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Title: Interactive effects of volatile fatty acids and Fe^{2+} and Mn^{2+} in suppressing *Fusarium oxysporum* implicated in black root rot complex of strawberry

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Public Abstract

Black root rot is a damaging disease complex in strawberry production, being responsible for reducing strawberry yields by 20-40% in affected fields. The pathogen *Fusarium oxysporum* (*Fo*) is one pathogen often implicated as a component of black root rot, and the pathogen can infect numerous other fruit and vegetable species. Soilborne pathogens such as those responsible for black root rot are normally managed by soil fumigation. While fumigation is effective for suppression of many soilborne pests (e.g., nematodes, pathogenic fungi, bacteria and oomycetes) in fruit and vegetable crops, synthetic chemical fumigants pose health and ecological dangers and as such require intensive safety training and certification for agricultural use. Therefore, alternatives to soil fumigation are needed. One such alternative is anaerobic soil disinfestation (ASD), in which soil is amended with labile organic materials, tarped, and saturated with water to induce anaerobic fermentation and with it production of fungicidal volatile fatty acids (VFAs) and reduced metals. To help better understand the mechanisms of ASD, *Fo* chlamydospores (survival structures) were incubated in aqueous solutions containing a VFA (i.e., formic, acetic, n-butyric, or isovaleric acid) and/or reduced metal [iron (Fe^{2+}) or manganese (Mn^{2+})]. These were followed by incubations of *Fo* spores in soils of three textures (sand, sandy loam, and silt loam) saturated with solutions containing a VFA (acetic, n-butyric, or isovaleric acid) and/or reduced metal (Fe^{2+} or Mn^{2+}), with both limed and unlimed replicates. Preliminarily, isovaleric acid was the most effective VFA for suppressing *Fo* in aqueous and soil-based media alike while reduced metals enhanced the ability of VFAs to suppress *Fo*. However, *Fo* suppression for a given concentration of VFAs and/or reduced metal cations was greatly reduced in limed and/or silt-loam soils.

Introduction

Anaerobic soil disinfestation is a promising alternative to soil fumigants for pre-plant disinfestation of soils in plasticulture strawberry production (Shennan *et al.*, 2017; Shrestha *et al*., 2016). Among the most important fungicidal compounds produced by the decomposition of organic matter amendments used in ASD are volatile fatty acids (VFAs) and we expect, reduced metals, particularly iron (Fe^{2+}) and manganese (Mn^{2+}). Volatile fatty acids are produced from the anaerobic decomposition of carbohydrates, proteins, and lipids found in organic amendments. VFAs are toxic to pathogenic soil fungi and nematodes likely because they diffuse across cell membranes in undissociated form and lower the cellular pH (Momma *et al*., 2006; Huang *et al*., 2015). However, deprotonated VFAs are less likely to diffuse across cell membranes than undissociated VFAs, and their disinfestation effectiveness is greater in acidic soil

solutions than in neutral or alkaline soil solutions (Oka 2010; McElderry *et al.,* 2005; Swilling *et al.,* 2021). The fungitoxicity of different VFAs also varies. VFA fungitoxicity toward *Fo* f. sp. *cubense,* as determined by 50 mM VFA applications to contaminated sandy soils, generally increased with greater molecular weight of VFAs evaluated (Huang *et al.,* 2015). Additionally, VFA fungitoxicity is affected by spore type, as *Fo* f. sp. *lycopersici* bud cells/conidia are easier to kill than chlamydospores (Momma *et al.,* 2006).

Besides VFAs, Fe^{2+} and Mn^{2+} ions can reduce viability of *Fo* bud cells and chlamydospores and suppress *Fo* growth in laboratory experiments (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 environments (Takai and Kamura, 1966; Finke *et al*., 2007). The fungitoxic effects of Fe^{2+} and Mn^{2+} ions are due to a combination of Fenton reactions, where the reduced metal cations react with peroxides to form reactive oxygen species (Touati, 2000) and displace the metals in the active sites of important metalloproteins (i.e. mismetallization) (Gerwien *et al.,* 2017). Additionally, acetic acid applications may solubilize soil Fe^{2+} and Mn^{2+} through formation of water-soluble iron and manganese acetates (Wildermuth *et al*., 2000; Reidies 2002).

While it is known that VFAs (especially acetic, butyric, and isovaleric acid) along with Fe^{2+} and Mn^{2+} ions suppress *Fo* in lab studies, little is known about the interactive fungitoxic effects of VFAs and Fe^{2+} and Mn^{2+} ions and how soil texture influences the fungitoxicity of these substances. Enhanced knowledge of the interactive effects of VFAs and reduced metal cations in suppressing *Fo* in different soil textures is important for developing models to explain the mechanisms by which ASD suppresses pathogenic fungi such as *Fo,* and for subsequent development of more targeted treatment recommendations for field applications of ASD in different soil environments.

Materials and Methods

Inoculum Preparation

Inoculum was prepared as according to Momma *et al.,* 2006. *Fusarium oxysporum* was grown on potato dextrose broth (PDB) for 7 days at 25 ^{o}C with shaking. To obtain chlamydospores, the mycelial mass grown in the PDB was suspended in potato sucrose broth (PSB) for seven days, transferred to sterilized soil water extract and incubated for three weeks at 25 ^{o}C. The soil water extract was prepared by thoroughly mixing 100 g of soil and 100 ml of distilled water, autoclaving the mix for 60 min at 121C, allowing the mixture to sit to settle the soil particles, then passing the supernatant through a set of 10 coffee filters to remove all soil particles but fine clay-sized soil particles. After the three-week incubation period, the mycelial mass and soil water extract were homogenized by five 30-second blending sessions using a Waring blender to obtain a chlamydospore-containing suspension and both chlamydospore and conidial density was determined using a haemocytometer.

Soil Conditions

Silt loam samples were collected from the University of Tennessee Organic Crops Unit at the East Tennessee Research and Education Center (ETREC), crushed and sieved through 10 mm mesh to remove large rocks and debris, then air-dried and the soil used for the experiment air-dried and sieved through 4 mm mesh. Some of the silt-loam was mixed with sand on a 50/50 w/w basis to make sandy loam. Three soil textures, sand, sandy loam, and silt loam were evaluated. Soil solution pH was measured after the

soil-based aqueous incubation experiments to assess if there is any relationship between soil solution pH and *Fo* suppression.

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

Chlamydospores were incubated in aqueous solutions containing $FeSO_4$ or $MnSO_4$ at 0, 0.01%, and 0.05% w/w along with 0, 5, and 10 mmol L^{-1} of a VFA (formic, acetic, propionic, n-butyric, or isovaleric acid) for two days, before transferring to media. 80 µl of chlamydospore suspension was added to 2400 µl of VFA/reduced metal solution after determining the chlamydospore density of the suspension using a haemocytometer. Viable propagules were grown in Nash-Snyder Media, prepared according to the instructions found in Nash and Snyder (1962), an agar medium which enhances *Fo* growth, for five days. for each reduced metal/VFA treatment. There were four replicates in each of two trials.

Table 1. Experimental Design for Fo Disinfestation Efficiency trials of VFAs and reduced Metals in Aqueous Media

Factor	**Level**
VFA Treatment	6
1. Water control	
2. Formic acid	
3. Acetic acid	
4. Propionic acid	
5. N-butyric acid	
6. Isovaleric acid	
VFA Concentration	2
1. 5 mmol L^{-1}	
2. 10 mmol L^{-1}	
Reduced metals	3
1. Fe^{2+}	
2. Mn^{2+}	
3. Control (water)	
Reduced metal concentration	2
0.01% w/w	
0.05% w/w	
Factorial combinations	4
# of replications total (2 trials)	288

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

To examine survival of *Fo* in soils amended with these voilatile fatty acids, 2 g dry soil of three textures; sand, sandy-loam, and silt-loam was twice-autoclaved and infested with 100 µl chlamydospore-containing suspension of *Fo*. The soil was treated with 1% w/w lime or had no lime added. 2 ml of volatile fatty acid (acetic acid, n-butyric acid, and isovaleric acid) with concentrations of 0, 10, and 20 mmol L^{-1} solutions with 0 or 0.05% w/w Mn(II)-EDTA or Fe(II)-EDTA were added to the infested soil at a rate sufficient to raise its water saturation level to field capacity (Table 2). While the original plan called for using formic and propionic acids as well, these were left out because formic acid is not a major product of organic

matter decomposition in soil and while propionic acid is one, its *Fo* suppressiveness was in preliminary work very similar to acetic acid, which is produced in substantially higher quantities from organic matter decomposition. Three replicates of the soil–acid—reduced metal mixtures were transferred to glass vials and incubated at 25°C and 28°C in an anaerobic chamber. After 5 days of incubation, viable population sizes of the pathogens were determined by the standard dilution method from Nishimura (2007).

Table 2. Experimental Design for Fo Disinfestation Efficiency trials of VFAs and Reduced Metals in Soil

Factor	**Level**
VFA Treatment	4
1. Water (control)	
2. Acetic acid	
3. N-butyric acid	
4. Isovaleric acid	
VFA Concentration	2
1. 10 mmol L^{-1}	
2. 20 mmol L^{-1}	
Liming	2
1. no lime	
2. 0.1% w/w dolomitic lime	
Reduced metals	3
1. Control (water)	
2. Fe^{2+}	
3. Mn^{2+}	
Reduced metal concentration	1
0.05% w/w soil	
Soil Texture	4
1. Silt Loam	
2. Sandy loam	
3. Sand	
Factorial combinations	4
# of replications total (2 trials)	388

Data Analysis

The quantified microbial population estimate data was $\log_{10}$ transformed before statistical analyses. Pooled data from trial 1 and trial 2 of the aqueous incubations of *Fo* in VFA solutions with Mn(II)-EDTA or Fe(II)-EDTA added was analyzed using a completely randomized design with factorials with the main factors being VFA type (acid) and concentration (acidconc) along with metal type (metal) and metal

concentration (metalconc). In addition, the water only, water + VFA, and water + metal controls were analyzed by a simple completely randomized design since they do not fit well in a completely randomized design model with factorials.

In the soil incubations, the differences in soil physicochemical (Mn^{2+} and Fe^{2+}) and microbial properties among treatments was 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 CEC as the main effect and trial as the random effect was performed to analyze the effects of VFA's, Mn^{2+} and Fe^{2+}, and soil texture in influencing disinfestation efficiency. The model used was a completely randomized design and the statistical analyses performed using SAS 9.4 software.

Preliminary Results

Aqueous Incubations

VFA type and concentration, metal type and concentration, and the three-way interactive effect between VFA, metal type, and metal concentration all significantly influenced *Fo* suppression (Table 3). In addition, the water only, water + VFA, and water + metal controls were analyzed by a simple completely randomized design since they do not conform to the factorials in the completely randomized design model. Overall, higher VFA and/or Mn^{2+} and Fe^{2+}concentrations are associated with higher *Fo* suppressiveness. Additionally, Fe^{2+} was more suppressive than Mn^{2+} at a given concentration. Of the VFAs, formic and acetic acid were the least suppressive of *Fo* on their own while isovaleric acid was the most suppressive. At the same time, all solutions containing a VFA and 0.05% w/w Fe^{2+} nearly eradicated all *Fo* colony forming units, including chlamydospores. VFA data from both concentrations (5 mmol and 10 mmol) are presented in Fig. 1.

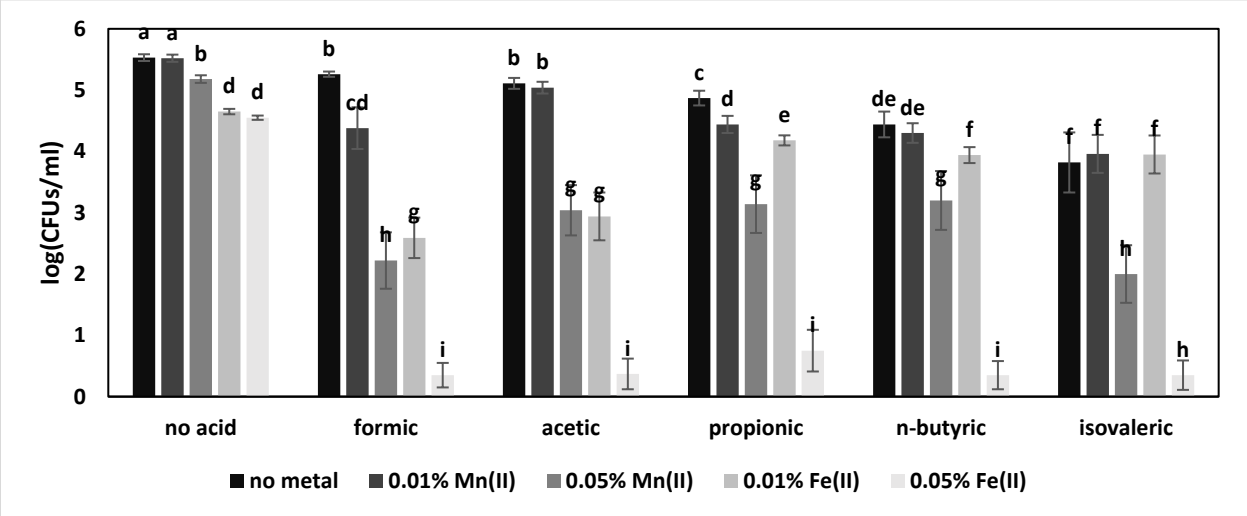

Fig. 1. Effect of volatile fatty acid and reduced metal type and concentration on the viability of *Fusarium oxysporum* colony-forming units (CFU's)

Soil Incubations

Though their effects were heavily diminished compared to in aqueous media, VFAs and reduced metals suppressed *Fo* in soils and of the VFAs, isovaleric acid was the most suppressive of *Fo*. For example, isovaleric acid in combination with Fe^{2+} and Mn^{2+} ions suppressed *Fo* spore viability by over 90% (Fig.

2), especially in sand (Fig. 3). However, both lime application and increased clay content is associated with reduced disinfestation efficacy for a given amendment (Fig. 2), likely due to adsorptive interactions with and/or pH buffering by lime and clay minerals.

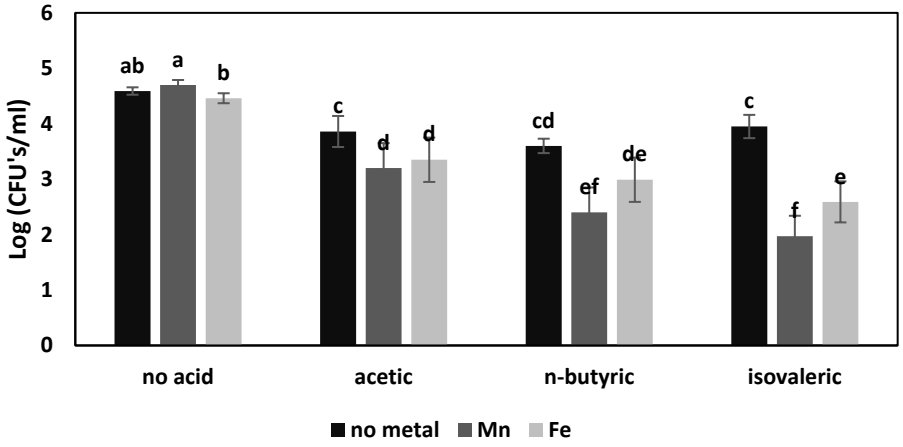

Fig. 2: Influence of VFA type and reduced metal type on *Fusarium oxysporum* chlamydospore suppression. Results are from data from both trials with involving aqueous solutions of 20 mM VFA concentrations on all soil texture types (sand, sandy loam, and silt loam) Reduced metal cations were added at a level of 0.05% w/w in aqueous solutions applied to soil.

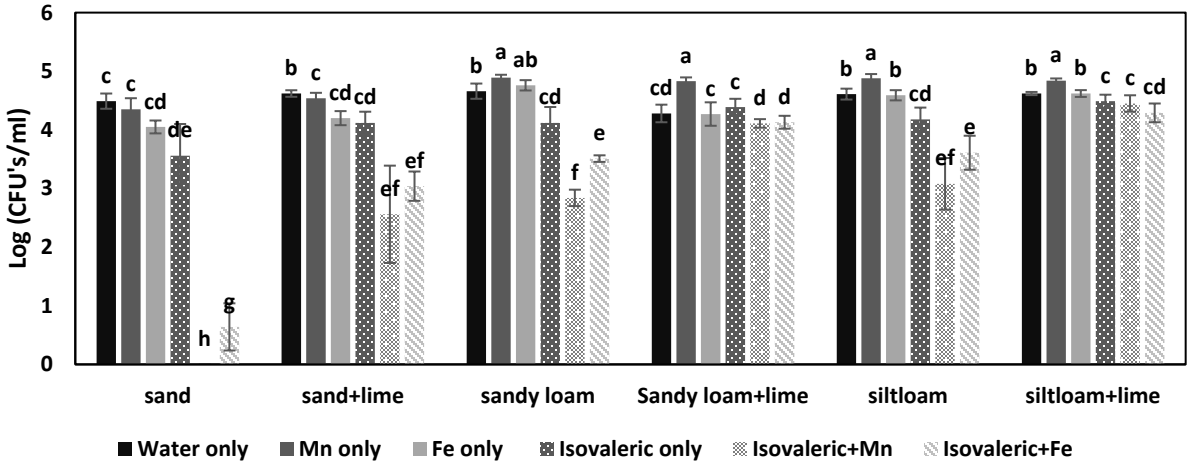

Fig. 3: Influence of liming and texture type on *Fusarium oxysporum* suppression by 20 mM Isovaleric acid and 0.05% w/w Fe^{2+} and Mn^{2+}. Limed treatments were amended with 0.1% w/w $CaCO_3$.

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