



12-2018

Role of Organic Acids in Suppression of *Sclerotium rolfsii* During Anaerobic Soil Disinfestation (ASD)

Keagan James Swilling
University of Tennessee, kswillin@vols.utk.edu

Follow this and additional works at: https://trace.tennessee.edu/utk_gradthes

Recommended Citation

Swilling, Keagan James, "Role of Organic Acids in Suppression of *Sclerotium rolfsii* During Anaerobic Soil Disinfestation (ASD). " Master's Thesis, University of Tennessee, 2018.
https://trace.tennessee.edu/utk_gradthes/5347

This Thesis is brought to you for free and open access by the Graduate School at TRACE: Tennessee Research and Creative Exchange. It has been accepted for inclusion in Masters Theses by an authorized administrator of TRACE: Tennessee Research and Creative Exchange. For more information, please contact trace@utk.edu.

To the Graduate Council:

I am submitting herewith a thesis written by Keagan James Swilling entitled "Role of Organic Acids in Suppression of Sclerotium rolfsii During Anaerobic Soil Disinfestation (ASD)." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant Sciences.

David M. Butler, Major Professor

We have read this thesis and recommend its acceptance:

Kimberly D. Gwinn, Bonnie H. Ownley

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Role of Organic Acids in Suppression of *Sclerotium rolfsii* During Anaerobic Soil Disinfestation (ASD)

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Keagan James Swilling
December 2018

Copyright © 2018 Keagan Swilling
All rights reserved.

ACKNOWLEDGEMENTS

First off, I would like to thank Dr. David M. Butler, Associate Professor in the Department of Plant Sciences at the University of Tennessee, Knoxville. Not only was Dr. Butler's door always open for any questions or concerns I had with both my research and my thesis, he also actively invested his off-campus time and attention to this project, for which I am very grateful. He has been an outstanding advisor and mentor throughout this whole process.

I would like to thank Dr. Bonnie H. Ownley, Professor and Research Plant Pathologist in the Department of Entomology and Plant Pathology at the University of Tennessee, Knoxville. Her expertise during the formation and steering of the project, her instruction in the editing process, and the use of her lab was invaluable. I am also grateful to Dr. Kimberly D. Gwinn, Associate Professor of the Department of Entomology and Plant Pathology at the University of Tennessee, Knoxville. Her perception and editing skills provided new insight into the study. Without their dedicated input and direction, my study would not be what it is today. I feel very fortunate to have them on my graduate committee.

I sincerely want to thank Dr. Utsala Shrestha, for her direct involvement in this project from beginning to completion. Her insight, recommendations, time, and expertise, as well as her previous works, were critical to all aspects of this study. Her dedication to both this project and her many other works in the field of sustainable agriculture has been an inspiration every step of the way.

I would like to thank Mary E. Dee, for her continued support and guidance throughout the project. Her support during the many lab trials was a vital part of their success. Her expertise in lab techniques as well her general advice was always very appreciated.

I would also like to acknowledge the indispensable contribution of those who helped me conduct the experiments that went into this study. Specifically, I would like to thank all the members of Dr. Butler's lab who played a role over the course of the project: Karen Leu, Ben Carter, Rachel Collins, Samantha Beal, Heather Toler, Amber Macdonald, Nick Ballew, McKenna Bates, Tyler Newton, and Ryan Vichich.

I would like to thank Sarah Stone for her constructive criticism, thoroughness, and dedication to the editing of this work. Her expertise has been pivotal to its completion.

Finally, I want to express my gratitude to my family, especially my inquisitive and involved wife Kayla Swilling, who has supported me throughout my years of study. Her encouragement, interest, and love have been invaluable.

Thank you all.

ABSTRACT

Anaerobic soil disinfestation (ASD) is a non-chemical method used for controlling soilborne plant pathogens. Individual elements of the ASD (also referred to as biological soil disinfestation or BSD) process, including application of organic amendments or soil saturation, have been studied for over 50 years for suppression of various soilborne plant pathogens. More recent research, primarily in the Netherlands, Japan, and the United States has been targeted at developing a soil disinfestation process based on anaerobic decomposition of labile soil amendments that can be integrated into modern horticultural production systems as a soil fumigant. The process leads to the creation of several fermentation by-products including the generation of short chain organic acids or volatile fatty acids (VFAs), other volatile compounds, and subsequent lowering of soil pH. These byproducts act as pesticides for soilborne pathogens. The saturated soil, changes in soil microbial communities, and byproducts of fermentation give rise to an environment inhospitable to many plant pathogens. Volatile fatty acids (VFAs), including acetic and *n*-butyric acid, are reported to play a role in the suppression of plant pathogen inoculum during anaerobic soil disinfestation (ASD), but it is unclear how VFAs affect sclerotia of *Sclerotium rolfsii*. To evaluate the effect of VFA, VFA concentration, soil pH and soil texture on germination of *S. rolfsii* sclerotia, a series of anaerobic growth chamber trials was conducted. Alongside anaerobic growth chamber trials, greenhouse trials were conducted to evaluate endemic soil populations of *Trichoderma* spp. following the same ASD treatments. These studies show Acetic and *n*-butyric acids in soil solution are likely a primary factor in suppression of germination and colonization of *S. rolfsii* during ASD treatment, and activity against these fungal propagules is dependent on concentration, solution pH and soil texture. Sclerotial germination was generally reduced by exposure to either acetic or *n*-butyric acids, and most notably reduced by VFAs in autoclaved soil (29% germination). Overall, mean germination rates in *n*-butyric acid were significantly lower than those of acetic acid (32% and 44%, respectively). Germination rates were reduced as acid concentration increased to 4, 8, and 16 mmol/kg concentration (56%, 38% and 19%, respectively).

TABLE OF CONTENTS

Chapter 1 <i>Literature review of ASD background and mechanisms</i>	1
Summary	2
Anaerobic soil disinfestation.....	2
<i>Trichoderma</i>	5
Soil microbial community.....	6
<i>Sclerotium rolfsii</i>	6
Conclusion	7
Works Cited	8
Chapter 2 <i>Volatile fatty acid concentration, soil pH and soil texture alter efficacy of anaerobic soil disinfestation in suppression of Sclerotium rolfsii germination</i>	13
Abstract	14
Introduction.....	14
Materials and Methods.....	15
Study 1.1. VFA concentration and soil solution pH effects on <i>S. rolfsii</i> germination	15
Study 1.2. VFA concentration and soil solution pH effect on <i>S. rolfsii</i> germination as affected by soil texture.....	16
Results.....	17
Study 1.1. VFA concentration and soil solution pH effects on germination and colonization of <i>S. rolfsii</i> sclerotia.....	17
Study 1.2. VFA concentration and soil solution pH effect on <i>S. rolfsii</i> germination as affected by soil texture.....	17
Discussion	19
Study 1.1. Germination	19
Study 1.1. Colonization.....	19
Study 1.2. Germination	20
Study 1.2. Colonization.....	20
Works Cited	22
Appendix: Figures and Tables	25
Chapter 3 <i>Role of VFA and concentration, carbon amendment, and presence of endemic soil microbial community during ASD in the suppression of S. rolfsii germination and endemic Trichoderma populations</i>	32
Abstract	33
Introduction.....	33
Materials and Methods.....	35
Study 2.1. Response of endemic soil populations of <i>Trichoderma</i> spp. to VFA, VFA concentration, and organic soil amendments during ASD.....	35
Study 2.2. Role of endemic soil microbial activity and carbon amendment on VFA-induced suppression of <i>S. rolfsii</i> germination during ASD.....	36
Results.....	37
Study 2.1. Response of endemic soil populations of <i>Trichoderma</i> spp. to VFA, VFA concentration, and organic soil amendments during ASD.....	37
Study 2.2. Role of endemic soil microbial activity and carbon amendment on VFA-induced suppression of <i>S. rolfsii</i> germination during ASD.....	38
Discussion	39

Study 2.1. Response of endemic soil populations of <i>Trichoderma</i> spp. to VFA, VFA concentration, and organic soil amendments during ASD.....	39
Study 2.2. Role of endemic soil microbial activity and carbon amendment on VFA-induced suppression of <i>S. rolfisii</i> germination during ASD.....	39
Works Cited	41
Appendix: Figures and Tables	44
Chapter 4 <i>Conclusion</i>	49
Summary	50
Vita.....	51

LIST OF TABLES

Table 2.1. Analysis of variance for response variables of germination and colonization of <i>Sclerotium rolfsii</i> sclerotia in study 1.1 as affected by the main effects of volatile fatty acid (VFA), VFA concentration, soil pH, and their interactions	25
Table 2.2. Analysis of variance of response variables germination and colonization of <i>Sclerotium rolfsii</i> sclerotia in study 1.2 as affected by main effects of volatile fatty acid (VFA), VFA concentration, soil pH, soil texture and their interactions	26
Table 3.1. Analysis of variance for response variables of <i>Trichoderma spp.</i> populations in study 2.1 as affected by the main effects of volatile fatty acid (VFA), VFA concentration, application of carbon amendments, and their interactions	38
Table 3.2. Analysis of variance of response variables germination and colonization of <i>Sclerotium rolfsii</i> sclerotia in study 1.2 as affected by main effects of volatile fatty acid (VFA) type, VFA concentration, soil amendment, soil type and their interactions	39
Table 3.3. Response variables percent germination and colonization of <i>Sclerotium rolfsii</i> sclerotia in study 2.2 as affected by main effects of volatile fatty acid (VFA) type, VFA concentration, soil amendment, soil autoclaving	40

LIST OF FIGURES

Figure 2.1. Mean percentage germination of <i>Sclerotium rolfsii</i> sclerotia in study 1.1 as affected by volatile fatty acid (VFA) concentration and soil pH.....	20
Figure 2.2. Mean percentage germination of <i>Sclerotium rolfsii</i> sclerotia in study 1.2 as affected by volatile fatty acid (VFA) concentration, soil pH, and soil texture.....	21
Figure 2.3. Mean percentage germination of <i>Sclerotium rolfsii</i> sclerotia in study 1.2 as affected by volatile fatty acid (VFA), VFA concentration, and soil texture.....	22
Figure 2.4. Mean percentage colonization of <i>Sclerotium rolfsii</i> sclerotia in study 1.2 as affected by volatile fatty acid (VFA) concentration and soil pH.....	23
Figure 2.5. Mean percentage colonization of <i>Sclerotium rolfsii</i> sclerotia in study 1.2 as affected by volatile fatty acid (VFA) concentration and soil texture.....	24
Figure 3.1. Mean percentage germination of <i>Sclerotium rolfsii</i> sclerotia in study 2.2 as affected by volatile fatty acid (VFA) concentration, and soil autoclaving.....	25
Figure 3.2. Mean percentage germination of <i>Sclerotium rolfsii</i> sclerotia in study 2.2 as affected by volatile fatty acid (VFA) concentration and soil amendment.....	26

CHAPTER 1

LITERATURE REVIEW OF ASD BACKGROUND AND MECHANISMS

Summary

Anaerobic soil disinfestation (ASD) is a non-chemical method used for controlling soilborne plant pathogens. Individual elements of the ASD (also referred to as biological soil disinfestation or BSD) process, including application of organic amendments or soil saturation, have been studied for over 50 years for suppression of various soilborne plant pathogens (Baker 1968, Shennan et al. 2014). More recent research, primarily in the Netherlands, Japan, and the United States has been targeted at developing a soil disinfestation process based on anaerobic decomposition of labile soil amendments that can be integrated into modern horticultural production systems as a soil fumigant alternative (Blok et al. 2000, Shinmura 2004). A predominant factor in research activity related to ASD was the necessity of developing alternatives to the soil fumigant methyl bromide, which is slowly being phased out of usage in over 197 countries following international adoption of the 1987 Montreal Protocol.

Methyl bromide had been used for decades as the primary soil fumigant due to its broad-spectrum activity against soilborne pests and weed propagules, and relative ease of use in plasticulture production systems. However, due to its ozone depleting nature it was due for complete prohibition in the United States in 2017. Several chemical fumigant alternatives have been researched and are commercially used in place of methyl bromide but all face limitations in terms of efficacy, regulatory restrictions on use, or potentially negative effects on environmental quality or human health. Further, desire for more ecologically friendly and sustainable crop production systems has led researchers to evaluate and optimize ASD methods that maximize potential for use in commercial horticultural crop production.

Anaerobic soil disinfestation

In past research, ASD has been shown to be as effective as methyl bromide against many soilborne pathogens (Butler et al. 2012, 2014). ASD has seen wide adoption in certain sectors (e.g., in-ground greenhouse production in Japan, organic strawberry in California), but the ASD treatment process is heavily influenced by amendment type and environmental conditions (e.g., soil type, temperature). To date most studies have focused on adaptation to regional cropping systems and pathogens. The lack of simple decision tools and protocols for ASD treatment implementation across environments, crops, and pathogens of interest makes it difficult to increase adoption among producers.

Anaerobic soil disinfestation is relatively simple in concept. The aim of the method is to induce a prolonged period (typically 2 to 6 weeks) of anaerobic soil conditions prior to planting a high value horticultural crop. To accomplish this, a labile carbon source (e.g., fresh cover crop residue, agricultural by-products) is incorporated into the soil then covered with polyethylene mulch film. Once covered, the area is irrigated to saturation with drip irrigation and left to ferment until planting. The readily-available carbon along with high moisture levels allow anaerobic bacteria such as *Clostridium* and *Enterobacter* to proliferate and begin the process of fermentation. The process leads to the creation of several fermentation by-products including the generation of short chain organic acids or volatile fatty acids (VFAs), other volatile compounds, and subsequent lowering of soil pH (Momma 2008). These byproducts act as pesticides for soilborne pathogens (Abbasi et al. 2009, Huang et al. 2015, McElderry et al. 2005, Momma et al. 2006, Samaniego-Gaxiola and Balagurusamy 2013, Tenuta et al. 2002). The saturated soil, changes in soil microbial communities, and byproducts of fermentation give rise to an

environment inhospitable to many plant pathogens (Huang et al. 2015, Momma et al. 2013). The major obstacles to adoptions of ASD as an alternative to soil fumigation include finding an effective and economical carbon source in a given region, and determining treatment implementation practices (e.g., amendment rate, amendment chemical properties, tarping time) that result in consistent control of pathogens of interest. Accordingly, it is necessary to fully understand mechanisms through which ASD controls soilborne pests under varying treatment factors and environmental conditions in order to provide end-users with detailed recommendations on treatment implementation and management for diverse environmental conditions and pathosystems.

To date, research on the specific mechanisms of control by ASD are fairly limited. As a whole, the ASD process has been observed to work as well or better than methyl bromide for suppressing some soilborne plant pathogens (Butler et al. 2012b, 2014, Roskopf et al. 2014), but the specifics as to how this level of suppression occurs is not well-understood. ASD's mechanisms of pathogen control are varied, and it is not likely a single aspect of the process that accounts for a majority of control. The essential aspect that separates ASD from other biofumigation and biological disinfestation methods is a period of anaerobic activity, wherein the various other processes that make it effective occur (Lamers et al. 2010). Overall the anaerobic period seems to be essential to the control of several pathogens (Blok et al. 2000). The anaerobic period is facilitated by soil saturation. While flooding as a means of pathogen control can be effective on its own as seen in rice cultivation, it is not practical for most cropping systems. Studies on flooding have observed that it can take more than 6 weeks of flooding to see significant reduction in populations of fungal plant pathogens (Pullman and DeVay 1981, Ioannou et al. 1977). In cases where plant matter or other amendments were added, time needed for suppression was greatly reduced.

The addition of organic amendments alone reduces some plant pathogens when incorporated into field soil (Abbasi et al. 2009, Huber and Watson 1970, Tenuta et al. 2002). Inhibitory effects may be due to the encouragement of a wide range of other soil organisms that may compete with or prey on the plant pathogens and thus act as direct biocontrols (Bulluck et al. 2002, Chet et al. 1981, Lori et al. 2017, Mäder et al. 1996). Studies done in Tennessee (Shrestha et al. 2018) have shown that a certain ratio of C/N is preferable when choosing a carbon source depending upon certain soil and planting conditions, and pests of concern. Another way that the incorporation of labile carbon can be inhibitory to plant pathogens is by facilitating the rise in levels of organic compounds such as volatile fatty acids (VFAs) these to have a suppressive effect on some plant pathogen propagules (Huang et al. 2015). The rise in VFA concentration has been attributed to the proliferation of microorganisms that exude them as by-products of anaerobic metabolism (Blok et al. 2000, Bulluck et al. 2002, Huang et al. 2015, Momma 2008). VFAs include acetic, *n*-butyric, isovaleric, and propionic acids, all of which are produced by organisms such as *Clostridium* and *Enterobacter* under anaerobic conditions (Momma 2008).

The process of biological or anaerobic soil disinfestation (ASD) has been reported to be an effective alternative to soil fumigation for control of plant pathogen inoculum in several environments (Butler et al. 2014, Roskopf et al. 2014, Shennan et al. 2014), but the precise mechanisms of control are not well understood across environments and pathosystems. It is likely that numerous changes in soil chemical, physical, and biological properties contribute to ASD treatment effects (Roskopf et al. 2015, Hewavitharana et al. 2015 and 2016). Volatile fatty

acids (VFAs), including acetic acid, *n*-butyric acid, isobutyric acid, valeric acid, and isovaleric acid, are found in varying levels in most biologically active moist soils, but particularly proliferate during the anaerobic decomposition of labile carbon during ASD (Blok et al. 2000, Momma et al. 2006, Runia et al. 2014). Acetic acid and *n*-butyric acid were found in relatively high concentrations in soil during ASD (Huang et al. 2015, Runia et al. 2014, Shrestha et al. 2018). These compounds were an important component of control for inoculum of some soilborne plant pathogens, including *Fusarium*, *oxysporum*, *Verticillium dahliae*, *Pratylenchus penetrans*, and *Pyrenochaeta terrestris* along with several nematode species (Blok et al. 1999, Browning et al. 2006, Huang et al. 2015, Momma et al. 2006, Runia et al. 2014, Shinmura 2004, Oka 2010). It is not clear how VFAs interact with different components of the soil and its ecology during ASD. In this study, we will focus on how VFAs affect the soilborne plant pathogen *Sclerotium rolfsii* and the soilborne fungi, in the genus *Trichoderma*. We will also look at how soil texture (clay content, organic matter) and labile carbon amendments affect the suppressive nature of VFAs during ASD.

Volatile fatty acids (VFAs) or short-chain fatty acids are fatty acids with 2 to 6 carbons in chain. These acids are important to many organisms and are a major source of energy for ruminants and other animals (Besten et al. 2013, Kristensen et al. 1998, Morrison and Preston 2016). These acids are usually formed as products of fermentation (e.g., breakdown of fiber in ruminant mammals or organic matter in wet soil) where they are then metabolized/excreted by various organisms (Besten et al. 2013, Kristensen et al. 1998). In soils, VFAs are produced by bacteria such as *Clostridium*, *Enterobacter*, and *Acetobacter* during phases of anaerobic decomposition of carbon sources. While several VFAs are seen during the fermentation process in soil, the two VFAs consistently seen in highest abundance during ASD are acetic and *n*-butyric acid (Huang et al. 2015; Momma et al. 2006, 2008).

Acetic acid, the undissociated form of acetate, chemical formula CH_3COOH , is a carboxylic acid with the molecular weight 60.052 g/mol, pKa of 4.76, and vapor pressure of 11.4 mm Hg at 20°C. It is the main component of vinegar and has antibacterial and antifungal properties (Doran 1928, Levine and Fellers 1940). Acetic acid is also crucial to most organisms as its acetyl group binds to Coenzyme A, which transfers carbon for energy production as part of the citric acid cycle. In soils, acetic acid is produced by anaerobic bacteria such as *Clostridium* as they break down carbon sources. Butyric acid, the undissociated form of butyrate, chemical formula $\text{CH}_3\text{CH}_2\text{CH}_2\text{COOH}$, is a carboxylic acid with the molecular weight of 88.106 g/mol, pKa of 4.82, and vapor pressure of 0.43 mm Hg at 20°C. Butyric acid has a characteristic odor (main smell in vomit) and is found in low levels in milk and cheese. Under ASD conditions, microorganisms that produce these VFAs can proliferate to the point that their combined exudates become lethal to many soilborne plant pathogens (Browning et al. 2004, 2006, Butler et al. 2012, Momma et al. 2006, Runia et al. 2014, Tenuta et al. 2002, Ushiwata et al. 2009). Their lethality is attributed to the ability to readily diffuse across cell membranes in their dissociated forms and cause debilitating levels of acidity in the cell cytoplasm (Browning et al. 2006).

Trichoderma

Trichoderma is a genus of fungi found commonly in soils, especially in soils rich in root systems. They are free living fungi that can reduce losses to several fungal and oomycete plant pathogens including *Fusarium*, *Phytophthora*, *S. rolfsii*, and several nematode species (Bae et al. 2016, Chet et al. 1981, Küçük and Kivanç 2003, Mishra et al. 2011, Zhang et al. 2015).

Trichoderma species have also been linked to the promotion of root growth and drought resistance in plants (Benitez et al. 2004, Yedidia et al. 2001). This genus of fungus has also been shown to play an important role in bioremediation of soil given its ability to break down components of several insecticides and herbicides (Arfarita et al. 2016). The addition of organic amendments to soil as fertilizer positively affects populations of *Trichoderma* and it has also been reported to survive anaerobic environments as a facultative anaerobe (Bulluck et al. 2002, Kurakov et al. 2008, 2011). As a biocontrol, *Trichoderma* spp. use several mechanisms to retard or inhibit the growth of plant pathogens. Their mycelium can directly parasitize and enzymatically lyse the mycelium of other fungi and integuments of nematodes (Chet et al. 1981, Sharon et al. 2001). Some species produce antibiotics that can affect soilborne organisms (Benitez et al. 2004, Mishra et al. 2011). *Trichoderma* spp. can act as direct competitors to other soil fungi, thus lowering the nutrients available to other more pathogenic species (Benitez et al. 2004). Alternatively, *Trichoderma* increased total plant growth in species such as wheat, cucumbers and tomatoes (Duffy et al. 1997, Gravel et al. 2007, Yedidia et al. 2001). Another way *Trichoderma* can directly affect crops is by stimulating their native defensive mechanisms thus raising their resistance to various plant pathogens (Naher et al. 2014). Therefore, *Trichoderma* spp. populations are likely an important consideration when evaluating soil disinfestation systems. If the natural populations of *Trichoderma* spp. could be maintained or even enhanced, it could potentially add to the suppressive ability of ASD treatments as a whole.

Trichoderma spp. (Bulluck et al. 2002, Küçük and Kivanç 2003, Mishra et al. 2011, Shennan et al. 2014) and VFA concentrations (Abbasi et al. 2009, Browning et al. 2006, Huang et al. 2015, Momma et al. 2006, Tenuta et al. 2002) inhibit germination and retard pathogenicity of fungal pathogens. *Trichoderma* filtrates suppress a wide range of fungal plant pathogens such as *Fusarium culmorum*, *F. oxysporum*, *R. moniliforme*, *Rhizoctonia*, and *S. rolfsii* (Küçük and Kivanç 2003, Mishra et al. 2011). These studies also reported that *Trichoderma* directly compete with other soil borne organisms for nutrients. Bulluck (2002) observed that suppressed *S. rolfsii* and that populations of *Trichoderma* were enhanced by addition of organic amendments. This was supported by the finding of Shrestha et al. (2018) who observed *Trichoderma* directly parasitizing *S. rolfsii* after ASD treatment. Studies by Abbasi et al. (2009) and Tenuta et al. (2002) both indicated that VFAs reduced the number of microsclerotia of *Verticillium dahliae* after several days, and the effect was more pronounced at lower soil pH. This is corroborated with the finding of Browning et al. (2006) who postulated that it is the undissociated form of the organic acids that make them able to diffuse readily across cell membranes, which leads to acidification of the cytoplasm. Huang et al. (2015) and Momma et al. (2006) both observed that acetic and *n*-butyric acids were found in the highest abundance during ASD and that they suppressed *F. oxysporum* along with other fungal and nematode species. In trials by Momma et al. (2006), bacterial wilt caused by *Ralstonia solanacearum* was reduced as well.

Soil microbial community

Soils used in farming typically contain a wide range of microorganisms. This is especially true for soils undergoing organic farming practices where fewer pesticides are used (Bulluck et al. 2002, Lori et al. 2017). Microbes increase the ability of the soil to form, accumulate, and retain soil nutrients (Insam and Seewald 2010, Mäder et al. 1996). The microbial community of the soil (*Trichoderma* spp. included) break down complex organic matter, thus making it more available for crops. Organically-managed soils have a high number of microbes and levels of microorganism biomass, organic carbon, nutrient transformation rate, higher fungi count, and biomass of earthworms and arthropods (Mäder et al. 1996, Wang et al. 2012). These microorganisms, along with the organic matter they feed upon, make up the bulk total of soil organic matter. Components of soil organic matter play a large role in the overall health and productivity of a soil system. Soil organic matter is the repository of active nutrients important to plant growth such as phosphorus, iron, zinc, manganese, sodium, and copper (Yedidia et al. 2001). Soil organic matter and clay content are the major components of soil pH buffering capacity since they offer many negatively charged sites that can bind with the positively charged hydrogen ions (Insam and Seewald 2010, McCauley et al. 2017).

During anaerobic soil disinfestation, the soil microbial community is transformed. The rapid depletion of oxygen post-tarping and soil saturation causes a shift in the soil ecology that promotes soil microbes that can anaerobically metabolize abundant labile carbon. Organisms such as *Clostridium*, *Enterobacter*, and *Acetobacter* rapidly reproduce and quickly start to break down the organic compounds into VFAs, alcohols, and CO₂ (Momma 2008). These products can then be metabolized by organisms such as facultative anaerobic species of *Bacillus*, which have been observed to be biocontrol organisms (Asaka and Shoda 1996, Huang et al. 2005, Nakano and Zuber 1998). Even so, VFAs eventually accumulate to concentrations lethal to many plant pathogens (Abbasi et al. 2009, Browning et al. 2006, Huang et al. 2015, Momma et al. 2006, Tenuta et al. 2002).

After the ASD process (3 to 4 weeks), holes are punched into the polyethylene mulch and the soil microbial community changes due to increased aerobic activity. Several populations of soil fungi rise quickly upon the return of an aerobic environment. The most important fungi post ASD treatment in terms of biocontrol are likely *Trichoderma*, which has been demonstrated to be a biocontrol of plant pathogens (Kurakov et al. 2008, 2011).

Sclerotium rolfsii

Sclerotium rolfsii is the causal fungal pathogen of southern blight disease. *S. rolfsii* is a tropical to subtropical pathogen and is found on several continents. It prefers warm moist soils and grows best at temperatures over 25°C, but can grow at temperatures as low as 8°C (Punja 1985, Mullen 2006). It has a wide host range including tomato, pepper, lettuce, and many other warm season vegetable crops (Mullen 2006). *S. rolfsii* can form long-term survival structures known as sclerotia. These structures form when mycelia condense and form a rind that hardens over time. Sclerotia can lay dormant in the soil for years and are easily dispersed by wind, water, and foot or farm vehicle traffic (Punja 1985, Xu 2008). As a plant pathogen, *S. rolfsii* symptoms normally start at the base of the plant and move towards the roots, but it can also attack the leaves and fruits if they are in contact with the soil. The first symptom usually noticed is general plant wilt and yellowing that is not remedied by watering. On close inspection at later stages, the entire

plant stem will appear girdled. *S. rolfsii* can cause significant economic losses due to its wide range of hosts and ability to lay dormant. Infestations range from a few plants to an entire crop (Mullen 2006) and disease severity ranges from minor to crop loss.

Conclusion

Many studies have demonstrated ASD's ability to control a wide array of plant pathogens, including *S. rolfsii*. While some aspects of the process may have a greater influence on pathogen control than others, a picture has emerged that the ASD process is best viewed as a complete biological cycle. My goal through my studies is to evaluate specific components (VFA type and concentration, soil pH, soil texture, microbial community presence) of the ASD process in lab and controlled environment settings that allows for control of many of the variables that cannot be controlled in a field or greenhouse environment in order to evaluate the role VFAs play in the suppression of *S. rolfsii*.

Works Cited

- Abbasi PA, Lazarovits G, Jabaji-Hare S (2009). Detection of high concentrations of organic acids in fish emulsion and their role in pathogen or disease suppression. *Phytopathology* 99 274–281 doi: 10.1094/PHYTO-99-3-0274
- Arfarita N, Imai T, Prasetya B (2016). The application of *Trichoderma viride* strain frp 3 for biodegradation of glyphosate herbicide in contaminated land. *Agrivita Journal of Agricultural Science* 38 275–281 doi: 10.17503/agrivita.v38i3.55
- Asaka O, Shoda M (1996). Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. *Applied Environmental Microbiology* 62 4081–4085 doi: 0099-2240/96/\$04.0010
- Bae SJ, Mohanta TK, Chung JY, Ryu M, Park G, Shim S, Hong SB, Seo H, Bae DW, Bae I Kim JJ (2016). *Trichoderma* metabolites as biological control agents against *Phytophthora* pathogens. *Biological Control* 92 128–138 doi: 10.1016/j.biocontrol.2015.10.005
- Baker R (1968). Mechanisms of biological control of soil-borne pathogens. *Annual Review Phytopathology* 6 263–294 doi: 10.1146/annurev.py.06.090168.001403
- Benítez T, Rincón A, Limón MC, Codón AC (2004). Biocontrol mechanisms of *Trichoderma* strains. *International Microbiology* 7 249–260 doi: 1139-6709
- Besten GD, Eunen KV, Groen AK, Venema K, Reijngoud D, Bakker BM (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research* 54 2325–2340 doi: 10.1194/jlr.R036012
- Blok WJ, Lamers JG, Termorshuizen AJ, Bollen GJ (2000). Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. *Phytopathology* 90 253–259 doi: 10.1094/PHYTO.2000.90.3.253
- Browning M, Dawson C, Alm SR, Gorrës JH, Amador JA (2004). Differential effects of butyric acid on nematodes from four trophic groups. *Applied Soil Ecology* 27 47–54 doi: 10.1016/j.apsoil.2004.03.005
- Browning M, Wallace DB, Dawson C, Alm SR, Amador JA (2006). Potential of butyric acid for control of soil-borne fungal pathogens and nematodes affecting strawberries. *Soil Biology and Biochemistry* 38 401–404 doi: 10.1112/S0024609305018412
- Bulluck LR, Ristaino JB (2002). Effect of synthetic and organic soil fertility amendments on southern blight, soil microbial communities, and yield of processing tomatoes. *Phytopathology* 92 181–189 doi: 10.1094/PHYTO.2002.92.2.181
- Butler DM, Kokalis-Burelle N, Albano JP, McCullum GT, Muramoto J, Shennan C, Roskopf EN (2014). Anaerobic soil disinfestation (ASD) combined with soil solarization as a Methyl Bromide alternative: Vegetable crop performance and soil nutrient dynamics. *Plant & Soil* 378 365–381 doi: 10.1007/s11104-014-2030-z

- Butler DM, Roskopf EN, Kokalis-Burelle N, Albano JP, Muranoto J, Shennan C (2012). Exploring warm-season cover crops as carbon sources for anaerobic soil disinfestation (ASD). *Plant & Soil* 355 149–165 doi: 10.1007/s11104-011-1088-0
- Chet I, Harman GE, Baker R (1981). *Trichoderma hamatum*: Its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. *Microbial Ecology* 7 29–38 doi: 10.1007/BF02010476
- Doran WL (1928). Acetic acid as a soil disinfectant. *Journal of Agricultural Research* 36 269–280
- Duffy BK, Ownley BH, Weller DM (1997). Soil chemical and physical properties associated with suppression of take-all of wheat by *Trichoderma koningii*. *Phytopathology* 87 1118–1124 doi: 10.1094/PHYTO.1997.87.11.1118
- Gravel V, Antoun H, Tweddell RJ (2007). Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonas putida* or *Trichoderma atroviride*: Possible role of indole acetic acid (IAA). *Soil Biology and Biochemistry* 39 1968–1977 doi: 10.1016/j.soilbio.2007.02.015
- Hewavitharana SS, Mazzola M (2016). Carbon source-dependent effects of anaerobic soil disinfestation on soil microbiome and suppression of *Rhizoctonia solani* AG-5 and *Pratylenchus penetrans*. *Phytopathology* 106 1015–1028 doi: 10.1094/PHYTO-12-15-0329-R
- Hewavitharana SS, Ruddell D, Mazzola M (2015). Carbon source-dependent antifungal and nematocidal volatiles derived during anaerobic soil disinfestation. *European Journal of Plant Pathology* 140 39–52 doi: 10.1007/s10658-014-0442-5
- Huang C-J, Wang T-K, Chung S-C, Chen C-Y (2005). Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28-9. *Journal of Biochemistry and Molecular Biology* 38 82–88 doi: 10.5483/BMBRep.2005.38.1.082
- Huang X, Wen T, Zhang J, Meng L, Zhu T, Cai Z (2015). Toxic organic acids produced in biological soil disinfestation mainly caused the suppression of *Fusarium oxysporum* f. sp. *cubense*. *BioControl* 60 113–124 doi: 10.1007/s10526-014-9623-6
- Huber DM, Watson RD (1970). Effect of organic amendment on pathogens. *Phytopathology* 60 22–26
- Insam H, Seewald MSA (2010). Volatile organic compounds (VOCs) in soils. *Biology and Fertility of Soils* 46 199–213 doi: 10.1007/s00374-010-0442-3
- Ioannou N, Schneider RW, Grogan RG (1977). Effect of flooding on the soil gas composition and the production of microsclerotia by *Verticillium dahliae* in the field. *Phytopathology* 77 651–656 doi: 10.1094/Phyto-67-651
- Kristensen NB, Danfaer A, Agergaard N (1998). Absorption and metabolism of short-chain fatty acids in ruminants. *Archives of Animal Nutrition* 51 165–75 doi: 10.1080/17450399809381916

Küçük Ç, Kivanç M (2003). Isolation of *Trichoderma* spp. and determination of their antifungal, biochemical and physiological features. *Turkish Journal of Biology* 27 247–253

Kurakov AV, Lavrent'ev RB, Nechitailo TY, Golyshin PN, Zvyagintsev DG (2008). Diversity of facultatively anaerobic microscopic mycelial fungi in soil. *Microbiology* 77 90-98 doi: 10.1134/S002626170801013X

Kurakov AV, Khidirov KS, Sadykova VS, Zvyagintsev DG (2011). Anaerobic growth ability and alcohol fermentation activity of microscopic fungi. *Applied Biochemistry and Microbiology* 47 169-175 doi: 10.1134/S0003683811020098

Lamers JG, Runia WT, Molendijk LPG, Bleeker PO (2010). Perspectives of anaerobic soil disinfection. *Acta Horticulturae* 883 277–284 doi: 10.17660/ActaHortic.2010.883.34

Levine AS, Fellers CR (1940). Action of acetic acid on food spoilage microorganisms. *Journal of Bacteriology* 39 499-515

Lori M, Symnaczyk S, Mäder P, De Deyn G, Gattinger A. (2017). Organic farming enhances soil microbial abundance and activity—a meta-analysis and meta-regression. *PLoS ONE* 12 1–25 doi: 10.1371/journal.pone.0180442

Mäder PL, Fliesshach A, Lützow M, Munch JCP (1996). Soil ecology—the impact of organic and conventional agriculture on soil biota and its significance for soil fertility. *Fundamentals of Organic Agriculture* 5 223-287 doi: 10.1080/1448765.1988.9755147

McCauley A, Jones C, Olson-Rutz K, (2017). Soil pH and organic matter. *Nutrient Management* 8 1-16

McElderry CF, Browning M, Amador JA (2005). Effect of short-chain fatty acids and soil atmosphere on *Tylenchorhynchus* spp. *Journal of Nematology* 37 71–77

Mishra BK, Mishra RK, Mishra RC, Tiwari AK, Singh RY, Dikshit A (2011). Biocontrol efficacy of *Trichoderma viride* isolates against fungal plant pathogens causing disease in *Vigna radiata* L. *Archives of Applied Science Research* 3 361–369

Momma N (2008). Biological soil disinfection (BSD) of soilborne pathogens and its possible mechanisms. *Japan Agricultural Research Quarterly* 42 7–12

Momma N, Yamamoto K, Simandi P, Shishido M (2006). Role of organic acids in the mechanisms of biological soil disinfection (BSD). *Journal of General Plant Pathology* 72 247–252 doi: 10.1007/s10327-006-0274-z

Momma N, Kobara Y, Uematsu S, Kita N, Shinmura A (2013). Development of biological soil disinfections in Japan. *Applied Microbiol Biotechnology* 97 3801–3809 doi: 10.1007/s00253-013-4826-9

Morrison DJ, Preston T (2016). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes* 7 189–200 doi: 10.1080/19490976.2015.1134082

Mullen J, (2006). Southern blight, Southern stem blight, White mold. The Plant Health Instructor doi: 10.1094/PHI-I-2001-0104-01

Naher L, Ismail A, Yusuf UK, Hossain K (2014). *Trichoderma spp.*: A biocontrol agent for sustainable management of plant diseases. Pakistan Journal of Biology 46 1489–1490

Nakano and Zuber (1998). Anaerobic growth of a “strict aerobe” (*Bacillus subtilis*). Annual Review of Microbiology 52 165-190 doi: 10.1146/annurev.micro.52.1.165

National Center for Biotechnology Information. PubChem Compound Database; CID=264, <https://pubchem.ncbi.nlm.nih.gov/compound/264> (accessed July 18, 2018).

Oka Y (2010). Mechanisms of nematode suppression by organic soil amendments-A review. Applied Soil Ecology 44 101-115 doi: 10.1016/j.apsoil.2009.11.003

Pullman GS, DeVay JE (1981). Effect of soil flooding and paddy rice culture on survival of *Verticillium dahliae* and incidence of Verticillium Wilt in cotton. Ecology and Epidemiology 71 1285-1289

Punja ZK (1985). The biology, ecology, and control of *Sclerotium rolfsii*. Phytopathology 23 97-127

Roskopf EN, Kokalis-Burelle N, Hong J, Butler DM, Noling JW, He Z, Booker B, Sances F (2014). Comparison of anaerobic soil disinfestation and drip-applied organic acids for raised-bed specialty crop production in Florida. Acta Horticulturae 1044 221–228 doi: 10.17660/ActaHortic.2014.1044.26

Runia WT, Molendijk LPG (2010). Physical methods for soil disinfestation in intensive agriculture: old methods and new approaches. Acta Horticulturae 883 249–258 doi: 10.17660/ActaHortic.2010.883.31

Runia WT, Thoden TC, Molendijk LPG, Berg W (2014). Unravelling the mechanism of pathogen inactivation during anaerobic soil disinfestation. Acta Horticulturae 1044 177–193 doi: 10.17660/ActaHortic.2014.1044.21

Samaniego-Gaxiola JA, Balagurusamy N (2013). Survival of soil-borne fungus *Phymatotrichopsis omnivora* after exposure to volatile fatty acids. Journal of General Plant Pathology 79 105–109 doi: 10.1007/s10327-013-0436-8

Sharon E, Bar-Eyal M, Chet I, Herrera-Estrella A, Kleifeld O, Spiegel Y (2001). Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Phytopathology 91 687-693 doi:10.1094/PHYTO.2001.91.7.687

Shennan C, Muramoto J, Lamers J, Mazzola M, Roskopf EN, Kokalis-Burelle N, Momma N, butler DM, Kobara Y (2014). Anaerobic soil disinfestation for soil borne disease control in strawberry and vegetable systems: Current knowledge and future directions. Acta Horticulturae 1044 165–175 doi: 10.17660/ActaHortic.2014.1044.20

Shinmura A (2004). Principle and effect of soil sterilization method by reducing redox potential of soil (in Japanese). The Phytopathological Society of Japan (PSJ) Soilborne Disease Workshop Report 22 2–12

Shrestha U, Dee ME, Ownley BH, Butler DM (2018). Anaerobic soil disinfestation reduces germination and affects colonization of *Sclerotium rolfsii* sclerotia. *Phytopathology* 108 342–351 doi: 10.1094/PHYTO-04-17-0152-R

Tenuta M, Conn KL, Lazarovits G (2002). Volatile fatty acids in liquid swine manure can kill microsclerotia of *Verticillium dahliae*. *Phytopathology* 92 548–552 doi: 10.1094/PHYTO.2002.92.5.548

Ushiwata SY, Amemiya Y, Inubushi K (2009). Inhibition of in vitro growth of *Rhizoctonia solani* by liquid residue derived from steam-treated grass clippings. *General Plant Pathology* 75 312-315 doi: 10.1007/s10327-009-0174-0

Wang S, Li Z, Fan G (2012). Soil quality and microbes in organic and conventional farming systems. *African Journal of Microbiology Research* 6 5077–5085 doi: 10.5897/AJMR11.1541

Xu Z (2008). Overwinter survival of *Sclerotium rolfsii* and *Sclerotium rolfsii* var. *delphinii*, screening hosta for resistance to *Sclerotium rolfsii* var. *delphinii*, and phylogenetic relationships among *Sclerotium* species. Graduate Theses and Dissertation, Iowa State University

Yedidia I, Srivasta AK., Kapulnik Y, Chet I (2001). Effect of *Trichoderma harzianum* on microelement concentration and increased growth of cucumber plants. *Plant and Soil* 235 235–242

Zhang S, Gan Y, Xu B (2015). Biocontrol potential of a native species of *Trichoderma longibrachiatum* against *Meloidogyne incognita*. *Applied Soil Ecology* 94 21–29 doi: 10.1016/j.apsoil.2015.04.010

CHAPTER 2

VOLATILE FATTY ACID CONCENTRATION, SOIL PH AND SOIL TEXTURE ALTER EFFICACY OF ANAEROBIC SOIL DISINFESTATION IN SUPPRESSION OF SCLEROTIUM ROLFII GERMINATION

Abstract

Volatile fatty acids (VFAs), including acetic and *n*-butyric acid, are reported to play a role in the suppression of plant pathogen inoculum during anaerobic soil disinfestation (ASD), but it is unclear how VFAs affect sclerotia of *Sclerotium rolfsii*. To evaluate the effect of VFA, VFA concentration, soil pH and soil texture on germination of *S. rolfsii* sclerotia, a series of anaerobic growth chamber trials was conducted. In the first, sclerotia were exposed to 4, 8, or 16 mmol/kg dry soil concentrations of acetic or *n*-butyric acids in a sandy soil; soil pH values were buffered to 4.5, 5.5, or 6.5. Sclerotial germination was generally reduced by exposure to either acetic or *n*-butyric acids, most notably at pH 4.5 (60% germination at 4 mmol/kg soil, 5% germination at 8 mmol/kg soil, and 0% germination at 16 mmol/kg soil). Germination in water or HCl controls was consistently above 90%. In a second series of trials, sclerotia in sandy or sandy loam soil were exposed to VFAs at concentrations of 4 or 16 mmol/kg of soil and soil pH buffered to 4.5 or 5.5. The effectiveness of VFAs for suppression of *S. rolfsii* germination was reduced in the sandy loam soil as compared to the sandy soil, especially at the lower VFA concentration of 4 mmol/kg soil (e.g., at pH 4.5, > 80% germination in sandy loam versus < 10% germination in sandy soil). Clay content may play a role in this reduction. Germination of sclerotia was similarly low in both soil textures at the 16 mmol/kg soil VFA concentration. Acetic and *n*-butyric acids in soil solution are likely a primary factor in suppression of germination and colonization of *S. rolfsii* during ASD treatment, and activity against these fungal propagules is dependent on concentration, solution pH and soil texture.

Introduction

While the process of biological or anaerobic soil disinfestation (ASD) has been reported as an effective alternative to soil fumigation for control of plant pathogens in several environments (Butler et al. 2014, Roskopf et al. 2014, Shennan et al. 2014), the precise mechanisms of control are not well understood across environments and pathosystems. It is likely that numerous changes in soil chemical, physical and biological properties contribute to treatment effects of ASD treatment (Hewavitharana et al. 2015, 2016; Roskopf et al. 2015, Shrestha et al. 2018). Short chain volatile fatty acids (VFAs), including acetic acid (2C), *n*-butyric acid (4C), isobutyric acid (4C), valeric acid (5C), and isovaleric acid (5C), are found in varying levels in most biologically active moist soils, but particularly proliferate during the anaerobic decomposition of labile carbon during ASD (Blok et al. 2000, Momma et al. 2006, Runia et al. 2014). Acetic acid and *n*-butyric acid are found in relatively high concentrations in soil during ASD (Huang et al. 2015, Runia et al. 2014, Shrestha 2016). These compounds are an important component of control of some soilborne plant pathogens, including *Fusarium oxysporum*, *Verticillium dahliae*, *Pratylenchus penetrans*, and *Pyrenochaeta terrestris* (Blok et al. 2000, Browning et al. 2006, Huang et al. 2015, Momma et al. 2006, Runia et al. 2014, Shinmura 2004, Oka 2010), but it is not clear how fungal plant pathogens with large, resistant sclerotia, such as *S. rolfsii*, may potentially be impacted by VFAs during ASD treatment.

Release of VFAs into soil during ASD leads to a reduction in soil pH (Momma et al. 2006). With the lower soil pH, a larger ratio of the VFAs are in a non-dissociated form due to pKa values near 4.8 for both acetic and *n*-butyric acids. The non-dissociated forms are generally more toxic to plant pathogens (Banage and Visser 1965, Oka 2010, McElderry et al. 2005, Runia et al. 2014). It follows that the activity of VFAs against plant pathogens would likely be affected by soil pH and soil buffering capacity; buffered soils would have more neutral soil pH during ASD

treatment. At the same time, adsorption of VFAs by clay minerals and soil organic matter could potentially remove the compounds from soil solution and essentially deactivate toxicity to plant pathogen inoculum (Helling et al. 1964, Insam and Seewald 2010).

The overall goal of our study was to evaluate the role of two VFAs at varying concentrations, soil pH, and soil texture on *S. rolf sii* germination following ASD treatment. Increased mechanistic understanding of ASD treatment will allow for more precise development of treatment recommendations for use of this biologically-based treatment. Our objectives were 1) to evaluate the role of concentration of acetic and *n*-butyric acid in suppression of germination of *Sclerotium rolf sii* sclerotia, 2) to evaluate the role of soil pH in suppression of germination of *S. rolf sii* sclerotia by acetic and *n*-butyric acid, and 3) to evaluate the effect of soil texture on suppression of *S. rolf sii* sclerotia germination by acetic and *n*-butyric acid. We hypothesized that: 1) acetic and *n*-butyric acid would suppress germination of *S. rolf sii* sclerotia, in a dose-dependent manner, 2) VFA-induced suppression of *S. rolf sii* sclerotia germination would increase as a function of lower soil pH, and 3) VFA-induced suppression of *S. rolf sii* sclerotia germination would be lessened by increasing soil clay content.

Materials and Methods

Study 1.1. VFA concentration and soil solution pH effects on S. rolf sii germination

Sclerotium rolf sii isolated from hybrid field tomatoes at the East Tennessee Research and Education Center, Knoxville, TN, was isolated and cultured at room temperature in 6-well plates of 32 g/L potato dextrose agar (PDA) with addition of 6.9 mg/L fenpropathrin as a miticide (Danitol, Valent Chemical, Walnut Creek, CA). After 5 to 7 days, mycelial plugs were transferred to freshly-prepared PDA plates to stimulate production of sclerotia. Plates were incubated at room temperature. After a period of 3 to 4 weeks, mature sclerotia of consistent large size and dark color had formed. The largest were harvested under a biosafety cabinet. The plates were incubated for another 2 to 3 weeks and additional sclerotia were collected. The sclerotia were dried and stored at 8°C in Petri dishes sealed with Parafilm until use.

Three VFA concentrations were selected based on preliminary data (4, 8, and 16 mmol/kg soil) to represent a typical range of the acids present in soil during the ASD process (Shrestha et al., unpublished data). Working solutions of VFAs were prepared by combining reagent-grade concentrated acids with autoclaved double-deionized water to achieve acid concentrations of 0.027, 0.053, and 0.107 M. These concentrations were equivalent to 4, 8, and 16 mmol/kg in dry soil at the rates of solution and soil used. Working solution pH was determined using a pH electrode. To determine soil solution pH, 0.75 mL of the respective acid solution was added to 5 g soil and left to equilibrate for 10 min. Once equilibrated, soil pH was measured using a pH electrode and soil mixed with 10 mL of 0.01M CaCl₂ solution (Kissel et al. 2009). To achieve desired soil pH values based on buffering of respective soil textures, calcium hydroxide or 10% HCl solution was added to VFA solutions to achieve a desired soil pH of 4.5, 5.5, or 6.5.

The study was a randomized complete block trial with four replicates per trial, which was repeated. To evaluate impact of VFA exposure on sclerotia germination, 10 g of autoclaved sand was added to 20-mL polypropylene scintillation vials, with 10 sclerotia added to each vial. Sclerotia and soil were mixed by light shaking. According to randomly assigned treatment, 1.5 mL of respective stock solution (acetic acid, *n*-butyric acid, HCl, or water) was added to each

vial of soil to bring the 10 g of soil to water holding capacity without standing water in the vials. Vials were then lightly shaken again to ensure that all areas of the sand were thoroughly moistened by the solution. Lidded vials were placed into a controlled atmosphere chamber (PLAS LABS Model 855-AC, Lansing, MI) with an atmosphere of 90% N₂, 5% CO₂, and 5% H₂, and the lids were removed for 5 min while the palladium molecular sieve of the anaerobic chamber removed existing oxygen from the chamber and vials. This process is accomplished by heating the canister of aluminized palladium pellets and passing chambered air through canisters; trace oxygen in the chamber comes into contact with hydrogen and the heated palladium pellets and is converted to water vapor. Vials were then re-capped and incubated in the chamber at room temperature for 4 days. A 4-day incubation period was used based on the cycles of VFA concentrations measured in previous ASD field experiments (Shrestha et al., unpublished data). Vials then were removed from the chamber, and sclerotia plated individually into 24-well plates with 32 g/L PDA amended with 6.9 mg/L Danitol and 10 mg/L rifampicin (Sigma-Aldrich, St. Louis, MO) and incubated at room temperature. Germination and colonization by other fungi was monitored and recorded over the course of 2 wks at 1, 3, 5, 7, 10, and 14 days. Most sclerotia germinated within the first 3 to 7 days.

Study 1.2. VFA concentration and soil solution pH effect on S. rolfsii germination as affected by soil texture

In study 1.2, effect of increased soil clay content was evaluated by comparing activity of VFAs in sandy soil to that in soil with a sandy loam texture. The study was a randomized complete block factorial design with two levels of soil texture (sandy, sandy loam), two VFAs (acetic, *n*-butyric acids), two concentrations of VFAs (4, 16 mmol/kg soil), and two soil pH levels (4.5, 5.5). An acid control of HCl at 16 mmol/kg and pH 4.5 was used in addition to a water control. There were four replicates in each of two (repeated) trials. Soil types included autoclaved sand as well as an autoclaved 50/50 mix of sand and field soil from the surface horizon of soil at the University of Tennessee Organic Crops Unit (Dewey silt loam, fine, kaolinitic, thermic Typic Paleudult), which resulted in a sandy loam soil texture with a clay content of 10%. Organic acid stock solutions were mixed to achieve desired concentrations of 4 and 16 mmol/kg soil, at 4.5 or 5.5 soil pH, as described previously. Sclerotia were added to vials with soil and respective treatment solutions, and then placed in the anaerobic chamber as described for study 1.1. Following a 4-day incubation period, sclerotia germination was assessed as described for study 1.1.

Statistical analysis. Data were subjected to mixed models analysis of variance using PROC GLIMMIX in SAS 9.4 (SAS Institute, Cary, NC). Study one experiment was a completely randomized factorial design with three factors. The main effects and interactions of VFA type, VFA concentration, and soil pH were treated as fixed effects and trial was treated as a random effect. Study two was similar with the addition of a soil texture main effect. Differences between means were determined using an F-protected LSD at $P < 0.05$. Arcsine square root transformations were used to satisfy the nonnormal and unequal variances. Untransformed means and standard error of the mean were reported.

Results

Study 1.1. VFA concentration and soil solution pH effects on germination and colonization of S. rolfsii sclerotia

For germination, significant main effects were observed for VFA, VFA concentration, and soil pH (*P*-values for main effects and interactions are shown in Table 2.1). A significant two-way interaction was observed for soil pH x VFA concentration. There were no differences between treatment means for VFA x VFA concentration, VFA x soil pH, or VFA x concentration x pH interactions. Germination of sclerotia in the sterile water control was 100%, and germination of sclerotia from the HCl control averaged 98% across all soil pH treatments and VFA concentrations.

For acetic and *n*-butyric acids, at 4 mmol/kg soil concentration, germination of sclerotia was 36% and 21% germination, respectively at soil pH of 4.5 compared to soil pH 5.5 (95% and 88% respectively), soil pH 6.5 (90% and 72% respectively), and the HCl control (97%). This effect was even more apparent at the higher acid concentrations where at soil pH of 4.5, sclerotia germination was 2.5% or less (Fig 2.1). As soil pH increased from 4.5 to 5.5, germination rates were higher for all VFA concentrations. VFA concentration affected germination rates similarly for both acetic and *n*-butyric acids at pH 5.5, where germination decreased stepwise as VFA concentration increased (95% and 88% at 4 mmol/kg, 73% and 66% at 8 mmol/kg, and 35% and 29% at 16 mmol/kg, respectively). Once the soil pH increased to 6.5, the predicted trend of suppression with increasing VFA concentration was not observed (Fig 2.1). For both acetic and *n*-butyric acids at this pH, the variation in germination rate seemed to be most affected by levels of colonization by other fungal organisms rather than that of acid concentration.

For microbial colonization, significant effects were observed for VFA, the interaction of VFA and VFA concentration, and the interaction of VFA and soil pH (Table 2.1). There were no significant effects observed for the main effect of soil pH, VFA concentration, or the interactions of VFA and VFA concentration, soil pH and VFA concentration, or VFA, VFA concentration and soil pH (Table 2.1). The HCl and sterile water controls had less than 3% colonization.

There was a significant difference in overall colonization levels between VFAs, acetic and *n*-butyric acid, across all levels of soil pH and VFA concentration (30% and 16%, respectively). The VFA interaction with VFA concentration was significant, with the highest colonization observed from acetic acid at 16 mmol/kg soil (43% colonization) and intermediate colonization for acetic acid at 8 mmol/kg (30%). Acetic and butyric acid at 4 mmol/kg soil concentration were equivalent (18% colonization). Treatment with *n*-butyric acid at 8, and 16 mmol/kg reduced colonization (14%). The interaction between VFA and soil pH was significant with the highest colonization rate observed for acetic acid at soil pH 4.5 (47% colonization) compared with that of acetic acid at soil pH 5.5 (29% colonization) and *n*-butyric acid at soil pH 5.5 and 6.5 (18% colonization). The lowest colonization was observed for acetic acid at soil pH 6.5 and *n*-butyric acid at soil pH 4.5 (13% colonization).

Study 1.2. VFA concentration and soil solution pH effect on S. rolfsii germination as affected by soil texture

For sclerotia germination, significant main effects were observed for VFA, VFA concentration, and soil texture (*P*-values for main effects and interactions are shown in Table 2.2). Significant

interaction effects were observed for VFA concentration x soil pH, VFA concentration x soil texture, VFA x VFA concentration x soil texture and VFA concentration x soil pH x soil texture (Table 2.2). Germination of sclerotia from the HCl and water controls averaged 97%.

For both acetic and *n*-butyric acids at 4 mmol/kg soil concentration, germination was significantly reduced at soil pH 4.5 in the sandy soil (6% and 1% germination, respectively) compared to that of the sandy loam soil (85% germination for both VFAs). Similarly, at 4 mmol/kg soil concentration, germination was significantly reduced at soil pH 5.5 in the sandy soil (51% germination for acetic and 19% for *n*-butyric) compared with the sandy loam soil (83% germination for both VFAs). At the 16 mmol/kg soil concentration, germination was significantly reduced at soil pH 4.5 for both acetic and *n*-butyric acids in both the sandy (20% and 19% germination, respectively) and sandy loam (10% and 4% germination, respectively) soils. This effect was also observed for the 16 mmol/kg soil concentration at the soil pH of 5.5 where the germination rate for the sandy and sandy loam soils was not different and averaged between 3% and 9% germination.

For the three-way interaction between VFA concentration, soil pH and soil texture, significant differences were observed at the 4 mmol/kg VFA concentration where the sclerotial germination rate in sandy soil at pH 4.5 was 4% compared to the 85% germination rate in sandy loam (Fig 2.2). At pH 5.5 the sclerotial germination rate in sandy soil was 35% compared with the 83% germination rate in sandy loam. At the 16 mmol/kg soil VFA concentration no significant differences were seen at either pH across soil textures (Fig 2.2).

For the three-way interaction between VFA type x VFA concentration x soil type, at the 4 mmol/kg soil VFA concentration both acetic and *n*-butyric acid had significantly higher levels of sclerotial germination (84%) in sandy loam compared to the sandy soil texture (29% and 10% respectively) (Fig 2.3). At the higher 16 mmol/kg VFA concentration no significant differences were observed between either VFA in interaction with soil texture (Fig 2.3).

For colonization of sclerotia, significant main effects were observed for VFA concentration and soil texture (*P*-values for main effects and interactions are shown in Table 2.2). Significant interaction effects were observed for VFA and soil pH, VFA concentration and soil pH, VFA and soil texture, VFA concentration and soil texture, and soil pH and soil texture. There was also a significant interaction of VFA, VFA concentration, and soil pH. For both the HCl and sterile water controls, colonization rates were 3% or less.

In this study, the most significant factor in colonization was soil texture. For all levels of soil pH and VFA concentration, the rate of colonization between the sandy loam and sandy soil were significant with differences increasing as both VFA concentration and soil pH increased. Both VFAs had much higher rates of colonization in the sandy loam soil compared to the sandy soil.

For both acetic and *n*-butyric acids at 4 mmol/kg soil concentration, colonization was significantly reduced (34% and 65% colonization, respectively) compared to the controls (3%). Soil texture played a significant factor in colonization rates with the sandy soil having a mean colonization rate of 21% compared to the sandy loam soil, which had a mean colonization rate of 78%. A soil pH of 4.5, acetic and *n*-butyric acids had significantly different colonization rates (57% and 46%, respectively) while at soil pH 5.5, they were similar (45% and 50%, respectively; data not shown). The VFA concentration interaction with soil pH had similar colonization rates

between both soil pH at 4.5 and 5.5 for VFA concentration of 4 mmol/kg soil (32% and 36%, respectively) but significantly different colonization rates between the two soil pH values at the 16 mmol/kg soil concentration (70% and 59%, respectively). The VFA interaction with soil texture showed that both acetic and *n*-butyric acid had similar colonization rates in sandy loam soil (75% and 81% colonization, respectively) but were significantly different in the sandy soil (27% and 16%, respectively). For the VFA concentration interaction with soil texture, colonization rate was significantly different for all four combinations of VFA concentration and soil texture. The 4 mmol/kg soil concentration in sandy soil differed from that of the sandy loam (10% and 58%, respectively). At the 16 mmol/kg VFA concentration, colonization rates differed between sandy soil and sandy loam as well (32% and 97%, respectively). The soil pH interaction with soil texture showed that the sandy loam soil had a similar colonization rate at soil pH of 4.5 and 5.5 (75% and 80%, respectively). However, the sandy soil had differing colonization rates between soil pH of 4.5 and 5.5 (28% and 14%, respectively; data not shown).

Colonization rates in acetic and *n*-butyric treatments at the 16 mmol/kg soil concentration were similar at pH 4.5 (70 and 71%, respectively) and soil pH 5.5 (64% and 54%, respectively). At the 4 mmol/kg soil concentration and soil pH 4.5, the VFAs had significant differences in colonization rate (43% and 21%, for acetic and *n*-butyric, respectively). This difference was also observed at soil pH 5.5 at the 4 mmol/kg soil concentration (25% and 46%, respectively). The interaction of VFA concentration and soil pH was also significant. The 4 mmol/kg VFA concentration had lower rates of sclerotial colonization compared to the 16 mmol/kg VFA concentration (Fig 2.4). At the 4 mmol/kg VFA concentration both pH 4.5 and 5.5 had similar colonization (32% and 36%, respectively) but at the 16 mmol/kg VFA concentration there was a significant difference with pH 4.5 having a colonization rate of 70% compared to the 59% observed at pH 5.5 (Fig 2.4). For the VFA concentration and soil texture interaction, at 4 mmol/kg soil VFA concentration colonization rates differed between the sandy and sandy loam soil textures (10% and 58%, respectively; Fig 2.5). This difference between soil textures was also observed at the 16 mmol/kg VFA concentration (32% and 97%, respectively; Fig 2.5).

Discussion

Study 1.1. Germination

Soil pH plays a significant role in activity of VFAs against *S. rolfsii* germination. The HCl control did not reduce sclerotia germination rates at equivalent soil pH and concentration to the VFAs, which indicates that suppression due to VFA exposure is not directly caused by acidic pH and high acid concentration. The lack of HCl-induced germination suppression leads us to hypothesize that it is the fungal uptake of the VFAs that allows for inhibition of germination. Both acetic and *n*-butyric acids are capable of being metabolized by fungi as a carbon source, and are able to diffuse into the cell while undissociated (non-ionized), which can lead to the acidification of the cell cytoplasm (Browning et al. 2006, Tenuta et al. 2002, Ushiwata et al. 2009) and eventually the death of the cell. Similarly, Doran (1928) found that bacterial organisms can live in lower pH if the pH is regulated by HCl compared to when pH is regulated with acetic acid.

Study 1.1. Colonization

For microbial colonization of sclerotia post exposure, the major trend that is apparent from study 1.1 is that *n*-butyric acid had less fungal colonization than those in acetic acid treatment. This was

most apparent at VFA concentration of 16 mmol/kg soil, but trends were similar across VFA concentration and soil pH. This may have been caused by several differences in the properties of *n*-butyric acid compared to acetic acid. First, *n*-butyric acid has a higher molecular weight than acetic acid (88 g/mol compared to 60 g/mol). Second, *n*-butyric acid has a longer carbon chain (four compared to two C). These factors combine to give *n*-butyric acid a significantly lower vapor pressure than that of acetic acid (0.43 mm Hg compared to 11.4 mm Hg) at 20°C. This could indicate that acetic acid may dissipate significantly quicker than *n*-butyric acid, which would likely hinder its ability to suppress more resistant organisms such as fungi with resistant survival structures (Tenuta et al. 2002).

Study 1.2. Germination

The hypotheses for study 1.2 were that suppression of sclerotia germination would increase as a function of both VFA concentration and decreased soil pH. In addition, it was hypothesized that increasing clay content would reduce the activity of VFAs against *S. rolf sii*. In each case, the results of the study support our hypotheses and further emphasize the significant role VFAs play during the ASD process. The results also support the major role that soil texture plays in how effective VFAs are at reducing germination rates of *S. rolf sii*.

Of the two soil types, the sandy loam contained a higher percentage of clay (10% vs. 0%) and slightly higher organic matter (1% vs. 0%). The sandy loam also harbored residual organisms that survived the autoclaving process. These factors enhanced sclerotial colonization by several fungal pathogens post VFA exposure. The most common microbial colonists of sclerotia throughout the study were *Trichoderma*, *Mucor* and *Penicillium*. These endemic soil microorganisms were likely able to make use of the higher organic matter in the sandy loam soil. Another possibility is that the higher clay content of the sandy loam soil promoted colonization by inhibiting the effectiveness of the VFAs. By binding VFAs in solution and making them unavailable to both the sclerotia and other soil organisms, the clay reduced the active amounts of VFAs in soil solution (Helling et al. 1964, Insam and Seewald 2010, Serrano and Gallego 2006). It is possible that the reduction in VFA concentration by clay and soil organic content, caused the remaining VFA concentration to be low enough that soil microorganisms metabolizing them could do so at a slow enough rate that the cytoplasm did not become acidic enough to disrupt cell function. This would allow the organisms to make use of other carbon sources naturally found in the sandy loam soil.

Study 1.2. Colonization

As in study 1.1, *n*-butyric acid was associated with a reduction in colonization rate compared to acetic acid. Specifically, it reduced the number of fungal colonizers. Also, there was a significant amount of *Bacillus*, rather than fungi, that contributed to the colonization of *n*-butyric acid. This may be due to the ability of *Bacillus* to metabolize *n*-butyric acid under anaerobic conditions better than most other soil organisms (Dwider et al. 2013, Massie et al. 1985).

Overall the results from this study indicate that volatile fatty acids such as acetic and *n*-butyric acid likely play a major role in the suppression of *Sclerotium rolf sii* during the anaerobic soil disinfestation process. Presence of these VFAs at lower soil pH (4.5 to 5.5) allows for a high proportion of the non-ionized form of each acid to be present in soil solution. This non-ionized state allows the acids to be taken into the cytoplasm of the fungal cells where the increased acidity halts the germination process. While the combination of high VFA concentration and low soil pH led to the most effective reduction in germination, lower concentrations of VFAs can still

be effective, but the mechanism is likely more complex. Inhibition at the lower concentrations may be a function of weakened sclerotia and enhanced sclerotial colonization by fungal antagonists such as *Trichoderma*.

Works Cited

- Banage WB, Visser SA (1965). The effect of some fatty acids and pH on a soil nematode. *Nematologica* 11 255-262.
- Blok WJ, Lamers JG, Termorshuizen, AJ, Bollen GJ (2000). Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. *Phytopathology* 90 253–259. doi: 10.1094/PHYTO.2000.90.3.253
- Browning M, Wallace DB, Dawson C, Alm SR, Amador JA (2006). Potential of butyric acid for control of soil-borne fungal pathogens and nematodes affecting strawberries. *Soil Biology and Biochemistry* 38 401–404. doi: 10.1112/S0024609305018412
- Butler DM, Kokalis-Burelle N, Albano JP, McCullum GT, Muramoto J, Shennan C, Roskopf EN (2014). Anaerobic soil disinfestation (ASD) combined with soil solarization as a methyl bromide alternative: vegetable crop performance and soil nutrient dynamics. *Plant & Soil* 378 365–381 doi: 10.1007/s11104-014-2030-z
- Doran WL (1928). Acetic acid as a soil disinfectant. *Journal of Agricultural Research* 36 269-280
- Dwidar M, Kim S, Jeon BS, Um Y, Mitchell RJ, Sang BI (2013). Co-culturing a novel *Bacillus* strain with *Clostridium tyrobutyricum* ATCC 25755 to produce butyric acid from sucrose. *Biotechnology for Biofuels* 6 1–10 doi: 10.1186/1754-6834-6-35
- Helling CS, Chesters G, Corey RB (1964). Contribution of organic matter and clay to soil cation-exchange capacity as affected by the pH of the saturating solution 1. *Soil Science Society of America Journal* 28 517 doi: 10.2136/sssaj1964.03615995002800040020x
- Hewavitharana SS, Mazzola M (2016). Carbon source-dependent effects of anaerobic soil disinfestation on soil microbiome and suppression of *Rhizoctonia solani* AG-5 and *Pratylenchus penetrans*. *Phytopathology* 106 1015–1028 doi: 10.1094/PHYTO-12-15-0329-R
- Hewavitharana SS., Ruddell D, Mazzola M (2015). Carbon source-dependent antifungal and nematicidal volatiles derived during anaerobic soil disinfestation. *European Journal of Plant Pathology* 140 39-52 doi: 10.1007/s10658-014-0442-5
- Huang X, Wen T, Zhang J, Meng L, Zhu T, Cai Z (2015). Toxic organic acids produced in biological soil disinfestation mainly caused the suppression of *Fusarium oxysporum* f. sp. cubense. *BioControl* 60 113–124 doi: 10.1007/s10526-014-9623-6
- Insam H, Seewald MSA (2010). Volatile organic compounds (VOCs) in soils. *Biology and Fertility of Soils* 46 199–213 doi: 10.1007/s00374-010-0442-3
- Kissel DE, Sonon L, Vendrell PF, Isaac RA (2009). Salt concentration and measurement of soil pH. *Communications in Soil Science and Plant Analysis* 40 179-187 doi: 10.1080/00103620802625377

- Massie J, Roberts G, White PJ (1985). Selective isolation of *Bacillus sphaericus* from soil by use of acetate as the only major source of carbon. *Applied and Environmental Microbiology* 49 1478–1481 doi: 0099-2240/85/061478-04\$02.00/0
- McElderry CF, Browning M, Amador JA (2005). Effect of Short-Chain Fatty Acids and Soil Atmosphere on *Tylenchorhynchus* spp. *Journal of Nematology* 37 71–77
- Momma N, Yamamoto K, Simandi P, Shishido M (2006). Role of organic acids in the mechanisms of biological soil disinfestation (BSD). *Journal of General Plant Pathology* 72 247–252 doi: 10.1007/s10327-006-0274-z
- Oka Y (2010). Mechanisms of nematode suppression by organic soil amendments-A review. *Applied Soil Ecology* 44 101-115 doi: 10.1016/j.apsoil.2009.11.003
- Roskopf EN, Kokalis-Burelle N, Hong J, Butler DM, Noling JW, He Z, Booker B, Sances F (2014). Comparison of anaerobic soil disinfestation and drip-applied organic acids for raised-bed specialty crop production in Florida. *Acta Horticulturae* 1044 221–228 doi: 10.17660/ActaHortic.2014.1044.26
- Roskopf EN, Serrano-Perez P, Hong J, Shrestha U, Rodrigues-Molina MC, Martin K, Kokalis-Burdle N, Shennan C, Muramoto J, Butler DM (2015). Anaerobic soil disinfestation and soilborne pest management. *Soil Biology* 46 277-305 doi: 10.1007/978-3-319-23075-7_13
- Runia WT, Thoden TC, Molendijk LPG, Berg W (2014). Unravelling the mechanism of pathogen inactivation during anaerobic soil disinfestation. *Acta Horticulturae* 1044 177–193 10.17660/ActaHortic.2014.1044.21
- Serrano A, Gallego M (2006). Sorption of 25 volatile organic compounds in several Mediterranean soils using headspace-gas chromatography-mass spectrometry. *Journal of Chromatography* 1118 261-270 doi: 10.1016/j.chroma.2006.03.095
- Shinmura A (2004). Principle and effect of soil sterilization method by reducing redox potential of soil (in Japanese). The Phytopathological Society of Japan (PSJ) Soilborne Disease Workshop Report 22 2–12
- Shennan C, Muramoto J, Mazzola M, Momma N, Kobara Y, Lamers J, Roskopf EN, Kokalis-Burelle N, Butler DM (2014). Anaerobic soil disinfestation for soil borne disease control in strawberry and vegetable systems: Current knowledge and future directions. *Acta Horticulturae* 1044 165–175 doi: 10.17660/ActaHortic.2014.1044.20
- Shrestha U, Auge RM, Butler DM (2016) A meta-analysis of the impact of anaerobic soil disinfestation on pest suppression and yield of horticultural crops. *Frontiers in Plant Science* 7 1-20 doi: 10.3389/fpls.2016.01254
- Shrestha U, Dee ME, Ownley BH, Butler DM (2018). Anaerobic soil disinfestation reduces germination and affects colonization of *Sclerotium rolfsii* sclerotia. *Phytopathology* 108 342–351 doi: 10.1094/PHYTO-04-17-0152-R

Ushiwata SY, Amemiya Y, Inubushi K (2009). Inhibition of in vitro growth of *Rhizoctonia solani* by liquid residue derived from steam-treated grass clippings. *General Plant Pathology* 75 312-315 doi: 10.1007/s10327-009-0174-0

Tenuta M, Conn KL, Lazarovits G (2002). Volatile fatty acids in liquid swine manure can kill microsclerotia of *Verticillium dahliae*. *Phytopathology* 5 548–552 doi: 10.1094/PHYTO.2002.92.5.548.

Appendix: Figures and Tables

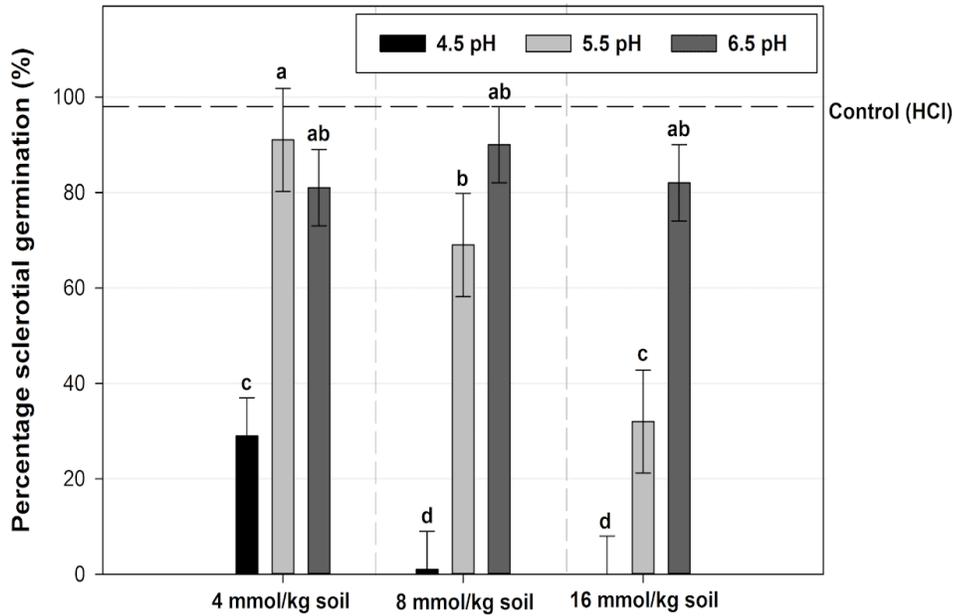


Figure 2.1. Mean percentage germination of *Sclerotium rolfsii* sclerotia in study 1.1 as affected by volatile fatty acid (VFA) concentration and soil pH. Bars indicated by the same letter are not significantly different according to an F-protected LSD at $P < 0.05$.

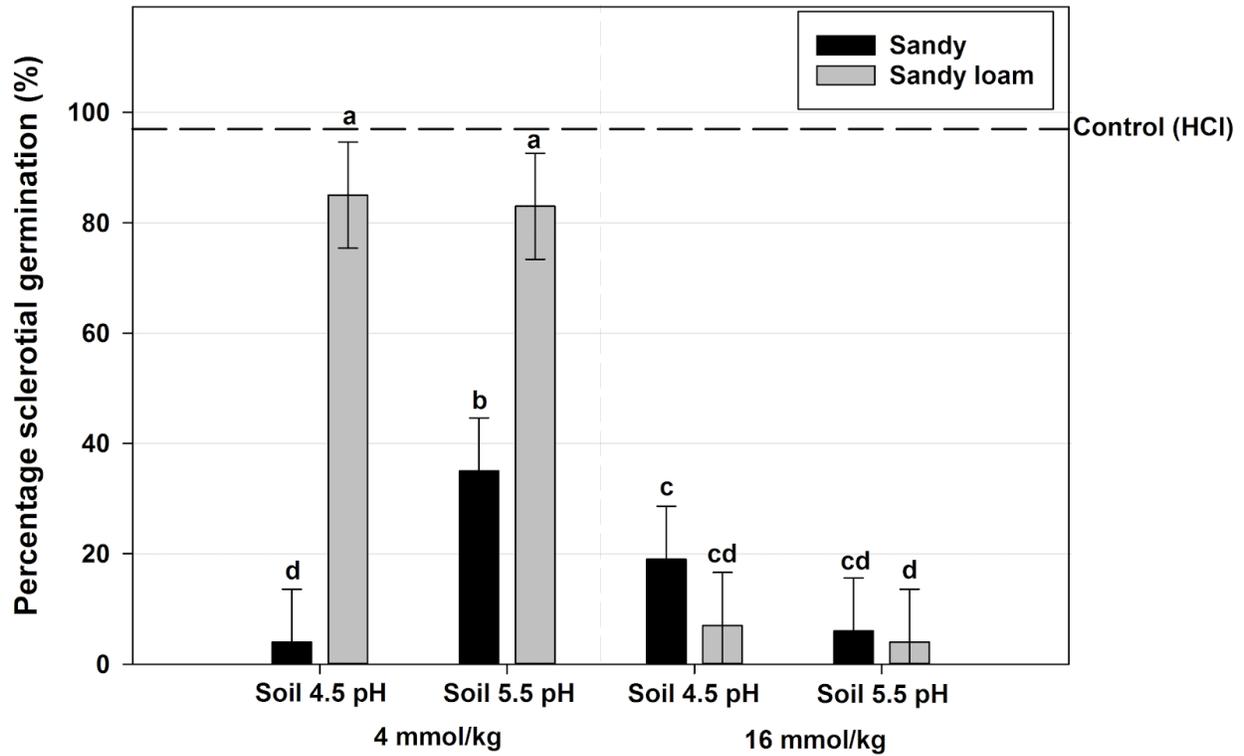


Figure 2.2. Mean percentage germination of *Sclerotium rolfsii* sclerotia in study 1.2 as affected by volatile fatty acid (VFA) concentration, soil pH, and soil texture. Bars indicated by the same letter are not significantly different according to an F-protected LSD at $P < 0.05$.

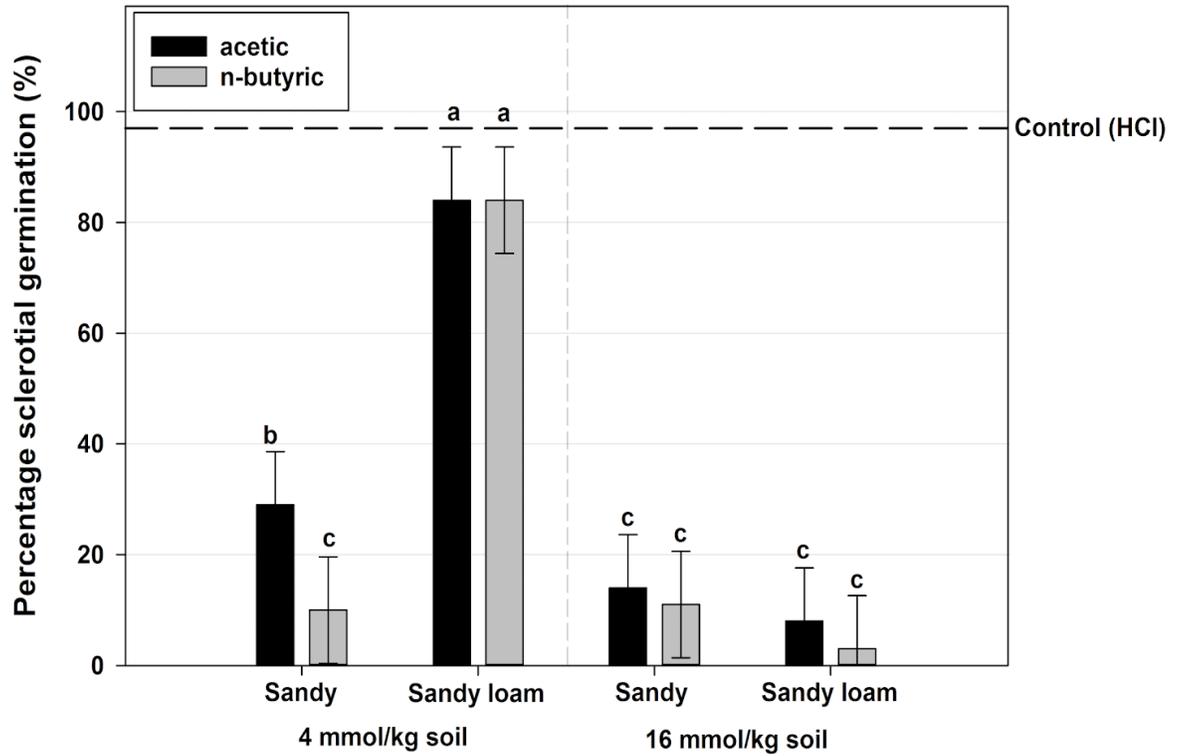


Figure 2.3. Mean percentage germination of *Sclerotium rolfii* sclerotia in study 1.2 as affected by volatile fatty acid (VFA), VFA concentration, and soil texture. Bars indicated by the same letter are not significantly different according to an F-protected LSD at $P < 0.05$.

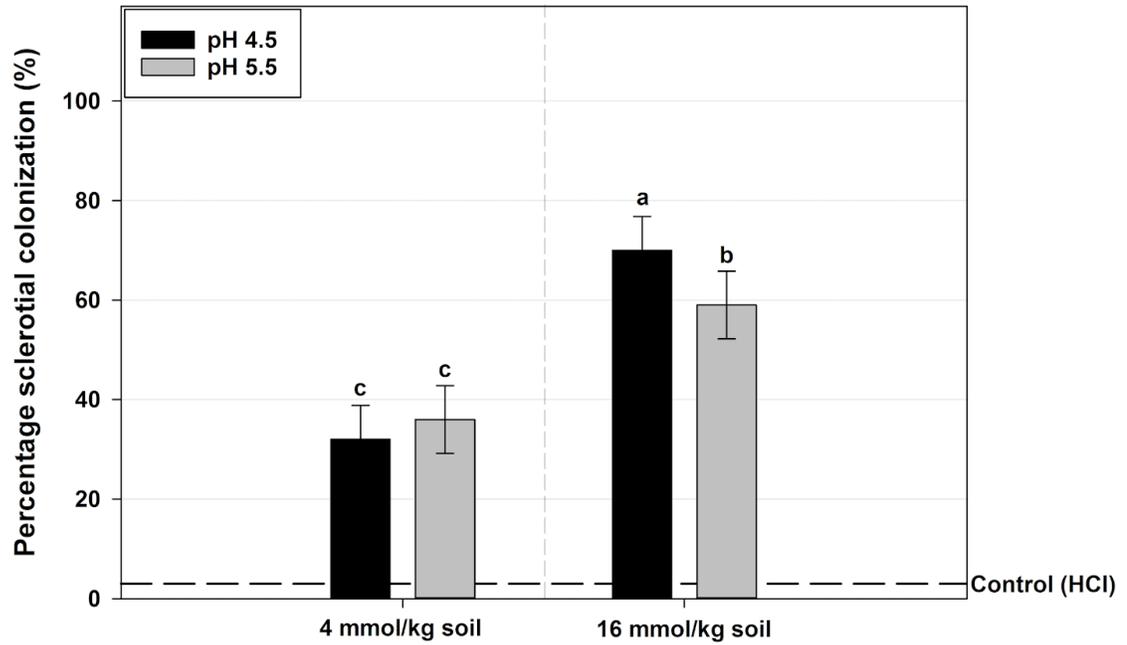


Figure 2.4. Mean percentage colonization of *Sclerotium rolfsii* sclerotia in study 1.2 as affected by volatile fatty acid (VFA) concentration and soil pH. Bars indicated by the same letter are not significantly different according to an F-protected LSD at $P < 0.05$.

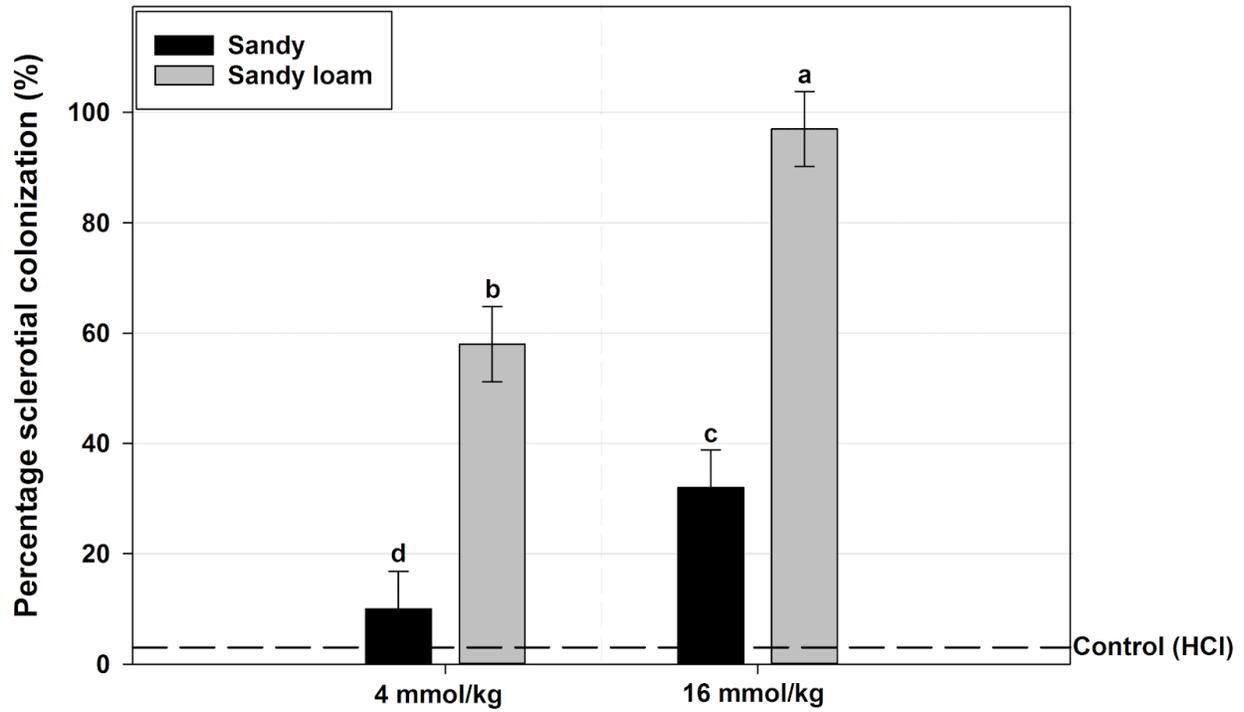


Figure 2.5. Mean percentage colonization of *Sclerotium rolfsii* sclerotia in study 1.2 as affected by volatile fatty acid (VFA) concentration and soil texture. Bars indicated by the same letter are not significantly different according to an F-protected LSD at $P < 0.05$.

Table 2.1. Analysis of variance for response variables of germination and colonization of *Sclerotium rolfsii* sclerotia in study 1.1 as affected by the main effects of volatile fatty acid (VFA), VFA concentration, soil pH, and their interactions. NS = not significant, $P > 0.05$.

	Germination	Colonization
Effect	----- <i>P</i> -values-----	
VFA	0.0497	0.002
VFA concentration	<0.0001	NS
VFA x VFA concentration	NS	0.002
Soil pH	<0.0001	NS
VFA x soil pH	NS	0.001
VFA concentration x soil pH	<0.0001	NS
VFA x VFA concentration	NS	NS
VFA x VFA concentration x soil pH	NS	NS

Table 2.2. Analysis of variance of response variables germination and colonization of *Sclerotium rolfsii* sclerotia in study 1.2 as affected by main effects of volatile fatty acid (VFA), VFA concentration, soil pH, soil texture and their interactions. NS = not significant, $P > 0.05$.

	Germination	Colonization
Effect	-----P-values-----	
VFA	0.008	NS
VFA concentration	<0.0001	<0.0001
VFA x VFA concentration	NS	NS
Soil pH	NS	NS
VFA x soil pH	NS	0.04
VFA concentration x soil pH	0.0004	0.04
VFA x VFA concentration x soil pH	NS	0.0003
Soil texture	<0.0001	<0.0001
VFA x soil texture	NS	0.01
VFA concentration x soil texture	<0.0001	0.008
VFA x VFA concentration x soil texture	0.05	NS
Soil pH x soil texture	NS	0.04
VFA x soil pH x soil texture	NS	NS
VFA concentration x soil pH x soil texture	0.0007	NS
VFA x VFA concentration x soil pH x soil texture	NS	NS

CHAPTER 3

ROLE OF VFA AND CONCENTRATION, CARBON AMENDMENT, AND PRESENCE OF ENDEMIC SOIL MICROBIAL COMMUNITY DURING ASD IN THE SUPPRESSION OF S. ROLFSII GERMINATION AND ENDEMIC TRICHODERMA POPULATIONS

Abstract

Volatile fatty acids (VFAs), such as acetic and *n*-butyric acid, are known to play a role in suppression of plant pathogen inoculum, e.g. *Sclerotium rolfsii*, during anaerobic soil disinfestation (ASD), but it is unclear how VFAs, organic amendments, and their interaction affect sclerotia of *S. rolfsii*. To evaluate effects of VFA concentration and soil amendments on the germination rates of *S. rolfsii* sclerotia post exposure, a series of anaerobic growth chamber trials were conducted. Alongside anaerobic growth chamber trials, greenhouse trials were conducted to evaluate endemic soil populations of *Trichoderma* spp. following the same ASD treatments. To evaluate effects of VFA concentration and organic amendment on sandy loam soil, soil amended with dry molasses or non-amended was exposed to 4, 8, or 16 mmol/kg dry soil concentrations of acetic and *n*-butyric acids at a soil pH buffered to 4.5. Soils were sampled post treatment and *Trichoderma* spp. Populations were assessed by standard dilution plating onto PDA plates. In carbon-amended soils there was a population increase of over 300% (3.4×10^6 colony forming units (CFU)/g soil) compared to the non-amended soil (9.6×10^5 CFU/g soil). In order to evaluate how these conditions affect germination rate of *S. rolfsii*, a second series of trials was conducted in an anaerobic growth chamber. Sclerotia of *S. rolfsii* were exposed to 4, 8, or 16 mmol/kg dry soil concentrations of acetic or *n*-butyric acids, in autoclaved or non-autoclaved sandy clay loam soil, which was either amended with dry molasses or non-amended. Sclerotial germination and colonization was measured. Sclerotial germination was generally reduced by exposure to either acetic or *n*-butyric acids, and most notably reduced by VFAs in autoclaved soil (29% germination). Overall, mean germination rates in *n*-butyric acid were significantly lower than those of acetic acid (32% and 44%, respectively). Germination rates were reduced as acid concentration increased to 4, 8, and 16 mmol/kg concentration (56%, 38% and 19%, respectively).

Introduction

While the process of biological or anaerobic soil disinfestation (ASD) has been reported to be an effective alternative to soil fumigation for control of plant pathogen inoculum in several environments (Butler et al. 2014, Roskopf et al. 2014, Shennan et al. 2014, Shrestha et al. 2016), the precise mechanisms of control are not well understood across environments and pathosystems. It is likely that numerous changes in soil chemical, physical, and biological properties contribute to ASD treatment effects (Roskopf et al. 2015, Hewavitharana et al. 2015, 2016). Volatile fatty acids (VFAs), including acetic acid, *n*-butyric acid, isobutyric acid, valeric acid, and isovaleric acid, are found in varying levels in most biologically active moist soils, but particularly proliferate during the anaerobic decomposition of labile carbon during ASD (Blok et al. 2000, Momma et al. 2006, Runia et al. 2014). There are multiple reports that acetic acid and *n*-butyric acid are found in relatively high concentrations in soil during ASD (Huang et al. 2015, Runia et al. 2014, Shrestha 2016). These compounds are an important component of control for inoculum of some soilborne plant pathogens, including *Fusarium oxysporum*, *Verticillium dahliae*, *Pratylenchus penetrans*, *Pyrenochaeta terrestris*, and several other nematode species (Blok et al. 2000, Browning et al. 2006, Huang et al. 2015, Momma et al. 2006, Runia et al. 2014, Shinmura 2004, Oka 2010), but it is not clear how VFAs and the presence of additional carbon amendments affect potential mycoparasites of plant pathogens, such as *Trichoderma* spp., during ASD treatment.

Release of VFAs into soil during ASD lowers soil pH (Momma et al. 2006). With the lower soil pH, a larger ratio of the VFAs are in a non-dissociated form due to pKa values near 4.8 for both acetic and *n*-butyric acids. The non-dissociated forms are generally, more toxic to plant pathogens (Runia et al. 2014). It follows that because the activity of VFAs suppress soil borne plant pathogen inoculum (such as *S. rolfisii*), that it would likely also affect other organisms present in the soil such as the potential fungal mycoparasite, *Trichoderma*.

Trichoderma is a fungal genus with numerous described species found commonly in soils, especially in soils rich in root systems. The free-living fungus is an effective biocontrol for several fungal plant pathogens including *Fusarium*, *Phytophthora*, and *Sclerotium rolfisii* (Mishra et al. 2011). *Trichoderma* species have also been linked to the promotion of plant growth and drought resistance in plants (Benitez et al. 2004, Duffy 1997, Yedidia et al. 2001). *Trichoderma* has also been shown to play an important role in bioremediation of soil given its ability to break down components of several insecticides and herbicides (Arfarita et al. 2016). As a biocontrol *Trichoderma spp.* use several mechanisms to retard or inhibit the growth of plant pathogens. Mycelium can directly parasitise and lyse the mycelia of other fungi and integuments of nematodes via enzymes (Chet et al. 1981, Sharon 2001) and produce antibiotics that negatively affect other soilborne organisms (Mishra et al. 2011). *Trichoderma* can also act as direct competitors to other soil fungi, thus reducing the nutrients available to other more pathogenic species (Benitez et al. 2004). It follows that populations of *Trichoderma spp.* may be an important consideration in evaluation of non-chemical and biological techniques of soil disinfestation. If endemic populations of *Trichoderma spp.* could be maintained or enhanced it could potentially add to the suppressive ability of ASD treatments as a whole.

Sclerotium rolfisii, causes southern blight. It has a wide host range including tomato, pepper, lettuce, and many other warm season vegetable crops (over 500 species) (Mullen 2001). Long term survival structures known as sclerotia are formed by *S. rolfisii* when mycelium condenses and forms a rind that hardens over time. Sclerotia can lay dormant in the soil for years and are easily dispersed by wind, water, and foot or farm vehicle traffic (Punja 1985, Xu 2008). Southern blight disease symptoms normally start from the base of the plant and move toward the roots, but the pathogen can also attack the leaves and fruits if they are in contact with the ground. The first sign is typically yellowing and general plant wilt that is not remedied by watering. In later stages, the entire plant stem will appear girdled. *Sclerotium rolfisii* occurs in tropical to subtropical regions and is found on several continents. It prefers warm moist soils and grows best at temperatures over 25°C, however it can grow at temperatures as low as 8°C (Punja 1985, Mullen 2001). Economic damage due to *S. rolfisii* is an issue since it has a wide range of hosts and the ability to remain dormant. Infestations range from localized on a few plants to decimating an entire crop (Mullen 2001). Both *Trichoderma spp.* (Bulluck and Ristiano 2001, Kucuk and Kivanc 2003, Mishra et al. 2011, Shennan et al. 2014, Shrestha et al. 2018) and VFA concentrations (Abbasi et al. 2009, Browning et al. 2006, Huang et al. 2015, Momma et al. 2006, Tenuta et al. 2002) affect germination and retard pathogenicity of fungal pathogens. Filtrates from *Trichoderma* suppressed a wide range of fungal plant pathogens, such as *Fusarium culmorum*, *F. oxysporum*, *F. moniliforme*, *Rhizoctonia*, and *Sclerotium rolfisii*. *Trichoderma* directly competes with other soilborne organisms for nutrients. Bulluck and Ristiano (2001) and Shrestha et al. (2018) observed that *Trichoderma* suppressed *S. rolfisii* and that populations of *Trichoderma* were enhanced by addition of organic amendments. This observation was supported by Shennan et al. (2014) who observed *Trichoderma* directly parasitizing *S. rolfisii*

post ASD treatment. Studies by Abbasi et al. (2009) and Tenuta et al. (2002) indicated that VFAs reduced the number of microsclerotia of *Verticillium dahliae* after several days, and the effect was more pronounced at lower soil pH. This was corroborated by Browning et al. (2006) who postulated that it is the undissociated form of the organic acids that make them able to readily diffuse across cell membranes leading to acidification of the cytoplasm. Acetic and butyric acids were found in the highest abundance during ASD and they suppressed *F. oxysporum* along with other fungal and nematode species (Huang et al. 2014 and 2015, Momma et al. 2006). In trials by Momma et al. (2006) bacterial wilt caused by *R. solanacearum* was also reduced. Therefore, methods of anaerobic soil disinfestation that enhances both mechanisms of control would likely enhance ASD treatment effects.

Based on preliminary data and previous studies, the goal of study 2.1 was to evaluate the role of two VFAs at varying concentrations on populations of endemic *Trichoderma* spp. following ASD treatment. Carbon amendments were also added as a treatment factor. The goal of study 2.2 was to evaluate the same treatment effect on germination of *S. rolfsii* sclerotia, with the addition of the treatment factor of autoclaved versus non-autoclaved soil. Increased mechanistic understanding of ASD treatment will allow for more precise development of treatment recommendations for effective use of biologically-based ASD treatment.

Our objectives were as follows: 1) evaluate impact of acetic and *n*-butyric acids and carbon amendment during ASD on *Trichoderma* spp. population dynamics post ASD treatment, and 2) evaluate effect of soil microbial community and carbon amendment on suppression of germination of *S. rolfsii* sclerotia during ASD. We hypothesized that: 1) acetic and *n*-butyric acids will increase the activity of *Trichoderma* spp. populations and activity will be a function of acid concentration and carbon amendment and 2) soil microbial activity and carbon amendments used during ASD will alter the effect of VFAs on *S. rolfsii* germination.

Materials and Methods

Study 2.1. Response of endemic soil populations of Trichoderma spp. to VFA, VFA concentration, and organic soil amendments during ASD

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

Concentrations of VFA were selected based on previous studies (4, 8, and 16 mmol/kg soil) to represent a typical range of the acid concentrations present in soil during the ASD process (Shrestha et al., unpublished data). Working solutions were created by combining reagent-grade concentrated VFA with autoclaved double deionized water to achieve acid concentrations of 0.027, 0.053, and 0.107 M. These concentrations were equivalent to 4, 8, and 16 mmol/kg dry soil given treatment application rates. Stock solution pH was determined using a pH electrode (Orion Star A221, Thermo Scientific, Waltham, MA). To determine soil solution pH, 0.75 mL of acid solution was added to 5 g soil and left to equilibrate for 10 min. Once equilibrated, soil pH was measured using a pH electrode in soil mixed with 10 mL of 0.01M CaCl₂ (Kissel et al. 2009). To achieve the desired pH value of 4.5, based on buffering of respective soil types, Ca(OH)₂ or 10% HCl solution was added to VFA solutions in small quantities while pH was continuously monitored.

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

Study 2.2. Role of endemic soil microbial activity and carbon amendment on VFA-induced suppression of S. rolfsii germination during ASD

To evaluate effect of endemic soil microbial activity and carbon amendment on VFA-induced suppression of *S. rolfsii* germination, the activity of VFAs in field soils that were either autoclaved (45 min at 121°C and 15 psi, twice, 24 hours apart) or not autoclaved, and amended with an organic amendment or not amended. The study was a randomized complete block factorial design with two levels of soil type (autoclaved, non-autoclaved), two amendment rates (amended, non-amended), two VFAs (acetic, *n*-butyric acids), and three VFA concentrations (4, 8, 16 mmol/kg soil at a single soil pH of 4.5). HCl was used as an acid control at a soil pH of 4.5 and 16 mmol/kg soil concentration, and sterile water was used as a baseline control. There were four replicates in each of two repeated trials of the study. The soil was air-dried field soil from the surface horizon at the University of Tennessee Organic Crops Unit (Dewey silt loam, fine, kaolinitic, thermic typic Paleudult) mixed in equal parts with sand by volume, which resulted in a sandy loam soil texture with a clay content of 10%. Organic acid stock solutions were mixed to achieve desired concentrations of 4, 8, and 16 mmol/kg soil, as described previously.

Sclerotium rolfsii sclerotia was prepared from a culture originally isolated from hybrid field tomatoes at the East Tennessee Research and Education Center, Knoxville, TN, cultured at room temperature on Felicity F1 pepper leaf and stem tissue. Briefly, frozen pepper tissue was chopped (< 2.5 cm) and placed into 1-L Erlenmeyer flasks corked with polyfill and cheesecloth and covered with aluminum foil. Tissue was autoclaved twice with 24-h between each treatment. After the second autoclave cycle, flasks were cooled to room temperature and ten sclerotia of *S. rolfsii* were added to each flask. The flasks were incubated for 3 to 4 weeks until large numbers of sclerotia were produced. Sclerotia were harvested under a biosafety cabinet and left in an open Petri dish in the hood to dry overnight. Sclerotia were stored at 8°C in parafilm Petri dishes

until needed. Using this method, with 300-cm³ pepper tissue (autoclaved volume) produced approximately 1,000 sclerotia per flask.

To evaluate sclerotia, germination after post exposure to VFAs, 10 g of soil and 10 sclerotia each were added to 20-mL polypropylene scintillation vials and mixed by light shaking. According to randomly assigned treatment, 1.5 mL of solution (acetic acid, *n*-butyric acid, HCl, or water) was added to each vial of soil to bring the 10 g of soil, the water holding capacity of the soil mixture (without standing water in the vials). Vials were then lightly shaken to ensure that all areas of the sand were thoroughly moistened by the solution. Lidded vials were placed into a controlled atmosphere chamber (Model 855-AC, PLAS LABS, Lansing, MI) with an atmosphere of 90% N₂, 5% CO₂, and 5% H₂ then lids were removed for five min while the palladium molecular sieve of the anaerobic chamber removed existing oxygen from the chamber and vials. This process is accomplished by heating the canister of aluminized palladium pellets and passing the chambered air through the cannisters; trace oxygen in the chamber reacts with hydrogen in the presence of the heated palladium pellets and is converted to water. Vials were then re-lidded and then incubated in the chamber at room temperature for 4 days. A 4-day period was selected based on the cycles of VFA concentrations seen in previous ASD field experiments (Shrestha et al., unpublished data). After the 4-day period, vials were removed from the chamber, and sclerotia plated individually into 24-well plates with 32 g/L PDA with 6.9 mg/L Danitol and 10 mg/L rifampicin (Sigma-Aldrich, St. Louis, MO) and incubated at room temperature. Germination and colonization by other fungi or bacteria was monitored and recorded over the course of 2 weeks at 1, 3, 5, 7, 10, and 14 days. Most sclerotia germinated within the first 3 to 7 days.

Statistical analysis. Data were subjected to mixed models analysis of variance using PROC GLIMMIX in SAS 9.4 (SAS Institute, Cary, NC). The experiment was a completely randomized factorial analysis design with three factors. The main effects and interactions of VFA, VFA concentration, soil amendment and/or soil type were treated as fixed effects and trial treated as a random effect. Differences between means were determined with an F-protected LSD at $P \leq 0.05$. Logarithmic (*Trichoderma* spp.) or arcsine square root (germination and colonization) transformations of data were used to satisfy the non-normal distribution and unequal variances. Untransformed means and standard error of the mean are reported.

Results

Study 2.1. Response of endemic soil populations of Trichoderma spp. to VFA, VFA concentration, and organic soil amendments during ASD

P-values for each main effect and interaction and shown in Table 3.1. Amended soil had a population of 3.4×10^6 CFU/g soil and non-amended soil had a population of 9.6×10^5 CFU/g soil. The other main effects of VFA and VFA concentration were not significant. The interactions between these effects were also not significant (Table 3.1). The population of the non-amended HCl control group was 1.6×10^5 while the amended HCl control had a population of 5.8×10^5 .

Study 2.2, Role of endemic soil microbial activity and carbon amendment on VFA-induced suppression of S. rolfsii germination during ASD

P-values for each main effect and interaction are shown in Table 3.2. Significant main effects on sclerotia germination were observed for VFA, VFA concentration, and soil autoclaving, but not soil amendment (Table 3.2). A significant interaction effect was observed between VFA concentration and soil autoclaving (Table 3.2). Germination of sclerotia in the water control and HCl control was 100% regardless of soil autoclaving or amendment.

For the main effect of VFA, the germination rate of acetic acid averaged 44% compared to 32% for *n*-butyric acid (Table 3.3). For the effect of VFA concentration, germination rate in the 4 mmol/kg treatment averaged 57% compared to the 38% at the 8 mmol/kg soil treatment and 19% at the 16 mmol/kg soil treatment, which all differed significantly. For soil autoclaving, germination averaged 47% for the autoclaved soil type which was significantly more than that for the non-autoclaved soil type (29% germination).

For the interaction effect between VFA concentration and soil autoclaving, at 4 mmol/kg soil sclerotia germination did not significantly differ between the autoclaved soil (57% germination) and the non-autoclaved soil (56% germination) when averaged across VFAs (Fig. 3.1). At 8 mmol/kg soil, soil autoclaving differed significantly between soil types with autoclaved soil having a germination rate of 49% compared with that of the non-autoclaved soil at 27%, averaged across VFA. At 16 mmol/kg soil concentration, the non-autoclaved soil had a sclerotia germination rate of 35% compared to the 4% in the autoclaved soil.

For colonization of sclerotia, significant main effects were observed for VFA, VFA concentration, soil amendment, and soil autoclaving (Table 3.2). A significant interaction effect was observed between soil amendment and soil autoclaving. Colonization of sclerotia in the non-autoclaved sterile water controls averaged (79%) and colonization of the non-autoclaved HCl control averaged (95%).

For the VFA main effect, overall colonization rates of sclerotia in acetic acid treatments averaged 49% compared to 27% colonization in *n*-butyric acid treatments (Table 3.3). The main effect of VFA concentration showed 49% colonization at 4 mmol/kg and 42% colonization at 8 mmol/kg soil, which were higher than that at 16 mmol/kg soil (23% colonization; Table 3.3). For soil amendment, 32% colonization was observed for the non-amended soil type compared to 44% colonization in the amended soil treatments. The mean colonization rate for the non-autoclaved soil type was 58%, which was higher than that observed in the autoclaved soil type (19% colonization).

For the significant interaction effect observed between soil amendment and soil autoclaving, mean colonization was highest in non-autoclaved soil treatments (54% to 61%), intermediate in autoclaved soils that were amended (28% colonization) and lowest in autoclaved soils that were not amended (10%; data not shown).

Discussion

Study 2.1. Response of endemic soil populations of Trichoderma spp. to VFA, VFA concentration, and organic soil amendments during ASD

There was not a significant difference between 4, 8, and 16 mmol/kg soil on populations of endemic *Trichoderma* in soil, which does not agree with that reported by Roskopf et al. (2014). This result may be due to different methods, including the use of different VFAs (Rосkopf et al. did not report the organic acids used in the proprietary mixture used in their study). In the present study, the addition of labile carbon to the field soil increased *Trichoderma* populations by more than 300% over that of the non-amended soil. This population increase under ASD conditions is consistent with other studies involving organic soil amendments (Bulluck and Ristiano 2002, Kurakov 2008, Kurakov 2011, Shrestha 2018). *Trichoderma* survived ASD conditions for up to a month, and along with *Mucor* and *Fusarium*, may be enhanced by ASD treatment (Bulluck 2001, Kurakov et al. 2008 and 2011). Bulluck and Ristiano (2002) and Kurakov et al. (2008, 2011) indicated that while these organisms grow at a reduced rate under ASD or similar anaerobic conditions they are not eliminated by the process and can utilize metabolites produced, such as sugars and alcohols. This, in turn, can give them an advantage over the other soilborne fungi when conditions return to normal, post ASD.

Study 2.2. Role of endemic soil microbial activity and carbon amendment on VFA-induced suppression of S. rolfsii germination during ASD

Our hypotheses were generally supported as related to the effects of VFAs on sclerotia germination post-exposure. While acetic and *n*-butyric acid were significantly different in terms of sclerotia germination, both VFAs significantly reduced sclerotia germination rates over that of the water and HCl control treatment groups. The hypothesis involving endemic soil microbial activity was also supported in that an average of 46% of sclerotia incubated in non-autoclaved soil germinated, compared with only 29% of sclerotia germinated when incubated in soil that had been sterilized by autoclaving. This suggests that there are processes in biologically active soil that reduce the effectiveness of VFAs against *S. rolfsii*. This is likely because both acetic and *n*-butyric acids are organic and can be readily metabolized by soil microorganisms such as *Bacillus* and *Clostridium* (Chauhan and Ogram 2006a and 2006b, Coates et al. 1998, Massie et al. 1985) thus increasing anaerobic activity. These results are further supported by the interaction effects of VFA concentration and soil type where it was observed that the effect of autoclaving on sclerotia germination rates was most pronounced as VFA concentrations increased. This suggests that the biology of active soil that reduces the effectiveness of VFAs against *S. rolfsii* through microbial metabolism are rate dependent.

As described in our previous study (Chapter 2), acetic acid consistently promoted higher fungal colonization over that of *n*-butyric acid at both the 8 and 16 mmol/kg soil concentrations. As a shorter carbon chain fatty acid, acetic acid (a two C compound) is both more readily broken down by microorganisms and is more volatile than butyric acid (a four C compound) thus it does not persist as long in the soil compared to *n*-butyric acid (Chauhan and Ogram 2006a and 2006b, Coates et al. 1998, Massie et al. 1985). This indicates that endemic soil microbes may more readily metabolize higher rates of acetic acid than *n*-butyric acid. VFA concentration effects were also consistent with earlier work (Chapter 2). As VFA concentrations increased, there was a steady decrease in the rate of fungal colonization of sclerotia. This is likely due to the same

biological factors previously mentioned. Addition of the molasses-based organic amendment also had predicted results. We hypothesized that the additional organic matter would increase microbial activity and raise the soils adsorptive capacity, thus potentially lessening the suppressive ability of VFAs due to physicochemical inaccessibility (Insam and Seewald 2010).

Works Cited

- Abbasi PA, Lazarovits G, Jabaji-Hare S (2009). Detection of high concentrations of organic acids in fish emulsion and their role in pathogen or disease suppression. *Phytopathology* 99 274–281 doi: 10.1094/PHYTO-99-3-0274
- Arfarita N, Imai T, Prasetya B (2016). The application of *Trichoderma viride* strain frp 3 for biodegradation of glyphosate herbicide in contaminated land. *Agrivita Journal of Agricultural Science* 38 275–281 doi: 10.17503/agrivita.v38i3.550
- Benítez T, Rincón A, Limón M.C., Codón AC (2004). Biocontrol mechanisms of *Trichoderma* strains. *International Microbiology* 7 249–260 doi: 1139-6709
- Blok, WJ, Lamers JG, Termorshuizen a J, Bollen, GJ (2000). Control of soilborne plant pathogens by incorporating fresh organic amendments followed by tarping. *Phytopathology* 90 253–259 doi: 10.1094/PHYTO.2000.90.3.253
- Browning M, Wallace DB, Dawson C, Alm SR, Amador JA (2006). Potential of butyric acid for control of soil-borne fungal pathogens and nematodes affecting strawberries. *Soil Biology and Biochemistry* 38 401–404 doi: 10.1112/S0024609305018412
- Bulluck LR, Ristaino JB (2002). Effect of synthetic and organic soil fertility amendments on southern blight, soil microbial communities, and yield of processing tomatoes. *Phytopathology* 92 181–189 doi: 10.1094/PHYTO.2002.92.2.181
- Butler DM, Kokalis-Burelle N, Albano JP, McCullum GT, Muramoto J, Shennan C, Roskopf EN (2014). Anaerobic soil disinfestation (ASD) combined with soil solarization as a methyl bromide alternative: Vegetable crop performance and soil nutrient dynamics. *Plant & Soil* 378 365–381 doi: 10.1007/s11104-014-2030-z
- Chauhan A, Ogram A (2006a). Fatty acid-oxidizing consortia along a nutrient gradient in the Florida Everglades. *Applied and Environmental Microbiology* 72 2400–2406 doi: 10.1128/AEM.72.4.2400-2406.2006
- Chauhan A, Ogram A (2006b). Phylogeny of acetate-utilizing microorganisms in soils along a nutrient gradient in the Florida everglades. *Applied and Environmental Microbiology* 72 6837–6840 doi: 10.1128/AEM.01030-06
- Chet I, Harman GE, Baker R (1981). *Trichoderma hamatum*: Its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. *Microbial Ecology* 7 29–38 doi: 10.1007/BF02010476
- Coates JD, Ellis DJ, Blunt-Harris EL, Gaw CV, Roden, EE, Lovley DR (1998). Recovery of humic-reducing bacteria from a diversity of environments. *Applied and Environmental Microbiology* 64 1504–1509 doi: 0099-2240/98/\$04.0010
- Duffy BK, Ownley BH, Weller DM (1997). Soil chemical and physical properties associated with suppression of take-all of wheat by *Trichoderma koningii*. *Phytopathology* 87 1118–1124 doi: 10.1094/PHYTO.1997.87.11.1118

Hewavitharana SS, Mazzola M (2016). Carbon source-dependent effects of anaerobic soil disinfestation on soil microbiome and suppression of *Rhizoctonia solani* AG-5 and *Pratylenchus penetrans*. *Phytopathology* 106 1015–1028 doi: 10.1094/PHYTO-12-15-0329-R

Hewavitharana SS, Ruddell D, Mazzola M (2015). Carbon source-dependent antifungal and nematicidal volatiles derived during anaerobic soil disinfestation. *European Journal of Plant Pathology* 140 39-52 doi: 10.1007/s10658-014-0442-5

Huang X, Wen T, Zhang J, Meng L, Zhu T, Cai Z (2015). Toxic organic acids produced in biological soil disinfestation mainly caused the suppression of *Fusarium oxysporum* f. Sp. cubense. *BioControl* 60 113–124. doi: 10.1007/s10526-014-9623-6

Insam, H, Seewald MSA (2010). Volatile organic compounds (VOCs) in soils. *Biology and Fertility of Soils* 46 199–213 doi: 10.1007/s00374-010-0442-3

Kissel DE, Sonon L, Vendrell PF, Isaac RA (2009). Salt concentration and measurement of soil pH. *Communications in Soil Science and Plant Analysis* 40 179-187 doi: 10.1080/00103620802625377

Küçük Ç, Kivanç M (2003). Isolation of *Trichoderma* spp. and determination of their antifungal, biochemical and physiological features. *Turkish Journal of Biology* 27 247–253

Kurakov AV, Lavrent'ev RB, Nechitailo TY, Golyshin PN, Zvyagintsev DG (2008). Diversity of facultatively anaerobic microscopic mycelial fungi in soil. *Microbiology* 77 90-98

Kurakov AV, Khidirov KS, Sadykova VS, Zvyagintsev DG (2011). Anaerobic growth ability and alcohol fermentation activity of microscopic fungi. *Applied Biochemistry and Microbiology* 47 169-175 doi: 10.1134/S0003683811020098

Massie J, Roberts G, White PJ (1985). Selective isolation of *Bacillus sphaericus* from soil by use of acetate as the only major source of carbon. *Applied and Environmental Microbiology* 49 1478–1481 doi: 0099-2240/85/061478-04\$02.00/0

Mishra BK, Mishra RK, Mishra RC, Tiwari AK, Singh, RY, Dikshit A (2011). Biocontrol efficacy of *Trichoderma viride* isolates against fungal plant pathogens causing disease in *Vigna radiata* L. *Archives of Applied Science Research* 3 361–369.

Momma N, Yamamoto K, Simandi P, Shishido M (2006). Role of organic acids in the mechanisms of biological soil disinfestation (BSD). *Journal of General Plant Pathology*, 72 247–252 doi: 10.1007/s10327-006-0274-z

Mullen J (2001). Southern blight, southern stem blight, white mold. *The Plant Health Instructor* doi: 10.1094/PHI-I-2001-0104-01

Punja ZK (1985). The biology, ecology, and control of *Sclerotium rolfsii*. *Phytopathology* 23 97-127 doi: 066-4286/85/0901-0097\$02.00

Oka Y (2010). Mechanisms of nematode suppression by organic soil amendments-A review. *Applied Soil Ecology* 44 101-115 doi: 10.1016/j.apsoil.2009.11.003

- Roskopf EN, Burelle N, Hong J, Butler DM, Noling JW, He Z, Booker B, Sances F (2014). Comparison of anaerobic soil disinfestation and drip-applied organic acids for raised-bed specialty crop production in Florida. *Acta Horticulturae* 1044 221–228
- Roskopf EN, Serrano-Perez P, Hong J, Shrestha U, Rodrigues-Molina MC, Martin K, Kokalis-Burdle N, Shennan C, Muramoto J, Butler DM (2015). Anaerobic soil disinfestation and soilborne pest management. *Soil Biology* 46 277-305 doi: 10.1007/978-3-319-23075-7_13
- Runia WT, Thoden TC, Molendijk LPG, Berg W (2014). Unravelling the mechanism of pathogen inactivation during anaerobic soil disinfestation. *Acta Horticulturae* 1044 177–193
- Sharon E, Bar-Eyal M, Chet I, Herrera-Estrella A, Kleifeld O, Spiegel Y (2001). Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. *Phytopathology* 91 687-693 P-2001-0516-01R
- Shennan C, Muramoto J, Mazzola M, Momma N, Kobara Y, Lamers J, Butler DM (2014). Anaerobic soil disinfestation for soil borne disease control in strawberry and vegetable systems: Current knowledge and future directions. *Acta Horticulturae* 1044 165–175 doi: 10.17660/ActaHortic.2014.1044.20
- Shinmura A (2004). Principle and effect of soil sterilization method by reducing redox potential of soil (in Japanese). The Phytopathological Society of Japan (PSJ) Soilborne Disease Workshop Report 22 2–12
- Shrestha U, Auge RM, Butler DM (2016). A meta-analysis of the impact of anaerobic soil disinfestation on pest suppression and yield of horticultural crops. *Frontiers in Plant Science* 7 1254 doi: 10.3389/fpls.2016.01254
- Shrestha U (2016). Anaerobic soil disinfestation: meta-analysis and optimization of amendment carbon rate and C:N ratio to control key plant pathogens and weeds. Graduate Dissertation, University of Tennessee, Knoxville
- Shrestha U, Dee ME, Ownley BH, Butler DM (2018). Anaerobic soil disinfestation reduces germination and affects colonization of *Sclerotium rolfsii* sclerotia. *Phytopathology* 108 342-351 doi: 10.1094/PHYTO-04-17-0152-R
- Tenuta M, Conn KL, Lazarovits G (2002). Volatile fatty acids in liquid swine manure can kill microsclerotia of *Verticillium dahliae*. *Phytopathology* 5 548–552 doi: 10.1094/PHYTO.2002.92.5.548
- Xu Z (2008). Overwinter survival of *Sclerotium rolfsii* and *Sclerotium rolfsii* var. *delphinii*, screening hosta for resistance to *Sclerotium rolfsii* var. *delphinii*, and phylogenetic relationships among *Sclerotium* species. Graduate Dissertation, Iowa State University
- Yedidia I, Srivasta AK, Kapulnik Y, Chet I (2001). Effect of *Trichoderma harzianum* on microelement concentration and increased growth of cucumber plants. *Plant & Soil* 235 235–242 doi: doi.org/10.1023/A:1011990013955

Appendix: Figures and Tables

Table 3.1. Analysis of variance for response variables of *Trichoderma spp.* populations in study 2.1 as affected by the main effects of volatile fatty acid (VFA), VFA concentration, application of carbon amendments, and their interactions. NS = not significant, $P > 0.10$.

Study 2.1 Effects	
VFA Type	NS
VFA Concentration	NS
VFA Type x VFA Concentration	NS
Amendment	0.05
VFA Type x Amendment	NS
VFA Concentration x Amendment	NS
VFA Type x VFA Concentration x Amendment	NS

Table 3.2. Analysis of variance of response variables germination and colonization of *Sclerotium rolfsii* sclerotia in study 1.2 as affected by main effects of volatile fatty acid (VFA) type, VFA concentration, soil amendment, soil type and their interactions. NS = not significant, $P > 0.05$.

Study 2.2 Effects	Germination	Colonization
	-----P-value----- ----	
VFA	0.02	<.0001
VFA Concentration	<.0001	<.0001
VFA x VFA Concentration	NS	NS
Soil Amendment	NS	0.03
VFA x Soil Amendment	NS	NS
VFA Concentration x Soil Amendment	NS	NS
VFA x VFA Concentration x Soil Amendment	NS	NS
Soil Autoclaving	0.0002	<.0001
VFA x Soil Autoclaving	NS	NS
VFA Concentration x Soil Autoclaving	0.02	NS
VFA x VFA Concentration x Soil Autoclaving	NS	NS
Soil Amendment x Soil Autoclaving	NS	0.05
VFA x Soil Amendment x Soil Autoclaving	NS	NS
VFA Concentration x Soil Amendment x Soil Autoclaving	NS	NS
VFA x VFA Concentration x Soil Amendment x Soil Autoclaving	NS	NS

Table 3.3. Response variables percent germination and colonization of *Sclerotium rolfsii* sclerotia in study 2.2 as affected by main effects of volatile fatty acid (VFA) type, VFA concentration, soil amendment, soil autoclaving, $P > 0.05$.

	Germination	Colonization
	-----percentage (%) -----	
<i>VFA</i>		
Acetic	44 a	49 x
Butyric	32 b	27 y
<i>VFA concentration</i>		
4 mmol/kg soil	57 a	49 x
8 mmol/kg soil	38 b	42 x
16 mmol/kg soil	19 c	23 y
<i>Soil amendment</i>		
Molasses	39 a	32 y
No Molasses	37 a	44 x
<i>Soil autoclaving</i>		
Autoclaved	47 a	58 x
Non-Autoclaved	29 b	19 y

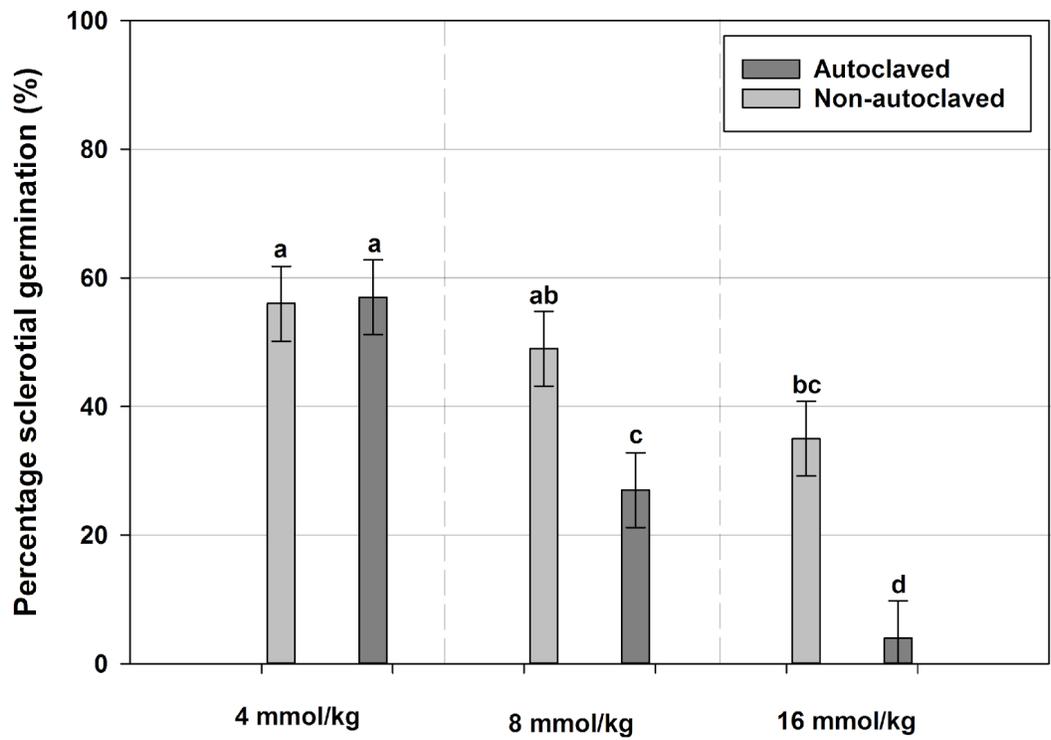


Figure 3.1. Mean percentage germination of *Sclerotium rolfii* sclerotia in study 2.2 as affected by volatile fatty acid (VFA) concentration, and soil autoclaving. Bars indicated by the same letter are not significantly different according to an F-protected LSD at $P < 0.05$.

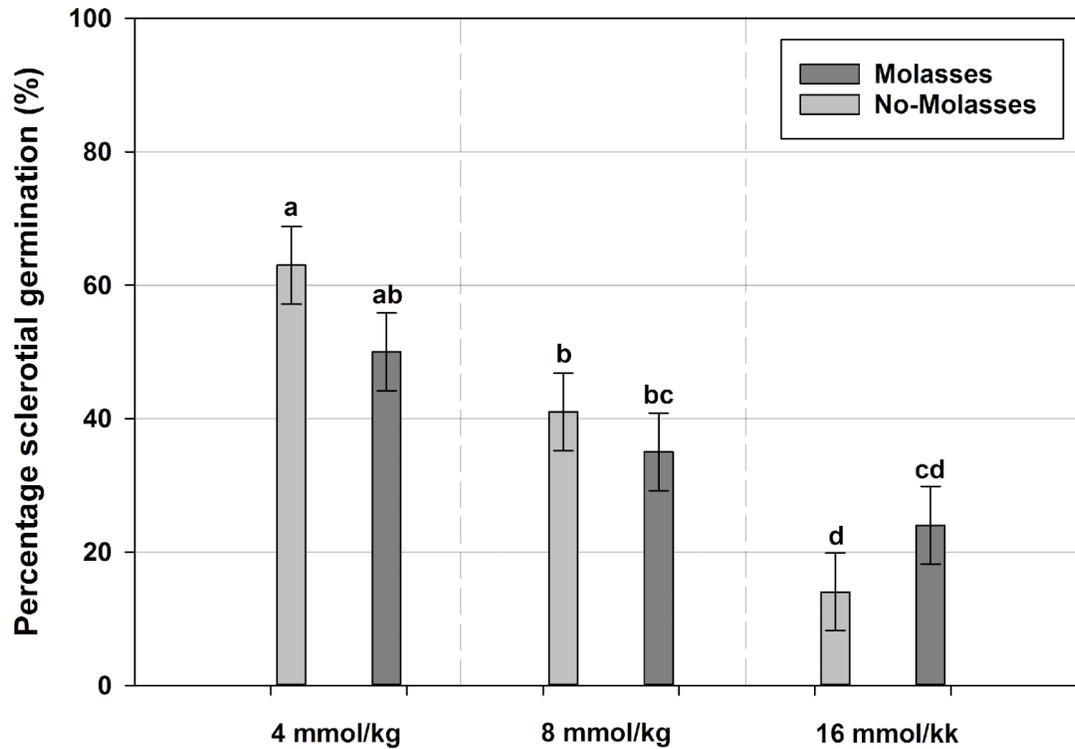


Figure 3.2. Mean percentage germination of *Sclerotium rolfsii* sclerotia in study 2.2 as affected by volatile fatty acid (VFA) concentration and soil amendment. Bars indicated by the same letter are not significantly different according to an F-protected LSD at $P < 0.05$.

CHAPTER 4
CONCLUSION

Summary

As a non-chemical method of soil fumigation, anaerobic soil disinfestation has a proven track record against many soilborne plant pathogens. The goal of our study was to better determine the role short chain volatile fatty acids such as acetic and *n*-butyric acid play during the ASD process. In order to better understand ASD's suppressive mechanisms, several trials were conducted using the plant pathogen *Sclerotium rolfsii* due to its hardy survival structures known as sclerotia. These studies were conducted as part of a continuing series of trials with the goal of formulating a more precise recommendations for farmers who seek to use ASD as a sustainable means of soil fumigation. Over the course of the trial series, several aspects of the relationship between VFA type, VFA concentration, soil pH, soil texture, and soil ecology were observed. Germination rates of *S. rolfsii* were reduced as acid concentration increased. Sclerotial germination was generally reduced by exposure to either acetic or *n*-butyric acids, most notably as pH decreased. Soil pH plays a significant role in activity of VFAs against *S. rolfsii* germination. The HCl control did not reduce sclerotia germination rates at equivalent soil pH and concentration to the VFAs, which indicates that suppression due to VFA exposure is not directly caused by acidic pH and high acid concentration. The lack of HCl-induced germination suppression leads us to hypothesize that it is the fungal uptake of the VFAs that allows for inhibition of germination. The effectiveness of VFAs for suppression of *S. rolfsii* germination was reduced in sandy loam soil as compared to sandy soil, especially at lower VFA concentrations. Clay content may play a role in this reduction. Overall, mean germination rates in *n*-butyric acid were significantly lower than those of acetic acid. This may be due to the lower vapor pressure of *n*-butyric acid. While acetic and *n*-butyric acid were significantly different in terms of sclerotia germination, both VFAs significantly reduced sclerotia germination rates over that of the water and HCl control treatment groups. Acetic acid consistently promoted higher fungal colonization of sclerotia over that of *n*-butyric acid. As a shorter carbon chain fatty acid, acetic acid (a two C compound) is both more readily broken down by microorganisms and is more volatile than butyric acid (a four C compound) thus it does not persist as long in the soil compared to *n*-butyric acid. As VFA concentrations increased, there was a steady decrease in the rate of fungal colonization of sclerotia. Additional organic matter increased microbial activity and raised the soils adsorptive capacity, thus potentially lessening the suppressive ability of VFAs due to physicochemical inaccessibility. In the greenhouse trials involving *Trichoderma* spp., populations were observed to drastically increase with the addition of labile carbon amendments to the soil, but were not affected by addition of VFAs. Overall, trials covered in this thesis increase our understanding of mechanisms during the ASD process. The combination of biological, chemical, and physical changes that occur in the soil during ASD suppress the germination of *S. rolfsii* sclerotia. VFAs do play a vital role in plant pathogen suppression during ASD especially at lower soil pH or higher concentration.

VITA

Keagan James Swilling

The author was born in October 1988, the first child to Jimmy and Robyn Swilling in Georgia. He pursued his Bachelor of Science in biology at Dalton State College in Georgia. After receiving his bachelor's degree in 2011, he began pursuing his Master of Science in Horticulture at the University of Tennessee Knoxville in 2015. He studied under the direction of Dr. Butler, Associate Professor in the Department of Plant Sciences while working under the same professor as a Graduate Research Assistant. During his time at UTK, in addition to his own research, he also co-authored publications with Dr. Utsala Shrestha and others. He attended and presented at the 11th International Congress of Plant Pathology Boston in 2018 with his research *Volatile fatty acid concentration, soil pH and soil texture alter efficacy of anaerobic soil disinfestation in suppression of Sclerotium rolfsii germination*. His interest and study focused on anaerobic and biological soil disinfestation mechanisms and application, soilborne plant pathogens, horticulture and vegetable crop production, and sustainable vegetable crop production.