

Optimizing Anaerobic Soil Disinfestation through Soil Fermentation Experiments and Modelling

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Abstract

Anaerobic soil disinfestation, a soil pest/pathogen control method where soil is amended with labile organic matter, saturated with water, and covered with plastic film to induce anaerobic conditions to suppress soilborne fungal, oomycete, bacterial, and nematode pathogens, was developed as an alternative to chemical fumigation, which has severe ecological and human health hazards. Known mechanisms of ASD include anaerobic soil conditions, release of toxic volatile fatty acids (VFAs) such as acetic, propionic, butyric, and isovaleric acid under low soil pH; and increased soil solution Fe^{2+} and Mn^{2+} concentrations. However, little is known about the interactive effects of VFAs, dissolved Fe^{2+} and Mn^{2+} , and lime applications in suppressing soilborne fungal pathogens. In this study, aqueous incubations of *Fusarium oxysporum* (*Fo*) chlamydospores and conidia in solutions containing VFAs and reduced metals followed by similar incubations in water-saturated soils of different textures amended with varying concentrations of different VFAs, Fe^{2+} or Mn^{2+} , both with and without CaCO_3 lime was done to examine any interactive fungicidal effects of these substances. This was followed by ASD experiments in which soils of different textures and liming conditions were amended with varying ratios of protein-rich and carbohydrate-rich amendments in centrifuge tubes followed by strawberry pot studies that involved performing ASD treatments using various amendments (dried molasses, wheat bran, spent brewer's yeast, and Azolla meal) on both *Fo* suppression and overall strawberry health. Overall, higher VFA and reduced metal concentrations were associated with greater *Fo* suppression while liming and finer soil texture decreases ASD efficacy. In addition, it was found that healthy and diverse root-associated

fungus communities played at least as important a role in safeguarding strawberry health as suppression of *Fo* by ASD did.

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Chapter 1: Literature Review

Introduction

Black root rot is one of the most damaging disease complexes in strawberry fields, being responsible for reducing strawberry yields by 20-40% in affected fields (NC State 2019). While Black Root Rot symptoms are very similar (i.e., blackened rat-tail like roots, damping off, stunted growth/low yields, etc.). It is caused by a wide variety of infectious organisms, including *Fusarium spp.*, *Rhizoctonia spp.*, *Pythium spp.*, and various parasitic nematodes (Koike and Gordon 2015). One of the most important pathogens responsible for black root rot is *Fusarium oxysporum* (Fo), which is currently found worldwide and in addition to strawberries also infects numerous other fruit and vegetable species including banana, tomato, lettuce, and cucumber (Agrios 2005).

Soilborne pathogens such as those responsible for black root rot are normally managed via fumigation (Agrios 2005). While fumigation is highly effective for broad spectrum suppression of soilborne pests (e.g., nematodes, pathogenic fungi, bacteria and oomycetes) in fruit and vegetable crops, synthetic chemical fumigants such as methyl bromide, chloropicrin, dazomet, and metam sodium pose enormous health and ecological dangers and as such require intensive safety training and certification for agricultural workers to use. Methyl bromide was phased out in 2008 due to its ozone-depleting properties and carcinogenicity. Other common chemical fumigants are hazardous in their own right as chloropicrin is a severe irritant and carcinogen (NIOSH 2013), dazomet an irritant and a deadly environmental toxin to aquatic animals (US EPA 2008), and metam sodium is acutely toxic to fish and aquatic invertebrates (PPDB 2019).

However, common alternatives to soil fumigation have their own severe drawbacks, as soil flooding takes several weeks to effectively reduce soil fungal pathogen populations while soil

solarization works only in temperatures of 30 °C or above, which limits crop growing seasons for farmers adopting this practice (Butler et al., 2012; McSorely et al., 1999). Soil steaming is also highly effective for controlling soilborne pathogens but has high associated energy costs (Fennimore and Goodhue 2016).

Anaerobic soil disinfestation (ASD), a broad-spectrum pest and pathogen management technique in which soils are amended with highly labile organic substrates, then wetted to saturation and covered with impermeable plastic films to induce anaerobic soil conditions is a promising alternative to chemical fumigation. Amendments used in ASD include cover crops, animal manures, molasses, wheat bran, and ethanol (Shrestha et al., 2016). Anaerobic soil disinfestation was researched first in the Netherlands (Blok et al., 2000), Japan (Okazaki et al., 1986; Shinmura 2004; Momma et al., 2013), and the U.S., particularly in California and Florida (Butler et al., 2012b; Roskopf et al., 2014; Shennan et al., 2014) where fruit and vegetable cultivation is responsible for a substantial portion of the region's agricultural economy and where high human population density makes it especially important to develop alternatives to toxic fumigants. In all of these studies, ASD was found to reduce soil pathogen populations, including fungal pathogens, oomycetes, bacteria, and nematodes by 59-94% if the soil is amended with at least 0.3 kg m⁻² organic C, as reported in a meta-analysis by Shrestha et al., (2016). Pest and pathogen suppression mechanisms in ASD include not just inducing anaerobic conditions but also the production of volatile fatty acids (VFAs) (Momma et al., 2006; Samaniego-Gaxiola and Balagurusamy 2013; Huang et al., 2015), increases in soil solution Mn²⁺ and Fe²⁺ concentrations (Momma et al., 2011), and the production of ammonia, nitrous acid, and nitrogen- and sulfur-containing volatile organic compounds such as isothiocyanates, as is

the case with biofumigation (Hewawitharana et al., 2014; Korthals et al., 2014; Dutta et al., 2019).

While it is well-known that VFAs, particularly acetic, propionic, *n*-butyric, isobutyric, valeric, and valeric acid play important roles in the suppression of soil fungal pathogens and that dissolved Fe^{2+} and Mn^{2+} also helps suppress soil fungal pathogens, little is known about any interactive fungitoxic effects of dissolved Fe^{2+} and Mn^{2+} and VFAs. Additionally, little is known about how co-application of lime influences the effectiveness of ASD. While alkaline pH was shown to enhance production of propionic, *n*-butyric, isobutyric, valeric, and isovaleric acids (Zhang et al., 2009; Kuruti et al., 2017; Esteban-Gutierrez et al., 2018; Strazzera et al., 2018), which are more fungitoxic than acetic acid (Samaniego-Gaxiola and Balagurusamy 2013; Huang et al., 2015), we also know that VFAs have greater toxicity in their non-dissociated forms as found in acidic soils (Conn et al., 2005; Swilling et al., 2019). Therefore, it is necessary to research the interactive effects of reduced metal cations, VFAs, and liming to not only help improve our understanding of the mechanisms of ASD but ultimately to develop improved ASD treatments geared toward maximizing effectiveness while minimizing input costs.

Mechanisms of ASD

The fungitoxic effects of ASD are largely driven by four main mechanisms besides oxygen starvation: 1) production of VFAs from organic matter decomposition, 2) increased proportions of Fe^{2+} and Mn^{2+} in the soil solution from the reduction of soil Fe and Mn oxides and oxyhydroxides, 3) production of other nitrogen- and/or sulfur-containing volatiles that can be

toxic toward soilborne pathogens, and 4) competition and/or parasitism of pathogenic fungi by *Trichoderma* spp. and other soil microbes. Of the VFAs produced from organic matter decomposition in anaerobic conditions, formic, acetic, propionic, (iso)butyric, and (iso)valeric acid play the most important roles in suppressing fungal pathogens (Momma et al., 2006; Samaniego-Gaxiola and Balagurusamy 2013; Huang et al., 2015). However, citric and lactic acid were minimally toxic toward *Fo* (Huang et al., 2015). The mechanism by which VFAs kill fungal and other soil pathogens is likely by diffusing across cellular barriers in undissociated form, then dissociating and lowering the pH within cells, thereby interfering with protein function and metabolism within them (Samaniego-Gaxiola and Balagurusamy 2013; Huang et al., 2015). However, the fungitoxicity of VFAs differ from one another as it was reported by Huang et al., (2015), who conducted experiments where 50 mM VFA solutions were applied to contaminated sandy soils that isovaleric acid and butyric acid were the most effective acids for killing *Fusarium oxysporum* f. sp. *cubense* followed by acetic acid and propionic acid, with formic acid having the lowest fungitoxicity. Likewise, Browning et al., (2006) reported that *n*-butyric acid vapors stopped all fungal endoparasite growth within tomato and strawberry roots when 0.1-1.0 M butyric acid was used to fumigate strawberry roots while drenching said roots in 0.1-1.0 *n*-butyric acid killed 98-100% of *Pratylenchus penetrans* nematode eggs. However, VFAs may affect different pathogens differently, as formic and propionic acids were the most effective for suppressing *Phymatotrichopsis omnivora* while acetic and butyric acid were both less effective, as reported by Samaniego-Gaxiola and Balagurusamy (2013). Besides VFAs, ASD also generates other volatile compounds that are capable of suppressing fungal, oomycete, and nematode pathogens in Petri dish trials, such as nitrous acid, nonanal, decanal, dimethyl trisulfide, and

specifically for brassicaceae seed meal, allyl isothiocyanate (Mazzola et al., 2007; Hewavitharana et al., 2014; Runia et al., 2014; Dutta et al., 2019).

Another mechanism by which ASD suppresses soil fungal pathogens is through increased concentrations of Fe^{2+} and Mn^{2+} ions in soil solution, which happens as a result of both the reduction of iron and manganese oxyhydroxides in anaerobic soil environments and the increased solubilization of these same soil minerals by VFAs. Unlike with VFAs, comparatively little research has been done on the fungitoxicity of Fe^{2+} and Mn^{2+} ions. While Fe^{3+} and Mn^{4+} , the predominant species of Fe and Mn in aerated soils exist as sparingly soluble iron and manganese oxyhydroxide minerals, these minerals are nevertheless reduced by a wide variety of bacteria, including those of genera *Geobacter* and *Shewanella* and the reduced metals stabilized through biologically-mediated reactions at the mineral interface (Lovely 2013). Many common organic substances found in ASD amendments, including carbohydrates, alcohols, long-chain fatty acids, VFAs, and even aromatic compounds can serve as electron donors for Fe- and Mn- reduction (Coates et al., 1998; Roden and Wetzel 2003; Lovely 2013). However, it was found by Momma et al., (2011) that dissolved Fe^{2+} and Mn^{2+} ions, both by themselves and together with acetic acid suppressed *Fo* populations in liquid media and soil incubation trials. While the mechanism of the fungitoxicity of Fe^{2+} and Mn^{2+} ions is unknown, it is hypothesized based on previous experiments done on bacterial and fungal pathogens that Fe^{2+} and Mn^{2+} ions induce cytotoxicity through reactive oxygen species and displacing metal ions in essential metalloproteins (Murata et al., 2008; Gerwien et al., 2017).

Lastly, ASD suppresses pathogenic fungi by promoting diverse soil microbial ecosystems, which contain not only large numbers of organisms that compete with soilborne pathogens for nutrients but also some fungal species, such as *Trichoderma* spp., which parasitize pathogenic fungi, preventing them from re-infecting crop roots (e.g., Roskopf et al., 2014; Shrestha et al., 2018). This is likely because unlike chemical fumigation, ASD does not sterilize the soil but instead alters soil conditions to favor anaerobic microbes, thereby leaving soil microbial ecosystems intact (Xie et al., 2015; Shrestha et al., 2018; Zhao et al., 2018). This also means ASD-treated soils have richer microbial, especially bacterial communities than chemically-fumigated soils (Streminska et al., 2014). As ASD favors the development of a more diverse non-pathogen soil microbiome compared to chemical fumigation, it also improves soil quality and with it crop yields for a similar level of pathogen suppression compared to chemical fumigation (Korthals et al., 2014; Shrestha et al., 2016).

Soil Conditions, Substrate Type, and ASD Effectiveness

The disinfestation efficiency of ASD is strongly influenced by soil temperature, texture, and pH along with the C:N ratios and bioavailability of organic amendments used. Overall, ASD is most effective in sandy soils where organic matter is degraded rapidly and where VFAs produced by organic matter biodegradation readily diffuse, in contrast to clayey soils where organic acid adsorption by clay minerals and the limited diffusion of VFAs restrict ASD effectiveness. This is demonstrated by studies that indicated far lower ASD effectiveness for given organic matter amendment levels compared to sandy soils or higher organic amendment levels to achieve the same level of ASD effectiveness as in sandy soils (Abd-El-Hareem, 2009; Runia et al., 2014; Shrestha et al., 2016).

Besides soil texture, soil temperature and chemical conditions also significantly influence ASD effectiveness. Anaerobic soil disinfestation is more effective at higher temperatures, requiring less than 10 days to work at temperatures of at least 30°C but over 2 weeks when temperatures are between 20-30°C as higher temperatures enhances biomass decomposition and therefore pore space oxygen depletion (Shrestha et al., 2016). There is also some limited research that indicates that lime application together with ASD decreases the effectiveness of ASD but that ASD together with liming is still significantly more effective for suppressing soilborne fungal pathogens than the unfumigated control (Meng et al., 2018). This can be explained by the strong adsorption of VFAs to calcareous minerals (Strom et al., 2001) and that at higher pH, a greater proportion of VFAs exist in dissociated form than at more acidic pH levels, therefore limiting the amount that diffuses across cell membranes (Meng et al., 2018). However, liming to near-neutral pH may also reduce the risk of infection, at least by *Fo* by reducing the proportion of dissolved micronutrients and/or increasing competition by other soil microorganisms with *Fo* (Gordon et al., 2019). Amendment type also matters greatly, as liquid amendments such as sugarcane molasses and ethanol are often more effective than cover crops such as alfalfa or wheat or rice straw for suppressing soil pathogens (Momma et al., 2010; Straus and Kluepfel, 2015; Shrestha et al., 2016). Not only are liquid amendments typically more bioavailable and therefore take shorter time to decompose and induce anaerobic and acidic soil conditions, but they are also easier to apply throughout the soil profile than solid amendments as they can be mixed with irrigation water rather than having to be incorporated into the soil (Straus and Kluepfel, 2015; Shrestha et al., 2016). However, wheat and rice bran are also considered to be highly effective amendments for suppressing fungal and other pathogens via ASD due to high

protein and polysaccharide concentrations (Katase et al., 2009; Momma et al., 2013; Straus and Kluepfel 2015). Likewise, fermented vegetable by-products (H7022/Herbie, Thatchtec) are also capable of reducing pathogenic fungi and nematode populations in contaminated soils by up to 100% if applied at a rate of 2 g raw protein L⁻¹ soil while no amount of grass amendments eliminated viable *Verticillium dahliae* sclerotia (Ludeking et al., 2011). Overall, effective ASD amendments are rich in labile (i.e., sugars, starches, proteins, lipids, or ethanol) and semi-labile organic compounds (i.e., cellulose) but have low proportions of recalcitrant hemicellulose or lignin (Mowlick et al., 2013; Butler et al., 2014b; Shrestha et al., 2016). Additionally, the C:N ratios of solid organic matter amendments used for ASD is 30:1 or less as C:N ratios are negatively correlated with disinfestation efficacy as lower C:N ratios in amendments favor greater decomposer populations (Liu et al., 2016; Shrestha et al., 2018).

Optimizing Fungitoxic Organic Acid Production

As some VFAs, particularly valeric, isovaleric, *n*-butyric, isobutyric and propionic acid are significantly more fungitoxic than acetic acid, which is produced in the highest quantities through anaerobic decomposition of organic matter, it will be important to research ways to tailor organic matter amendments used in ASD to favor the production of these acids. While there has been no research yet to investigate methods for enhancing the production of (iso)valeric, (iso)butyric, and propionic acid in the context of ASD, there is some research in tailoring industrial fermentation processes to favor the production of these acids. For the purpose of this literature review, industrial fermentation studies that utilize mixed cultures of natural bacteria rather than monocultures of single organisms or genetically engineered

organisms and utilize food and/or agricultural wastes are included as these studies may be used to help develop improved ASD methods.

The most important factors that determine the VFA balance produced from fermentation are substrate composition, pH, and chemical oxygen demand (COD). Butyric acid production is favored in the fermentation of sugar-based media at pH > 6.3 while lactic and acetic acids are favored at pH < 5.7 (Zhang et al., 2009). Likewise, treating rice straw with NaOH enhances *n*-butyric acid production (Ai et al., 2014). Brassica residues, wheat bran, and black oat residues (*Avena strigose*) are also highly effective for broad spectrum fungal pathogen suppression in ASD because these soil amendments favor the growth of bacteria in the genus *Clostridium*, which ferments organic biomass into acetic and *n*-butyric acid (Mowlick et al., 2013). Food waste and sewage sludge fermentation is affected differently by pH changes. For mixed food waste, lactic acid fermentation is favored at pH 3.2-4.5, *n*-butyric acid fermentation at pH 4.7-5.0, and mixed acid (i.e., acetic, propionic, *n*-butyric, isobutyric, valeric, and isovaleric acids) at pH 5.0-6.0 (Feng et al., 2018). In turn, sewage sludge fermentation at pH ~5.5 and 35°C favors the production of propionic and isovaleric acids (Esteban-Gutierrez et al., 2018). Additionally, *n*-butyric acid production from glucose is favored at COD levels above 4000 mg O₂ L⁻¹ while protein-rich soybean effluent media is associated with higher propionic acid production rates than glucose-based media at all COD levels (Trevisan et al., 2014). Protein-based media may favor higher propionic acid production levels than glucose-based media because propionate is produced from degradation of the amino acids valine, methionine, isoleucine, and threonine (Gonzales-Garcia et al., 2017).

While numerous industrial fermentation studies reported that near-neutral pH favors the production of the more fungitoxic propionic, *n*-butyric, and isovaleric acids, it is probable that high pH reduces their effectiveness. According to Meng et al., (2018), liming was found to reduce the effectiveness of ASD in reducing *Fo* infection of cucumber roots for the same given amount of alfalfa amendments, a result attributed to liming reducing the proportion of undissociated VFAs. VFAs are more likely to be undissociated at $\text{pH} < 6$ and therefore more lethal to pathogens, as the fungicidal mechanism of VFAs is through their diffusion across cell membranes in undissociated form, then lowering pH to lethal levels within the cytoplasm (e.g., Momma et al., 2006; Huang et al., 2015). However, it is also worth noting that the Meng et al., (2018) study involved using lime to raise soil pH to 7.30 while the optimum soil pH for strawberries is 6.0-7.0 (Cornell 2015), so the effect of liming to raise pH to within this range on soil pathogens may be different.

Besides substrate type, pH, and COD, an additional factor influencing the organic acid balance resulting from organic matter fermentation is the concentration of trace metal nutrients, particularly Fe, Mn, Cu, Zn, and Co. These trace metals are necessary for the production of key enzymes involved in metabolizing the various compounds found in organic substrates used as fermentation feedstocks (Fermoso et al., 2009; Lo et al., 2012; Veronica et al., 2013). For the production of butyric acid from sewage sludge, the optimum trace metal concentrations (w/w) are 0.112% Fe, 0.005% Co, 0.0055% Zn, 0.0042% Cu and 0.112% Mn with Fe and Mn being the most important of the trace metal nutrients, with *n*-butyric acid representing the majority of VFAs produced under these conditions (Liu et al., 2015). This is because protein, one of the main components of sewage sludge, is the main contributor to *n*-butyric acid production while

the aforementioned trace metal concentrations favored the growth of *Clostridium spp.*, the dominant bacterial genus involved in *n*-butyric acid production (Liu et al., 2015).

Challenges of ASD and Knowledge Gaps

The biggest challenges to the wider adoption of ASD are dependence on warm temperatures, the time it takes for ASD amendments to be used to successfully suppress soil pathogens, and the amount of amendment required, especially if the amendments are crop residues rather than liquids. Anaerobic soil disinfestation typically takes roughly 3 weeks to effectively suppress soil pathogens but can take over 10 weeks if temperatures are consistently below 16°C (Mowlick et al., 2013; Shrestha et al., 2016). This is a potential inconvenience for farmers, as long incubation times may cut into the growing season and as a result, limit ASD usage to regions with warm or mild temperate climates unless the production system already incorporates long fallow periods during which ASD can be performed. Additionally, grass, brassica, and legume amendments have shown more limited (~64%) pathogen suppression and require relatively high amendment rates of roughly 5-6 kg m⁻² soil to effectively suppress soil pathogens as their biomass contains significant quantities of recalcitrant compounds that have slow decomposition rates (Mowlick et al., 2013; Butler et al., 2014b; Shrestha et al., 2016), and can incur high transportation costs unless these amendments are cover crop residues. However, significantly less amendments need to be applied (~300 g m⁻² soil) if the amendments are highly labile liquids or solutions (i.e., molasses, ethanol, or glucose) (Shennan et al., 2014; Daugovish et al., 2015; Shrestha et al., 2016). Solid amendments would still be necessary in some cases, such as sandy soils, where leaching of liquid amendments and/or decomposition products can limit ASD effectiveness (Jones et al., 2003).

In light of the challenges associated with ASD, studies are needed on the interactive fungicidal effects of Fe^{2+} and Mn^{2+} and different organic acids (i.e., lactic, formic, acetic, propionic, *n*-butyric, isobutyric, valeric, and isovaleric acids) in suppressing fungal pathogens such as *Fo*, which causes root rot in strawberries. By doing so, it may be possible to not only improve our understanding of the mechanisms of ASD but also help identify improved ASD methods and to develop alternative low-risk soil fungicide mixtures to replace the use of hazardous fumigants. In addition, it would be worthwhile to investigate the interactive effects of amendment type (i.e., molasses, brewer's spent yeast, or *Azolla* meal) and CaCO_3 lime applications on both soil organic acid balance and disinfestation effectiveness as it is likely that soil pH would greatly influence the relative proportions of different VFAs produced via anaerobic decomposition. Additionally, *Azolla* meal would be a potential ASD amendment to research because of its usefulness with combining on-farm atmospheric nitrogen fixation, fertilization, and anaerobic soil disinfestation if used for that purpose. *Azolla* is a rapidly-growing freshwater fern which has symbiotic cyanobacteria of the genus *Anabaena* as nitrogen fixers capable of producing 37.8 t ha^{-1} fresh biomass annually (Hasan et al., 2009). *Azolla* also contains ~20% protein per unit dry mass along with 3900 mg Fe kg^{-1} dry mass and 762 mg Mn kg^{-1} dry mass (Tran 2015).

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Chapter 2: Interactive Effects of Volatile Fatty Acids and Fe²⁺ and Mn²⁺ in Suppressing

Fusarium oxysporum* f. sp. *fragariae

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Abstract

Less hazardous alternatives to chemical soil fumigants for suppressing soilborne pathogens such as *Fusarium oxysporum* (*Fo*), one causative agent of strawberry black root rot complex prevalent in the southeastern U.S., are urgently needed. A promising alternative is anaerobic soil disinfestation (ASD), in which soil is amended with labile organic materials, irrigated to field capacity, and tarped to induce anaerobic fermentation for a brief period before planting. Pathogen-suppression mechanisms of ASD include anaerobic conditions and generation of reduced metal cations (Fe^{2+} and Mn^{2+}) and volatile fatty acids (VFAs; e.g., acetic, *n*-butyric, isovaleric, and others). However, little is known about how the interaction between VFAs, reduced metals, soil texture, and liming influences suppression of *Fo*. We investigated *Fo* suppression by VFAs and reduced metal cations in both aqueous and soil-based incubation trials. Inoculum containing *Fo* chlamydospores was added to aqueous medium containing either 5 or 10 mmol/liter VFAs and either 0.01% or 0.05% (w/w) reduced metals. In soil-based incubations, chlamydospore-containing inoculum was applied to sandy, sandy loam, and silty clay soil saturated by solutions containing 10 or 20 mmol/liter VFAs with or without 0.05% (w/w) reduced metals. VFAs, particularly in combination with Fe^{2+} in aqueous solutions and Mn^{2+} in soils significantly reduced *Fo* viability. At the same time, liming and higher soil clay content reduced the effectiveness of VFAs and reduced metals for suppressing *Fo*, highlighting the influence of soil pH and soil texture on ASD effectiveness.

Introduction

Black root rot is a damaging disease complex in strawberry production in the southeastern U.S. that can reduce strawberry yields by 20 to 40% in affected fields (Louws and Cline, 2019). The causal pathogens of strawberry black root rot differ among sites. Environmental conditions that favor plant injury or poor growth, coupled with various fungal, oomycete, and nematode pathogens, including the fungal pathogen *Fusarium oxysporum* (*Fo*) have been implicated (McManus, 2004). Pathogens responsible for black root rot are most typically managed by soil fumigation with synthetic chemical fumigants, primarily mixtures of 1,3-dichloropropene and chloropicrin, which are effective for broad spectrum suppression of soilborne pathogens in strawberry crops but pose limitations in terms of human health and environmental safety (Blok et al. 2000; Momma et al. 2006; US-EPA, 2008). Accordingly, research into alternative, non-fumigant systems with low human and environmental health risks are needed as they would be beneficial to strawberry production systems, particularly organic systems, or those systems existing at the interface with urban and suburban residential areas.

Anaerobic soil disinfestation (ASD), or biological soil disinfestation, is a biologically-based soil disinfestation process with potential for use across a broad spectrum of pathogens, environments and cropping systems (e.g., Blok et al. 2000; Momma et al. 2006; Shrestha et al. 2016). In ASD, labile organic matter is incorporated in soil, which is then tarped and saturated to field capacity by irrigation to induce anaerobic decomposition of the organic matter. Through anaerobic decomposition, various pathogen-suppressing compounds including volatile fatty acids (VFAs), especially acetic, *n*-butyric and isovaleric acid; and reduced metals, particularly

Fe^{2+} and Mn^{2+} are formed (e.g., Momma et al. 2006; Momma et al. 2011). Volatile fatty acids are toxic to many pathogenic soil microorganisms as they diffuse across cell membranes in undissociated form and lower the cellular pH to fatal levels (Momma et al. 2006; Huang et al. 2015). However, as deprotonated VFAs are less likely to diffuse across cell membranes than undissociated VFAs, their disinfestation effectiveness is greater in acidic soil solutions than in neutral or alkaline soil solutions (Banage and Visser, 1995; Oka, 2010; Runia et al. 2014; McElderry et al. 2015; Swilling et al. 2022). The fungitoxicity of different VFAs also varies; for example, isovaleric and *n*-butyric acid were both more suppressive of *Fusarium oxysporum* f. sp. *cubense* than acetic acid (Huang et al. 2015). Additionally, different fungal spore types respond differently to VFAs, as *Fusarium oxysporum* f. sp. *lycopersici* microconidia were rendered non-viable by significantly lower VFA concentrations than chlamydospores (Momma et al. 2006).

Reduced metal ions, such as Fe^{2+} and Mn^{2+} , have similarly been demonstrated in various laboratory experiments to reduce viability of *Fo* conidia and chlamydospores (Peng et al. 1999; Momma et al. 2011). Unlike Fe^{3+} and Mn^{4+} ions, which exist as sparingly soluble oxyhydroxides or are adsorbed strongly to clay minerals, Fe^{2+} and Mn^{2+} ions are highly soluble and are the dominant Fe and Mn species in highly reducing soil and sediment environments (Takai and Kamura, 1966; Finke et al. 2007). The suppressive effects of Fe^{2+} and Mn^{2+} ions are due to a combination of Fenton reactions; reduced metal cations react with peroxides to form reactive oxygen species (Touati, 2000) and also interference with the function of essential metalloproteins by displacing the metals in their active sites (Gerwien et al. 2017). Additionally,

acetic acid applications may solubilize soil Fe^{2+} and Mn^{2+} through the formation of water-soluble iron (II) and manganese (II) acetates (Wildermuth et al. 2000; Reidies, 2002).

The disinfestation efficiency of ASD is likely to be strongly influenced by soil texture and soil pH. We expect that ASD is most effective in sandy soils where organic matter is degraded rapidly and VFAs and water-soluble reduced metal cations produced during organic matter biodegradation readily diffuse through large pore spaces. This contrasts with soils with higher clay content where potential VFA adsorption and removal from soil solution by clay minerals and the limited diffusion of VFAs may restrict ASD effectiveness (Shrestha et al. 2016). This is demonstrated by a few studies and a meta-analysis that suggested far lower ASD effectiveness for given organic matter amendment rates in finer-textured compared to sandy soils (Abd-El-Hareem, 2009; Runia et al. 2014; Shrestha et al. 2016). Previous work similarly suggests that higher amendment rates for ASD treatment may be needed in finer-textured soils to achieve the same level of ASD effectiveness as observed in sandy soils (Shrestha et al. 2016). There is also limited research that indicates that lime (calcium or magnesium carbonates) application together with ASD decreases the effectiveness of ASD, but that ASD coupled with liming is still significantly more effective for suppressing soilborne fungal pathogens than unfumigated controls (Meng et al. 2018). This can be explained by the strong adsorption of VFAs to calcareous minerals (Strom et al. 2001) and that at higher soil pH, a greater proportion of VFAs exist in dissociated form than at more acidic soil pH, therefore limiting VFA diffusion across cell membranes (Meng et al. 2018). However, liming in the absence of ASD was also found to reduce the growth of a pathogenic *Fo* strain (Cruz et al. 2019).

While it is known that VFAs and $\text{Fe}^{2+}/\text{Mn}^{2+}$ ions suppress *Fo*, little is known about the interactive fungitoxic effects of VFAs and Fe^{2+} and Mn^{2+} ions and how soil texture and liming influences suppression of *Fo*. Improved knowledge of the interactive effects of VFAs and reduced metal cations in suppressing *Fo* in different soil textures is needed to develop improved ASD treatment recommendations for various soil conditions. Our objectives were to (i) evaluate the interactive effects of VFAs and reduced metal cations in suppressing *Fo*; and (ii) evaluate how soil texture and liming influences the suppression of *Fo* by VFAs and reduced metal cations. We hypothesized that: (i) VFAs, especially *n*-butyric and isovaleric acid, would be far more suppressive of *Fo* in combination with reduced metal cations than alone; and (ii) greater soil clay content and/or liming would reduce the effectiveness of VFAs and reduced metals in suppressing *Fo*.

Materials and Methods

Soil Collection and Preparation

Soil was collected from the Organic Crops Unit at the University of Tennessee East Tennessee Research and Education Center in Knoxville, TN, USA from the surface horizon (Dewey silt loam, fine, kaolinitic, thermic Typic Paleudult). The soil was sieved (10-mm mesh) to remove large rocks and debris, then air-dried. The exact soil texture (silty clay; 44% clay, 38% silt, and 18% sand) was determined using the hydrometer method (Bouyoucos 1962). For sandy loam soil (22% clay, 19% silt, and 59% sand), the silty clay was mixed 50/50 (by weight) with 100% sand (sand particle sizes 62.5 to 1500 μm). Soil pH values before treatments were near neutral for all soil textures; 6.94 for sand, 7.16 for sandy loam, and 7.19 for silty clay.

Inoculum Preparation

An isolate of pathogenic *Fusarium oxysporum* ('SC5', GenBank Accession numbers ON703236, OR472390) causing strawberry black root rot was initially collected from strawberry roots on a strawberry farm in Tennessee was cultured in potato dextrose broth (PDB) for 7 days at 25°C with shaking. To obtain a chlamydospore-containing inoculum suspension, mycelial mass grown in PDB was suspended in potato sucrose broth for 7 days, then homogenized and added to sterile soil water extract and incubated for 3 weeks at 25°C (Momma et al. 2006). The soil water extract was prepared by thoroughly mixing 100 g of soil and 100 ml of deionized water, autoclaving the mix for 60 min at 121°C, allowing the soil particles to settle, then passing the supernatant through a 0.44-µm filter with a vacuum (Dismic-25cs, Advantec, Tokyo, Japan). After the 3-week incubation period, the mycelial mat and soil water extract were homogenized in five 30-s blending sessions in a sterile blender. The concentration of chlamydospores and conidia in the resultant inoculum suspension were counted using a hemocytometer before each *Fo* incubation experiment.

Aqueous Incubations with Fe²⁺, Mn²⁺, and VFAs

To evaluate *Fo* chlamydospore susceptibility to VFAs and reduced metal ions, an *Fo* inoculant containing chlamydospores was incubated in aqueous solutions containing experimental treatment combinations of either FeSO₄-EDTA or MnSO₄-EDTA at 0%, 0.01%, and 0.05% w/w concentrations, factorially combined with 0, 5, and 10 mmol/liter concentrations of a VFA (acetic, *n*-butyric, or isovaleric acid) for 2 days at 20 to 25°C. Methods were based on those of Momma et al. (2011) along with results from preliminary trials with EDTA added to retain the Fe²⁺ and Mn²⁺ in solution. The samples were incubated within this narrow temperature range as

temperature is known to affect ASD efficacy (e.g., Shrestha et al. 2016; Mazzola et al. 2018; Shrestha et al. 2021). In each 20-ml glass scintillation vial, 80 µl of chlamydospore-containing inoculum suspension (~80,000 chlamydospores and ~800,000 conidia per vial) was added to 2.4 ml of treatment solution (i.e., VFA/reduced metal or control) solution. Inoculant propagule density was determined using a hemocytometer. Following treatment, viable propagules of *Fo* in each vial were determined by standard dilution plating on Nash-Snyder medium (Nash and Snyder, 1962). There were four replicates of each treatment in each of two trials.

Soil Incubations with Fe^{2+} , Mn^{2+} , and VFAs

To examine survival of *Fo* in soils when exposed to VFAs and reduced metals and its interaction with soil liming, 2 g of dry soil of three textures (sand, sandy loam, silty clay) were autoclaved and infested with ~25,000 chlamydospores/g soil and ~ 10^5 conidia/g soil using a chlamydospore-containing suspension prepared as described previously. Volatile fatty acid (acetic acid, *n*-butyric acid, and isovaleric acid) solutions with concentrations of 0, 10, and 20 mmol VFA/liter, $FeSO_4$ or $MnSO_4$ at 0% or 0.05% w/w concentrations, and laboratory-grade calcium carbonate (lime) at 0% or 0.1% w/w concentration were added to infested soil at 1 ml solution/g soil for the equivalent of 0, 10, and 20 mmol VFA/kg dry soil and 0% and 0.05% w/w reduced metals in dry soil. These concentrations were chosen based on preliminary soil incubation trials and work by Swilling et al. (2022). Four replicates of each treatment combination in glass scintillation vials were incubated at 20 to 25°C in an anaerobic chamber (PLAS LABS Model 855-AC, Lansing, MI, USA) as described by Swilling et al. (2022). The vials were placed in the anaerobic chamber with lids off, purged of oxygen by alternate application of vacuum and pumping in a deoxygenated gaseous mixture of nitrogen, carbon dioxide, and

hydrogen in ten cycles, followed by recapping for the incubation. After 5 days of incubation, viable *Fo* population in each vial was determined by standard dilution plating on Komada's medium (Komada 1975). Soil solution pH was determined in three replicates of each treatment at the end of incubation by measurement with a laboratory pH electrode (Orion 3-Star Plus pH Benchtop Meter; Thermo Scientific, Waltham, MA). The soil incubation trial was repeated.

Data Analysis

The quantified microbial population estimate data was $\log_{10}$ transformed before statistical analyses. The differences in soil physicochemical (Mn^{2+} and Fe^{2+}) and microbial properties among treatments were compared using the LSD test at $P < 0.05$ after a one-way ANOVA to test the significant difference. Additionally, a three-way ANOVA test with different VFA concentrations, Mn^{2+} and Fe^{2+} concentrations, and soil texture as the main effect and trial as the random effect were performed to analyze the effects of VFAs, Mn^{2+} and Fe^{2+} , and soil texture in influencing disinfestation efficiency. The model used was a completely randomized design and the statistical analyses was performed with SAS 9.4 software.

Results

Aqueous Incubations of *Fo* in VFAs and Reduced Metals

The independent variables VFA, VFA concentration, reduced metal, and reduced metal concentration all significantly influenced *Fo* suppression ($P < 0.0001$; Table 2.1). Likewise, the two-way interactive effects of VFA and VFA concentration ($P = 0.0168$), VFA concentration and reduced metal concentration ($P = 0.0163$), and reduced metal and reduced metal concentration ($P < 0.0001$) also significantly influenced *Fo* suppression (Table 2.1). However, the lower-order

effects are masked by the three-way interactive effects of VFA, reduced metal, and concentration of reduced metal ($P = 0.0077$), and the interactive effects of VFA concentration, reduced metal, and reduced metal concentration ($P = 0.0031$; Table 2.1).

Overall, isovaleric acid was the most suppressive of *Fo* of all the VFAs tested at 10 mmol/liter, followed by *n*-butyric and acetic acids. At 10 mmol/liter, isovaleric acid reduced *Fo* viability from 5.1 to 1.8 $\log_{10}(\text{CFU}+1)/\text{ml}$ while acetic and *n*-butyric acid both reduced *Fo* CFU viability from 5.2 to ~ 2.6 -2.8 $\log_{10}(\text{CFU}+1)/\text{ml}$ compared to the VFA-free control (Fig. 2.1). However, there was no significant difference in *Fo* suppressiveness between 5 mmol/liter acetic, *n*-butyric, and isovaleric acid solutions, which all reduced *Fo* viability by 1.5 $\log_{10}(\text{CFU}+1)/\text{ml}$ compared to the VFA-free control (Fig. 2.1).

Solutions containing 0.05% w/w Mn^{2+} or 0.01% or 0.05% w/w Fe^{2+} were significantly more suppressive than solutions without reduced metals. For instance, 0.05% w/w Mn^{2+} reduced *Fo* viability from 5.5 to 5.2 $\log_{10}(\text{CFU}+1)/\text{ml}$ in solutions without VFAs, from 5.0 to 3.7 $\log_{10}(\text{CFU}+1)/\text{ml}$ in 5 mmol/liter VFA solutions, and from 4.8 to 1.8 $\log_{10}(\text{CFU}+1)/\text{ml}$ in 10 mmol/liter VFA solutions (Fig. 2.2). Effects were concentration-dependent, as 0.01% Mn^{2+} solutions did not significantly reduce viability of *Fo* in solutions without VFAs and suppressed *Fo* viability by a lesser degree in combination with VFAs compared to solutions with 0.05% Mn^{2+} (Fig. 2.2). The suppressive effects of Fe^{2+} in combination with VFAs were stronger than that of Mn^{2+} . For instance, 0.01% Fe^{2+} reduced *Fo* viability by 0.7 $\log_{10}(\text{CFU}+1)/\text{ml}$ in solutions without VFAs, 1.8 $\log_{10}(\text{CFU}+1)/\text{ml}$ in 5 mmol/liter VFA solutions, and by 2.7 $\log_{10}(\text{CFU}+1)/\text{ml}$ in 10

mmol/liter VFA solutions compared to controls without VFAs or Fe^{2+} / Mn^{2+} . Solutions containing 10 mmol/liter of VFA and 0.05% Fe^{2+} resulted in near non-detectability of *Fo*, reducing *Fo* viability from ~ 5.0 to $5.5 \log_{10}(\text{CFU}+1)/\text{ml}$ as is the case in VFA-free controls to less than $1.0 \log_{10}(\text{CFU}+1)/\text{ml}$ (Fig. 2.2).

Additionally, the suppression of *Fo* was significantly influenced by the interactive effects of VFA type, reduced metal type, and concentration ($P = 0.0077$; Table 2.3). In the absence of VFAs, 0.05% Mn^{2+} resulted in suppression of *Fo* viability by $0.5 \log_{10}(\text{CFU}+1)/\text{ml}$ while both 0.01% and 0.05% Fe^{2+} suppressed *Fo* viability by ~ 0.9 to $1.0 \log_{10}(\text{CFU}+1)/\text{ml}$ (Fig. 2.3). In contrast, 0.05% Mn^{2+} combined with acetic or *n*-butyric acid suppressed *Fo* viability by approximately $2.5 \log_{10}(\text{CFU}+1)/\text{ml}$ compared to the VFA- and reduced metal-free control and suppressed *Fo* viability by $3.5 \log_{10}(\text{CFU}+1)/\text{ml}$ compared to the VFA- and reduced metal-free control when combined with isovaleric acid (Fig. 2.3).

Soil-based Incubations of Fo in VFAs and Reduced Metals

Suppression of *Fo* in soil was strongly influenced by the first-order effects of VFA, soil texture, and liming ($P < 0.0001$; Table 2.2). Suppression of *Fo* in soils was also strongly influenced by the interactions of soil texture and liming ($P < 0.0001$); soil texture and VFA concentration ($P < 0.0001$); VFA, VFA concentration, and reduced metals ($P < 0.0001$; Table 2.2); and the five-way interaction between VFA, VFA concentration, reduced metals, soil texture, and liming ($P = 0.0006$; Table 2.2). Soil solution pH at the end of the incubation period was strongly influenced by the first-order effects of VFA concentration, soil texture, liming, and reduced metals ($P <$

0.002); and multiple interactions including the four-way interaction among these effects ($P < 0.0001$). Most notably, soil pH in unlimed sandy soils was highly responsive to effects of VFA application (Table 2.3), indicative of low pH buffering capacity. At the same time, limed sandy soils generally had increased soil pH or no change in soil pH with VFA application compared to untreated soil. Changes in soil pH were minimal in unlimed sandy loam and silty clay soil textures even at high VFA concentrations and increased as much as 1 pH unit in limed soils (Table 2.2).

Overall, *Fo* suppression was greater in coarser-textured soils (i.e., sandy) than in finer-textured soils (i.e., silty clay) as *Fo* viability in unlimed sandy soil was reduced by $1.9 \log_{10}(\text{CFU}+1)/\text{g}$ soil compared to unlimed silty clay soil (Fig. 2.4). Additionally, liming by application of 0.1% w/w CaCO_3 largely negated the suppressive effects of VFAs in silty clay and sandy loam soil while reducing the level of *Fo* suppression in sand compared to unlimed silty clay (Fig. 2.4).

While higher concentrations of VFAs were more suppressive than lower concentrations in all soil textures, the effect was more pronounced in soils with lower clay content. For instance, 20 mmol/kg soil VFA solutions reduced *Fo* viability by $0.5 \log_{10}(\text{CFU}+1)/\text{g}$ soil in silty clay soil (44% clay), 0.6 log units in sandy loam soil (22% clay), and 1.8 log units in sandy soil (0% clay) (Fig. 5) compared to VFA-free controls for the same corresponding soil texture. When no VFAs were applied, *Fo* viability was significantly greater in the silty clay and sandy loam soils than in sandy soil (Fig. 2.5).

As with aqueous incubations, VFA and reduced metal type significantly ($P < 0.0001$) influenced *Fo* suppression in soil solution (Table 2.2). Overall, solutions containing VFAs reduced *Fo* viability by 0.2 to 0.5 $\log_{10}(\text{CFU}+1)/\text{g}$ soil compared to solutions without VFAs in the absence of reduced metals while in contrast, reduced metal cations did not significantly suppress *Fo* in solutions without VFAs in soils (Fig. 2.6) unlike in purely aqueous incubations (Fig. 2.2). However, the presence of reduced metals enhanced *Fo* suppression in combination with VFAs, as 0.05% w/w Mn^{2+} reduced *Fo* viability by 0.7 $\log_{10}(\text{CFU}+1)/\text{g}$ when combined with acetic acid and by ~ 1.1 to 1.2 $\log_{10}(\text{CFU}+1)/\text{g}$ soil when combined with *n*-butyric or isovaleric acids compared to controls without VFAs or reduced metals (Fig. 2.6). While 0.05% w/w Fe^{2+} also generally reduced *Fo* in combination with VFAs as compared to VFA-only treatments, it was less effective than 0.05% w/w Mn^{2+} in combination with *n*-butyric or isovaleric acid in soils (Fig. 2.6).

Lastly, the interactive effects of VFA, reduced metals, VFA concentrations, soil texture, and liming significantly influenced *Fo* suppression rates ($P = 0.0006$; Table 2.2). The greatest differences in *Fo* suppression rates between different VFA types, concentrations, and whether or not lime or reduced metals were applied was observed in sand followed by sandy loam and silty clay soils (Fig. 2.7). In unlimed sandy soils, addition of 0.05% w/w Mn^{2+} enhanced the suppressiveness of all VFAs tested and resulted in undetectable levels of *Fo* in 20 mmol/kg soil *n*-butyric and isovaleric acids (Fig. 2.7a). However, application of 0.1% w/w CaCO_3 lime not only reduced the suppressive effects of all VFAs and reduced metals but also resulted in no significant difference in the suppressiveness of VFA-containing solutions with Mn^{2+} and VFA-containing solutions with Fe^{2+} in sandy soils (Fig. 2.7a). However, solutions containing both

VFAs, and reduced metals were generally more suppressive of *Fo* than metal-free solutions with VFAs even in limed sandy soil. Just as with sandy soils, *Fo* suppression was greatest in unlimed sandy loam soils treated with 20 mmol/kg soil *n*-butyric or isovaleric acid together with 0.05% w/w Mn^{2+} (Fig. 2.7b). However, only solutions containing 10 or 20 mmol/kg soil *n*-butyric acid in the absence of reduced metals or 20 mmol/kg soil *n*-butyric or isovaleric acid together with 0.05% w/w Mn^{2+} or Fe^{2+} suppressed *Fo* to any extent in limed sandy loam soils (Fig. 2.7b). In silty clay soils, the difference in the suppressiveness of different VFAs at differing concentrations in combination with reduced metals was even less than in sandy loam soils while the effect of liming was also less pronounced considering treatment effects were limited compared to coarser-textured soils (Fig. 2.7c). However, the application of 20 mmol/kg soil isovaleric acid suppressed *Fo* by $1.5 \log_{10}(CFU+1)/g$ in combination with 0.05% w/w Mn^{2+} and $1.0 \log_{10}(CFU+1)/g$ in combination with 0.05% w/w Fe^{2+} in unlimed silty clay soil (Fig. 2.7c).

Discussion and Conclusions

Suppression of *Fo* was strongly affected by differences in VFAs and reduced metal cations and their concentrations in soil solutions, but also by soil texture and soil liming. Similarly, Huang et al. (2015) and Abd-el-Kareem (2009) reported that *n*-butyric and isovaleric acids were more suppressive of *Fo* than acetic acid in both soil-based and aqueous incubations. Likewise, the stronger suppressiveness of *Fo* by Fe^{2+} compared to Mn^{2+} in aqueous solutions is consistent with past research by Momma et al. (2011) who reported greater toxicity of Fe^{2+} toward *F. oxysporum* f. sp. *lycopersici* than Mn^{2+} . VFAs also help solubilize metal cations such as Fe^{2+} and Mn^{2+} (Roden and Wetzels 2003), likely enabling far higher *Fo* suppression than by reduced metal

cations or VFAs alone. This is especially likely given that the mechanisms of pathogen suppression for VFAs and reduced metals are very different. VFAs kill *Fo* cells and spores by lowering the internal cellular pH (e.g., Momma et al. 2006; Huang et al. 2015), while reduced metal cations cause mortality by inducing the production of reactive oxygen species and causing interference with the production and function of metalloproteins (Touati, 2000; Gerwien et al. 2017). However, the effects of soil texture and liming complicates the suppression of *Fo* by VFAs and reduced metal cations in soils.

While Fe^{2+} was more suppressive of *Fo* than Mn^{2+} in aqueous solutions, this was not the case in soil-based water solutions. In soils, there was either no significant difference between *Fo* suppression by Mn^{2+} and Fe^{2+} as was the case in solutions containing acetic or *n*-butyric acid, or in the case of VFA-free and isovaleric solutions, greater suppression of *Fo* by Mn^{2+} than Fe^{2+} . This may be due to not only the greater oxidation potential of Fe^{2+} compared to Mn^{2+} , making it easier for it to be oxidized into sparingly soluble Fe^{3+} compounds, but also that the adsorptivity of Fe^{2+} on common clay minerals such as kaolinite is higher than the adsorptivity of Mn^{2+} (Tufekci et al. 2010; Shaban and Nassif, 2014). Additionally, finer-textured soils have greater adsorptivity and buffering capacity than coarser-textured soils, therefore requiring higher concentrations of reduced metal cations and/or VFAs in soil water for a given level of *Fo* suppression. Likewise, lime application also significantly impacts the suppression of *Fo* by VFAs and reduced metal cations by altering soil chemical conditions. Liming can affect VFA toxicity by increasing soil pH, which increases VFA dissociation as the pH increases above the pKa of the VFA (pKa of ~4) or by direct neutralization through interaction of VFAs with calcareous minerals

in recently limed soils (Strom et al. 2001; Tenuta et al. 2002). Together, this makes it less likely that VFAs will cross fungal cellular membranes, unlike in their undissociated form, and accordingly reduces *Fo* suppression by VFAs (Fig. 2.2) (e.g., Momma et al. 2006; Meng et al. 2018; Swilling et al. 2022). This effect was most pronounced in sandy soil, likely due to low pH buffering capacity and relatively high suppression rates in unlimed sandy soils (Fig. 2.2, Table 2.3). The reduction of the ability of VFAs to suppress *Fo* in soils with high clay content and/or less acidic soil pH is also consistent with past studies involving other soilborne plant pathogens. For instance, the germination of *Agroathelia (Sclerotium) rolfsii* sclerotia was also suppressed by VFAs, but VFAs were less suppressive at higher soil clay content and as pH increased (Swilling et al. 2022). This was attributed to the increasing dissociation of VFAs as soil pH increased above that of the VFA pKa which can be approximated using the Henderson-Hasselbalch equation (Tenuta et al. 2002; Swilling et al. 2022).

While the present work was conducted under laboratory conditions, it is important to consider how the results may relate to impacts on *Fo* inoculum under field conditions. Previous studies with *Fo* suggest that inoculum density of $3 \log_{10}(\text{CFU}+1)/\text{g}$ soil and higher generally leads to consistent disease development (e.g., Navas-Cortés et al. 2000; Elmer 2002; Hao et al. 2009). In our soil-based incubations, *Fo* inoculum density was reduced to or below this key threshold most often in unlimed sandy soil, but also in unlimed sandy loam and silty clay with 20 mM isovaleric acid and 0.05% Mn^{2+} (Fig. 2.7). However, the *Fo* inoculum densities used in the present study likely exceed more typical inoculum densities of infested fields. At a lower initial inoculum density, reductions of $\sim 1 \log_{10}(\text{CFU}+1)/\text{g}$ soil may be sufficient for disease

management, especially when using other integrated disease management tactics. Lastly, this work does not address potentially additive effects of other disease control mechanisms of ASD treatment, such as the activity of biocontrol microbes during and after ASD treatment incubation (Strauss et al. 2017; Shrestha et al. 2020).

While our work further supports the importance of VFAs and $\text{Fe}^{2+}/\text{Mn}^{2+}$ cations as key mechanisms by which *Fo* is suppressed by ASD, it is important to consider potentially adverse effects if soils are not returned to aerobic conditions prior to crop production. High concentrations of VFAs and $\text{Fe}^{2+}/\text{Mn}^{2+}$ in soil solutions were also found to be associated with enhanced Fusarium wilt severity in banana crops (Orr and Nelson 2018) as anaerobic soil conditions are detrimental to plant health and also because despite its toxicity at high concentrations, *Fo* requires water-soluble Fe and Mn as micronutrients. This is typically less of a problem in raised bed vegetable and small fruit production where soils are better-drained (Orr and Nelson 2018).

As the effectiveness of a VFA treatment depends not only on the concentrations of VFAs and reduced metal cations released through organic matter fermentation by soil microbial communities, but also on soil physical and chemical properties, it is likely necessary to tailor ASD treatments for *Fo*-infested fields. For instance, higher soil clay content and/or more alkaline soil pH may require higher organic amendment rates or amendments of differing substrate biochemical quality to generate adequate rates and types of VFAs and reduced metals for suppression of soilborne plant pathogens in the context of ASD. Similarly, it may be

advisable to lime soils after rather than before performing ASD and where possible, to maintain soil pH in the more acidic part of the recommended range for the given crop type(s) to lessen interference with the suppression of *Fo* by ASD fermentation byproducts. While the present work provides a framework for understanding how types and rates of VFAs and reduced metal cations affect survival of *Fo* inoculum during ASD treatments, more work is needed to better understand how biochemical quality and rate of organic amendments used for ASD treatments affect the production of these fermentation by-products during amendment fermentation under varying soil biological, chemical, and physical conditions. This would help facilitate development of field-specific treatment recommendations based on soil properties and needed rates of fermentation by-products to suppress particular soilborne plant pathogens.

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Appendix I

Table 2.1. Analysis of variance for the main effects and interaction effects influencing *Fusarium oxysporum* suppression in aqueous media; volatile fatty acid (VFA) type and concentration, $\text{Fe}^{2+}/\text{Mn}^{2+}$ type and concentration, and their interactions. NS = not significant, $P > 0.05$.

Effect	P-values
VFA	<0.0001
VFA concentration	<0.0001
$\text{Fe}^{2+}/\text{Mn}^{2+}$	<0.0001
$\text{Fe}^{2+}/\text{Mn}^{2+}$ concentration	<0.0001
VFA $\times$ $\text{Fe}^{2+}/\text{Mn}^{2+}$	NS
VFA $\times$ VFA concentration	0.0168
VFA concentration $\times$ $\text{Fe}^{2+}/\text{Mn}^{2+}$	NS
VFA concentration $\times$ $\text{Fe}^{2+}/\text{Mn}^{2+}$ concentration	0.0163
$\text{Fe}^{2+}/\text{Mn}^{2+} \times \text{Fe}^{2+}/\text{Mn}^{2+}$ concentration	<0.0001
VFA $\times$ VFA concentration $\times$ $\text{Fe}^{2+}/\text{Mn}^{2+}$	NS
VFA $\times$ VFA concentration $\times$ $\text{Fe}^{2+}/\text{Mn}^{2+}$ concentration	NS
VFA $\times$ $\text{Fe}^{2+}/\text{Mn}^{2+} \times \text{Fe}^{2+}/\text{Mn}^{2+}$ concentration	0.0077
VFA concentration $\times$ $\text{Fe}^{2+}/\text{Mn}^{2+} \times \text{Fe}^{2+}/\text{Mn}^{2+}$ concentration	0.0031
VFA $\times$ VFA concentration $\times$ $\text{Fe}^{2+}/\text{Mn}^{2+} \times \text{Fe}^{2+}/\text{Mn}^{2+}$ concentration	NS

Table 2.2. Analysis of variance for the main effects and interaction effects influencing *Fusarium oxysporum* suppression in soil solution; volatile fatty acid (VFA) type and concentration, Fe²⁺/Mn²⁺, liming, soil texture, and their interactions. NS = not significant, *P* > 0.05.

Effect	<i>P</i> -values
VFA	<0.0001
Fe ²⁺ / Mn ²⁺	NS
Soil texture	<0.0001
Liming	<0.0001
VFA × Fe ²⁺ / Mn ²⁺	<0.0001
VFA × Soil texture	NS
VFA × Liming	0.002
Fe ²⁺ / Mn ²⁺ × VFA concentration	NS
Fe ²⁺ / Mn ²⁺ × Soil texture	0.002
Fe ²⁺ / Mn ²⁺ × Liming	<0.0001
Soil texture × Liming	<0.0001
VFA concentration × Soil texture	<0.0001
VFA concentration × Liming	<0.0001
VFA × Fe ²⁺ / Mn ²⁺ × VFA concentration	<0.0001
VFA × Fe ²⁺ / Mn ²⁺ × Soil texture	0.005
VFA × VFA concentration × Soil texture	NS
Fe ²⁺ / Mn ²⁺ × VFA concentration × Soil texture	<0.0001
VFA × Fe ²⁺ / Mn ²⁺ × Liming	0.0001
VFA × VFA concentration × Liming	NS
Fe ²⁺ / Mn ²⁺ × VFA concentration × Liming	0.04
VFA × Soil texture × Liming	NS
Fe ²⁺ / Mn ²⁺ × Soil texture × Liming	<0.0001
VFA concentration × Soil texture × Liming	<0.0001
VFA × VFA concentration × Fe ²⁺ / Mn ²⁺ × Soil texture	<0.0001
VFA × Fe ²⁺ / Mn ²⁺ × Soil texture × Liming	0.007
VFA × VFA concentration × Soil texture × Liming	NS
Fe ²⁺ / Mn ²⁺ × VFA concentration × soil texture × Liming	NS
VFA × Fe ²⁺ / Mn ²⁺ × VFA concentration × Soil texture × Liming	0.0006

Table 2.3. Soil solution pH as influenced by the interactive effects of volatile fatty acid (VFA) concentration, reduced metals, soil texture, and liming. Within soil texture, means indicated by different letters are significantly different at $P < 0.05$ according to Fisher's protected least significant difference test.

Reduced metal	VFA concentration	Sand		Sandy loam		Silty clay	
		no lime	limed	no lime	limed	no lime	limed
No metal	No VFA	6.94 cd	8.16 ab	7.16 cd	7.91 b	7.19 fgh	8.24 abc
	10 mmol	5.80 e	8.37 a	7.34 c	8.30 a	7.41 efg	8.33 a
	20 mmol	5.34 e	8.28 a	7.36 c	8.36 a	7.43 ef	8.26 ab
Mn ²⁺	No VFA	3.78 fg	7.82 abcd	6.98 de	7.81 b	7.16 h	8.02 bcd
	10 mmol	3.95 f	7.76 abc	7.16 d	8.41 a	7.44 e	8.08 cd
	20 mmol	3.67 fg	7.04 d	7.33 c	8.34 a	7.40 efgh	7.97 d
Fe ²⁺	No VFA	4.14 fg	8.12 abc	6.80 e	7.83 b	7.28 efgh	8.00 cd
	10 mmol	3.95 f	7.45 bcd	7.37 c	8.27 a	7.25 gh	8.21 abc
	20 mmol	3.18 g	5.53 e	6.80 e	8.35 a	7.24 gh	8.06 cd

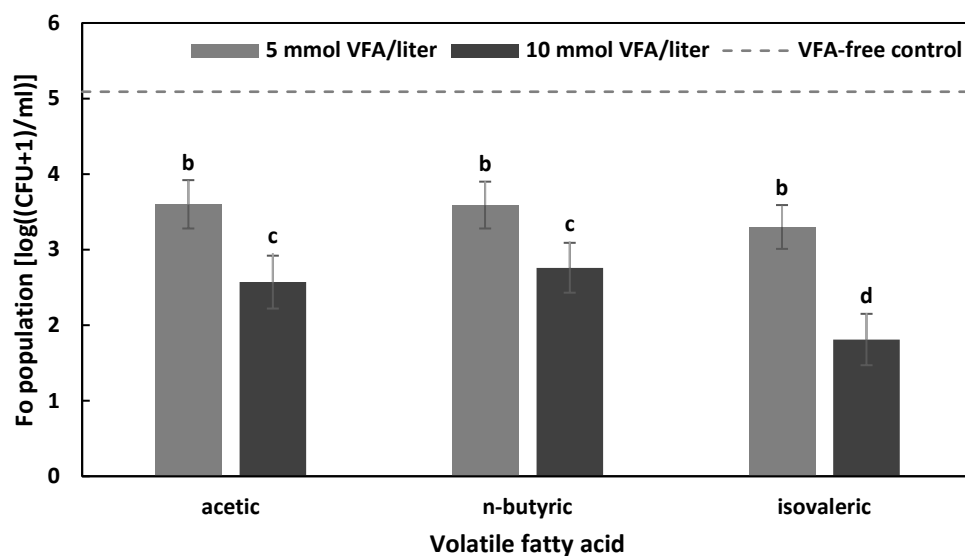


Figure 2.1. Effect of volatile fatty acid (VFA) type and concentration on suppression of *Fusarium oxysporum* (*Fo*) colony forming units (CFUs) in aqueous incubations. The control line represents no VFA (sterile water only). Bars indicated by different letters for each category are significantly different at $P < 0.05$ according to Fisher's protected least significant difference test. Error bars indicate standard error, with four replicates in each (repeated) trial.

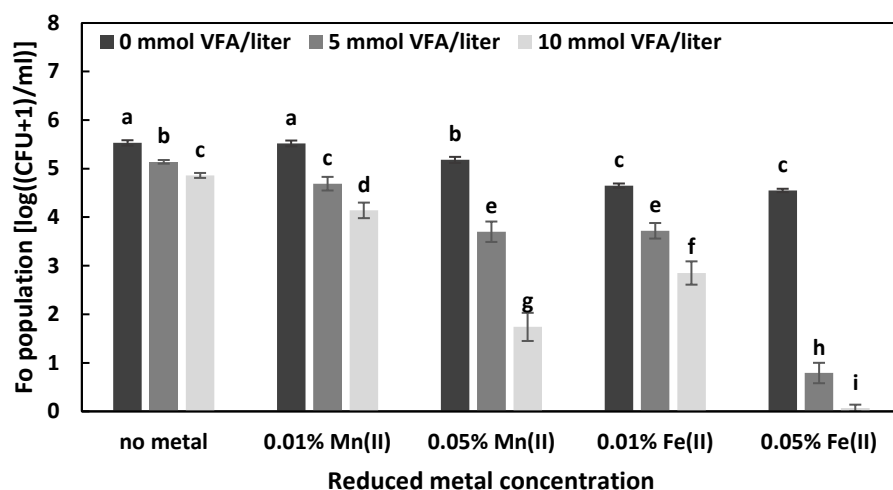


Figure 2.2. Effect of volatile fatty acid (VFA) concentration and Fe^{2+} / Mn^{2+} concentration on suppression of *Fusarium oxysporum* (*Fo*) colony forming units (CFUs) in aqueous incubations. Bars with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$). Error bars indicate standard error, with four replicates in each (repeated) trial.

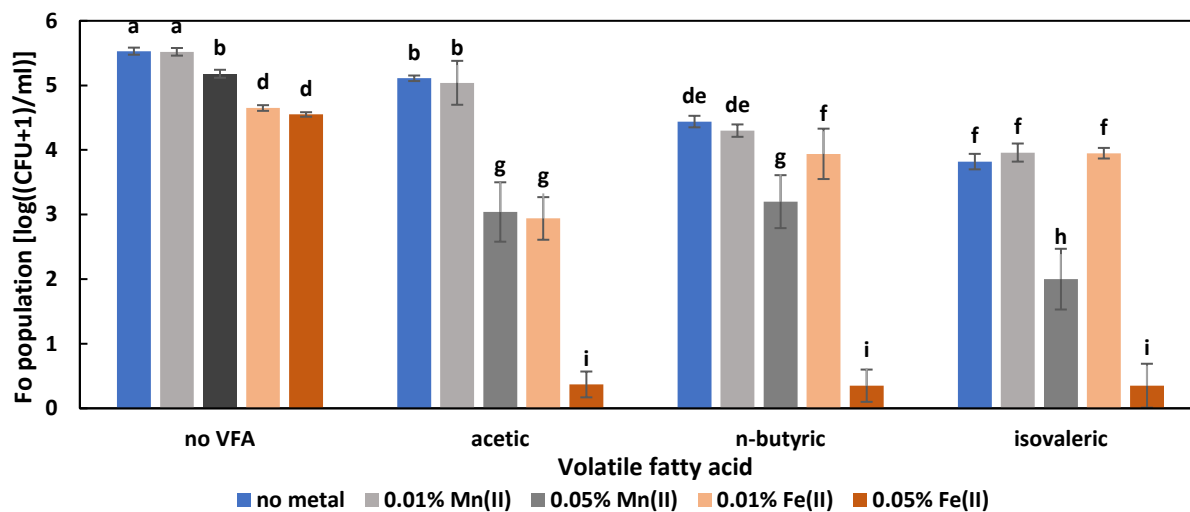


Figure 2.3. Effect of volatile fatty acid (VFA) type and $\text{Fe}^{2+}/\text{Mn}^{2+}$ concentration on suppression of *Fusarium oxysporum* (*Fo*) colony forming units (CFUs) in aqueous incubations. Bars indicated by different letters for each category are significantly different at $P < 0.05$ according to Fisher's protected least significant difference test. Error bars indicate standard error, with four replicates in each (repeated) trial.

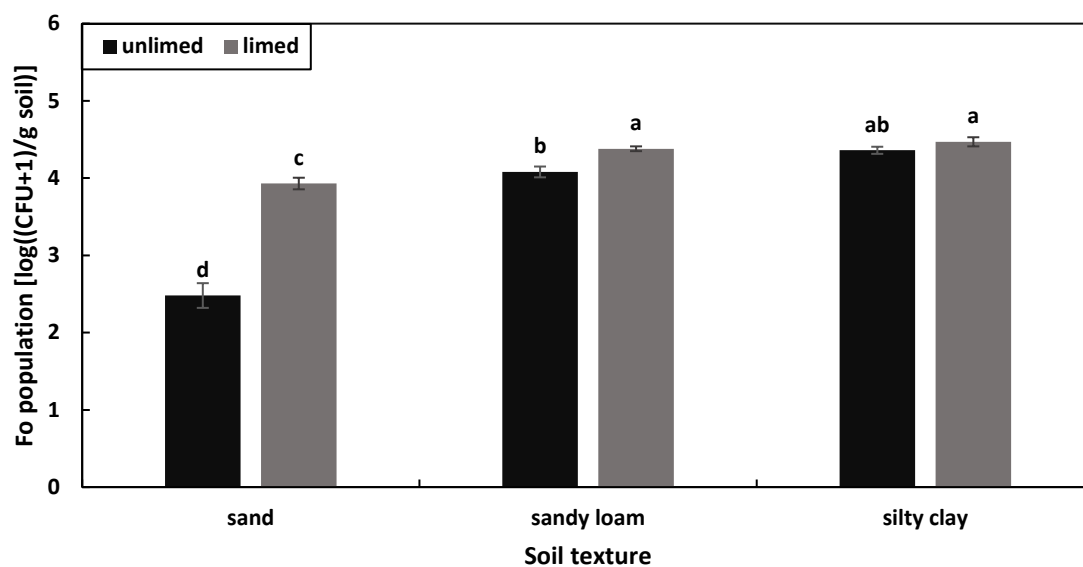


Figure 2.4. Effect of soil texture and 0.1% by weight CaCO_3 lime application on suppression of *Fusarium oxysporum* (*Fo*) population in colony forming units (CFUs). Limed soils were amended with 0.1% CaCO_3 by weight. Bars indicated by different letters for each category are significantly different at $P < 0.05$ according to Fisher's protected least significant difference test. Error bars indicate standard error, with four replicates in each (repeated) trial.

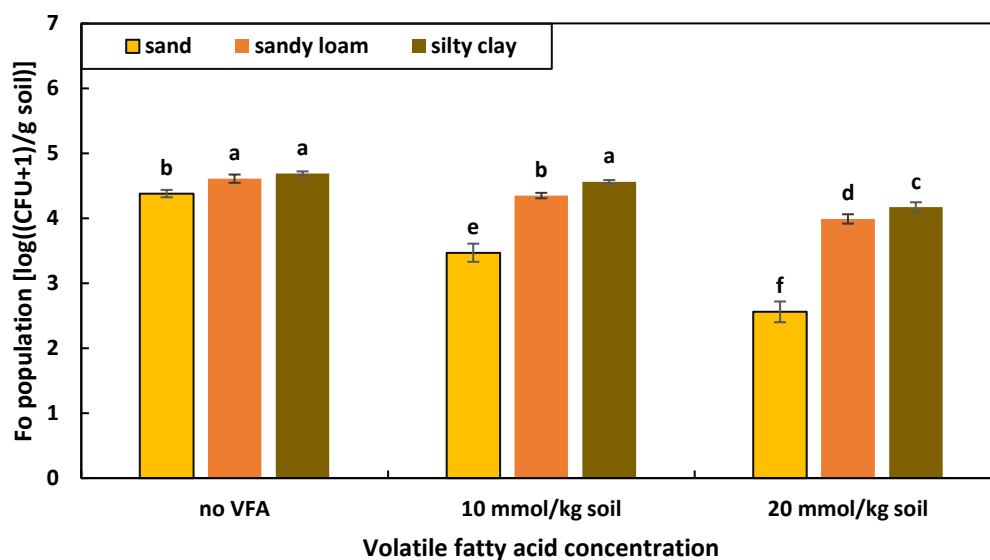


Figure 2.5. Effect of soil texture and volatile fatty acid (VFA) concentration on suppression of *Fusarium oxysporum* (*Fo*) population in colony forming units (CFUs). Bars indicated by different letters for each category are significantly different at $P < 0.05$ according to Fisher's protected least significant difference test. Error bars indicate standard error, with four replicates in each (repeated) trial.

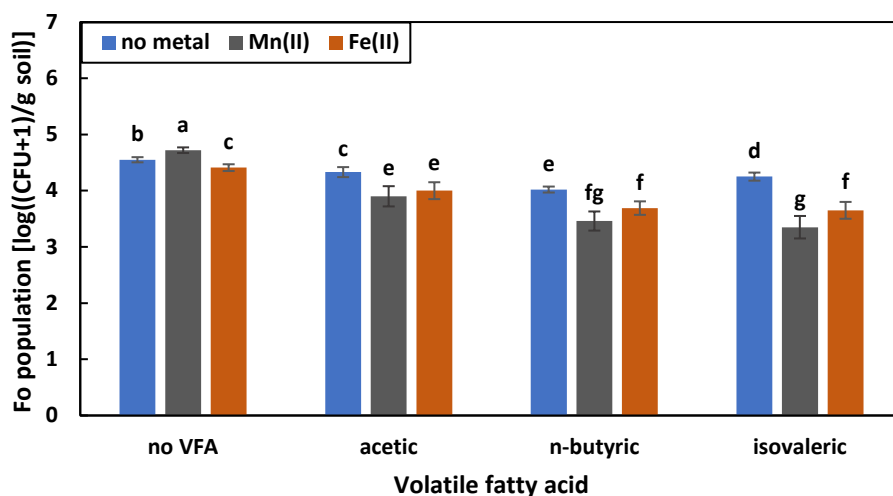


Figure 2.6. Effect of volatile fatty acid (VFA) type and application of 0.05% w/w reduced metal cations on suppression of *Fusarium oxysporum* (*Fo*) colony forming units (CFUs) in soils of all textures and liming rates. Bars indicated by different letters for each category are significantly different at $P < 0.05$ according to Fisher's protected least significant difference test. Error bars indicate standard error, with four replicates in each (repeated) trial.

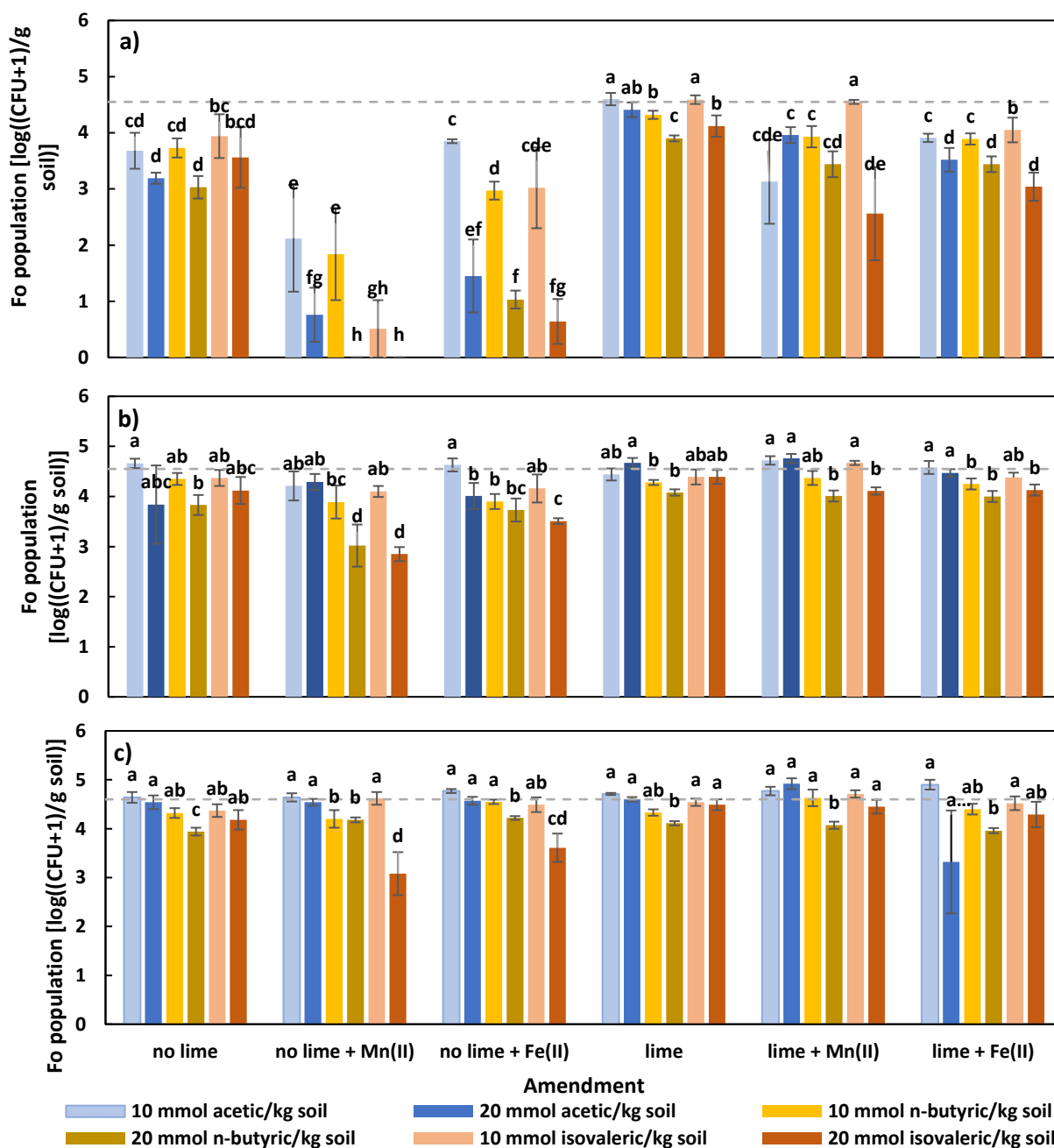


Figure 2.7. Effect of volatile fatty acid (VFA) type and concentration, application of 0.05% w/w reduced metal cations, and liming on suppression of *Fusarium oxysporum* (*Fo*) colony forming units (CFUs) in a) sand, b) sandy loam, and c) silty clay soil. Bars indicated by different letters for each category are significantly different at $P < 0.05$ according to Fisher's protected least significant difference test. Error bars indicate standard error, with four replicates in each (repeated) trial.

Chapter 3: Optimizing Substrate Composition for Suppressing *Fusarium oxysporum* during Anaerobic Soil Disinfestation

Abstract

Less hazardous alternatives to soil fumigants for suppressing soilborne pathogens such as *Fusarium oxysporum* (Fo), one causative agent of the strawberry black root rot complex, are urgently needed. A promising alternative is anaerobic soil disinfestation (ASD), in which soil is amended with labile organic materials, irrigated to field capacity, and tarped to induce anaerobic fermentation for a brief period before planting. ASD suppresses soilborne plant pathogens via the generation of reduced metal cations (Fe^{2+} , Mn^{2+}) and volatile fatty acids (VFAs, e.g., acetic, *n*-butyric, and isovaleric). However, little is known about how the interaction between soil texture, liming, or organic amendment composition influences *Fo* suppression via soil biogeochemistry. Suppression of *Fo* by VFAs and reduced metal cations was investigated in soil-based ASD incubation trials in which *Fo*-inoculated sand, sandy loam, and silty clay soils with and without lime (0.1% CaCO_3) were amended at 1% w/w basis with varying levels of soybean protein isolate (SPI) and dried molasses (DM) to initiate ASD treatments. Soil solution VFA concentrations and soil pH measurements were taken post-ASD. Total soil solution VFA concentrations were negatively correlated with viability of *Fo* populations (colony forming units; CFU) in sandy and sandy loam soils, while amendment protein concentration was positively correlated with soil solution VFA concentrations only in sandy soils. However, pH minimally influenced *Fo* suppression. In general, finer textures and limed soils were associated with lower *Fo* suppression. This highlights the importance of soil texture, soil pH and amendment composition in influencing ASD effectiveness.

Introduction

Strawberry black root rot disease complex is one of the most damaging disease complexes for strawberries, reducing yields by 20 to 40% in affected fields (Louws and Cline 2014). While black root rot symptoms are similar from site to site (i.e., blackened and woody rat-tail like roots, stunted growth, dead and wilted older leaves, lack of response to fertilization and other improved growing conditions), the causal pathogens vary and may include *Fusarium* spp., *Rhizoctonia* spp., *Pythium* spp., and plant parasitic nematodes (Koike and Gordon 2015). Soilborne plant pathogens such as those responsible for black root rot were normally managed in the past by soil fumigation with gaseous pesticides (Agrios 2005). While fumigation is highly effective for broad spectrum suppression of soilborne pests in fruit and vegetable crops, synthetic chemical fumigants present significant risks to human and ecological health and accordingly require extensive safety training and certification for use (NIOSH 2013) (US EPA 2008) (PPDB 2019). However, common alternatives to soil fumigation (e.g., soil solarization, biofumigation, crop rotation, and cultural control practices) also have limitations related to cost, efficacy, length of treatment, and optimal environmental conditions for application (e.g., Koike and Gordon 2015; Panth et al. 2020).

Anaerobic soil disinfestation (ASD), a broad-spectrum pest and pathogen management technique in which soils are amended with highly labile organic substrates, then wetted to field capacity and covered with plastic mulch films to induce anaerobic soil conditions is a promising alternative to chemical fumigation. Amendments used in ASD include cover crops, animal manures, molasses, wheat bran, and ethanol (Shrestha et al. 2016). Anaerobic soil

disinfestation was studied first in the Netherlands (Blok et al. 2000), Japan (Okazaki et al. 1986; Shinmura 2004; Momma et al. 2013), and the U.S. states of California and Florida (Butler et al. 2012b; Roskopf et al. 2014; Shennan et al. 2014) where fruit and vegetable cultivation is responsible for a substantial portion of regional agricultural economies and where high human population density can increase regulatory and social pressures to adopt non-fumigant alternatives. Across these studies, ASD was found to reduce soil pathogen populations, including fungi, oomycetes, bacteria, and nematodes by 59 to 94% if at least 0.3 kg m⁻² of organic amendment is added to soil, as reported in a meta-analysis by Shrestha et al. (2016). Pest and pathogen suppression mechanisms in ASD include not just inducing anaerobic conditions, but also the formation of volatile fatty acids (VFAs) (Momma et al. 2006; Samaniego-Gaxiola and Balagurusamy 2013; Huang et al. 2015) and increased solubilization of soil solution Mn²⁺ and Fe²⁺ under anaerobic conditions (Momma et al. 2011).

While it is well-known that VFAs, particularly acetic, *n*-butyric, and isovaleric acid play important roles in the suppression of soil fungal pathogens and that dissolved Fe²⁺ and Mn²⁺ also help suppress soil fungal pathogens, little is known about interactive fungitoxic effects of dissolved Fe²⁺ and Mn²⁺ and VFAs. Additionally, little is known about how co-application of lime influences the effectiveness of ASD. While alkaline pH was shown to enhance production of *n*-butyric and isovaleric acids (Katase et al. 2009; Kuruti et al. 2017; Esteban-Gutierrez et al. 2018; Strazzera et al. 2018), which are more fungitoxic than acetic acid (Samaniego-Gaxiola and Balagurusamy 2013; Huang et al. 2015), we also know that VFAs have greater toxicity in their non-dissociated forms as found in acidic soils (Momma et al. 2006; Swilling et al. 2022).

Additionally, it was reported that organic substrate composition affects the type of VFAs produced from organic matter fermentation in the context of industrial fermentation. For instance, protein-rich substrates are associated with enhanced production of *n*-butyric and/or isovaleric acids while carbohydrate-dominated substrates are typically associated with high acetic acid production rates in the context of industrial fermentation (i.e., Mowlick et al. 2013; Liu et al. 2015; Esteban-Gutierrez et al. 2018). Likewise, work by Swilling et al. (2022) determined that higher soil clay content is associated with reduced suppression of *Agroathelia* (*Sclerotium*) *rolfsii* sclerotial germination by VFAs, but little is known about how soil clay content influences suppression of *Fo* by VFAs and reduced metals. Therefore, more studies are needed to determine how organic substrate composition affects VFA production and balance in the context of ASD to help identify the best amendments to use for pathogen suppression and soil fertility enhancement and moreover, how soil clay content influences *Fo*-suppressive effects of VFAs and reduced metals. As such, the objectives of this study are to determine which amendment combination is best for maximizing *Fo* suppression, and to examine how soil texture influences the amount of VFAs, and reduced metals required to suppress *Fo* adequately for a soil with a given clay concentration.

Materials and Methods

Soil Preparation and Conditions

Soil was collected from the University of Tennessee Organic Crops Unit at the East Tennessee Research and Education Center (ETREC) in Knoxville, TN, U.S.A. The soil was sieved (10-mm mesh) to remove large rocks and debris, then air-dried. The soil texture (silty clay; 44% clay,

38% silt, and 18% sand) was determined using the hydrometer method (Bouyoucos, 1962). For sandy loam soil (22% clay, 19% silt, and 59% sand), the silty clay was mixed 50/50 (by weight) with sand (particle sizes 62.5 to 1500 μm).

Inoculum Preparation

A pathogenic isolate of the *Fusarium oxysporum* species complex ('SC5', GenBank Accession No. ON338265), recently confirmed as *Fusarium cugenangense* (Shrestha et al., 2024), was collected from strawberry roots on a farm in Tennessee and cultured in potato dextrose broth (PDB) for 7 days at 25°C with shaking. The mycelial mass grown in PDB was suspended in potato sucrose broth for 7 days, homogenized, and incubated for 3 weeks at 25°C, followed by incubation in sterile soil water extract, as described by Momma et al. (2006), to obtain chlamydospores. The soil water extract was prepared by thoroughly mixing 100 g of soil and 100 ml of deionized water, autoclaving the mix for 60 min at 121°C, allowing the soil particles to settle, then passing the supernatant through a 0.44- μm filter with a vacuum (Dismic-25cs, Advantec, Tokyo, Japan). After the 3-week incubation period, the mycelial mat and soil water extract were homogenized in five 30-s blending sessions in a sterile blender and the numbers of chlamydospores and conidia in the resultant inoculum suspension were counted using a hemocytometer.

Soil Fermentation Studies

Centrifuge tubes (50-ml) were filled with 30 g of silty clay, sandy loam (50% silty clay + 50% sand), or sandy soil, half of which were treated with 0.1% w/w laboratory-grade CaCO_3 lime

(Table 3.1). For each combination of texture, amendment and lime/no-lime, there were four replicates and the studies were repeated for a total of two trials. Soil texture was determined using the hydrometer method. The soil was inoculated with ~25,000 chlamydospores/g of soil and ~1,300,000 conidia/g of soil from the inoculum suspension, mixed with an organic amendment at 1% w/w basis, then saturated with water by adding 30 ml of deionized water, and incubated for 2 weeks at 35 to 30°C. There were four amendments evaluated, including: 1) 100% soybean protein isolate, which has a C:N ratio of ~5:1 (Yoshiki et al. 2013), 2) 75% soybean protein isolate and 25% dried molasses, 3) 50% soybean protein isolate and 50% dried molasses, 4) 25% soybean meal and 75% dried molasses, and 5) 100% dried molasses, in addition to unamended controls (Table 3.1). Ammonium nitrate was used to adjust the C:N ratios of all amendment mixtures except for the 100% soybean protein isolate amendment to 5:1.

Soil Solution Chemical Analysis

Organic acids (i.e., acetic, *n*-butyric, and isovaleric) produced during ASD were measured by Shimadzu Prominence UFLC HPLC analyser (Shimadzu, Japan) using a modified version of the method described by Ling et al. (2011b). Soils from the soil fermentation experiments were immersed in 1 ml water/g soil, vortexed for 30 s, then centrifuged at 15,000 rpm for 10 min and the soil solution filtered first through filter paper, then syringe filter tips before being transferred to clean HPLC vials and acidified with 30 µl 0.5 N H₂SO₄ per ml soil water extract. Organic acid from each fraction was identified by comparing their retention times and the areas of their peaks with those of standards (Sigma, U.S.A.). Soil solution Fe²⁺, Mn²⁺, and alkaline

cation (Ca^{2+} , Mg^{+} , and K^{+}) concentrations were measured using inductively-coupled plasma atomic emission spectroscopy (ICP-AES) at the University of Tennessee Soil and Pest Center in Nashville, TN, U.S.A. In addition, soil solution pH was measured after the soil fermentation stage of the experiment was concluded.

Assessment of *Fusarium oxysporum* Populations and Disease Symptoms of Plants

Fusarium oxysporum quantification was performed through serial dilution plating, where 1 g of pathogen-contaminated soil from each sample from both the soil fermentation and greenhouse studies was resuspended in 9 ml of sterile dH_2O , with this dilution step repeated twice more to make 10^{-1} and 10^{-2} dilutions. A 100- μL aliquot of each dilution for each sample was plated on Komada Medium (Komada, 1975) plates and incubated for 5 days. The effect of different ASD treatments was analyzed by enumerating the number of colonies for each dilution level associated with each ASD treatment. Identification was confirmed as needed with compound microscopy at 100X magnification to identify microconidia and monophialids that are characteristics of *Fo*. Additionally, strawberry plants in each pot were assessed for disease symptoms, including stunting, number of dead older leaves, and collapse/damping off.

Strawberry roots were weighed after washing.

Data Analysis

The quantified *Fo* population data was $\log_{10}$ transformed before statistical analyses. Differences in soil VFA, reduced metal concentrations, and population of viable *Fo* CFUs among treatments were compared using Fisher's LSD test at $P < 0.05$. A three-way ANOVA test evaluating the main

effects of amendment composition, soil texture, presence or absence of CaCO_3 lime, and interaction effects as the main effects and replicate as the random effect were performed to analyze the effects of VFAs and Mn^{2+} and Fe^{2+} in influencing disinfestation efficiency. Two trials were performed for both the soil fermentation and greenhouse studies. The model was a completely randomized design, and the statistical analyses were performed using SAS 9.4 software. Additionally, correlation analysis modelling was performed to investigate links between amendment composition, soil texture, VFAs, and reduced metals in soil solution, and *Fo* suppression. This was followed by performing 3D regression analysis in SAS 9.4 using the proc g3d and proc reg functions to generate 3D scatter plots and regression equations to illustrate how various soil conditions (i.e., soil texture, pH, amendment properties) interactively influence key factors that influence *Fo* suppression, such as total soil solution reduced metal and VFA concentrations. For the purposes of 3D regression analysis, the combined total VFA and Fe^{2+} and Mn^{2+} concentrations for each treatment were used after converting the Fe^{2+} and Mn^{2+} concentration values from mg/kg soil to mmol/kg soil.

Results

Soil and Amendment Properties and *Fo* Suppression

The suppression of *Fo* was significantly influenced by amendment composition, soil texture, and their interaction ($P = 0.001$) (Table 3.2). There was no observed main effect of soil liming on *Fo* suppressiveness. The suppressive effects of ASD were strongest in sandy soils, in which amendments containing 25 to 100% soybean protein isolate (SPI) and the balance as dried molasses (DM) reduced viable *Fo* CFUs by 1.1 to 2.6 $\log_{10}(\text{CFU}+1)/\text{g}$ soil compared to the

unamended control (Fig. 3.1). An even mixture with 50% SPI and 50% DM was the most suppressive. On the other hand, 100% DM amendment reduced the number of viable *Fo* CFU by $\sim 1.2 \log_{10}(\text{CFU}+1)/\text{g}$ soil (Fig. 3.1). As soil clay content increased, the suppressive effect of ASD became less pronounced. In sandy loam soils, amended soils exhibited average reductions of viable CFU by 1.2 to 1.8 $\log_{10}(\text{CFU}+1)/\text{g}$ soil compared to unamended soil, and the largest reductions were observed when amended with 100% DM (Fig 3.1). The effectiveness of ASD was lowest in silty clay soils, with reductions in *Fo* CFU viability by 0.4 to 0.7 $\log_{10}(\text{CFU}+1)/\text{g}$ soil compared to unamended silty clay soil with minimal differences among amendments (Fig. 3.1). The main effects of amendment composition ($P < 0.0001$) and soil texture ($P = 0.0033$) but not their interaction also significantly influenced total VFA concentrations (Table 3.3). As with *Fo* suppression, there was no significant effect of liming on soil VFA concentrations. Overall, soils of all textures amended with 100% SPI had the highest total VFA concentration (~ 30 mmol/kg soil) followed by 75% SPI/25% DM (~ 22.4 mmol/liter soil solution) while soils amended with 50% or less SPI combined with 50% or more DM had ~ 16 to 17 mmol/kg soil total VFAs in soil solution (Fig. 3.2). Sandy loam soils exhibited higher total VFA concentrations (20 to 25 mmol/kg soil) than sandy (14 to 16 mmol/kg soil) or silty clay (12 to 16 mmol/kg soil) soils (Fig. 3.3). Additionally, acetic acid dominated the VFA balance for all treatments, though the degree of its dominance varied. Acetic acid dominance of VFA fractions ranged from 70% in treatments amended with 100% soybean protein isolate to 88% in treatments amended with 100% dried molasses and the degree of acetic acid dominance of the VFA balance increases as the proportion of protein in the amendment decreases (Fig. 3.2). Additionally, the degree of acetic acid dominance is the lowest in sandy soils at 68% and greatest in silty clay soils at 80% (Fig.

3.3). Of the other VFAs, isovaleric acid is the most commonly produced by organic amendment fermentation, followed by *n*-butyric acid and the other VFAs (i.e., propionic, isobutyric, and valeric acids) for a given treatment (Fig. 3.2 and Fig. 3.3).

Soil solution Fe^{2+} and Mn^{2+} were significantly influenced by individual effects of amendment composition, soil texture, and liming ($P < 0.0001$) as well as the interactive effects of amendment composition and soil texture ($P < 0.0001$) and amendment composition and lime ($P = 0.01$) (Table 3.4). Treatments in sandy soils exhibited the lowest overall total combined Fe^{2+} and Mn^{2+} concentrations, ranging from 0.27 to 3.22 mg/kg soil for unamended treatments and amendments containing 0 to 50% dried molasses to sand amended with 100% dried molasses at 33.6 mg/kg soil (Fig. 3.4). Combined soil solution Fe^{2+} and Mn^{2+} concentrations were significantly higher overall in sandy loam and silty clay treatments. In both cases, unamended treatments exhibited the lowest combined Fe^{2+} and Mn^{2+} concentrations (19 mg/kg soil in sandy loam and 41 mg/kg soil in silty clay) while soils amended with 75% dried molasses exhibited the highest Fe^{2+} and Mn^{2+} concentrations in sandy loam (301 mg/kg soil) and 100% dried molasses in silty clay soils (297 mg/kg soil) (Fig. 3.4). Additionally, the reduced metal balance in soil solutions were overwhelmingly dominated by Mn^{2+} , except for sandy soils amended with 100% dried molasses (0.9 mg/kg soil Mn^{2+} and 30 mg/kg soil Fe^{2+}), 25% soybean protein isolate and 75% dried molasses (1.8 mg/kg soil Mn^{2+} and 9 mg/kg soil Fe^{2+}) and 50% soybean protein isolate and 50% dried molasses (1.1 mg/kg soil Mn^{2+} and 2.1 mg/kg soil Fe^{2+}) (Fig. 3.4). Additionally, Mn^{2+} represents 98% or more of the reduced metals in soil solution in all the sandy loam and silty clay treatments (Fig. 3.4). Likewise, treatments amended with 100%

dried molasses had the highest Fe^{2+} fraction in the reduced metals in soil solution, at 7% for unlimed and 5% for limed treatments (Fig. 3.5). However, differences between amended treatments were greater in sandy loam than in silty clay soils. Additionally, unamended soils, both limed and unlimed had the lowest Fe^{2+} and Mn^{2+} concentrations (~16-24 mg/ kg soil) while unlimed soils amended with mixtures containing 75 to 100% dried molasses had the greatest combined Fe^{2+} and Mn^{2+} concentrations (235 to 243 mg/kg soil) but there were no significant differences between the combined Fe^{2+} and Mn^{2+} concentrations in soils amended with mixtures containing 50% or more SPI and/or were limed (123 to 171 mg/ kg soil) (Fig. 3.5).

Analysis of Soil Texture and pH Influence of *Fo* suppression by VFAs and Reduced Metals

Overall, the proportion of clay in soil was significantly correlated with combined soil solution Fe^{2+} and Mn^{2+} concentrations ($r = 0.67$, $P < 0.001$) and the population of viable *Fo* ($r = 0.71$, $P < 0.001$) (Table 3.5). Likewise, soil pH was negatively correlated with soil solution reduced metal concentrations, largely dominated by manganese overall ($r = -0.23$, $P < 0.001$) as well as in all three soil textures tested. The relationship between soil pH, clay percentage, and total soil solution Fe and Mn concentrations in mg/kg soil is depicted in Fig. 3.6 and follows the equation:

$$\text{reduced metal concentrations} = 455 + 5.7 * (\% \text{ clay in soil}) - 56 * (\text{soil solution pH})$$

(equation 1)

Amendment protein concentrations were positively correlated with total soil solution VFA concentrations overall (Pearson correlation coefficient (r) = 0.20, $P < 0.01$) with the strongest correlation in sandy ($r = 0.31$, $P < 0.001$) and sandy loam soils ($r = 0.43$, $P < 0.001$) but with no correlation in silty clay soils (Table 3.5). The relationship between the proportion of protein in the amendment, soil clay percentage, and the combined total soil solution VFA and reduced metal concentrations (in mmol/kg soil) is depicted by Fig. 3.7 and follows the equation:

$$\text{Total VFA and reduced metal concentrations} = 12 + 0.1 * (\% \text{ protein in amendment}) + 0.3 * (\% \text{ clay in soil}) \quad (\text{equation 2})$$

Total soil solution VFA concentrations were negatively correlated with *Fo* CFU populations in silty clay soils ($r = -0.35$, $P < 0.01$) but not in coarser-textured soils (Table 3.3). The relationship between total soil solution VFA and reduced metal concentrations (mmol/kg soil solution), percent clay in soil and *Fo* propagule viability (in $\log((\text{CFUs} + 1)/\text{g soil})$) is depicted by Fig. 3.8 and follows the equation:

$$\# \text{ of viable } Fo \text{ propagules} = 1 - 0.005 * (\text{total VFA and reduced metal concentration}) + 0.07 * (\% \text{ clay in soil})$$

Discussion and Conclusions

The contribution of different organic amendments to *Fo* suppression was found to be determined by a variety of interactive factors, particularly amendment composition, soil texture/clay content, and the resulting balance of VFAs, Fe^{2+} and Mn^{2+} cations, and alkaline cations in soil solution. One of the most important factors determining the ability of a given ASD treatment to suppress *Fo* is soil texture/clay content. Unlike the quartz minerals that dominate the beach sand used in this experiment that has little reactive surface area for cation exchange, clay minerals have far higher reactive surfaces where various cations, including alkaline (i.e., K^+ , Mg^{2+} , and Ca^{2+}) and transition metal cations (i.e., $\text{Fe}^{3+}/\text{Fe}^{2+}$ and $\text{Mn}^{4+}/\text{Mn}^{2+}$) bind. Likewise, finer-textured soils are richer in Fe and Mn oxyhydroxides than sandy soils. Fe^{3+} and Mn^{4+} cations are sparingly soluble while their reduced counterparts, Fe^{2+} and Mn^{2+} , which form as a result of reductive processes associated with organic matter fermentation in soil as part of ASD, are highly soluble (Takai and Kamura, 1966; Finke et al. 2007; Momma et al. 2011).

Overall, both soil water alkaline cation and reduced metal cation concentrations were positively correlated with soil clay content. In turn, the higher level of alkaline cations in clay-enriched soils may counteract the suppressive effects of VFAs produced by organic matter fermentation, as dissociated VFAs bound to alkaline cations have greater difficulty crossing cellular barriers, such as the cell wall and membrane of *Fo* than undissociated VFAs (e.g., Momma et al. 2006). Therefore, while the soil solution of more clay-rich soils is enriched with *Fo*-suppressive Fe^{2+} and Mn^{2+} , the higher alkaline cation content means that a given total VFA concentration is less suppressive in comparison to coarser-textured soils. Likewise, the addition of CaCO_3 lime also

counteracted suppression of *Fo* by VFAs produced by organic matter fermentation, both in this study and in some previous studies (e.g., Meng et al. 2018). Additionally, soil liming may have reduced the concentration of Fe^{2+} and Mn^{2+} in some of the soil solutions by neutralizing some of the VFAs in solution and preventing them from reacting with and solubilizing these cations, another possible mechanism by which liming reduces ASD efficacy. However, even though *Fo* viability was high in all soils in terms of their ability to grow on Komada Medium, this does not mean that *Fo* germinates readily in all soil types tested. Past studies have demonstrated that overall, sandy soils are more conducive for *Fo* germination than soils containing more clay particles, particularly montmorillonites (e.g., Amir and Alabouvette 1993; Li et al. 2018). This is attributed to a combination of factors, including greater adsorption of root exudates and other nutrients necessary for inducing *Fo* germination to clay minerals and that finer-textured soils also tend to have more microbial life, which competes with *Fo* for nutrients, which also prevents their germination. Additionally, all of the strawberries were irrigated with tap water rather than irrigation water with added fertilizers so the nutrients in the sandy soils may have been leached out at much higher proportions compared to the sandy loam and silty clay soils, in turn contributing to the significantly reduced health of strawberries grown in sandy soils.

Besides soil texture and mineralogy, soil amendment composition also strongly influenced soil solution chemistry and with it, suppression of *Fo*. Amendments dominated by proteins (i.e., soybean protein isolate) were associated with greater soil solution VFA concentrations compared to carbohydrate-dominated (i.e., dried molasses) amendments, likely because of the greater ratio of carbon-to-oxygen of proteins compared to carbohydrates. This is also reflected

in other industrial fermentation studies where at low levels of chemical oxygen demand, sugar fermentation produces more VFAs such as acetic, butyric, and isovaleric acids than fermentation of carbohydrates while carbohydrate fermentation produces lactic acid in addition to VFAs (i.e., Trevisan et al. 2014; Liu et al. 2015). In turn, the greater rate of VFA production in soils amended with protein-dominated amendments translates to improved rates of *Fo* suppression, particularly in unlimed coarser-textured soils. Together with the influence of soil texture/mineralogy on ASD efficacy, the differences in effectiveness of protein-dominated vs. carbohydrate-dominated amendments illustrates the need to tailor the ASD method to be used based on not only the best amendment to be used based on ASD efficacy and the nutritional needs of subsequent crops but also local soil conditions. Likewise, ASD is best performed on unlimed soils to maximize its benefits while the use of protein-rich amendments can be a way to combine nitrogen fertilization with ASD.

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Appendix 2

Table 3.1 ASD amendment trials: the effect of protein to carbohydrate ratio and soil texture on VFA balance and disinfestation efficiency

Factor	Level
Amendment	6
1. Unamended control	
2. 100% soybean protein isolate	
3. 75% soybean protein isolate, 25% dried molasses	
4. 50% soybean protein isolate, 50% dried molasses	
5. 25% soybean protein isolate, 75% dried molasses	
6. 100% dried molasses	
Soil Texture	3
1. Sand	
2. Sandy loam	
3. silty clay	
Liming	2
1. no lime	
2. 0.1% w/w CaCO ₃ lime	
Factorial combinations	3
# of replications total (2 trials)	288

Table 3.2. Analysis of variance for the main effects and interaction effects influencing *Fusarium oxysporum* suppression in soil solution; amendment composition, soil texture, liming, and their interactions. NS = not significant, $P > 0.05$

Effect	<i>P</i> -values
Amendment	<.0001
Texture	<.0001
Lime	NS
Amendment × texture	0.0007
Amendment × lime	NS
Texture × lime	NS
Amendment × texture × lime	NS

Table 3.3. Analysis of variance for the main effects and interaction effects influencing total soil solution VFA concentrations; amendment composition, soil texture, liming, and their interactions. NS = not significant, $P > 0.05$

Effect	<i>P</i>-values
Amendment	<.0001
Texture	0.0033
Lime	NS
Amendment × texture	NS
Amendment × lime	NS
Texture × lime	NS
Amendment × texture × lime	NS

Table 3.4. Analysis of variance for the main effects and interaction effects influencing total soil solution Fe^{2+} and Mn^{2+} concentrations; amendment composition, soil texture, liming, and their interactions. NS = not significant, $P > 0.05$

Effect	<i>P</i>-values
Amendment	<.0001
Texture	<.0001
Lime	<.0001
Amendment × texture	<.0001
Amendment × lime	0.0095
Texture × lime	NS
Amendment × texture × lime	NS

Table 3.5. Pearson correlation coefficients showing the relationship between soil texture and amendment properties, soil biogeochemical properties, and the population of viable *Fusarium oxysporum* (*Fo*) propagules in soil fermentation experiments. Significant values are in bold and asterisks indicate **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Soil Parameters	Soil biogeochemical factors			
	Total VFAs (mmol/liter)	Total Fe ²⁺ /Mn ²⁺ (mg/liter)	Soil solution pH	<i>Fo</i> population (log((CFU+1)/g soil))
<i>All textures</i>				
Clay proportion (%)	0.14	0.67***	-0.036	0.71***
Amendment protein (%)	0.20**	-0.20**	0.23***	-0.071
Soil solution pH	0.087	-0.23***	N/A	-0.040
<i>Fo</i> population (log((CFU+1)/g soil))	0.039	0.22*	-0.040	N/A
<i>Sand</i>				
Amendment protein (%)	0.31***	-0.33**	0.026	-0.197
Soil solution pH	0.018	-0.28*	N/A	-0.037
<i>Fo</i> population (log((CFU+1)/g soil))	-0.018	0.055	-0.037	N/A
<i>Sandy loam</i>				
Amendment protein (%)	0.43***	-0.19	0.39***	0.070
Soil solution pH	0.20	-0.53***	N/A	0.15
<i>Fo</i> population (log((CFU+1)/g soil))	-0.11	-0.30**	0.15	N/A
<i>Silty clay</i>				
Amendment protein (%)	0.049	-0.19	0.64***	0.008
Soil solution pH	-0.12	-0.30**	N/A	-0.043
<i>Fo</i> population (log((CFU+1)/g soil))	-0.35**	-0.14	-0.043	N/A

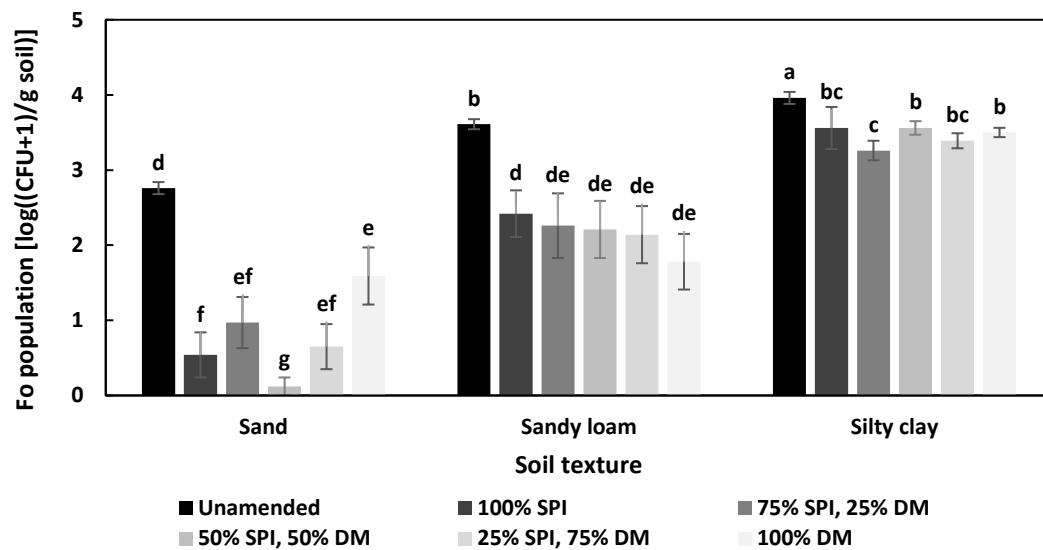


Figure 3.1. Effect of soil texture and amendment soybean protein isolate (SPI) and dried molasses (DM) proportions on suppression of *Fusarium oxysporum* (*Fo*) colony forming units (CFUs). CFU counts per treatment with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

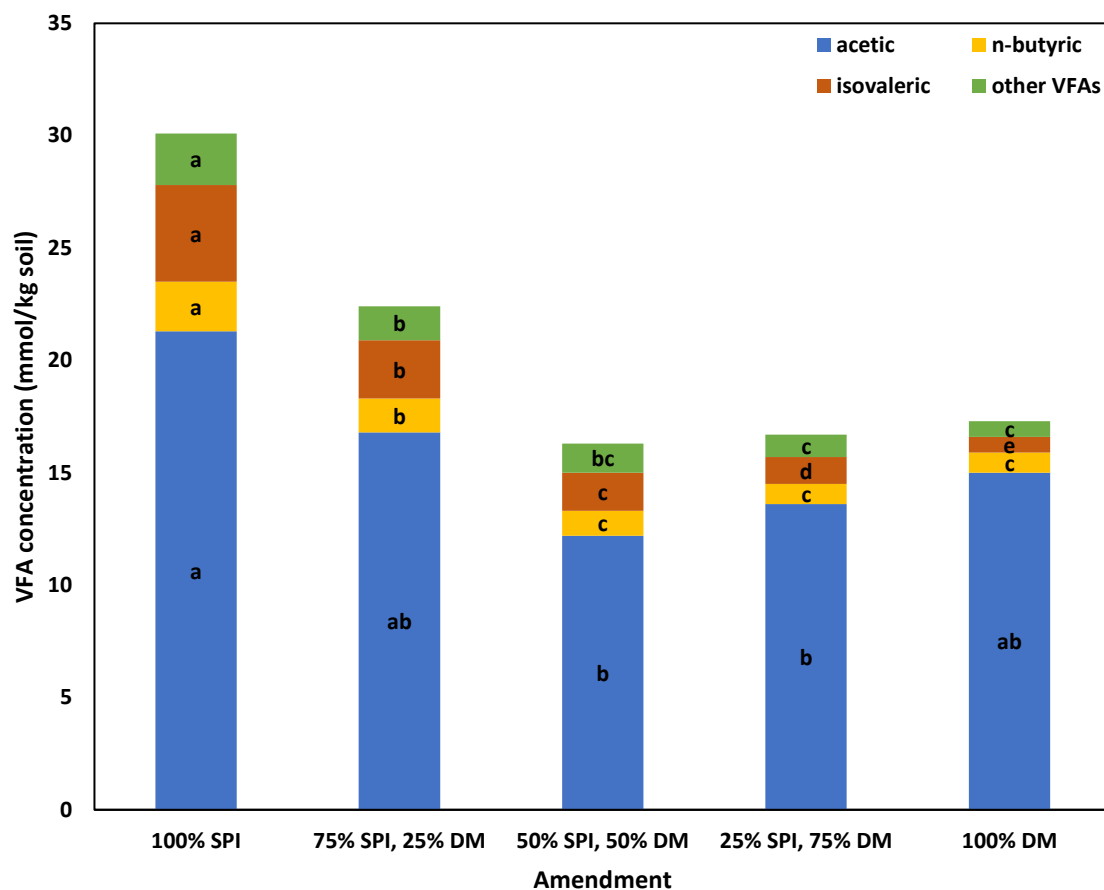


Figure 3.2. Effect of amendment soybean protein isolate (SPI) to dried molasses (DM) ratio on soil solution volatile fatty acid (VFA) concentrations. Total VFA concentrations per treatment with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

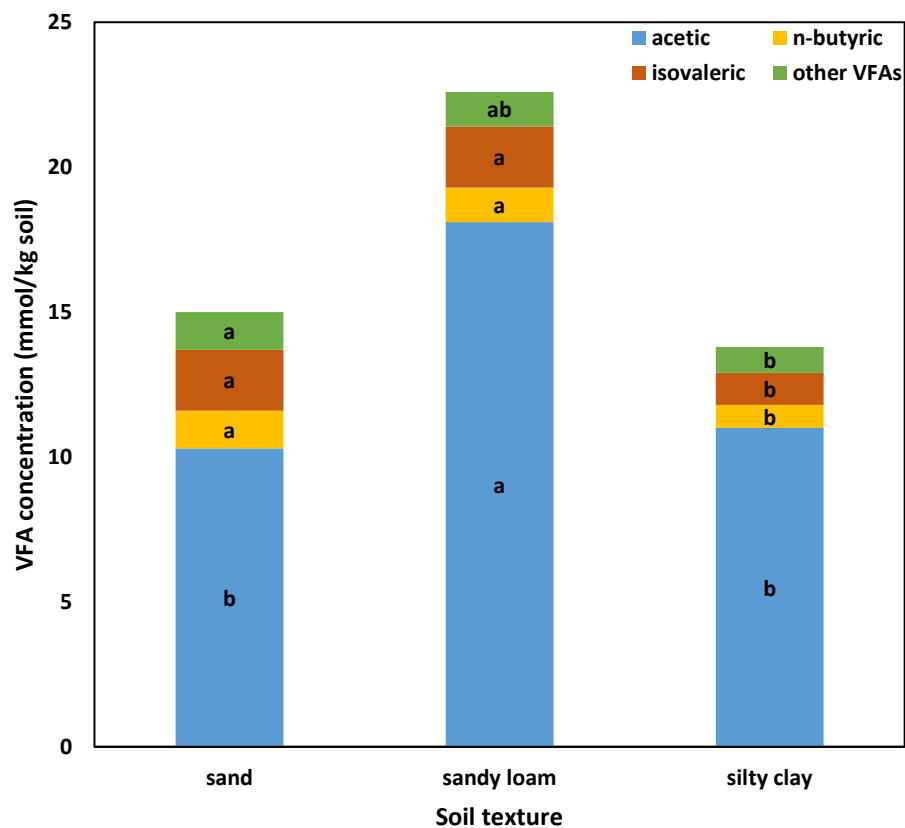


Figure 3.3. Effect of soil texture on soil solution volatile fatty acid concentrations in the soil solution. Total soil solution volatile fatty acid concentrations with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

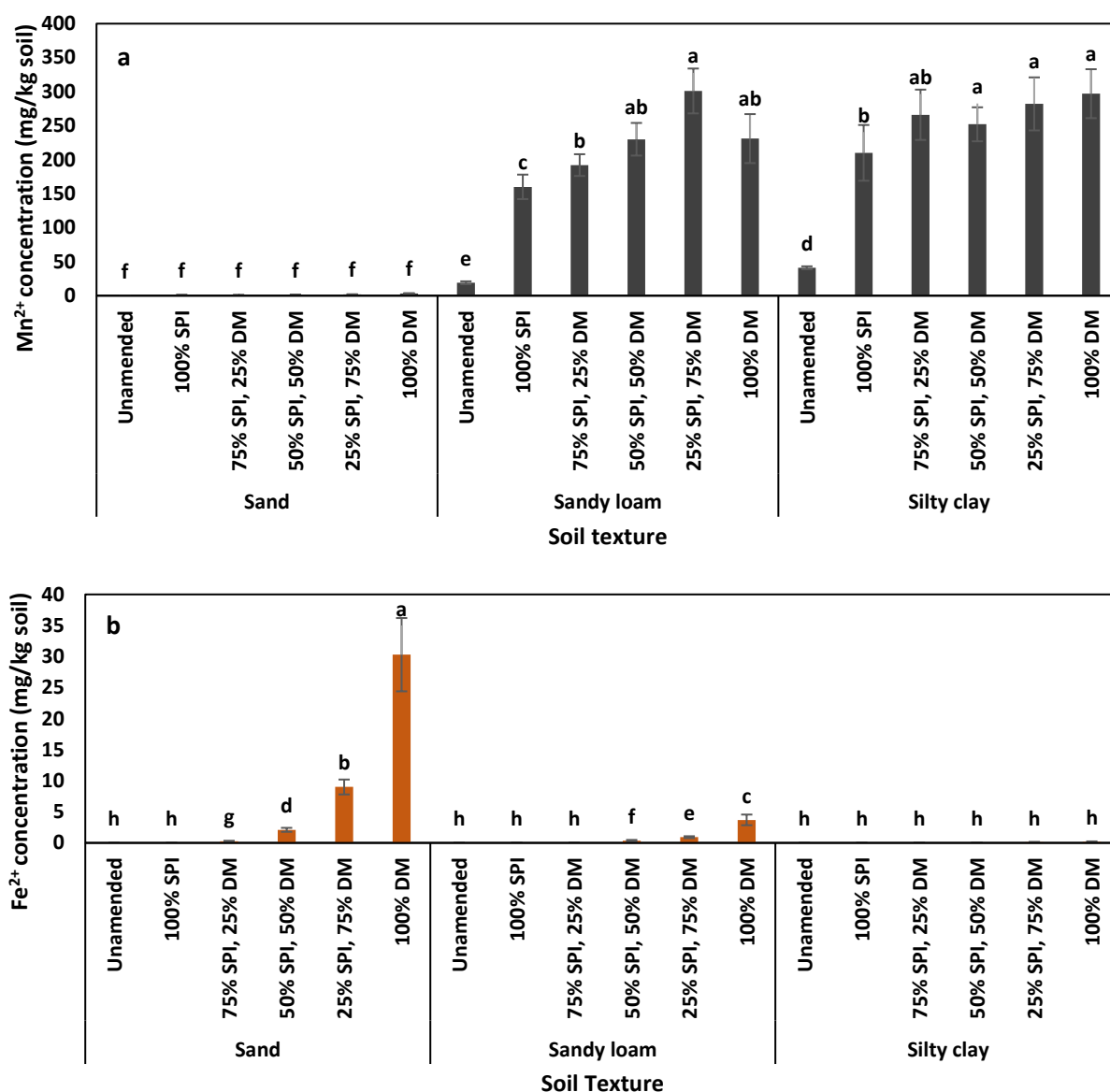


Figure 3.4. Effect of soil texture and amendment soybean protein isolate (SPI) percentage on soil solution Mn²⁺(a) and Fe²⁺(b) concentrations. Treatment concentrations (upper-case letters) with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

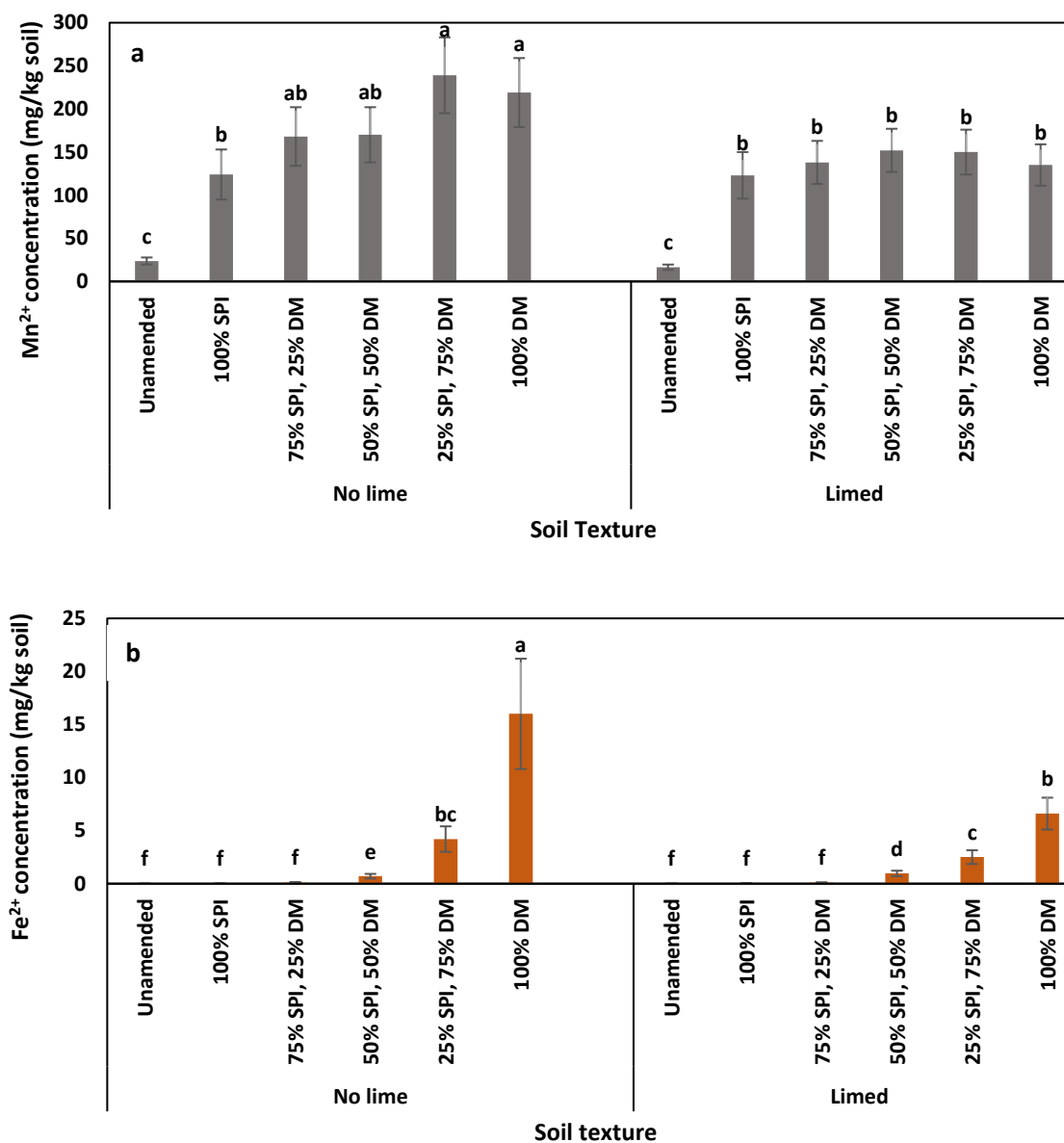


Figure 3.5. Effect of 0.1% CaCO₃ lime application and amendment soybean protein isolate (SPI) percentage on soil solution Mn²⁺(a) and Fe²⁺(b) concentrations. Treatment concentrations (upper-case letters) with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

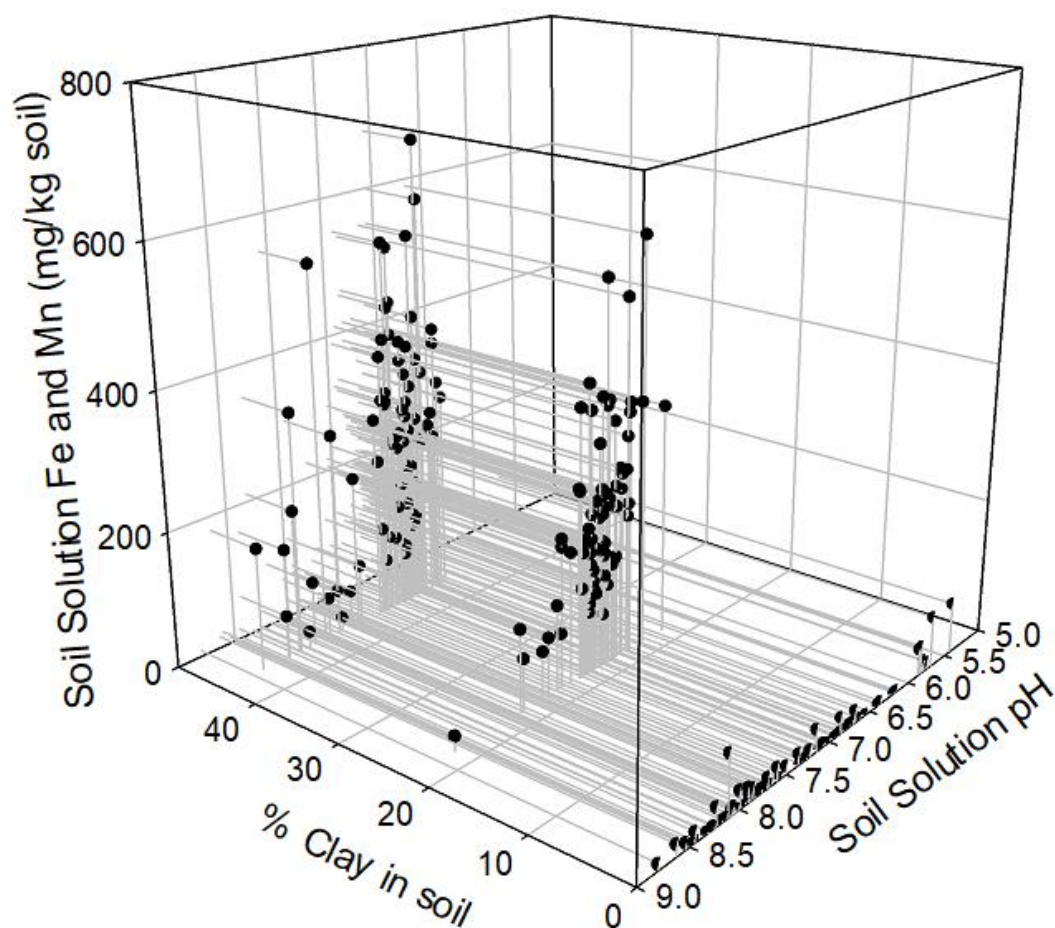


Figure 3.6. The effect of soil clay percentage and pH on the concentration of Mn²⁺ and Fe²⁺ in the soil solution.

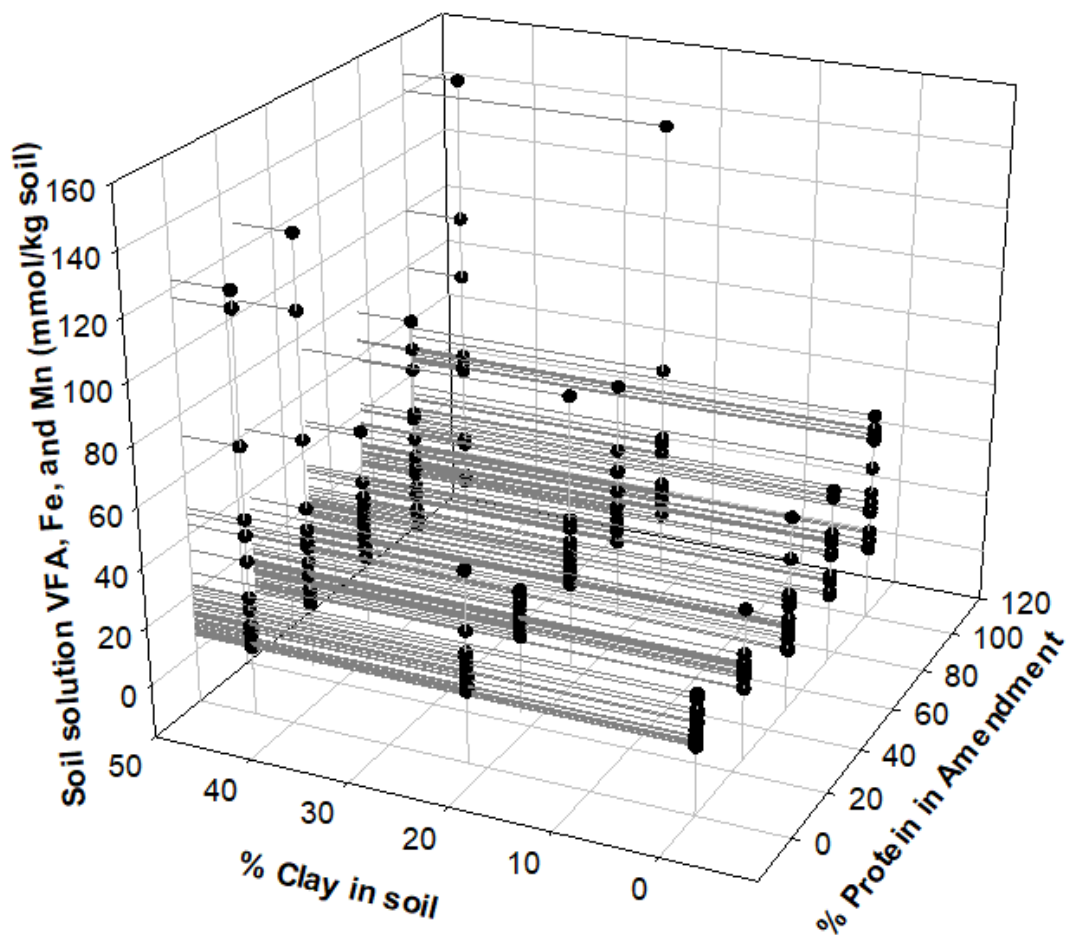


Figure 3.7. The effect of soil clay percentage and percentage of protein in amendment on combined volatile fatty acid (VFA) and reduced metal concentrations in the soil solution.

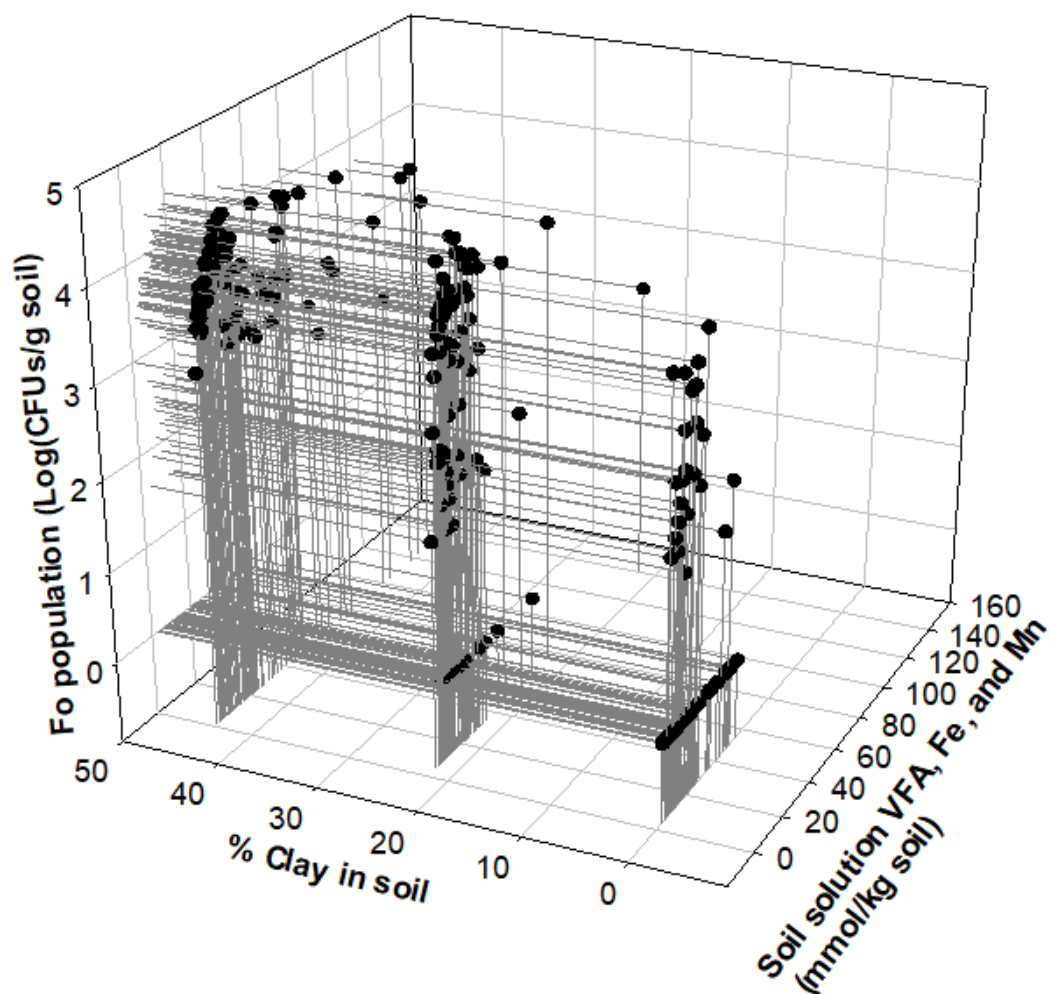


Figure 3.8. The effect of total combined soil solution volatile fatty acid (VFA) and reduced metal concentrations and soil clay percentage on the suppression of *Fusarium oxysporum* (*Fo*) colony forming units (CFUs).

Chapter 4: Amendment Composition, Soil Properties, and Plant Health in Strawberry Pot Studies

Abstract

Less hazardous alternatives to soil fumigants for suppressing soilborne pathogens such as *Fusarium oxysporum* (*Fo*), one causative agent of the strawberry black root rot complex, are urgently needed. A promising alternative is anaerobic soil disinfestation (ASD), in which soil is amended with labile organic materials, irrigated to field capacity, and tarped to induce anaerobic fermentation for a brief period before planting. ASD suppresses soilborne plant pathogens via the generation of reduced metal cations (Fe^{2+} , Mn^{2+}) and volatile fatty acids (VFAs, e.g., acetic, *n*-butyric, and isovaleric). However, little is known about how the interaction between soil texture, liming, or organic amendment composition influences not just *Fo* suppression but also the health of subsequent strawberry crops and root-associated fungal communities. The influence of amendment composition and soil texture on *Fo* suppression, crop health, and fungal community composition was studied in strawberry pot trials in a greenhouse, where dried molasses, wheat bran, spent brewer's yeast, and Azolla meal were used as amendments followed by planting each of the pots with strawberry plants and letting them grow for 9 weeks following ASD termination. Soil solution VFA concentrations and soil pH measurements were taken post-ASD. Additionally, roots, dried shoots, and fruits were weighed, and root samples set aside for fungal ITS2 amplicon sequencing to determine the effect of amendment type on root-associated fungal communities and how the abundance of key beneficial fungal clades (i.e., *Trichoderma spp.*, *Clonostachys rosea*, Agaricales, Glomeromycota, and Serendipitaceae) are correlated with overall plant health and how their abundance differs between treatments. Total soil solution VFA concentrations were negatively correlated with *Fo* colony forming unity (CFU) viability in sandy and sandy loam soils while amendment protein

concentration was positively correlated with soil solution VFA concentrations only in sandy soils. However, pH minimally influenced *Fo* suppression. In general, finer textures and limed soils were associated with lower *Fo* suppression. Moreover, strawberry plants grown in silty clay and sandy loam soils were significantly healthier than those grown in sand, particularly if amended with dried molasses or wheat bran. Additionally, greater abundance of agaricomycetes of the order Agaricales and family Serendipitaceae was positively associated with plant health, especially root and fruit biomass. In turn, dried molasses amendments were associated with the highest abundance of Agaricales and Serendipitaceae of any treatment. This highlights the importance of soil texture, soil pH and amendment composition in influencing ASD effectiveness and crop health via root-associated fungal communities.

Introduction

The overall goal of this study is to analyze the effect of amendment composition on *Fo* suppression and overall crop health as mediated by soil texture and fungal communities. This study was done following an investigation of the VFA balance resulting from the fermentation of protein (i.e., soybean protein isolate) and carbohydrate (i.e., dried molasses), both with and without CaCO₃ lime (Chapter 3 of this dissertation). In the present study, the relative effectiveness of three different ASD amendments (molasses + NPK, wheat bran, *Azolla* meal, and brewer's spent yeast), with or without lime applications for suppressing *Fusarium oxysporum* (*Fo*) and enhancing strawberry plant health were investigated, while accounting for soil chemical conditions in each treatment. Each of these amendments were selected due to their low cost (i.e., sugarcane molasses and brewer's spent yeast) or in the case of rapidly-

growing and nitrogen-fixing *Azolla*, its potential usefulness for combined on-farm nitrogen fixation, as well as ASD and fertilization in high-density organic strawberry or vegetable farms with minimal external inputs. Each of these amendments have significantly different concentrations of carbohydrates, proteins, Fe, and Mn, which may in turn influence disinfestation efficiency due to differences in VFA balance and Fe^{2+} and Mn^{2+} concentrations in their anaerobic decomposition byproducts. While Fe^{2+} and Mn^{2+} can be fungitoxic by themselves (Momma *et al.*, 2011), they also enhance the growth of butyric acid-producing bacteria (Liu *et al.*, 2015). In addition, amendments with lower C:N ratios have greater disinfestation efficiencies than those with higher C:N ratios both through inducing anerobic soil conditions faster and through greater production of fungitoxic VFAs, as high protein concentrations favors increased production of propionic, butyric, and isovaleric acids (Liu *et al.*, 2015; Esteban-Gutierrez *et. al.*, 2018). Finally, VFA balance and disinfestation efficiency are affected by soil solution pH as high soil pH reduces disinfestation efficiency (Meng *et al.*, 2018) Alkaline pH also enhances butyric acid production from carbohydrate-based feedstocks (Zhang *et al.*, 2009; Ai *et al.*, 2014).

The biochemical composition and therefore resulting VFA balance of the three ASD amendments to be studied differs greatly from one another. Brewer's yeast has the highest protein ($49\% \pm 8\%$ dry solids) concentrations of the three amendments to be tested (Heuze *et al.*, 2018). In contrast, sugarcane molasses is predominantly sugars (55 to 79% dry mass) and has the lowest protein (2.1 to 9.3% dry mass), Fe (123 to 277 mg kg^{-1} dry matter), and Mn (29 to 121 mg kg^{-1} dry matter) concentrations of all the amendments to be tested (Heuza *et al.*,

2015a). *Azolla* contains 13.9 to 28.1% protein per unit dry mass, 9.3 to 13.5% lignin per unit dry mass, 711 to 8,200 mg Fe kg⁻¹ dry mass, and 208 to 1,429 mg Mn kg⁻¹ dry mass (Tran, 2015). Wheat bran has a roughly similar nutritional profile, albeit with far less Fe and Mn at 14.1 to 20.5% protein per unit dry mass, 1.9 to 5.3% lignin per unit dry mass, 58 to 253 mg Fe kg⁻¹ dry mass, and 41 to 188 mg Mn kg⁻¹ dry mass (Heuze et al., 2015b).

While it is well-known that VFAs, particularly acetic, *n*-butyric, and isovaleric acid, play important roles in the suppression of soil fungal pathogens and that dissolved Fe²⁺ and Mn²⁺ also help suppress soil fungal pathogens, little is known about any interactive fungitoxic effects of dissolved Fe²⁺ and Mn²⁺ and VFAs. Additionally, little is known about how co-application of lime influences the effectiveness of ASD. While alkaline pH was shown to enhance production of *n*-butyric and isovaleric acids (Katase et al. 2009; Kuruti et al. 2017; Esteban-Gutierrez et al. 2018; Strazzera et al. 2018), which are more fungitoxic than acetic acid (Samaniego-Gaxiola and Balagurusamy 2013; Huang et al. 2015), we also know that VFAs have greater toxicity in their non-dissociated forms as found in acidic soils (Momma et al. 2006; Swilling et al. 2022). Additionally, it was found that organic substrate composition affects the type of VFAs produced from organic matter fermentation in the context of industrial fermentation. For instance, protein-rich substrates are associated with enhanced production of *n*-butyric and/or isovaleric acids while carbohydrate-dominated substrates are typically associated with high acetic acid production rates in the context of industrial fermentation (i.e., Mowlick et al. 2013; Liu et al. 2015; Esteban-Gutierrez et al. 2018). Likewise, work by Swilling et al. (2022) determined that higher soil clay content is associated with reduced suppression of *Agroathelia* (*Sclerotium*)

rolfsii sclerotial germination by VFAs, but little is known about how soil clay content influences suppression of *Fo* by VFAs and reduced metals. Therefore, more studies are needed on how organic substrate composition affects VFA production and balance in the context of ASD to help identify the best amendments to use for pathogen suppression and soil fertility enhancement, and how soil clay content influences *Fo*-suppressive effects of VFAs and reduced metals. As such, the objectives of this study are to determine which amendment combination is best for maximizing *Fo* suppression and to examine how soil texture influences the amount of VFAs, and reduced metals required to suppress *Fo* adequately for a soil with a given clay concentration.

Likewise, it is important to study the interactive effects of pH, VFA and dissolved Fe^{2+} and Mn^{2+} concentrations in suppressing *Fo* populations in the context of strawberry production to both gain an enhanced understanding of the mechanisms of ASD and to formulate the ideal ASD strategy for suppressing *Fo* infections of strawberries while enhancing strawberry yields. Besides assessing the impact of different lime/amendment combinations on *Fo* populations, the greenhouse studies will also include studies of *Trichoderma* and arbuscular mycorrhizal fungal abundance. Both play key roles in healthy agroecosystems, as *Trichoderma* parasitizes pathogenic soil fungi (e.g., Shrestha et al., 2018) while arbuscular mycorrhizal fungi enhance crop resilience and nutrient uptake efficiency (Douds and Millner 1999). The specific objectives and hypotheses are listed below:

Objective 1: Investigate the effects of substrate composition and lime application on soil VFA balance.

Hypothesis 1: Brewer's spent yeast will have the highest disinfestation efficiency as its high protein concentration would allow it to produce greater proportions of butyric and isovaleric acid than molasses while its high Fe concentrations would enhance the fungitoxicity of brewer's spent yeast amendments. However, CaCO₃ lime is expected to reduce disinfestation efficiency no matter the amendment by raising pH.

Objective 2: Investigate the effects of different amendments on soil quality (i.e., nutrient availability and beneficial microbes).

Hypothesis 2: *Azolla* + lime amendments will be the most beneficial to overall soil quality as *Azolla* is mostly highly labile but is also rich in nitrogen, trace metals, and lignin, which not only enriches the soil, but the high diversity of different biochemicals found in *Azolla* meal will be beneficial to the development of diverse fungal communities, including root-associated ones. Additionally, lime may stabilize pH in the ideal range for soil phosphate availability.

Objective 3: Investigate the effect of different amendments on plant health.

Hypothesis 3: Brewer's spent yeast amendments will maximize overall plant health and minimize *Fo* infection rates and severity due to the greater production of fungitoxic *n*-butyric and isovaleric acids compared to other amendments. Likewise, the high nitrogen and phosphorus concentration in spent brewer's yeast will be expected to benefit strawberry growth. However, *Azolla* amendments are also expected to benefit plant health due to its high trace metal and nitrogen concentrations.

Objective 4: Investigate the effects of different amendments on *Trichoderma* and arbuscular mycorrhizal fungal populations.

Hypothesis 4: *Azolla* amendments will be the most beneficial for *Trichoderma* and arbuscular mycorrhizal fungal population due to its high concentration of trace metals and greater diversity of biochemical substrate types compared to brewer's spent yeast and molasses.

Materials and Methods

Soil Preparation and Conditions

Soil was collected from the University of Tennessee Organic Crops Unit at the East Tennessee Research and Education Center (ETREC) in Knoxville, TN, USA. The soil was sieved (10-mm mesh) to remove large rocks and debris, then air-dried. The soil texture (silty clay; 44% clay, 38% silt, and 18% sand) was determined using the hydrometer method (Bouyoucos, 1962). For sandy loam soil (22% clay, 19% silt, and 59% sand), the silty clay was mixed 50/50 (by weight) with sand (particle sizes 62.5 to 1500 μm).

Inoculum Preparation

An isolate of pathogenic *Fusarium oxysporum* ('SC5', GenBank Accession No. ON338265) collected from strawberry roots on a strawberry farm in Tennessee was cultured in potato dextrose broth (PDB) for 7 days at 25°C with shaking. The mycelial mass grown in PDB was suspended in potato sucrose broth for 7 days, homogenized, and incubated for 3 weeks at 25°C and incubated in sterile soil water extract following a method by Momma et al. (2006) to obtain chlamydospores. The soil water extract was prepared by thoroughly mixing 100 g of soil and

100 ml of deionized water, autoclaving the mix for 60 min at 121°C, allowing the soil particles to settle, then passing the supernatant through a 0.44- μm filter with a vacuum (Dismic-25cs, Advantec, Tokyo, Japan). After the 3-week incubation period, the mycelial mat and soil water extract were homogenized in five 30-s blending sessions in a sterile blender and the numbers of chlamydospores and conidia in the resultant inoculum suspension were counted using a hemocytometer.

Strawberry Pot Studies

Pots (23-cm tall and 12-cm diameter) were filled with approximately 1 kg soil with a bulk density of $\sim 1.2 \text{ g cm}^{-3}$. The soils were silty clay, sandy loam (50% silty clay and 50% sand) and sandy soil prepared using a mixture of silty clay collected from the ETREC Organic Crops Unit and sand (Table 4.1). Each pot was inoculated with $\sim 25,000$ *Fo* chlamydospores/g soil and roughly 100X the amount of conidia using the inoculum suspension prepared in the previous step, planted with two strawberry seedlings, and fertilized with urea (control and molasses + N), brewer's spent yeast, or *Azolla* meal. For each soil texture type, there were eight treatments (four amendments and two lime treatments) with three replicates per treatment; no-lime (just N), limed (NPK + 1.0 g kg^{-1} soil CaCO_3 lime), dried molasses (NPK + 10 g C kg^{-1} soil molasses), dried molasses + lime (NPK + 10 g C kg^{-1} soil molasses + 1.0 g kg^{-1} soil CaCO_3 lime), brewer's spent yeast (10 g C kg^{-1} soil brewer's spent yeast), brewer's spent yeast + lime (10 g C kg^{-1} brewer's spent yeast + 1.0 g kg^{-1} soil CaCO_3 lime), *Azolla* meal (10 g C kg^{-1} soil *Azolla*), *Azolla* meal + CaCO_3 lime (10 g C kg^{-1} soil *Azolla* + 1.0 g kg^{-1} soil CaCO_3 lime), wheat bran (10 g C kg^{-1} soil wheat bran) and wheat bran + lime (10 g C kg^{-1} soil wheat bran + 1.0 g kg^{-1} soil CaCO_3 lime)

(Table 4.1). The macro- and macronutrient profile of the amendments are listed in (Table 4.2). Each pot was inoculated with ~25,000 chlamydospores/g of chlamydospores and roughly 100 times the amount of conidia from the chlamydospore-containing suspension, wetted to saturation, the soil covered with plastic tarp, and incubated at 25-30 °C for 14 days in the greenhouse. The trial was repeated.

Assessing root colonization by beneficial fungi

Root samples were taken from strawberry plants grown in unlimed silty clay soils, where overall crop health was considered optimal yet there were great variations between amendment type. Unlimed soils were chosen as liming prior to ASD is considered detrimental to ASD effectiveness in suppressing *Fo* and that the goal of this portion of the study was to analyze how ASD affects strawberry rhizosphere fungal communities. Roots were washed with tap water, frozen in liquid nitrogen, and ground to a powdery consistency with a mortar and pestle. DNA was extracted from 0.25 g of each sample using the DNeasy PowerSoil kit (Qiagen, USA) and stored at -80°C before analysis. The strawberry root DNA extracts were sent to Innomics (2904 Orchard Parkway San Jose, CA 95134) for ITS2 amplicon sequencing as ITS2 is highly specific for soil fungi for the purpose of this study after the removal of strawberry sequences. The data from the amplicon sequencing was then filtered, analyzed, and classified into Operational Taxonomic Units (OTUs) ranging from Kingdom- to Species- level using the mothur software (Schloss 2020) and the Unite ITS database (Nilsson et al. 2019). After the sequences were filtered, classified, and summarized as counts per OTU, special attention was paid to the abundance of counts from the genus *Fusarium* and specifically *Fusarium oxysporum* as well as to beneficial fungi such

as the mycoparasites of the species *Clonostachys rosea* and members of the genus *Trichoderma*, and beneficial mycorrhizal fungi from the phylum Glomeromycota and the order Agaricales and the family Serendipitaceae within the phylum Basidiomycota.

Data Analysis

The quantified *Fo* population data was $\log_{10}$ transformed before statistical analyses. Differences in soil VFA and reduced metal concentrations on one hand and the population of viable *Fo* CFU among treatments were compared using Fisher's LSD test at $P < 0.05$. A three-way ANOVA test evaluating the main effects of amendment composition, soil texture, presence or absence of CaCO_3 lime, and interaction effects as the main effects, and replicate as the random effect were performed to analyze the effects of VFAs and Mn^{2+} and Fe^{2+} in influencing disinfestation efficiency. Two trials were performed for both strawberry pot studies. The model was a completely randomized design, and the statistical analyses were performed using SAS 9.4 software. Additionally, correlation analysis modelling was performed to investigate links between amendment composition, soil texture, VFA and reduced metals in soil solution, and key values of interest (*Fo* suppression, abundance of key fungal clades, and strawberry root, shoot, and fruit biomass). To analyze the strength and direction of how soil solution VFA and reduced metal concentrations influence *Fo* CFU viability, a path and mediation analysis was conducted in R using the lavaan package (Rosseel 2012) and the PROCESS function (Hayes 2013). It was hypothesized that the influence of total VFA and reduced metal concentrations on *Fo* viability is mediated by soil clay concentrations and soil pH. In this model, total reduced Fe and Mn and VFA concentrations were summed up for each treatment after conversion and data

from all the soil fermentation and strawberry pot trials were pooled. Total soil solution VFA, Fe, and Mn concentrations were used as the exogenous (independent) variable, *Fo* populations as the endogenous (independent) variable, and soil solution pH and soil texture (i.e., percentage clay in soil) as the mediator variables. The model individually evaluates a series of linear structured equations and standardized parameter estimates were obtained. The model fit was assessed by a non-significant P (> 0.05) value and high scores (> 0.90) in Comparative Fit Index (CFI) and Tucker-Lewis Index (TLI). Standardized direct, indirect, and total effects of total VFAs, Fe, and Mn concentrations on *Fo* populations were also estimated. This was followed by performing 3D regression analysis in SAS 9.4 using the proc g3d and proc reg functions to generate a 3D scatter plot and regression equation to illustrate how the interactive effects of combined soil solution VFA and reduced metal concentrations and soil texture, particularly the percentage of clay in soil, affects the viability of *Fo* populations.

Results

Influence of Amendment Type and Soil Texture on *Fo* Suppression and Strawberry Health

Fo suppression in the strawberry pot studies were significantly influenced by the first-order effects of amendment type ($P < 0.0001$), soil texture ($P = 0.0001$), and liming ($P < 0.002$); the second-order interactive effects of amendment and texture ($P < 0.0001$) and amendment type and liming ($P = 0.0034$); and most importantly, the third-order interactive effects of amendment type, soil texture, and liming ($P = 0.0039$) (Table 4.3). *Fo* viability was generally higher in finer-textured sandy loam and silty clay soils than sandy soils, particularly unlimed sandy soils. In addition, differences in *Fo* suppression based on amendment type and whether

or not the soil was limed were minimal in fine-textured sandy loam and silty clay soils. In both limed and unlimed silty clay soils, *Fo* CFU counts ranged from 4.2 $\log_{10}(\text{CFU}+1)/\text{g}$ to 4.5 $\log_{10}(\text{CFU}+1)/\text{g}$ soil for soils amended with dried molasses, spent brewer's yeast, and wheat bran while for soils amended with *Azolla* meal or controls, both inoculated and uninoculated, *Fo* CFU counts ranged from 4.1 – 4.3 $\log_{10}(\text{CFU}+1)/\text{g}$ soil (Fig. 4.1). However, the effects of liming are greater in coarse-textured than in finer-textured soil. For instance, liming in sandy soils was associated with an increase in *Fo* CFU counts by 0.9 $\log_{10}(\text{CFU}+1)/\text{g}$ soil in sandy soil amended with wheat bran and by 2.1 $\log_{10}(\text{CFU}+1)/\text{g}$ soil in soils amended with *Azolla* meal (Fig. 4.1). On the other hand, liming was associated with a decrease in *Fo* CFUs by 0.7 $\log_{10}(\text{CFU}+1)/\text{g}$ soil in sandy soils amended with spent yeast. Additionally, the number of viable *Fo* CFUs in inoculated sandy loam and silty clay soils exceeded that of sandy soils by 2.7 to 3.6 $\log_{10}(\text{CFU}+1)/\text{g}$ soil while amended sandy soils, both limed and unlimed exhibited significantly higher rates of *Fo* viability compared to inoculated controls, ranging from an increase of 1.7 $\log_{10}(\text{CFU}+1)/\text{g}$ soil for *Azolla* meal to 3.1 $\log_{10}(\text{CFU}+1)/\text{g}$ soil for wheat bran (Fig. 4.1).

Soil solution VFA concentrations were significantly influenced by amendment type ($P < 0.0001$), soil texture ($P < 0.0001$) and the interactive effects of amendment type and soil texture ($P = 0.0021$) (Table 4.4). In general, soil solution VFA concentrations were higher in soils with greater sand content than soils with greater clay content with sandy soils amended with spent brewer's yeast (5.3 mmol/kg soil) or dried molasses (4.3 mmol/kg soil) along with sandy loam soils amended with spent brewer's yeast (4.11 mmol/kg) having the highest VFA concentrations (Fig. 4.2). In contrast, soil solution VFA concentrations were lowest in silty clay soils, where

average soil solution VFA concentrations for all amendment types was <1 mmol/kg soil with the pots amended with Azolla meal having the lowest VFA concentrations (0.1 mmol/kg soil) (Fig. 4.2). Soil solution Fe and Mn concentrations were significantly influenced by amendment type ($P = 0.0240$) and soil texture ($P < 0.001$) but not the interaction of these factors (Table 4.5). Soil solution Fe and Mn concentrations were highest in soils amended with dried molasses (8.2 mmol/kg soil) and lowest in soils amended with spent brewer's yeast (6.3 mmol/kg soil) (Fig. 4.3). Unlike with VFAs, greater soil clay content is associated with greater soil solution Fe and Mn concentrations as both silty clay (8.2 mg/kg soil) and sandy loam (8.7 mg/kg soil) had significantly higher soil solution Fe and Mn concentrations than sandy soils (5.2 mg/kg soil) (Fig. 4.4).

Strawberry fruit biomass was significantly influenced by soil texture ($P = 0.0031$), the second-order interactive effects of soil amendment and liming ($P = 0.044$) and the third-order interactive effects of amendment type, soil texture, and liming ($P = 0.025$) (Table 4.6). In general, average fruit yields were lowest in sandy soils (0.15 g - 2.8 g strawberries/ plant) except for those amended with spent yeast and lime (5.1 g strawberries/plant) (Fig. 4.5). Average strawberry fruit biomass was highest in limed sandy loam and silty clay soils amended with wheat bran, and unlimed inoculated controls in silty clay soils, with 8.5 g – 10.6 g fruit/plant (Fig. 4.5). This is in contrast to unlimed sandy loam and silty clay soils amended with wheat bran (1.0 g – 2.5 g fruit/plant) and limed inoculated controls in silty clay soil (4.0 g fruit/plant) (Fig. 4.5). However, while liming enhanced strawberry fruit biomass for plants grown in silty clay soil inoculated with *Fo* and amended with wheat bran, it reduced strawberry

fruit biomass for plants grown in silty clay soils amended with spent yeast from 6.9 g to 1.0 g fruit/plant (Fig. 4.5).

As with average strawberry fruit biomass per plant, the third-order interactive effects of amendment type, soil texture, and liming significantly influenced dried shoot biomass ($P = 0.0143$) (Table 4.7). Average dried shoot biomass values were the lowest for plants grown in sandy soils ranging from 0.13 g - 0.65 g/plant in limed sandy soils amended with dried molasses, spent yeast, or Azolla meal to 1.9 g/plant for limed inoculated controls (Fig. 4.6). Average dried shoot biomass was highest in plants grown in unlimed silty clay soils amended with dried molasses and unamended, limed inoculated controls (5.2 g - 5.7 g/plant) (Fig. 4.6). Additionally, liming increased average dried shoot biomass by 1.2 g/plant in sandy loam and by 1.7 g/plant in silty clay soils and likewise, by 1.1 g/plant in both sandy loam and silty clay soils amended with wheat bran (Fig. 4.6). However, liming was associated with reductions of average strawberry plant shoot biomass by 1.3 g/plant in silty clay soil amended with dried molasses, 2.0 g/plant for silty clay soils amended with spent yeast, and 2.3 g/plant for silty clay soils amended with *Azolla* meal (Fig. 4.6).

Strawberry fresh root biomass was significantly influenced by amendment type ($P = 0.0012$) and soil texture ($P < 0.0001$) but not by lime application or any of the interactive effects (Table 4.8). Average strawberry fresh root biomass was highest in unamended inoculated controls (~5.41 g/plant) and lowest in plants grown in soil amended with spent yeast (~2.58 g/plant) (Fig. 4.7). The average fresh root biomass of plants grown in soils amended with Azolla meal, wheat bran,

and dried molasses did not differ significantly from one another and ranged from ~3.51 g to 4.11 g/plant (Fig. 4.7). Additionally, strawberries grown in sandy loam soil had the highest average root masses (~5.08 g/plant) while those grown in sandy soils had the lowest (~2.53 g/plant) (Fig. 4.8). Those grown in silty clay soils had an intermediate average root mass of ~3.85 g/plant (Fig. 4.8).

Relations Between Soil Biogeochemical Factors and Strawberry Health

Overall, while the proportion of clay in soil was positively correlated with the population of viable *Fo* CFUs ($r = 0.18$, $P < 0.05$), it was also positively correlated with root biomass ($r = 0.25$, $P < 0.001$), shoot biomass ($r = 0.57$, $P < 0.0001$), and fruit biomass ($r = 0.19$, $P < 0.01$) (Table 4.9). In contrast, soil solution pH was negatively correlated with the population of viable *Fo* CFU ($r = -0.53$, $P = 0.001$), shoot biomass ($r = -0.31$, $P < 0.001$) and root biomass ($r = -0.16$, $P < 0.05$) (Table 4.9). Similarly, soil solution VFA concentration was negatively correlated with the population of viable *Fo* CFU ($r = -0.16$, $P < 0.05$) and shoot biomass ($r = -0.27$, $P < 0.001$), while total soil solution Fe and Mn concentration was positively correlated with the population of viable *Fo* CFU ($r = 0.15$, $P < 0.05$) (Table 4.9).

The overall path and mediation analysis model combining the data from this study and the Chapter 3 study on analyzing how amendment composition and soil texture influences *Fo* suppression via ASD explained 37% of the variability in *Fo* suppression by VFAs and reduced metals and identified significant suppression of *Fo* by soil solution VFAs and reduced metals via direct effects (path coefficient -0.26 , $P < 0.0001$) (Fig. 4.9). On the other hand, the indirect

influence of total soil solution VFA and reduced metals on *Fo* CFU viability as mediated by soil clay content (path coefficient 0.58, $P < 0.0001$) and pH (path coefficient 0.18, $P < 0.0001$) was significant and positive, indicating that higher soil clay content and pH counteracts the suppressive effects of VFAs and reduced metals (Fig. 4.9). Likewise, it was found via 3D regression analysis that both the total soil VFA and reduced metal concentrations ($P < 0.0001$) and the percentage by weight of clay in soil ($P < 0.0001$) significantly influenced the viability of *Fo* CFUs (Fig. 4.10). The overall 3D regression model has an R^2 value of 0.34. The relationship between total soil solution VFA and reduced metal concentrations (in mmol/L), weight percentage clay in soil, and viable *Fo* CFU populations (in log ((CFUs+1)/g soil)) is: depicted in Fig. 4.10 and follows the equation:

$$\text{Viable } Fo \text{ CFUs} = 2.3 + 0.048 * (\% \text{ clay in soil}) - 0.022 * (\text{total soil VFA and reduced metal concentrations})$$

Analysis of Root-Associated Fungal Communities and Their Relationship to Strawberry Health

Overall, the dominant root-associated fungal classes in all the samples are Sordariomycetes (20.4 to 43.1%), Leotiomyces (12.7 to 23.3%), Dothideomycetes (6.8 to 12.1%), and Eurotiomycetes (2.8 to 6.8%) from the phylum Ascomycota, and Agaricomycetes (1.8 to 6.0%) from the phylum Basidiomycota (Fig. 4.11). None of the strawberry plants grown in silty clay were diseased, regardless of the amendment added or whether or not it was inoculated with *Fo*. In all these samples *Fo* represented only 0.25 to 0.64% of the total ITS2 counts associated with the genus *Fusarium* and 0.003 to 0.023% of the total root-associated fungal counts (Table

4.10). Additionally, there was no significant correlation between the abundance of *Fo* and other members of the genus *Fusarium* and strawberry health, as indicated by root, shoot, and fruit biomass (Table 4.11). On the other hand, the abundance of many beneficial fungal clades was positively associated with plant health. While there was no significant correlation between any strawberry health measure and the total abundance of beneficial fungi of the phylum Glomeromycota and the species *Clonostachys rosea*, higher total abundance of members of the order Agaricales and family Serendipitaceae and genus *Trichoderma* were correlated with enhanced crop health. The abundance of *Trichoderma* spp. was positively correlated with fresh root biomass ($r = 0.726$, $P < 0.0001$) and dried shoot biomass ($r = 0.515$, $P < 0.05$), Agaricales abundance was positively correlated with fresh root biomass ($r = 0.769$, $P < 0.0001$) and dried shoot biomass ($r = 0.515$, $P < 0.01$), and Serendipitaceae abundance was positively correlated with fresh root biomass ($r = 0.423$, $P < 0.05$) and fruit biomass ($r = 0.512$, $P < 0.05$) (Table 4.11). Of these beneficial fungal clades found to be positively associated with strawberry health, only Serendipitaceae abundance was significantly influenced by differences in amendment (Table 4.11). The highest abundance of Serendipitaceae counts was found in strawberry roots from pots amended with dried molasses (~7,300 ITS2 OUT counts/g root tissue) and Azolla meal (~5,700 ITS2 counts/g root tissue) while strawberry roots from pots amended with spent yeast had the least abundance of Serendipitaceae counts (~700 ITS2 counts/g root tissue) (Fig. 4.12).

Discussion and Conclusions

The results of this experiment demonstrates the importance of both soil texture and soil fungal communities in influencing the health of strawberry crops in heavily-infested soils even if ASD

fails to kill a meaningful proportion of pathogens, in this case *Fo*. Overall, even though higher soil clay content was associated with greater *Fo* CFU viability, higher soil clay content was also associated with healthier plants. This may be due to a wide variety of reasons. Clay adsorbs not just the VFAs produced by ASD but also the extracellular enzymes and effector molecules utilized by soilborne plant pathogens to infect crop roots in addition to adsorbing root exudates that trigger the germination of pathogen resting spores, therefore restricting pathogen germination (Smith 1977; Alabouvette 1993; Li et al. 2018). Additionally, clay-enriched soils are also enriched in key micronutrients essential for crop health such as Fe, Mn, Mo, B, and Si compared to sandy soils (Siddiqui et al. 2016; Orr and Nelson 2018). Likewise, even though suppression of *Fo* by ASD was greater in sandy soils, owing in part to greater VFA production (Fig. 4.2), overall crop health, as demonstrated by vastly reduced root, shoot, and fruit biomass was also the worst in sandy soils (Fig. 4.5, 4.6, and 4.8). This may be in part because none of the strawberries were fertilized beyond the original ASD amendment and application of aqueous nutrients to the pots upon transplantation so the nutrients within the sandy soils was leached out when the pots were irrigated.

Competition between *Fo* and other fungi and the presence of beneficial fungi has also proven far more important than *Fo* suppression by VFA for safeguarding the health of strawberry plants grown in heavily infested soils. For instance, while *Fo* CFU viability levels were among the highest in all unlimed silty-clay soils, the strawberry plants growing in them were healthier than those grown in sandy soils, where the lack of nutrients may have stunted the growth of strawberry plants and likely made them more susceptible to fusarium wilt. However, in the

unlimed silty clay samples selected for amplicon sequencing, *Fo* represents only 0.008-0.02% of total root-associated fungal sequences and 0.25-0.64% of all root-associated fungi of the genus *Fusarium*, demonstrating that *Fo* was outcompeted by other fungi and prevented from causing disease in strawberry roots in those particular samples. Besides competition, the presence of beneficial fungi also benefited crop growth. These include *Trichoderma* spp., which were demonstrated in other studies to not just be mycoparasites vs. plant pathogenic fungi but also enhance crop phosphate availability as endophytes (i.e., Alabouvette et al. 2009; Devi et al., 2012; Saravanakumar et al. 2013); Agaricales, which includes many ectomycorrhizal species; and Serendipitaceae, which can live as saprophytes but can also form ectomycorrhizal relationships with a wide variety of host plants, including various trees, cereals, and fruit crops (i.e., Weiß et al. 2016; Cheng et al. 2020). In fact, even though both Serendipitaceae (ectomycorrhizae) and Glomeromycetes (arbuscular mycorrhizae) are present in the strawberry root microbiomes analyzed by amplicon sequencing, Serendipitaceae but not Glomeromycetes had significant positive impact on crop growth (Table 4.11). This may be because of the greater versatility of members of the family Serendipitaceae, which can live as saprophytes when they are unable to form mycorrhizal partnerships and can also be cultured on carbohydrate-based media like Potato Dextrose Agar, unlike Glomeromycetes, which cannot be cultured independently as they are largely obligate mycorrhizal symbionts (i.e., George et al. 1995; Gill et al. 2016; Weiß et al. 2016; Cheng et al. 2020). This may explain why Serendipitaceae were favored in pots amended with dried molasses or Azolla meal, which are carbohydrate-dominated amendments but not the protein-rich spent brewer's yeast treatments.

Lastly, it is very important to keep in mind the limits of ASD for treating soilborne crop diseases such as black root rot of strawberry caused by *Fo*. Past studies demonstrating the effectiveness of ASD against *Fo* typically utilized fresh hyphal biomass and/or conidia/bud cells (Blok et al. 2000; Butler et al. 2012; Hewawitharana et al. 2021), or infested field soil, which can be expected to contain a mixture of macro- and microconidia, and chlamydospores (e.g., Huang et al. 2015; Meng et al. 2018). However, the resistance of conidia and hyphal biomass to environmental stresses, including VFAs is significantly lower than that of chlamydospores (e.g., Smith 1977; Momma et al. 2006; Momma et al. 2010) and in turn, *Fo* chlamydospores have higher inoculum potential than conidia and hyphae (Cal et al. 1997). This may explain the relative lack of success of ASD in suppressing *Fo*, particularly in sandy loam and silty clay soils compared to past ASD experiments. Likewise, the fact that ASD, particularly with amendments rich in protein in addition to carbohydrates actually led to reduced strawberry crop health compared to inoculated controls, as shown by root, shoot, and fruit biomass may be because the amendments actually enhanced the germination of chlamydospores that survived ASD treatment. This is because *Fo* chlamydospores need to be induced to germinate by the presence of a combination of glucose, amino acids (i.e., asparagine), and dissolved Fe (Smith 1977; Peng et al. 1999; Were et al. 2023). This in turn is corroborated by studies indicating that increased soil solution Fe and Mn levels are associated with increased *Fo* disease severity in banana crops (Orr and Nelson 2018). Therefore, while ASD is often effective in treating *Fo*-infested soils, other disease management techniques are necessary as well to prevent the buildup of inoculant, particularly chlamydospores. For example, infected plants can be rouged when found and residues of infected crops should be removed from the field post-harvest to

minimize the chance of overwintering pathogens infecting future crops. Crop rotation can be used to prevent the buildup of the inoculum of *Fo* forma speciales specialized for a particular crop. Additionally, ensuring high root colonization levels by beneficial fungi such as members of the order Agaricales or family Serendipitaceae can protect crops from infection and help enable their healthy growth even in soils with high pathogen population densities.

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Appendix III

Table 4.1. ASD strawberry pot trials: the effect of amendment type, soil texture, and liming on plant health.

Factor	Level
Amendment	5
1. Unamended control	
2. Dried molasses	
3. Wheat Bran	
4. <i>Azolla</i> meal	
5. Spent brewer's yeast	
Soil Texture	3
1. Sand	
2. Sandy Loam	
3. Silty clay	
Liming	2
1. no lime	
2. 0.1% w/w CaCO ₃ lime	
Factorial combinations	3
# of replications total (2 trials)	240

Table 4.2. Macronutrient and micronutrient profile of the amendments utilized for the strawberry pot studies

Sample	Macronutrients (% dry biomass)							Micronutrients (ppm dry biomass)			
	Ca	K	Mg	P	C	N	S	Fe	Mn	Si	Zn
Dried Molasses	0.80	3.24	0.38	0.12	42	1.81	0.33	302.0	28.7	349.5	25.0
Wheat Bran	0.10	1.11	0.45	1.07	43.7	2.94	0.17	263.8	95.2	106.8	111.0
Spent Yeast	0.06	1.21	0.27	1.40	49.2	7.57	0.33	98.8	33.0	92.3	112.6
Azolla Meal	1.53	5.47	0.64	1.46	35.6	3.46	1.32	29016	325.8	321.3	3333.8

Table 4.3. Analysis of variance for the main effects and interaction effects influencing *Fusarium oxysporum* (Fo) colony forming unit (CFU) viability in strawberry pot studies; amendment composition, soil texture, liming, and their interactions. NS = not significant, $P > 0.05$.

Effect	<i>P</i>-values
Amendment	<.0001
Texture	<.0001
Lime	0.002
Amendment × texture	<.0001
Amendment × lime	0.0034
Texture × lime	NS
Amendment × texture × lime	0.0039

Table 4.4. Analysis of variance for the main effects and interaction effects influencing soil solution volatile fatty acid (VFA) concentrations in strawberry pot studies; amendment composition, soil texture, liming, and their interactions. NS = not significant, $P > 0.05$.

Effect	<i>P</i>-values
Amendment	<.0001
Texture	<.0001
Lime	NS
Amendment × texture	0.0021
Amendment × lime	NS
Texture × lime	NS
Amendment × texture × lime	NS

Table 4.5. Analysis of variance for the main effects and interaction effects influencing soil solution Fe and Mn concentrations in strawberry pot studies; amendment composition, soil texture, liming, and their interactions. NS = not significant, $P > 0.05$.

Effect	<i>P</i>-values
Amendment	0.0240
Texture	<.0001
Lime	NS
Amendment × texture	NS
Amendment × lime	NS
Texture × lime	NS
Amendment × texture × lime	NS

Table 4.6. Analysis of variance for the main effects and interaction effects influencing strawberry fruit biomass in strawberry pot studies; amendment composition, soil texture, liming, and their interactions. NS = not significant, $P > 0.05$.

Effect	<i>P</i>-values
Amendment	NS
Texture	0.0031
Lime	NS
Amendment × texture	NS
Amendment × lime	0.044
Texture × lime	NS
Amendment × texture × lime	0.025

Table 4.7. Analysis of variance for the main effects and interaction effects influencing strawberry dried shoot biomass in strawberry pot studies; amendment composition, soil texture, liming, and their interactions. NS = not significant, $P > 0.05$.

Effect	<i>P</i>-values
Amendment	<.0001
Texture	<.0001
Lime	NS
Amendment × texture	0.0109
Amendment × lime	0.0006
texture × lime	0.0271
Amendment × texture × lime	0.0143

Table 4.8. Analysis of variance for the main effects and interaction effects influencing strawberry fresh root biomass in strawberry pot studies; amendment composition, soil texture, liming, and their interactions. NS = not significant, $P > 0.05$.

Effect	<i>P</i>-values
Amendment	0.0012
Texture	<.0001
Lime	NS
Amendment × texture	NS
Amendment × lime	NS
texture × lime	NS
Amendment × texture × lime	NS

Table 4.9. Pearson correlation coefficients showing the relationship between soil biogeochemical properties and strawberry heath. Significant values are in bold and asterisks indicate * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Soil conditions	<i>Fo</i> population (log((CFU+1)/g soil))	Crop health parameters		
		dried shoot biomass (g)	root biomass (g)	fruit biomass (g)
Clay proportion (%)	0.18*	0.57***	0.25***	0.19**
pH	-0.53***	-0.31***	-0.16*	NS
<i>Fo</i> population (log((CFU+1)/g soil))	N/A	0.27***	NS	NS
Total VFAs (mmol/kg soil)	-0.16*	-0.27***	NS	NS
Total Fe ²⁺ /Mn ²⁺ (mg/kg soil)	0.15*	NS	NS	NS

Table 4.10. The proportion of *Fusarium oxysporum* (*Fo*) relative to total populations of all members of the genus *Fusarium* and the total root-associated fungal population for each amendment treatment.

Treatment	<i>Fo</i>: total <i>Fusarium</i> (%)	<i>Fo</i>: Total Fungi (%)
Dried Molasses	0.64	0.023
Wheat Bran	0.43	0.019
Spent Yeast	0.52	0.02
Azolla Meal	0.36	0.008
Uninoculated Control	0.46	0.015
Inoculated Control	0.25	0.003

Table 4.11. Pearson correlation coefficients showing the relationship between the abundance of key root-associated fungal clades of interest and strawberry health, as determined by root, shoot, and fruit biomass. Significant values are in bold and asterisks indicate * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Fungal clade of interest	Strawberry health measure		
	Fresh root biomass	Dry shoot biomass	Fruit biomass
Total <i>Fusarium</i>	-0.284	-0.334	-0.052
<i>Fusarium oxysporum</i>	-0.226	-0.24	-0.003
Glomeromycota	0.123	-0.005	-0.139
Agaricales	0.769***	0.525**	0.236
Serendipitaceae	0.423*	0.402	0.512*
Trichoderma	0.726***	0.515*	0.264
<i>Clonostachys rosea</i>	-0.079	-0.236	-0.149

Table 4.12. Analysis of variance for the effects of the abundance of beneficial fungal clades found to be positively associated with strawberry health and if their abundance is significantly affected by differences in amendment (i.e. dried molasses, wheat bran, spent yeast, or Azolla meal). NS = not significant, $P > 0.05$.

Beneficial fungal clade	<i>P</i>-value
Agaricales	NS
Serendipitaceae	0.0353
Trichoderma	NS

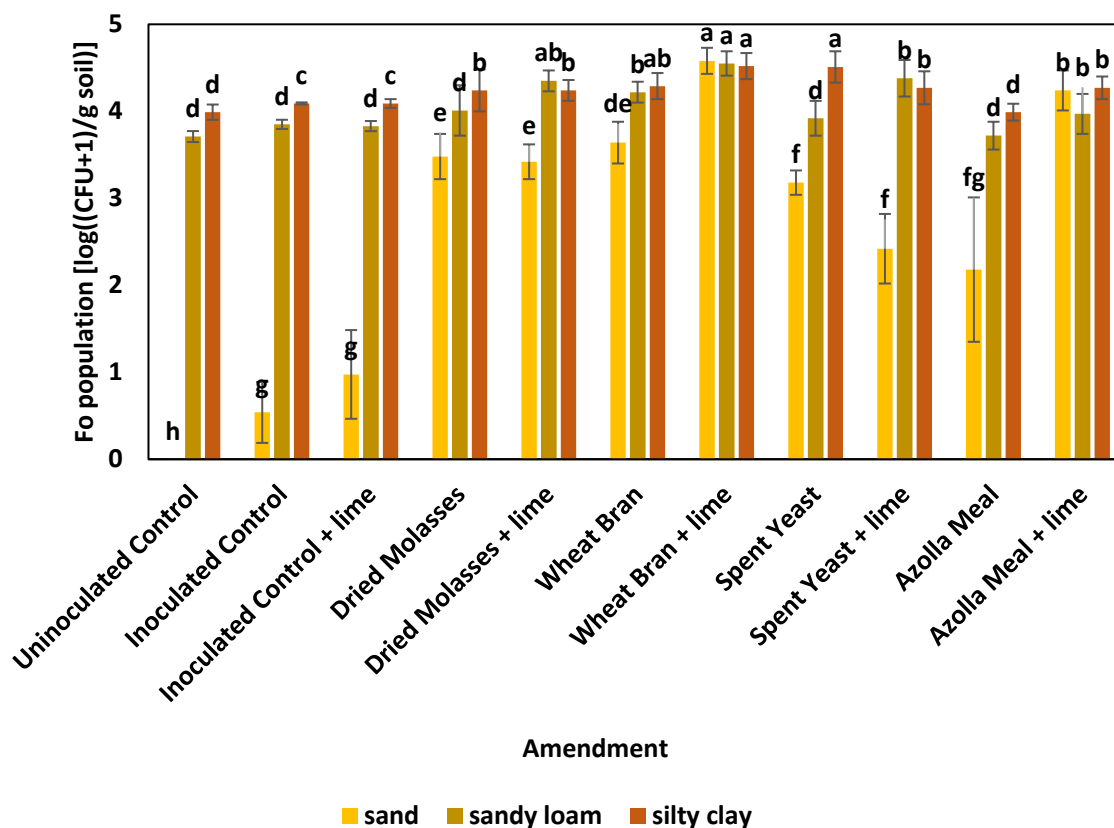


Figure 4.1. Effect of soil texture, liming, and amendment type on suppression of *Fusarium oxysporum* (*Fo*) colony forming units (CFUs) in strawberry pot studies. Amendments tested include none/inoculated control (IC), uninoculated control (UC), dried molasses (DM), wheat bran (WB), spent yeast (SY) and *Azolla meal* (AM). CFU counts per treatment with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

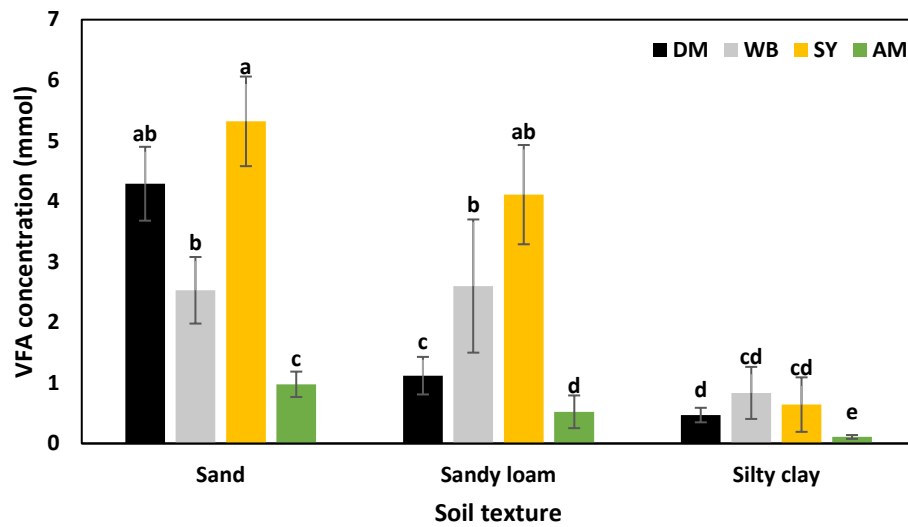


Figure 4.2. Effect of soil texture and amendment type on total VFA concentrations in strawberry pot studies. Amendments tested include dried molasses (DM), wheat bran (WB), spent yeast (SY) and *Azolla meal* (AM). VFA concentrations per treatment with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

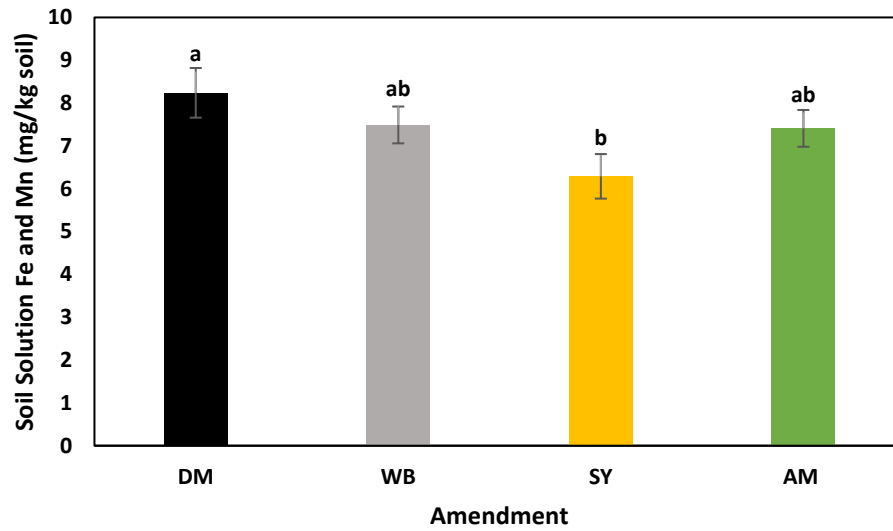


Figure 4.3. Effect of amendment type on soil solution Fe and Mn concentrations in strawberry pot studies. Amendments tested include dried molasses (DM), wheat bran (WB), spent yeast (SY) and *Azolla meal* (AM). Total soil solution Fe and Mn concentrations per treatment with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

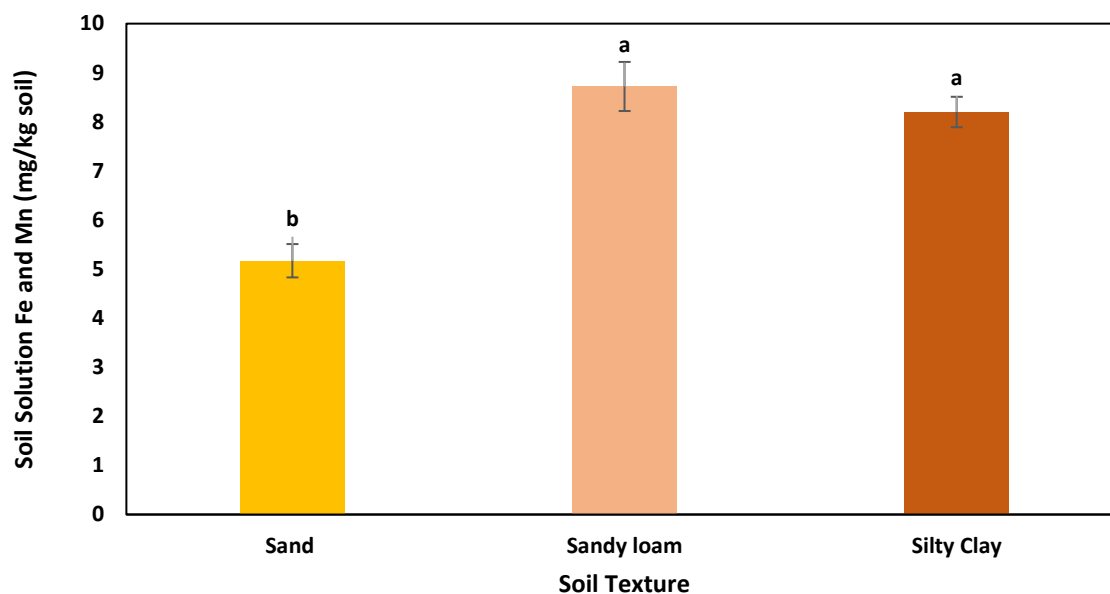


Figure 4.4. Effect of soil texture on soil solution Fe and Mn concentrations in strawberry pot studies. Amendments tested include dried molasses (DM), wheat bran (WB), spent yeast (SY) and *Azolla meal* (AM). Total soil solution Fe and Mn concentrations per treatment with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

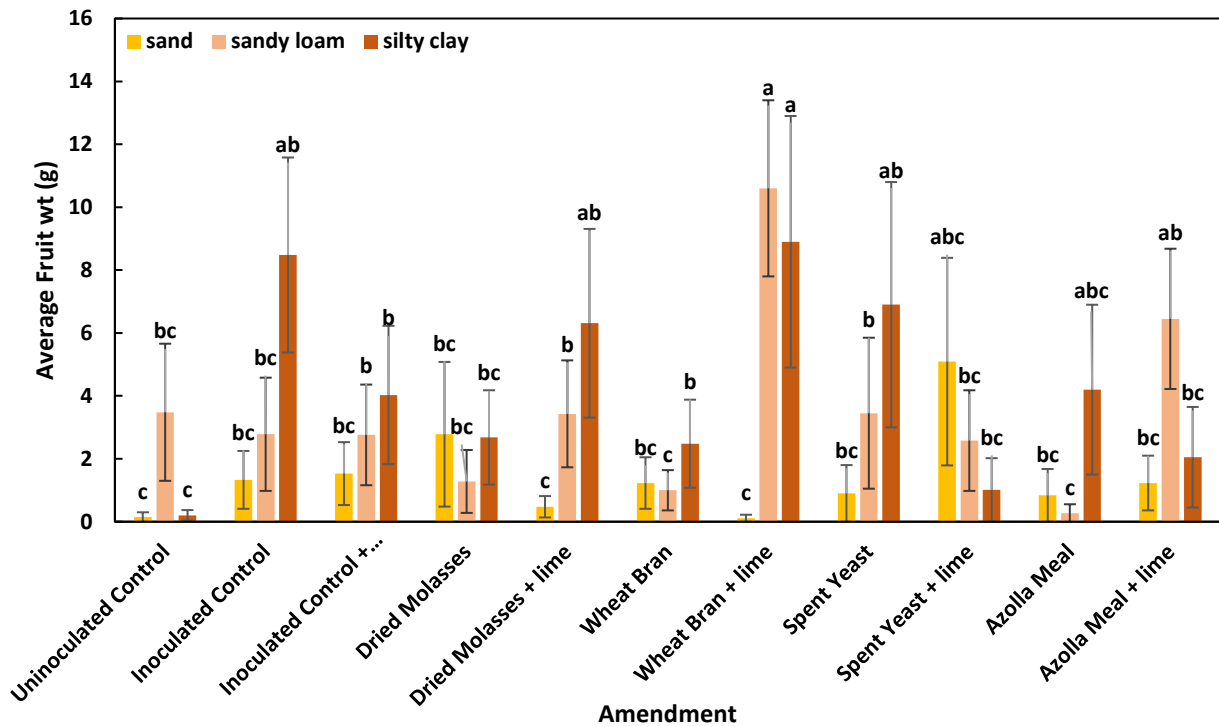


Figure 4.5. Effect of soil texture, liming and amendment type on strawberry fruit biomass in strawberry pot studies. Amendments tested include none/inoculated control (IC), uninoculated control (UC), dried molasses (DM), wheat bran (WB), spent yeast (SY) and *Azolla meal* (AM). Strawberry fruit biomass values per treatment with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

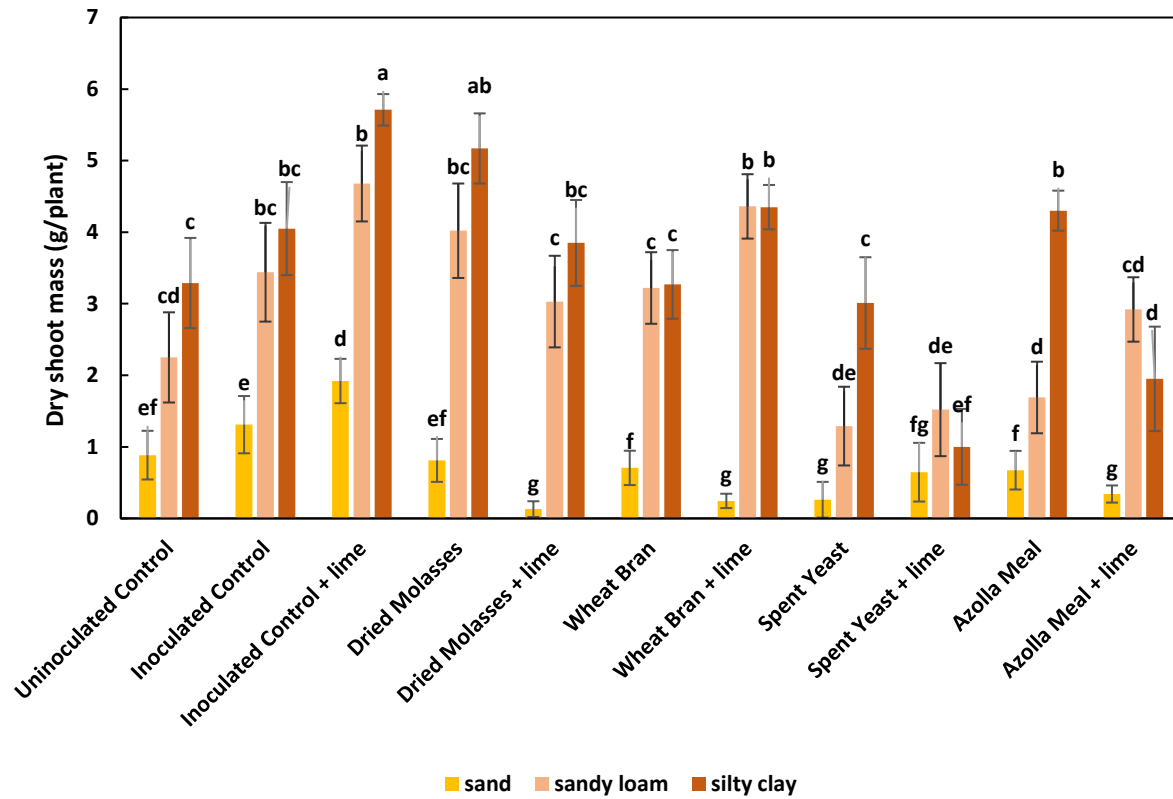


Figure 4.6. Effect of soil texture, liming and amendment type on strawberry dried shoot biomass in strawberry pot studies. Amendments tested include none/inoculated control (IC), uninoculated control (UC), dried molasses (DM), wheat bran (WB), spent yeast (SY) and *Azolla meal* (AM). Strawberry dried shoot biomass values per treatment with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

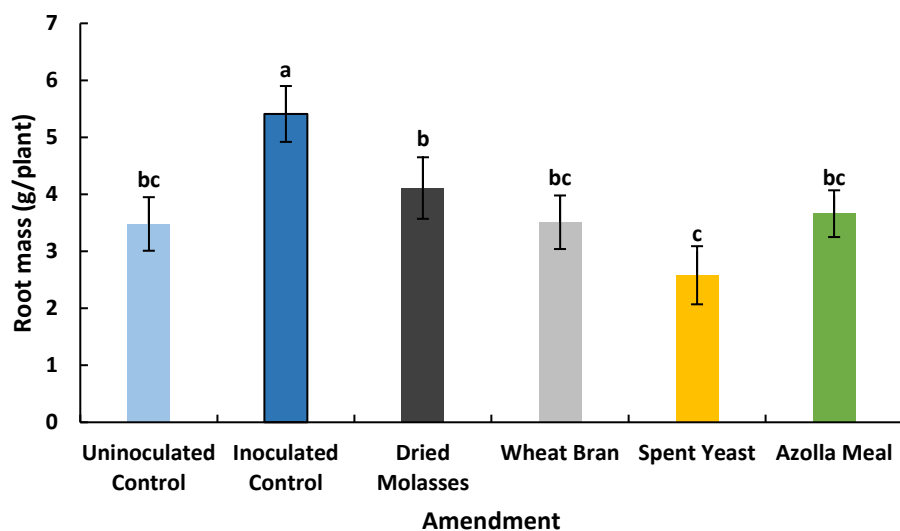


Figure 4.7. Effect of amendment type on strawberry fresh root biomass in strawberry pot studies. Amendments tested include none/inoculated control (IC), dried molasses (DM), wheat bran (WB), spent yeast (SY) and *Azolla meal* (AM). Strawberry dried shoot biomass values per treatment with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

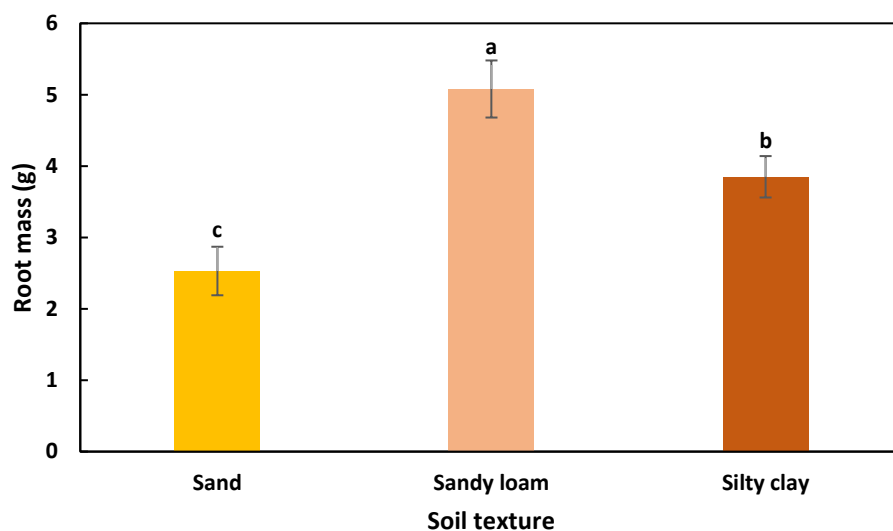


Figure 4.8. Effect of soil texture on strawberry fresh root biomass in strawberry pot studies. Amendments tested include none/inoculated control (IC), uninoculated control (UC), dried molasses (DM), wheat bran (WB), spent yeast (SY) and *Azolla meal* (AM). Strawberry dried shoot biomass values per treatment with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

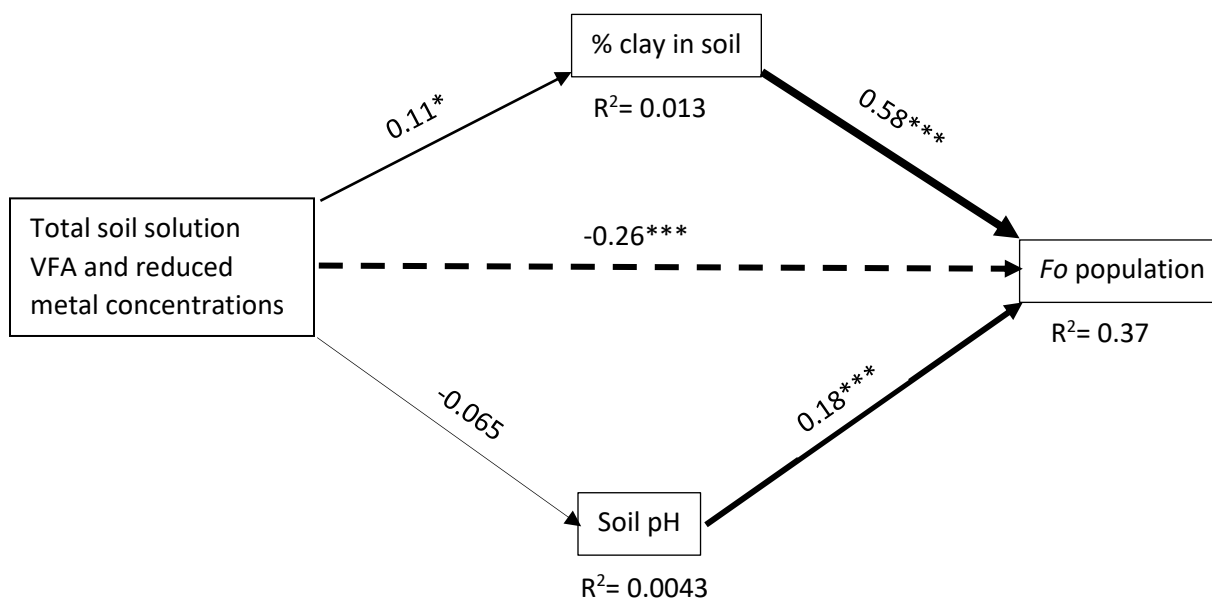


Figure 4.9. Soil physical and chemical properties influencing the suppression of *Fusarium oxysporum* (*Fo*) by volatile fatty acids (VFAs) and reduced metals. The path and mediation diagram represents the strength and direction of connections between combined soil solution VFA and reduced metal concentrations, soil clay concentration and pH, and *Fo* populations. The thickness of the lines represents the estimates of standardized path coefficients, with solid lines reflecting positive relationships and dashed lines negative relationships. Non-significant connections are shown in grey lines. The R² represents the model's explanatory powers for direct and indirect effects of soil solution VFA and reduced metal concentrations on *Fo* suppression. Significance of path coefficients are * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

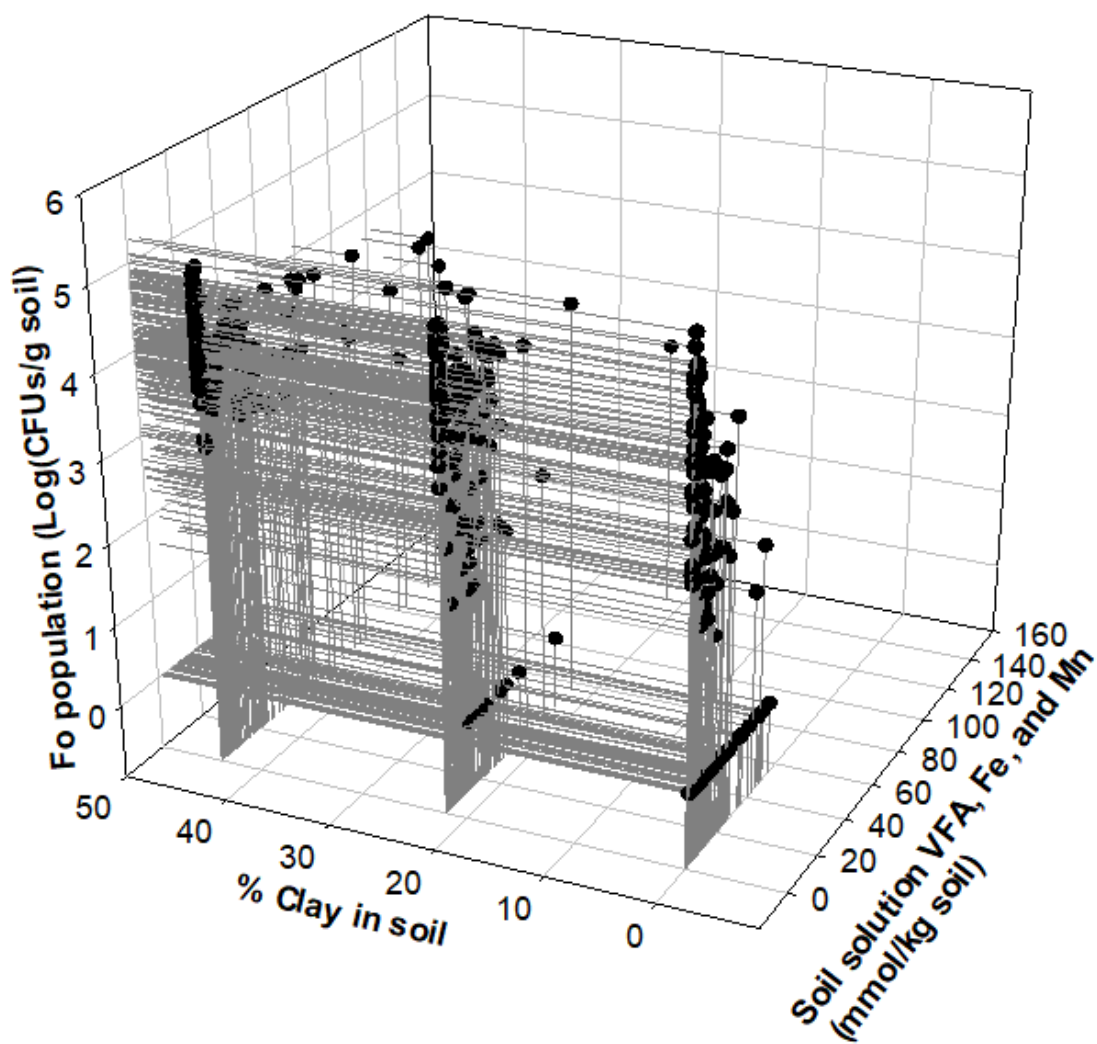


Figure 4.10. The effect of total combined soil solution volatile fatty acid (VFA) and reduced metal concentrations and soil clay percentage on the suppression of *Fusarium oxysporum* (*Fo*) colony forming units (CFUs) in both soil fermentation studies and strawberry pot ASD studies.

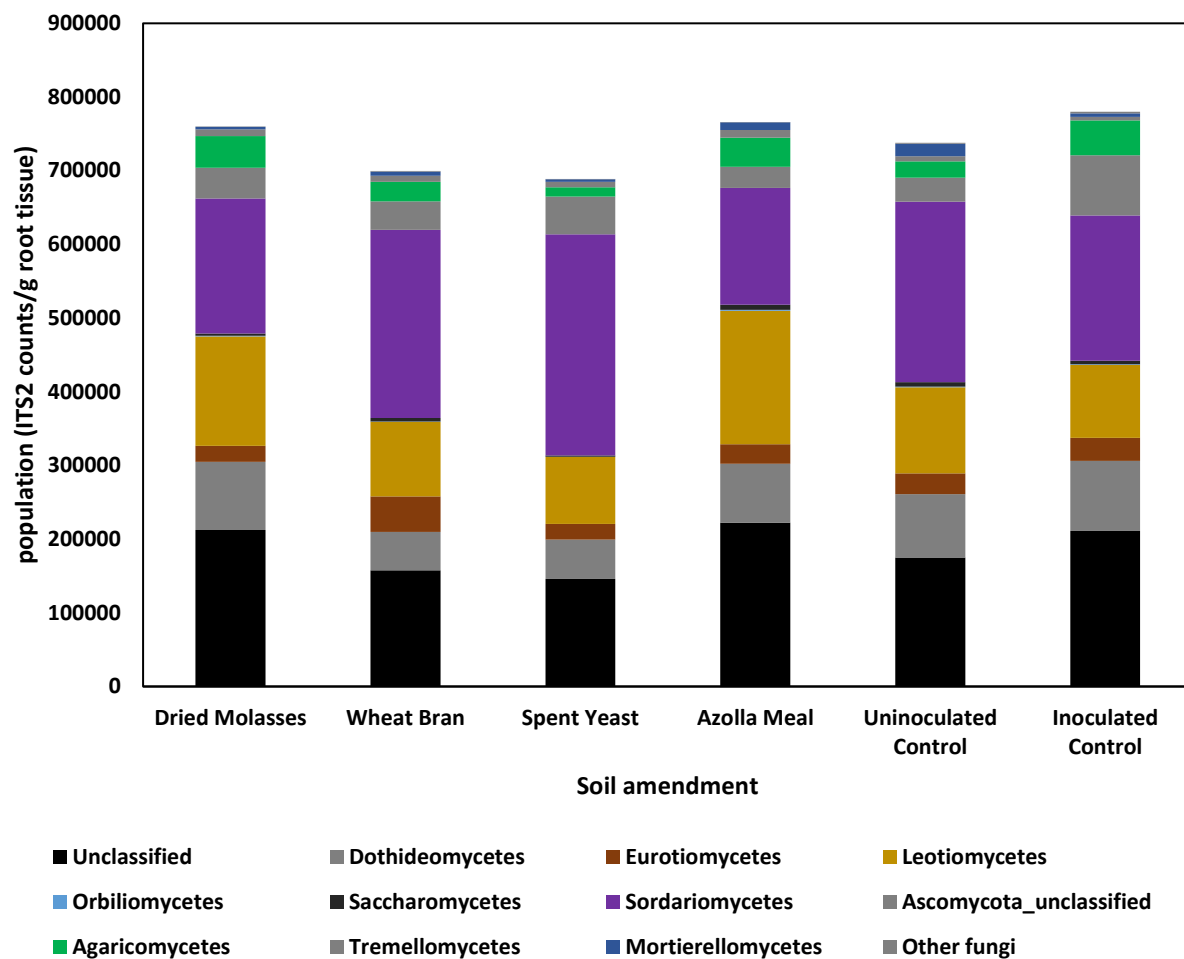


Figure 4.11. Root-associated fungal community composition of strawberry plants grown in unlimed silty clay soil at the class-level.

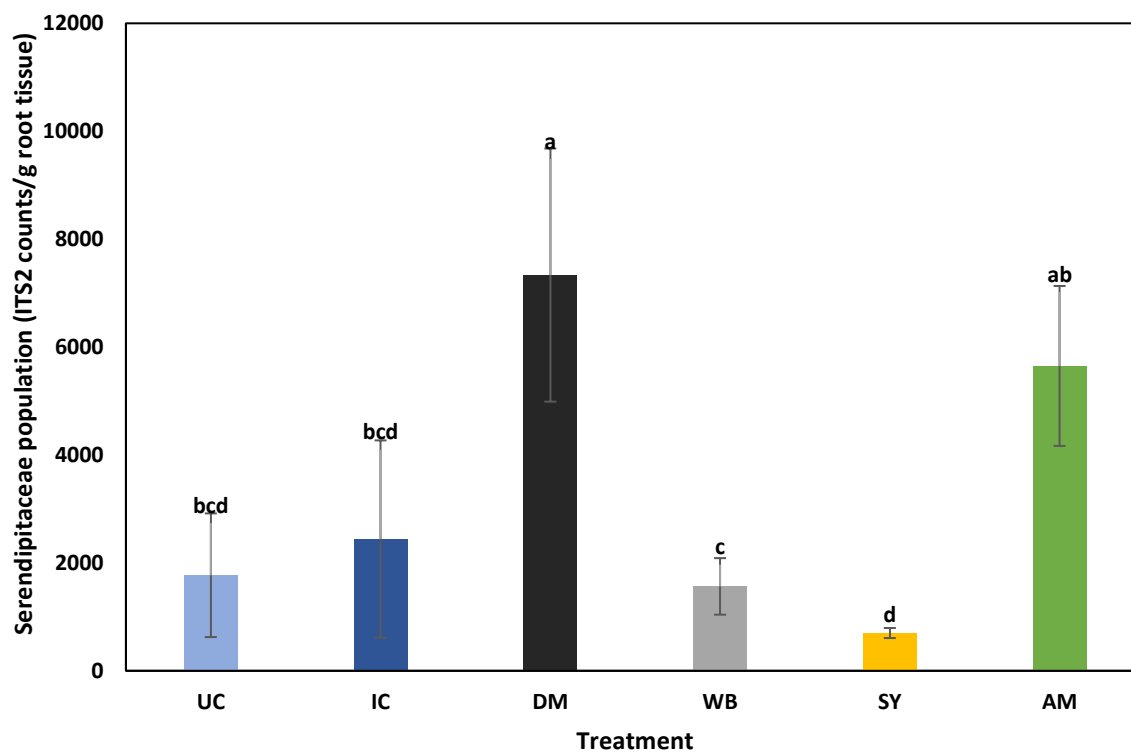


Figure 4.12. Effect of amendment type used for ASD on the abundance of Serendipitaceae associated with strawberry roots. Amendments tested include none/inoculated control (IC), uninoculated control (UC), dried molasses (DM), wheat bran (WB), spent yeast (SY) and *Azolla meal* (AM). Serendipitaceae ITS2 counts per treatment with the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

Summary

The ecotoxic and negative human health effects of soil fumigants and many other agrochemicals used to control crop diseases and pests makes the development of safe alternatives of paramount importance. This is particularly true for small fruits and vegetables, high value crops that are best grown in close proximity to consumer markets as they are prone to bruising and spoilage in transit/storage. As such, numerous alternatives to soil fumigation has been developed, including soil steaming, soil solarization, soil drenches involving organic acids, and biofumigation/anaerobic soil disinfestation (ASD). Each of these fumigation alternatives has benefits and limitations. For instance, both soil steaming and solarization are highly effective for all-around suppression of soilborne pathogens, pests, and weed seeds but steaming is very energy-intensive, largely limiting much of its use to greenhouses while solarization is mainly applicable in hot climates. Meanwhile, ASD is applicable in not just the tropics but also in warm temperate zones as long as water is plentiful. Together, these regions produce much of the world's fruits and vegetables.

Anaerobic soil disinfestation is a fumigation alternative that takes advantage of the pathogen-suppressive effects of organic fermentation by-products, particularly volatile fatty acids (VFAs) and reduced metal cations (i.e., Mn^{2+} and Fe^{2+}). In ASD, readily decomposable organic matter, usually various food/agricultural by-products like wheat bran, poultry litter, spent brewer's yeast, or ground up grasses are applied to soil, which is then tarped with impermeable plastic film and saturated to field capacity with water to favor anaerobic fermentation of the applied organic matter. However, because it is still unclear how soil texture and liming influence the

effectiveness of ASD, we conducted a series of studies to determine which VFA/reduced metal combinations are most effective for suppressing *Fusarium oxysporum* (*Fo*), a common causative agent of black root rot in strawberries and many other crops, then analyzed how soil clay content and liming affects ASD efficacy. We started with aqueous incubations of *Fo* inoculum in sterile aqueous media containing varying levels of different VFAs (acetic, *n*-butyric, or isovaleric acids) and Fe^{2+} -EDTA or Mn^{2+} -EDTA, followed by similar experiments done by saturating soils of different textures (i.e., silty clay, sandy loam, and sand), both with or without CaCO_3 lime added, with aqueous solutions containing VFAs and/or Fe^{2+} -EDTA or Mn^{2+} -EDTA. This was in turn followed by ASD experiments utilizing non-sterile soils, first involving amending soils of different textures (i.e., silty clay, sandy loam, and sand) both with and without CaCO_3 lime with organic amendments containing different ratios of protein in the form of soy protein isolate and carbohydrates in the form of dried molasses to determine how amendment composition influences the biogeochemistry of ASD and the efficacy of *Fo* suppression. Finally, ASD strawberry pot studies were performed to test how different ASD amendments (i.e., dried molasses, wheat bran, spent brewer's yeast, and Azolla meal), soil texture, and liming influences *Fo* suppression and overall strawberry health and productivity.

Overall, it was found that in addition to soil solution chemistry, soil texture and whether or not lime was added, significantly influenced *Fo* suppression and strawberry health, as demonstrated by root and shoot biomass along with fruit yields. In both aqueous media and soils, higher VFA and reduced metal concentrations were associated with enhanced suppression of *Fo*. However, while Fe^{2+} more effectively enhanced the suppression of *Fo* by

VFAs in aqueous incubations, Mn^{2+} more effectively enhanced *Fo* suppression by VFAs in soils. Additionally, it was found that increased soil clay content as well as liming counteracts the suppression of *Fo* by VFAs and reduced metals, with the effect of high clay content being especially strong. Additionally, it was found that the effectiveness of ASD may be more limited in settings that approximate real-life crop production conditions, such as the infested soils used in the strawberry pot studies, as maintaining perfectly anaerobic conditions necessary for the production of VFAs and reduced metals from organic matter fermentation can be very difficult to achieve. Under these circumstances, it was found that soil texture was the most important factor influencing strawberry health, as strawberries grown in sandy loam and silty clay fared far better than strawberries grown in sand, especially if they were from treatments amended with wheat bran. This was despite that these treatments exhibited high *Fo* populations. Moreover, it was found that the composition of root-associated fungal communities played a role as greater populations of ectomycorrhizal basidiomycetes, such as members of the order Agaricales and family Serendipitaceae, were associated with healthier crops, as indicated by their higher root, shoot, and fruit biomass in spite of the high initial population of *Fo*. Likewise, *Fo* populations were extremely low compared to even other *Fusarium* species in all strawberry roots from plants grown in silty clay, even in inoculated controls, illustrating the importance of competition with other microbial species in preventing *Fo* from infecting strawberry roots in heavily contaminated soils. Therefore, while ASD may not always be effective in killing *Fo* chlamydospores and/or conidia, especially if water availability is limiting or maintaining anaerobic condition in soil proves too difficult, soil drenches involving VFAs and/or solutions containing Fe^{2+} and/or Mn^{2+} may be another safe alternative to consider for reducing *Fo*

populations. Likewise, it would not be advisable to perform ASD or soil drenches involving VFAs/reduced metals before rather than after liming soil to maximize disinfestation efficacy. Additionally, it is crucial to maintain healthy soil microbial communities, especially ones that favor mycorrhizal fungi, which enhance crop nutrient access, stress tolerance, and protection against pathogen infection.

Vita

James Littrell was born in 1992 in Shady Grove, Maryland. He received his Bachelor of Science in Marine Science from Boston University. He then earned his Master of Science in Environmental and Soil Science with a focus on soil organic carbon cycling at the University of Tennessee. His Ph.D. work on the biogeochemical processes involved in anaerobic soil disinfestation is a collection of four chapters that are presented as journal publications. Additionally, he has presented his work in annual meetings of the Soil Science Society of America and the American Phytopathological Society and contributed research reports to the Southern Region Small Fruits Research Consortium and Sustainable Agriculture Research and Education Program.