A, 2B).

### *3.1.2 Anaerobic soil conditions*

There was a significant effect of organic amendment, C:N on cumulative soil anaerobic conditions ($p<0.001$) as well as a significant interaction of organic amendment with C:N ($p<0.03$) on both trials. For organic amendments, mean cumulative soil anaerobic conditions were higher (i.e., more anaerobic) for dry molasses (222,780 – 233,098 mV h) than wheat bran (179,338 – 205,104 mV h) and lowest for the control (142,324 – 92,823 mV h). In treatments with dry molasses, cumulative anaerobic conditions were lowest at C:N 40:1 in both trials; while amendments with wheat bran showed lowest cumulative anaerobic conditions at 20:1 C:N and 30:1 C:N for trial 1 and trial 2, respectively. The greatest difference between organic amendments in accumulation of anaerobic conditions was observed for 20:1 C:N (72,469 mV h; Figure 3-2A) and 30:1 C:N (67,662 mV h; Figure 3-2B).

### *3.1.3 Fusarium population*

Across all amended treatments, *Fol* populations were significantly lower at the 15-cm depth than at 5-cm (19% in trial 1 and 35% in trial 2), but there were no interactions of depth with organic amendment or C:N ratios in both trials. *Fol* counts were lowest in control treatment at 15-cm depth in trial 2 (Table 2). There was a significant interaction of organic amendment and C:N in *Fol* counts in trial 1 ($p<0.001$), with fewer *Fol* colonies recovered from the lower C:N ratios (10:1, 20:1 and 30:1) for dry molasses compared to the C:N 40:1 and the control (2.6 to 2.7 $\log_{10}$ [CFU+1] $g^{-1}$). There was no difference among C:N ratios and the control for wheat bran and mean *Fol* counts ranged from 3.5 to 3.7 $\log_{10}$[CFU+1] $g^{-1}$ (Figure 3-3A). We did not observe any interaction of organic amendment and C:N and differences in trial 2. Nevertheless fewer *Fol* colonies were observed in control and dry molasses treated pots, especially in the 10:1 and 20:1 (2.8 and 2.6 $\log_{10}$[CFU+1] $g^{-1}$ respectively) than wheat bran treated pots (3.7 $\log_{10}$[CFU+1] $g^{-1}$, Figure 3-3B).

## *3.2 Field study*

During the treatment period, mean soil temperature was 22.04 to 24.7°C in 2013 and 23.6 to 24.5°C in 2014. In 2013, at termination of ASD the gravimetric soil moisture content ranged

from 1.5 to 2.9 g $g^{-1}$ across treatments with highest moisture content observed for ASD20 (3.1 g $g^{-1}$) and lowest for fumigated plot (1.5 g $g^{-1}$). In 2014 soil moisture content ranged from 2.9 to 3.9 g $g^{-1}$, and was highest for ASD30 (3.9 g $g^{-1}$). Soil pH, as in the pot study, did not differ among organic amendments and control in both years. However, soil pH change was higher after three weeks of ASD implementation in 2013 (0.51 to 0.64 units) than in 2014 (-0.01 to 0.06 units). Similar to trial 1, no significant relationship between oil pH, anaerobic condition and *Fusarium* population was observed.

We did not observe any galling during field trials. The total number of pepper fruits was significantly higher in amended plots with lower C:N (10 and 20) than fumigated plots and non-amended plots. However, this difference was seen only in the year 2014. Marketable and fancy fruit numbers per hectare was higher in all amended plots with 4 mg C $g^{-1}$ soil (Figure 3-6). Similarly, total fruit yield (61 to 71 mt $ha^{-1}$) and marketable yield (54 to 61 mt $ha^{-1}$) was significantly higher in the second year in amended plots than in fumigated and non-amended plots (<50 mt $ha^{-1}$).

### *3.2.1 Anaerobic soil conditions*

Across the treatments, the percentage of oxihydroxide paint removal was higher for all amended treatments with 4 mg C $g^{-1}$ of soil in both years than non-amended control, indicating enhanced anaerobic conditions driven by soil microbial activity. Percentage of oxihydroxide paint removal in reduced C rate amended treatment (LCASD30) was intermediate between high C rate amended plot and non-amended plot. However, cumulative anaerobic soil condition of LCASD30 was found similar to high-amended treatments ($p$<0.002, Figure 3-4). The accumulated anaerobic condition in 2014 also revealed a similar trend across all treatments with the lowest mean anaerobic condition recorded for control plots (105,737 mV h).

### *3.2.2 Fusarium population*

Inoculum packets retrieved from soil after ASD termination showed no differences in population count in 2013. However, highest suppression of *Fol* inoculum was observed in amended treatments at C:N 20 and C:N 30 (1 to 1.4 $\log_{10}$[CFU+1] $g^{-1}$ soil) and lowest suppression in the

non-amended control (2.5 $\log_{10}$[CFU+1] $g^{-1}$ soil). A similar trend in *Fol* suppression with significant differences was observed in 2014 with significant reduction of *Fol* population in amended plots (3.2 $\log_{10}$[CFU+1] $g^{-1}$ soil) though *Fol* population retrieved were higher in all treatment than in the first year study with highest *Fol* population (5.7 $\log_{10}$[CFU+1] $g^{-1}$ soil) in non-amended control ($p<0.001$, Figure 3-5). The *Fol* populations from packets that was not buried in soil was 6.07 $\log_{10}$[CFU+1] $g^{-1}$ soil.

At the end of ASD termination *Fusarium* counts from soil samples was significantly higher in ASD20 and lower in fumigated plots ($p<0.001$) in 2013. However, across all treatments in 2014, there was no interaction and no significant differences for soil *Fusarium* counts (Table 3-4), but reported low after ASD few weeks of fumigation treatment. The endemic *Fusarium* population identified was *F. solani*, *F. foetens*, and *F. concolor.*

## 4. Discussion

Separate analysis was performed for pot trials as we observed variation in trials in terms of interaction between organic amendment and C:N treatment, soil pH, cumulative anaerobic condition, and *Fusarium* inoculum survival. In addition, separate analysis for field trials was conducted due to separate fields selected for ASD study.

Soil pH reduction in ASD treatment is one of the indicators of organic acids production, such as acetic acid and butyric acid (Momma et al., 2006; Momma et al., 2011) that is attributable to breaking down of amendment carbon. Several studies have reported reduced pH after amendment incubation in ASD (Katase et al., 2009; Butler et al., 2012b; Hewavitharana and Mazzola, 2013) and in our pot studies, we also observed lowering of soil pH by 0.8 to 0.9 units across all treatments including non-amended control; lowest soil pH was recorded at C:N 10:1. On the contrary, significant lowering of soil pH after the ASD treatment is not necessary (Butler et al., 2014b; Huang et al., 2016). Soil pH in 2013 was higher than initial soil pH, which was similar to the first year field study of McCarty et al. (2014) however, the soil pH change in the second year though negative, was not significant. Generally, not only amendment types and soil microbial activity influences soil pH, soil types also control pH instability. In the field study, the clay loam soil type may have a higher buffering capacity than soil collected for the pot study

(sandy soil), which limited any pH changes. Also recorded soil pH value in the field study, where sampling covers a large area than in the pot study was expected to have a lower pH change than at the site of microbial activity (Strong et al., 1997; Katase et al., 2009) as low pH contributers, i.e organic acid generated during ASD, can be readily escape once soil is exposed (Jones et al., 2003). Studies have reported that *Fusarium oxysporum* survival is observed in wide pH range (Manandhar and Bruehl, 1972) and control is observed only if pH of greater than nine units is obtained (Jones and Woltz, 1969).

As discussed above, overall soil pH may or may not be critical factors for reduction of the number of *Fusarium* propagules during ASD treatment, but reduced soil conditions due to loss of oxygen by soil microbes and limited gas exchange inside plastic mulch may play a significant role in the survival of *Fusarium*. Though our soil temperature in both studies was not high enough to provide any lethal effect, increased accumulation of anaerobic conditions was considered to create unfavorable soil conditions for pathogens. In our pot trials, anaerobic conditions was higher in all of our amended pots along with control (>140,000 mV h). Application of organic amendments as carbon source significantly increased accumulation of anaerobic conditions (>179, 000 mV h) indicating high microbial activity. This was approximately three- to four-fold greater than the set threshold level of 50,000 mV h that is expected to have such effect (Shennan et al., 2010). Similarly, in our field study, iron oxyhydroxide paint removal indicated high anaerobic condition in amended plots suggesting that microbial activity is more prevalent in amended plots than in control. Low anaerobic condition in plot amendment with 2 mg C $g^{-1}$ soil compared to 4 mg C $g^{-1}$ soil indicated that anaerobic condition is largely affected by the rate of carbon than C:N ratios.

Significant reduction of *Fol* populations (44 to 87%) by ASD with high anaerobic condition was observed when dry molasses at the rate of 4 mg C $kg^{-1}$ of soil at various C:N ratios was applied in the field plots. Surprisingly, suppression of *Fol* in pot trials with the same rate of carbon at different C:N ratios was not consistent among two pot trials and the field study. Use of dry molasses showed higher suppression of the pathogen in pot trials than wheat bran. However, lack of clear effects of amendments as compared to control treatment in trial 2 suggests that initial soil properties and accumulation of anaerobic condition may have influenced inoculum survival. As mentioned above, the anaerobic condition for pot studies was high enough to suppress *Fol*

inoculum at moderate soil temperature and this is attributed to an increase in anaerobic bacteria especially Clostridia (Mowlick et al., 2013a; Mowlick et al., 2013b). We believe even in the covered but non-amended treatments there could be similar conditions and other synergistic effects of organic acid, volatiles like allyl alcohol and reduced form of Fe and Mn, which are usually reported during ASD treatment (Momma et al., 2011; Hewavitharana et al., 2014) may also exist. Though production of these toxic product for non-amended control may not reach the suppressive threshold level, we believe that the higher anoxic condition, which is prevalent at the greater depth may have significantly reduced *Fol* survival (see Table 2). Ebihara and Uematsu (2014) also reported that anaerobic condition without any amendments can make significant contribution in the reduction of *Fusarium oxysporum* propagules but higher incubation period (>30 weeks) required for complete eradication at 30°C. In trial 2, besides the anaerobic condition, the relationship between *Fusarium* survival and C:N of soil at the end of treatment with high C:N in control may have attributed to no difference. Also, soil nutrients present in different forms impacts *Fusarium* survival and disease development (Jones and Woltz, 1969; 1970; Huber and Thompson, 2007) and higher nitrate N and ammonium N in trial 2 than trial 1 explain lower *Fusarium* populations in control in an anaerobic condition.

Usually, saturating and covering the soil without any amendments have failed to control *Fo* in field studies (Blok et al., 2000; Mowlick et al., 2012b; Mowlick et al., 2014). However, ASD is not always effective against *Fusarium* suppression. For instance, similar to our pot studies, artificial infestation of *Fo* in soil treated with cover crop amendments cowpea and pearl millet (Butler 2012a) incubated for 3 weeks failed to suppress *Fol*. Similarly, a previous pot study on *Fusarium* root rot of common bean also produced inconsistent result in disease suppression that utilized cool-season cover crops as a C source Butler et al. (2014b). When by-products like wheat bran at the rate of 1 kg $m^{-2}$ (Horita and Kitamoto, 2015) and rice bran, or a combination of rice bran and mustard applied as a C source (Daugovish et al., 2013), also failed to control the *Fo* population under soil temperatures that ranged between 25 to 35°C indicating a high carbon requirement for disease suppression.

In this research, although higher C rates at different C:N were maintained for organic amendments, it is possible that different forms of organic C and their relative recalcitrance to microbial decomposition might have influenced our results. Soil suppression and microbial

activity is influenced by amendment quality (Steiner and Lockwood, 1970; Senechkin et al., 2014) and *Fol* suppression in soil has always been challenging (Momma et al., 2006; Huang et al., 2015). Addition of amendments creates confounding effects in soil, i.e., amendment may protect or even increase the *Fusarium* population nullifying the fungistatic property of soil beforehand when some air is present (Zakaria and Lockwood, 1980; Kamble and Bååth, 2014; Morauf and Steinkellner, 2015) and after anoxic condition *Fol* may exist by nitrate respiration (Zhou et al., 2001); thus, preventing complete eradication of *Fol*. Further, instead of pathogen incorporation in soil, use of small bags for *Fol* propagules in our study may facilitate the propagules to escape the toxic effect of organic acids and other volatiles generated during ASD. While the effect of amendments could have been different if propagules were not colonized in rice and placed in media plates as in Hewavitharana et al. (2014), exposing propagules to volatiles from amended pots.

Only a few studies on pathogens and nematodes have reported C:N of amendments used in the ASD. The amendments C:N applied to suppress *Fo* inocula were found effective at ranges of 10 to 40 C:N ratios. Cover crops utilized in Butler et al. (2012a) ranged from 10 to 40. The lowest ratio found for cowpea (C:N 10), highest for sorghum sudan (C:N 40) and dry molasses, pearl millet and sunn hemp had C:N 21 to 30. Mustard seed meal, composted steer manure, grass residues and rice bran used in Hewavitharana et al. (2014) had C:N of 10 to 20. These studies were conducted in similar environment to the present study with sandy soil, soil temperatures not exceeding 25°C and soil depth in pots of 5- to 15-cm depth. Only composted steer manure at lower C:N, and cowpea and liquid molasses at higher C:N failed to suppress *Fusarium* populations. In our pot trials also at higher C:N of 40 in our pot trials *Fol* was not suppressed and between two amendment types wheat bran was not effective in controlling *Fol* at all C:N ratios. Dry molasses showed 24 to 30% of suppression in trial 1, though no significant results were observed in trial 2. It was not surprising to see higher *Fol* decreased in field conditions (<35% at C:N 10, 36 to 58% in other C:N ratios) at higher C rate when compared with control since pathogen inoculum is better exposed (Butler et al. 2012a) and the readily decomposable nature of dry molasses was more effective against pathogen inoculum than in pots.

We analyzed soil samples collected from the field for endemic *Fusarium* populations and these were similar across all treatments in 2014. Not all endemic *Fusarium* spp. are pathogens and soil

samples contained a mixture of other pathogens, saprophytes, and beneficial endophytes. Similar results, without possible effect of ASD on the relative abundance of endemic *Fusarium* populations have been reported in Rosskopf et al. (2014) and Huang et al. (2016). However, we observed a negative effect of fumigation on endemic *Fusarium* population after application of fumigation in field trials indicating greater chance of potential loss of other beneficial organisms.

Pepper yield data from the field study showed increased productivity in ASD treatment compared to non-amended covered treatment and the fumigant treatment. It is not surprising to observe higher yield from ASD treated plots than fumigated plots as addition of amendments in soil is reported to increase microbial response to promote plant health. Our meta-analysis review (Chapter 1) also pointed to the role of increased yield in ASD. However, ASD may have more potential as a disease control practice in lower temperature regimes. More ASD studies with naturally infested field soil need to be explored to allow growers' confidence to rely on this technique.

## 5. Conclusion

To summarize, dry molasses was the first amendment to be studied for potential control of *Fusarium* population and it was found to be more potent than wheat bran maintained at C:N 20 to 30. *Fol* suppression using dry molasses with a lower C:N and a lower rate is not recommended, which was also evident from poor bacterial pathogen suppression in study conducted by McCarty et al. (2014). However, soil nutrient analysis after ASD treatment could be helpful in drawing conclusive recommendations (Butler et al., 2014a). At this time, our results can help growers decide on the amendment rate and C:N for *Fusarium* control.

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# Appendix

Table 3-1. Gravimetric soil moisture content (g $g^{-1}$) of soil after 3 weeks of ASD treatment, pot study

| Carbon[y] | No. of observations | Moisture content (g $g^{-1}$) [a] | |
|---|---|---|---|
| | | Trial 1 | Trial 2 |
| Control | 8 | 0.23 ± 0.01 b | 0.23 ± 0.01 c |
| DM | 16 | 0.26 ± 0.01 a | 0.24 ±0.01 b |
| WB | 16 | 0.25 ± 0.01 a | 0.26 ± 0.01 a |
| *p*-value | | <0.0001 | <0.0001 |
| C:N | | | |
| CN10 | 8 | 0.24 ± 0.01 bc | 0.25 ± 0.01 a |
| CN20 | 8 | 0.26 ± 0.01 ab | 0.25 ± 0.01 ab |
| CN30 | 8 | 0.26 ± 0.01 a | 0.26 ± 0.01 a |
| CN40 | 8 | 0.26 ± 0.01 ab | 0.25 ± 0.01 a |
| Control | 8 | 0.23 ± 0.01 c | 0.23 ± 0.01 b |
| *p*-value | | <0.0001 | .01 |

[a]Numbers represent the soil moisture mean ± standard error respective to number of observation. Different letters represent significant differences at $p<0.05$ within columns for each category according to Fisher's protected LSD test.

[y]Dry molasses (DM), Wheat bran(WB), C:N ratio 10:1 (10), C:N ratio 20:1 (20), C:N ratio 30:1 (30) and C:N ratio 40:1(40)

Table 3-2. Effect of location of *Fusarium* inoculum packet in the pot on *Fusarium* populations, pot assays

| Location treatment | *Fusarium* population[a] | |
|---|---|---|
| | Trial 1 | Trial 2 |
| 15-cm | 3.06+0.17 b | 3.12+0.15 b |
| Control, 15-cm | 3.52+0.27 ab | 2.27+0.57 c |
| 5-cm | 3.67+0.13 a | 4.2+0.1 a |
| Control, 5-cm | 3.67+0.31 ab | 4+0.17 a |
| *p*-value | <0.025 | <0.001 |

[a]Numbers represent the mean soil Fusarium populations ($\log_{10}$[CFU+1]$g^{-1}$ inoculum) ± standard error of four replicated pots. Different letters represent significant differences at $p<0.05$ within columns for each category according to Fisher's protected LSD test.

Table 3-3. Effect of C: N ratio soil treatment on soil gravimetric moisture content (g $g^{-1}$) in field condition

| C:N[a] | Year 2013 | | | Year 2014 | | |
|---|---|---|---|---|---|---|
| | Pre ASD | End ASD | Post ASD | Pre ASD | End ASD | Post ASD |
| ASD10 | 2.5 ± 0.16 | 2.9 ± 0.05 ab | 3.0 ± 0.2 | 2.7 ± 0.19 | 3.4 ± 0.24 | 2.5 ± 0.17 |
| ASD20 | 2.6 ± 0.09 | 3.1 ± 0.1 a | 2.9 ± 0.09 | 2.9 ± 0.11 | 2.9 ± 0.29 | 2.7 ± 0.31 |
| ASD30 | 2.7 ± 0.07 | 2.8 ± 0.15 b | 3.1 ± 0.15 | 3.1 ± 0.26 | 3.9 ± 0.55 | 2.4 ± 0.27 |
| ASD40 | 2.5 ± 0.05 | 2.5 ± 0.17 cd | 2.7 ± 0.14 | 3.0 ± 0.08 | 2.9 ± 0.35 | 2.9 ± 0.25 |
| Fum | 2.5 ± 0.13 | 1.5 ± 0.53 d | 2.1 ± 0.72 | 2.7 ± 0.05 | 3.3 ± 0.33 | 1.8 ± 0.28 |
| LCASD30 | 2.7 ± 0.10 | 2.9 ± 0.08 ab | 2.8 ± 0.13 | 2.8 ± 0.11 | 3.1 ± 0.31 | 2.4 ± 0.09 |
| UTC | 2.5 ± 0.05 | 2.8 ± 0.03 bc | 2.9 ± 0.05 | 2.8 ± 0.10 | 3.2 ± 0.29 | 2.4 ± 0.18 |

[a]Carbon to nitrogen (C:N) C:N ratio 10:1 (ASD10), C:N ratio 20:1 (ASD20), C:N ratio 30:1 (ASD30), C:N ratio 40:1 (ASD40), Low carbon C:N ratio 30:1, C rate 2 mg C g-1 soil (LCASD30) Untreated, non-amended control (UTC) and Fumigated of Methyl bromide and chloropicrin (67:33), 200 lbs $acre^{-1}$

Table 3-4. Effect of C: N ratio soil treatment on endemic soil *Fusarium* populations in field assays

| C:N[a] | Year 2013 | | | Year 2014 | | |
|---|---|---|---|---|---|---|
| | Pre ASD | End ASD | Post ASD | Pre ASD | End ASD | Post ASD |
| ASD10 | 2.5 ± 0.16 | 2.9 ± 0.05 ab | 3.0 ± 0.2 | 2.7 ± 0.19 | 3.4 ± 0.24 | 2.5 ± 0.17 |
| ASD20 | 2.6 ± 0.09 | 3.1 ± 0.1 a | 2.9 ± 0.09 | 2.9 ± 0.11 | 2.9 ± 0.29 | 2.7 ± 0.31 |
| ASD30 | 2.7 ± 0.07 | 2.8 ± 0.15 b | 3.1 ± 0.15 | 3.1 ± 0.26 | 3.9 ± 0.55 | 2.4 ± 0.27 |
| ASD40 | 2.5 ± 0.05 | 2.5 ± 0.17 cd | 2.7 ± 0.14 | 3 ± 0.08 | 2.9 ± 0.35 | 2.9 ± 0.25 |
| Fumigated | 2.5 ± 0.13 | 1.5 ± 0.53 d | 2.1 ± 0.72 | 2.7 ± 0.05 | 3.3 ± 0.33 | 1.8 ± 0.28 |
| LCASD30 | 2.7 ± 0.1 | 2.9 ± 0.08 ab | 2.8 ± 0.13 | 2.8 ± 0.11 | 3.1 ± 0.31 | 2.4 ± 0.09 |
| UTC | 2.5 ± 0.05 | 2.8 ± 0.03 bc | 2.9 ± 0.05 | 2.8 ± 0.10 | 3.2 ± 0.29 | 2.4 ± 0.18 |
| *p*-value | 0.4648 | 0.0006 | 0.526 | 0.3346 | 0.3159 | 0.0806 |

Numbers represent the mean soil Fusarium populations ($\log_{10}$[CFU+1]$g^{-1}$ soil) ± standard error of four replicated plots. Different letters represent significant differences at $p<0.05$ within columns for each category according to Fisher's protected LSD test.

[a]Carbon to nitrogen (C:N) C:N ratio 10:1 (ASD10), C:N ratio 20:1 (ASD20), C:N ratio 30:1 (ASD30), C:N ratio 40:1 (ASD40), Low carbon C:N ratio 30:1, C rate 2 mg C g-1 soil (LCASD30) Untreated, non-amended control (UTC) and Fumigated of Methyl bromide and chloropicrin (67:33), 200 lbs $acre^{-1}$

Table 3-5. Growth characteristics of 8-week-old tomato plant transplanted at 5 weeks old in pots after termination of ASD treatment (Trial 1)

| Carbon[y] | Fruit weight (g) | Fruit (no.) | Flower (no.) | Shoot height (cm) | Stem diameter (mm) | Dry shoot biomass (g) | Dry root biomass (g) |
|---|---|---|---|---|---|---|---|
| Control | 29.8 ± 6.6 b | 3.6 ± 1.2 b | 16.5 ± 3.2 ab | 35.6 ± 1.7 ab | 8.6 ± 0.3 b | 7 ± 1.3 ab | 0.8 ± 0.1 a |
| DM | 65.2 ± 8.6 a | 6.4 ± 3.1 a | 17.2 ± 1.9 a | 37.3 ± 1.6 a | 9.4 ± 0.3 a | 7 ± 0.4 a | 0.8 ± 0.1 a |
| WB | 48.5 ± 7.4 ab | 4.4 ± 2.4 b | 10.1 ± 2.3 b | 30.6 ± 1.7 b | 8.8 ± 0.2 b | 5.7 ± 0.6 b | 0.7 ± 0.1 a |
| *p*-value | 0.031 | 0.0309 | 0.0234 | 0.0129 | 0.0446 | 0.0312 | 0.6301 |
| C:N | | | | | | | |
| 10 | 86.9 ± 5.7 a | 6.9 ± 0.9 a | 20.1 ± 2 a | 37.9 ± 2.2 a | 9.9 ± 0.3 a | 8.8 ± 0.4 a | 1.1 ± 0.1 a |
| 20 | 69 ± 10.1 ab | 5.8 ± 0.7 ab | 10.1 ± 3 b | 33.3 ± 2.9 a | 9.1 ± 0.3 ab | 4.9 ± 0.5 b | 0.7 ± 0.1 b |
| 30 | 47.1 ± 11.8 bc | 5.9 ± 1.4 ab | 14.9 ± 3.3 ab | 33.1 ± 3.2 a | 9.1 ± 0.3 abc | 6.3 ± 0.7 b | 0.7 ± 0.1 b |
| 40 | 24.4 ± 2.4 c | 3.1 ± 0.6 c | 9.5 ± 3.3 b | 31.6 ± 1.8 a | 8.3 ± 0.3 c | 5.4 ± 0.6 b | 0.6 ± 0.1 b |
| Control | 29.8 ± 6.6 c | 3.6 ± 0.4 bc | 16.5 ± 3.2 ab | 35.4 ± 1.7 a | 8.6 ± 0.3 bc | 7 ± 1.5 b | 0.8 ± 0.1 b |
| *p*-value | <0.0001 | 0.0193 | 0.0421 | 0.3051 | 0.006 | 0.0002 | 0.0087 |

Numbers represent the mean ± standard error of four replicates of tomato plant for fruit weight, fruit number, flower number, shoot height, stem diameter, dry shoot biomass, dry root biomass. Different letters represent significant differences at $p<0.05$ within columns for each category according to Fisher's protected LSD test.

[y]Dry molasses (DM), Wheat bran(WB), C:N ratio 10:1 (10), C:N ratio 20:1 (20), C:N ratio 30:1 (30) and C:N ratio 40:1(40)

Table 3-6. Growth characteristics of 8-week-old tomato plant transplanted at 5 weeks old in pots after termination of ASD treatment (Trial 2)

| Carbon[y] | Fruit weight (g) | Fruit (no.) | Flower (no.) | Shoot height (cm) | Stem diameter (mm) | Dry shoot biomass (g) | Dry root biomass (g) |
|---|---|---|---|---|---|---|---|
| Control | 9.5 ± 3.6 c | 3.3 ± 0.6 b | 19.5 ± 3.1 ab | 31.9 ± 1 b | 8.6 ± 0.3 b | 5.6 ± 0.8 b | 0.9 ± 0.1 a |
| DM | 49 ± 5.4 a | 6.4 ± 0.5 a | 19.9 ± 3.5 a | 36.4 ± 1.5 a | 9.4 ± 0.2 a | 7.1 ± 0.6 a | 1 ± 0.1 a |
| WB | 25.4 ± 4.5 b | 6.4 ± 1 a | 20 ± 2.8 b | 32.6 ± 1.3 b | 8.4 ± 0.2 b | 5.4 ± 0.6 ab | 0.8 ± 0.1 a |
| *p*-value | <0.0001 | 0.011 | 0.9836 | 0.0463 | 0.0075 | 0.0496 | 0.2913 |
| C:N | | | | | | | |
| 10 | 53.1 ± 6.4 a | 7.4 ± 1.6 a | 17.9 ± 4.3 a | 34.6 ± 2.5 a | 9.5 ± 0.3 a | 6.6 ± 0.8 a | 1 ± 0.1 a |
| 20 | 28.4 ± 10 b | 6.9 ± 1.1 a | 22 ± 4.5 a | 35.9 ± 1.1 a | 8.8 ± 0.4 a | 6.1 ± 1.5 a | 1 ± 0.1 a |
| 30 | 34.9 ± 6 b | 7.4 ± 0.9 a | 20.5 ± 2.7 a | 35.6 ± 1.5 a | 8.6 ± 0.2 a | 6.4 ± 0.3 a | 0.8 ± 0.1 a |
| 40 | 32.3 ± 7.9 b | 4 ± 0.5 b | 19.5 ± 6.2 a | 31.9 ± 2.8 a | 8.6 ± 0.4 a | 5.8 ± 0.5 a | 0.8 ± 0.1 a |
| Control | 9.5 ± 3.6 c | 3.3 ± 0.6 b | 19.5 ± 3.1 a | 31.9 ± 1 a | 8.6 ± 0.3 a | 5.6 ± 0.3 a | 0.9 ± 0.1 a |
| *p*-value | 0.0003 | 0.0046 | 0.9854 | 0.2529 | 0.1717 | 0.2039 | 0.5686 |

Numbers represent the mean ± standard error of four replicates of tomato plant for fruit weight, fruit number, flower number, shoot height, stem diameter, dry shoot biomass, dry root biomass. Different letters represent significant differences at $p<0.05$ within columns for each category according to Fisher's protected LSD test.

[y]Dry molasses (DM), Wheat bran(WB), Carbon to nitrogen (C:N) C:N ratio 10:1 (10), C:N ratio 20:1 (20), C:N ratio 30:1 (30) and C:N ratio 40:1(40)

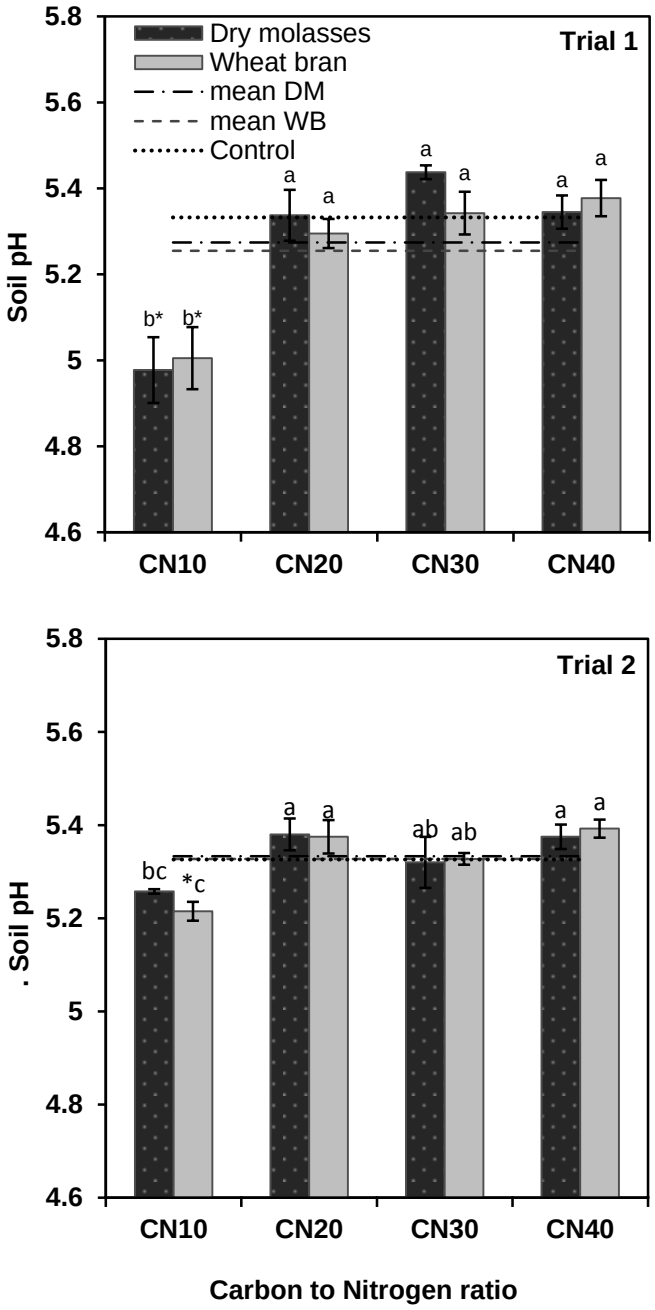


Figure 3-1. Effect of amendment C:N ratio on soil pH at treatment termination, pot study. Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's protected LSD test. Error bars indicate standard error with four replicates. Control=non-amended control, CN10=C:N ratio 10:1, CN20=C:N ratio 20:1, CN30=C:N ratio 30:1 and CN40=C:N ratio 40:1, DM=dry molasses, WB=wheat bran

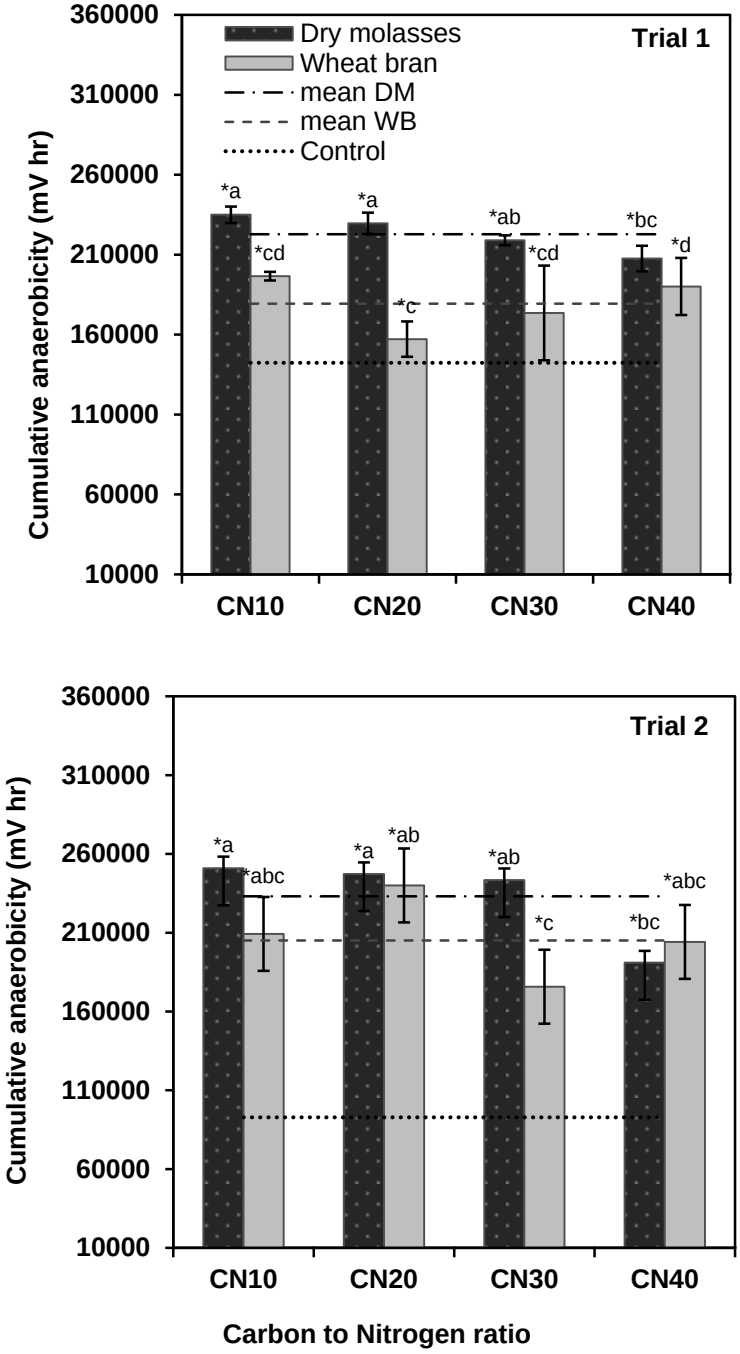


Figure 3-2. Effect of amendment C:N ratio on cumulative anaerobic condition during ASD, pot study.

Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's protected LSD test. Error bars indicate standard error with four replicates. Control=non-amended control, CN10=C:N ratio 10:1, CN20=C:N ratio 20:1, CN30=C:N ratio 30:1 and CN40=C:N ratio 40:1, DM=dry molasses, WB=wheat bran

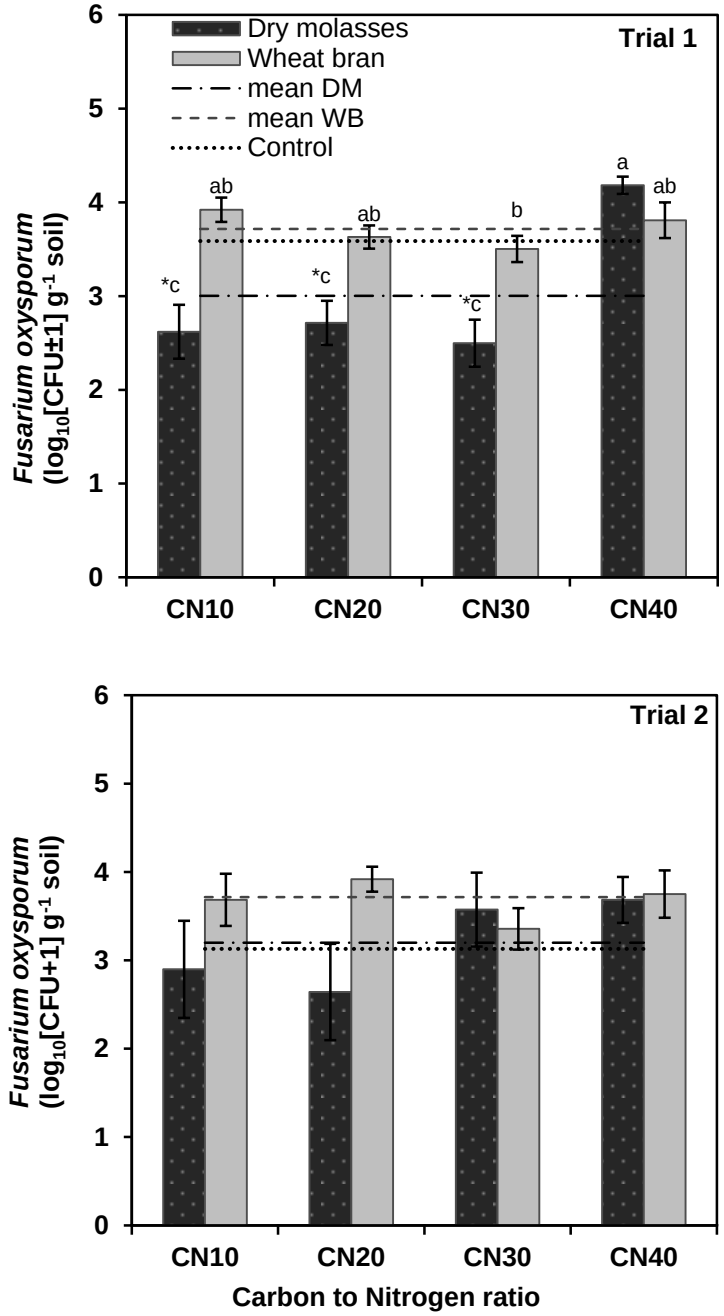


Figure 3-3. Effect of amendment and amendment C:N ratio on *Fusarium* populations during ASD treatment, pot study.

Bars indicated by different letters are significantly different, $p<0.05$ according to Fisher's protected LSD test. Error bars indicate standard error with four replicates. Control=non-amended control, CN10=C:N ratio 10:1, CN20=C:N ratio 20:1, CN30=C:N ratio 30:1 and CN40=C:N ratio 40:1, DM=dry molasses, WB=wheat bran

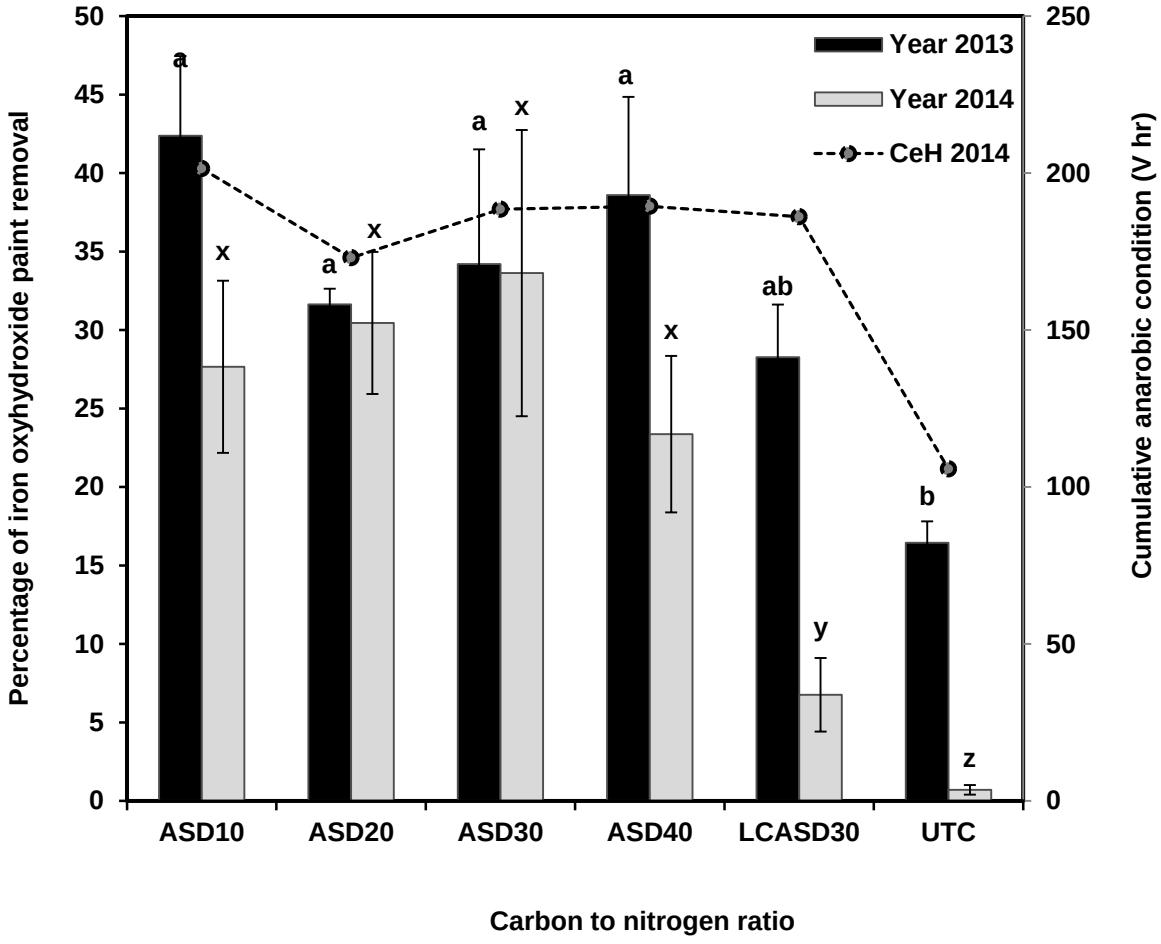


Figure 3-4. Effect of amendment C:N ratio on percentage of iron oxyhydroxide paint removal following ASD treatment, field study, 2013-2014.
Bars indicated by different letters are significantly different, $p<0.05$ according to Fisher's protected LSD test. ASD10=C:N ratio 10:1, ASD20=C:N ratio 20:1, ASD30=C:N ratio 30:1, ASD40=C:N ratio 40:1, LCASD30=C:N ratio 30:1, C rate 2 mg C g-1 soil (LC ± 'low carbon') and UTC=Untreated, non-amended control

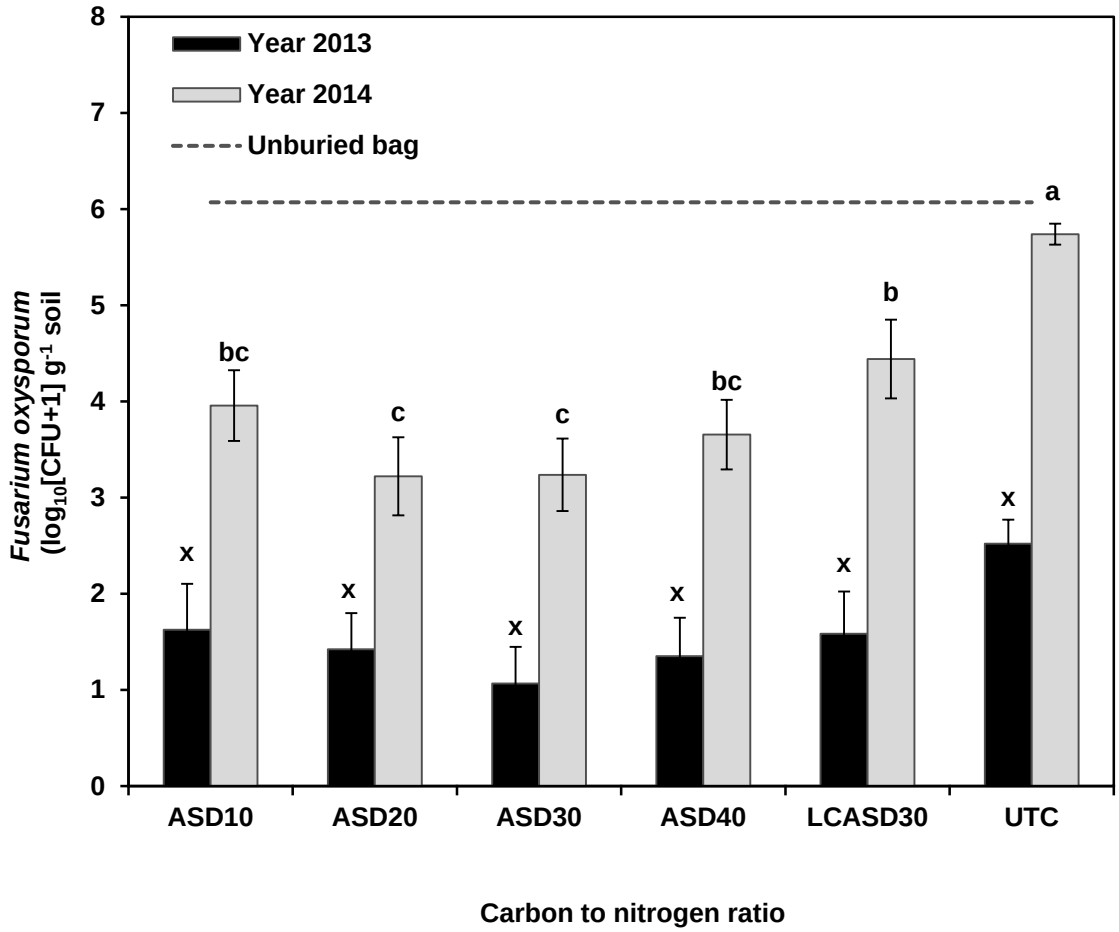


Figure 3-5. Effect of amendment C:N ratio on *Fusarium oxysporum* inoculum populations following ASD treatment, field study, 2013-2014.
Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's protected LSD test. The dashed line represents Fo populations (6.07 $\log_{10}$[CFU+1]$g^{-1}$ soil) from packets not buried in the field. Bars indicated by different letters are significantly different, $p<0.05$ according to Fisher's protected LSD test. ASD10=C:N ratio 10:1, ASD20=C:N ratio 20:1, ASD30=C:N ratio 30:1, ASD40=C:N ratio 40:1, LCASD30=C:N ratio 30:1, C rate 2 mg C g-1 soil (LC ± 'low carbon') and UTC=Untreated, non-amended control

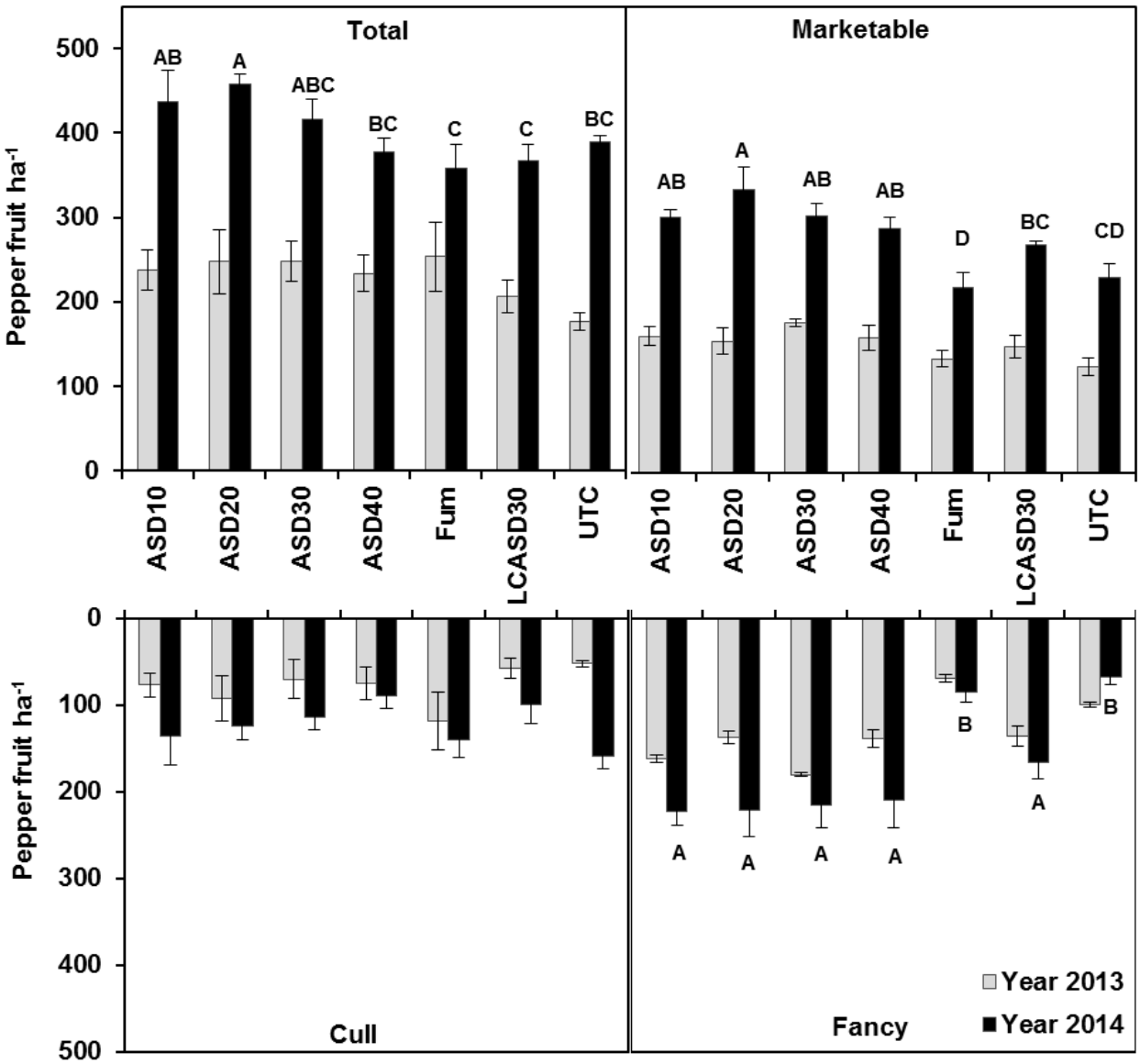


Figure 3-6. Mean pepper fruit numbers ha$^{-1}$ harvested for the year 2013 and 2014. i. total fruit, ii. marketable fruit (Fancy+US No.1 + US No.2), iii. Fancy and iv. culled fruit
Within yield class and year, means indicated by different letters are significantly different, Tukey test, $p<0.05$. Error bars represent std. error at n=4.

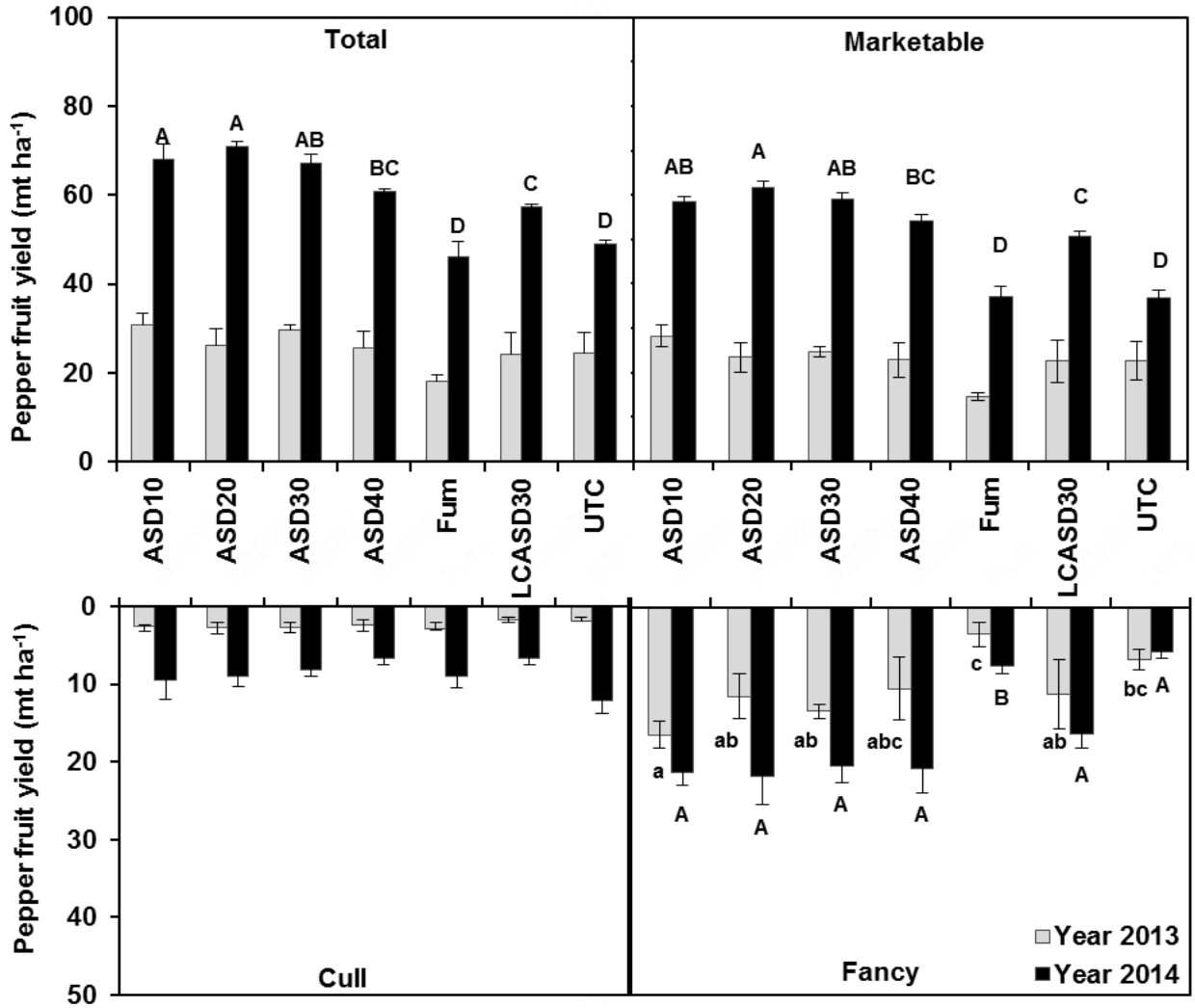


Figure 3-7. Mean pepper yield (mt $ha^{-1}$) for the year 2013 and 2014. i. total yield, ii. marketable yield (Fancy + US No.1 + US No.2), iii. Fancy and iv. culled yield.
Within yield class and year, means indicated by different letters are significantly different, Tukey test, $p<0.05$. Error bars represent std. error at n=4.

# Chapter 4

# Effects of amendment C:N ratio and carbon rate on germination and parasitism of sclerotia of *Sclerotium rolfsii* following anaerobic soil disinfestation

A version of this chapter is a manuscript in preparation for *Plant Disease* by Utsala Shrestha, Bonnie H. Ownley and David M. Butler.

My primary contributions to this manuscript include experimental setup, data collection and analysis, results interpretation and writing.

## Abstract

Growth chamber and field studies were carried out with dry molasses and/or wheat bran maintained at different C:N ratios (10:1, 20:1, 30:1 and 40:1) to evaluate the ASD effect on mortality and parasitism of *Sclerotium rolfsii*. A growth chamber study with dry molasses amendment at rates of 2, 4, 6, and 8 mg C $g^{-1}$ soil (C:N ratio 30:1) was also carried out with sclerotia of *S. rolfsii* to examine the optimum ASD amendment rate. Two polyethylene mesh bags with 10 sclerotia each were buried at 5- to 10-cm depths in amended field soil and in pots containing field soil and sand. Sclerotial germination and parasitism were accessed after three weeks of ASD treatment. In the pot study, there were no significant interactions among carbon amendment, C:N ratio and soil depth observed for sclerotial germination, decomposition and parasitism, while significant relationships were observed between sclerotial germination and parasitism by *Trichoderma* treatments. Sclerotial germination was significantly reduced in all amended pots regardless of C:N ratios (0.6-8.5%) and carbon rates (7.5-46%) as compared to non-amended controls (21-36% and 61-96%, respectively). In the field study, sclerotial germination was significantly reduced to 50% and ranged from 0.8-11%. Similarly, during ASD treatment, amendments had a significant positive impact on colonization of sclerotia by *Trichoderma* spp., with higher parasitism in all C:N ratio treatments, and at 2 or 4 mg C $g^{-1}$ soil (>80%) compared to the control. In the field study, sclerotial parasitism by *Trichoderma* spp. was predominant, however, other mycoparasites, i.e., *Aspergillus* spp., *Fusarium* spp., *Mucor* spp., and other fungi were present. Our results suggest that ASD with carbon amendment application at the rate of at least 4 mg C $g^{-1}$ soil induces optimum anaerobic soil conditions, facilitating suppression of sclerotia of *S. rolfsii* and enhances parasitism by beneficial fungi including *Trichoderma* spp.



**Keywords:** Anaerobic, amendments, C:N ratio, carbon rate, parasitism, *Sclerotium rolfsii*, *Trichoderma*

# 1. Introduction

With the mandated phase out of the ozone depleting fumigant, methyl bromide (MeBr), vegetables and small fruit growers are seeking for an alternative method to control plant diseases, pests, and weeds in their production system. This has led to the introduction of several alternatives to MeBr for high value crops. However, these alternatives have not met the technical superiority and satisfaction of the growers compared to the use of MeBr fumigation (Belova et al., 2013). In addition, these alternatives also face regulatory restrictions and limitations (Martin, 2003) because of one or more constraints like geographic limitations, lack of efficacy, human safety concerns, and accumulation of phytotoxic materials (Csinos et al., 2002). In this scenario, anaerobic soil disinfestation (ASD) has potential as a non-chemical alternatives to MeBr (Butler et al., 2012b;Shennan et al., 2014; Rosskopf et al., 2015). Initiated in Japan as biological disinfestation and as anaerobic disinfestation in the Netherland sixteen years ago, ASD has evolved as one of the effective measures to control soilborne pathogens and nematodes. Application of organic amendments in soil as a disease suppressor has been widely studied (Bonanomi et al., 2010; Bonanomi et al., 2013) but using this technique to control soilborne pathogens has increased after significant disease suppression was observed with ASD (Blok et al., 2000; Shinmura 2004). ASD emphasizes using easily available organic amendments (OAs) as a source of labile carbon (C) in saturated and covered soil to create anaerobic conditions, enhance biocontrol agents, and release organic acids and volatiles as toxic compounds to soilborne pathogens. Thus far, ASD effectiveness has been shown against soilborne fungal pathogens. There are few studies on oomycetes and bacterial pathogens.

*Sclerotium rolfsii* Saccardo [Teleomorph: *Athelia rolfsii* (Curzi)] is a sclerotia forming necrotrophic soilborne pathogen known to cause seedling damping off, root rot or stem rot. The disease is commonly called southern blight. *Sclerotium rolfsii* is economically important as it is cosmopolitan and infects on more than 500 plant species. In USA, *S. rolfsii* is reported mainly in the southern region with tropical and sub-tropical areas and a warm temperate climate (Punja, 1985; Xu et al., 2009; Gao et al., 2015; Mehta et al., 2015); however, *S. rolfsii* is also an active saprophyte surviving two to three years (Aycock, 1966) in a wide range of soil temperature and

soil pH (Coley-Smith et al., 1974). The fungus survives as sclerotia and can resist microbial attack (Smith et al., 2015).

The pathogen was reported to cause stem rot in peanut as early as 1919 in Tennessee (USDA, 1919), and to date the infection from sclerotia of *S. rolfsii* is reported to cause huge losses in specialty vegetables and small fruits (U. Shrestha, A.L. Wszelaki, and D.M. Butler, 2014, a UT Extension Publication). After the phase-out of MeBr fumigation, chemical control practices offer alternative options to control *S. rolfsii*. However, a gradual shift of consumer preferences to organic food and USDA increasing their financial support to organic growers, and better pricing of organic products (~$39 billion) in the US retail market (Herrick, 2016) have encouraged farmers towards organic farming. In Tennessee, more than 50% of organic food consumers wanted to increase their organic food option (Bhavsar et al., 2016) indicating increased opportunities for organic growers. However, management of diseases such as southern blight can be great concern to these growers including Tennessee local and non-chemical growers if other alternatives to conventional chemicals are not available.

Alternative options such as solarization are not feasible in Tennessee due to insufficiency of lethal temperature regime during the growing season. Although, biofumigation using brassica plants can be a feasible alternative to inhibit mycelial growth, the inhibition of sclerotial germination using crucifers and allyl isothiocynate seems to be cost-ineffective (Harvey et al., 2002; Reddy, 2012). As mentioned above, ASD can be a cost effective option for Tennessee and southeastern growers to control sclerotia of *S. rolfsii* as it utilizes locally available agriculture by-products in a plastic culture system. Most of the studies showed that using various types of amendments in ASD has reduced the viability of many sclerotial pathogens. Various amendments, ranging from agricultural by products, cereal brans, grasses, cruciferous crops to animal and poultry manures were incorporated in soil to test ASD effectiveness on sclerotial/microsclerotial germination (Rosskopf et al., 2015; Strauss and Kluepfel, 2015) of *Verticilium dahlia* (Blok et al., 2000; Thaning and Gerhardson, 2001; Goud et al., 2004; Shennan et al., 2007; Shennan et al., 2009), *Macrophomina* (Rosskopf et al., 2010; Rosskopf et al., 2014), and *Rhizoctonia solani* (Blok et al., 2000; Hewavitharana and Mazzola, 2013; McCarty et al., 2014). All of these pathogens were well suppressed by ASD treatment. Only a few studies on ASD technique with warm season cover crops in Florida (Butler et al., 2012b) and cool season

cover crop in Tennessee (McCarty, 2012) have been carried out in pots to see sclerotial germination. Reduction in sclerotial germination was reported in Florida trials with liquid molasses; however, the result was inconsistent between trials (Butler et al., 2012b). Similarly, in Tennessee trials only cereal rye showed a consistent reduction in sclerotial germination in both trials indicating that the forms of carbon supplements may have an effect on the sclerotial germination (McCarty, 2012). Thus, it is imperative to identify suitable carbon amendments and rate to control *S. rolfsii* because amendments play a critical role in determining suitable microbial population structure, decomposition rates (Akhtar and Malik, 2000), plant growth (Rodriguez-Kabana et al., 1987) and collectively the effectiveness of ASD (Butler et al., 2012b).

In this study, we tested efficacy of ASD using organic amendments at different rates and different carbon to nitrogen ratios (C:N) on the survival of sclerotia of *S. rolfsii*. In particular, our aims were: (i) to evaluate redox potential of soils treated with carbon amendments for ASD treatment, (ii) to examine suppression and parasitism of introduced sclerotia of *S. rolfsii* using dry molasses and wheat bran at different C:N ratios, and (iii) to examine suppression and parasitism of introduced sclerotia of *S. rolfsii* using dry molasses at different C rates (2 to 8 mg $Cg^{-1}$ of soil ) in growth chamber. We also examined the application of dry molasses at different C:N ratios and rates in field conditions.

## 2. Materials and Methods

### *2.1 Sclerotia production and inoculum preparation*

*Sclerotium rolfsii* was isolated from hybrid field tomatoes grown at the UT East Tennessee Research and Education Center, Knoxville, TN, in 2005. Sclerotia were grown in petri dishes (100 mm x 15 mm) with full-strength potato dextrose agar (PDA) for 7 days, and plugs were transferred to the center of freshly prepared PDA plates. Cultures were allowed to grow in the dark for 7 days at room temperature or until the mycelium grew to the edge of the petri dish. Cultures were placed in a cold room at 3.3°C for 4 hour, and then returned to room temperature and kept in the dark and cultures were allowed to dry to encourage formation of sclerotia. After 4 to 6 weeks, matured sclerotia were harvested, dried overnight under a laminar flow hood, and stored in a sterile glass vial until use in the experiment. Before setting up the growth chamber

study, ten sclerotia were sealed in a 5-cm × 5-cm permeable packet (aperture Delnet® polyolefin fabric, DelStar Technologies, Austin, TX) and string was attached for easy retrieval from soil.

### *2.2 Growth chamber study*

#### *2.2.1 Amendment and C:N ratio effect*

Soil (Dewey silt loam) was collected from a field at the UT Organic Crops Unit, Knoxville, TN, sieved through 10 mm aluminum metal mesh and mixed with fine sand at a 1:1 proportion. The basic properties of the air-dried soil mixture, such as soil pH, total C, total N, and total P, were recorded. The soil and sand mixture were placed in tall pots (12-cm diameter and 23-cm height, 2.5 l) with two pathogen packets at 5-cm and 15-cm depths. Soil treatments consisted of two main treatments i) dry molasses and ii) wheat bran amended with four C:N ratios 10:1, 20:1, 30:1, and 40:1; see Table 1). Control pots without any amendments were included for each main treatment. All the pots were saturated with tap water, covered with polyethylene, and secured with a heavy-duty rubber band. The pots were randomly positioned on the wire rack in the environmental growth chamber with relative humidity maintained at 50%, and temperature at 25°C during day and 15°C at night. The study was completely randomized design with four replications. Trials were carried out on 30 April and repeated on 22 May 2013.

#### *2.2.2 Amendment C rate effect*

To determine the effect of C rates on sclerotial germination and parasitism, a growth chamber study was conducted as previously described above on June 2014 and May 2015. The treatments included different carbon rates of dry molasses mixed with corn starch to maintain a C:N ratio of 30:1 (Table 4-1). Two non-amended controls, with or without sclerotial packets were included also. After 3 weeks of ASD incubation, two sclerotial packets were removed from each pot to examine sclerotial germination and parasitism, and 50 loose sclerotia were mixed within the top 2-cm of pot soil before treatment initiation to access disease pressure on tomato plant for which six-week-old tomato seedlings (‘Florida Lanai’) planted in each pot. The design was a completely randomized with four replications.

### *2.3 Field study*

Two packets of sclerotia were inserted in soil at 10-cm depth in the field established at the UT Plateau Research and Education Center in Crossville, TN, during the second week of May in 2013 and 2014, to assess the effect of ASD on the sclerotial germination and parasitism. The soil is classified in the Lily series (Fine-loamy, siliceous, semiactive, mesic Typic Hapludult). The detail experimental layout and treatment application is described in Chapter 3. In short, our experimental design was randomized complete block design with four replications and treatments are listed in Chapter 3.

### *2.4 Assessment of sclerotia following ASD treatment*

After ASD treatment (3 weeks), packets were collected from the pots and fields and were stored in sealed bag (Ziploc®) at 4°C until examined. Packets containing sclerotia were washed with tap water to remove adhering soil, sonicated for 1 min, and then surface-sterilized in 10% commercial bleach for 1 min, followed by 1 minute in 10% ethanol. Sclerotia were carefully removed from packets and plated onto PDA amended with 6.9 mg fenpropathrin/liter (Danitol 2.4 EC, Valent Chemical, Walnut Creek, CA) and 10 mg/liter rifampicin (Sigma-Aldrich, St. Louis, MO), and incubated at room temperature for 4 to 6 weeks. Germination of sclerotia was confirmed with mycelial growth and production of new sclerotia. Parasitism by *Trichoderma* spp. and other microbial antagonists were observed simultaneously.

### *2.5 Measuring soil moisture, temperature, pH, and anaerobicity*

Each pot in the growth chamber study was equipped with oxidation-reduction electrodes (Combination ORP Electrode, Sensorex Corp., Garden Grove, CA, USA) at a15-cm depth to measure cumulative redox potential. The temperature of the soil mixture of each representative treated pots (two replicates) were recorded using temperature-moisture sensors at 10-cm (5TM Soil Moisture Probe, Decagon Devices, Pullman, WA, USA). Soil samples (0- to 10-cm depth) from each pot were collected for gravimetric soil moisture content and air-dried soil were used to determine soil pH, which was done using 0.01 M $CaCl_2$ buffer using a pH electrode (Orion 3-Star Plus pH Benchtop Meter, Thermo Scientific, Waltham, MA, USA). The soil pH taken in $CaCl_2$ was later adjusted to soil water pH by adding 0.6 units. The cumulative anaerobic

condition of soil over a three-week treatment period was calculated as described in Butler et al. (2012a). In field trials, anaerobic condition was determined by iron oxihydroxide coated IRIS tubes. Tubes were inserted in each plot at a 5- to 15-cm depth before irrigation and were retrieved after the three-week treatment, cleaned with tap water and removal of paint was assessed as described by Rabenhorst (2012). Ten soil cores from 5- to 15-cm depth were collected and composited from each plot for soil moisture and soil pH.

### *2.6 Data analysis*

Data were analyzed with Mixed Model Analysis of Variance (MMAOV) macro program in SAS 9.4 (SAS Institute, Cary, NC) and differences between means were determined using Tukey's test ($p<0.05$). In repeated trials in the growth chamber study, carbon and C:N ratio effects were analyzed separately as to completely randomized factorial analysis designs, where C amendments, C:N ratios and depth were treated as fixed factors. Data were analyzed separately by C amendment and depth to compare treatments with untreated control.

The growth chamber study with carbon rate was a completely randomized design with carbon rate as fixed factor, and field study was a randomized complete block design. The data were checked for outliers before analysis and non-normal and unequal variances were transformed using arcsinsqrt or rank transformations specified (DAWG 2004). Untransformed means and standard error of the mean were reported. Relationships between soil moisture, cumulative anaerobic condition and sclerotia germination and parasitism were assessed with correlation analysis at $p<0.05$.

## 3. Results

### *3.1 Growth chamber examination of ASD amendment and C:N ratio effect*

A significant negative moderate correlation between sclerotial germination and Ceh (-0.4, $p<0.002$) was observed in Pot assay 1, while only a weak relationship between soil pH, and Ceh and gravimetric soil moisture was observed in pot assay 2 with correlation value of 0.3 ($p<0.01$). No interaction among carbon amendment, C:N, and soil depth were observed for sclerotial germination, decomposition, and parasitism in both trials.

### *3.1.1 Soil characteristics and anaerobic conditions*

Mean soil temperatures did not differ across treatment and ranged from 28.4 to 29.4°C during the day and 18.4 to 19.3°C during the night. The volumetric water content of soil was higher in control pots and lowest in wheat bran treated pots (data not shown). In contrast, gravimetric soil moisture content was lowest in controls in pot assay 2 (Table 4-2). Gravimetric soil moisture content was lower in pot assay 1 than pot assay 2. Soil pH after the anaerobic condition was attained and did not differ across carbon amendment, but differed across C:N ratio. The lowest soil pH was recorded in C:N10 (4.9 and 5.3, pot assay 1 and pot assay 2, respectively, Figure 4-1A). The interaction between amendment and C:N ratio was observed for soil pH but was only significant in pot assay 1 ($p<0.04$) with high pH for dry molasses treated pots. Soil pH increased as C:N ratio increased from C:N10 to C:N30 for dry molasses. Mean cumulative anaerobic condition did not differ among carbon treatments in both assays, but was significantly different from untreated non-amended pots with the lowest value of 62.48 V h (Figure 4-1B). No interaction was observed between amendments and C:N ratio and the highest mean anaerobic condition for both assays were recorded for dry molasses (191V h) and C:N10 (210 V h).

### *3.1.2 Sclerotial germination and parasitism*

Carbon amendments reduced sclerotial germination compared to untreated controls in both assays ($p<0.001$). In pot assay 1, wheat bran amendment gave the highest sclerotial mortality (Figure 4-3A). Both C amendments had a significant positive impact on colonization of sclerotia by *Trichoderma* spp., with higher parasitism in all C:N ratio treatments (93 to 95% in pot assay 1 and 80 to 84% in pot assay 2) compared to controls (80.8 and 54% respectively, Table 4-2A). Parasitism by other mycoparasites *Fusarium* and other unidentified fungi was observed, but were not different among carbon treatments (Table 4-3) and C:N ratios, except for unidentified fungi in pot assay 1 (Figure 4-2). When percentage sclerotial parasitism by *Trichoderma* was assessed for pathogen packets retrieved from soil depths of 5-cm (top) and 15-cm (bottom), parasitism by *Trichoderma* was greater at deeper soil depth (97%) in all treatments than from lower depth (88%) in pot assay 2 (Figure 4-3B). In contrast, in the same study greater percentage of *Fusarium* and other fungi was determined for sclerotia retrieved from the 5-cm depth (Figure 4-3C, D). Among the treatments, sclerotial germination was lowest for carbon amendments at 5-

and 15-cm depths compared to untreated controls in pot assay 2, while for dry molasses sclerotial germination did not differ significantly from controls in pot assay 1 (Figure 4-3A). Colonization of *Trichoderma* was highest at the 5-cm depth for pot assay 2 for dry molasses (95%) and wheat bran (97%), and in pot assay 1, colonization ranged from 90 to 98% for both carbon treatments at both depths (Figure 4-3B). Sclerotial parasitism by *Fusarium* was the highest for wheat bran at 15-cm depth (Figure 4-3C). Sclerotial decomposition was not significantly affected by carbon, C:N ratio or depth of soil (Figure 4-1D).

### *3.2 Growth chamber examination of ASD amendment C rate*

There was a significant moderate negative correlation of anaerobic condition on sclerotial germination and a positive correlation on *Trichoderma* parasitism (±0.6, $p<0.0001$) in both carbon rate studies. Only *Trichoderma* parasitism was negatively correlated with sclerotial germination ranging from 0.6 to 0.7 at $p<0.005$. *Mucor* had a negative, moderate correlation with sclerotial germination in both trials and positive correlation with *Trichoderma* only in trial 2. *Fusarium* and bacterial parasitism on sclerotia showed moderate positive correlation (0.4-0.5, $p<0.05$).The data for the two depths were pooled as no difference was observed for the depth of sclerotial packets in the soil.

#### *3.2.1 Soil characteristics and anaerobic conditions*

Average volumetric soil moisture in trial 1 ranged from 0.27 to 0.28 $cm^3$ $cm^{-3}$, and in trial 2, soil moisture was 0.23-0.27-$cm^3$-$cm^{-3}$ in covered pots. Uncovered pots had 0.01 $cm^3$ $cm^{-3}$. Mean soil temperature did not differ among carbon treatments during the treatment period, ranging from 21.3 to 26.2°C for trial 1, and 19.9 to 22°C for trial 2. Average volumetric soil moisture content differed among treatments in both trials ranging from 0.2 to 0.3 g $g^{-1}$. Gravimetric soil moisture content was not recorded for this study. We did not see any effect of depth of sclerotial burial on sclerotial germination, or parasitism by *Trichoderma* for this study. No differences were observed among carbon rate treatments for soil pH (Figure 4-4A). The cumulative anaerobic condition was not affected by the amendment carbon rates of 2 to 8 mg $kg^{-1}$; however; it was significantly higher (141 to158 V h in trial 1 and 107-150 V h in trial 2) than anaerobic condition in the covered irrigated control and the uncovered irrigated control pots (0 to18 V h, Figure 4-

4B). Soil pH in trial 2 was relatively higher than trial 1 but none of the treatments differed in soil pH value in both studies.

### *3.2.2 Sclerotial germination and parasitism*

All ASD conditions, especially with carbon supplement, were effective in suppressing sclerotial germination compared to the control. However, sclerotial germination over two studies showed contrasting results. In trial 1, higher carbon level (>2 mg C $g^{-1}$ of soil) was the most effective in reducing sclerotial germination averaging 7.5% in C4 to 13.9% in C8. While in trial 2, carbon rates at 8 mg C $g^{-1}$ of soil or lower were more effective in repressing sclerotial germination (Figure 4-4C).

Sclerotial parasitism by *Trichoderma* was higher at all levels of carbon amendment compared to the controls in both trials (Figure 4-4D). However, in trial 1, the anaerobic condition supplemented with any level of carbon amendments was more effective for parasitism of sclerotia (54 to 98%) compared to controls. In the anaerobic condition without carbon supplement, in trial 2 there were no significant differences between the anaerobic condition without carbon supplement and the anaerobic condition supplemented with a higher carbon source (82%, C6). Nevertheless, anaerobic conditions supplemented with a lower level of carbon (89% with C6 to 98% with C4) were significantly more effective in parasitism of sclerotia suggesting that a higher carbon rate (<6 mg C $g^{-1}$ soil) is not required to control sclerotia. Sclerotial parasitism by bacteria and *Mucor* were observed in all carbon treatments with the highest percentage of parasitism recorded for C6 (32% and 14%, respectively in trial 1) and C8 (25% and 15%, respectively in trial 2). *Fusarium* colonization in both trials and other unidentified fungi in trial 1 were observed (Figure 4-5). This indicates that besides *Trichoderma*, other diverse microbes parasitize sclerotia, especially when carbon rates are higher.

After termination of the ASD treatment in growth chamber studies, pots were transferred to the greenhouse where 5-week-old tomato seedling were transplanted into potting mix and wilting and stem colonization were examined each week. Although, some yellowing and dry rot leaf symptoms of early blight (*Alternaria solani*) were observed in both trials, occurrence of southern

stem blight was not observed. The mean fruit number was significantly higher for pots amended with carbon rate 4 mg C $g^{-1}$ of soil (Table 4-4).

### *3.3 Field examination of ASD effect on anaerobic conditions, sclerotial germination and parasitism*

In the field studies, no significant correlations of sclerotial germination with soil moisture, soil pH, and parasitism by mycoparasites were observed in trial 1. However, there was a moderate negative relationship of *Trichoderma* parasitism and IRIS tube paint removal ($p<0.04$) and positive relationship with soil moisture, initial soil pH, other fungi and *Aspergillus* parasitism of sclerotia in 2013 (Pearson correlation = 0.4, $p<0.04$). In year 2014, sclerotial germination had a moderate negative correlation with *Trichoderma* parasitism of sclerotia (Spearman correlation = 0.7, $p<0.001$). In addition, soil pH and IRIS tube paint removal was moderately correlated (Spearman correlation = 0.4, $p<0.01$).

Average soil temperatures did not differ among ASD treatments in either field study. In field assay 1, mean soil temperatures ranged from 22 to 25°C and in field assay 2, slightly higher mean soil temperatures were observed with a range between 24°C and 25°C. Before treatment application, gravimetric soil moisture ranged between 2.5 to 2.7 g $g^{-1}$ and slightly higher gravimetric soil moisture were recorded in field assay 2, ranging from 2.7 to 3.1 g $g^{-1}$ (Table 4-5). Soil moisture content in field assay 1 after a three-week incubation period was significantly high in ASD20 (3.1 g $g^{-1}$) while high gravimetric soil moisture ranged from 2.9 to 3.9 g $g^{-1}$. No decline in soil pH among treatments was observed in both studies. Cumulative anaerobic condition for field indicated by the IRIS paint removal percentage study is reported in Chapter 2 (Figure 3-4). The maximum paint removal in carbon treated pots with 4 mg C $g^{-1}$ of soil regardless of C:N ratio with 31 to 42% in 2013, and 23 to 33% in 2014. These results suggest enhanced anaerobic condition. Lower anaerobic activity was observed for ASD treatments, with a carbon rate of 2 mg C $g^{-1}$ soil (6 to 28%) compared with the untreated control (1 to 16%).

In 2013, sclerotial germination was relatively low among C-amended treatments, ranging from 1% in ASD40 to 11% in ASD10 and 20, compared to the untreated control (27%), but this difference was not significant ($p=0.06$). However, in the 2014 study, the germination of sclerotia

was significantly low in all carbon treated plots (2 to 31%) as compared to control (31%, Figure 4-6A). This is attributed to higher sclerotial parasitism by *Trichoderma* spp., *Fusarium* spp., *Mucor* and other fungi in all carbon amended pots (Figure 4-6B, C, D). We also observed *Aspergillus* parasitism of sclerotia in 2013 and bacterial parasitism in 2014. Surprisingly, the results were not significant in both studies ($p$=0.07 for *Trichoderma* and $p$>0.1 for all fungal parasitism) except for *Mucor* in 2014 ($p$<0.02).

## 4. Discussion

Results from various studies have provided strong evidence that ASD is effective for control of various soilborne pathogens (see Chapter 1). Application of appropriate amendments at appropriate rates is crucial to increase ASD effectiveness. Amendments and soil C:N ratios always impact microbial populations (Shaban et al., 1998; Akhtar and Malik, 2000), and plant growth (Rodriguez-Kabana et al., 1987). Our previous study showed that ASD with different amendment C:N ratios was effective for control of yellow nutsedge (see Chapter 2). More specifically, a C:N ratio of 10:1 was found to be the best for yellow nutsedge tuber germination and production. In this study, similar to yellow nutsedge and the *Fusarium* study, we tested the effectiveness of ASD using dry molasses or wheat bran as a carbon source that had been adjusted with soybean meal or corn starch to form different C:N ratios. In parallel with our previous studies, this study also provided evidence that ASD is effective in controlling *S. rolfsii*.

Effectiveness for control of sclerotia was measured in terms of sclerotial germination and sclerotial parasitism by various soil microorganisms. The majority of sclerotia recovered from ASD treatments failed to germinate. Among the sclerotia germinated, most of sclerotia were incapacitated by mycoparasites and only a few germinated without any parasitism. The presence of endemic *Trichoderma*, *Fusarium*, and other fungi as mycoparasites of sclerotia revealed that sclerotia were either weaken or lysed post-ASD treatment. Sclerotial parasitism by *Trichoderma* was abundant in all studies; however, we also observed *Aspergillus* parasitism in field assay 1 and bacterial parasitism in field assay 2. *Trichoderma* spp. are the most studied mycoparasites that are known to produce bioactive metabolites (Ownley et al., 2009), and produce enzymes like β-1,3-glucanase and chitinase as a means to degrade sclerotial protective outer layer (Elad et al., 1984) or reduce of hyphal growth (Madi et al., 1997). It has been reported that eruptive

germination of sclerotia in soil or breaking of the rind due to treatment effect can release nutrients that stimulate the colonization of sclerotia (Smith, 1972). We also observed zygomycetes (*Rhizopus*, *Mucor*) as sclerotial parasites from the field soil that might have attributed more nitrate accumulation in the soil. The presence of *Mucor* is supposed to cause ammonium toxicity to sclerotia in soil. However, we did not observe any significant suppression of sclerotia by other mycoparasites as compared to the control treatment. In addition, it is also expected that sclerotia can be targeted by anaerobic bacteria especially Clostridia (Adandonon et al., 2015); however, our experiment did not test sclerotial decomposition by anaerobic bacteria.

With the application of amendments, regardless of C:N ratio and C rates, high cumulative anaerobic conditions were recorded within 3 weeks of ASD treatment, which was similar to our previous studies. Consistent relationships among anaerobic conditions, sclerotial germination, and *Trichoderma* parasitism of sclerotia was observed only in the growth chamber studies with carbon rate effect. In the pot study, C:N ratio10:1 had the lowest soil pH, which was similar to previous pot studies conducted for nutsedge and *Fusarium*. We also found that in ASD treatments, endemic *Trichoderma* parasitized higher number of sclerotia compared to the control. However, under field condition, sclerotial colonization by *Trichoderma* was lower than in pot studies and not significant. This could be due to diverse microorganism present in field soil that could lead to more competition for parasitism. In addition, we did not observe significant differences among different C:N ratios to repress sclerotial germination or sclerotial parasitism by *Trichoderma* in both pot and field conditions, although *Trichoderma* populations are reported to increase in soil with high soil C:N ratio (40:1) when amendment with glucose or cellulose is applied (Shaban et al., 1998). Overall, our growth chamber studies showed that ASD was effective in reducing sclerotial germination.

We observed that a carbon rate of 4 mg C $g^{-1}$ soil was the optimal amount of carbon to suppress sclerotial germination. Likewise, using 2 or 4 mg C $g^{-1}$ soil with ASD treatment resulted in the highest sclerotial parasitism by *Trichoderma*. Overall, these results suggest that 4 mg C $g^{-1}$ soil was the optimum amount of carbon to be used with ASD for sclerotial mortality. Further, studies with 4 mg C $g^{-1}$ soil with different amount of nitrogen to adjust different C:N ratio also showed higher mortality of sclerotia.

Studies have suggested that increased depth of sclerotial burial increased the mortality of the sclerotia (Imolehin and Grogan, 1980; Smith et al., 1989) as increased in soil pressure resulted in sclerotial substrate leakage (Punja et al., 1984) and presence of soil moisture further compacted the soil during ASD. In our study, the effectiveness of carbon supplements at different C:N ratio to suppress sclerotia was not effective at deeper depth (15-cm) in pot assay 1 which may be attributable to low moisture (Table 4-2) as sclerotia are susceptible to exposure to excess moisture for more than 50 days (Moore, 1949; Abawi et al., 1985). Overall, ASD treatment using both dry molasses and wheat bran as carbon amendments was more effective in inducing sclerotial mortality. In the carbon rate study with dry molasses, we observed higher sclerotial germination in trial 2, compared to trial 1.This could be due to the use of fresher sclerotia in trial 2 that were not as dry as sclerotia that were used in trial 1 and Smith et al. (1989) reported that dried sclerotia may have reduced longevity. Besides the trial 2 carbon rate study, we used sclerotia produced on PDA in our experiments, rather than sclerotia produced in soil which confirms the presence of parasites after ASD treatment were solely from soil in which they were buried.

## 5. Conclusion

In short, our results showed that ASD can effectively reduce sclerotial germination percentage. With ASD treatments, the majority of sclerotia failed to germinate, and if they germinate, *Trichoderma* in most of the cases parasitized those sclerotia and in some instances, other fungi parasitized sclerotia. We observed that this phenomenon was more efficient at lower soil depth (5-cm) than higher soil depth (15-cm). We also observed that 4 mg C $g^{-1}$ soil was the optimal amount of carbon amendments to add. However, as we presumed, we did not observe any difference in the effect of different C:N ratios on sclerotial mortality. Thus, our results support the notion that ASD can be one of the alternatives to control *S. rolfsii*. Addition of dry molasses or wheat bran as organic amendments not only reduce pathogen inoculum, but also contribute to increased population of beneficial organisms that suppress *S. rolfsii* in the soil (Beute and Rodriguez-Kabana, 1981). Moreover, organic matter enrichment obtained with addition of amendments, which is important to ameliorate soil structure and soil fertility (Bulluck et al., 2002; Bailey and Lazarovits, 2003; Blum and Rodríguez-Kábana, 2004).

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# Appendix

Table 4-1. Treatment: carbon to nitrogen ratio, carbon amendments, and amendments rates

| Treatments label | C:N ratio | Carbon rate | Rate of amendments | | |
|---|---|---|---|---|---|
| | | | Wheat bran | Soybean meal | Corn starch |
| Carbon:Nitrogen pot study | | mg C $g^{-1}$ soil | | g $kg^{-1}$ of soil | |
| C:N10 | 10:1 | 4 | 7.8 | 1.8 | - |
| C:N20 | 20:1 | 4 | 6.4 | - | 3.3 |
| C:N30 | 30:1 | 4 | 4.2 | - | 5.6 |
| C:N40 | 40:1 | 4 | 3.2 | - | 6.7 |
| | | | Dry molasses | Soybean meal | Corn starch |
| C:N10 | 10:1 | 4 | 6.4 | 3.6 | - |
| C:N20 | 20:1 | 4 | 9.4 | 0.9 | - |
| C:N30 | 30:1 | 4 | 10.3 | - | 0.1 |
| C:N40 | 40:1 | 4 | 7.7 | - | 2.6 |
| Carbon rate pot study | | | | | |
| ASD2 | 30:1 | 2 | 5.1 | - | 0.04 |
| ASD4 | 30:1 | 4 | 10.3 | - | 0.09 |
| ASD6 | 30:1 | 6 | 15.4 | - | 0.13 |
| ASD8 | 30:1 | 8 | 20.5 | - | 0.17 |
| Field Study | | | | kg $m^{-2}$ | |
| ASD10 | 10:1 | 4 | 0.86 | 0.48 | - |
| ASD20 | 20:1 | 4 | 1.26 | 0.12 | - |
| ASD30 | 30:1 | 4 | 1.38 | 0.01 | - |
| ASD40 | 40:1 | 4 | 1.03 | 0.35 | - |
| lowASD 30 | 30:1 | 2 | 0.69 | 0.01 | - |

Table 4-2. Mean gravimetric soil moisture, soil pH and anaerobic condition during treatment and post treatment from C:N ratio pot assay conducted in the growth chamber

| Carbon treatments | Gravimetric soil moisture | Soil pH | Soil anaerobic condition |
|---|---|---|---|
| | $g\ g^{-1}$ | units | V h |
| | | Pot assay 1 | |
| CTRL | 0.22 ± 0 | 6 ± 0 | 42.7 ± 15.3 b |
| Dry molasses | 0.22 ± 0 | 5.9 ± 0.1 | 185.3 ± 10.6 a |
| Wheat bran | 0.22 ± 0 | 5.8 ± 0.1 | 157.6 ± 12.8 a |
| *p value* | 0.607 | 0.4923 | <0.0001 |
| | | Pot assay 2 | |
| CTRL | 0.24 ± 0 b | 5.3 ± 0 | 82.3 ± 20.3 b |
| Dry molasses | 0.27 ± 0 a | 5.4 ± 0.1 | 196 ± 10.4 a |
| Wheat bran | 0.26 ± 0 a | 5.3 ± 0.1 | 165.2 ± 10 a |
| *p value* | 0.0026 | 0.1889 | <0.0001 |

Within column, means ± standard error indicated by different letters are significantly different, Tukey Test $p<0.05$. CTRL= non-amended, covered control without sclerotia.

Table 4-3. Effect of carbon soil treatment on sclerotia of *Sclerotium rolfsii* in pot assay, C:N ratio growth chamber study, 2013

| Carbon treatment | Sclerotial germination | *Trichoderma* parasitism of sclerotia | Fusarium parasitism of sclerotia | Sclerotial decomposition | Parasitism by unidentified fungi | Parasitism by fungi other than *Trichoderma* |
|---|---|---|---|---|---|---|
| | Percentage | | | | | |
| | Trial 1 | | | | | |
| CTRL | 21.5 ± 5 a | 81 ± 4.3 b | 2.5 ± 1.4 | 2.5 ± 1.9 | 1.9 ± 1 b | 4.4 ± 1.6 |
| Dry molasses | 11.2 ± 3.2 b | 91.8 ± 2.6 a | 0.7 ± 0.5 | 4.1 ± 1.4 | 4.5 ± 1.4 a | 5.2 ± 1.4 |
| Wheat bran | 2.5 ± 1.1 c | 96.1 ± 1.7 a | 3.6 ± 2.1 | 2.2 ± 1.2 | 1.6 ± 0.8 b | 5.2 ± 2.4 |
| *p value* | <0.0001 | 0.002 | 0.44 | 0.38 | 0.144 | 0.41 |
| | Trial 2 | | | | | |
| CTRL | 38.5 ± 9 a | 54.2 ± 9.2b | 8.8 ± 2.8 | 5 ± 2.2 | 3.2 ± 1.5 | 12 ± 3.2 |
| Dry molasses | 3.8 ± 1.5 b | 77.4 ± 5.2 a | 11 ± 3.1 | 5.3 ± 2.3 | 13.3 ± 3.7 | 24.2 ± 5.2 |
| Wheat bran | 3.4 ± 2.6 b | 84.8 ± 4.5 a | 12.6 ± 3.7 | 4.1 ± 1.8 | 5.9 ± 1.8 | 18.5 ± 4.4 |
| *p value* | <0.0001 | 0.004 | 0.9 | 0.6 | 0.1 | 0.49 |

Within column, means ± standard error indicated by different letters are significantly different, Tukey Test $p<0.05$. CTRL= non-amended, covered control without sclerotia.

Table 4-4. Growth characteristics of 8-week-old tomato plant transplanted at 5 weeks after termination of ASD treatment sclerotia inoculated pots, carbon rate growth chamber study

| Carbon rate[a] | Mean fruit number | | Mean fruit Weight | |
|---|---|---|---|---|
| | per pot | | g pot$^{-1}$ | |
| | Trial 1 | Trial 2 | Trial 1 | Trial 2 |
| C-2 | 7.3 ±0.9 b | 2.3 ± 1.1 | 164.9 ± 19.7 | 15.6 ± 10.5 |
| C-4 | 17.7 ± 6.6 a | 3.3 ± 1.8 | 159.1 ± 14.1 | 21.8 ± 11.9 |
| C-6 | 6 ± 0.4 b | 5 ± 1.8 | 138.5 ± 17 | 22.1 ± 7 |
| C-8 | 5.5 ± 0.9 b | 5.8 ± 2.1 | 136 ± 27.2 | 26.9 ± 10.3 |
| CTRL | 4.5 ± 1.9 b | 1 ± 0.4 | 82.9 ± 9.3 | 10.1 ± 4.2 |
| CTRL_S | 8.3 ± 2.4 b | 1 ± 0.7 | 152.2 ± 9.5 | 5.5 ± 3.2 |
| C-0 | 6 ± 1.1 b | 1 ± 1 | 130.7 ± 39.6 | 6.1 ± 6.1 |

Within column, means ± standard error indicated by different letters are significantly different, Tukey Test $p<0.05$.

[a]CTRL=non-amended, uncovered control without sclerotia, CTRL_S=non-amended covered control with sclerotia=C-2=Carbon rate 2 mg C g$^{-1}$ soil, C-4=Carbon rate 4 mg C g$^{-1}$ soil, C-6=Carbon rate 6 mg C g$^{-1}$ soil, C-8=Carbon rate 8 mg C g$^{-1}$ soil and C-0=Carbon rate 0 mg C g$^{-1}$ soil

Table 4-5. Effect of C: N ratio soil treatment on soil gravimetric moisture content (g $g^{-1}$) in field conditions

| C:N[a] | Field assay 1 Year 2013 | | Field assay 2 Year 2014 | |
|---|---|---|---|---|
| | Pre ASD | Post ASD | Pre ASD | Post ASD |
| ASD10 | 2.5 ± 0.16 | 2.9 ± 0.05 ab | 2.7 ± 0.19 | 3.4 ± 0.24 |
| ASD20 | 2.6 ± 0.09 | 3.1 ± 0.1 a | 2.9 ± 0.11 | 2.9 ± 0.29 |
| ASD30 | 2.7 ± 0.07 | 2.8 ± 0.15 b | 3.1 ± 0.26 | 3.9 ± 0.55 |
| ASD40 | 2.5 ± 0.05 | 2.5 ± 0.17 cd | 3 ± 0.08 | 2.9 ± 0.35 |
| Fum | 2.5 ± 0.13 | 1.5 ± 0.53 d | 2.7 ± 0.05 | 3.3 ± 0.33 |
| LCASD30 | 2.7 ± 0.1 | 2.9 ± 0.08 ab | 2.8 ± 0.11 | 3.1 ± 0.31 |
| UTC | 2.5 ± 0.05 | 2.8 ± 0.03 bc | 2.8 ± 0.1 | 3.2 ± 0.29 |

[a]Treatments are ASD10=C:N ratio 10:1, ASD20=C:N ratio 20:1, ASD30=C:N ratio 30:1, ASD40=C:N ratio 40:1, LCASD30=C:N ratio 30:1, C rate 2 mg C g-1 soil (LC ± 'low carbon'), Fum= Fumigated control, (LC ± 'low carbon') and UTC= Untreated, non-amended control.

Within column, means ± standard error indicated by different letters are significantly different, Tukey Test $p<0.05$.

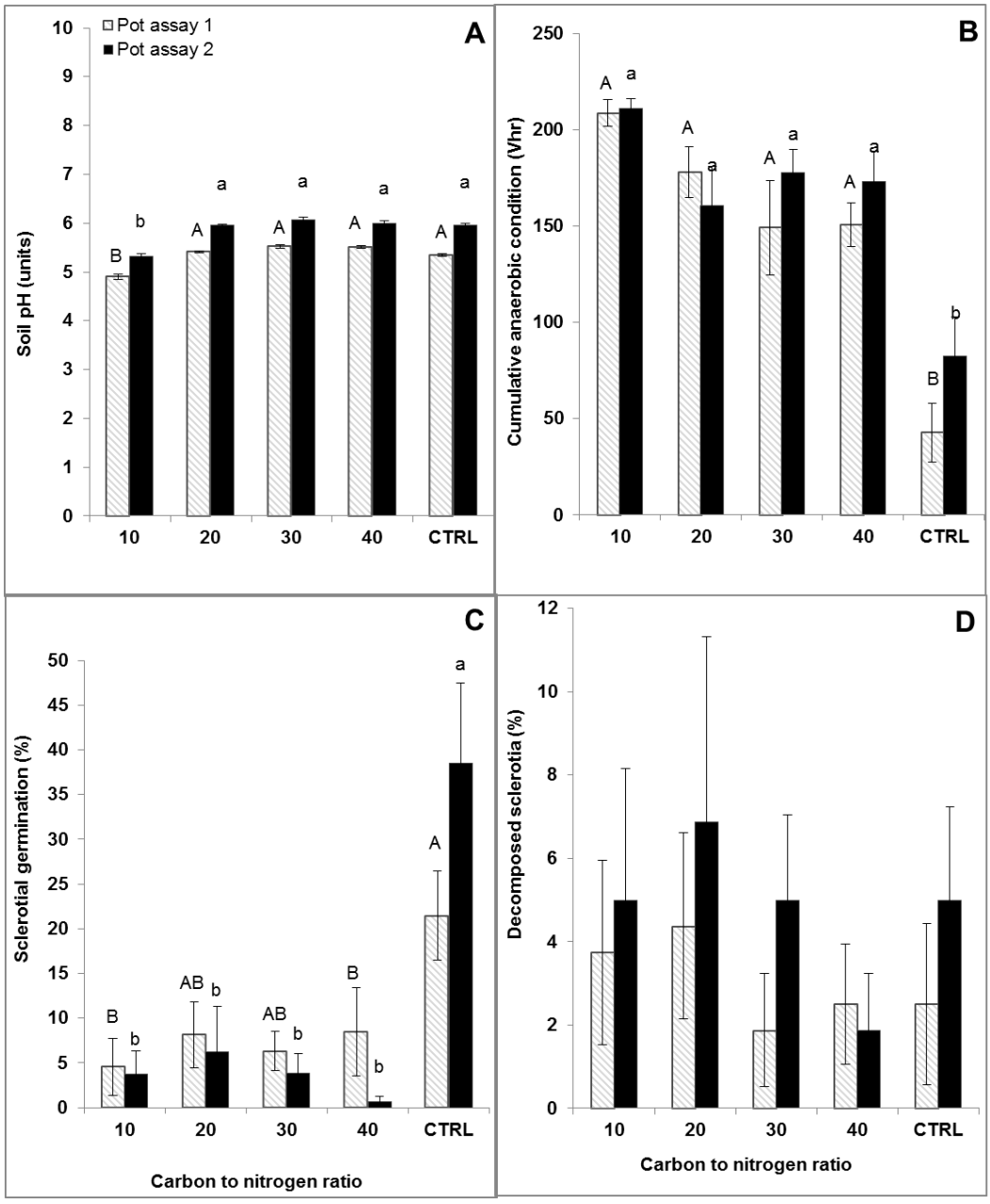


Figure 4-1.Effect of amendment C:N ratio on soil pH (A), cumulative anaerobic condition (B), percentage sclerotial germination (C), and percentage decomposed sclerotia (D) during ASD treatment, pot study.

Bars indicated by different letters are significantly different at $p<0.05$ according to Tukey's test. Capital letters are used to compare the respective means of pot assay 1 and small letters are used to compare respective means of pot assay 2. Error bars indicate standard error with four replicates. CTRL=non-amended control, 10=C:N ratio 10:1, 20=C:N ratio 20:1, 30=C:N ratio 30:1 and 40=C:N ratio 40:1.

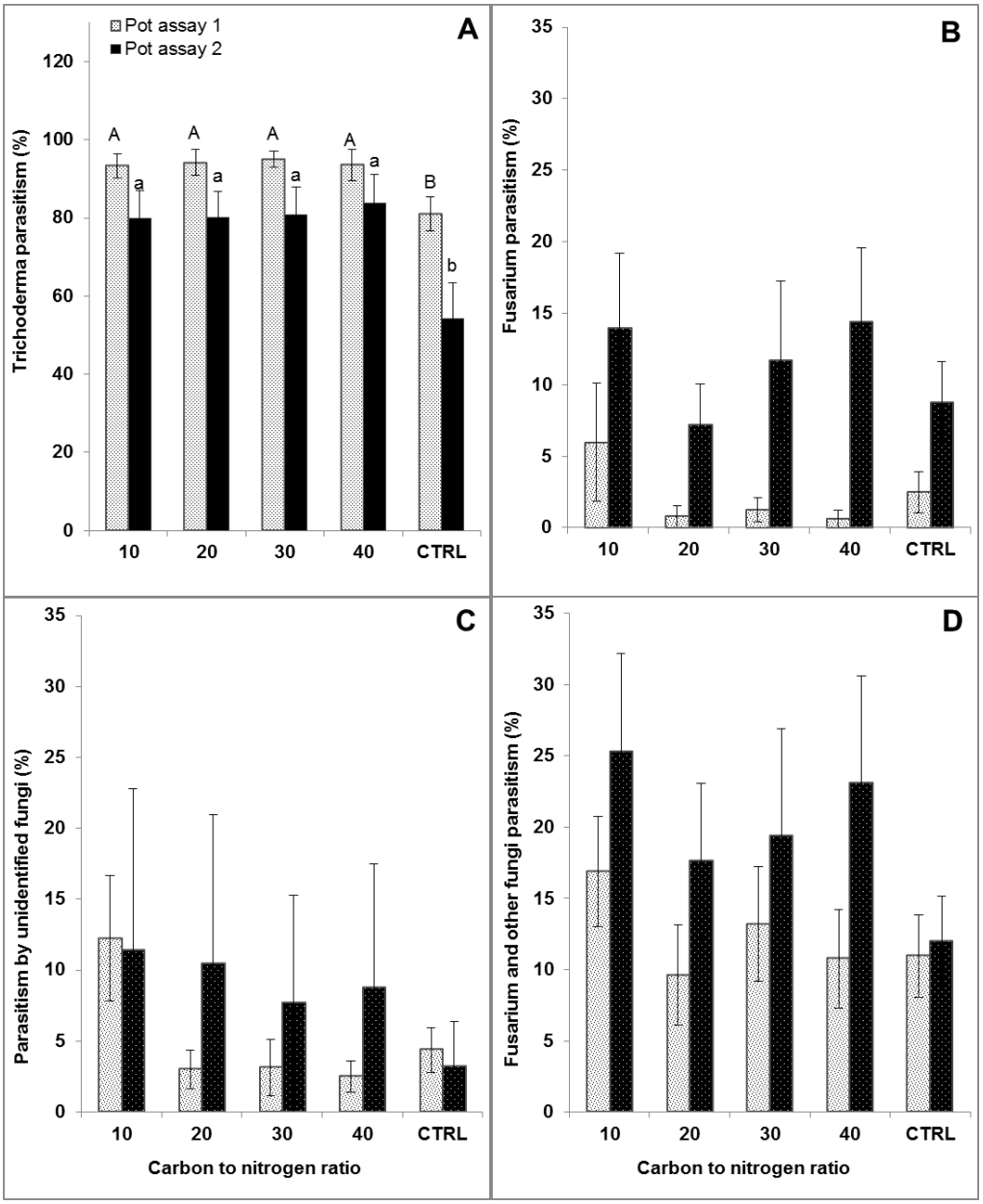


Figure 4-2. Effect of amendment C:N ratio on percentage sclerotial parasitism by *Trichoderma* (A), *Fusarium* (B), other fungi (C), and *Fusarium* and other fungi (D) after ASD treatment, pot study.

Bars indicated by different letters are significantly different at $p<0.05$ according to Tukey's test. Capital letters are used to compare the respective means of the pot assay 1 and small letters are used to compare respective means of the pot assay 2. Error bars indicate standard error with four replicates. CTRL=non-amended control, 10=C:N ratio 10:1, 20=C:N ratio 20:1, 30=C:N ratio 30:1 and 40=C:N ratio 40:1

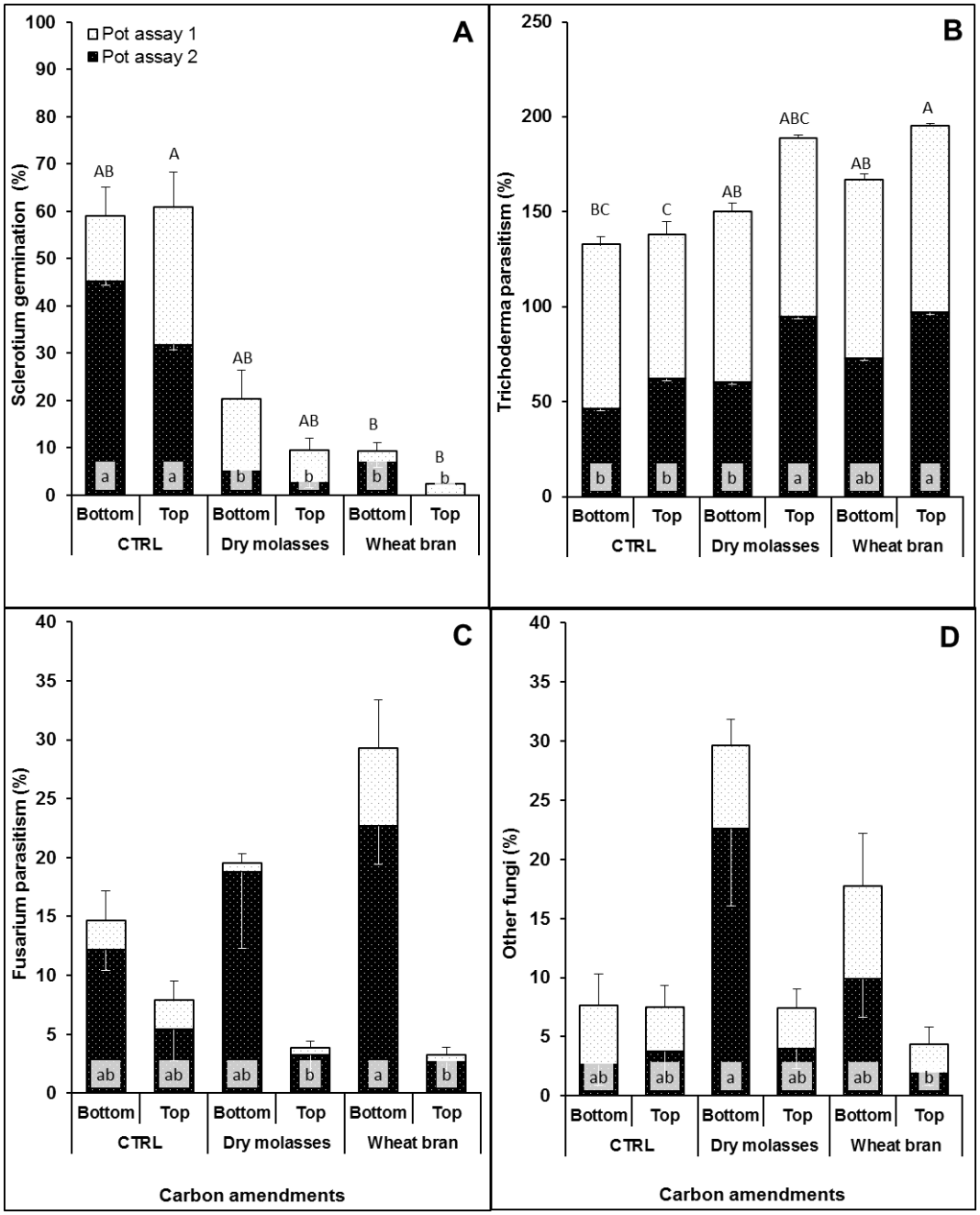


Figure 4-3. Effect of carbon amendments at depths 5-cm (top) and 15-cm (bottom) on percentage sclerotial germination (A), percentage sclerotial parasitism by *Trichoderma* (B), *Fusarium* (C), and other fungi (D) after ASD treatment, pot study.
Bars indicated by different letters are significantly different at $p<0.05$ according to Tukey's test. Capital letters are used to compare the respective means of the pot assay 1 and small letters are used to compare respective means of the pot assay 2. Error bars indicate standard error with four replicates. CTRL=non-amended control

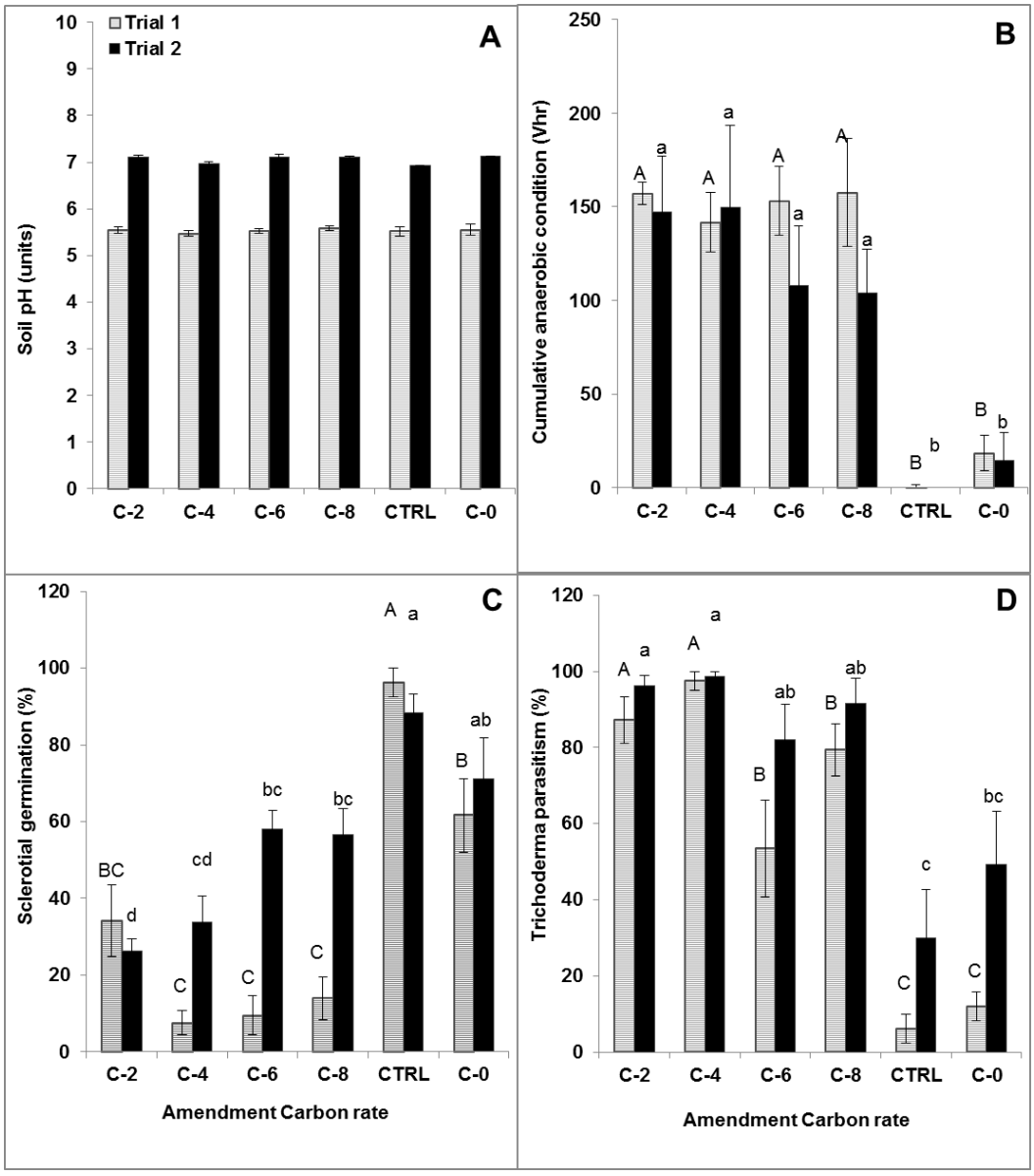


Figure 4-4. Effect of amendment C rates on soil pH (A), cumulative anaerobic condition (B), percentage sclerotial germination (C), and percentage *Trichoderma* parasitism (D) during ASD treatment, pot study.

Bars indicated by different letters are significantly different at $p<0.05$ according to Tukey's test. Capital letters are used to compare the respective means of the trial 1 and small letters are used to compare respective means of the trial 2. Error bars indicate standard error with four replicates. CTRL=non-amended, uncovered control, C-2=Carbon rate 2 mg C $g^{-1}$ soil, C-4=Carbon rate 4 mg C $g^{-1}$ soil C-6=Carbon rate 6 mg C $g^{-1}$ soil, C-8=Carbon rate 8 mg C $g^{-1}$ soil and C-0=Carbon rate 0 mg C $g^{-1}$ soil

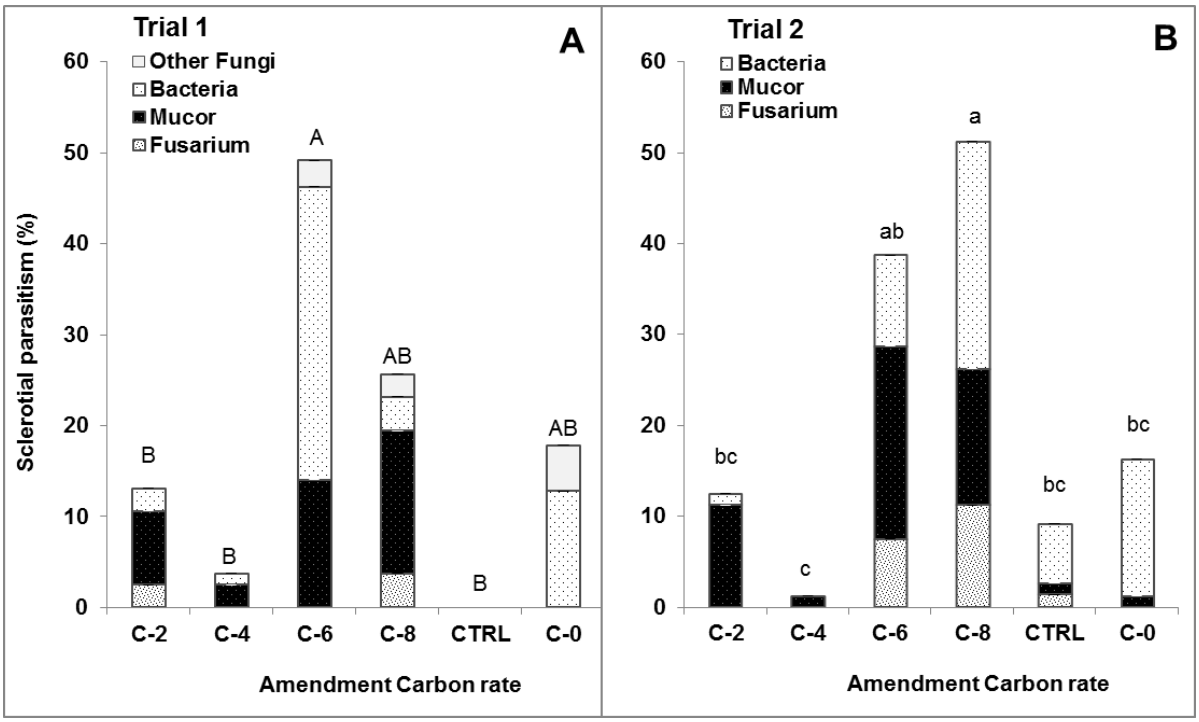


Figure 4-5. Effect of amendment C rates on percentage sclerotial parasitism in pot trial 1 (A) and trial 2 (B).

Bars indicated by different letters are significantly different at $p<0.05$ according to Tukey's test. Capital letters are used to compare the respective means of the trial 1 and small letters are used to compare respective means of the trial 2. Error bars indicate standard error with four replicates. CTRL=non-amended uncovered control, C-2=Carbon rate 2 mg C $g^{-1}$ soil, C-4=Carbon rate 4 mg C $g^{-1}$ soil, C-6=Carbon rate 6 mg C $g^{-1}$ soil, C-8=Carbon rate 8 mg C $g^{-1}$ soil and C-0=Carbon rate 0 mg C $g^{-1}$ soil

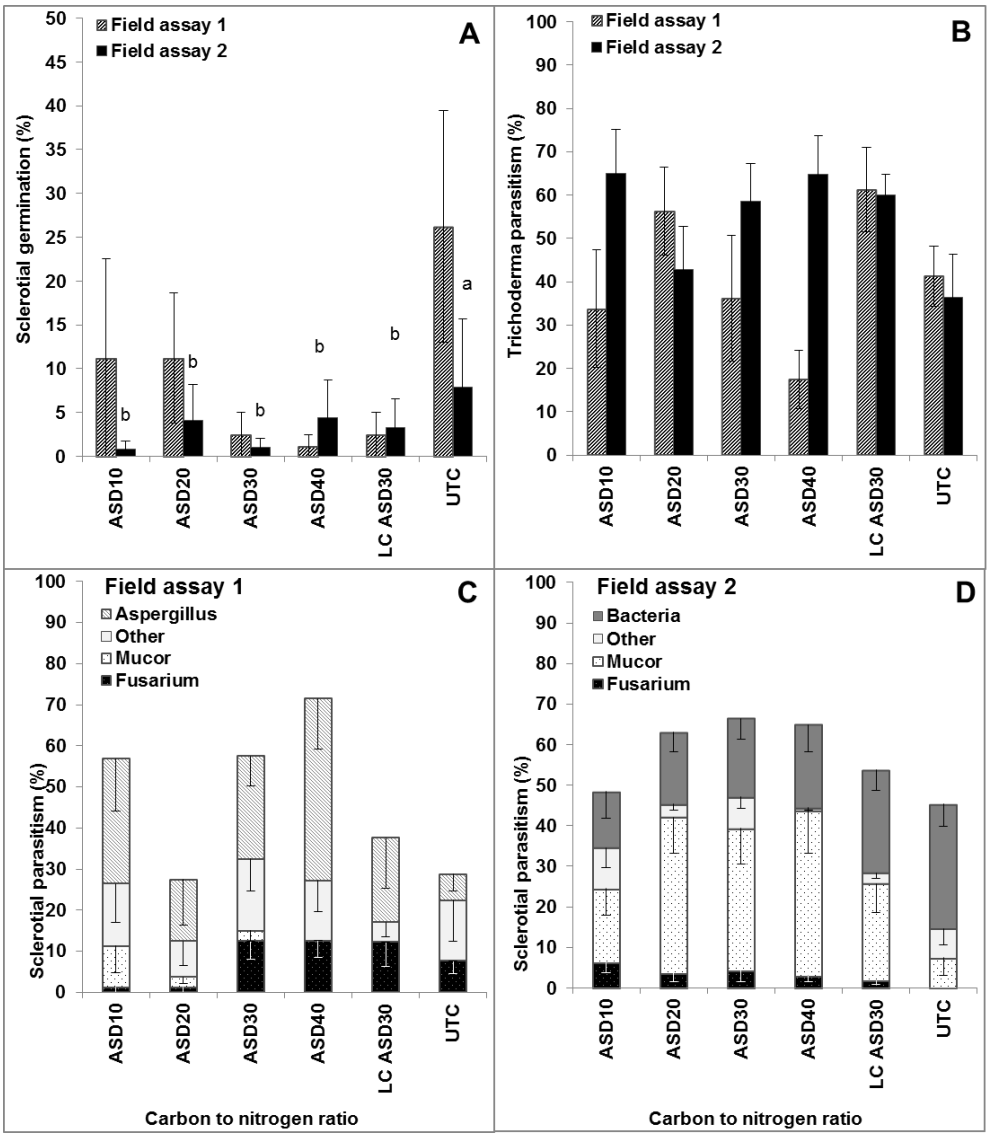


Figure 4-6. Effect of dry molasses amendment at different C:N ratios on percentage sclerotial germination (A), percentage sclerotial parasitism by *Trichoderma* (B) in the year 2013 (field assay 1) and 2014 (field assay 2); percentage sclerotial parasitism other than *Trichoderma* in year 2013 (C) and 2014 (D) after ASD treatment.
Bars indicated by different letters are significantly different at $p<0.05$ according to Tukey's test. Capital letters are used to compare the respective means of the pot assay 1 and small letters are used to compare respective means of the pot assay 2. Error bars indicate standard error with four replicates. ASD10=C:N ratio 10:1, ASD20=C:N ratio 20:1, ASD30=C:N ratio 30:1, ASD40=C:N ratio 40:1, LCASD30=C:N ratio 30:1, C rate 2 mg C g-1 soil (LC ± 'low carbon') and UTC=Untreated, non-amended control.

# Chapter 5

## Assessment of beneficial microorganisms: *Trichoderma*, actinomycetes, *Bacillus* and root colonizers in anaerobic soil disinfestation.

A version of this chapter is a manuscript in preparation for *Phytopathology* by Utsala Shrestha, Mary Dee, Bonnie H. Ownley and David M. Butler.

My primary contributions to this manuscript include experimental setup, data collection and analysis, results interpretation and writing. Mary Dee helped in examination of sclerotial germination, parasitism and soil serial dilutions of beneficial organisms.

## Abstract

Studies on anaerobic soil disinfestation (ASD), a non-chemical alternative to soil fumigants for controlling many soilborne diseases, have shown that it enhances populations of beneficial microorganisms against plant pathogens, including increased presence of the biocontrol agent *Trichoderma* as sclerotial parasites of *Sclerotium rolfsii*. However, studies on ASD effectiveness paired with beneficial mycoparasites and commercial biofungicide applications are lacking. This study compared the effect of ASD and incorporation of antagonists separately or in combination, at the initiation of ASD treatment, against the sclerotial germination and parasitism. The effect of ASD amendment on soil populations of endophytic isolates of *Trichoderma*, actinomycetes, and *Bacillus* spp. were also assessed. The anaerobic condition was also determined during ASD treatment in growth chamber studies. The root nodules of cowpea and plant biomass (cowpea and tomato) after ASD treatment were also recorded in greenhouse study. Arbuscular mycorrhizae were quantified from pepper plant in field study. In contrast to the negative effect of ASD on sclerotial population, we observed positive or no effect on the population of beneficial microorganisms. Further, ASD enhanced the mycoparasitic and bacterial colonization of sclerotia; however, ASD followed by addition of antagonists did not increase sclerotial mortality or parasitism of sclerotia.



**Keywords:** Actinomycetes, arbuscular mycorrhizae, anaerobic soil disinfestation, *Bacillus*, cowpea, Mycostop®, nodules, parasitism, RootShield®, sclerotia, *Trichoderma*, tomato

# 1. Introduction

Worldwide awareness in sustainable and chemical-free farming and food concepts has encouraged farmers to pursue the best non-chemical techniques to control pests. The ASD approach to soil disinfestation reduces chemical pollutants, reduces human health risks, and enhances safety of farmers and residential areas. Optimizations of ASD with various organic amendments at different rates have been examined in varied temperature regimes against different soilborne pathogens (*Fusarium*, *Ralstonia*, *Rhizoctonia*, *Verticillium*, *and Phytophthora*). ASD also induces changes in soil physical characteristics especially, soil pH, soil moisture and soil nutrients, due to addition of organic matter (Bonanomi et al., 2010). It is also reported to cause a significant shift in the microbial community composition (Mazzola et al., 2012), especially the composition of the bacterial community or soil aerobes (Messiha et al., 2007; van Agtmaal et al., 2015), and does not re-establish the original community structure during aerobic incubation, following ASD treatment. Bacterial activity in the early stage of soil treatment by ASD is considered more important for ASD effectiveness than in the later stages, emphasizing the fact that indigenous microorganisms play important roles in the biological control capacity of ASD. Anaerobic microorganisms, including *Bacillus* and *Clostridium* spp., are well-known antibiotic and toxin producers and bacterial community studies, particularly Clostridia species, have suggested that their effectiveness to suppress soilborne diseases (Momma et al., 2013; Mowlick et al., 2013a; Huang et al., 2016), is due to production of volatile fatty acids (Mowlick et al., 2013b). Hong et al. (2013) revealed that ASD treatment increases beneficial populations of the bacterial genera *Bacillus* and *Paenibacillus*, that may act as biocontrol agents in ASD.

Soil microbial community also comprises fungal mycoparasites and endophytic beneficial microbes; however, fungal activities and their interaction with the ASD treatment or with beneficial populations have not been investigated in detail. Studies on *Trichoderma* spp. as biocontrol fungi against soilborne diseases (Chet et al., 1979; Kredics et al., 2003) have been extensively studied (Lewis and Papavizas, 1983; Lewis and Papavizas, 1991), and *Trichoderma* spp. are commercially used to control soilborne pathogens such as *Fusarium*, *Pythium*, *Rhizoctonia*, *Sclerotinia*, *Alternaria*, and more (see Verma et al. (2007), Table 1). Various

*Trichoderma* spp. have been studied as sclerotial parasites (Papavizas and Lewis, 1989; John et al., 2015; Pacheco et al., 2016). *Trichoderma* is also present as a sclerotial parasite after ASD treatment (McCarty, 2012; Shrestha et al., 2013; Rosskopf et al., 2015). Organic amendments play important role in the proliferation of *Trichoderma* (Yossen et al., 2008; Bonanomi et al., 2010) and ASD treated soil might also support its growth. However, successful soil treatment by ASD is a function of change in soil pH, metal ions, and facultative and anaerobic bacteria. These parameters are also known to affect the biocontrol efficacy of *Trichoderma* (Duffy et al., 1997; Kredics et al., 2003), and better understanding of how these parameters affect biocontrol with *Trichoderma* in ASD would be helpful, while optimizing the ASD amendment sources. *Trichoderma harzianum* applied to tomato transplants in combination with *Bacillus subtilis* resulted in greater control of soilborne diseases and yield of tomato, than when used singly (Morsy et al., 2009). However, *Trichoderma* is highly competitive with other soil microbes (Kaur et al., 2005), and its effect on other potential biocontrol organisms during ASD treatment is unknown. In previous ASD studies, isolates of bacteria *Bacillus*, actinomycetes, *Trichoderma*, zygomycetes and nonpathogenic endophytic *Fusarium* were recovered from field soil following ASD treatment (Shrestha et al., 2013) and found *Bacillus* and actinomycetes (*Streptomyces* spp.) as new biocontrol for sclerotial parasites (Adhilakshmi et al., 2014; Gholami et al., 2014). Our hypothesis for this study was that integration of these potential biocontrol agents along with carbon amendments into the ASD system will improve effectiveness of biocontrol against soilborne plant pathogens.

Many *Trichoderma* spp. have been identified and used as active ingredient of commercial bio-pesticides (Woo et al., 2014). In USA, several *Trichoderma* based products have been registered for crop protection such as: *T. hamatum,* Floragard (Sellew Associates, LLC); *T. harzianum* DB 103, T-Gro (Dagutat Biolab); *T. harzianum* Rifai Strain T-22, RootShield® WP biological fungicide; *T. virens* strain G-41, BW240 G, BW240 (Bioworks inc); *T. polysporum* Rifai ATTC 20475 and *T. viride* sensu Bisby , *T. viride* ATCC 20476 (Binab Bioinnovation eftr ab ). There are several other *Trichoderma* species based product that is under registration process. Among various spp., *T. asperellum* has been identified as a potent biopesticides against various pathogens and was recently commercialized as active ingredients under the commercial name Ecohope, Ecohope-Dry (Japan), Quality WG, Trichodermax EC (Brazil) and Trichotech

(Kenya). Recognizing the commercial importance of biofungicidal properties of *T. asperellum*, and we selected local isolate of *T. asperellum* recovered from *S. rolfsii* sclerotia as one of our antagonists in this study. Two commercially available biofungicides (Table 1) with active spores of *Trichoderma harzianum* (RootShield®) and *Streptomyces riseoviridis* (Mycostop®) were selected to incorporate in soil with ASD treatment to see how these antagonists impact the efficacy of ASD. We determined the impact of these biocontrols by analyzing the germination and parasitism of sclerotia of *S. rolfsii* in ASD with C rates of 4 mg C $g^{-1}$ at C:N ratio 30:1. In addition, we also evaluated the impact of ASD amendment on soil populations of *Trichoderma*, actinomycetes, and *Bacillus.*

Rhizobia are nitrogen-fixing beneficial microbes that induce nodule formation where atmospheric nitrogen is fixed (Shantharam and Mattoo, 1997). To gain insights into the effect of ASD on indigenous rhizobium populations, we quantified the number and mass of cowpea root nodules because nodule mass is directly related to bacteroid numbers (Wadisirisuk and Weaver, 1985). The symbiotic association between arbuscular mycorrhizal (AM) fungi and pepper root is reported to increase plant growth as AM fungi helps in uptake of phosphorus nutrient in exchange for photosynthates (Davies et al., 1992; Martin and Stutz, 2004). Very little is known about the impact of ASD on AM fungi. To determine the ASD effect on this beneficial organism, we quantified total root colonization by AM fungi on pepper after ASD treatment.

## 2. Material and Methods

### *2.1 Sclerotia inoculum preparation*

An isolate of *S. rolfsii* from tomatoes was grown in petri dishes (100 mm x 15 mm) with full-strength potato dextrose agar (PDA; BD BBL™, Fisher Scientific) for 7 days, and plugs were transferred to partitioned Quad PDA plate. Cultures were then allowed to grow to obtain mature sclerotia as described in Chapter 4. Mature sclerotia were harvested from PDA plates after 1 to 2 weeks. Sclerotia were placed in 5-cm × 5-cm bags made from Delnet® aperture film. The film allows water, and air to pass through. Bags containing 10 sclerotia were prepared for different depth before ASD treatment. Bag containing 100 loose sclerotia were prepared for burial at 2-cm depth.

### *2.2 Isolation and spore suspension of Trichoderma asperellum*

The culture of *T. asperellum* was isolated from parasitized sclerotia in soil previously treated with ASD. *Trichoderma asperellum* was identified by extracting genomic DNA from colonies grown on potato dextrose broth for at least a week using the Qiagen plant extraction kit. Elongation factor 1 (EF1) and EF2 regions were amplified by PCR and the amplicon was sequenced. The sequences obtained were used to blast in the NCBI database. The EF1and EF2 sequences were 99% identical to those of GenBank accessions of *T. asperellum.* Spore suspensions from *T. asperellum* isolates were prepared in double deionized water by harvesting green spores from a 2-week-old culture. Number of spores was quantified using a haematocytometer and then diluted to obtained 1.3 x $10^5$spores $ml^{-1}$.

### *2.3 ASD treatments and/or biocontrol treatments*

Dry molasses (C:N~29.7, Westway, New Orleans, LA) that was mixed with corn starch (C:N~0, Tate & Lyle ingredients Americas, INC. Decatur, IL) was adjusted to a C:N ratio 30:1 of ASD treatments. Two commercial biocontrol agents RootShield® (Bioworks Inc. Geneva, New York, USA) and Mycostop® (Verdera, Espoo, Finland) and *T. asperellum* isolated from parasitized sclerotia were selected to evaluate impact of ASD on biocontrol populations (Table 1). ASD treatments included combination of ASD with i) RootShield®, ii) Mycostop®, iii) RootShield® and Mycostop® and iv) *T. asperellum* and non-ASD treatments included i) RootShield®, ii) Mycostop®, iii) RootShield® and Mycostop® and iv) *T. asperellum*. Amended ASD control and two non-amended controls without carbon supplement, with and without plastic covering, were included.

### *2.4 Pot setup*

Growth chamber study conditions were maintained similar to previous studies (25°C for 14 h, 15°C for 10 h with 50% relative humidity). ASD was  was carried out for 3 weeks with soil (Fine-loamy, siliceous, semiactive, mesic Typic Hapludult ) collected from the ‘Ap’ horizon at the Plateau Research and Education Center, Crossville, TN, where wheat was planted. Soil to fill 20-cm square polyethylene pots (~10.2-cm width x 24.1cm height, 1.5 L) was sieved (<10 mm) to remove organic debris and mixed with white fine sand at 1:1 proportion (pH 6, trial1 and pH

6.7, trial 2). Soil mixture was mixed with dry molasses and corn starch and placed in pots. Three bags with ten sclerotia of *S. rolfsii* each were buried at 5-, 10- and 15-cm depths in each pot. Bags with 100 sclerotia were buried at 2-cm depth. Oxidation-reduction electrodes (ORP) and temperature-moisture sensors were inserted at 10- to 15-cm depths to measure cumulative redox potential and temperature of soil. Three replicates of each treatment received ORP probes and two replicates received temperature probes. Pots for each biocontrol treatments were carefully drenched with ~500 ml spore suspension prepared in sterile deionized water to attain complete saturation. Other pots were saturated with deionized water. All pots were covered with black polyethylene (0.03 mm) with a heavy-duty rubber band, except a non-amended treatment for 3 weeks. The design was completely randomized with four replicates that started on the 5 to 25 of March 2015 and was repeated on 24 June to 15 July 2015.

When ASD was completed, plastic, ORP, and temperature probes were removed. Bags with string were carefully removed and soil samples adhered to each bags along with soil within 15-cm depth were collected in sampling bag. A soil samples was collected for estimation of biocontrol agents populations. Subsamples were oven-dried (105°C for 48 h) to determine gravimetric moisture content and the remaining sample was air-dried and sieved (<2 mm) for soil pH. Soil pH and Cumulative soil anaerobic condition was calculated as described previously (Butler et al., 2012b; McCarty et al., 2014).

### *2.5 Pathogenicity testing*

The top bag of each pot was opened and sclerotia were mixed with the top 2-cm soil. Pots were moved to the greenhouse bench and arrranged in randomized block design with four replications. The mean temperature of the greenhouse and the mean relative humidity of the greenhouse was 25°C (ranging 18 to 38°C ) and 55% during trial 1 and 30°C (39 to 23°C) and 90% during trial 2, respectively. Three-week-old tomato plants (cv. Florida Lanai) and two pre-sprouted cowpea seeds (cv. California black-eyed pea) that were later thinned to one were planted in each pot. Sclerotial survival and disease pressure were evaluated weekly on plants and the disease severity index was measured for tomato and cowpea plants as described in Guzmán-Valle et al. (2014) and Errakhi et al. (2007), respectively. The nominal scale used to quantify disease severity was 1 = healthy plants, 2 = plants without symptoms but with sclerotia in the soil, 3 = yellowish

colored plants with surrounding mycelium and sclerotia, 4 = plants with symptoms of wilt and rot with mycelium and sclerotia. Each pot was drip irrigated 1 min for the first week and then increased to 2 min every morning to maintain proper moisture content. After 8 weeks, shoots and roots of tomato and cowpea plants from each pot were oven-dried at 65°C for dry biomass. Before drying cowpea roots, root nodules were cleaned with tap water, counted and dried at room temperature before taking the weight.

### *2.6 Assessment of sclerotial germination and parasitism*

To access germination and parasitism of sclerotia, bags of sclerotia were retrieved from bags at 5-, 10- and 15-cm depths, and sclerotia were plated onto 24 well plates containing i. PDA (Difco™ Potato Dextrose Agar) amended with 6.9 mg $l^{-1}$fenpropathrin (Danitol 2.4 EC, Valent Chemical, Walnut Creek, CA) and 10 mg $l^{-1}$ rifampicin (Sigma-Aldrich, St. Louis, MO), ii. AIA (Difco™ Actinomycete Isolation Agar, 22 g $l^{-1}$) mixed with 5g Difco™ Glycerol, and iii. TSM (*Trichoderma* selective medium) *Trichoderma* modified PDA adapted from Gil et al. (2009) containing 39 g $l^{-1}$ PDA amended with 0.02 g $l^{-1}$, rose bengal, 0.3 g $l^{-1}$ chloramphenicol, 0.02 g $l^{-1}$ streptomycin sulfate maintained at pH 6 (Figure 5-1). We used different media for different depths as our objective was to only access the sclerotial germination and parasitism on a respective medium and not intended to make any comparison among depth of inoculation. Plates were incubated at room temperature for at least 3 weeks and observation were made for mycelial growth, germination of sclerotia, parasitism of sclerotia by *Trichoderma*, zygomycetes, *Fusarium* spp. and actinomycetes or other bacteria.

### *2.7 Quantification of Trichoderma*, **actinomycetes**, *and Bacillus from soil*

Media plates for quantification of *Trichoderma* and actinomycetes were prepared using TSM and AIA. Media plates for *Bacillus*, facultative anaerobic spore formers (*Bacillus* isolation medium = BIA), were prepared by adding 15 g potato dextrose agar, 5 g glucose (Dextrose), 5 g peptone, 3 g beef extract and 1 g yeast extract. One gram of composited soil samples from each pot were suspended in 9 ml of sterile double-deionized water and serial dilutions from $10^{-2}$ to $10^{-4}$ were prepared for *Trichoderma* and actinomycetes, and dilutions of $10^{-3}$ to $10^{-6}$ were prepared for *Bacillus*. An aliquot of 0.1 ml of each dilution was spread on plates containing selective media

until liquid was absorbed into the TSM and AIA media. Before plating *Bacillus* in BIA, serial dilution tubes were heated (80 to 85°C) in a water bath for 30 min to activate heat-resistant spores and kill non-heat tolerant vegetative cells in the soil sample, and then spread onto agar plates. Inoculated plates for three biocontrols were duplicated and incubated at room temperature for 18 to 24 hr for *Bacillus*, 1 to 2 weeks for *Trichoderma* and 4 to 6 weeks for actinomycetes. Emerging colony forming units (CFU) of each biocontrols from each plates were counted and expressed as CFU per gram of soil, and re-isolated in selective medium for identification (Figure 5-2). *Trichoderma* isolates were preserved in slant amended PDA tubes. Fifty percent (v/v) glycerol stock solution was prepared for preservation of actinomycetes in actinomycetes vegitone broth (AVB) and *Bacillus* in Nutrient broth yeast extract (NBY), and was stored at -20°C for future study.

### *2.8 Molecular identification*

To identify *Trichoderma*, the fungus with green appearance on PDA was observed under the microscope. Further identification of *Trichoderma* spp. was done by extracting genomic DNA from colonies of the isolate grown on PD broth using the QiagenDNeasy DNA extraction kit. ITS regions 1 and 2 of *Trichoderma* isolates (White et al., 1990) amplified by PCR using the appropriate primers pairs (5'-TCCGTAGGTGAACCTGCGG-3' / ITS2: 5'-GCTGCGTTCTTCATCGATGC-3'). PCR was carried out in a 50-µl reaction mixture containing 50 ng genomic DNA, 5 µl each of 0.5 µM forward and reverse primers, 1 µL dimethylsulfoxide and 25 µL of 5 PRIME HotMasterMix (VWR International). The PCR conditions was an initial denaturation of 94°C for 2 min followed by 42 cycles of 1 min denaturation at 94°C, 1 min of annealing at $42^0$C and 2 min extension at 72°C, and final extension of 3 min at 72°C. The PCR products was purified using ExoSAP-IT® PCR Product Cleanup ExoSAP-IT (Affymetrix, Santa Clara, CA), and sequencing was done by the Molecular Biology Resource Facility, UTK, Knoxville, TN. The resultant sequences were used to blast in the NCBI GenBank database and were edited with Sequncher (v 5.1) to configure for highest accuracy.

### *2.9 ASD effect on* AM *fungi*

To quantify the root colonization of pepper root, three root systems at the end of harvesting selected randomly from the field experiment at the UT Plateau Research and Education Center in Crossville (see experiment layout in chapter 3). Roots were cleaned with tap water and stored at 4°C until analyzed. The modified staining procedure of Grace and Stribley (1991) was followed to stain the root samples. Briefly, cleaned root samples were cut into 1-cm pieces to fit in a histology tissue cassette and boiled at 70-80°C in KOH (10%) solution for 10-15 min. The roots were cooled at room temperature and then rinsed with tap water before acidifying with HCl (2%) for 1.5 hr. Roots were stained in trypan blue (0.05%) for 1 hour. Cassettes with root were rinsed 2-3 times with distilled water and then immersed in lactoglycerol to destain. The root segments were then observed under the microspce to visualize AM fungal hyphae, vesicles, and or arbuscules.

### *2.10 Data analysis*

Data was subjected to analysis of variance using mixed model in SAS (Glimmix procedure, SAS Institute, Cary, NC) to find the relationship between applied beneficial organisms and anaerobic conditions in ASD, and to determine the impact on sclerotial germination and crop performance. Our design was randomized compete block design with trial as a random factor and treatments as a fixed factor. DNA sequences were edited using Sequencher 5.0 (GeneCodes). These sequences were compared with sequences in the NCBI GenBank nucleotide database to identify the bacterial or fungal species. Sequences were aligned using ClustalX (Larkin et al., 2007). Phylogenetic analysis was done using the program MEGA 5.2 (Tamura et al., 2012).

## 3. Results

In our previous field study, ASD treatment with 4 mg C $g^{-1}$ soil used at 30:1 C:N ratio was the most effective in increasing sclerotial mortality, although, we observed no significant difference among C:N treatments in both pot and field studies. In this study, we tested wheather the effectiveness of ASD against sclerotia could be improved by incorporating commercial bio-fungicide or endemic *Trichoderma* populations before ASD treatment. We also performed serial

soil dilutions on ASD treated soil to observe the effect of ASD on populations of beneficial microbes. We observed a significant weak negative relationship between sclerotial germination with cumulative anaerobic condition and sclerotial parasitism by *Trichoderma*, zygomycetes and bacteria (-0.3 to -0.5, $p<0.001$). *Trichoderma* parasitism also had a positive weak relationship with soil pH and cumulative anaerobic condition. Parasitism (%) by "other fungi" and bacterial parasitism showed moderate positive relationships and both showed a negative relationship with soil pH and soil moisture (Table 5-2).

Cumulative redox potential (Ceh) was measured as an indicator of cumulative anaerobic conditions. We observed that the ASD treatments had Ceh readings of 128,858 to 141,019 mV hr which were significantly higher than non-ASD treatments (1,033 to 29,464 mV hr), providing evidence for the generation of the anaerobic condition in ASD treatments (Figure 5-3A). Soil pH ranged from 6.12 to 6.61 units across treatments and theses were significantly higher soil pH values for *T. asperellum.*Soil pH was lowest for untreated controls and non-ASD treatments except for Mycostop®+RootShield® (Figure 5-3B). The post gravimetric soil moisture content was significantly higher in the covered control, ASD + Mycostop®, and Mycostop® treatments (0.18 g $g^{-1}$) than other treatments (~0.16g $g^{-1}$) and lowest for the uncovered, unirrigated control (0.03 g $g^{-1}$).

### *3.1 Germination of sclerotia*

Previously we observed that ASD is effective in increasing sclerotial mortality. In order to test if the incorporation of antagonists such as Mycostop®or RootShield® or the endophytic isolates (*T. asperellum*) could further improve effectiveness of ASD, sclerotia were inoculated in soil at different depths. After ASD completion, sclerotial germination was tested. We observed that germination of sclerotia retrieved from ASD treatments from 5-, 10- and 15-cm depth were, in general, significantly lower than non-ASD treatments (Figure 5-4). At 15-cm, sclerotial germination from non-ASD RootShield® treatment was significantly lower and similar to ASD treatments (0 to 7.5%). *Trichoderma. asperellum* with ASD had a lower percentage of germinated sclerotia (14%) than non-ASD *T. asperellum* (33%), but it was not significant. Mycostop® alone, in non-ASD, failed to suppress sclerotia and was similar to the covered control (63-67%). There were no significant differences in the suppression of sclerotial germination

among the ASD treatments (Figure 5-4C). Similarly, at the 10-cm depth, all ASD treatments alone or with inoculated antagonists, except Mycostop®, significantly contributed to sclerotial mortality (0-5%). The non-ASD treatments with biofungicides were not effective in sclerotial suppression (18-30%) and had the highest germination recorded for *T. asperellum* (38%, Figure 5-4B). At greater depth (15-cm) under non-ASD conditions, addition of organic amendments plays an important role in sclerotial suppression.Addition of Mycostop® biofungicides and endophytic *Trichoderma* under anaerobic condition were not effective, and may have been affected adversely by high gravimetric soil moisture (Figure 5-3C) and gravitational pressure. Interestingly, at 5-cm depth sclerotial germination in the ASD only treatment (18%) was lower than ASD treatments into which other anatagonists were also added. At 5-cm, mortality of sclerotia was lower than greater depth (10-15-cm) and use of only anatagonists did not reduce sclerotial germination compared to the plastic covered or non-covered control (57-75%). At 5-cm, we observed that sclerotial germination was highest for Mycostop®+ RootShield® (88%) followed by two controls and non-ASD *T. asperellum*. Our results showed that the use of commercial bio-fungicides such as Mycostop® or RootShield®, or endophytic isolate - *T.* asperullum did not reduce sclerotial germination under covered condition at any depths. Addition of these antagonists into ASD also did not promote further suppression of sclerotial germination, compared to ASD only treatment.

### *3.2 Sclerotial parasitism*

Sclerotial bags at 5-, 10- and 15-cm depths were retrieved and cultured in PDA, AIA and TSM, to test sclerotial parasitism by fungi and bacteria parasites. Although our objective was not to test the sclerotial germination or parasitism differences across different depths or media, we observed that *Trichoderma*, zygomycetes, bacteria and other fungal parasites of sclerotia occured across soil of depths of 5-, 10- and 15-cm. However, sclerotial parasitism by actinomycetes was only observed in sclerotia retrieved from 10-cm and plated on AIA plates.

Sclerotia retrieved from 5-cm depth plated on PDA showed relatively higher percentage *Trichoderma* parasitism of sclerotia in ASD treatments compared to the control. Incorporation of antagonists with ASD did not increase sclerotial parasitism by *Trichoderma*. Sclerotial parasitism by *Trichoderma* in these treatments with antagonist was equivalent to their

corresponding antagonists in non-ASD treatments except treatment that used both Mycostop® and RootShield® (Figure 5-5A). Our results showed that sclerotial parasitism by zygomycetes in ASD treatments with antagonists were higher in non-ASD treatments with corresponding antagonists. For sclerotia retrieved from 5-cm, parasitism by other fungi was highest for the control, but we did not observe any significant difference among any treatments in sclerotial parasitism by bacteria.

Sclerotia retrieved from 10-cm, plated on AIA (Figure 5-7) showed significantly highest bacterial parasitism in ASD alone and ASD with antagonists, except Mycostop®, when compared with non-ASD with antagonists (72-83%). Zygomycetes parasitism of sclerotia was significantly greater in all ASD treatment with the highest parasitism observed in ASD + Mycostop® (98%) compared with both non-ASD and control treatments. In contrast, *Trichoderma* colonization of sclerotia was significantly higher in non-ASD treatment and the control (45-73%,). Figure 5-6 represents the actinomycete parasitism of sclerotia, which was significantly higher in Mycostop® treatments (65-88%) even though we observed high percentage of sclerotial germination. This also suggests that actinomycetes from addition of Mycostop® competed with other mycoparasites, especially *Trichoderma*, bacteria and other fungi for sclerotial parasitism in ASD treatments. This study does not provide any evidence of significant parasitism of sclerotia by endemic actinomycetes in ASD. These results indicate the significance of using isolation media such as AIA for actinomycetes and bacterial parasitism of sclerotia.

Surprisingly, sclerotial parasitism on TSM, i.e. sclerotia retrieved from the 15-cm depth showed significantly higher *Trichoderma* parasitism of sclerotia in non-ASD and control (32-72%) than ASD treatment with or without antagonists. The zygomycetes parasitism of sclerotia was significantly higher in ASD alone (40%) and addition of the antagonists increased sclerotial parasitism by zygomycetes for ASD with Mycostop®+Rootshield®. Intriguingly, we observed significant sclerotial parasitism by other fungi (for e.g., *Myrothecium* spp., *Penicillium* spp.) at 15-cm for ASD.We not only observed highest mortality of sclerotia at 15-cm depth but also noticed limited *Trichoderma* colonization of sclerotia in all ASD treatments revealing an unfavorable environment for *Trichoderma* at greater depth (Figure 5-8).

### *3.4 Effects on beneficial organisms*

Our previous studies showed that ASD had negative effects on soilborne pathogens and altered microbial composition. Addition of relatively high amendment rate under covered and irrigated condition induces the anaerobic environment and alters soil properties as well (Inglett et al., 2005; Butler et al., 2014) with the production of organic acids (Momma et al., 2006) and other volatiles (Hewavitharana et al., 2014). Therefore, we were interested to determine the impact of ASD on soil beneficial microorganisms. To test the effect of ASD on beneficial microorganisms, such as actinomycetes, *Bacillus* and *Trichoderma*, we counted the number of colony forming units with standard dilution plating of soil samples on selective media. Our analysis showed that ASD had no effect or a positive effect on the beneficial microorganism populations. We observed that use of different antagonists reduced the populations of actinomycetes during ASD treatment (Figure 5-9). We observed enhanced populations of *Bacillus* in ASD treatments with highest CFU recorded for ASD with *T. asperellum* and the combination of Mycostop® and RootShield® (5.6 log CFU+1 $g^{-1}$ of soil, Figure 5-10). *Trichoderma* spp. populations were similar across the ASD treatments and was highest in ASD when compared to ASD with antagonist, except for *T. asperellum* with and without ASD (Figure 5-11). Different endophytic fungi identified from soil are presented in Figure 5-16.

### *3.5 Effect on nodule and colonization of AM*

We observed that cowpea root nodule number and mass did not change in ASD treatment compared to the controls suggesting that ASD has no negative effect on the population of rhizobium. We also observed that incorporation of *T. asperellum* enhanced cowpea nodule mass compared to the controls under ASD conditions indicating an enhanced symbiotic relationship among cowpea root, *T. asperellum*, and indigenous nodule forming bacteria (Figure 5-13). Root colonization by AM was significantly higher in all ASD treatments with 4 mg C $g^{-1}$ of soil in the field study (Figure 5-14), when compared to fumigated and non-amended plots.

### *3.6 Effect on root and shoot biomass*

There was no significant difference in the total root biomass of cowpea and tomato among all non-ASD treatments and control treatments. Only ASD with *T. asperellum* showed higher root

biomass when compared to ASD + Rootshield®, non-ASD treatments with antagonists and control treatments. Assessment of dry shoot biomass showed higher biomass only in ASD with *T. asperellum* (17 g/pot) compared to ASD, ASD + Mycostop® + RootShield® and non-ASD and control treatments.

## 4. Discussion

Anaerobic soil disinfestation has been widely studied to control soilborne pathogens and it has been demonstrated that the ASD is efficient for control of some nematodes and weed propagules and ultimately contributes to yield increment (increases when combined with solarization) when compared to non-amended controls (Butler et al., 2012a). Nevertheless, the pepper yield data from Chapter 3 had significantly higher marketable yield than both fumigated and control treatments. Besides pathogen control and yield, many studies have focused on alternation in microbial community composition during ASD treatment (Mowlick et al., 2012; Streminska et al., 2014; van Agtmaal et al., 2015; Huang et al., 2016).These studies used ethanol, wheat bran, crucifers, commercial protein-rich vegetal by-product (Herbie®) as carbon amendments for ASD. The microbial study of ASD is of interest as temporary anaerobic condition generated during the process stressed the microbial population and resulted in the microbial shift which risks the pathogen suppressive ability of soil (van Agtmaal et al., 2015). These studies mainly focused on bacterial composition and results showed increased populations of Bactereroidetes, *Bacillus*, and Clostridia spp. Only a few studies have focused on endemic soil populations of beneficial fungi. *Trichoderma* spp. and endemic *Fusarium* spp. are predominant after organic acid addition in ASD (Rosskopf et al., 2014); however, results were not consisted among during trials. Since our amendment in this study was dry molasses, it is noteworthy to see an effect of ASD on the endemic population of *Trichoderma* and other potential sclerotial parasites, such as actinomycetes and *Bacillus*. Biological based pesticides coupled with other pathogen control mechanisms have shown promising results in pathogen suppression (Cook and Baker, 1983). The *Trichoderma* based biopesticides are available worldwide (Woo et al., 2006; Woo et al., 2014) and are primarily used for seed and transplant treatments. Similarly, Mycostop® is also marketed as a seed dressing or soil treatment (White et al., 1990). For our study, we selected RootShield® with fungal spores of *T. harzianum* Rifai Strain T-22, and Mycostop® with actinomycetelspores

of *Streptomyces riseoviridis* and active spores of the sclerotial mycoparasite *T. asperellum* i.e. isolated and grown on PDA.

Application of biofungicides and endophytic mycoparasites before ASD treatment, in combination with irrigation supplement, successfully suppressed sclerotial germination when compared with antagonists added to pots with non-ASD conditions (i.e. non-amended , irrigated and covered) and control treatments. However, the lowest sclerotial germination suppression was observed at 5-cm depth as compared to 10-cm and 15-cm. Individual addition of Mycostop® at 10-cm and *T. asperellum* at 15-cm in ASD did not improve the efficacy of ASD against *Sclerotium* germination. At all depths, regardless of added antagonists, the sclerotial parasitism by *Trichoderma*, zygomycetes and bacteria were apparent, but actinomycete parasitism was only observed in AIA medium. After 3-weeks of incubation of soil under ASD treatment, populations of beneficial soil organisms, *Trichoderma* and *Bacillus* increased and there were no negative effects on actinomycetes observed. However, addition of antagonists in ASD treatment impacted the filamentous spore-formers population and we hypothesize that actinomycetel spores might retard their growth and failed to compete with other mycoparasites. In soil alone, actinomycetes (especially *Streptomyces* spp.) are considered poor competitors when they do not colonize the plant root (Lahdenperä et al., 1991; Lahdenpera, 2000). Actinomycetes populations under anaerobic conditions increase their population when chitin is present; however, due to presence of high organic matter, active chitin decomposers, such as *Trichoderma*, may compete with actinomycetes under anaerobic condition (Manucharova et al., 2006). Chitinase and glucanase, which is reported to control *S. rolfsii* (El-Katatny et al., 2001) from *Trichoderma* spp. inhibit *S. rolfsii* by cell wall lysis (Elad et al., 1983; Sivan and Chet, 1989; Chet and Inbar, 1994; El-Katatny et al., 2000). *Bacillus*, on the other hand, is a facultative anaerobe and previous studies showed that *Bacillus* is present in soil treated with ASD (Mowlick et al., 2013a). *Trichoderma* spp. colonization of sclerotia induced greater mortality of sclerotia in ASD alone or ASD in combination with antagonists. Soil *Trichoderma* populations were also highest in ASD- treated soil. Besides *Trichoderma*, we observed significant contribution of zygomycetes as mycoparastism. Zygomycetes have been isolated from sclerotia of *Sclerotinia sclerotiorum* (Ćosić et al., 2012) and produce glucanase and chitinase (Elad et al., 1985). Surprisingly, *Trichoderma* and zygomycetes grow slowly in anaerobic conditions, however, these fungi are

active and in the presence of organic matter, they revive and recover in aerobic conditions indicating a facultative nature. In addition, after isolation of these organisms from anaerobic conditions their relative abundance increases (Kurakov et al., 2008).

Growers are concerned about the phytotoxic effect of ASD on root growth (McCarty et al., 2014; van Agtmaal et al., 2015) and our study showed no negative impact of ASD on root growth. Further, it does not influence nodule formation in cowpea and amendment applied during ASD treatment may have enhanced root colonization of AM fungi in pepper plant (Gosling et al., 2006), and may have contributed to yield increment (see Figure 3-7). Antagonists addition before ASD did not enhanced the total root and shoot biomass, although the presence of *Trichoderma* populations is supposed to enhance plant growth (Harman et al., 2004). Overall, we did not see any significant impact on plant growth in controls, compared to non-ASD treatments, even though we added 100 sclerotia in the top 2-cm of soil before ASD treatment. Soil in pots may have enhanced beneficial organisms as these might be opportunistically feeding on sclerotia that were feebled by the absence of a host infection zone during the three-week period.

## 5. Conclusion

We observed no effect of antagonists when incorporated during ASD treatment. Therefore, we suggest studying the effect of the antagonist in ASD by adding antagonist after the completion of ASD. Identification of *Bacillus* and actinomycete species is ongoing and quantification of the *T. harzianum* population with qPCR might further elucidate the *Trichoderma* parasitism and actual quantification of the *Trichoderma* population. Further research on the impact of ASD on beneficial organisms in field condition could enhance the effects of ASD and carbon amendments on plant disease.

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# Appendix

Table 5-1. List of biocontrol agents and ASD treatment

| Biocontrol agents | Active ingredients | Concentration $g^{-1}$ | Application rate |
|---|---|---|---|
| *Trichodema asperellum* | *T. asperellum* isolated from parasitized sclerotia | 1.3 x $10^5$CFU $ml^{-1}$ | 1,000 spores $sclerotium^{-1}$ |
| RootShield® | *T. harzianum*Rifai strain KRL-AG2 (1.15%) | 3,000 CFU $ml^{-1}$ | 0.3 g $l^{-1}$ |
| Mycostop® | *Streptomyces* Strain K61 (4%) | 1,000 CFU $ml^{-1}$ | 0.01 g $l^{-1}$ |
| RootShield® + Mycostop® | *T. harzianum*Rifai strain KRL-AG2 (1.15%) + *Streptomyces* strain K61 | 1,500 + 500 CFU $ml^{-1}$ | 0.15 + 0.005 g $l^{-1}$ |

Table 5-2. Correlation among sclerotial germination, sclerotial parasitism and soil properties during ASD treatment, pot study, 2015

| Variables | *Trichoderma* parasitism | Zygomycetes parasitism | Other fungal parasitism | Bacterial parasitism | Total parasitism* | Soil pH | Ceh | Post soil moisture |
|---|---|---|---|---|---|---|---|---|
| Sclerotial Germination[@] | -0.46 | -0.33 | 0.06 | -0.32 | -0.09 | 0.05 | -0.47 | 0.01 |
| | **<0.0001** | **0.002** | 0.610 | **0.003** | 0.388 | 0.628 | **<0.0001** | 0.911 |
| | *Trichoderma* parasitism | -0.04 | -0.26 | -0.17 | -0.15 | 0.25 | 0.23 | 0.20 |
| | | 0.678 | **0.014** | 0.123 | 0.171 | **0.022** | **0.046** | 0.062 |
| | | Zygomycetes parasitism | -0.09 | 0.21 | 0.49 | 0.05 | 0.18 | -0.16 |
| | | | 0.420 | 0.052 | **<0.0001** | 0.679 | 0.112 | 0.144 |
| | | | Other fungal parasitism | 0.55 | -0.11 | -0.44 | -0.07 | -0.31 |
| | | | | **<0.0001** | 0.300 | **<0.0001** | 0.527 | **0.004** |
| | | | | Bacterial parasitism | 0.05 | -0.73 | 0.14 | -0.52 |
| | | | | | 0.672 | **<0.0001** | 0.217 | **<0.0001** |
| | | | | | Total parasitism* | 0.18 | 0.10 | 0.04 |
| | | | | | | 0.108 | 0.388 | 0.699 |
| | | | | | | Soil pH | 0.10 | 0.67 |
| | | | | | | | 0.360 | **<0.0001** |
| | | | | | | | Ceh | 0.09 |
| | | | | | | | | 0.442 |
| | | | | | | | | Post soil moisture |

*Total parasitism including zygomycetes, other fungi, and bacteria without *Trichoderma*

[@]Each top row within each variable represents spearman correlation coefficients and bottom row represents the *p* value. Correlation is significant at $p<0.05$ level. Bold text represents significant *p* value.

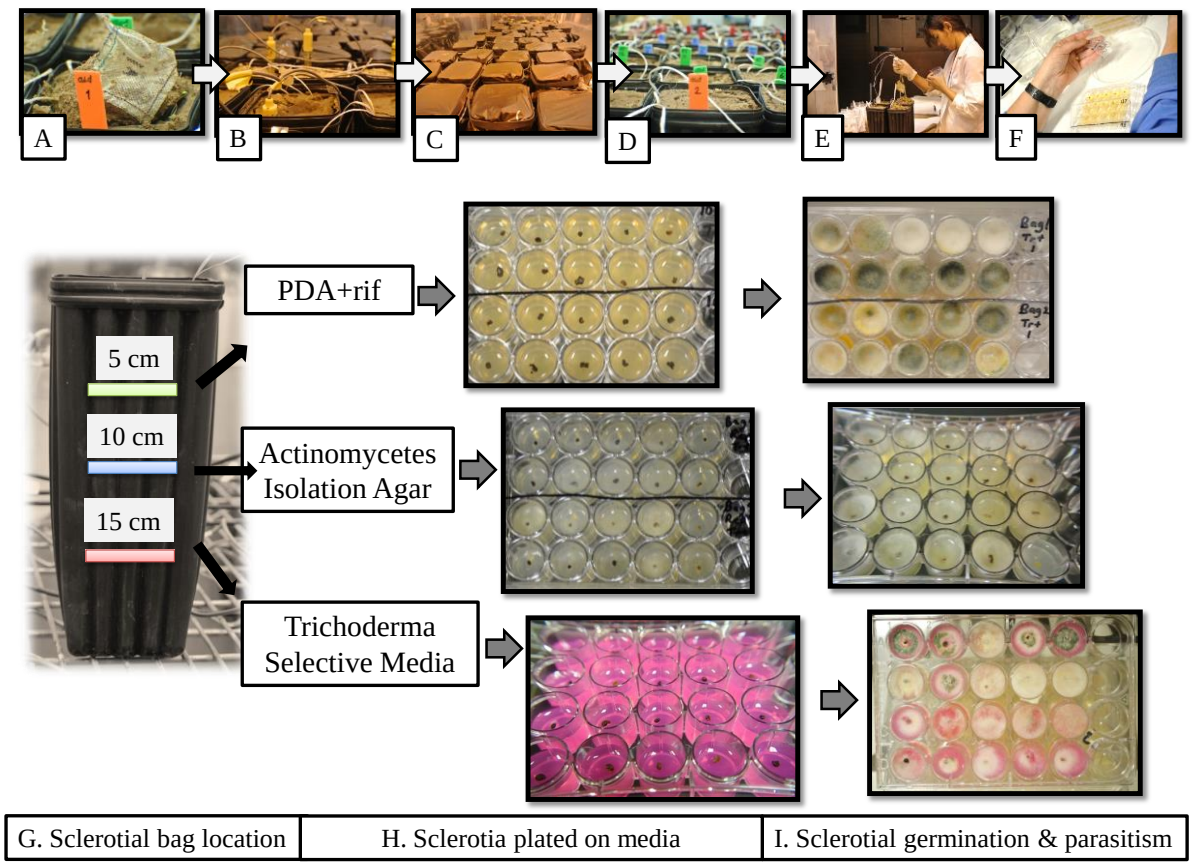


Figure 5-1. Pot set-up in the growth chamber for ASD study on beneficial organism.
Pot set-up with oxidation-reduction electrode and pathogen bags (A-B), pots saturated with deionized water (or deionized water with *Trichoderma* spore suspension), covered with polyethylene (C), ASD termination at 3 wks.(D), bag removal, from 5-, 10- and 15-cm depths (E), surface-sterilization and plating of sclerotia onto plate well plate (F), location of sclerotial bag in the pots (G), sclerotia plated on respective media (H), and observation of sclerotial germination and parasitism by parasites.

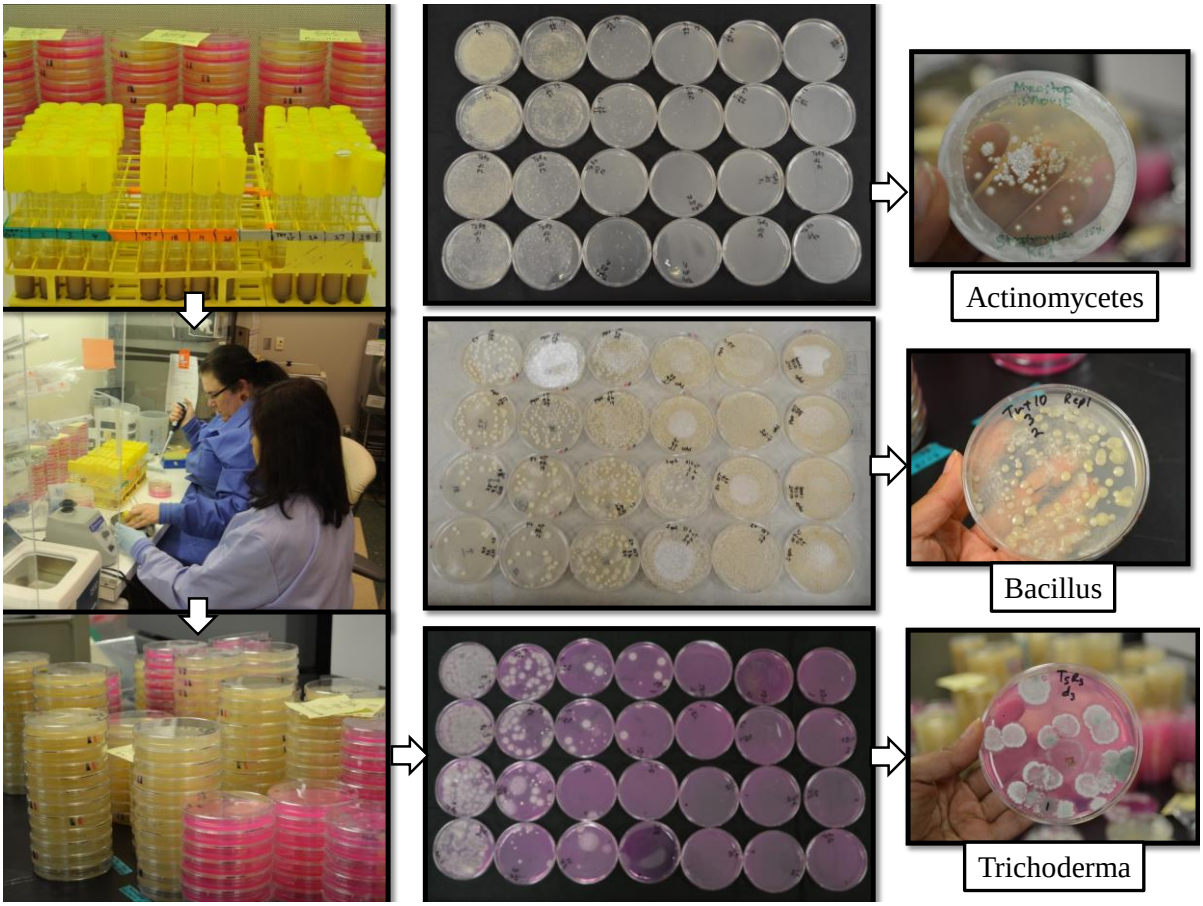


Figure 5-2. Process of soil serial dilutions to measure actinomycetes, *Bacillus* and *Trichoderma* populations.

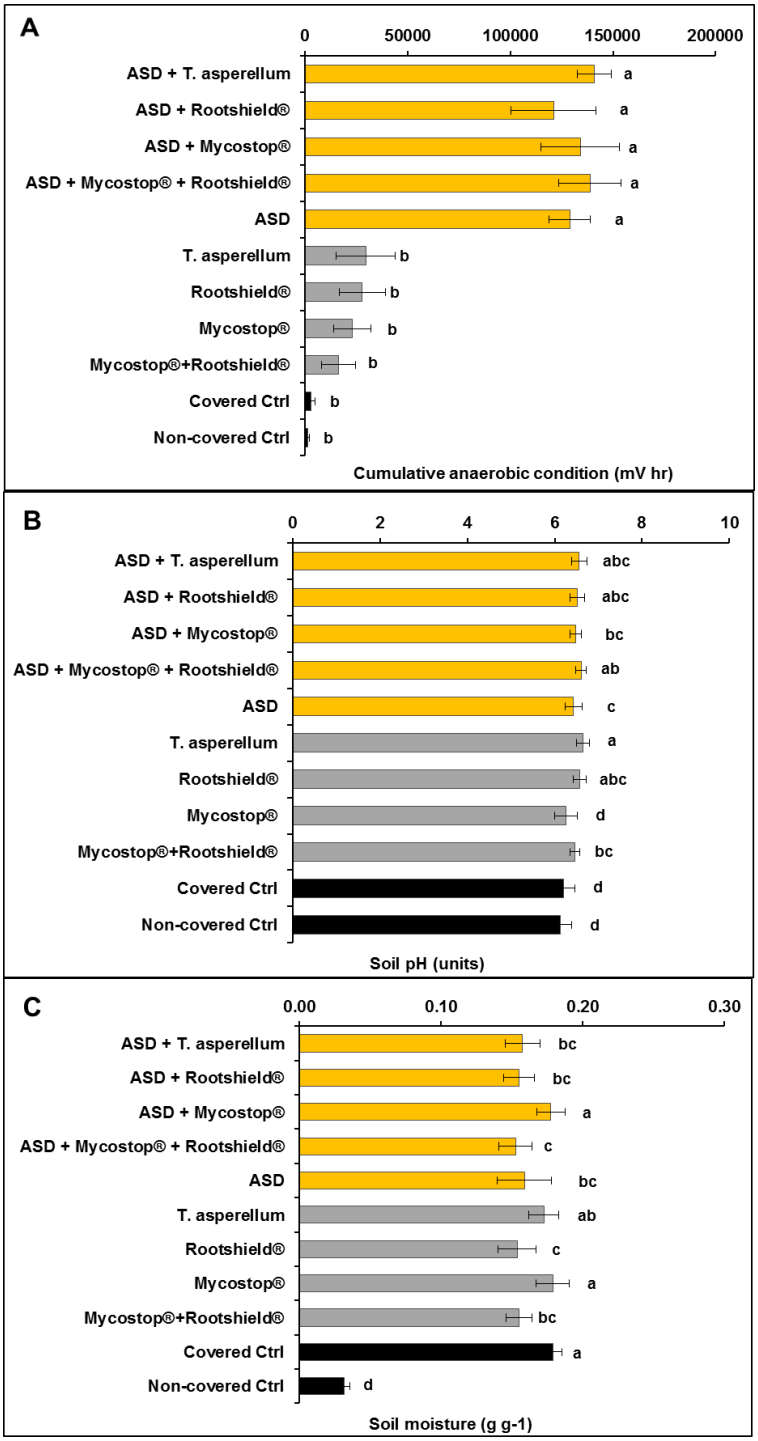


Figure 5-3. Effect of ASD (with/without biofungicides) and biofungicides on (A) cumulative anaerobic condition, (B) soil pH, and (C) gravimetric soil moisture content.
Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's PLSD test. Error bars indicate standard error with four replicates.

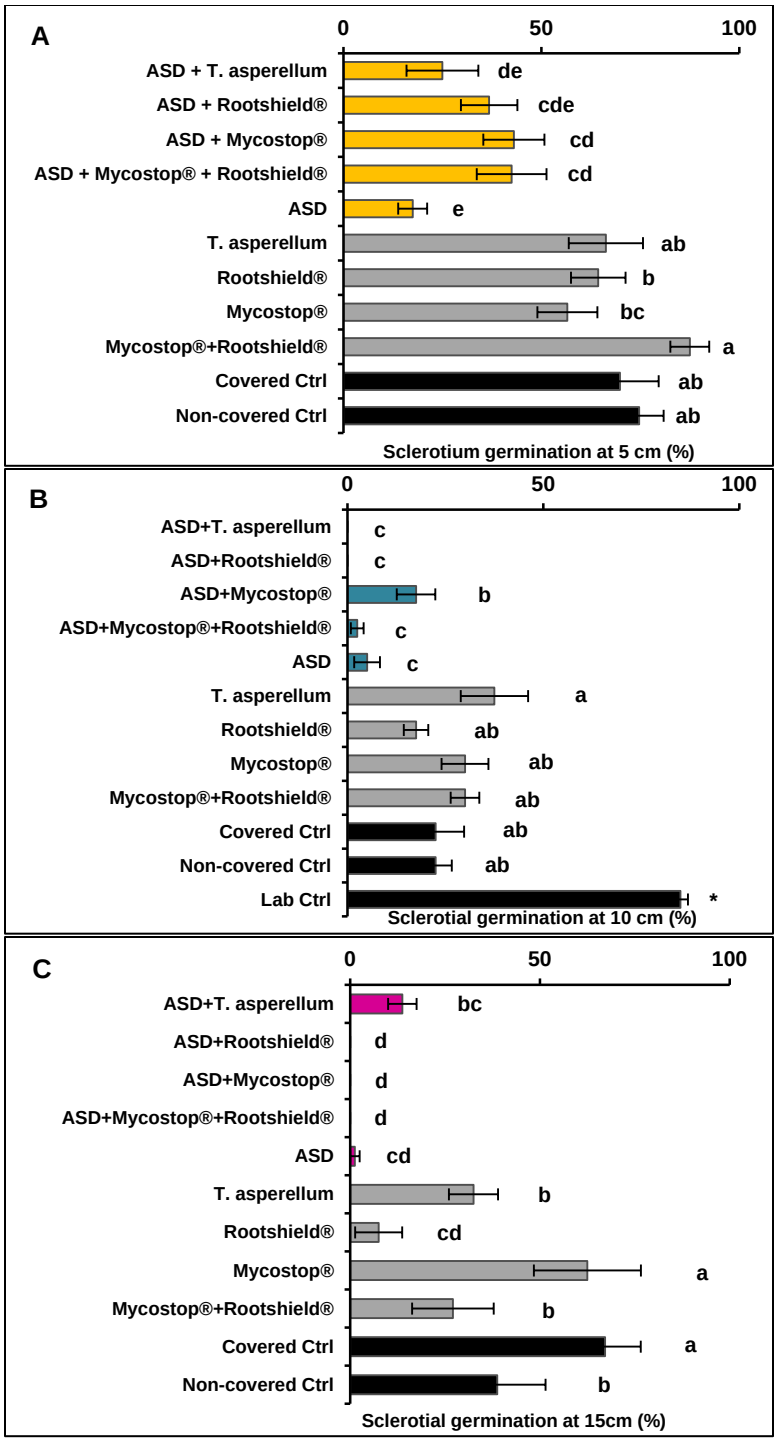


Figure 5-4. Effect of ASD (with/without biofungicides) and biofungicides on percentage sclerotial germination from sclerotia recovered from (A) 5-cm examined on PDA, (B) 10-cm examined on AIA, and (C) 15-cm examined on TSM.
Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's PLSD test. Error bars indicate standard error with four replicates.

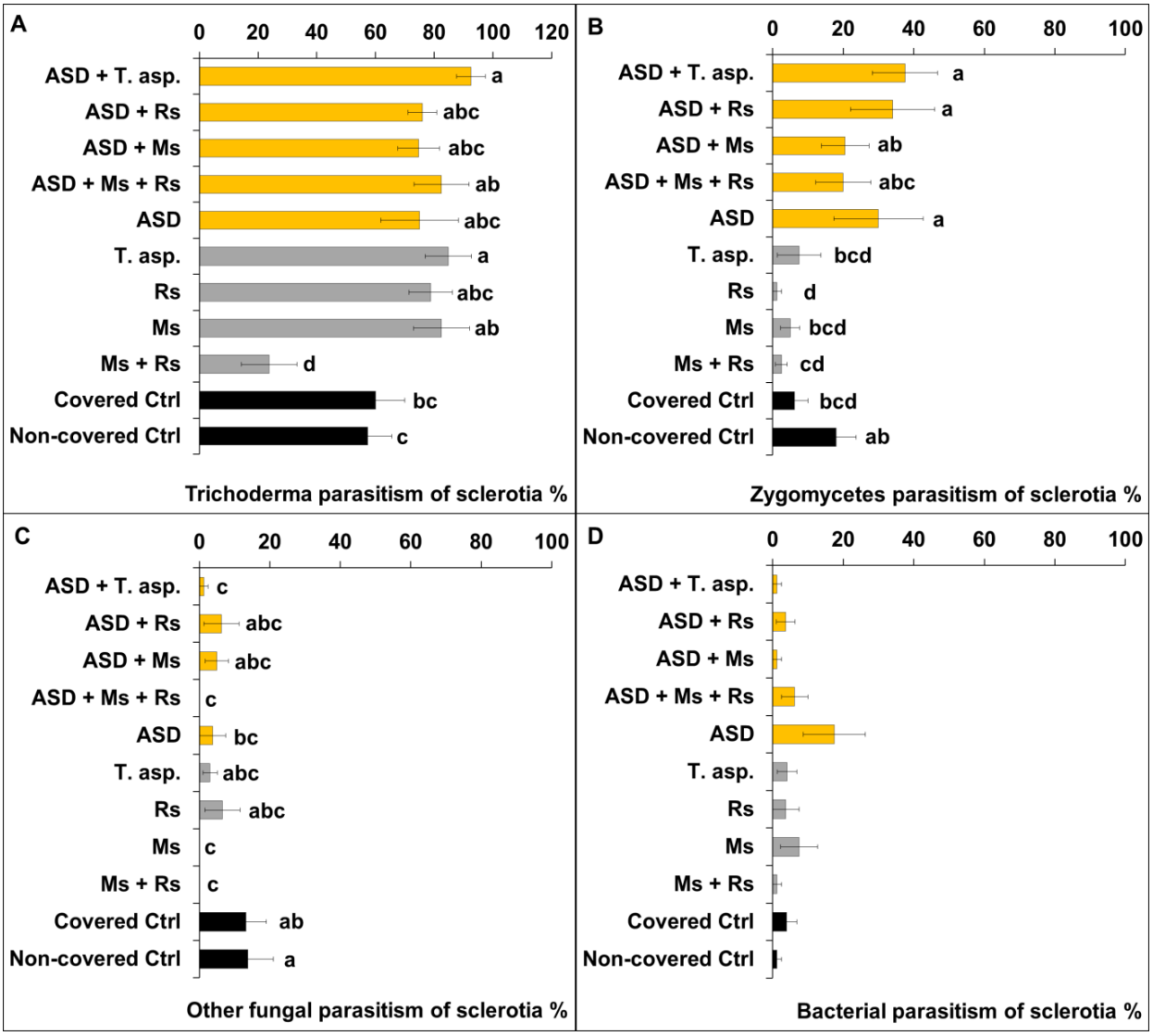


Figure 5-5.Effect of ASD (with/without biofungicides) and biofungicides on (A) percentage sclerotial parasitism by *Trichoderma*, (B), zygomycetes, (C) other fungi, and (D) bacteria examined on PDA. Sclerotia were recovered from a depth of 5-cm.
Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's PLSD test. Error bars indicate standard error with four replicates. ASD=Pots amended with dry molasses, T. asp.=*T. asperellum*, Rs=Rootshield®, Ms=Mycostop®, Ctrl=control

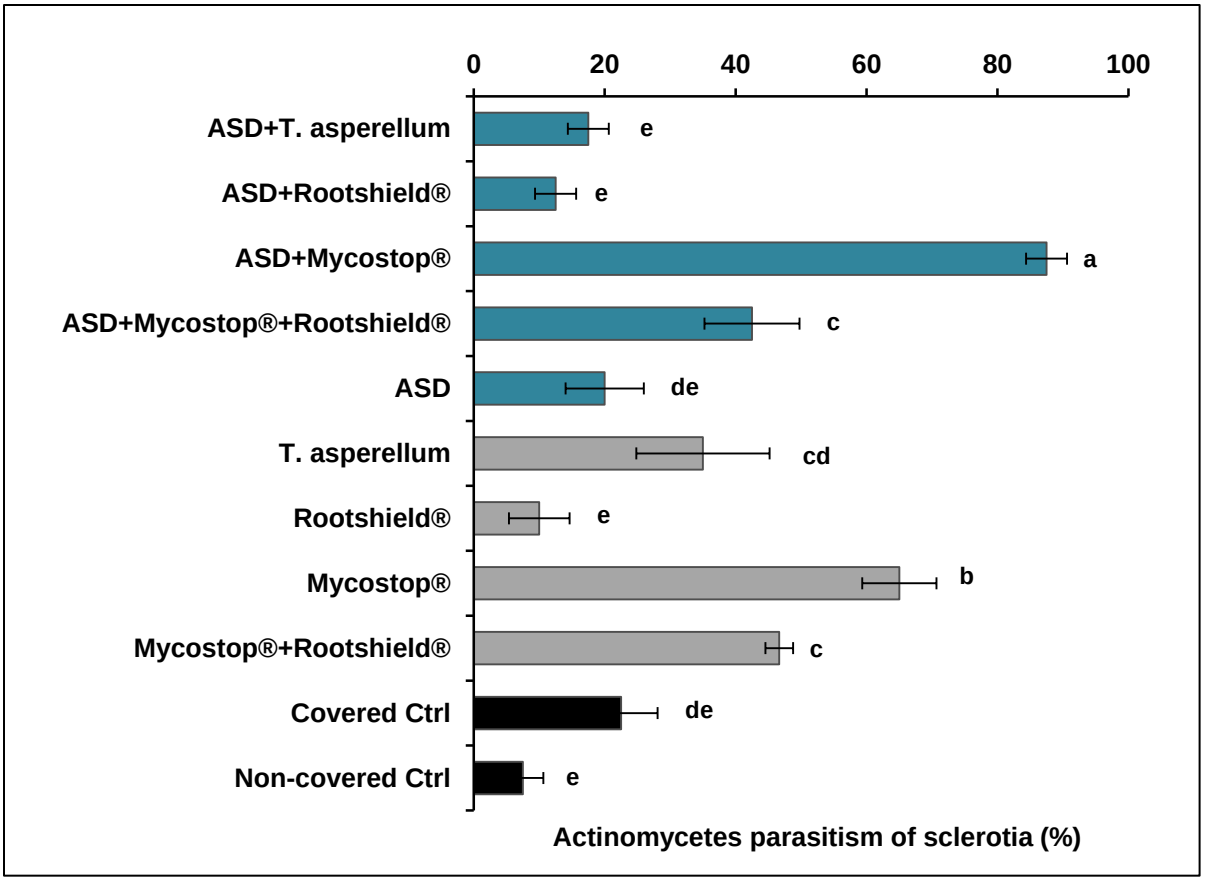


Figure 5-6. Effect of ASD (with/without biofungicides) and biofungicides on percentage sclerotial parasitism by actinomycetes on AIA. Sclerotia were recovered from a depth of 10-cm. Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's PLSD test. Error bars indicate standard error with four replicates.

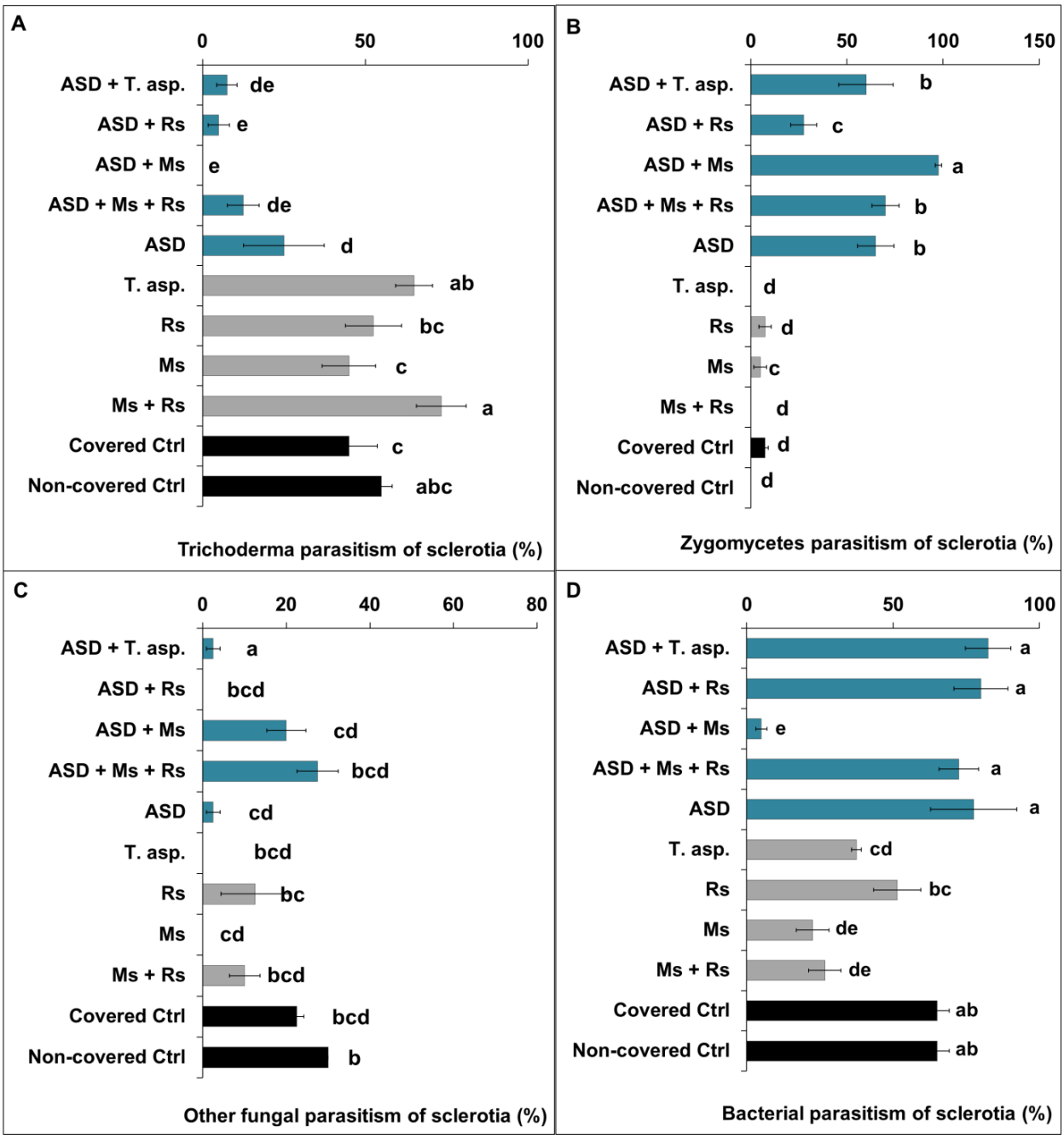


Figure 5-7. Effect of ASD (with/without biofungicides) and biofungicides on percentage sclerotial parasitism by (A) *Trichoderma* (B), zygomycetes (C) other fungi, and (D) bacteria on AIA. Sclerotia were recovered from a depth of 10-cm.
Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's PLSD test. Error bars indicate standard error with four replicates. ASD=Amended with dry molasses, T. asp.=*T. asperellum*, Rs=Rootshield®, Ms=Mycostop®, Ctrl=control

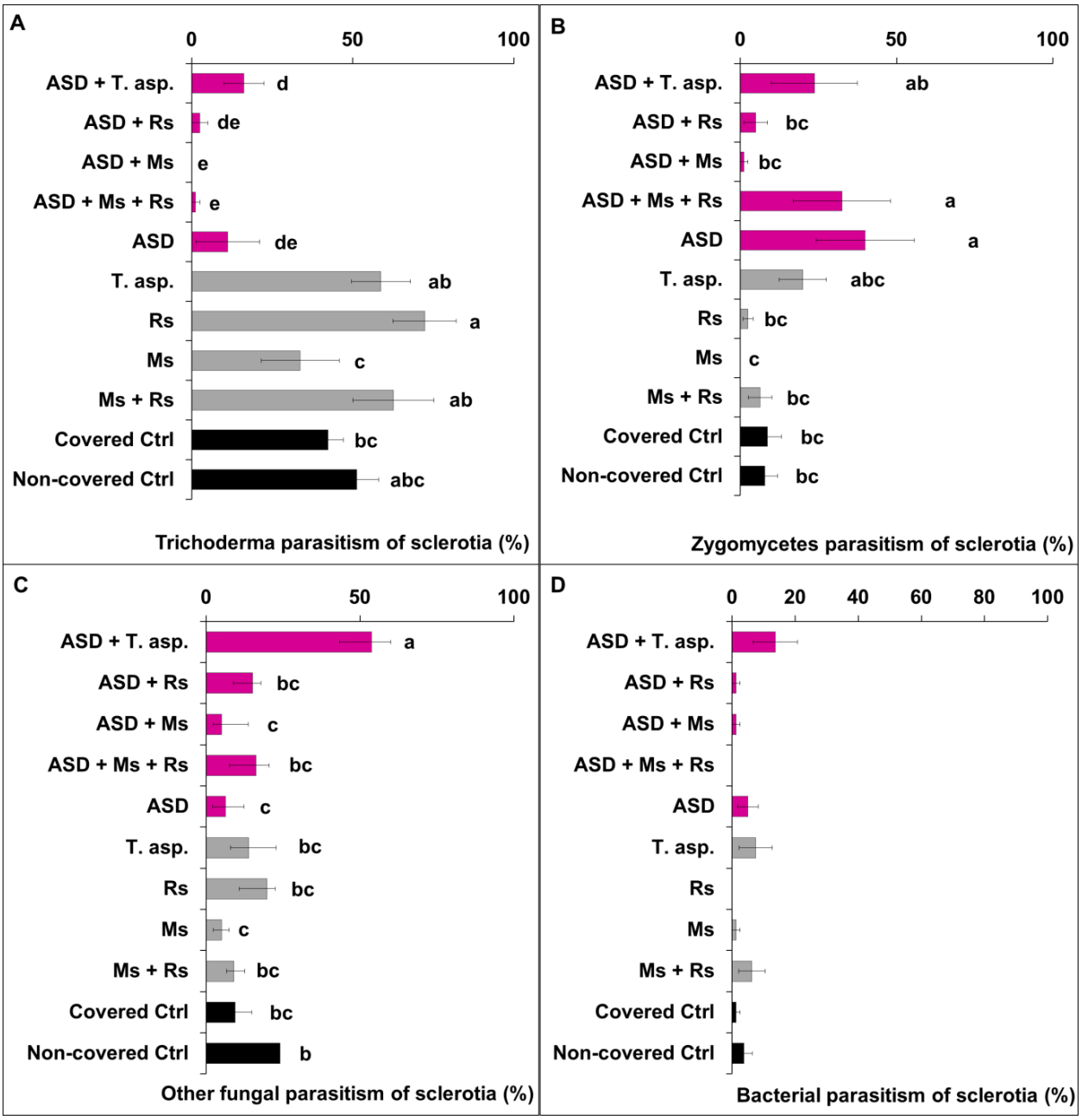


Figure 5-8. Effect of ASD (with/without biofungicides) and biofungicides on (A) percentage sclerotial parasitism by *Trichoderma* (B), zygomycetes (C) other fungi and (D) bacteria examined on TSM. Sclerotia were recovered from a depth of 15-cm.
Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's PLSD test. Error bars indicate standard error with four replicates. ASD=Pots amended with dry molasses, T. asp.=*T. asperellum*, Rs=Rootshield®, Ms=Mycostop®, Ctrl=control

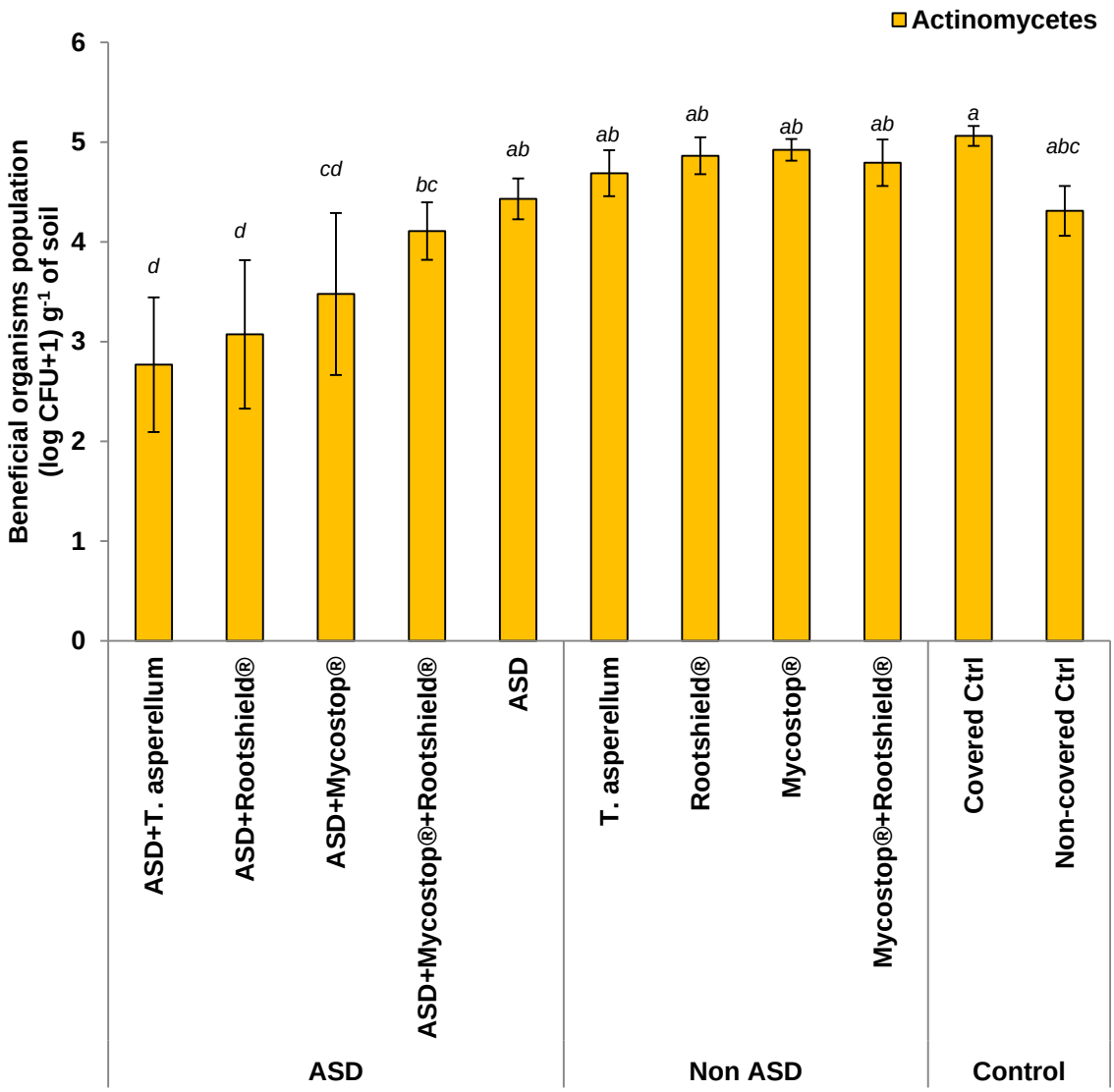


Figure 5-9. Effect of ASD (with/without biofungicides) and biofungicides on soil population of actinomycete spp.
Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's PLSD test. Error bars indicate standard error with four replicates.

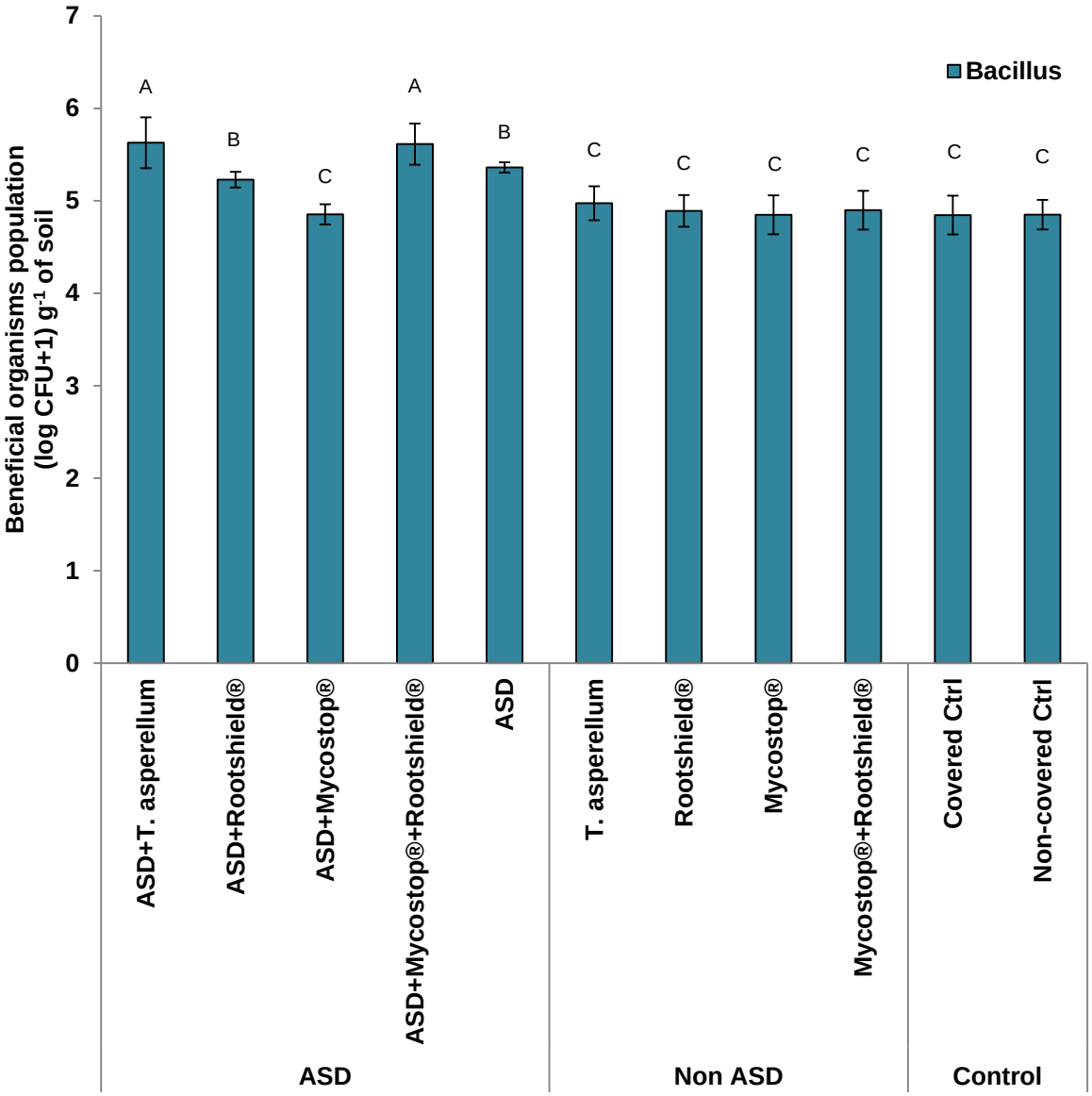


Figure 5-10. Effect of ASD (with/without biofungicides) and biofungicides on soil populations of *Bacillus* spp.

Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's PLSD test. Error bars indicate standard error with four replicates.

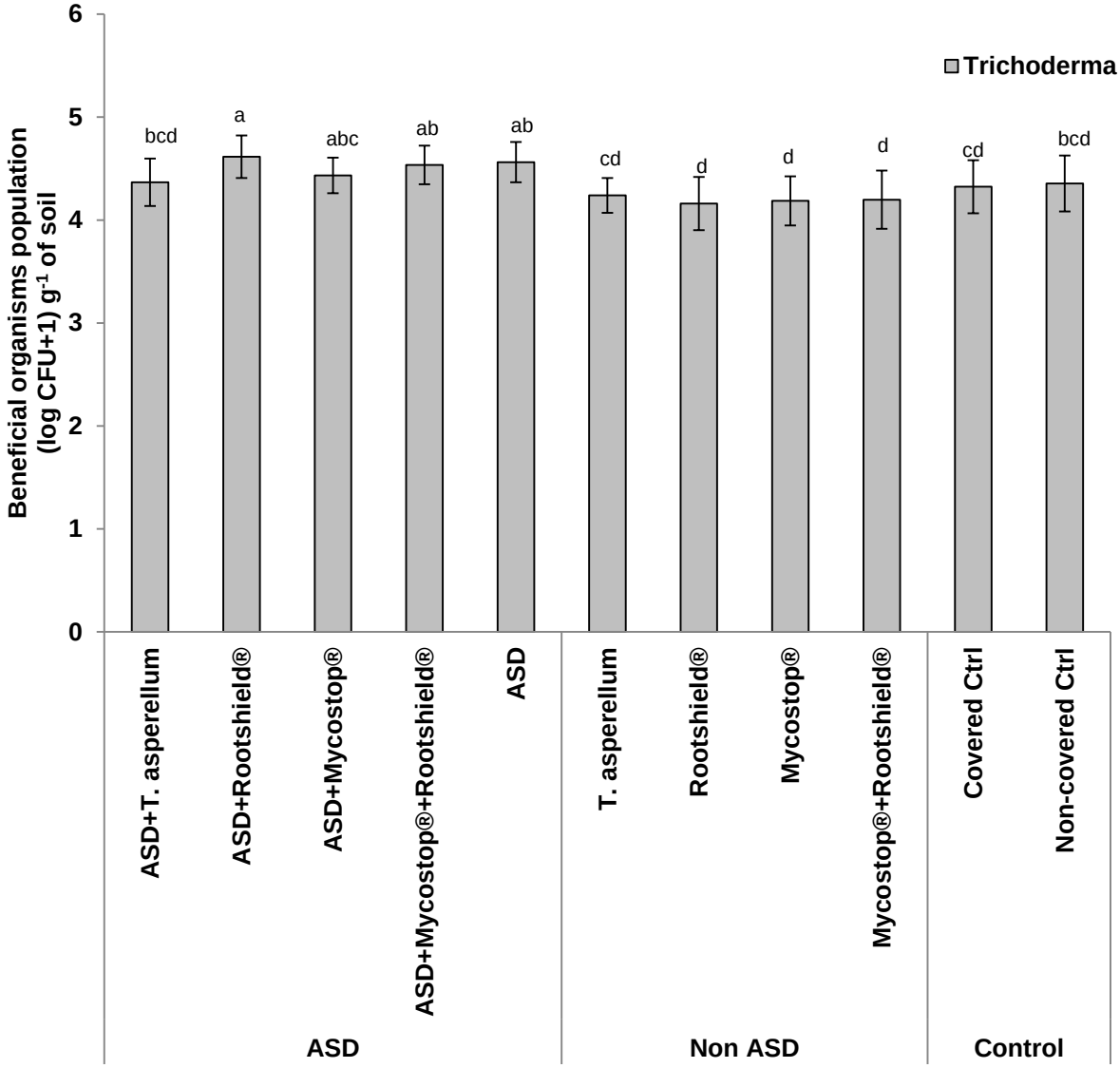


Figure 5-11. Effect of ASD (with/without biofungicides) and biofungicides on soil populations of *Trichoderma* spp.

Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's PLSD test. Error bars indicate standard error with four replicates.

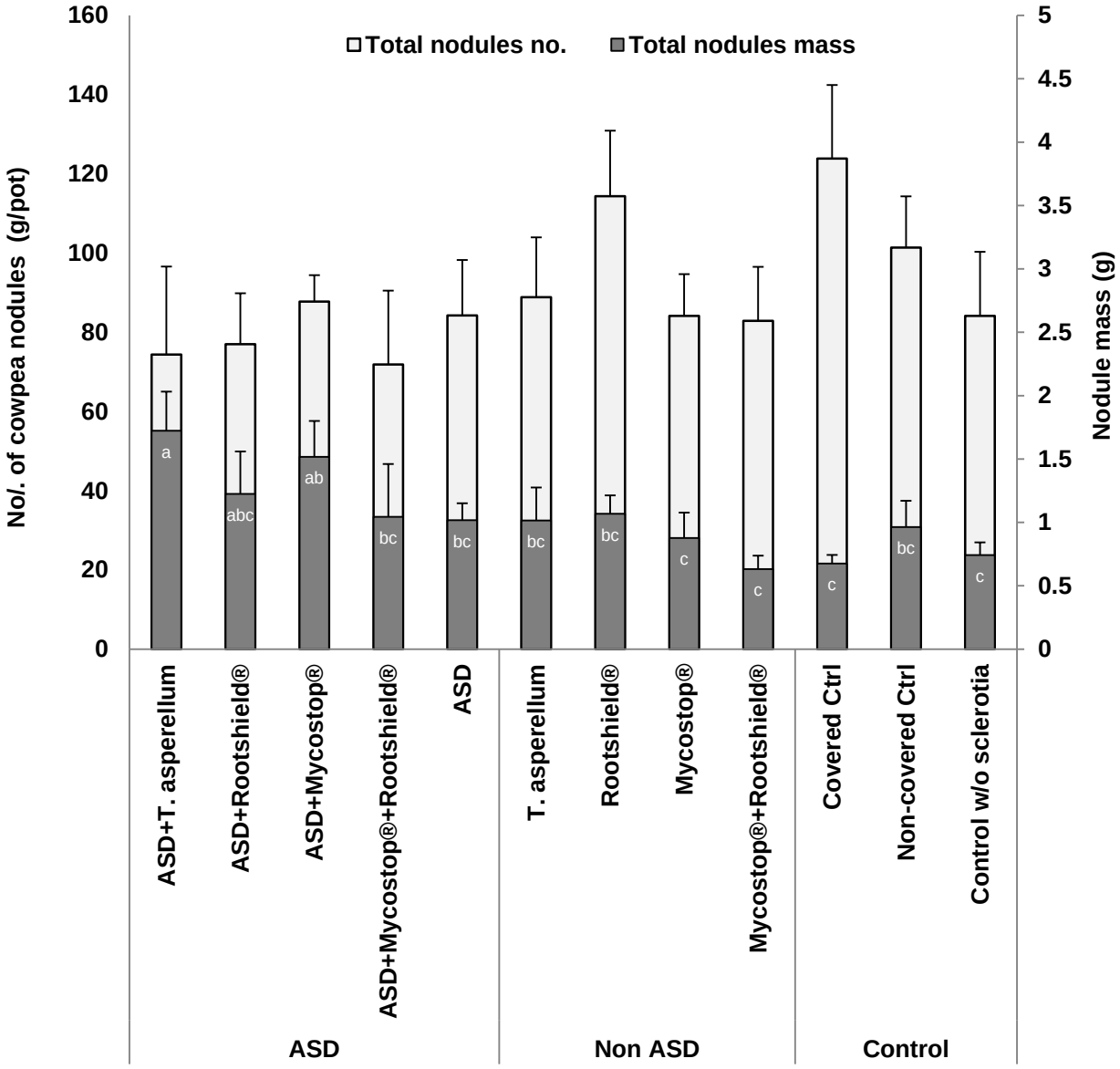


Figure 5-12. Effect of ASD (with/without biofungicides) and biofungicides on cowpea nodules number and mass.

Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's PLSD test. Error bars indicate standard error with four replicates.

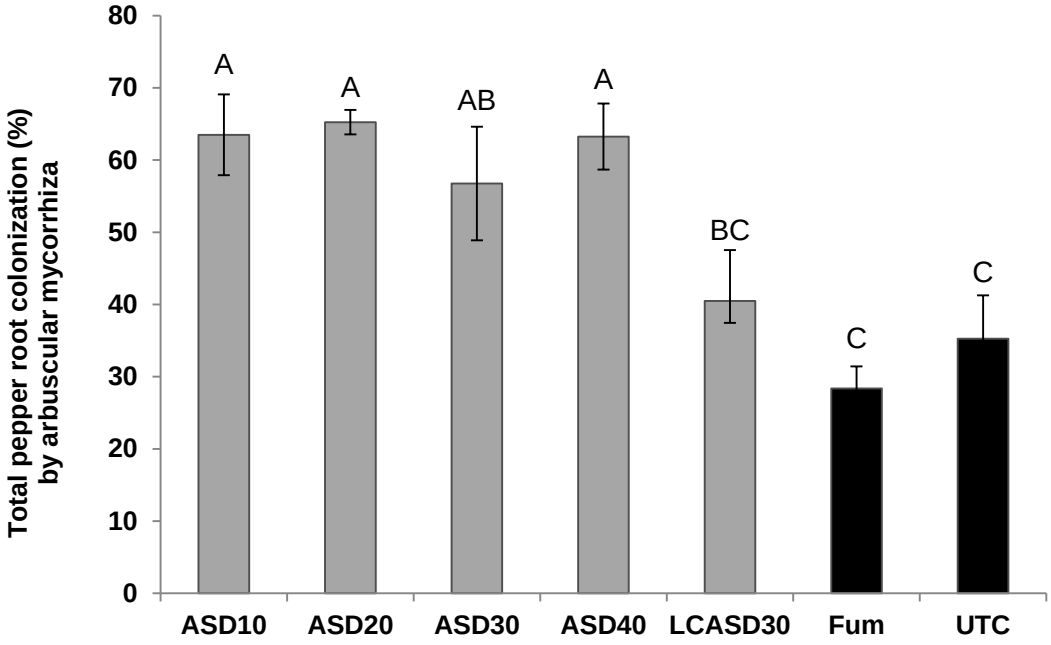


Figure 5-13. Effect of amendment C:N ratio on percentage root colonization after ASD treatment, 2014.

Bars indicated by different letters are significantly different, $p<0.05$ according to Fisher's protected LSD test. ASD10=C:N ratio 10:1, ASD20=C:N ratio 20:1, ASD30=C:N ratio 30:1, ASD40=C:N ratio 40:1, LCASD30=C:N ratio 30:1, C rate 2 mg C g-1 soil (LC ± 'low carbon') and UTC=Untreated, non-amended control.

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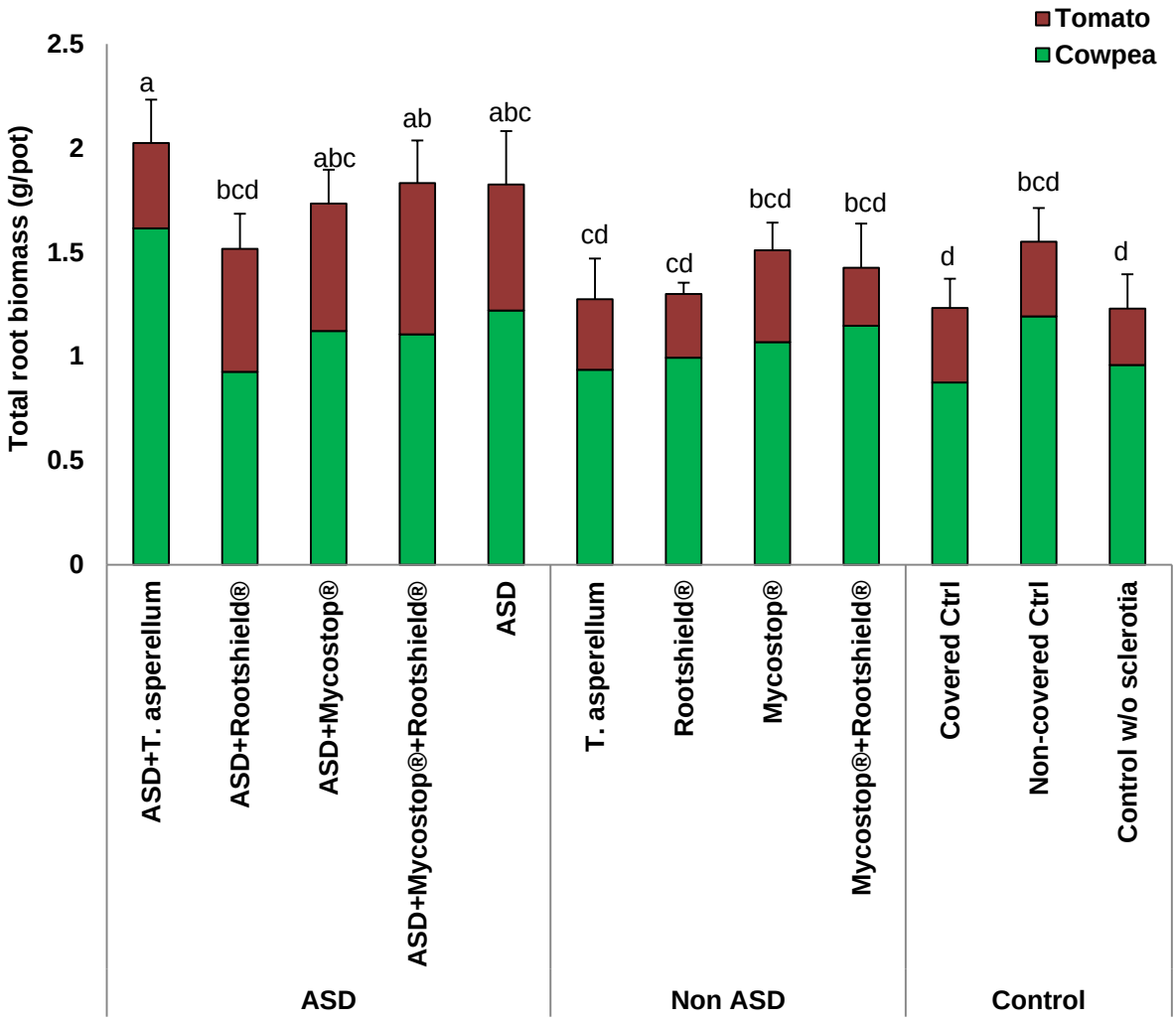


Figure 5-14. Effect of ASD (with/without biofungicides) and biofungicides on root biomass of cowpea and tomato.
Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's PLSD test. Error bars indicate standard error with four replicates.

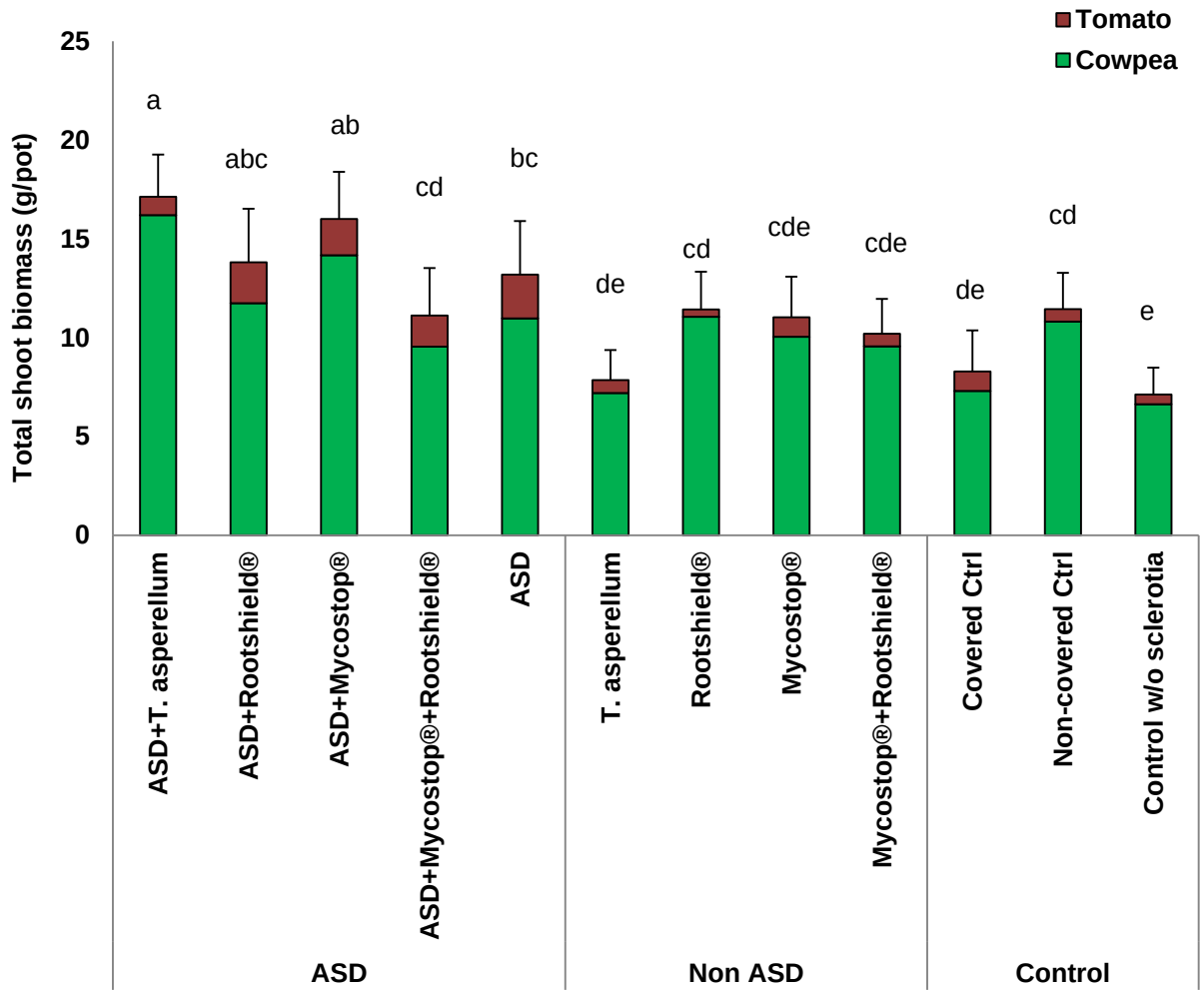


Figure 5-15. Effect of ASD (with/without biofungicides) and biofungicides on shoot biomass of cowpea and tomato.
Bars indicated by different letters are significantly different at $p<0.05$ according to Fisher's PLSD test. Error bars indicate standard error with four replicates.

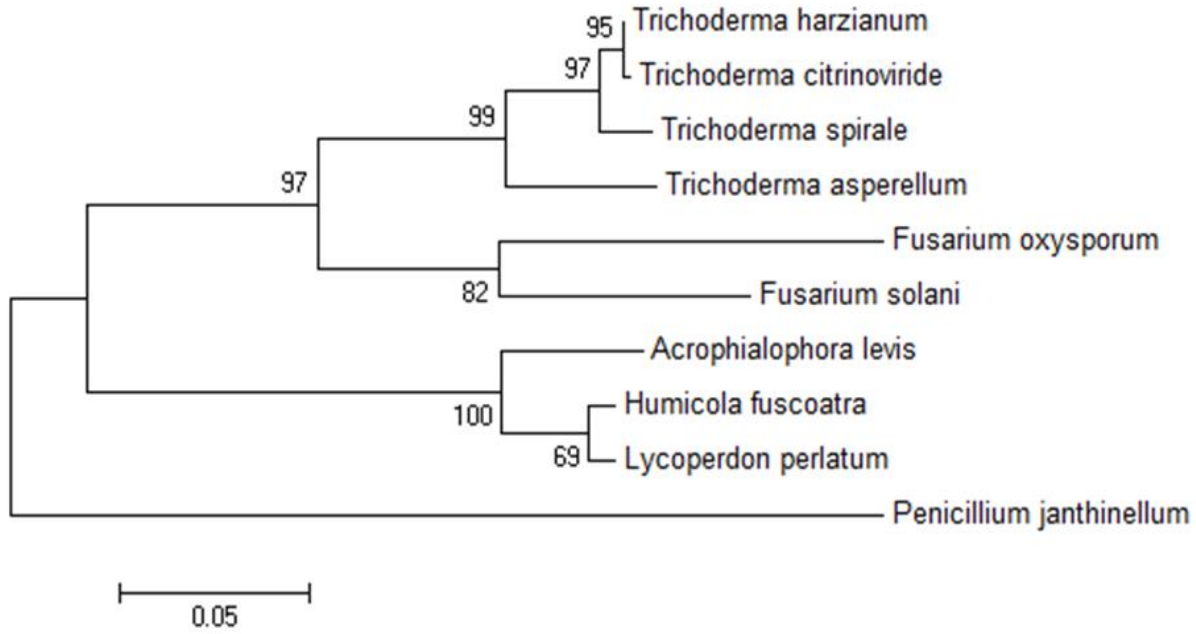


Figure 5-16. Phylogenetic analysis of major soil microbial fungi identified in ASD treated soil. Sequences were aligned using ClustalW and phylogenetic tree was constructed using Mega 5.6. The values in the tree represent bootstrap value for 100 replications.

# Summary

The harmful effects of agro-chemicals on the environment, agro-biodiversity, and human beings have been well established. Therefore, there is an increasing concern for the production methods of horticultural products, which has motivated farmers to shift from current production practices that utilize chemical soil fumigants to non-chemical practices. However, the lack of promising non-chemical alternatives is a major problem for growers even if they intend to shift toward a non-chemical approach. In addition, the phase out of the broad-spectrum fumigant methyl bromide has compelled additional growers to seek alternatives and it is imperative that we develop reliable non-chemical options that will control pests and maintain crop yields. Flooding, solarization, steam sterilization, and bio-fumigation are options that can be adopted in regions where water availability, high temperature, high investment, and site-specific crucifer production are not limiting, respectively. For regions with any of these limitations, anaerobic soil disinfestation (ASD) could be an alternative to management of pathogens, weeds, and nematodes in high-value horticultural crops. However, before adopting ASD production systems, it is imperative to optimize the conditions of ASD to fit in a given production system and prevailing environmental conditions. In this study, we carried out meta-analysis of ASD to show that ASD is effective against pathogens and weeds, and we identified the ideal C:N ratio and C rate of ASD amendment that is effective to control two key soilborne pathogens (*Fusarium oxysporum* and *Sclerotium rolfsii*) and nutsedge tubers for a moderate soil temperature regime.

Our meta-analysis study on the effectiveness of ASD on pest suppression and yield of horticultural crops showed that ASD is effective in suppressing soilborne bacterial, oomycete, and fungal pathogens under different environmental regimes, with various soil types, soil temperatures, and different incubation periods of treatments, compare to non-amended controls. Analysis of ASD effectiveness on pathogen, nematodes, and weeds across a range of amendment types (agricultural by-products – cereal bran and crop residue, cruciferous, legume, grass, protein by-product, manure, organic acid) demonstrated that pathogen suppression was significant. However, due to variability in reported research, more studies on nematodes and weeds are warranted to draw conclusive results on the overall effectiveness of ASD.

To evaluate the impact of organic amendment C:N ratio (dry molasses or wheat bran) and C rate (dry molasses) on ASD treatment at moderate soil temperatures (15 to 25°C), we conducted growth chamber, greenhouse, and field studies at the University of Tennessee. Results showed high soil anaerobic activity in all amended treatments, regardless of amendment type and rate, in both pot and field study and lowest soil pH at amendment C:N ratio 10:1 for pot studies. Similarly, the survival and production of introduced yellow nutsedge tubers, survival of inoculum of *Fusarium oxysporum* and *Sclerotium rolfsii* were lowered in all amended pots at 4 mg C $g^{-1}$ of soil when compared to a non-amended control. The lowest populations of *F. oxysporum* were generally observed at amendment C:N ratios of 20:1 and 30:1 for the dry molasses amendment. However, amendment type and C:N ratio did not affect the mortality of sclerotia and a carbon rate of 2 to 4 mg C $g^{-1}$ at C:N 30:1 was optimum to control the pathogen. ASD amendment application increased parasitism of sclerotia by *Trichoderma*, zygomycetes, *Aspergillus*, *Fusarium*, actinomycetes, bacteria and other unidentified fungi. The occurrence of these mycoparasites varied across studies and was primarily driven by soil type and isolation media in the lab. ASD with dry molasses (4 mg C $g^{-1}$ of soil) at C:N 30:1, did not alter nodule formation by rhizobia on cowpea, and soil population of beneficial actinomycetes. In addition, ASD enhanced populations of key fungal antagonists, *Trichoderma*, and bacteria, *Bacillus* and total root colonization by arbuscular mycorrhizae. We also observed that use of RootShield® and/or Mycostop® or *T. asperellum* during ASD treatment did not enhance sclerotial mortality. Future research focusing on the application of antagonists as seed treatment or by soil drenching after the termination of ASD is recommended.

As reported before, organic acid assay of soil solution from pot trials at various C:N ratios showed that acetic and butyric are the primary organic acids generated during ASD treatment, along with isobutyric, isovaleric, methyl butryric, and propionic acid. ASD, however, did not show any significant improvement in tomato biomass in pot studies, we observed increased pepper fruit yield in all ASD treatments compared to both fumigated and control treatments. Our results showed that the higher C rate (4 mg C $g^{-1}$ of soil) and a C:N ratio <30:1 gave the highest yield, suggesting C:N ratio and C rates are important determinants for yield.

Along with the above-mentioned works on optimization of organic amendments for ASD to control pathogens and weeds, as an effort to disseminate this technique, we also set up

demonstration trials of ASD in Nepal to control common plant pathogens in tomato production areas. The trials were established at the Central Horticulture Center, Kirtipur, Nepal on May 2016 and 25 farmers from various districts were trained to use this technique. For the demonstration, a locally available agricultural by-product (rice hulls; 1 kg $m^{-2}$) was used as the carbon supplement in the soil and tarped after irrigation.

In short, we showed that ASD is effective for control of various plant pathogens and weeds under controlled and partially controlled environment. We recommend testing this optimized ASD technique on farmers' fields to evaluate constraints in working production systems.

# Vita

The author was born on January 1985 as the first daughter of Mr. Uttam Raj Shrestha and Mrs. Sheela Shrestha in Kalimati-14, Kathmandu, Nepal. She pursued her Bachelor Degree in Agricultural Science majoring in Horticulture from Tribhuvan University, Institute of Agriculture and Animal Science, Rampur, Chitwan in 2007. After receiving her B.S. degree, she started her Master of Science in Agriculture majoring in Conservation Ecology in 2009. She worked as a Horticulture Development Officer for the Government of Nepal for three years and then in 2013, she began her Doctor of Philosophy program in the Department of Plant Sciences at the University of Tennessee, Knoxville, under the direction of Dr. David M. Butler. Her dissertation research focused on optimization of organic amendments used in anaerobic soil disinfestation to control key soilborne pathogens and weeds.

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