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AN EXAMINATION OF ORGANIC OPTIONS IN TOMATO SYSTEMS AND THEIR USE AS ALTERNATIVES TO COPPER-BASED PRODUCTS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture, Food and Environment at the University of Kentucky

By
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Lexington, Kentucky
Director: Dr. Paul Vincelli, Professor of Plant Pathology
Lexington, Kentucky
2022

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ABSTRACT OF DISSERTATION

AN EXAMINATION OF ORGANIC OPTIONS IN TOMATO SYSTEM AND THEIR USE AS ALTERNATIVES TO COPPER-BASED PRODUCTS

Organic farming is an ever-increasing segment of tomato production. Currently, limited information is available which directly compares conventional to organic treatment programs for disease control in tomato production. Furthermore, many methods available rely on the use of copper products which may be contribute to high Cu levels in agricultural soils. In this study, the efficacies of current conventional and organic methods were compared. In addition, newer disease-control programs, with and without copper were examined, which potentially could reduce over-reliance on copper products.

Standard organic and conventional spray programs were conducted over a four year period targeting two pathogens, *Alternaria tomatophila* (tomato early blight) and *Xanthomonas euvesicatoria* (tomato bacterial spot). Both programs contained a copper product (Nordox). Field trials with these programs were found to reduce the disease severity of both pathogens. Additionally, these two programs were not distinguishable statistically ($p>0.05$) throughout the four years.

Subsequently, in a tomato high tunnel study, alternative bioproducts to copper were used to control powdery mildew (*Oidium neolycopersici*). These alternatives included a *Bacillus* sp. product and a novel microbial fermentation product (MFP). Although the *Bacillus* sp. treated tomatoes had statically ($p<0.05$) lower disease severity than the control, it did not match the performance of copper products. Alternatively, in most trials, the MFP performed statistically ($p>0.05$) similar to the copper product, making it a viable candidate for further study.

The MFP was further investigated in an open field setting against *X. euvesicatoria*. The MFP and copper product were used in single product spray programs as well as tank-mixed with each other. In these field trials, as opposed to the high tunnel studies, although MFP treatment resulted in statistically ($p<0.05$) lower disease severity than the untreated control, it did not lower tomato disease severity to the same extent as the copper product. In addition, when MFP was tank-mixed with copper, efficacy was not statistically ($p>0.05$) better than either isolated product alone, indicating possible antagonistic behavior.

To understand these differences in efficacy of the MFP against *O. neolycopersici* and *X. euvesicatoria*, possible modes of action (MOA) were examined. A lack of detectable fast-growing organisms within the MFP indicated the MOA was likely not associated with either competition or hyperparasitism. Alternatively, MFP inhibited spore germination in *Botrytis cinerea*, *Magnaporthe oryzae*, and *Colletotrichum higginsianum* and reduced

mycelial expansion in *B. cinerea*, *Sclerotinia sclerotiorum*, and *C. higginsianum* in-vitro assays indicating antibiosis/antimicrobial properties. MFP also reduced bacterial growth of *X. euvesicatoria* and *Pseudomonas syringae* at 8% concentration or higher in liquid culture. Northern blot and RNA-seq results indicate possible plant defense induction from the application of MFP at 8% v/v.

Results of these studies indicate that the MFP may be a potential alternative to copper in tomato cropping systems. However, the MFP's efficacy appears limited based on either the environment or the target pathogen. Further investigation revealed the possibility of multiple MOAs. The primary mode of action appears to be an antibiosis/antimicrobial effect which may differ based on the resistance of the target pathogen. The secondary MOA may be induction of plant defense genes.

KEYWORDS: Copper, Plant Pathology, Microbial Fermentation Product, Tomato, Organic.

Erica Ann Fealko

(Name of Student)

04/28/2022

Date

AN EXAMINATION OF ORGANIC OPTIONS IN TOMATO SYSTEMS AND THEIR USE AS ALTERNATIVES TO COPPER-BASED PRODUCTS

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Date

DEDICATION

I dedicate this dissertation to my family. Their support and encouragement have helped me through this long process.

A special acknowledgment to my mother, Dr. Susanne Keller, a person who has shown me time and again what it means to be a formidable woman in science.

I would also like to dedicate this dissertation to my partner, Lucas Pinheiro de Araújo. His love and support have been beyond what I could have hoped for, he has helped me through the unorthodox challenges of my graduate education and life.

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[Supplemental Document 1.2 Early Blight and Septoria Leaf Spot of Tomato Management for Commercial Growers] …..……………………………………………... [PDF 1.4 MB]
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CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

As plants are critical sources of food, clothing, furniture/housing, and feed for animals, the control of plant diseases is vitally important. Plant diseases can have immense impact on human survival. The gravity of plant disease can be seen in the plant pandemic in 1845 called the Irish potato famine caused by *Phytophthora infestans*. This event resulted in a famine causing the starvation of 1.5 million people and the migration of another 1.5 million people (Fry and Goodwin 1997). Plant disease can also impact the cost of food production, and the health of humans and animals, and destroy environments (Omotayo et al. 2019). Fusarium species that cause root, stem and ear rot in maize kernels can cause the occurrence of mycotoxins. Mycotoxins then get into feeds for animals and food for human consumption. The consumption of mycotoxins is often associated with chronic or acute mycotoxicosis in livestock and humans, in addition to cancer in humans (Bullerman 1979; Logrieco et al. 2002; Ostry et al. 2017). Overall, at least 10% of global food production is lost due to plant disease (Strange and Scott 2005).

However, mitigation of plant disease and reduction of pathogen load in order to reduce food losses is not without issue. In an attempt to mitigate plant disease, billions of pounds of pesticides are deposited into the environment and water leading to additional environmental costs (Tiryaki and Temur 2010; Tudi et al. 2021; Werf 1996). Environmentally, some pesticides, such as methyl bromide, are of growing global concern. Methyl bromide is listed as an ozone depleting substance. Concern over this issue prompted a worldwide ban (Katan 1999; Whipps and Lumsden 2001). One additional example of

detrimental pesticide application is the use of antibiotics. Initially, in the 1950's, approximately 40 antibiotics were screened for use in plant disease management. The potency at low doses and the negligible toxicity in plants made them more palatable than the metal-based bactericides that were available to growers at the time (McManus et al. 2002). However, there has been an emergence of antibiotic-resistant strains limiting the effectiveness of current products such as streptomycin, which has been used to control both *Pseudomonas syringae* (Tomato Bacterial Speck) and *Xanthomonas euvesicatoria* (Tomato Bacterial Spot) (McManus et al. 2002). There are limited data on the effect of heavy use of antibiotics in plant-related agriculture with respect to antibiotic resistance in human pathogens, which is another emerging issue (World Health Organization 2019). Some of these environmental concerns form part of the reasoning behind the shifting trend towards organic and sustainable agriculture production methods (Gomiero et al. 2011).

1.2 Tomatoes

Tomatoes have an economic importance worldwide. They are the second most consumed vegetable in the world after the potato (Bergougnoux 2014). They can be sold not only as fresh produce but processed as paste, soup, juice, sauce, powder, concentrate or whole. Worldwide production reached almost 160 million tons in 2011 alone (Anwar et al. 2019; Bergougnoux 2014). Overall tomatoes are the seventh most important crop species after corn, rice, wheat, potatoes, soybeans and cassava.

Tomato cultivation occurs world-wide; however, their temperature tolerance limits cultivation as temperature below 50°F (10°C) may damage the plant (Foolad and Lin 2001; Lyons 1973). Tomato cultivation in protected environments, such as greenhouses and high

tunnels, has increased dramatically as such environments can provide more consistent growth conditions and longer growing seasons (Baskins et al. 2019; NASS 2021). Interestingly, tomato yields in northern climates such as Iceland, have higher yields per harvested areas then countries with less climate limitations such as Brazil (Bergougnoux 2014). This is attributable to the greater use of protected environments such as greenhouses for cultivation in these countries. In the U.S., in 2014, tomatoes grown in protected environments totaled 42,587,000 square feet (Bergougnoux 2014). By 2019, this volume increased to 52,576,000 square feet. This increase in high tunnel and greenhouse usage is fueled by the desire to extend the growing season (Bergougnoux 2014). Extending the growing seasons presents multiple economic advantages allowing farmers to plant earlier in the spring, and harvest later into the fall. In some cases, harvests can extend year-round. This extended growing season can result in increased marketable yields and better product consistency (Galinato 2013; LaMondia 2018). Other advantages include reduction in fertilizer and pesticide transportation costs and the ability to utilize soil health techniques that can prevent erosion, suppress weeds, increase soil water content, as well as reduced pesticide applications (Burlakoti et al. 2014; De Villiers et al. 2009; Lamont 2009). There are also state and federal cost-sharing programs in place that incentivize their use such as NRCS EQIP High Tunnel Systems Initiative. However, these protected environments can present unique disease management issues due to factors such as increased humidity (Bruce et al. 2019).

The importance of tomatoes and their disease burden generates considerable research interest (Anwar et al. 2019). As such, they stand alongside *Arabidopsis* and Tobacco as a model plant system used in research (Anwar et al. 2019; Gebhardt 2016).

For example, the tomato has been used previously to study induction pathways for systemic acquired resistance (SAR) to disease (Lin et al. 2004). Lin et al introduced the *Arabidopsis* NPR1 gene into tomatoes and found increased resistance to a broad spectrum of different disease. The increased resistances were stably inherited.

1.3 Factors of the Disease Triangle

A tomato plant, similar to any plant, can become diseased either when it is attacked by a pathogen or when it is affected by an abiotic stress factor. In the case of pathogen attack, for disease to occur three components must be in place (De Wolf and Isard 2007; Grulke 2011). The first is the correct environmental conditions; second is a susceptible host and the third is the virulent pathogen. Together these components (pathogen, host, and environment) make up what is commonly referred to as the plant disease triangle. Each component can vary, and those variabilities can affect the disease severity. To illustrate, if a host is highly susceptible to a particular pathogen the disease severity is greater than if the host were less susceptible. Plants can mount a defense against many pathogens. For example, in cultivated tomatoes, only a few varieties are available with resistance to the tomato early blight disease, which is caused by several species of *Alternaria*. Some of this resistance is sourced from *Solanum lycopersicum* accession PI138630. Additional genetic resistance has been identified in wild tomatoes (Adhikari et al. 2017). Tomatoes without these resistance genes can experience yield reduction and, in severe cases, plant death (Adhikari et al. 2017). The same early blight pathogen does not affect (or infect) peppers (Tsedaley 2014).

Additionally, if the environment is not conducive for pathogen growth the disease severity can be reduced. Alternatively, a poor plant growth environment may enhance the frequency and severity of disease. The environment can do more than influence disease severity, it may also impact the types of diseases observed. In field tomatoes, it is not common to see the significant disease severity of Powdery Mildew, caused by *Oidium neolycopersici*. However, this pathogen can cause far more damage in high tunnel or greenhouse production systems (Jones et al. 2001). The difference can be partially attributed to the high humidity and particular light conditions maintained in a high tunnels and greenhouses that allow for a more favorable growth environment for the pathogen. Despite some higher incidence of disease and disease severity, these different environments can have some advantages over open field systems. They can allow for exclusion techniques to limit the introduction of pathogens, as well as other sanitation techniques that would otherwise be impractical on a field scale.

As opposed to the more controlled environment in a greenhouse or high tunnel, field environments lack the same consistent conditions. For example, exclusion of pathogens from the environment is not always possible. One example is some Rust pathogens (*Pucciniales sp.*), which have the ability to spread over long distances given the right conditions. In addition, geographical region and even climate change can create more favorable environment for pathogen development resulting in greater spread of disease and higher disease severity. Again, this may be illustrated with Rust diseases. Typically, Rusts are favored by milder winters, and some do not survive temperature below −13°C (i.e. *Puccinia graminis* subsp. *graminicola*) (Helfer 2014). These condition restraints generally limit the spread of the pathogen in fields that are in colder climates. However, due to the

shift in plant hardiness zones through global climate change and some natural variations in field conditions, many types of rust diseases develop earlier and occur earlier and extend later into the year than previously reported (Helfer 2014). This extension of this disease occurrence has led to greater disease severity in colder zones than in previous years.

1.4 Pathogen Disease Cycles

With most pathogens there is a series of similar events that occurs leading to the development and spread of disease. This process is commonly referred to as the disease cycle of a pathogen (De Wolf and Isard 2007). The primary steps in this cycle are inoculation, penetration, establishment of infection, colonization, growth, reproduction of the pathogen, dispersal, and survival of the pathogen in the absence of the host (over wintering stage). With pathogens such as *Alternaria tomatophila*, *X. euvesicatoria*, and *O. neolycopersici* this process may include multiple (secondary) infections.

The first step in the disease process, the inoculation stage, is when the pathogen makes initial contact with the host and is generally the site where the infection begins. The inoculum itself can be any part of the pathogenic organism that can initiate infection. For a fungus this maybe a spore, sclerotia, or possible a fragment of mycelia. One unit of inoculum is called a propagule. There are two types of inoculum or propagule. First is the primary inoculum which causes the primary infection or original infection of the host and can originate from overwintering structures and plant debris from the previous year. The secondary inoculum is produced from tissues that had been infected during the original plant infection. A primary inoculum can originate from surrounding plants, plant debris, soil, insects, seeds, transplants, or other propagative tissues. Most inoculum is dispersed to

the host by wind, water, or insect (De Wolf and Isard 2007). The initial inoculation step is frequently enabled through adhesion of the inoculum to the plant surfaces. Adhesion, in the case of fungal pathogens, occurs primarily through the development of intermolecular forces between the host surface and the hyphae and radicles. With some fungal pathogens, an adhesion pad forms when the spore itself comes into contact with a moist surface (Nicholson and Epstein 1991). Cutinase and cellulase enzymes released from the spore help it adhere to plant surfaces (Nicholson 1996). Other fungal spores carry adhesive substances at their tips that, once hydrated, allow the spore to attach. After contact is established, some fungi, such as *O. neolycopersici,* form a structure (usually circular) called the appressorium, that provides additional anchorage for the pathogen to the plant (Nonomura et al. 2010).

The second stage of the disease cycle is penetration by the pathogen. Some pathogens only have one mode of entry while others can utilize multiple methods of penetration and entry (Bellincampi et al. 2014; De Gara et al. 2003; Tucker and Talbot 2001). When appressorium are present, such as with *O. neolycopersici*, a penetration peg will form and punch through the plant cuticle and cell wall. Physical penetration is accomplished by a build-up of turgor pressure within the appressorium (Nicholson 1996; Nicholson and Epstein 1991). A secretion of chemicals that may influence components or mechanisms of the host or penetration of the host cells may also occur (Huang 1986; Mendgen et al. 1996). As an alternative to direct plant cell wall penetration, some pathogens enter the host through natural openings or wounds. Bacteria such as, *P. syringae,* enter the host by the use of natural openings like stomata or wounds that have been created by insects or other mechanical means (Bellincampi et al. 2014; De Gara et al. 2003).

Nematodes can penetrate plant surfaces with the aid of a stylet (mouthpiece), which moves back and forth to create a mechanical pressure to break through (Endo 1975). Once a fungus or nematode has successfully entered a plant host, they may secrete an enzyme to soften or break down the plant cells to continue to penetrate the host with greater ease (Endo 1975).

Infection, which follows penetration, is a process by which the pathogen establishes contact with susceptible tissue of the host and begins to extract nutrients from them. After infection, colonization of the plant occurs. During this stage pathogens typically will grow and/or multiply to colonize the plant (Tucker and Talbot 2001). During colonization there is continued growth and reproduction of the pathogen (Peyraud et al. 2019). The pathogen may then go on to colonize both within the host and then spread to new hosts through dispersal. Dispersal may occur through wind currents, where air picks up and spreads fungal spores (Numminen and Laine 2020). They can also disperse through water. For example, bacteria, nematodes, and spores can be lodged in the soil or on fallen plant debris. Rain and irrigation can move the inoculum through the soil (Fitt et al. 1989). Pathogen dispersal can also occur by other vectors such as insect or mechanical/human factors. The dispersal of pathogens can also be enhanced though infected seeds, transplants, or other stock, or through the use of infested agricultural tools such as trowels, pruners and shears.

Frequently, pathogens can survive from season to season by overwintering, which represents the final step in the disease cycle. In particular, fungi have developed multiple different methods for overwintering. They can survive as mycelia in diseased plant tissues, such as in cankers, or as spores near the infected plant surface (Paul and Ayres 1986). They also occasionally develop structures called sclerotia which is a hardened mass of mycelia. *Alternaria tomatophila,* for example, survives as mycelia, conidia, or chlamydospores in

the plant debris and soil (Vloutoglou and Kalogerakis 2000). Another example, *O. neolycopersici* survives overwintering as either mycelia in plant tissue or as cleistothecia (closed, globose fungal fruiting bodies) (Jacob et al. 2008). As opposed to some fungi, many bacteria are well-known to survive low-temperatures and can also survive in plant debris. *X. euvesicatoria* can survive in this manner as well as on seeds (Momol et al. 2002). As opposed to fungi and bacteria, viruses can only survive in living plant tissue, consequently they may remain on roots of perennial plants, seeds, or sometimes within insects.

1.5 Diseases of Tomato: Early Blight

Early blight of tomato can be one of the most destructive fungal diseases in the tomato cropping system (Chaerani and Voorrips 2006). Early blight can cause up to 79% yield losses (Chaerani and Voorrips 2006). Early blight of tomato is caused by either *Alternaria solani* or *Alternaria tomatophila.* Collar rot, which is caused by the same pathogen, can cause seedling losses between 20%-40% in the field (Chaerani and Voorrips 2006). The importance of this disease and relative lack of resistant varieties generates research interest with respect to means of control.

Alternaria tomatophila, as opposed to *A. solani*, is the more virulent of the two on tomatoes and the primary causal pathogen of early blight. *Alternaria tomatophila* can also affect other members of the Solanaceae family such as potato (Kemmitt 2013). The disease symptoms of early blight can occur on foliage, fruit, and stems at any stage of development. It is more commonly seen in the field as opposed to controlled environments such as greenhouses or high tunnels. The lesions will first develop on the older lower leaves as

brownish-black spots. These lesions can expand to about 0.64 – 1.27 cm in diameter with concentric rings in the darkened area. The area surrounding the lesions can become yellow and chlorotic. As the disease progresses to the upper foliage, yellowing of the leaves may occur as well as defoliation. This may lead to increased susceptibility of the fruit to sunscald. Fruits may also be directly impacted by the pathogen, becoming infected through the calyx near the stem at either the immature or mature stages. The lesions may expand to the entire fruit and are sunken, and leathery. The lesions will have a dark brown to black appearance with the concentric rings associated with the pathogen (Jones et al. 2014).

The primary inoculum source can be from soil, plant debris, seed, or an alternate host (Adhikari et al. 2017). Inoculum propagules can come in the form of either conidia or mycelial fragments (Adhikari et al. 2017). The tomato penetration will normally occur in warm and humid conditions. Conidia germinate at temperatures anywhere from 8-32°C in cool and humid conditions and requires the presence of moisture in order for the germ tube to develop. The germ tubes will penetrate the tomato tissues by directly pushing though the leaf tissue or by entering stomata or wounds (Adhikari et al. 2017). During host infection, *A. tomatophila* will produce alternaric acid as one of its major toxins or mechanism of attack on the tomato cells (Patel et al. 2011). Although when sprayed alone on tomatoes it does not cause phytotoxicity, alternaric acid does enhance the infection process and the development of necrotic/chlorotic symptoms when mixed with *A. tomatophila* conidial spores (Patel et al. 2011).

Typically, symptoms will develop after pathogen infection or about two to three days after initial infection of host. Production of secondary inoculum requires a long period of leaf wetness. This secondary inoculum, or conidia, will be produced with alternating

wet and dry conditions. During these wet dark periods the conidiophores are produced. The conidiophore will form conidia in subsequent wet periods after a period of light and dry weather. The spores are typically dispersed primarily by wind, air currents, or rain splash.

This disease can be partially managed by removing alternative host plants (e.g. volunteer tomatoes, nightshades, potatoes etc.) and plant debris from the previous season. Other cultural practices include sanitation, tolerant variety selection, and crop rotation. There are also various chemical control options that can be utilized by growers. *A. tomatophila* will overwinter as conidia, mycelia, or chlamydospores in soil, plant debris, seeds, or in an alternative host (Adhikari et al. 2017; Vloutoglou and Kalogerakis 2000).

1.6 Diseases of Tomato: Powdery Mildew

Powdery mildew of tomato can also be an important fungal disease. Although not as relevant for the open field setting, it does present a challenge in protected environments. Tomato powdery mildew is caused by either *Leveillula taurica* or *Oidium neolycopersici,* although, of these two fungi, *O. neolycopersici* is the more important pathogen. Both pathogens favor higher humidity environments that are more common in protected settings such as greenhouses and high tunnels. Powdery mildew has very distinct signs of powdery white lesions that form on the upper leaf surface. This pathogen is also an obligate biotroph, requiring a living host. The primary inoculum from this pathogen can be conidial spores or mycelia from a living or dormant volunteer host plant (pepper, eggplant, potato, tobacco, etc.). The conidial spores are ellipsoidal-shaped approximately 30 μm x 15 μm in size. The surfaces of the conidia are covered by irregular arrays of ribbon-like projections which are rounded on the ends. They are easily dislodged from the infected host tissue and dispersed

by air/wind. When the pathogen comes into contact with the tomato, a germ tube will develop smooth and elongated at the growing apex. This tip then becomes lobed in a cloverleaf like configuration. Appressoria are frequently found at the junction of three epidermal cells. The penetration peg then emerges from the center of the appressorium and enters the plant through direct cell wall penetration (Nonomura et al. 2010; Tucker and Talbot 2001).

Normally, rapid colonization of the leaf occurs after penetration of the cell wall. The secondary appressoria develop either singly or in pairs from the hyphae that develop over the host. This cycle is then completed with the formation of conidiophores. The conidiophores stand at a 90° degree angle to the host surface with a straight cylindrical foot cell. This supports a meristematic zone of immature conidia that carry a single mature ellipsoidal conidium at the top of the column. Germination of spores will normally occur within three to five hours after inoculation. Appressoria will develop at six to eight hours after inoculation and penetration will occur approximately 11 hours after inoculation (Jones et al. 2001). *Oidium neolycopersici* can overwinter as cleistothecia, a globose completely closed fruiting body with no special opening to the outside (Glawe 2008).

1.7 Diseases of Tomato: Bacterial Spot

Another disease of importance to tomato systems is tomato bacterial spot. This is an important disease around the world with economic impact. In some ideal conditions as much as 50% yield loss in tomatoes can occur in addition due to a reduction in product overall quality caused by this disease (Kunwar et al. 2018).

Bacterial spot of tomato can be caused by *Xanthomonas vesicatoria*, *Xanthomonas euvesicatoria*, *Xanthomonas gardneri* and *Xanthomonas perforans*. These pathogens are gram-negative rod shaped and strictly aerobic. *X. euvesicatoria* favors temperatures about 75° to 86° F (24°-30° C) with high precipitation and/or humidity. The longer the period of leaf wetness the higher the likelihood infection will occur (Momol et al. 2002). Having colonized host tissues, *X. euvesicatoria* can induce a hypersensitive response in the plant.

Symptoms appear initially as brown circular spots approximately < 0.32 cm. These lesions will develop chlorosis around the edge and the centers may fall out, producing small holes. Lesions can also occur on the stems and fruit calyx in the form of small brown circular spots. The fruits may also develop spots about 0.64 cm in size. On the fruit, these lesions will be slightly raised brown and scabby. The fruit may also develop a waxy white halo surrounding the fruit lesion. These lesions will occur on both mature and immature fruit (Sharma and Bhattarai 2019).

The primary inoculum for *Xanthomonas euvesicatoria* can be from infected/infested plant tissues such as seeds, transplants, volunteer hosts, and crop debris (Momol et al. 2002). The inoculum can reach the host through rain/irrigation dispersal, infested tools, and contaminated plant material (Momol et al. 2002; Sharma and Bhattarai 2019). Once in contact with the host, *X. euvesicatoria* enters though natural openings in the plant such as stomates and hydathodes. The bacteria can also enter by wounds created from wind-driven sand, insect punctures, or by mechanical injury (Momol et al. 2002). *Xanthomonas euvesicatoria* will overwinter in seeds or plant debris. In colder regions the survivability of the pathogen may be impacted negatively by temperatures (Momol et al. 2002).

This pathogen is managed by the use of resistant varieties as well as other cultural practices. The grower can utilize certified disease-free seed, maintain proper sanitation practices, and crop rotation. There are also a variety of chemical controls available to growers.

1.8 Disease Progression within Hosts

Once in a host, such as a tomato plant, pathogens continue their attack. This attack can be aided by different chemical agents. These substances can by enzymes, toxins, growth regulators, and polysaccharides (Kubicek et al. 2014). Pathogen enzymes can disintegrate structural components of the host cells. The purpose is to break down host cell components for use as nutritional sources or to be able to affect the host membrane and protoplast more directly. These actions by the pathogen can interfere with the normal functions of the plant (Kubicek et al. 2014). Some fungi will produce enzymes which focus on the degradation of cellulose, xylan, and pectin, which are components of plant cell walls (Kubicek et al. 2014). The fungus *Sclerotinia sclerotiorum,* which causes timber rot and white mold of tomato, produces cellulolytic enzymes that can break down the cellulose in plant cell wall (Riou et al. 1991). Toxins produced by the pathogen will directly interact with the protoplast components of the host and interfere with the permeability of its membranes impacting the cell's function. *Alternaria* pathogens are known to produce host-specific toxins. The toxins can cause necrosis on leaves of susceptible cultivars at concentrations as low as 10^{-8} to 10^{-9} M (Tsuge et al. 2013). Growth regulators produced by pathogens will exert a hormonal effect on the plant by influencing either the increase or decrease of the host cell's ability to divide and enlarge. This is the case with bacterial plant

pathogens. Bacterial pathogens can secrete type III effector proteins that impact hormone biology as well as plant hormones and hormone analogs produced by the pathogens (Kunkel and Harper 2018). Some bacteria can produce a naturally occurring auxin or indole-3-acetic acid (IAA) (Spaepen and Vanderleyden 2011). IAA can play a role in several different plant-microbe interactions, including some beneficial microbial interactions such as plant growth promoting rhizobacteria and nitrogen-fixing symbiosis (Patten et al. 2013; Spaepen and Vanderleyden 2011). There are several pathogenic *Pseudomonas syringe* pathovars that can produce IAA (Fett et al. 1987; Glickmann et al. 1998; Kunkel and Harper 2018; McClerklin et al. 2018; Spaepen and Vanderleyden 2011).

Additionally, polysaccharides can have multiple roles in a pathogen's life cycle. During pathogen attack, for example, polysaccharides can be involved in vascular disease. Slimy polysaccharides will interfere passively with water translocation of a host by blocking vascular tissues. In vascular wilt pathogens, large polysaccharide molecules are released into the xylem which can lead to blockage of vascular bundles and create wilting symptoms (Mace 2012; Yadeta and Thomma 2013).

In the course of the normal disease cycle of any particular pathogen, any one of these types of substances may play a role. Depending on the type of pathogen, multiple substances may be involved in attack. For example, *Rhizopus* spp. produce numerous enzymes including amylase, pectinase, and cellulase that make it particularly efficient at breaking down the cell walls necessary for the pathogen to colonize the host plant (Scruggs and Quesada-Ocampo 2016).

However, in each case plants are not entirely without some sort of protection. Production of many of these substances can result in the induction of various plant defense mechanisms. The production or induction of such defense mechanisms may short-circuit the normal pathogen life cycle, resulting in plant disease resistance.

1.9 Plant Host Defense Mechanisms

All plants, including tomatoes, defend themselves from pathogen attacks by two primary methods. The first means of defense is through preexisting characteristics or constitutive defenses, which consists of both physical and chemical aspects (Tariq and Saleem 2018). The physical characteristics typically act as physical barriers and prevent the pathogen from entering and colonizing the host. These can include preformed barriers such as cell walls, waxy epidermal cuticles, and bark. An example of the importance of these defenses can be observed with the tomato fruit cuticles. In the immature stages, the fruit cuticle serves as vital defense against *Botrytis cinerea* (Botrytis gray mold of tomato). However, as the fruit matures and the cuticular wax breaks down, the fruit becomes increasingly susceptible to *B. cinerea* (Blanco-Ulate et al. 2016; Ziv et al. 2018).

There are also preexisting molecules that provide defense against disease. These take place in host cells and tissues where the plant produces substances that are either toxic to the pathogen or create an environment that inhibits the pathogen growth. These can act as a second line of defense as they protect against pathogens that were able to make it past the initial physical barriers. These biochemical defenses can include a variety of substances. One such group of substances are fungitoxic compounds. Fungitoxic compounds may be excreted on the surface of some plants like tomatoes and can inhibit

germination of the spore of some fungi such as *Botrytis* (Singh et al. 2005). Tomatoes produce a saponin called α-Tomatine, which has been shown to have potent broad-spectrum antifungal activity. It is present in the healthy tomato plant with levels as high as 1 mM in leaf tissue even without the presence of pathogens, (Arneson and Durbin 1968; Arneson 1968; Martin-Hernandez et al. 2000).

Another method by which a plant may deter pathogen infection is through ambient pH. A plant may have a lower cellular pH as a way to create an undesirable environment for pathogen growth. Some pathogens such as *Alternaria* can have increased pathogenicity with more alkaline environments which modulates its pathogenicity. The host ambient alkalization is caused by an ammonia secretion from the pathogen. Cell tissue pH surrounding the pathogen is increased which then results in an increased elicitation of virulence factors secreted by the pathogen. This in turn allows the pathogen to select specific virulence factors required for the desired host without utilizing resources to express all virulence genes (Akimitsu et al. 2004).

1.10 Induced Defense Mechanisms

The alternative to previously discussed pre-existing defense mechanisms in tomatoes are inducible defenses. These can be either structural or biochemical. Induced resistance is expression of defense mechanisms genetically available to the plant that are not constitutively expressed (Hammerschmidt 2009). These defenses can be highly dependent on the plant's ability to recognize the pathogen.

Plant pathogen recognition occurs when a pathogen-associated molecular pattern (PAMP), microbe-associated molecular pattern (MAMP), and effector triggered immunity

(ETI) is recognized by the plant's pattern recognition receptors (PRRs). This triggers a multifaceted immune response resulting in increased disease resistance (Bigeard et al. 2015; Jones and Dangl 2006). Some examples of PAMPs are bacterial molecules like lipopolysaccharides and flagellin and fungal molecules like chitin (Bigeard et al. 2015). However, these do not all occur in all plant species and, when they do, are not at the same levels between plant species. As an example, tomatoes can specifically recognize a 15-amino-acid flagellin peptide from *Escherichia coli* as opposed to *Arabidopsis* which cannot (Felix et al. 1999; Meindl et al. 2000; Nguyen et al. 2010).

Induced resistances may occur in stages. When a plant has been stimulated into an induced resistance (IR) state, but has not yet been attacked by a pathogen, it is in what is referred to as the priming phase (Pastor et al. 2014). Typically defense responses appear or begin after the priming phase or recognition of the pathogen. Induced structural defense may then occur through toughening or hardening of epidermal walls, waxy cuticles, cell wall thickness, size and shape of stomata, lenticels modification and morphology of thorns and spines. Not all changes occur in every plant, nor are all such changes associated with increased pathogen resistance, some are simply related to increased stress. Other changes can occur in plasma membrane permeability and hypersensitive responses which are visible at the cellular level (Darvill and Albersheim 1984; Tariq and Saleem 2018). Inducible biochemical defense can include an increase in concentration of compounds that could be antimicrobial in nature (Tariq and Saleem 2018; Yedidia et al. 1999). In response to B*otrytis cinerea*, tomatoes increase lignin metabolism levels and thicken cell walls (Yang et al. 2018). Another induced response can be the formation of necrotic lesions due to a hypersensitive response (HR) (Zhou et al. 1995). A HR is localized rapid plant cell death,

normally associated with the site of pathogen infection (Balint-Kurti 2019). Other immune responses can consist of lignification, synthesis of proteins, deposition of callose, the accumulation of antimicrobial low-molecular-weight substances, and induction of pathogen defense-related genes (Zhou et al. 1995). These induced defenses can be specific to a pathogen or more general and can last from weeks to entire seasons depending upon the target crop (Conrath 2006). In tomatoes, HR is used to defend against multiple pathogens such as those causing bacterial spot (Scott et al. 2001). *Oidium neolycopersici* uses enzymatic breakdown of plant cells to colonize host tissue (Jones et al. 2001). The use of HR is critical in restricting the pathogen's colonization from progressing to other leaf tissue (Jones et al. 2001; Li et al. 2007).

Inducible plant pathogen defense systems can be a systemic acquired defense (SAR) and/or a localized acquired defense response (LAR). SAR is a broad spectrum or whole plant immune response and involves a variety of genes and plant hormones in its mechanism. This immune response is frequently mediated by one or more plant hormone signaling pathways. The salicylic acid (SA) pathway is one that can be involved in both local defenses and SAR (Bernsdorff et al. 2016; Huot et al. 2014; Shah et al. 2001). There is also the jasmonic acid (JA) pathway which has been associated with powdery mildew defense in wheat (Duan et al. 2014; Gao et al. 2011). The primed state, resulting from the induction both local and/or systemic defense, can cause an accelerated response to external stress (Balmer et al. 2015)

SAR genes have some overlap with known genes for pathogenesis-related (PR) proteins (Conrath 2006). The plant hormone SA (salicylic acid) appears to contribute more to SAR than PR proteins. SA is required for the appearance of SAR in distal tissues of the

infected plant. This was confirmed through the use of plants constitutively expressing SA hydroxylase (Conrath 2006). These plants were unable to accumulate SA in high levels and also unable to produce an SAR response. In contrast, in mutants where there is an overproduction of SA there is also an increased resistance to pathogens (Conrath 2006)

In tomatoes, there are numerous examples of induced disease responses which include systemic resistance (ISR) and systemic acquired resistance (SAR) (Gao et al. 2014) as well as localized acquired resistance (LAR). These induced defense systems are targeted as a form of pathogen control by some marketed products. Some products such as benzothiadiazole (BTH, a synthetic analog of SA) stimulate the SA pathway and "prime" the tomato plant prior to infection. When a tomato enters the primed state, only a low level of stimulation is required to initiate a defense response (Conrath 2009). However, pathogen recognition and priming are not the only methods to stimulate defense genes. Other factors that can produce this affect include injury or exposure to adverse environments or chemical in the environment.

1.11 Cultural Control of Tomato Plant Disease and Disease Responses

There are multiple means through which plant disease progression may be manipulated and controlled in tomato production. One of the methods of control is described as exclusion or the management of plant disease by preventing the introduction of pathogens to the crop production area, such as is accomplished through quarantines (Fry 2012; Koike et al. 2000).

Quarantine refers to the restriction of movement of plants and plant materials such as seeds, propagative tissues, soil, machinery, or any other materials that may harbor

pathogens (Fry 2012). This is an important strategy when there is an infected tomato field and a non-infected tomato field. Alternatively, if a grower has a protected environment such as a greenhouse or high tunnel, they may start their work/management in the protected environment first and then move to the open field. Protected environments can utilize management techniques not applicable to open fields but require higher attention to pathogen introduction into the environment and dispersal. For example, in a tomato greenhouse 'clean' material can be used and protected from pathogen spread that happens through rain or wind dispersion. However, if an infected field is traversed, pathogen inoculum can be picked up on boots, hands or equipment, and brought into the greenhouse. Powdery mildew is present naturally in the environment but does not present major disease issues in field tomatoes. However, in protected environments this pathogen thrives and once established, is very difficult to manage.

Another way to exclude pathogens is to use pathogen-free seed/plants and pathogen-free vegetative propagation materials. Since some seeds and propagative materials may have pathogen inoculum, using certified disease-free materials can limit the introduction of pathogens into the production area (Fry 2012; Koike et al. 2000). Failure to utilize disease-free materials is a common way for tomato pathogens to enter an environment. This method of control can be extremely important in a protected environment since they are typically free of environmental pathogens—or nearly so--at the start of production.

If tomatoes plants purchased for transplant into the field carry pathogens, the grower can risk contaminating their field. In cases such as *X. euvesicatoria*, the host range

include peppers, and any surrounding pepper (or other alternative hosts) could start to show disease symptoms.

Another method of disease management is through cultural practices. These involve controlling pathogen disease through the cultural manipulation of plants such as crop rotation. Crop rotation along with exclusion are recommended to control tomato bacterial spot with the addition of crop rotation to a non-host species (Momol et al. 2002).

Crop rotation involves the rotation from a pathogen host plant to a non-host plant similar as is done to avoid nutrient depletion in soils, such as is done with corn production (Ma et al. 2012). This control removes the host from the environment causing the pathogen to either die off or enter its survival/overwintering stage. Another method to accomplish the same result is by fallowing or leaving a field free of crops. This is normally incorporated into a crop rotation plan (Koike et al. 2000). A common practice in tomato rotation is to alternate with a legume such as beans or alfalfa, to promote nitrogen fixation (Moura et al. 2020). Crop rotation isn't always a viable option in protected systems so growers should focus on other management methods like sanitation.

Sanitation is also a cultural practice that can retard the pathogen's life cycle. Sanitation includes the removal of plant debris from the environment to reducing pathogen overwintering from season to season. Disinfesting tools, machinery, boots or anything that may carry pathogens that come in contact with the host plant is also included in basic sanitation practice (Fry 2012; Koike et al. 2000). Since plant debris is a common source of inoculum this can be a very important management method. In tomato bacterial spot, tomato bacterial speck, powdery mildew, and tomato early blight, plant debris is frequently the source of inoculum from a previous year. Stakes used in trellising tomatoes, as well as

other re-used equipment such as clippers or trowels and rakes, can be a source of inoculum and should be properly disinfested (Stirling et al. 2004; Toro et al. 2012). This may be especially difficult when porous materials such as wood are used.

Barriers and mulches are also of use in the prevention of plant disease infection. These techniques help prevent the spread of pathogen by creating a physical barrier to the pathogen. Mulches can reduce the pathogens' ability to be splashed-up and be carried by runoff water from the soil onto the plant (Jabran 2019). Mulches are often use in both protected and open field systems. In addition to barriers to prevent pathogen dispersion, an irrigation line (drip tape) can be used under the barrier limiting the spread of pathogens encouraged by irrigation.

Intercropping plants can also create a physical barrier to reduce the spread of pathogens. Growers may also choose to physically spread out their plants. This creates distance between plants that may be infected and make it more difficult for a pathogen to spread throughout a field (Yang et al. 2014). An essential component of intercropping is the use of a plant that is not a host of the target pathogen (Trenbath 1993). With respect to *X. euvesicatoria*, a grower should avoid Solanaceae plants or peppers. For *A. tomatophila* management intercropping with potatoes would not be recommended as they are also a host crop. The *O. neolycopersici* host range can be wide including plants in the Cucurbitaceae. Although many physical methods such as these are available to reduce pathogen spread and infection of the plants, chemical methods are frequently still required for management of plant disease.

1.12 Chemical Disease Management Methods

One of the most common means to control plant diseases in field, greenhouse, and high tunnel cultivation is the use of various chemical antimicrobials/fungicides. Chemicals used in the control of plant pathogens can inhibit fungal germination, pathogen growth, pathogen multiplication and colonization or kill the pathogen altogether. Depending on the target pathogen, the chemical can be classified as a fungicide, bactericide, nematicide, or viricide (Fry 2012). Insecticides may also be used to control insect pathogen vectors as a means to control diseases. Compounds/chemicals used can be pathogen-specific or broad-spectrum. A significant portion of compounds are used to control disease of above-ground plant tissue. However, some are used to disinfest or protect plant starting materials like seeds, tubers, and blubs. Many compounds used in the past were contacts and have historically focused on plant surfaces. Frequently such compounds of historical use, although locally effective, encountered issues related to potential phytotoxicity (Dias 2012). Currently, there are newer products that have a more systemic mode of action (Edgington 1981). Of these, there are several main groups of systemic fungicides: benzimidazole, sterol demethylation inhibitors, strobilurin-related, phenylamides, and SDHIs (succinate dehydrogenase inhibitor) fungicides. Benzimidazole and sterol demethylation inhibitors require an interaction between the chemical component and a fungal component. This is addressed by identifying the target pathogen and optimizing its interaction with different inhibitors (Davidse 1986; Karaoglanidis et al. 2000). The inhibitor fungicides have been used for the control of *Botrytis cinerea*, a pathogen that causes grey mold in tomatoes (Leroux 2007). Strobilurin-related fungicides are inhibitors of pathogen respiration. Their specific mechanism of efficacy is the secondary responses

of respiration such as a pathogen's alternative respiration pathway or detoxification (Avila-Adame et al. 2003). An example is azoxystrobin, which is commonly used in tomatoes to control *Alternaria tomatophila* (Rosenzweig et al. 2008).

Among the frequently applied fungicides applied to plant surfaces are those which contain cuprous oxides as the active ingredient. Cuprous oxide formulations function as a prophylactic protectant to inhibit pathogen infection (Horsfall et al. 1937; McCallan 1949; Walter et al. 2015). Cuprous oxide releases cuprous ions that quickly convert to cupric ions, both of which have bactericidal and fungicidal activity. Organisms in contact with cuprous and cupric ions take these up, which pass through their cell walls and disrupt their cellular enzymes (Horsfall et al. 1937; McCallan 1949; Walter et al. 2015). Unfortunately, copper uptake is not restricted to pathogens, and may harm beneficial microbiota found in the soil (Giller et al. 1998). In addition, copper may build up in soils, resulting in continuing harm to beneficial microbiota as well as to sensitive plants.

A multi-site mode of action (M) fungicide, mancozeb, is also often used in conventional spray programs as a contact pesticide. Mancozeb belongs to a class of compounds known as ethylene bisdithiocarbamates (Thind and Hollomon 2018). On exposure to moisture, ethylene bisisothiocyanate sulfide (EBIS) is released which is converted via the action of UV light into ethylene bisisothiocyanate (EBI). Both EBIS and EBI interfere with enzymes containing sulphydryl groups and result in fatal disruption of core enzymatic processes that interferes with at least six different biochemical processes within the fungal cell cytoplasm and mitochondria. One of the results of this active ingredient can also be inhibited fungal spore germination. While this product primarily remains on the leaf, penetration of the leaf as would be seen with systemics does occur and

may cause phytotoxic affects to the plant. Overall, it has a broad spectrum activity (Gullino et al. 2010; Thind and Hollomon 2018).

There are also chemical-based methods for inducing defense. One such product, Actigard®, has an active ingredient call acibenzolar-S-methyl (Benzo (1,2,3) thiadiazole-7-carbothiotic acid-S-methyl ester) which is be referred to as either ASM or BTH (Obradovic et al. 2005). This active ingredient is a SA functional analog belonging to the benzothiadiazole (BTH) family (Gozzo and Faoro 2013; Marolleau et al. 2017). Generally, the major function of SA is its association in the induction of *PR-1* (pathogen resistance 1) genes which are involved in the essential mediation of NPR1 protein (Gozzo and Faoro 2013). There is evidence supporting that the application of SA, or SA analogs, will cause the induction of plant defense priming (Gao et al. 2014; Klessig et al. 2000; Wu et al. 2012). Other metabolites have also been identified in their relation to defense induction and are being studied in their possible use in the field. These include glycerol-3-phospate (G3P) which is the alcoholic matrix of glycerolipids that are essential in growth and defenses. This metabolite can induce a mild systemic acquired resistance response when applied locally (Kachroo and Robin 2013). Azelaic acid (AzA) can be involved as well. AzA is a nonandioic acid and another mobile metabolite with priming properties related to systemic immunity (Kachroo and Robin 2013). AzA does not directly induce SA but has been related to priming plants to produce high levels of SA and SA-associated signaling marker *PR1* when challenged by *Pseudomonas syringae* (Gozzo and Faoro 2013; Jung et al. 2009). Pipecolic acid (Pip) has also been identified as a possible metabolite application capable of induce resistance in plants. Pip is a lysine catabolite. When *Arabidopsis* leaves are inoculated with *P. syringae*, there is an accumulation of Pip that appears not completely

associated with SA (Bernsdorff et al. 2016; Kachroo and Robin 2013; Návarová et al. 2012; Wang et al. 2018).

1.13 Disease Management with Living Organisms

One alternate type of disease management that attempts to eschew the use of chemicals is termed “biocontrol”. Biocontrols can be implemented instead of, or in congruence with, chemical products in agriculture. Biocontrols can involve the use of biological organism(s) or organically made chemical(s) to manage pests, weeds or diseases. This method of plant defense is currently used by a variety of different products on the market such as Companion® (Growth Products, Liberty, MO) which contains *Bacillus subtilis.*

Biocontrols can utilize microorganisms along with other integrated pest management (IPM) strategies in order to modulate disease severity (Baker and Cook 1974; Ram et al. 2018). There are several different modes of action and some biocontrols will implement more than one mode of action at a time. With respect to the management of plant diseases, biocontrol modes of action include: antibiosis, competition, parasitism, cell wall degrading enzymes or other compounds, and plant defense induction (Ram et al. 2018). These modes of action can be further categorized into either direct or indirect modes of action (Ram et al. 2018).

Antibiosis is an antagonistic interaction between two microorganisms such that an antibiotic or compound produced by one organism negatively impacts a target organism. Antimicrobials, for example, can be produced by one fungus but impact other fungi or bacterial pathogens. This can be observed with *Aspergillus flavus,* which produces an

antibiotic that affects *Candida albicans* (Makut and Owolewa 2011). *Bacillus subtilis* GB03 produces a broad-spectrum antibiotic called Iturin which can disrupt the cell wall formation of different plant pathogens (Haidar et al. 2016a; Lastochkin et al. 2019a; Moyne et al. 2001b; Romero et al. 2007). Antibiotic production has also been studied through the use of mutant antagonistic microorganisms deficient in the production of antibiotics. For example, phenazine, (an antimicrobial produced by the *Pseudomonas fluorescens strain 2-79*), was believed to play a role in the reduced disease severity in take-all disease of wheat (*Gaeumannomyces graminis* var. *tritici*) (Handelsman and Stabb 1996; Thomashow and Weller 1988; Weller 1988). By comparing phenazine minus *Pseudomonas fluorescens* strain 2-79 (created by the use of single-site Tn5 insertions), which do not produce the phenazine antibiotic, with the wild-type, the authors demonstrated higher incidence of take-all disease of wheat (*Gaeumannomyces graminis* var. *tritici*) than with the phenazine producing strain (Handelsman and Stabb 1996; Thomashow and Weller 1988; Weller 1988). In bacterial wilt of tomato caused by *Ralstonia* (previously *Pseudomonas*) *solanacearum,* it has been demonstrated that beneficial (non-pathogenic) pseudomonads may provide sufficient disease management. Furuya used specific *Pseudomonas aeruginosa* to colonize tomato roots. This led to increased tomato seedling survival in laboratory conditions (Furuya et al. 1997).

Competition is an interaction where two organisms compete for resources. In disease management, the biocontrol organism outcompetes the target pathogen for either substrates or physical space, hindering its ability to cause disease in the host (Lorito et al. 1994). Demonstrating nutrient uptake dynamics among microorganisms can be a daunting challenge (Handelsman and Stabb 1996; Nelson 1991; Nelson and Craft 1991). There has

been conflicting data on biosynthesis of pyoverdine (a siderophore) by *P. fluorescens* and its contributions to pathogen suppression (Baker and Cook 1974; Handelsman and Stabb 1996; Nelson and Craft 1991). Some studies have shown the efficacy of siderophore production on damping off caused by *Pythium ultimum* (Baker and Cook 1974; Loper 1988; Loper and Buyer 1991). In these studies, siderophore production was shown to control *P. ultimum* when researchers used a single Tn5 insertion in the *P. putida* strain WC358 that activated both pyoverdine and plant growth promotion (Baker and Cook 1974). Baker et al. showed a 13% yield increase in some fields with the addition of WCS358 isolates. The manipulation of siderophores through the use of Tn5 manipulation has not always proven so efficacious. For instance, some studies indicate little or no efficacy using Tn5 mutants of the *P. putida* strain N1R in suppression of *Pythium* species (Hamden 1991, (Keel et al. 1989; Paulitz and Loper 1991). In Paulitz et al., a Tn5 mutant of *P. putida* strain N1R, deficient in pyoverdine production, appeared to have no influence on *Pythium* damping off in cucumber (Paulitz and Loper 1991).

Parasitism occurs when one organism uses another as a nutrient source without an exchange of resources. Parasitism between two fungi is referred to as mycoparasitism (also known as hyperparasitism) (Baker and Cook 1974). Mycoparasitism is a four-step process starting with chemotropic growth (Handelsman and Stabb 1996; Lam and Gaffney 1993; Lo 1998; Tunlid et al. 1992). Chemical stimuli produced by the pathogenic fungus are recognized by the biocontrol fungus, stimulating growth towards it. Chemotropic growth is followed by a recognition step where there is recognition of the target pathogenic fungi by the biocontrol fungi through lectins on the pathogenic fungi and carbohydrates receptors located on the biocontrol fungi (Deacon and Berry 1992; Inbar and Chet 1992; Inbar and

Chet 1994). The biocontrol fungus then attaches to the target and begins cell wall degradation with enzymes such as chitinases and β-1,3-glucanase. The last step is penetration of the target organism. The biocontrol fungus produces an appressorium-type structure in order to penetrate the target fungus.

Cell wall degrading enzymes also represent a mode of action. These hydrolytic cell wall-degrading enzymes are produced by microbes extracellularly (Lam and Gaffney 1993). These enzymes target fungal cell wall components such as chitin and b-1,3-glucans with enzymes such as chitinase and β-1,3-glucanase either alone or in combination (Lam and Gaffney 1993).

The last mode of action available for a biocontrol is the induction of plant defense. This can happen at either the local or systemic level (Junaid et al. 2013). This mode of action has been observed with the use of *B. subtilis* in fungicide formulations. The presence of *B. subtilis* was shown to stimulate phytohormones to induce plant disease defense mechanisms (Haidar et al. 2016a; Lastochkin et al. 2019a). The induced defenses remained for anywhere from a few days to weeks in the plant. In tomatoes, some *Trichoderma* strains have been shown to induce resistance. This resistance can be seen up to 14 days post *Trichoderma* inoculation (Junaid et al. 2013; Saksirirat et al. 2009).

1.14 Novel Chemical Treatments

Another potential example of a non-living microorganism serving as a biopesticide is microbial fermentation products (MFP). MFP contain multiple components that have been identified as elicitors of plant defense. One such component is yeast cell walls which are derived from the brewing process (Yaguchi et al. 2017). Budding yeasts, such as

Saccharomyces pastorianus, a bottom fermentation yeast commonly used in the brewing industry, are utilized for cell-wall components. Both polysaccharides glucan and mannan are components of yeast cell walls. These display similarities with polysaccharides produced by pathogens which may elicit a defense response (Minami et al. 2011; Yaguchi et al. 2017). Yeast cell wall components produced as part of an MFP have an additional advantage for organic crop production. MFP may be viewed as organic, whereas isolated products such as glucan or mannan may not be. Consequently, MFP may be allowed for organic crop production whereas isolated inducers of plant defenses may not be.

Another microbial product derived from prokaryotic glutamate fermentation was also found to elicit a defense response (Chen et al. 2014; Twamley et al. 2019). Peptidoglycan is an essential component specific to bacteria and has been found to elicit a defense response in tobacco, tomato, and rice (Chen et al. 2014). Additionally, lactic acid bacteria, also involved with the fermentation processes, have been shown to prevent fungal disease in the field in cereal crops (Oliveira et al. 2014). Some cyclic dipeptides isolated from lactic acid bacteria (LAB) broth have shown potential antifungal activity (Oliveira et al. 2014). However, as whole cultures or an MFP, this activity is dependent on growth media, the temperature and incubation time, the pH, nutritional factors, etc. (Oliveira et al. 2014).

Although many products marketed have a clearly defined ingredients and mode(s) of action, this is not true for MFPs. With an increasing amount of bioproducts such as MFPs, that have more than one ingredient, defining the specific mode of action becomes difficult. In some cases, these product components can work independently or in an

additive or synergistic nature (Bernsdorff et al. 2016; Conrath 2009; Conrath et al. 2015; Návarová et al. 2012; Sharma et al. 2014).

1.15 Integrated Pest Management Programs

Management strategies for tomato systems typical involve the utilization of multiple different methods of control. Cultural controls, as previously mentioned, are used as preventative measures before the employment of chemical strategies. Nonetheless, additional treatment is frequently required in the control of plant diseases. When creating a treatment program there are a few key guidelines recommended by the Fungicide Resistance Action Committee (FRAC 2021) one of which is to not use one product exclusively. FRAC recommends to apply products as a mixture with multiple different modes of action or as a single product in a rotation or alternation with other products (Brent and Hollomon 2007). This method will reduce the selection pressure produced by an 'at-risk' modes of action and prevent the development of resistant strains. An 'at-risk' mode of action is an active ingredient that has a higher likelihood of pathogen resistance development, such as QoI (quinone outside inhibitors) fungicides. They also recommend not to overuse a product but to reduce the number of applications and apply only when necessary (Brent and Hollomon 2007; Brent and Hollomon 1995). To ensure the use of alternate modes of action, the FRAC code of a product should be referenced. If the FRAC codes are the same, then the product listed has the same mode of action.

1.16 Tomato Disease Management

1.16.1 Early Blight

Early blight can be managed to some extent through cultural methods. There are resistant varieties of tomatoes available, however these do not have complete resistance (Batista et al. 2006). Other forms of cultural control available for early blight management include crop rotation for at least two years into a non-host crop. It is also necessary to control host weeds such as black nightshade and hairy nightshade. Avoiding leaf wetness also reduces the favorable environment for the pathogen. This is achieved by reducing any overhead irrigation or using drip tape under plastic or mulch. Mulch can also reduce rain dispersal. Leaving adequate space between plants can also reduce leaf wetness. Products such as coppers and mancozeb have shown efficacy in reducing disease severity, but other products are other available that have varying degrees of efficacy. The University of Kentucky 'Vegetable Production Guide for Commercial Growers' (ID-36) recommends increased application frequency during times of prolonged leaf wetness (Coolong et al. 2009; Jones et al. 2014).

1.16.2 Powdery Mildew

Powdery mildew is an obligate biotrophic pathogen requiring a living host to survive. Removal of any susceptible host plants can prevent further spread of the pathogen. A crop rotation is also recommended. Powdery mildew conidial spores do not have the same leaf wetness requirements as early blight making irrigation control less vital (Warren

et al. 2015). However, humidity levels can have a great influence on either conidial spore production and mycelial expansion (Jacob et al. 2008). There are very few tomato varieties with described powdery mildew resistance (Warren et al. 2015). Chemical management of systemic fungicides typically show some efficacy along with the use of copper-based products (Coolong et al. 2009; Jones et al. 2014).

1.16.3 Bacterial Spot

Bacterial spot is also managed through the use of resistant varieties of tomato, although these are limited. Other methods include the use of disease-free transplants and seeds. Management of leaf wetness is critical for breaking the bacterial spot disease cycle, consequently the use of overhead irrigation methods should be limited and mulches used when available. Mulches have the added advantage of limited rain splash dispersal as well adding a barrier between the plants and other soil associated pathogens. High pressure sprays can also cause plant injury which allows the pathogen to enter the plant. Tools should be cleaned in between uses as injury is a method of entry into the tomato for this pathogen. Trellising lines can also cause plant injury which become source of entry into the plant. For chemical management of bacterial spot in Kentucky, Actigard®, copper-based products, mancozeb are recommended on seven to 14 day schedules (Coolong et al. 2009; Jones et al. 2014).

1.17 Summary

Continuously developing new efficacious pathogen management methods is vital to the long-term sustainability of agriculture. Current treatments available for many tomato

diseases such as bacterial spot, early blight, or powdery mildew are frequently dependent on direct modes of action on the bacterial pathogen. While these modes of action can be efficacious, they also have a greater risk resistance development in the plant pathogen. With some treatments over-reliant on copper-based products, environmental contamination with high levels of copper can occur. Newer type products frequently target the natural disease responses of plants as opposed to exhibiting a direct mode of action on the pathogen. Some of these products include the use of living microorganisms such as *Bacillus* sp., which may provide multiple modes of action including the induction of natural plant defenses. Other newer products include MFPs. The actual function of MFPs is not well understood. MFPs may contain cell walls or other components/chemicals related to microbial fermentation. Although hypothesized to induce plant defenses, their complex nature may involve multiple modes of action which can include both direct and indirect action on plant pathogens. In this study, the efficacy in the field and modes of action of one MFP was examined. The MFP was tested using tomatoes as the model plant system. Tomatoes are grown in a variety of different environments such as open fields and protected environments like high tunnel allowing a broad examination of the efficacy of this product. Several important diseases of tomatoes were followed to establish efficacy over a wide range of pathogens. Further examination of mode of action was also examined in a laboratory environment using tomato model systems.

CHAPTER 2. CONVENTIONAL AND ORGANIC SPRAY PROGRAM EFFICACY IN BACTERIAL AND FUNGAL TOMATO PATHOSYSTEMS

2.1 Introduction

Public interest in organic farming, and the importance of disease management in conventional farming, have increased in importance in recent years (Van Bruggen et al. 2016). Attention to organic farming methods can be connected to public concern over reducing negative impacts of agriculture (Van Bruggen et al. 2016). The global value of the organic market reached $72 billion in 2013 (Willer and Lernoud 2019). Specific organic farming standards can be seen in detail in the USDA's National Organic Program 'Organic Regulations' (USDA Agricultural Marketing Service 2021). Organic plant disease management consists of more than just application of (Organic Materials Review Institute) OMRI-certified products. It can involve cultivating diverse plant populations with increased spacing, soil health promotion through crop rotation, use of living organisms for disease and pest control, and the use of organic fertilizers (USDA Agricultural Marketing Service 2021). Often the use of chemical controls is a last resort for organic growers. Chemical options available to organic growers are often sourced from plant extracts or substances produced by bacteria or other organisms. Some can also be mined, like some natural ore products.

One commodity of considerable importance to organic food production is tomato, particularly those destined for the fresh market. Both conventional and organic tomatoes have an economic importance worldwide. They are the second most consumed vegetable in the world after potato (Bergougnoux 2014). Worldwide production reached almost 160

million tons in 2011 alone (Anwar et al. 2019; Bergougnoux 2014). Tomatoes also have high popularity within organic production (Kaiser 2016). Furthermore, consumers are constantly seeking new varieties with unique flavors (Kaiser 2016).

Both organic and conventional tomato production face the same disease issues. Fungal pathogens such as *Alternaria tomatophila*, one of the pathogens that can cause tomato early blight, can be extremely destructive to tomato production (Chaerani and Voorrips 2006). *Alternaria tomatophila* can cause as much at 79% yield loss in tomatoes (Chaerani and Voorrips 2006). Methods for disease management include standard practices such as sanitation, use of resistant or partially resistant varieties, and crop rotation (Zhan et al. 2014). All these methods are available to both conventional and organic growers.

In addition to fungal pathogens such as *A. tomatophila*, bacterial pathogens are also of concern to both organic and conventional growers. *Xanthomonas euvesicatoria* can cause tomato bacterial spot, resulting in up to 50% yield loss (Kunwar et al. 2018). Cultural practices for disease management can be similar to those mentioned for early blight (Obradovic et al. 2005).

Although implementation of cultural practices for disease management in both conventional and organic agriculture may be preferred, additional chemical approaches may still be required to reduce yield losses. Unsurprisingly, conventional and organic systems can have different products available to them. Many products available to conventional farming, while efficacious, are not permitted for organic production. As a consequence, far fewer efficacious products are available in organic production. Of the products currently marketed for organic use, as seen on the Organic Materials Review Institute (OMRI) list (https://www.omri.org/omri-lists), two that enjoy popular usage are

living organisms and copper-based products such as *Bacillus* sp. and Nordox®, respectively. Living organisms for disease management may function through competition, hyperparasitism, or more directly through production of antimicrobial compounds that may inhibit the growth of plant pathogens (Ram et al. 2018). Such application of living microorganisms may also function indirectly through activation of plant defenses (Ram et al. 2018). The copper-based products can provide a more direct form of protection by directly inhibiting pathogen life cycles. A direct comparison of organic and conventional spray programs in comparable tomato growth environments has not been well documented.

In this study, two spray programs were compared: a standard conventional spray program similar to what would be used commercially in Kentucky and a spray program designed to represent a typical organic-based spray program. The comparison was based on each method's ability to reduce the disease severity of either *A. tomatophila* or *X. euvesicatoria.* Both disease severity and yields effects were examined.

2.2 Methods/Materials

2.2.1 Culture, cultivation, and inoculum preparation

2.2.1.1 Alternaria tomatophila

Alternaria tomatophila was isolated from diseased tomatoes in Lincoln County, KY, in 2006. *A. tomatophila* samples were inoculated on ¼ potato dextrose agar (PDA) media. Stocks were originally stored on autoclaved filter paper at -20° C. Plates were grown in 23° C, 12 hour day/night light conditions with a combination of florescent and

black light/blue light bulbs. After seven days slits were aseptically cut in the cultures to promote sporulation. The plates were then harvested ten days later. Conidial spores were collected by flooding the plates with 10 +/- 0.5 mL autoclaved DI water. Conidia were dislodged with an autoclaved pestle. The solution was poured into a secondary container through sterile cheesecloth. Inoculum concentration was determined using a hemocytometer. The concentration for inoculum in 2017 was $1x10^4$ conidia per mL. The inoculum concentration for 2018 was $5.5x10^5$ conidia per mL Each plot received approximately ~35mL +/- 2mL per plot.

2.2.1.2 Xanthomonas euvesicatoria

Isolates of *X. euvesicatoria* were originally isolated from Calloway County, KY from tomato fields exhibiting bacterial spot. Cultures were streak-plated (Wise 2006) on LB agar (VWR Radnor, PA) and grown in a dark temperature-controlled chamber (VWR Personal Low Temperature Incubator VWR Cat. No 89511-416) at 27 +/- 1° C for two days. Inoculum was collected by flooding plates with 10 mL of a sterile potassium phosphate buffer pH 7.4 +0.15 (VWR, 0.05M) and gently rubbing an autoclaved pestle on the surface of the media. Inoculum concentration was determined by using a spectrophotometer at OD_{600} and by diluting appropriately in phosphate buffer (VWR) and spread plating (Wise 2006) on LB agar. Final concentrations were based on 48-h colony counts on LB agar. The concentration of inoculum used in all fields was 2 $x10^8$ CFU/mL. The 2019 field was inoculated on June 19th 2019 and both 2020 fields were inoculated on June 19th 2020.

2.2.2 Plant material

2.2.2.1 2017-2018

Tomatoes used were ‘Rutgers’ variety (W. Atlee Burpee & Co., Warminster, PA). Plants were grown in 72 cell flats for six weeks (15.2 cm – 20.3 cm tall) in a greenhouse. Plants were left to harden outside for 2-3 days then transplanted into the field.

2.2.2.2 2019-2020

The tomato variety used for the field experiments in 2019 and 2020 was ‘Sunstart’ variety (W. Atlee Burpee & Co., Warminster, PA). Plants were grown in 72 cell flats for six weeks (15.2 cm – 20.3 cm tall) in a greenhouse. Plants were left to harden outside for 2-3 days then transplanted into the field.

2.2.3 Field sites

2.2.3.1 2017-2018

Fields were located on the University of Kentucky Spindletop Farm Lexington, KY. Plots were arranged in a randomized complete block design, with four blocks (replicates) for each application. Tomato plants were set at 0.46 meters (18 inches) in-row spacing. Plots were comprised of six plant per plot. Each plot was separated by 1.8 +/- 0.1 meters apart.

2.2.3.2 2019-2020

A single location was used, the University of Kentucky Spindletop Farm Lexington, KY, during 2019. In 2020, two separate locations were used; one field located at University of Kentucky Spindletop Farm and a second at the University of Kentucky Horticulture Research Farm in Lexington, KY. Tomato plants were set with the same spacing as for the 2017-2018 season. Each plot was considered a single replicate for each spray program. Four plots for each spray program were used at each site, resulting in 48 plants per spray program over two sites.

2.2.4 Spray Programs

The copper-based product used in this study was Nordox® 75WG (Brandt, Springfield, IL). Nordox® has an active ingredient of cuprous oxide at 83.9%. Nordox® was sprayed at the recommended concentration for tomatoes, 0.84 kg ai per 100 L water (2.79 kg/ha).

Companion® (Growth Products, Liberty, MO) was used as an application to investigate the use of a living organism. The active ingredient in Companion® is *Bacillus subtilis* GB03 (00.03% concentration in the formulated product). Companion® rates were also based on the product label for greenhouse use: 3.75 ml ai per 100 L water (125 ml product per 100 L water).

Actigard® (Syngenta, Basel, Switzerland) was used to represent a currently marketed plant systemic resistance inducer using the active ingredient BTH (50%

concentration in the formulated commercial product). It was sprayed at the recommended rates for tomatoes by the product label at 27.2 g ai/ha (4.94 g/ha).

The mancozeb product used for these trials was Dithane® (Corteva, Wilmington, Delaware). The mancozeb concentration within the product was 75%. The manufacturer's recommended tomato rates used were 0.45 kg ai per 100 L of water (1.68 kg/ha). The approximate area in each location that was treated was 3.5 meter^2 +/- 0.5.

The conventional spray program was comprised of Actigard®, Dithane® F-45 Rainshield, and Nordox®. All products were sprayed at the manufacturer's recommended rates as previously stated. The organic spray program was comprised of Companion® and Nordox® both of which are OMRI-certified.

All spray programs were applied at a spray volume of 30 gallons per acre (e.g. 1 gallon/acre = 9.35 L/Hectare) every 7-10 days at 45 PSI with a single-nozzle (TeeJet® conejet hollow cone spray tip TXVS-18) boom over the top of tomato plants for the first two applications. When the tomato plants were approximately two feet tall, the application volume was increased to 50 gallons per acre (1 gallon/acre = 9.35 L/Hectare) for all applications. When spray volumes were increased, a three-nozzle boom was used to ensure full coverage of the foliar tissue.

2.2.5 Field set-up and maintenance

2.2.5.1 2017-2018

As noted earlier, fields in both years were arranged in a randomized complete block design except that the in 2017 field trial, each spray program replicated five times and

whereas the 2018 field trail was replicated four times. The following applications: untreated control, conventional grower spray program (comprised of a tank-mix of Dithane®, Actigard®, and Nordox®), and organic grower spray program (comprised of a tank-mix of Nordox® and Companion®).

2.2.5.2 2019-2020

The 2019 field included the following applications: untreated control, conventional grower spray program, and organic grower spray program. All applications in 2019 were replicated four times whereas in 2020 they were replicated five times in each location. The 2020 field applications included untreated control, conventional spray program, and organic spray program. Additionally in 2020, the Spindletop irrigation drip tape was set about 10 cm +/- 5 cm deeper than the Horticulture Research Farm (set at about 10 cm +/- 1 cm. Insecticide (Radiant SC®, 365.4 mL to 730.79 mL product amt/ha) throughout 2017-2020 were distributed through the drip irrigation with the exception of the Horticulture Research Farm in 2020 which was done by bucket drench.

2.2.6 Disease rating

2.2.6.1 2017-2018

Plant disease severity ratings were recorded once per week after the initial inoculation until termination of the experiment. Ratings were based on visual assessment of disease coverage of the plant. The ratings were based on the Horsfall-Barratt rating system (Hebert 1982; Kranz 1988). These rating were then converted to a 0-100% scale.

2.2.6.2 2019-2020

Plant disease severity ratings were recorded as indicated for the 2017-2018 season with the following variation. Fields were rated on a continuous scale of 0-100% based on affected plant area, with 0% being no symptoms of disease and 100% being complete disease coverage or plant death.

2.2.7 Harvest evaluations

Fields were harvested three times over the course of the season. The first two harvests when taken fruits had begun to turn, and the remaining harvest was a "pick plants clean." Fruits were divided into three categories: marketable, unmarketable, and affected by bacterial spot. Marketability was evaluated based on USDA guidelines (USDA Agricultural Marketing Service 2022). The USDA has three separate grades of tomatoes, U.S. No. 1-3. Tomato marketability was determined by the fruit's ability to meet or exceed the requirements for U.S. No. 3 grade tomatoes. Any tomatoes that did not meet these requirements were considered unmarketable with the exception of any tomato displaying symptoms of bacterial spot. Tomatoes exhibiting such symptoms were placed into the bacterial spot category. Total number of fruits, the total weight of the tomatoes, and the average weight (calculated) of a single tomato from the specified spray program were recorded.

2.2.8 Statistical analysis

2.2.8.1 2017-2018

Field data were analyzed via analysis of variance using PROC GLM in SAS 9.3 (Cary, NC). Means were separated using Fisher's Protected Least Significant Difference (LSD, $P<0.05$).

2.2.8.2 2019-2020

Field data were analyzed via analysis of variance using PROC GLM in SAS 9.3 (Cary, NC). Means were separated using Fisher's Protected Least Significant Difference (LSD, $P<0.05$). If the P value was significant than all pairwise comparisons between treatments were examined through LSD.

2.3 Results & Discussion

2.3.1 Early Blight

In 2017 and 2018 (Table 2.1) the conventional and organic spray programs both showed statistically lower disease severity ($P<0.05$) against early blight than the untreated control. Additionally, in both years the disease severity ratings of the conventional and organic spray programs were statistically similar ($P>0.05$). It appears that both the conventional and organic spray programs reduced early blight disease severity to the same degree. Organic growers are limited in their options of products that can be used for control.

This program appears to be a possible option for control of *A. tomatophila* disease severity that is comparable to the conventional grower option.

In 2017 yield data (Figure 2.1) there were no statistically differences ($P>0.05$) between any of the spray programs in measures of total harvest. This applies to all variables that were measured: total number of tomatoes, total weight of tomatoes, and the average weight per tomato.

In the 2018 field season (Figures 2.2, 2.3, 2.4), some bins in the second harvest had been mis-labeled. Therefore, some replications in that harvest were dropped resulting in insufficient replication for statistical comparison in the second harvest. In the first harvest, the conventional and organic spray programs grouped statistically ($P<0.05$) the same in all categories.

In the organic spray program, the product Companion® was included. This product has been shown to induce plant defense genes (Haidar et al. 2016b; Lastochkin et al. 2019b; Moyne et al. 2001a; Romero et al. 2001). Plant defense inducers have been shown in other studies to commonly cause a yield drag effect (Adhikari et al. 2017; Egel et al. 2018). A yield drag is when there is a negative influence on yield based on a product application or genetic trait change through breeding. In these studies, with *A. tomatophila*, we did not observe any indications of yield drag in the organic spray program. Considering these yield and disease severity results, the proposed organic program may be a competitive option in comparison to conventional standards.

2.3.2 Bacterial Spot

In all locations in both 2019 and 2020 (Table 2.2), both conventional and organic spray programs had statistically significant ($P<0.05$) lower bacterial spot severity than the untreated control. Additionally in all locations over both years, the conventional and organic spray programs were statistically similar ($P>0.05$) to each other.

In 2020, there were slight differences among trials in field management. At Spindletop Farm, the drip tape had been laid 10 cm +/- 5 cm deeper than at the Horticulture Research Farm. This likely resulted in less water uptake by the tomato plants early in the season. The insecticide was also run through the drip tape at Spindletop so with the deeper drip-tape, the plants had likely received less insecticide during setting, resulting in higher insect pressures. Additionally, the Spindletop location exhibited increased weed pressure as compared to the Horticulture Research Farm. These overall management differences probably resulted in higher plant stress levels at the Spindletop location in comparison to the Horticulture Research Farm. Despite these stress differences the conventional and organic tank-mixes still performed statistically similar ($P>0.05$).

In all yield metrics measured in both 2019 and 2020 (Figures 2.5, 2.6, 2.7, 2.8) there was no statistical difference between the conventional spray program, the organic spray program, and the untreated control ($P>0.05$). The metrics included were the same as the 2017 and 2018 field trials. As stated with the *A. tomatophila* trials, the organic program contained Companion® which has been shown to induce plant defense genes. Based on these harvest results, there does not appear to be any yield drag effects from the organic program at the concentrations and application timings tested here. Had there been a yield

drag, we would expect to see a reduction in yield as compared to the conventional control. To further elucidate if yield drag may be a factor to consider additional experiments with increasing amounts of Companion® would be necessary. However, in these experiments only the recommended rates were used, and it would be unlikely a grower would use a vastly differing rate.

2.3.3 Organic program vs Conventional program

Regardless of trial year, there were no differences between the conventional and organic spray programs between any of the metrics used in this study. Additionally, the inoculation of fungal or bacterial pathogen did not have an influence on the effectiveness of these spray programs. In the 2020 bacterial spot trial, the difference in field management also did not appear to create any differences between these two disease management methods. Additionally, during the applications conducted prior to pathogen inoculations, no adverse phytotoxic effects were observed. This indicates that there is unlikely to be adverse impact form the conventional and organic spray programs evaluated here.

The specific organic spray program that we are testing proved to be as effective as the proposed spray program for conventional growers against both *A. tomatophila* and *X. euvesicatoria*. It should be noted in the controls, disease pressure had reached a minimum of 45%, indicating high disease pressure. These experiments do not rule out the possibility of application effects at higher disease pressure or different environmental conditions. Nonetheless, this organic program could be useful for either conventional or organic growers. For conventional growers interested in more environmentally sustainable practices without sacrificing disease management ability, this may also be a viable option.

Other studies have also indicated the use of these efficacious OMRI-certified products in conventional programs to reduce resistance development in pathogen populations (Pethybridge et al. 2017).

In 1995 Drinkwater et al, had a similar comparison study where they looked at conventional and organic tomato practices (Drinkwater et al. 1995). Their study examined multiple factors that may influence efficacy differences between organic and conventional practices such as; soil type influences, disease severity, soil microbial communities, yield, and arthropod damage/communities. There are advantages to examining all these influential factors as they can impact overall efficacy, however, it makes it difficult to determine if a single factor or multiple factors influence product efficacy. Additionally, alternative locations may be required to provide adequate environmental differences for more comprehensive testing. In this study spray programs were applied at the same time to the same field. This allows for the control of factors such as soil type, management practices, and disease/insect pressures. This, in turn, allows for a more controlled examination of disease product efficacy. In order to determine if these other factors may have influenced efficacy with the examined products, additional studies would be required.

This study examines only one conventional spray program and one organic spray program. To make an accurate comparison of efficacy between conventional and organic spray programs in tomatoes more types of programs should be included. Programs could be chosen based on surveys of Kentucky conventional and organic tomatoes growers. This survey would help determine what are the most typical programs used in both systems so these could be compared.

Another consideration with respect to efficacy of the spray programs is the inclusion of Nordox® W75 (copper) in both. This product, which includes cuprous oxide as the active ingredient, could have caused the similar efficacy of the spray programs used here. Further research would be required to determine if copper alone caused the efficacy observed in our organic program. Copper-based products are available in both conventional and organic systems and are recommended for use against a variety of pathogens. However, programs that reduce pathogen resistance development should contain multiple modes of action. Further studies could be undertaken to examine how efficacious these presented programs would be with a decreased reliance on copper-based products.

Copper-based products, such as Nordox®, have been under increased scrutiny for contributions to increased Cu levels in soil (Lamichhane et al. 2018). These increased Cu levels can have negative impacts on some soil-borne biota such as some microorganisms, earthworms, nematodes, and snails (Eijsackers et al. 2005; Giller et al. 1998; Jaworska and Gorczyca 2002; Rogevich et al. 2008; Van Zwieten et al. 2004). These adverse accumulations have a high chance of remaining in certain types of soils for generations (Van Zwieten et al. 2004). Drinkwater et. Al had suggested that the soil microbiome community is both influenced by the management method and leads to a reduction in specific disease pressures such as pythium and corky root rot. Due to issues surrounding repeated use of copper it is suggested that further studies aim at the identification of alternative organic products to replace the use of copper.

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Table 2.1 Means of Early Blight Disease Severity in Tomatoes for Evaluation Dates at Spindletop Farm for 2017 and 2018

a. Means of early blight severity for each date (2017)

Spray Program	June 29th	July 12th	July 21st	Aug. 2nd	Aug. 7th	AUDPC
UTC	0 a	33 b	67 b	92 b	95 b	1039 a
Conventional	0 a	2 a	4 a	12 a	35 a	341 b
Organic	0 a	2 a	5 a	14 a	36 a	430 b

b. Means of early blight severity for each date (2018)

Spray Program	June 28th	July 5th	July 10th	July 20th	July 27th	AUDPC
UTC	2 a	27 b	37 b	54 b	94 b	1186 a
Conventional	1 a	4 a	4 a	8 a	8 a	142 b
Organic	1 a	7 a	5 a	11 a	10 a	245 b

Table 2.1. Means of early blight disease severity in tomatoes for evaluation dates ($P<0.05$) at Spindletop Farm for 2017 and 2018. 1a: 2017. 1b: 2018.Ratings were based on a Horsfall-Barratt scale and then converted to percentages with 0% being no disease symptoms and 100% being complete plant death. Analysis of variance (ANOVA) was completed using PROC GLM Statistical Analysis System, SAS). Means were separated using Fisher's Protected Least Significant Difference (LSD). UTC is the uninoculated control. AUDPC is the area under the disease progress curve. The conventional spray program was made up of a tank-mix of Nordox® (ai cuprous oxide), Actigard® (ai BTH), and Dithane® (ai mancozeb). The organic spray program was comprised of a tank-mix of Companion® (ai *Bacillus subtilis*) and Nordox®. Applications were made every 7-10 days.

Table 2.2 Means of Bacterial Spot Disease Severity in Tomatoes for Evaluation Dates at Spindletop Farm for 2019 and 2020

a. Means of bacterial spot severity for each date from Spindletop Farm (2019)

Treatment	June 25th (6 dpi)	July 1st (12 dpi)	July 9th (20 dpi)	July 16th (27 dpi)	July 23rd (34 dpi)	July 29th (40 dpi)	Aug. 8th (50 dpi)	AUDPC
UTC	0 a	6 a	5 a	5 a	10 a	35 a	44 a	710 a
Conventional	0 a	2 b	1 b	1 b	2 b	14 b	17 b	251 b
Organic	0 a	1 b	1 b	1 b	2 b	17 b	20 b	285 b

b. Means of bacterial spot severity for each date from Spindletop Farm (2020)

Treatment	July 10th (21 dpi)	July 15th (26 dpi)	July 21st (32 dpi)	July 28th (39 dpi)	Aug. 7th (49 dpi)	Aug. 13th (55 dpi)	AUDPC
UTC	4 a	4 a	13 a	22 a	49 a	87 a	953 a
Conventional	0 b	1 b	3 b	6 b	8 b	17 b	195 b
Organic	0 b	1 b	5 b	5 b	18 b	23 b	295 b

c. Means of bacterial spot severity for each date from Horticulture Research Farm (2020)

Treatment	July 10th (21 dpi)	July 15th (26 dpi)	July 21st (32 dpi)	July 28th (39 dpi)	Aug. 7th (49 dpi)	Aug. 13th (55 dpi)	AUDPC
UTC	4 a	4 a	7 a	19 a	39 a	48 a	683 a
Conventional	0 b	1 b	0 b	6 b	10 b	21 b	166 b
Organic	1 b	0 b	1 b	8 b	16 b	26 b	258 b

Table 2.2. Means of bacterial spot severity from Spindletop Farm bacterial spot trials in 2019 and 2020. Ratings were done on a 0-100% basis with 0% being no disease symptoms and 100% being complete plant death. Analysis of variance (ANOVA) was complete using PROC GLM. Means were separated using Fisher's Protected Least Significant Difference (LSD). UTC is the uninoculated control. The conventional spray program was made up of Nordox® (ai cuprous oxide), Actigard® (ai BTH), and Dithane® (ai mancozeb). The organic spray program was comprised of Companion® (ai *Bacillus subtilis*) and Nordox®. Spray programs were applied as tank mixing with all listed products being applied every 7-10 days. DPI stands for days post inoculation. 2a. bacterial spot disease severity rates for the 2019 tomato field. 2b. bacterial spot disease severity rates for the 2020 Spindletop Farm tomato field. 2c. bacterial spot disease severity rates for the 2020 Horticulture Research Farm tomato field.

Figure 2.1 Tomato Harvest Data from Spindletop Farm 2017 Early Blight Disease Severity Trial

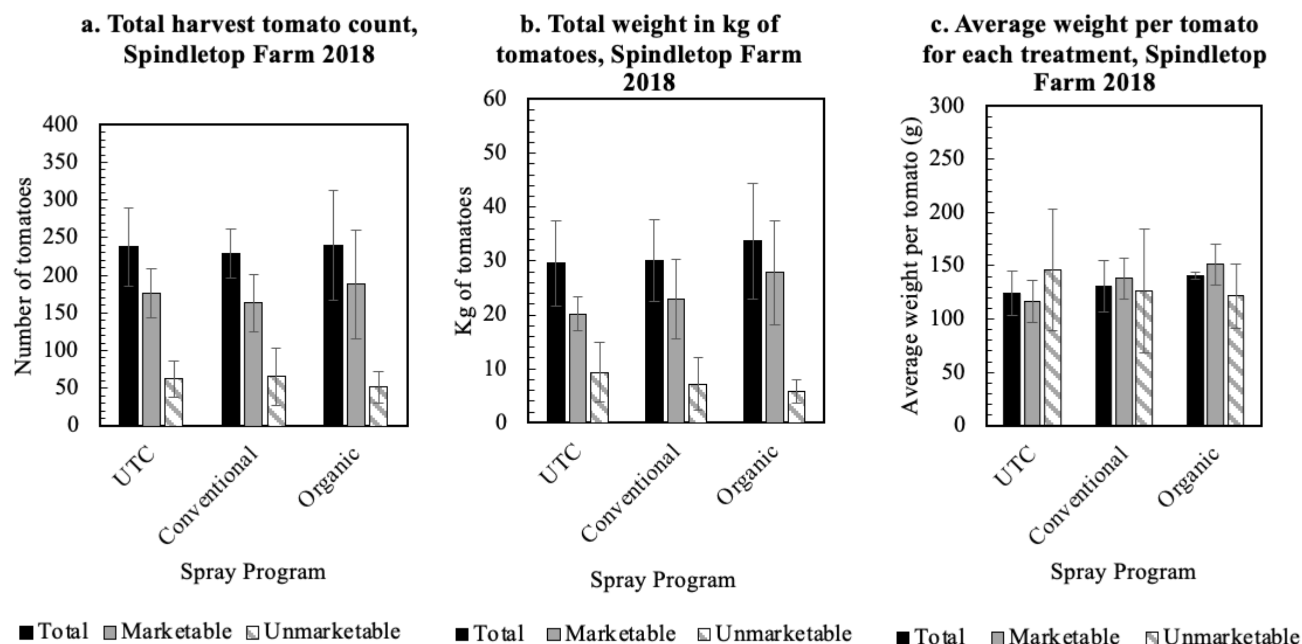


Fig. 2.1. Tomato harvest data from Spindletop Farm 2017 early blight disease severity trial. Marketable tomatoes were any tomatoes that were U.S. grade 1-3. All other tomatoes were considered unmarketable. Bars displayed show standard deviation. 1a. total number of tomatoes harvests for each treatment. 1b. total weight in kg of tomatoes harvested from each treatment. 1c. average weight in kg of a single tomato from each treatment. Spray program calculations were done by plot. The conventional spray program was made up of a tank mix of Nordox® (ai cuprous oxide), Actigard® (ai BTH), and Dithane® (ai mancozeb). The organic spray program was comprised of a tank-mix of Companion® (ai *Bacillus subtilis*) and Nordox®. Spray programs were applied as tank mixing with all listed products being applied every 7-10 days. Numbers and weights calculated by plot.

Figure 2.2 First Tomato Harvest Data from Spindletop Farm Early Blight Trial in 2018

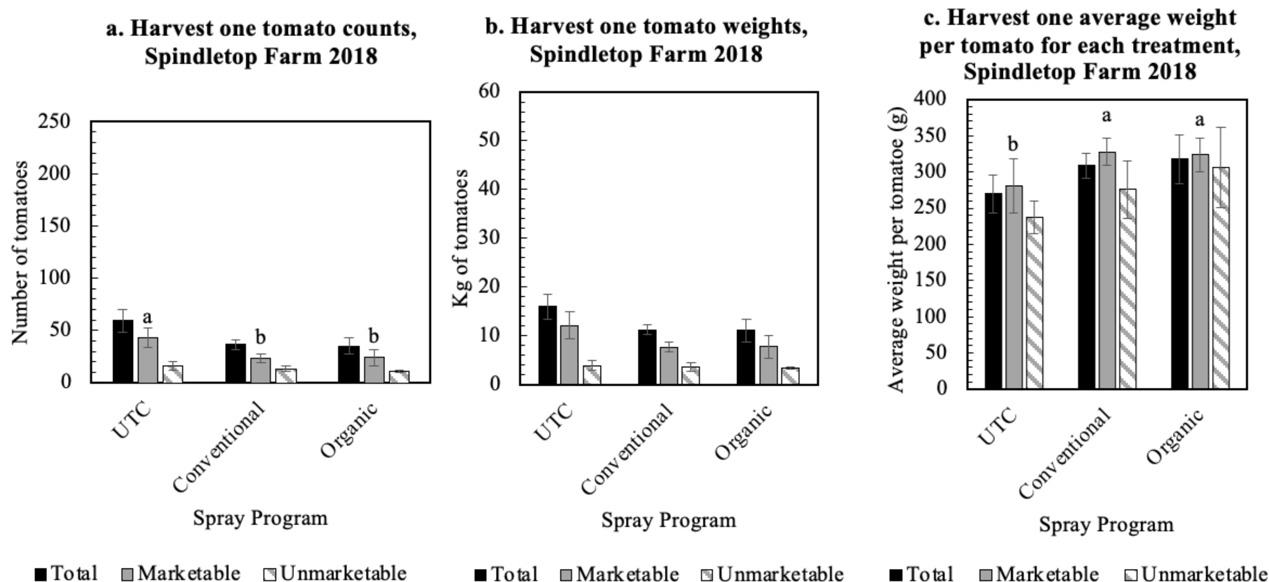


Fig. 2.2. The first tomato harvest (July 19th) data from the Spindletop Farm early blight trial in 2018. Marketable tomatoes were any tomatoes that were U.S. grade 1-3. All other tomatoes were considered unmarketable. Bars displayed show standard deviation. 2a. total number of tomatoes harvested for each treatment within the first harvest in 2018. 2b. total weight in kg of tomatoes harvested from each treatment for the first harvest in 2018. 2c. average weight of a single tomato from each treatment within the first harvest of 2018. Spray programs calculations were done by plot. The conventional spray program was made up of Nordox® (ai cuprous oxide), Actigard® (ai BTH), and Dithane® (ai mancozeb). The organic spray program was comprised of Companion® (ai *Bacillus subtilis*) and Nordox®. Spray programs were applied as tank mixes with all listed products being applied every 7-10 days.

Figure 2.3 Second Tomato Harvest Data from Spindletop Farm Early Blight Trial in 2018

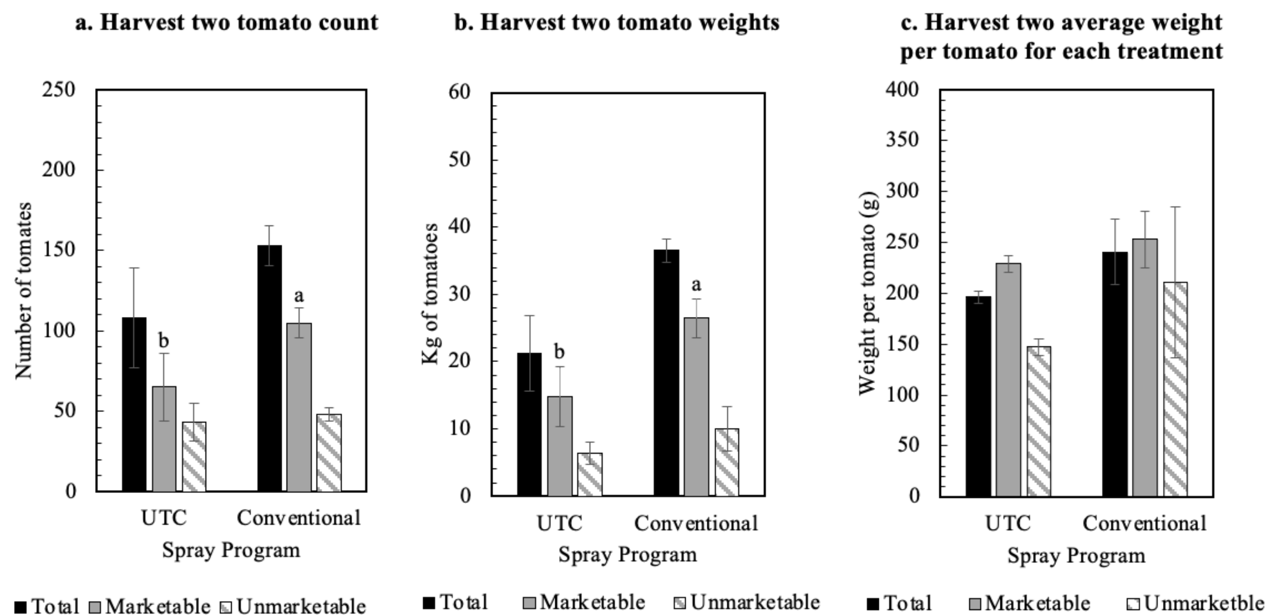


Fig. 2.3. The second tomato harvest (July 28th) from Spindletop Farm 2018 early blight severity trials. Marketable tomatoes were any tomatoes that were U.S. grade 1-3. All other tomatoes were considered unmarketable. Bars displayed show standard deviation. 3a. total number of tomatoes harvests for each treatment within the second harvest in 2018. 3b. total weight in kg of tomatoes harvested from each treatment for the second harvest in 2018. 3c. average weight in g of a single tomato from each treatment within the second harvest of 2018. Organic spray program was omitted from these data as some tomatoes were mislabeled and therefore were removed from statistical analysis. This and resulting in insufficient data for statistical analysis. Spray programs calculations were done by plot. The conventional spray program was made up of Nordox® (ai cuprous oxide), Actigard® (ai BTH), and Dithane® (ai mancozeb). Spray programs were applied as tank mixing with all listed products being applied every 7-10 days.

Figure 2.4 Total Harvest Data from Spindletop Farm 2018 Early Blight Trials

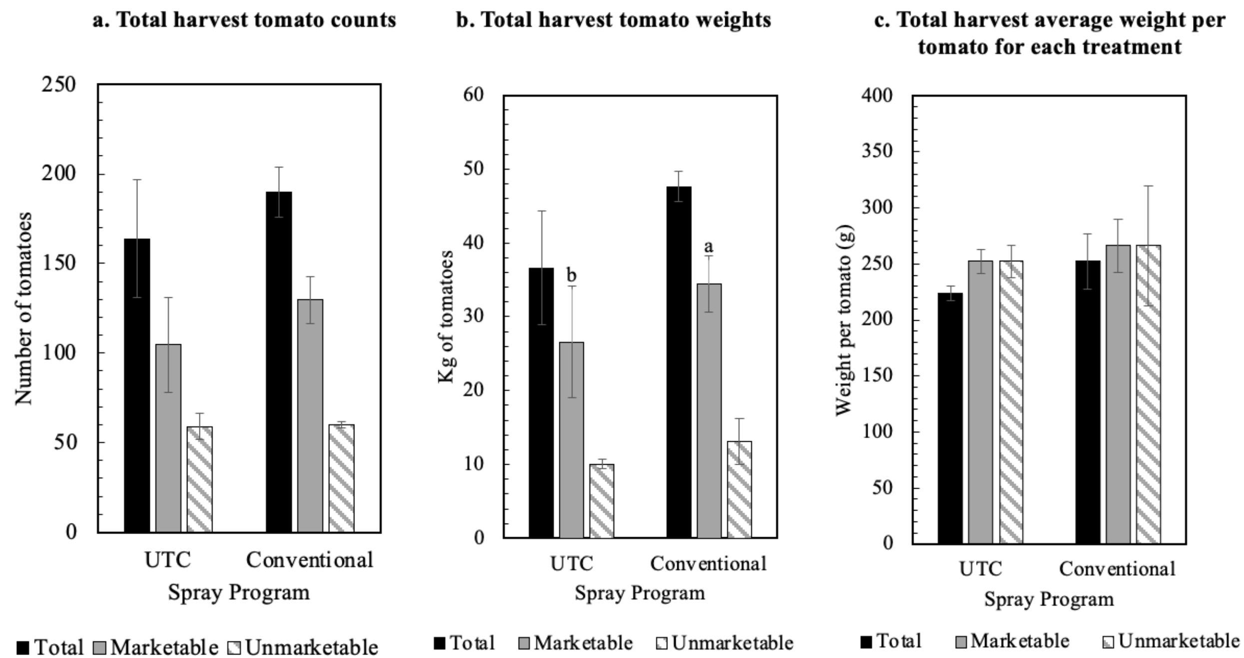


Fig. 2.4. Total harvest data from Spindletop Farm 2018 early blight trials. Marketable tomatoes were any tomatoes that were U.S. grade 1-3. BS was any fruit displaying bacterial spot symptoms. All other tomatoes were considered unmarketable. Bars displayed show standard deviation. 4a. total number of tomatoes harvests for each treatment. 4b. total weight in kg of tomatoes harvested from each treatment. 4c. average weight in g of a single tomato from each treatment. Spray programs calculations were done by plot. Organic spray program was omitted from these data as some tomatoes were mislabeled and therefore were removed from statistical analysis. This and resulting in insufficient data for statistical analysis. Spray programs calculations were done by plot. The conventional spray program was made up of Nordox® (ai cuprous oxide), Actigard® (ai BTH), and Dithane® (ai mancozeb). Spray programs were applied as tank mixing with all listed products being applied every 7-10 days.

Figure 2.5 Total Harvest Data from Spindletop Farm Bacterial Spot Tomato Trials in 2019

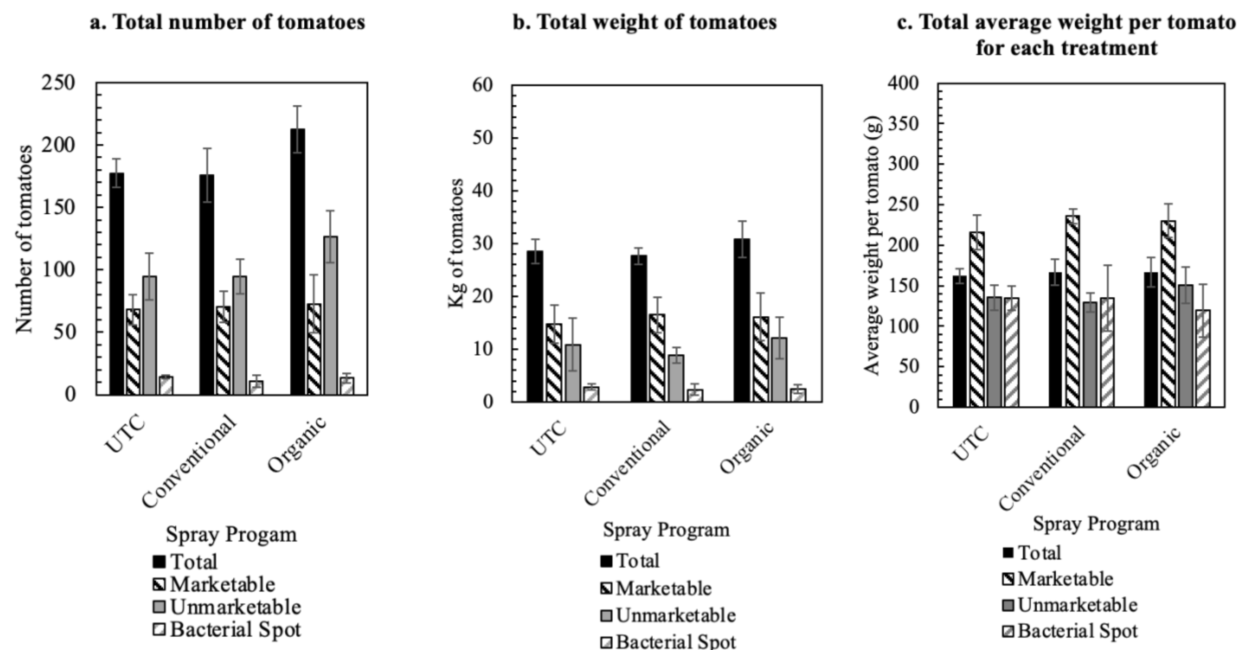


Fig. 2.5. Total harvest data from the Spindletop Farm bacterial spot tomato trials in 2019. Marketable tomatoes were any tomatoes that were U.S. grade 1-3. BS was any fruit displaying bacterial spot symptoms. All other tomatoes were considered unmarketable. Lines indicate standard deviation. 5a. total number of tomatoes harvests for each treatment. 5b. total weight in kg of tomatoes harvested from each treatment. 5c. average weight in g of a single tomato from each treatment. Spray programs calculations were done by plot. The conventional spray program was made up of Nordox® (ai cuprous oxide), Actigard® (ai BTH), and Dithane® (ai mancozeb). The organic spray program was comprised of Companion® (ai *Bacillus subtilis*) and Nordox®. Spray programs were applied as tank mixing with all listed products being applied every 7-10 days.

Figure 2.6 Total Number of Tomato from Spindletop Farm and Horticulture Research Farm Bacterial Spot Trials in 2020

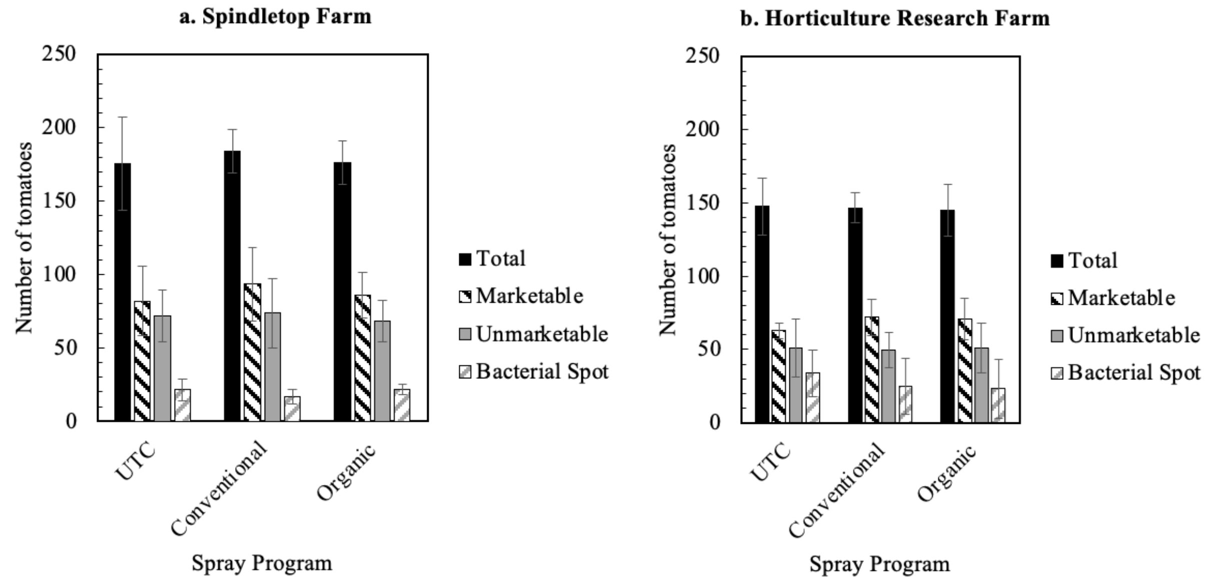


Fig. 2.6. Total number of tomatoes from 2020 Spindletop Farm and Horticulture Research Farm trials. Marketable tomatoes were any tomatoes that were U.S. grade 1-3. BS was any fruit displaying bacterial spot symptoms. All other tomatoes were considered unmarketable. 6.a tomato counts from the Spindletop Farm trial. 6.b. tomato counts from the Horticulture Research Farm trial. Lines indicate standard deviation. Spray programs calculations were done by plot. The conventional spray program was made up of Nordox® (ai cuprous oxide), Actigard® (ai BTH), and Dithane® (ai mancozeb). The organic spray program was comprised of Companion® (ai *Bacillus subtilis*) and Nordox®. Spray programs were applied as tank mixing with all listed products being applied every 7-10 days.

Figure 2.7 Total Weight of Tomato from Spindletop Farm and Horticulture Research Farm Bacterial Spot Trials in 2020

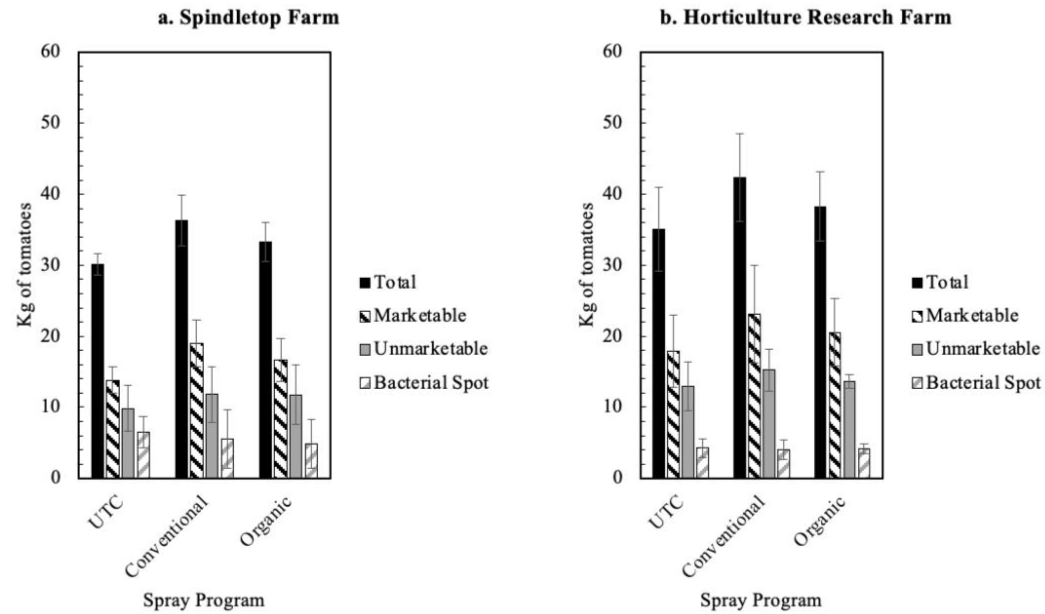


Fig. 2.7. Total weight of tomatoes from 2020 Spindletop Farm and Horticulture Research Farm field trials. Marketable tomatoes were any tomatoes that were U.S. grade 1-3. BS was any fruit displaying bacterial spot symptoms. All other tomatoes were considered unmarketable. 7.a tomato weights from the Spindletop Farm trial. 7.b. tomato weights from the Horticulture Research Farm trial. Lines indicate standard deviation. Spray programs calculations were done by plot. The conventional spray program was made up of Nordox® (ai cuprous oxide), Actigard® (ai BTH), and Dithane® (ai mancozeb). The organic spray program was comprised of Companion® (ai *Bacillus subtilis*) and Nordox®. Spray programs were applied as tank mixing with all listed products being applied every 7-10 days.

Figure 2.8 Average Weight per Tomato from Spindletop Farm and Horticulture Research Farm Bacterial Spot Trials in 2020

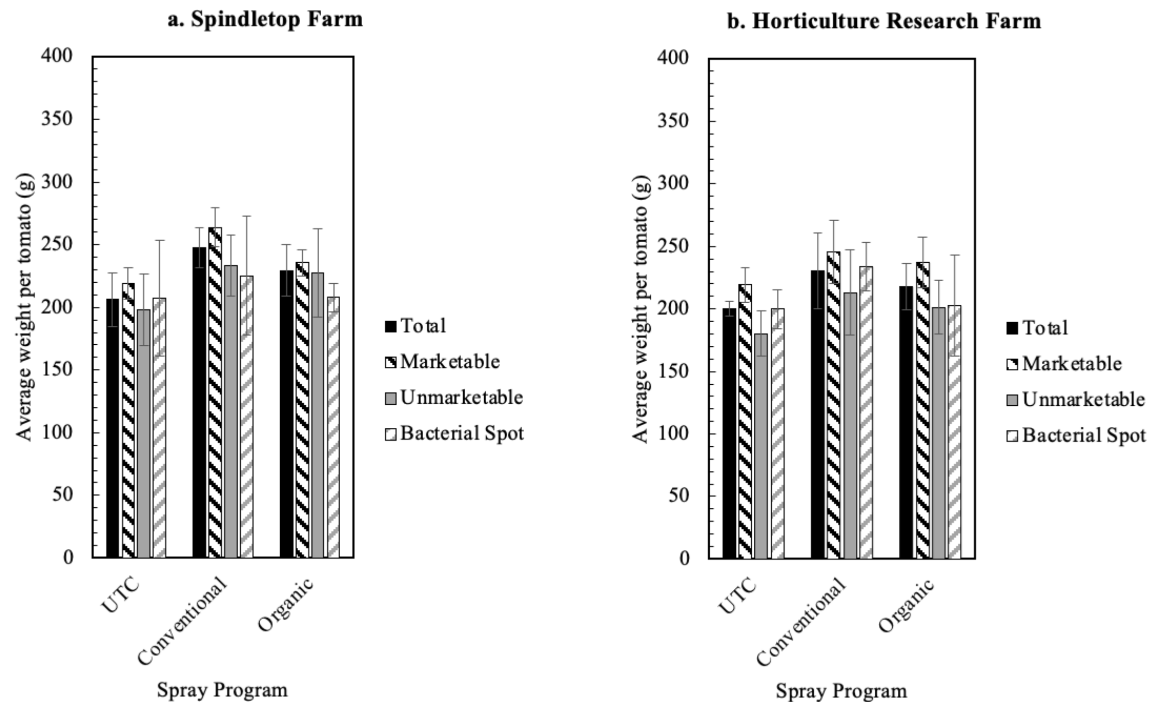


Fig. 2.8. Average weight per tomato from Spindletop Farm and Horticulture Research Farm 2020 field trials. Marketable tomatoes were any tomatoes that were U.S. grade 1-3. BS was any fruit displaying bacterial spot symptoms. All other tomatoes were considered unmarketable. 8.a average weight per tomato from the Spindletop Farm trial. 8.b. average weight per tomato from the Horticulture Research Farm trial. Lines indicate standard deviation. Spray programs calculations were done by plot. The conventional spray program was made up of Nordox® (ai cuprous oxide), Actigard® (ai BTH), and Dithane® (ai mancozeb). The organic spray program was comprised of Companion® (ai *Bacillus subtilis*) and Nordox®. Spray programs were applied as tank mixing with all listed products being applied every 7-10 days.

CHAPTER 3. EFFICACY OF BIOPESTICIDES AS ALTERNATIVES TO COPPER FOR MANAGEMENT OF *OIDIUM NEOLYCOPERSICI* (POWDERY MILDEW) IN TOMATO HIGH TUNNEL SYSTEM

3.1 Introduction

Consumer demand for organically grown produce has shown double digit growth since 2020 (Donaldson 2021), with fruits and vegetables accounting for 37% of all organic sales in the USA (Kapoulas et al. 2011). Tomatoes are the second most consumed vegetable among organic produce sold (LaMondia 2018). However, in much of the US, tomato production in the open field is limited based on seasonal temperature, disease susceptibility, and light fluctuations.

To meet the growing market demand, in recent years there has been an increase of tomato production in protected environments (Baskins et al. 2019; NASS 2021). In 2014, US tomatoes grown in protected environments totaled 42,587,000 square feet. By 2019, this volume increased to 52,576,000 square feet. For national statistics, protected environments typically include both structures such as a greenhouse or a high tunnel. However, significant differences do exist. A high tunnel, as opposed to a greenhouse, does not have a cement slab foundation or semi-permanent flooring (like gravel) and cost-sharing programs such as the NRCS EQIP high tunnel systems initiative, make them considerably less costly, thus accelerating their adoption.

Extending the growing season presents multiple economic advantages, allowing farmers to plant earlier in the spring, harvest later into the fall, and in some cases produce a diversity of crops for longer than a typical field season (Galinato 2013; LaMondia 2018).

The structure also allows for a reduction in foliar disease seen in open fields and the ability to control water through irrigation which can also result in reduction of favorable environments for disease development. Even so, these protected environments can present unique disease management challenges (Bruce et al. 2019).

Certain fungal pathogens can pose a significant risk in high tunnel production due to extended periods of high humidity. Some of these pathogens include those that cause gray mold (*Botrytis cinerea*), leaf mold (*Passalora fulva*), and powdery mildew (*Oidium neolycopersici*) (Bicici et al. 2000; Menzel et al. 2014; Warren et al. 2015). Although these diseases also occur in the field, the protected environment of high tunnels increases the risk, and thus occurrence, of these pathogens as compared to the open field. Among these disease risks, powdery mildew of tomato, caused by *O. neolycopersici,* can result in up to 90% disease severity on 100% of affected plants (Jones H. 2001; LaMondia 2018), which can result in up to 50% yield loss (Li 2013). *Oidium neolycopersici* can thrive in these protected environments as the humidity common in these environments favors the growth of the pathogen. Of note, 80% relative humidity, which is not uncommon in protected environments, is optimal for disease proliferation (Whipps and Budge 2000).

There are limited commercial products effective against powdery mildew that are registered for application both in greenhouses and in high tunnels. This limitation is particularly acute for organic production, with few treatments listed by the Organic Materials Review Institute (Institute 2021; Pottorff 2009). Of these limited control options, the modes of action of products can be distinguished as direct and indirect. A direct mode of action product directly impacts the pathogen by inhibition of growth and development,

parasitizing the pathogen, or inhibition of a metabolic process or pathway. As such, the direct effect on the pathogen can prevent infection of the plant.

Well-known products having such direct effects contain active ingredients such as Cuprous oxides. Cuprous oxide formulations function as a prophylactic protectant to inhibit pathogen infection (Horsfall et al. 1937; McCallan 1949; Walter et al. 2015). Oxide releases cuprous ions that quickly convert to cupric ions, both of which have bactericidal and fungicidal activity. Organisms in contact with cuprous and cupric ions take these up, which pass through their cell walls and disrupt their cellular enzyme functions (Horsfall et al. 1937; McCallan 1949; Walter et al. 2015). Copper products have a FRAC (Fungicide Resistance Action Committee) code M due to their multi-site mode of action. Although copper products have multiple modes of action, which may reduce that chance of pathogen resistance development, there have still been cases of development of resistance to copper pesticides (Lamichhane et al. 2018). Additionally, copper products have been in use since the late 1800s and may be a contributing factor to high Cu agricultural soil levels world-wide (Lamichhane et al. 2018). High levels of Cu in the soil can be toxic to soil biota and some microorganisms are specifically sensitive to heavy metal accumulations (Giller et al. 1998). Other organisms have been shown to be impacted by Cu accumulation such as earthworms, nematodes, and snails (Eijsackers et al. 2005; Jaworska and Gorczyca 2002; Rogevich et al. 2008; Van Zwieten et al. 2004). Depending on the soil, high Cu accumulations are likely to remain for the foreseeable future, continually impacting overall soil health (Van Zwieten et al. 2004).

Indirect modes of action have an effect on the plant such as the induction or upregulation of plant defense compounds, increasing plant disease resistance defense or

can, in some way, impact the pathogen growth environment, thus indirectly affecting the pathogen. Possible indirect modes of action might include niche exclusion or induction of plant defense. This induction can result in the production of substances, such as phytoalexins, that are toxic to the pathogen. *Bacillus* spp. are examples of organisms used for their indirect modes of action, although they can also be considered direct modes of action as well. *Bacillus* spp. can produce toxins and occupy physical space, preventing the pathogen from becoming established on the plant.

Bacillus spp. can also provide an additional mode of action through the upregulation of plant defenses thereby increasing plant natural resistance. In particular, *Bacillus subtilis* has been identified as a species which may control pathogens through multiple modes of action. For example, *Bacillus subtilis* GB03 produces a broad-spectrum antibiotic (Iturin) which can disrupt the cell wall formation of different plant pathogens (Haidar et al. 2016a; Lastochkin et al. 2019a; Moyne et al. 2001b; Romero et al. 2007) and stimulates phytohormones to induce plant disease defense mechanisms for prolonged periods of time (Haidar et al. 2016a; Lastochkin et al. 2019a). *Bacillus* spp. may also quickly colonize plant tissue, outcompeting resources, such as substrates or physical space, from potential pathogens. Although not well studied, some possible downsides to this method of control include that the organism may also exclude some potentially beneficial microbes from the microbiome (Nishad et al. 2020; Pieterse et al. 2014).

Live microorganisms may not be required to provide indirect effects that enhance disease resistance. Similar indirect modes of action, such as plant extracts or metabolic by-products that are produced during fermentation, (Haidar et al. 2016a; Lastochkin et al. 2019a) can also act as elicitors of plant defense (Twamley et al. 2019).Yeast cell wall

extracts derived from the beer-brewing process may act as one such elicitor (Yaguchi et al. 2017). Some derivatives from yeast cell walls such as glucan, mannon, and chitin have been previously investigated for the potential to function as plant defense inducers (Minami et al. 2011; Narusaka et al. 2015; Reglinski et al. 1994, 1995; Yaguchi et al. 2017). Similarly, lactic acid bacteria, involved in a variety of different food fermentations, have been shown to prevent fungal disease in some field settings (Oliveira et al. 2014). As opposed to some fungicides that have defined active ingredients with specific modes of action, fermentation products often have complex components, making identification of mode of action difficult but also providing multiple avenues for plant disease suppression making pathogen resistance less likely. In some cases a product's antimicrobial activities may work either independently or have synergistic effects (Twamley et al. 2019). Additionally, biological products can show direct as well as indirect anti-fungal effects that can deter fungal development (Hansjakob et al. 2010; Nesler et al. 2015). Microbial fermentation products could serve as an additional avenue for disease management in organic farming systems.

Although there is potential for MFPs to serve as an additional disease management options, particularly for organic producers, there have been few studies on their efficacy compared to other organic control options. Currently, there is some evidence indicating fermentation products have efficacy against powdery mildew of wheat in laboratory settings (Twamley et al. 2019). However, this same efficacy has yet to be confirmed in protected systems such as high tunnels, particularly for tomatoes. Consequently, the objectives of this study were to examine the efficacy of copper alternatives in reducing tomato powdery mildew severity in high tunnels, including the use of a commercially

available microbial fungicide (*Bacillus* spp.) and an MFP to reduce plant disease severity and adverse effects on yields.

3.2 Materials/Methods

3.2.1 High tunnel site

The study was repeated four times, from Y1 (Fall 2018) to Y3 (Spring 2020) at University of Kentucky research farms in Lexington, KY, USA. The first three repetitions of the study (Y1 and Y2 (2019)) were conducted at the University of Kentucky Horticulture Research Farm (lat 37.37, long -84.53). High tunnels at the Horticulture Research Farm were managed according to National Organic Program (NOP) guidelines and were USDA certified organic since 2012. The fourth repetition (Y3) was located at the University of Kentucky Spindletop Farm (Spindletop, lat 38.14, long -84.5). High tunnels at Spindletop were not certified organic but were managed according to NOP guidelines. The soils on both sites were Maury silt loam (deep well drained, moderately permeable, solid, formed in silty material over residuum weathered from phosphatic limestone) (USDA Soil Series 2022).

All high tunnels were arranged in a randomized complete block design. The Horticulture Research Farm (Y1-3), 3 replicated blocks were present in each of two high tunnels, for a total of 6 replication per treatment. Experimental management dates and operations for the Y1 and Y2 high tunnel trials can be found in Table 3.1. In the Spindletop high tunnel trial (Y3) each treatment was replicated four times within a single high tunnel.

Fertilizer was incorporated with a rototiller on a walk behind tractor (BCS 853, BCS Incorporated, Portland, OR). Integrated fertilizer included a NutriSafe organic fertilizer (8-5-5), 2.8 kg per bed. Planting beds were covered with woven polypropylene groundcover (Sunbelt, Dewitt Co., Sikeston, MO) measuring 1.3 m wide and running the length of the bed. Drip tape was placed underneath the fabric and all crops were irrigated using municipal water sources. Tomato plants were set at 45.7 cm spacing through holes burned through the groundcover fabric. Plots were comprised of three plants per plot for the first three repetitions at the Horticulture Research Farm and six plants at the Spindletop Farm. Plants were trellised four times over the season beginning when plants were about 45 cm tall. No pruning was done on the plants.

3.2.2 Plant material

Tomato transplants ('Early Girl') were produced on site in a certified organic greenhouse using untreated seed grown in an organically-approved compost-based potting media (Fort Vee, Vermont Compost, Montpelier VT). Plants were grown in 72 cell flats for six weeks (15 cm to 20 cm) in a greenhouse before transplanting into the high tunnels. For inoculum purposes, some tomatoes were grown in a clean chamber until producing the first true leaves then were transferred to the chamber for maintaining infected plants.

3.2.3 Pathogen culture and cultivation

Oidium neolycopersici was selected due to its commonality in high tunnel production (Salvucci et al. 2016). Infected leaves were collected from infected whole plant samples from the study region (Muhlenberg and Fayette Counties, Kentucky) and were

stored at -20° C until used for inoculation (two days). Infected leaves were gently rubbed onto non-inoculated tomatoes and returned to the chamber for infected plants. Chamber conditions for inoculated plants were 12 h light/12 h dark light cycle with a day temperature of 25 +/- 1°C and a night temperature of 23 +/- 1°C. The relative humidity was maintained at roughly 70% +/- 10%. Plants were maintained for 14 +/-2 days.

Oidium neolycopersici field inoculum was harvested from chamber-grown tomatoes. Infected leaves were transported to the field in sealed containers plastic Tupperware containers. Inoculum was prepared in the field by washing the conidia from leaves harvested from the chamber-grown tomatoes with autoclaved DI water. Spore concentrations were quantified by hemacytometer. Conidial suspensions were sprayed on the plants using a hand-pump spray bottle calibrated to deliver ~3 ml per 10 squirts, using a $1x10^4$ conidia/mL concentration. Approximately 35 mL of conidial suspension was uniformly applied per plant. Inoculation occurred two days after the first foliar fungicide treatment (Table 3.1).

3.2.4 Disease management treatments

Experimental treatments included an inoculated water-treated treatment (IC), a non-inoculated treatment (UIC), cuprous oxide (Nordox®), *Bacillus subtilis* GB03 (Companion®), unfiltered MFP (U-MFP) at an 8% v/v concentration, and a filtered MFP (F-MFP) at an 8% v/v concentration. The copper treatment used was a cuprous oxide-based, OMRI-approved formulation (Nordox®, Brandt Co., Springfield, IL, 83.9% cuprous oxide). The commercial biopesticide used was a formulation of *Bacillus subtilis* (0.03%) (Companion®, Growth Products, Liberty, MO). A novel microbial fermentation

product (MFP) was also included, which is labeled as consisting of a proprietary blend of yeast cell walls and "inactive" fermentation media (Alltech, Inc., Nicholasville, KY). This product is reported to contain no viable microorganisms. Used directly, the UF-MFP had a high viscosity, required repeat agitation, and left visible residues when applied directly. To reduce the viscosity and enable easier application, a filtered treatment was created by centrifugation for 15 min at 6,000 rpm (3226 Xg), then the supernatant was removed and vacuum-filtered twice using an autoclaved Whatman 90 mm Grade 1 filter paper (Whatman Co, Fisher Scientific, Waltham, MA, cat No 1001-090). Both the filtered and unfiltered variation of the product were sprayed at an 8% (v/v) concentration. Cuprous oxide was sprayed at the manufacturer's recommended concentration for tomatoes of 29.6 oz ai per 100 L water (2 ½ lbs/acre). *Bacillus subtilis* spray rates were also based on manufacturer's recommendations for greenhouse use: 14.38 ml of ai per 100 L water (16 fl. oz per 100 gal. water). All treatment spray mixes were applied in a volume of 280.5 L/ha spray mix rate every 7-10 days at 45 pound-force per square inch (PSI) with a single-nozzle hollow cone spray tip (TXVS-18, TeeJet® Conejet, Glendale Heights, IL) boom directed over the top of the tomato plants for the first two applications. When the tomato plants were approximately 0.6 m tall, the application volume was increased to 467.5 L/ha for all treatments. In subsequent applications, a single nozzle was used with a uniform spray applied on both sides of each plot to ensure full coverage of foliar tissue.

3.2.5 Disease rating

Plant disease severity ratings were recorded once per week after the initial inoculation until termination of the experiment. Plant disease severity ratings used a

continuous 0-100% scale with 0 being no disease symptoms and 100% being total plant area affected. Plants were visually divided in half by height and rated by upper foliage (new growth) and lower foliage (old growth) separately., rather than rating disease severity based on whole-plant area. The plant height was visually divided in half. All surfaces below the halfway point were considered the lower foliage and the tissue above the point was considered the upper foliage. Upper foliage (new growth) and lower foliage (old growth) were rated for disease severity separately. Each plant per plot (three) was rated individually for plant disease severity and averaged for the plot. Data were analyzed using analysis of variance in SAS 9.3 (PROC GLM, SAS Institute, Cary, NC). Means were separated using Fisher's Protected Least Significant Difference (LSD, $P<0.05$).

3.2.6 Yield evaluations

Tomatoes were harvested from all trials except Fall Y2, which ended due to early frost before fruits were harvestable. Harvests were completed three times in each trial, removing fruit that was full size and breaking in color, with the exception of the last harvest as a 'pick clean' and all fruit were removed (including immatures).

Yields were separated into two categories: either mature or immature fruits. Maturity was based on USDA standards, any tomato that was any of the following USDA stages of maturity were identified as mature (tomato grades U.S. No 1-3); turning, pink, hard ripe, and firm ripe (USDA Agricultural Marketing Service). Data were recorded on the number of tomatoes and total weight of tomatoes, and the average weight per tomato was calculated. Data were analyzed using analysis of variance in SAS 9.3 (PROC GLM,

SAS Institute, Cary, NC). Means were separated using Fisher's Protected Least Significant Difference (LSD, $P<0.05$).

3.3 Results & Discussion

3.3.1 Disease assessments

Over most years, despite fall vs spring season difference, the MFP appear to reduce powdery mildew disease severity equal to that of the copper product treatment. In Y1 in the lower foliage, disease severity differed by treatment beginning on 27th day post-inoculation (dpi) ($P<0.05$) (Table 3.2). On 34 dpi, in the upper foliage, both the F-MFP and UF-MFP remained statistically ($P>0.05$) similar to both the cuprous oxide and the UIC All treatments on the lower foliage were statistically ($P<0.05$) similar to the UIC for the duration of the experiment with the exception of the *B. subtilis*. In the upper foliage, statistical differences appear on 14 dpi ($P<0.05$). Both F-MFP and UF-MFP had significantly lower powdery mildew severity than the inoculated treatment and *B. subtilis* treated plants. From 28 dpi until the termination of the experiment, the F-MFP and UF-MFP grouped statistically ($P>0.05$) with the UIC and the cuprous treatment. Both MFP treated groups also had significantly lower disease severity than the IC and the *B. subtilis* treatment.

On 57 dpi, Spring Y2 (Table 3.3), in the lower foliage, both the cuprous oxide and the F-MFP had lower powdery mildew severity ratings than the UF-MFP ($P<0.05$), *B. subtilis*, and both the IC and UIC (Table 3.3). In the upper foliage, all treatments were statistically ($P<0.05$) different from the IC on 57 dpi ($P<0.05$). However, the IC was not

statistically (*P*>0.05) different from the UIC. This could have been due to either an inefficient inoculation or inoculum spread from inoculated plants to the non-inoculated treatment. Regardless of the lack of difference between the IC and UIC, the results still indicated there was efficacy from our treatments.

In Fall Y2 (Table 3.4) the experiment was terminated early due to a frost event before powdery mildew severity levels exceeded 50%. The trial was inoculated Oct. 3rd but the onset of symptoms did not begin until 19 dpi. By termination date, 34 dpi, powdery mildew severity levels were no higher than 10%. The cuprous oxide and both F-MFP and UF-MFP lacked disease symptoms until date of termination (Table 3.4).

In the Y3 experiment, there were no statistical differences (*P*>0.05) observed between treatments or IC/UIC in either the upper or lower foliage ratings (Table 3.5). In Y3 (Table 3.5), one block was dropped from the experiment due to herbicide damage. This may have contributed to high levels of variance, resulting in no significant (*P*>0.05) differences between treatments throughout the season.

3.3.2 Efficacy of alternative products for powdery mildew control in high tunnel tomatoes

The results discussed above indicate the MFP and the cuprous oxide were the most effective in reducing powdery mildew disease severity. Tomatoes treated with the *B. subtilis* had consistently greater disease severity than the cuprous oxide and MFP treatments. As the *B. subtilis* product rely on the establishment of a *B. subtilis* population on the plant surface (epiphytic), there is more dependency on the presence of the organism for expression of the plant resistance to the pathogen. This may delay the impact of the

treatment, since the efficacy of *Bacillus* spp. is partially dependent on out-competing the attacking pathogen for resources. The MFP used here lacked living organisms and therefore does not require time to colonize the surface of the plant. The logistics of a program involving cuprous and a living organism would require further investigation. Since copper products are non-specific, they may have an adverse effect on the beneficial pathogen such as the *B. subtilis* when applied together.

As opposed to the efficacy of the *B. subtilis*, the MFP's efficacy mirrored that of the cuprous. Therefore, for powdery mildew control in high tunnels, it would appear these treatments could be substituted for cuprous oxide treatments, reducing the use of copper. Reduction in the use of cuprous oxide treatments could result in less soil contamination as well as a reduction in the development of copper resistant pathogens (Giller et al. 1998; Lamichhane et al. 2018; Van Zwieten et al. 2004).

3.3.3 Alternative products efficacy with respect to tomato yield

No differences were observed in total number of tomatoes harvested by treatment in Spring Y2 (Figure 3.1) Similarly, no difference in total harvest weight was observed (Figure 3.2) nor in average weight per tomato (Figure 3.3) in Spring Y2. In Y3, there were numerical differences but these were not significant (P>0.05) in yield measurements between the MFP treated tomatoes and the inculcated and non-inoculated treatments., This was likely due to high variability resulting in the lack of significant (P>0.05) differences seen in the number of tomatoes (Figure 3.4), weight of tomatoes (Figure 3.5), and average weight per tomato (Figure 3.6).

There were no visual phytotoxic affects from the MFP however, large variability in measured parameters were evident. These apparent differences and larger variation may be due to herbicide damage. Several plots were dropped due to plant death caused by herbicide damage which may also have resulted in the greater variability observed.

When evaluating plant defense activators, any decrease in yield due to treatments would clearly off-set any benefit with respect to lowered disease incidence. Products whose proposed mode of action is plant defense induction have often been associated with reduced yields when compared to inoculated and non-inoculated treatments (Louws et al. 2001; Romero et al. 2001). As *B. subtilis* products have been suggested to possess a partial plant induction mode of action and MFP mode(s) of action remain unidentified, evaluation of yields and their relationship to disease severity is critical in determination of product efficacy. Products possessing a plant induction mode of action have been connected with yield drag affects in the past (Desmedt et al. 2021; Huang et al. 2012; Louws et al. 2001). In this work, no effect on yield was observed for any proposed copper alternatives. This may be due to the relatively low application rates of active ingredients used in this work, as compared to other studies where yield drag was observed with BTH-based products (Louws et al. 2001). Nonetheless, the lack of any yield drag, coupled with decreased severity of disease, is noteworthy in the MFPs.

3.4 Conclusions: Viable alternatives to copper

The MFPs had shown potential to be used as cuprous oxide alternatives. MFPs had reduced powdery mildew disease severity similar to that of the cuprous oxide treatment, a treatment selected to represent a commercial standard for organic growers. The MFPs also

had lower disease severity than the organism-based product, *B. subtilis*. However, to be of practical use, treatments such as MFP and *B. subtilis* must also result in similar yields as obtained in untreated plants or in those treated with copper or other methods. Consequently, yields were examined and no yield drag was observed on treated plants. These results indicate that MFP may offer potential as a viable alternative to the heavily used copper in organic high tunnel tomato systems. If used in alternation with a copper product or as a substitute for a cuprous oxide product, MFP could contribute to the reduction of copper usage and therefore a reduction in copper accumulation in soils under high tunnel.

Although copper is heavily used in agricultural, the issues related to copper use become more prevalent in high tunnel systems. Unlike open field, crop rotation is not always a realistic option in high tunnels. The additional restrictions placed on organic growers make effective powdery mildew management options limited. There is a lack of data regarding MFPs' overall efficacy in different systems and within integrated management programs. In this study MFPs proved to be a viable copper alternative; however, further research should be done regarding its efficacy in integrated programs, efficacies in open fields, and against other pathogens in order to have a greater impact on copper use.

Table 3.1 Experimental Timeline of High Tunnels

	Dates			
Event	**Fall (Y1) 2018**	**Spring (Y2) 2019**	**Fall (Y2) 2019**	**Spring (Y3) 2020**
Seeded Tomatoes	9/3/18	3/14/19	8/21/19	4/4/20
Tomatoes transplanted	9/21/18	4/4/19	9/23/19	5/1/20
Initial treatment application	9/26/18	4/11/19	10/1/19	5/9/20
Tomatoes inoculated	0 dpi* (9/28/18)	0 dpi* (4/13/19)	0 dpi* (10/3/19)	0 dpi* (6/10/20)
Tomatoes treated	10 dpi, 19 dpi, 31 dpi	13 dpi, 27 dpi, 37 dpi, 47 dpi, 55 dpi	8 dpi, 19 dpi, 20 dpi	6 dpi, 16 dpi, 25 dpi
Experiment terminated	38 dpi (frost)	64 dpi	34 dpi (frost)	36 dpi

*dpi: days post inoculation

Table 3.1 Experimental timeline of Fall (Y1) 2018, Spring (Y2) 2019, Fall (Y2) 2019, and Spring (Y3) 2020 high tunnels. Shows dates of significant management events of high tunnel tomatoes.

Table 3.2 Powdery Mildew Disease Severity on Tomato for Fall 2018

	Oct. 12th (20 dpi)		Oct. 19th (27 dpi)		Oct. 26th (34 dpi)		Oct. 31st (39 dpi)		Nov. 7th (46 dpi)		AUDPC	
Treatment	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Inoculated control	25 e	6 a	3 c	18 a	4 b	32 d	5 b	28 b	16 c	46 b	307 a	663 a
Bacillus spp.	18 d	5 a	3 c	14 a	4 b	17 c	4 b	24 b	8 b	38 b	230 b	543 ab
Filtered MFP	7 c	6 a	3 c	8 a	1 a	13 bc	1 a	12 a	2 a	18 a	86 c	381 bc
Unfiltered MFP	5 bc	5 a	2 bc	7 a	1 a	9 ab	2 a	10 a	3 ab	14 a	83 c	287 c
Copper-based	2 ab	4 a	1 ab	4 a	0 a	6 a	0 a	8 a	2 a	11 a	37 c	237 c
Non-inoculated control	1 a	4 a	0 a	3 a	0 a	4 a	1 a	6 a	5 ab	15 a	37 c	226 c

Table 3.2. High tunnel powdery mildew severity for each date (Means, Fall 2018, Y1). Ratings were based on a 0-100% scale with 0% being no disease symptoms and 100% being complete plant death. dpi: days post inoculation. Means, N = 6. Different letters within each column indicate differences at $P<0.05$. AUDPC units are percent-days. Statistical analysis was done using analysis of variance in SAS 9.3 using LSD. Dpi: days post inoculation

Table 3.3 Powdery Mildew Disease Severity on Tomato for Spring 2019

	May 31st (44 dpi)		June 7th (51 dpi)		June 13th (57 dpi)		June 20th (64 dpi)		AUDPC	
Treatment	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Inoculated control	3 abc	19 b	12 a	68. a	34 a	70 a	64 a	99 a	551 a	1283 a
Bacillus spp.	2 a	11 a	8 a	44 a	28 bc	68 a	55 a	100 a	421 abc	1032 ab
Filtered MFP	0 bc	5 b	6 a	30 a	14 c	46 b	57 a	100 a	296 c	772 b
Unfiltered MFP	1 c	7 b	7 a	31 a	21 bc	54 ab	58 a	100 a	381 bc	873 b
Copper-based	2 ab	10 b	8 a	36 a	22 bc	46 b	63 a	99 a	390 bc	801 b
Non-inoculated control	1 abc	9 ab	11 a	52 a	34 a	74 a	58 a	100 a	476 ab	1127 ab

Table 3.3. High tunnel powdery mildew severity for each date (Means, Spring 2019, Y2). Ratings were based on a 0-100% scale with 0% being no disease symptoms and 100% being complete plant death. dpi: days post inoculation. Means, N= 6. Different letters within each column indicate differences at $P<0.05$. AUDPC units are percent-days. Statistical analysis was done using analysis of variance in SAS 9.3 using LSD. Dpi: days post inoculation

Table 3.4 Powdery Mildew Disease Severity on Tomato for Fall 2019

	Oct. 9th (6 dpi)		Oct. 17th (14 dpi)		Oct. 22nd (19 dpi)		Oct. 31st (28 dpi)		Nov. 6th (34 dpi)		AUDPC	
Treatment	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Inoculated control	0 a	0 a	0 a	0 a	0 a	1 a	0 a	1 a	0 a	2 a	1 a	29 a
Bacillus spp.	0 a	0 a	0 a	0 a	0 a	1 a	0 a	1 a	0 a	2 a	0 a	21 a
Filtered MFP	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	1 a	0 a	5 a
Unfiltered MFP	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	1 a	0 a	6 a
Copper-based	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a	3 a	3 a
Non-inoculated control	0 a	0 a	0 a	0 a	0 a	0 a	0 a	1 a	0 a	1 a	0 a	24 a

Table 3.4. High tunnel powdery mildew severity for each date (Means, Fall 2019, Y2). Ratings were based on a 0-100% scale with 0% being no disease symptoms and 100% being complete plant death. dpi: days post inoculation. Mean, N = 6. Different letters within each column indicate differences at $P<0.05$. AUDPC units are percent-days. Statistical analysis was done using analysis of variance in SAS 9.3 using LSD. Dpi: days post inoculation

Table 3.5 Powdery Mildew Disease Severity on Tomato for Spring 2020

	June 23rd (13 dpi)		June 30th (20 dpi)		July 7th (27 dpi)		July 16th (36 dpi)		AUDPC	
Treatment	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower	Upper	Lower
Inoculated control	0 a	3 a	3 a	12 a	11 a	44 a	39 a	16 a	156 a	487 a
Bacillus spp.	0 a	1 a	0 a	10 a	3 a	14 a	9 a	3 a	144 a	472 a
Filtered MFP	0 a	1 a	0 a	4 a	5 a	11 a	18 a	15 a	65 a	207 a
Unfiltered MFP	0 a	3 a	1 a	8 a	0 a	15 a	34 a	15 a	43 a	195 a
Copper-based	0 a	0 a	0 a	5 a	6 a	21 a	11 a	3 a	53 a	130 a
Non-inoculated control	0 a	2 a	2 a	12 a	12 a	47 a	54 a	28 a	21 a	205 a

Table 3.5. High tunnel powdery mildew severity for each date (mean Spring 2020, Y3). 6Ratings were based on a 0-100% scale with 0% being no disease symptoms and 100% being complete plant death. Dpi: days post inoculation. Mean, N= 3. Different letters within each column indicate differences at $P<0.05$. AUDPC units are percent-days. Statistical analysis was done using analysis of variance in SAS 9.3 using LSD. Dpi: days post inoculation

Figure 3.1 Total Number of Tomatoes from Spring 2019 High Tunnels

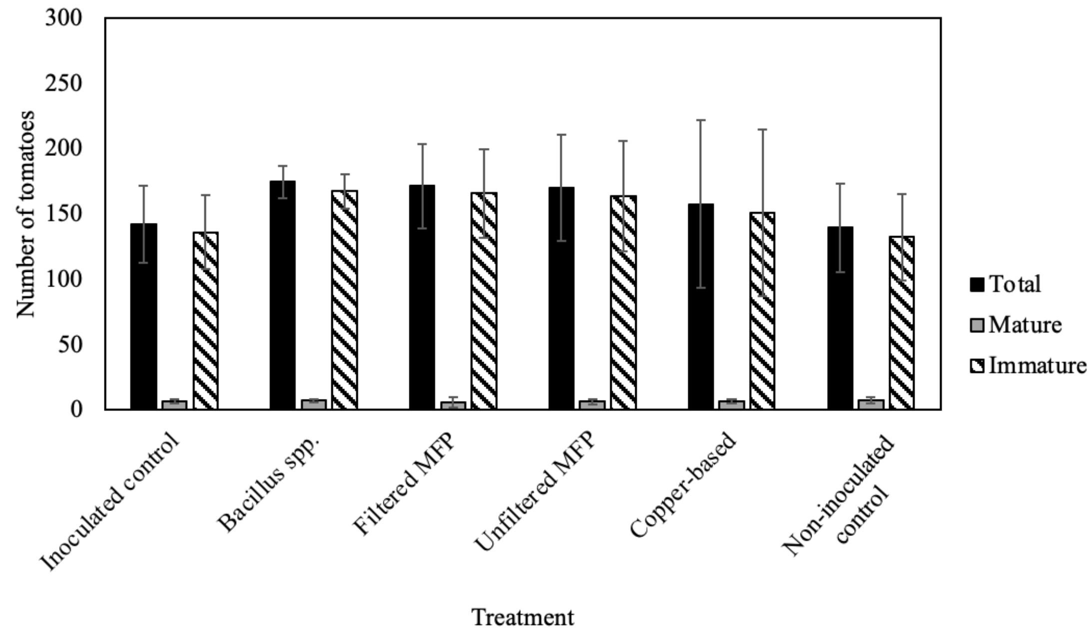


Fig. 3.1. Total number of tomatoes from Spring 2019 (Y2) high tunnels. Total: The combination of both mature and immature tomatoes. Mature: any fruit that had reached full size, was about to turn color, or had already begun to ripen. Immature: any fruit that had not yet reach full size or had not begun to ripen. Mean ± standard deviation is shown, N = 6

Figure 3.2 Total Weight of Tomatoes from Spring 2019 High Tunnels

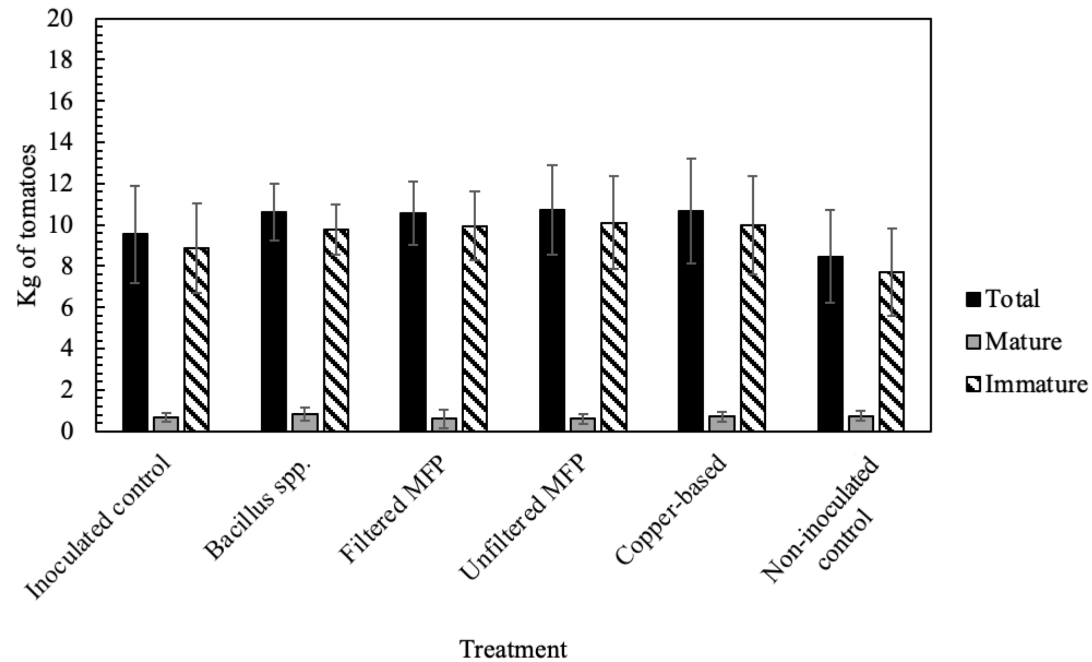


Fig. 3.2. Total weight of tomatoes from Spring 2019 (Y2) high tunnel. Total: The combination of both mature and immature tomatoes. Mature: any fruit that had reached full size, was about to turn color, or had already begun to ripen. Immature: any fruit that had not yet reach full size or had not begun to ripen. Mean ± standard deviation is shown, N = 6

Figure 3.3 Average Weight per Tomato from Spring 2019 High Tunnels

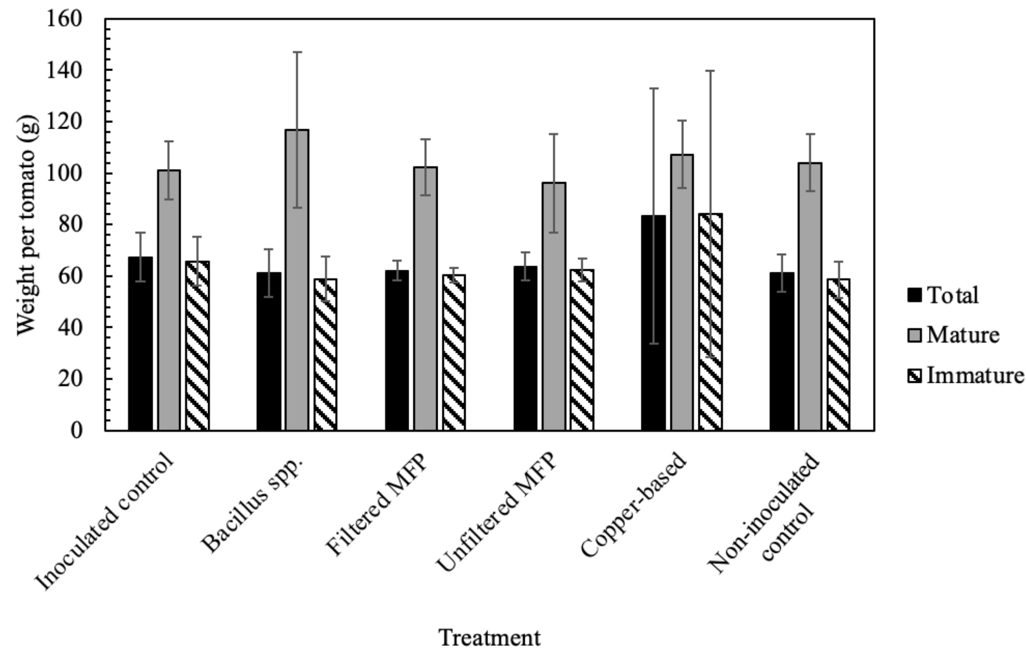


Fig. 3.3. Average weight per tomato from Spring 2019 (Y2) high tunnel. Total: The combination of both mature and immature tomatoes. Mature: any fruit that had reached full size, was about to turn color, or had already begun to ripen. Immature: any fruit that had not yet reach full size or had not begun to ripen. Mean ± standard deviation is shown, N = 6

Figure 3.4 Total Number of Tomatoes from Spring 2020 High Tunnel

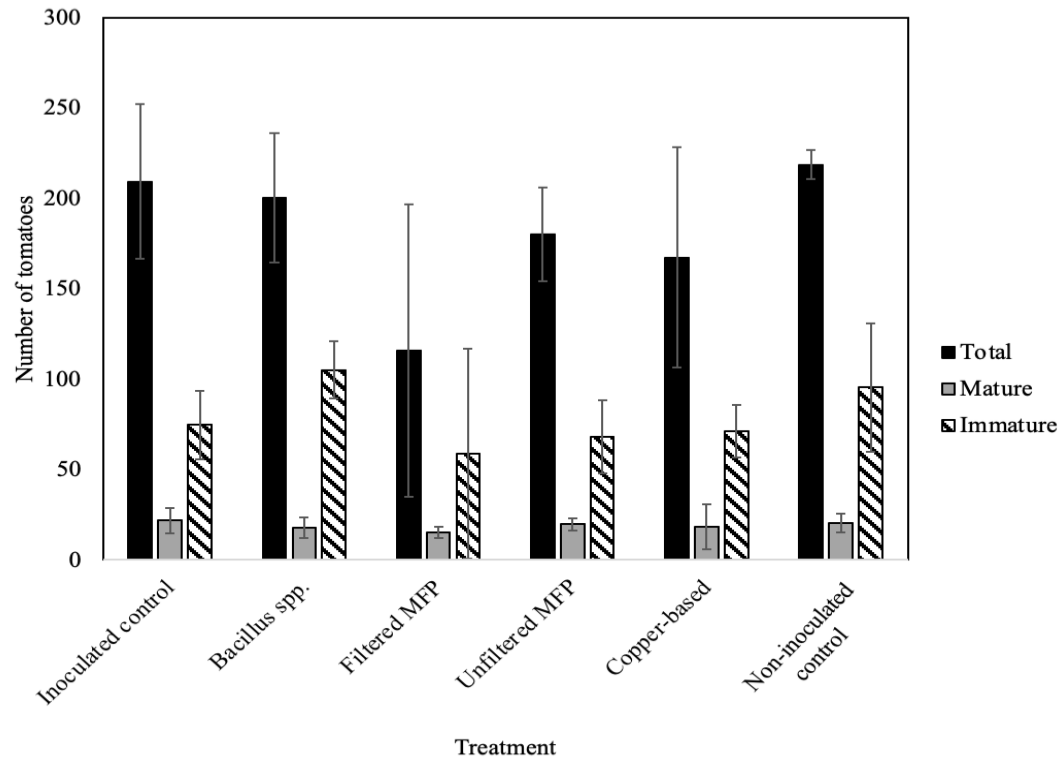


Fig. 3.4. Total number of tomatoes from Spring 2020 (Y3) high tunnels. Total: The combination of both mature and immature tomatoes. Mature: any fruit that had reached full size, was about to turn color, or had already begun to ripen. Immature: any fruit that had not yet reach full size or had not begun to ripen. Mean ± standard deviation is shown, N = 6

Figure 3.5 Total Weight of Tomatoes from Spring 2020 High Tunnel

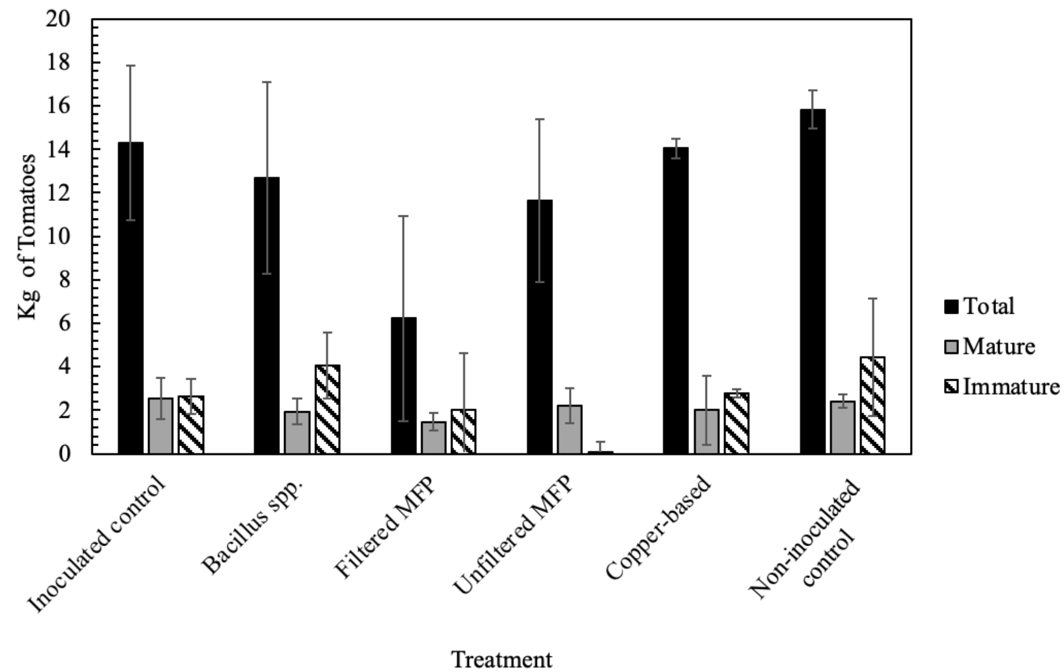


Fig. 3.5. Total weight of tomatoes from Spring 2020 (Y3) high tunnel. Total: The combination of both mature and immature tomatoes. Mature: any fruit that had reached full size, was about to turn color, or had already begun to ripen. Immature: any fruit that had not yet reach full size or had not begun to ripen. Mean ± standard deviation is shown, N = 6

Figure 3.6 Average Weight Per tomato from Spring 2020 High Tunnel

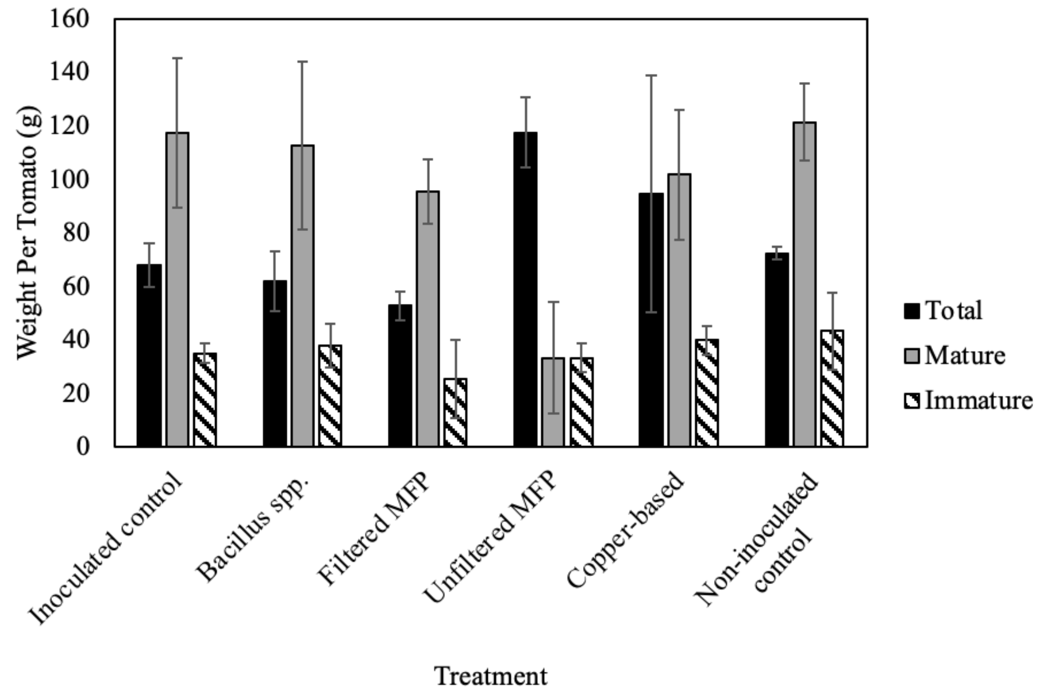


Fig. 3.6. Average weight per tomato from Spring 2020 (Y3) high tunnel. Total: The combination of both mature and immature tomatoes. Mature: any fruit that had reached full size, was about to turn color, or had already begun to ripen. Immature: any fruit that had not yet reach full size or had not begun to ripen. Mean ± standard deviation is shown, N = 6

CHAPTER 4. MICROBIAL FERMENTATION PRODUCT IN THE CONTROL OF *XANTHOMONAS EUVESICATORIA*: INVESTIGATION OF INDIVIDUAL USE AND INDICATIONS OF POSSIBLE INTERACTION WITH COPPER

4.1 Introduction

Tomato bacterial spot is an economically important disease in most regions where tomatoes are grown. In some highly disease-conducive conditions as much as 50% yield loss can be seen, in addition to a reduction in product overall quality (Kunwar et al. 2018). Bacterial spot of tomato can be caused by *Xanthomonas vesicatoria*, *Xanthomonas euvesicatoria*, *Xanthomonas gardneri* and *Xanthomonas perforans*. *X. euvesicatoria* can affect both tomato and pepper and has a worldwide distribution (Timilsina et al. 2015).

Currently, control of tomato bacterial soft spot includes the use of certified disease-free seed, maintaining proper sanitation practices, and crop rotation. However, in regions or locations with high humidity and appropriate growth temperatures that favor disease development, additional treatments may be required (Obradovic et al. 2005). Many types of treatments involved include the use of cuprous compounds. Unfortunately, exclusive use of such treatments, including those containing cuprous compounds can lead to pathogen resistance (Areas et al. 2017; Van Bruggen et al. 2016). Additionally, over-usage of copper based products may be a contributing factor to high copper in soil levels (Lamichhane et al. 2018). Not all soil-borne biota are tolerant to high levels of copper including microorganisms as well as earthworms, nematodes and snails. (Eijsackers et al. 2005; Giller et al. 1998; Jaworska and Gorczyca 2002; Rogevich et al. 2008; Van Zwieten

et al. 2004). Furthermore, copper accumulations are likely to remain in some types of soils for generations, continually impacting overall soil health (Van Zwieten et al. 2004).

These issues regarding the use of copper-based products have increased the focus on identifying products that may be used as an alternative or in combination with copper to reduce the adverse effects of copper usage. The most efficacious spray programs involve multiple different modes of action revolving around fungicides with different Fungicide Resistance Action Committee (FRAC) codes (FRAC 2021). FRAC is an organization that indicates the mode of actions of various products as to better determine the optimum combinations to employ for crops and field conditions.

A novel product that may have potential to control bacterial spot disease with comparable efficacy to copper-based products are microbial fermentation products (MFP). Although such products have not been tested against bacterial pathogens, efficacy against fungal pathogens has been documented. For example, such products have previously been shown to induce disease resistance in wheat against powdery mildew (Twamley et al. 2019). An MFP was also found to enhanced resistance against *Botrytis cinerea* infection in rice presumably through activation of JA and AzA signaling systems (Laluk and Mengiste 2010). Additionally, in the previous work here (Chapter 3), MFP had shown statistically similar ($P>0.05$) efficacy to a copper-based product to reduce the disease severity of powdery mildew in high tunnels. As the mode of action of MFPs is not well-defined and may be more general, they may provide protection against bacterial as well fungal diseases.

In this study, MFP's ability to be used in combination with and as an alternative to copper-based product for treatment of bacterial pathogens was examined. Comparisons

were made based *X. euvesicatoria* disease severity and tomato yields. MFP was used both as an isolated treatment and in a tank-mixture with a copper-based product.

4.2 Methods/Materials

4.2.1 Culture, cultivation, and inoculum preparation

Isolates of *X. euvesicatoria* were originally isolated from Calloway County, KY from tomato fields exhibiting bacterial spot. Cultures were plated on LB agar (VWR Radnor, PA) and grown in a dark temperature-controlled chamber (VWR Personal Low Temperature Incubator VWR Cat. No 89511-416) at 27 +/- 1° C for two days. Inoculum was collected by flooding plates with 10 mL of a sterile potassium phosphate buffer (VWR, 0.05M) and gently rubbing an autoclaved pestle on the surface of the media. Inoculum concentration was determined by using spectrophotometer at $OD_{(600)}$ and by diluting appropriately in phosphate buffer (VWR) and spread plating (Wise 2006) on LB agar. Concentrations were based on colony counts. Concentration of inoculum used in all fields was 2 $x10^8$ CFU/mL. Both 2020 fields were inoculated on June 19th 2020.

4.2.2 Plant material

The tomato variety used for the field experiments in 2022 was 'Sunstart' variety (W. Atlee Burpee & Co., Warminster, PA). Plants were grown in 72-cell flats for six weeks (15-20 cm tall) in a greenhouse. Plants were left to harden outside for 2-3 days then transplanted into the field.

4.2.3 Field Sites

In 2020, two separate locations were used; one field located at University of Kentucky Spindletop farm and a second at the University of Kentucky Horticulture Research farm in Lexington, KY. Tomato plants were set at 45.72 cm (18 inch) apart. Plots were comprised of six plant per plot. Each plot was separated by approximately six feet. Each plot was considered a single replicate for each treatment type. Four plots for each application were used at each site in a randomized complete block design, resulting in 48 plants per treatment.

4.2.4 Treatments

The copper-based treatment used as a control in this study was Nordox® 75WG (Brandt, Springfield, IL). Nordox® has an active ingredient of cuprous oxide at 83.9%. Nordox® was sprayed at the recommended concentration for tomatoes, 0.84 kg oz ai per 100 L water (2 ½ lbs/acre).

Companion® (Growth Products, Liberty, MO) was used as a treatment to investigate the use of a living organism having indirect modes of action. The active ingredient in Companion® is *Bacillus subtilis* GB03 (00.03% concentration within the product). Companion® rates were also based on the product label for greenhouse use, 125 ml per 100 L water (16 fl. oz per 100 gal. water). Approximate area in each location that was treated with the applications was 3.5 meter^2 +/- 0.5.

Actigard® (Syngenta, Basel, Switzerland) was used to represent a currently marketed plant systemic inducer using the active ingredient BTH (50% concentration

within the product). It was sprayed at the recommended rates for tomatoes by the product label at 27.2 g ai/ha. Approximate area in each location that was treated was 3.5 meter^2 +/- 0.5.

The mancozeb product used for these trails was Dithane® (Corteva, Wilmington, Delaware). The mancozeb concentration within the product was 75%. The manufacturer's recommended tomato rates used were 0.45 kg ai per 100 L of water. Approximate area in each location that was treated was 3.5 meter^2 +/- 0.5.

The conventional spray program was comprised of Actigard®, Dithane® F-45 Rainshield, and Nordox®. All products were sprayed at the manufacturer's recommended rates as previously stated. The organic spray program was comprised of Companion® and Nordox® both of which are OMRI-certified.

A novel MFP from Alltech, Inc. (Nicholasville, KY) was examined in these trials. As indicated on the product label, it consists of a proprietary blend of yeast cell walls and "inactive" fermentation media. No viable microorganisms were present as determined by Axenic culture. MFP is highly viscous and left visible residues when applied directly to plants and required repeated agitation to remain in uniform solution. Due to concerns with application, a filtered and unfiltered MFP treatment were used. To create the filtered product, reduce the viscosity and enable easier application to plants, the product was first centrifuged for 15 mins at 6,000 rpm (3226 Xg). The supernatant was then removed and run through a vacuum filter twice using an autoclaved Whatman 90mm filter paper (cat No 1001 090). Both the filtered and unfiltered variation of the product were sprayed at an 8% concentration (v/v).

In 2020, Nordox® plus the filtered MFP and Nordox® plus the unfiltered MFP were used. In both of these mixtures, the MFP was used at an 8% concentration and the Nordox® was used at 2.79 ai/ha.

4.2.5 Field set-up and maintenance

The field application included; untreated control, conventional grower spray program, organic grower spray program, copper product, unfiltered MFP 8%, filtered MFP 8%, unfiltered MFP 8% with a copper product, and filtered MFP 8% with a copper product. These applications were locationally replicated five times in a randomized complete block design (RCBD) at both farms with five blocks in each field

For irrigation purposes, the Spindletop irrigation drip tape was set about 10 cm +/- 5 cm deeper than the Horticulture Research Farm (set at about 10 cm +/- 1cm. Insecticide (Radiant SC®, 365.4 mL to 730.79 mL product Amt/ha) was distributed through the drip irrigation at the Spindletop Farm and done by bucket drench at the Horticulture Research Farm.

4.2.6 Disease rating

Plant disease severity ratings were recorded once per week after the initial inoculation until termination of the experiment. Ratings were based on visual assessment of disease coverage of the whole plant. Fields were rated on a continuous scale of 0-100% based on affected plant area, with 0% being no signs of disease and 100% being complete disease coverage or plant death.

4.2.7 Harvest evaluations

Fields were harvested three times over the course of the season. Fruits were divided into three categories, marketable, unmarketable, and bacterial spot. Marketability was evaluated based on USDA guidelines (USDA Agricultural Marketing Service). The USDA has three separate grades of tomatoes, U.S. No. 1-3. (both size and quality). Tomato marketability was determined by the fruit's ability to meet or exceed the requirements for U.S. No. 3 grade tomatoes. Any tomatoes that did not meet these requirements was considered unmarketable with the exception of any tomato displaying symptoms of bacterial spot. Tomatoes exhibiting symptoms were placed into the bacterial spot category. Data were recorded in three ways: the total number of fruits, the total weight of the tomatoes, and the average weight of a single tomato from the specified treatment.

4.2.8 Statistical analysis

Field data were analyzed via analysis of variance using PROC GLM in SAS 9.3 (Cary, NC). Means were separated using Fisher's Protected Least Significant Difference (LSD, $P<0.05$). If P values were significant than all pairwise comparisons were completed between treatments.

4.2.9 Synergy calculations

The data used for synergy calculations were the field disease severity ratings. This was a preliminary assessment of possible synergistic effects of the copper product and the filtered and unfiltered MFP. The Abbott formula (Expected % control = A + B – (AB/100)

was used to calculate the expected control. A and B were the control levels seen by the single products and used for the estimations (Gisi 1996). The actual percentage of control was calculated by using the following formula: PDS = 100*(1-(A_n/A_0)). A_n is the disease severity from a given spray program and A_0 is the disease severity of the untreated control.

4.3 Results/Conclusions

Both the unfiltered MFP and the filtered MFP did not consistently differ from the untreated control at the *P*>0.05 level, although occasionally they had statistically lower (*P*<0.05) disease severity than the untreated control (Table 4.1). This was in contrast to the copper treatment which had consistently statistically lower (*P*<0.05) disease severity than the untreated control. A similar trend can be seen with the AUDPC.

The spray program applications of a copper treatment and either the filtered MFP or the unfiltered MFP both performed statistically similarly (*P*>0.05). Additionally, they both had statistically lower disease severity than the untreated control. They also grouped statistically (*P*>0.05) with both the conventional and the organic treatments. However, the individual copper treatment also grouped statistically with the conventional and organic standard treatment. There were no statically significant (*P*>0.05) differences among any of the treatments for any of the production metrics (harvest total, marketable, unmarketable, and bacterial spot) used to analysis harvest date (Figures 4.1, 4.2, 4.3). These overall results indicate that the MFP (either filtered or unfiltered) is unlikely to have a synergistic interaction with copper products.

To further elucidate the potential for synergistic activity between the MFP (filtered of unfiltered) and the copper product, calculations were performed that indicate

antagonistic, additive, or synergistic potential. These results (Table 4.2) did not show any potential for the MFP to have any synergistic activity with the copper. However, it did show some potential for an antagonistic effect. A possible antagonistic effect could be seen due to possible chelation of copper by the MFP. Copper chelation could reduce the copper's overall efficacy. This should be further investigated as it could influence whether these products could be used in a tank mix together or whether they should be used together at all.

Although the filtered and unfiltered MFPs did show efficacy in reducing the disease severity of *X. euvesicatoria,* they did not perform as well individually as the isolated copper treatment. Also, the addition of either filtered or unfiltered MFP to copper did not appear to increase its efficacy in reducing plant disease severity. The MFP does not appear to be a viable candidate for increasing copper's efficacy. The synergy calculations also suggest that there is no additional benefit to be expected.

However, at the Horticulture Research farm, the MFPs grouped statically (P>0.05) with the organic spray program perhaps demonstrating itself to be a viable option to organic growers. The same effect was not observed at Spindletop. Although, the Horticulture research farm had some management difference to the Spindletop farm. At the Spindletop, the drip tape had been unintentionally placed lower. This led to two possible issues. First, the plants may have received less water in the initial growing stages. Second, the insecticide used was applied through drips tape and taken up by the roots. Since the drip tape had been placed lower, the insecticide was possibly not taken up by the roots adequately. This may have been a contributing factor to the observed higher levels of insect damage at the Spindletop location. In contrast to Spindletop farm, the Horticulture

Research farm had drip tape closer to the plant roots. Additionally, the insecticide was applied by drench at the base of the plant and not through the drip tape. These management differences could have led to reduced overall health of plants at Spindletop and in turn led to a reduced efficacy of the MFPs. These initial observations could indicate that external stresses may reduce overall efficacy of the MFP. Crop management conditions could therefore factor into the efficacy of the MFP and should be investigated further. Experiments to help determine the extent of this factors influence on efficacy may include the inclusion of multiple locations. Other stressors/factor to include in these experiments would be insect pressure, drought stress, and weed pressure.

This study indicates that the MFPs could reduce plant disease severity of bacterial spot. However, this efficacy maybe dependent on the overall health of the plant before pathogen presence (as observed in difference seen between Table 3.1). Also, the MFPs do not increase the efficacy of copper products, indicating that it is unlikely there is any synergistic interactions between the products. The synergy calculations (Table 4.2) support this hypothesis and may suggest there is possible antagonistic interaction.

MFP may not be as efficacious as copper but there is an increased public desire to reduce copper applications in agriculture. MFP could be a product used in rotation with copper, not to eliminate the use of copper, but as an alternative option to reduce the overall amount of copper applied during a season. To examine this possibility a few factors should be taken into consideration. Assays should include varying number of days in-between copper applications. This would help elucidate how much copper application could be reduced before loss of efficacy is observed. Additionally, multiple spray programs with different MFP amounts and application frequency could be used to help determine what

would be the best copper/MFP spray programs for further examination. Also, when examining multiple copper/MFP spray programs, other comparable spray programs should be included allowing efficacy to be actively compared to programs currently in use in Kentucky. Lastly, the possible antagonistic interaction between copper and MFP should be elucidated as this can heavily impact further use with copper, a much relied on product in both conventional and organic agriculture.

The target pathogen's influence over the MFPs efficacy should also be examined. In a previous study (Chapter 3), MFPs had shown statistically similar ($P>0.05$) efficacy to copper against *Oidium neolycopersici* in tomato high tunnel environments. This same level of efficacy compared to copper was not seen in our open field study with *X. euvesicatoria*. This may indicate that either the pathogen or the environment (high tunnel vs open field) had a significant influence on the efficacy of MFP. To enable full utilization of MFP as an agricultural product, investigation should be aimed at a determination of its limitations as a viable copper alternative. These investigations could include further investigations in the field as well as assays in more controlled settings. The mode of action should also be investigated. Elucidation of mode of action could help explain varying efficacy seemingly based on target pathogen.

Table 4.1 Tomato Bacterial Spot Severity for Spindletop and Horticulture Research Farms in 2020

a. Horticulture Research Farm

Application	July 10th (21 dpi)	July 15th (26 dpi)	July 21st (32 dpi)	July 28th (39 dpi)	Aug. 7th (49 dpi)	Aug. 13th (55 dpi)	AUDPC
UTC	4 a	34 a	7 a	19 a	39 a	48 a	683 a
Conventional Program	0 b	1 c	0 c	6 d	10 d	21 b	166 c
Copper	1 b	1 c	1 c	9 cd	13 cd	22 b	229 c
Un-MFP 8%	3 a	3 b	6 a	14 b	21 bc	28 b	432 b
F-MFP 8%	3 a	3 b	5 ab	12 bc	22 b	32 b	416 b
Un-MFP 8%/Copper	0 b	0 c	2 bc	8 cd	11 d	21 b	233 c
F-MFP 8%/Copper	1 b	0 c	1 c	6 d	10 d	22 b	176 c
Organic Program	1 b	0 c	1 c	8 cd	16 bcd	26 b	258 bc

b. Spindletop Farm

Application	July 10th (21 dpi)	July 15th (26 dpi)	July 21st (32 dpi)	July 28th (39 dpi)	Aug. 7th (49 dpi)	Aug. 13th (55 dpi)	AUDPC
UTC	4 a	4 a	13 a	22 a	49 a	87 a	953 a
Conventional Program	0 c	1 d	3 c	6 bc	8 d	17 c	195 c
Copper	0 c	1 cd	4 c	10 bc	19 d	25 c	285 c
Un-MFP 8%	2 b	2 bcd	8 bc	12 b	40 ab	75 b	715 ab
F-MFP 8%	3 b	3 ab	12 ab	12 b	30 bc	80 ab	682 b
Un-MFP 8%/Copper	0 c	1 d	3 c	10 bc	12 d	19 c	256 c
F-MFP 8%/Copper	0 c	3 bc	6 c	10 bc	19 cd	26 c	363 c
Organic Program	0 c	1 d	5 c	5 c	18 cd	23 c	295 c

Table 4.1. Means of bacterial spot severity for Horticulture Research and Spindletop Farms in 2020.1a. Bacterial spot disease severity rates for the 2020 Horticulture Research Farm tomato field. 1b. Bacterial spot disease severity rates for the 2020 Spindletop Farm tomato field. Ratings were done on a 0-100% basis with 0% being no disease symptoms and 100% being complete plant death. Analysis of variance (ANOVA) was complete using PROC GLM. Means were separated using Fisher's Protected Least Significant Difference (LSD). UTC: non-inoculated control. The conventional spray program was made up of Nordox® (ai cuprous oxide), Actigard® (ai BTH), and Dithane® (ai mancozeb). The organic spray program was comprised of Companion® (ai *Bacillus subtilis*) and Nordox®. Un-MFP is the unfiltered MFP and F-MFP is the filtered MFP. Spray programs were applied as tank mixing with all listed products being applied every 7-10 days. DPI: days post inoculation. ($P<0.05$)

Table 4.2 Efficacy of Copper and MFP in combination

a. Horticulture Research Farm														
	July 10th		July 15th		July 21st		July 28th		Aug. 7th		Aug. 13th		AUDPC	
Application	Act.	Exp.	Act.	Exp.	Act.	Exp.	Act.	Exp.	Act.	Exp.	Act.	Exp.	Act.	Exp.
Copper	78	.	82	.	85	.	55	.	66	.	47	.	67	.
Un-MFP 8%	10	.	26	.	22	.	27	.	46	.	41	.	37	.
F-MFP 8%	-12	.	31	.	19	.	37	.	40	.	28	.	39	.
Un-MFP 8%/Copper	92ns	97	96ns	99	65ns	97	58ns	84	72*	90	55ns	73	66ns	89
F-MFP 8%/Copper	70ns	90	96ns	99	78ns	97	71*	88	72*	90	55ns	75	74*	91

a. Spindletop Farm														
	July 10th		July 15th		July 21st		July 28th		Aug. 7th		Aug. 13th		AUDPC	
Application	Act.	Exp.	Act.	Exp.	Act.	Exp.	Act.	Exp.	Act.	Exp.	Act.	Exp.	Act.	Exp.
Copper	87	.	54	.	65	.	53	.	76	.	72	.	70	.
Un-MFP 8%	32	.	39	.	29	.	38	.	15	.	14	.	24	.
F-MFP 8%	27	.	12	.	-13	.	40	.	42	.	8	.	28	.
Un-MFP 8%/Copper	94ns	99	79ns	89	69*	90	51ns	77	74*	94	79*	94	72*	92
F-MFP 8%/Copper	98ns	99	28ns	65	58*	86	56*	81	57*	90	70*	92	62*	89

Table 4.2. Efficacy of copper and MFP alone and in combination based on plant disease severity from field ratings of tomatoes. Un-MFP is the unfiltered MFP and F-MFP is the filtered MFP. The copper is Nordox®. Percentage of control was determined using PDS = 100*((1-A_n/A_0)). A_n is the disease severity of the program and A_0 is the disease severity of the untreated control. The expected control was calculated using the Abbott formula (percentage control expected = A + B – (AB/100)). A and B are the control seen by the single products. Actual values that are significantly (*t* test, $P<0.05$) lower than the expected indicate antagonism and actual values that are significantly (*t* test, $P<0.05$) higher indicate a synergistic effect. Additivity occurs when not significant difference is observed (represented by ns = not significant).

Figure 4.1 Total Number of Tomatoes Harvested for Each Treatment at Spindletop and Hortilcuture Research Farms in 2020

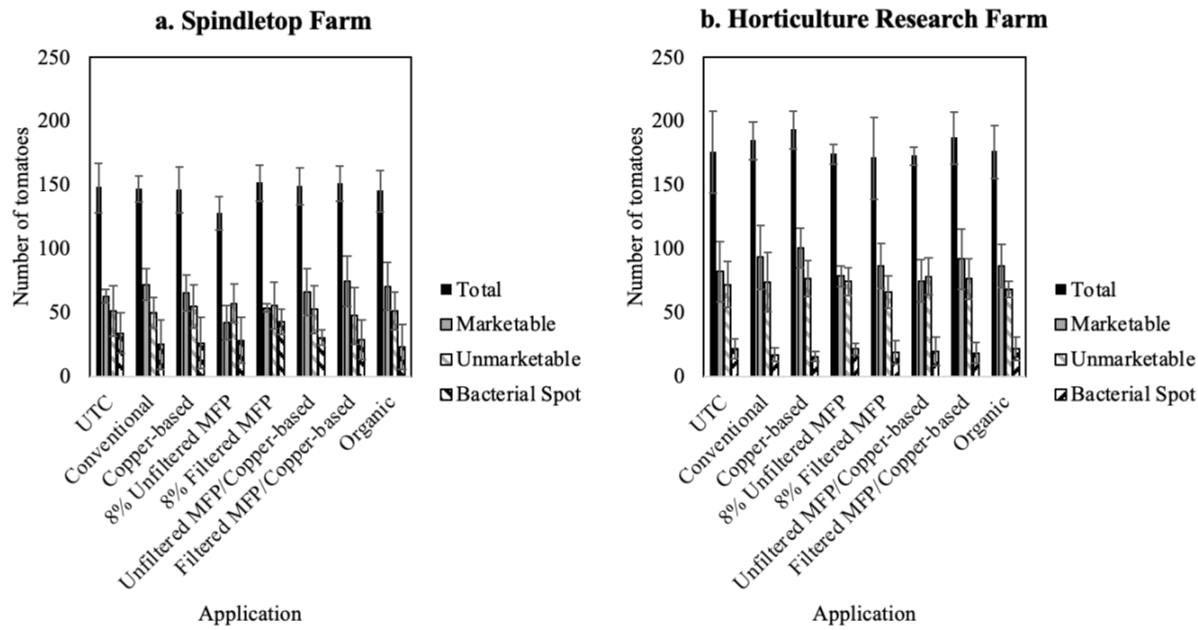


Fig. 4.1. Total number of tomatoes harvests for each treatment at the Horticulture Research and Spindletop Farms in 2020. Marketable tomatoes were any tomatoes that were U.S. grade 1-3. BS was any fruit displaying bacterial spot symptoms. All other tomatoes were considered unmarketable. 4.a: tomato counts from the Spindletop Farm trial. 4.b.: tomato counts from the Horticulture Research Farm trial. Lines indicate standard deviation. The copper-based product is Nordox®. (P<0.05)

Figure 4.2 Total Weight of Tomatoes Harvested for Each Treatment at Spindletop and Hortilcuture Research Farms in 2020

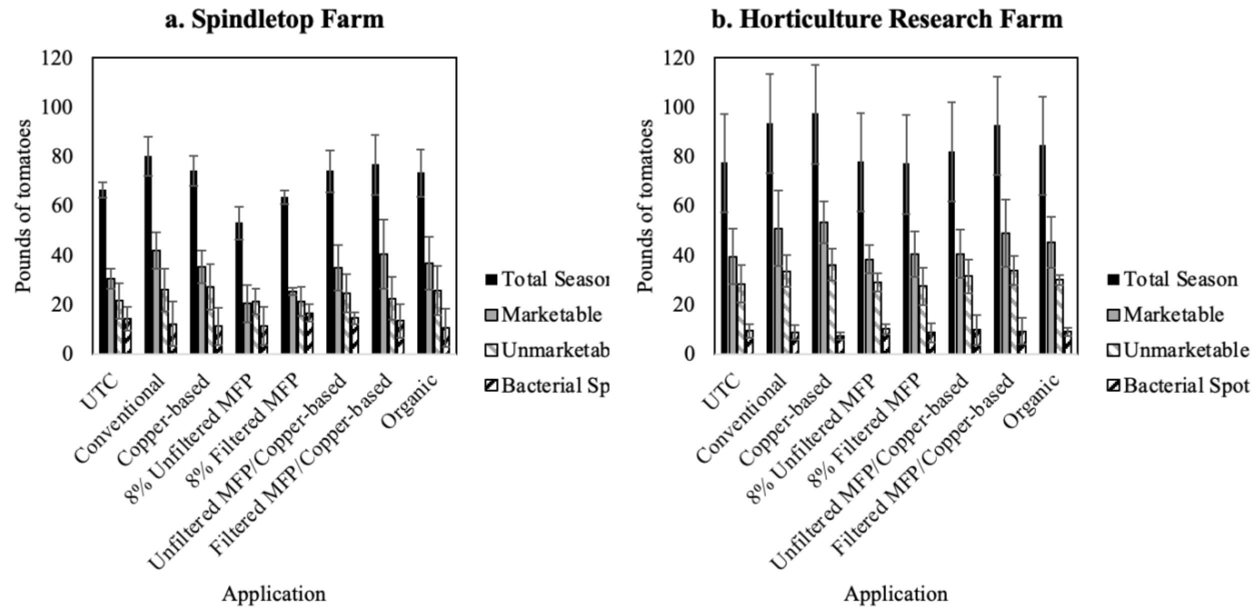


Fig. 4.2. Total weight in pounds of tomatoes harvested from each treatment at both Horticulture Research and Spindletop Farms in 2020. Marketable tomatoes were any tomatoes that were U.S. grade 1-3. BS was any fruit displaying bacterial spot symptoms. All other tomatoes were considered unmarketable. 5.a: tomato weights from the Spindletop Farm trial. 5.b.: tomato weights from the Horticulture Research Farm trial. Lines indicate standard deviation. Copper-based product is Nordox®. ($P<0.05$)

Figure 4.3 Average Weight Per Tomato for Each Treatment at Spindletop and Hortilcuture Research Farms in 2020

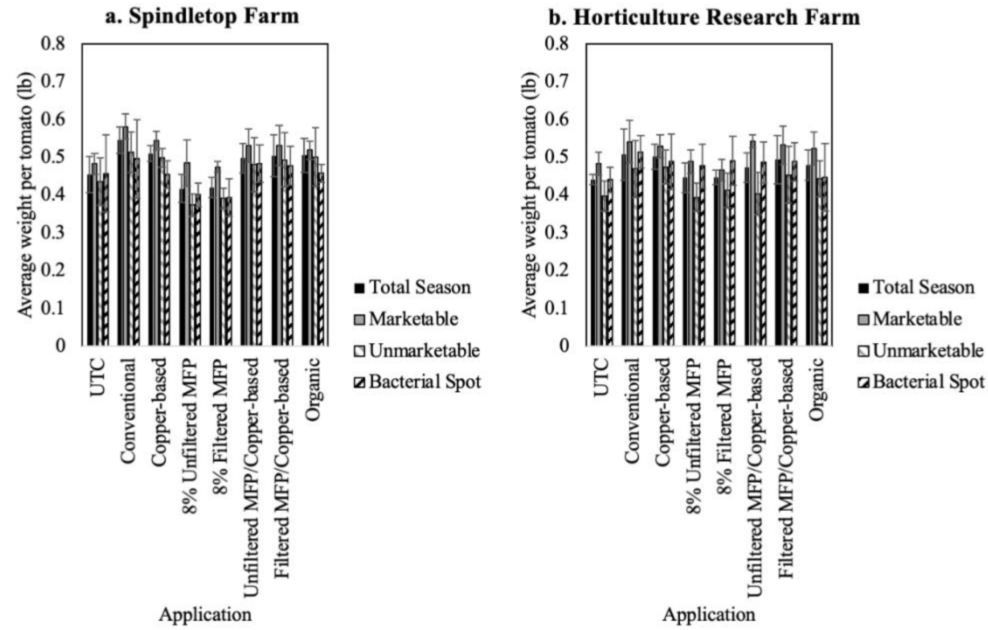


Fig. 4.3. Average weight per tomato harvested from each treatment at the Horticulture Research and Spindletop Farms in 2020. Marketable tomatoes were any tomatoes that were U.S. grade 1-3. BS was any fruit displaying bacterial spot symptoms. All other tomatoes were considered unmarketable. 6.a : average weight per tomato from the Spindletop Farm trial. 6.b.: average weight per tomato from the Horticulture Research Farm trial. Lines indicate standard deviation. The copper-based product was Nordox®. (P<0.05)

CHAPTER 5. PRELIMINARY INVESTIGATION OF POTENTIAL MODES FOR EFFICACY FOR A PROPRIETARY MICROBIAL FERMENTATION PRODUCT (MFP)

5.1 Introduction

There is potential for microbial fermentation products (MFP) to be a viable option for organic growers for management of plant disease. In previous studies (Chapters 2, 3, & 4) efficacy of the MFP was variable, particularly when used against powdery mildew and tomato bacterial spot. Previous experiments with high-tunnel tomatoes in Chapter 3, both the filtered and unfiltered MFP reduced disease severity of powdery mildew to a level similar to a copper-based product. In particular, when the MFP was used in an open tomato system (field production), it reduced the severity of tomato bacterial spot. Although this reduction in disease severity was not as prominent as the copper-based control, the reduction was significant ($P<0.05$). The basis for this activity is unknown. Given the product's efficacy in disease suppression in both fungal and bacterial pathosystems, the MFP has the potential to be a highly desirable aspect of integrated pest management.

Understanding the MFP's mode of action can assist growers in appropriate use and application. Bioproduct modes of actions are complex, with one or more properties. These modes of action may include competition, hyperparasitisim, antibiosis/antimicrobial, or plant defense induction. The MFP has the potential to control plant disease via one or more of these modes of action.

Previous efficacy studies in this dissertation have indicated that MFP may function as a multisite fungicide falling under FRAC Code M, which may also reduce the risk of pathogen resistance development. Products with multisite fungicide modes of action may

also be used to lessen or reduce the use of other products without such functionality. However, proper integration into different spray programs requires a deeper understanding of its mode of action.

MFPs are naturally occurring products and are comprised of multiple fermentation by-products including bacterial or fungal components such as yeast cells or lactic acid bacteria (LAB), or portions of these same cells and the spent fermentation media from which they were grown. These types of microorganisms are frequently employed in food fermentation and are considered non-pathogenic (Hutkins 2008). As some MFPs may include living organisms, modes of action may also be related to either competition or hyperparasitism.

Some components of microorganisms used for MFPs or their production have also shown the potential for antibiosis/antimicrobial effects. These include products such as chitins and polysaccharides and typical end-products of fermentation, such as alcohols, organic acids and antibiotics (Juturu and Wu 2018; Özel et al. 2018). Many of these components are well-documented to inhibit the growth of different pathogens. For example, nicin, a bacitracin, is produced by LAB and has a direct inhibitory effect on many different genera of bacteria (Juturu and Wu 2018; Özel et al. 2018). In MFPs that contain yeast cell wall components, such as chitin, direct inhibition of fungal growth and development can occur (Hadrami et al. 2010).

Additionally, many components of bacterial and fungal cells, as well their fermentation end-products, can contain bioactive components that may induce plant defense mechanisms. For example, both LAB and yeasts can produce β-aminobutyric acid, which is also found in plants (Dhakal et al. 2012). This compound can act as an

inducer of plant defense (Jakab et al. 2001; Jakab et al. 2005), as well as exhibit a direct inhibitory effect on plant pathogens (Elsherbiny et al. 2021).

Components of yeast cell walls such as glycan and mannan have been thought to elicit defense responses in plants due to their similarity to pathogenic polysaccharides (Basse et al. 1993). Activation of the *PR-1a* and *PDF1.2* genes was reported in *Arabidopsis* in response to application of yeast cell wall extract (Minami et al. 2011; Narusaka et al. 2015). Yeast cell wall extract applied to *Arabidopsis* was found to increase resistance to *Botrytis cinerea* infection (Yaguchi et al. 2017). Fragments of chitin, another component of yeast cell wall, have been shown to induce the accumulation of phytoalexins, pathogen-related (PR) proteins and proteinase inhibitors, lignin synthesis, and callose formation (Hadrami et al. 2010).

A similar product studied by Twamley et al. (2019), consisted of a proprietary blend of bacteria and yeast from a fermentation brewing media (Twamley et al. 2019). Those results indicated that MFPs may possess multiple modes of action. When applied to wheat, the MFP induced resistance by endogenous defense-related genes (Twamley et al. 2019). Significantly higher expression of PR genes was documented in MFP treated plants. Additionally, plants treated with MFP had a significant reduction of pustule as compared to the control. This group also noticed the powdery mildew in contact with the MFP had inhibited germination and spore differentiation.

The proprietary MFP used in assays conducted in our study contains yeast cell wall and inactive lactobacillus media. However, the product has not been well studied. It may contain intact microorganisms or components that may cause competition, hyperparasitism, antibiosis/antimicrobial, or plant defense induction. Consequently,

exploratory assays to examine multiple possible modes of action for the MFP are a logical next step.

In this study, we first aimed to elucidate the possible main modes of action of bioproducts; competition, hyperparasitism, antibiosis/antimicrobial, and plant defense induction. First, we examined the product for any viable organisms determine the possibility of competition or hyperparasitism. Our next steps were to assess the potential for pathogen sensitivity to the MFP through *in vitro* studies with both fungal and bacterial pathogens. Lastly, we examined the potential for induced resistance effects of this MFP on tomato.

5.2 Materials/Methods

5.2.1 Microbial Fermentation Product (MFP)

MFP was provided by Alltech, Inc. (Nicholasville, KY) and described as a microbial fermentation product. The provided product had high viscosity that created concerns with potential application. To address these concerns, a filtered and unfiltered MFP treatment were used for some experiments. To create the filtered product, reduce the viscosity and enable easier application to plants, the product was centrifuged for 15 min at 6,000 rpm (3226 Xg). The supernatant was then removed and run through a vacuum filter twice using an autoclaved Whatman 90mm filter paper (cat No 1001 090).

5.2.2 Bacterial Pathogens

5.2.2.1 Xanthomonas euvesicatoria

Xanthomonas euvesicatoria (UK, Calloway Co, KY) was utilized for RNA-seq inoculations. The culture was originally isolated from tomato plants exhibiting bacterial spots from commercial fields in Calloway Co, KY. Cultures were plated onto LB agar (VWR Radnor, PA) and grown in a dark temperature-controlled chambers (VWR Personal Low Temperature Incubator VWR Cat. No 89511-416) at 27 +/- 1° C for two days. For tomato inoculations, a single colony was transferred to LB Broth at 23°C for 48 h. Culture was centrifuged at for 15 mins at 4000 rpm (2151 X*g*). The supernatant was removed and pellet was re-suspended in 10 mM $MgCl_2$. This process was repeated a total of three times before bacterial count was calculated using spectrometer (OD_{600}). A concentration of $1x10^7$ CFU per mL in a buffer solution (10 mM $MgCl_2$) solution was used for inoculations.

5.2.2.2 *Pseudomonas syringae* pathovar tomato

Pseudomonas syringae (UK, location unknown) was used in the local and systemic induction assays. Cultures were stored in 15% glycerol in -80° C. *P. syringae* was grown on King's B agar (VWR) with kanamycin (GoldBio, St. Louis, MO) and rifampin (GoldBio) two days at 23° C for 48 h. Inoculum preparation was the same as for *X. euvesicatoria.*

5.2.3 Fungal Pathogens

5.2.3.1 Botrytis cinerea

Botrytis cinerea (isolate number 21KN001, Knox Co, KY,) was used in both spore inhibition assays and mycelial expansion assays. The cultures were stored as sclerotia at -

20° C and resuscitated on PDA plates (VWR) at 23 +/- 0.5° C. Conidia were harvested by flooding culture plates with 5.0 ± 0.2 mL autoclaved DI water and gently dislodging the conidia with a small pestle. The solution was then filtered through a cheesecloth. Initial harvested conidial concentrations averaged $1x10^6$ for *B. cinerea* and was determined using a hemocytometer.

5.2.3.2 *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum cultures (isolate number 20P73, CO unknown, KY) were used in mycelial expansion assays. Mycelial samples were stored in 15% glycerol solution at -80° C and resuscitated on PDA plates (VWR) at 23 +/- 0.5° C.

5.2.3.3 *Oidium neolycopersici*

Oidium neolycopersici (Muhlenberg Co. and Fayette Co., Kentucky) was used as the fungal inoculation in the RNA-seq experiments. The *O. neolycopersici* infected leaves were collected from infected whole tomato plants (Muhlenberg Co. and Fayette Co.) and stored at -4° C until used for inoculation (two days). Infected leaves were gently rubbed onto non-inoculated tomatoes leaves and returned to the chamber. Chamber conditions for inoculated plants were 12 light/12 dark light cycle with a day temperature of 25 +/- 1° C and a night temperature of 23 +/- 1° C (73.4 +/- 1° F) The relative humidity was maintained at 70% +/- 10%. Plants were maintained for 14 +/-2 days. *Oidium neolycopersici* inoculum was prepared by washing conidia from leaves harvested from the chamber-grown tomatoes with sterile DI water. Conidial concentrations were quantified by hemacytometer.

5.2.3.4 *Colletotrichum higginsianum*

Colletotrichum higginsianum (IMI 349063, obtained from CABI Bioscience, Wallingford, United Kingdom) cultures were stored at -80 on silica gel (Fisher, Hamptom, NH) and resuscitated on oatmeal agar plates (VWR) at 23 +/- 0.5° C (Tuite, 1969). Working stock plates were stored refrigerated at 4° C prior to use in experimentation. Cultures were transferred from working stock plates to oatmeal agar plates and cultivated for 2 weeks in continuous illumination at 23 +/- 0.5° C prior to harvesting. Conidia were harvested by flooding culture plates with 5.0 ± 0.2 mL autoclaved DI water and gently dislodging the conidia with a small pestle. After filtering through cheesecloth, the *C. higginsianum* conidial suspension was centrifuged at 6,000 rpm for 15 m (3226 X*g*). The supernatant was decanted, then the pellet was resuspended in autoclaved DI water. The centrifuging and resuspending step was repeated a total of three times. Initial harvested conidial concentrations averaged $1x10^7$ for *C. higginsianum* and was determined using a hemocytometer.

5.2.3.5 Magnaporthe oryzae

Magnaporthe oryzae isolate LpKY97-1 (Spindletop farm, Lexington, KY) was taken from a perennial ryegrass at Spindletop Farm, Lexington, KY. *Magnaporthe oryzae* stock was stored on autoclave filtered paper at -80C. M. oryzae was resuscitated on oatmeal agar and grown for two weeks in a 24 h light chamber at 23° C. The *M. oryzae* conidial suspension was not washed only filtered through cheesecloth after dislodging using the same method as described for *C. higginsianum.* Initial harvested spore concentrations averaged $1x10^5$ for *M. oryzae.*

5.2.4 Tomatoes

Plants ('Early Girl' or 'Rutgers') were grown in a Caron plant growth chamber (Caron Products, Marietta, OH) at 23° C, with 65% relative humidity during day light, and 20° C with 68% relative humidity at night. There was a 12 h light cycle. Plants were grown on autoclaved Pro-Mix soil (Premier Horticulture Inc., PA, USA). Soil was fertilized once using Scotts Peter's 20:10:20 peat lite special general fertilizer that contained 8.1% ammoniacal nitrogen and 11.9% nitrate nitrogen (Scottspro.com). Plants were irrigated using DI water.

5.2.5 Competition & Hyperparasitisim

5.2.5.1 Examination for viable organisms

Based on the description of the product (yeast cell walls, lactobacilli inactive media, and inert) PDA, YPD, Lactobacilli MRS, water agar, PCA, and King's B were used to determine if viable organisms were present within the product. The YPD and Lactobacilli MRS media allow the growth of either the yeast or lactobacillus. The PDA, PCA, and King's B were used to determine general fungal and bacterial growth. A 50 µL aliquot of unfiltered MFP was plated onto all of the following; PDA, YPD, Lactobacilli MRS, and water agar, duplicated three times, then incubated at 23° C for 48 h to observe the presence of colonies as an indication of viable colony producing cells presence. Experiments were repeated twice.

5.2.6 Antibiosis/Antimicrobial

5.2.6.1 Bacterial interactions

5.2.6.1.1 Pathogens

Xanthomonas euvesicatoria (Lexington, KY) and *Pseudomonas syringae* pathovar tomato were utilized for bacterial inhibition experiments. The *X. euvesicatoria* was used previously in the Ch2 and Ch4 studies. The *P. syringae* was used in induced resistance assays. Both cultures were handled as described above.

5.2.6.1.2 Liquid media inhibition

Bacterial inhibition assays used 25mL glass tubes with 5mL of amended King's B broth. Broth amendments included; 10% H_2O_2 (v/v), copper (11.5g/L), *Bacillus* (4.3g/L), filtered MFP at 12%, 8%, 4%, 2%, 1%, and 0.5% (v/v). Tubes were inoculated with the pathogens such that the initial concentration was $1x10^6$ CFU/ mL. Tubes were kept in Excella E25 incubator (New Brunswick Scientific, Enfield, CT) at 23° C dark. OD_{600} was taken at 24 and 48 h to estimate growth. Samples were enumerated by plating onto King's B at both 24 and 48 h and incubated at 23° C for up to 48 hr.

5.2.6.1.3 Solid media inhibition

Bacterial inhibition using solid agar was tested using *X. euvesicatoria* and *P. syringae*. An unamended King's B was divided into quarters. All plates were inoculated with the respective pathogen by applying 100 µl of $1x10^5$ CFU solution and spreading

evenly on the plates. Inoculum was prepared as previously described. In each quarter an autoclaved filter paper disk saturated with one of the treatments; autoclaved DI water, 10% H_2O_2, copper (Nordox®) filtered MFP, and unfiltered MFP was placed on the agar surface. Plates were incubated a 23° C dark bacterial growth chamber for 48 h.

5.2.6.2 Fungal interactions

5.2.6.2.1 Pathogens

The pathogens utilized for examining spore germination inhibition were *B. cinerea*, *C. higginsianum*, and *M. oryzae*. The pathogens chosen for the mycelial expansion assays were *B. cinerea*, *S. sclerotiorum*, and *C. higginsianum*. The samples were taken from the field infested the *O. neolycopersici*. All pathogens were handled as previously described.

5.2.6.2.2 High tunnel samples

Leaf samples were taken from 'Early Girl' tomatoes grown in an experimental high tunnel. High tunnels were inoculated with *O. neolycopersici*. In 2019 disease severity levels reached 100% by the end of the season in the water-treated controls. The description of 2019 Spring high tunnel maintenance and site set-up can be reviewed in Chapter 3. Representative disease samples (leaves) were selected from each treatment group to examine mycelial spread in a field setting. The leaves were then immediately stained for microscopic observations. The leaf samples were stained using a trypan blue staining protocol described by (Chandra-Shekara et al. 2006).

5.2.6.2.3 Mycelial growth

Initial examination of fungal mycelia was conducted on PDA agar. The pathogens used were *B. cinerea*, *S. sclerotiorum*, and *C. higginsianum*. PDA media was amended with the following treatments for *B. cinerea* and *S. sclerotiorum*: copper (Nordox®, Brandt, Springfield, IL, 11.5g/L), unfiltered MFP (Alltech, Inc., Nicholasville, KY) 8% v/v, and filtered MFP 8% v/v. For the *C. higginsianum* assays the treatments were: organic standard (contained copper (Nordox®, Brandt, Springfield, IL, 11.5g/L and *B. cinerea* (Companion®, Growth Products, Liberty, MO, 4.3g/L), conventional standard (contained copper, BTH, and mancozeb (Dithane®, Corteva, Wilmington, DE, 14.4 mL/L), unfiltered MFP (Alltech, Inc., Nicholasville, KY) 8% v/v, and filtered MFP 8% v/v. An unamended PDA media was used as a control. A plug of the pathogen was placed in the middle of each plate. Mycelial spread was determined by measuring the length and width of the leading edges and was assessed daily for one week or until the pathogen on the unamended control reached the edge of the plate. Percentage of inhibition was calculated on a day-by-day basis. Calculations were by dividing cm growth in treatment cultures (T) by cm growth in control (C) and multiplying by 100. This number was then subtracted from 100 for percentage of inhibition (100-((T/C)*100)).

5.2.6.2.4 Spore germination

Spore germination assays demonstrated fungal spore development with *B. cinerea*, *C. higginsianum*, and *M. oryzae*. The conidial concentration was quantified using a hemocytometer and first adjusted to $1x10^6$ conidia per ml for *C. higginsianum* and $1x10^5$

for *B. cinerea* and *M. oryzae*. Spore treatments included a negative water control, a positive control with 10% hydrogen peroxide (Fischer Scientific, Waltham, MA). Conidia ($1x10^6$ conidia per mL) were treated with Nordox® (Brandt, Springfield, IL), Companion® (Growth Products, Liberty, MO), and MFP (filtered, Alltech, Inc., Nicholasville, KY) individually for both *C. higginsianum* and *M. oryzae*. For the *B. cinerea* spore assays, all but the Companion® treatment was used. Six different concentrations of the filtered MFP treatment were used for *C. higginsianum* and *M. oryzae*: 12%, 8% (field concentration), 4%, 2%, 1%, and 0.5% (v/v, final concentrations). The *B. cinerea* used all the same concentrations, except that the lowest concentration was omitted (0.5% v/v, final concentrations). After mixing with a vortex, 15 µL of each concentration of each treatment were placed in a sterile empty petri dish (Falcon, København, Denmark). Petri dishes placed in a secondary container to prevent evaporation and maintained at ambient temperature. Spore germination was evaluated at 24 h. Percent germination was determined as the germinated/total conidia X 100. Each experiment was replicated three times.

5.2.7 Plant Defense

Plant defense induction was examined thorough multiple assays. Initially, the possibility of both local resistance and systemic acquired resistance were tested through standard assays to provide an indication if one or both was activated. Northern blots were then used as another confirmation of plant defense induction using SA mediated defense indicator genes. Then, a more in-depth RNA-seq assays was done to identify other genes that may be up- or down-regulated by the application of the MFP. These genes can then be compared to known plant defense genes. As there were exploratory studies, a model plant

system was used. Tomato was also a focus as this is the crop was used in previous field studies. An array of pathogens was used since variable efficacy was observed in the field against both bacterial and fungal pathogens in studies as described in previous chapters of this dissertation related to powdery mildew and bacterial spot.

5.2.7.1 Tomatoes

Tomato plants were used in the plant defense assays as this was the cropping system utilized in the field experiments described in Ch2, Ch3, and Ch4. Plant maintenance was as described previously.

5.2.7.2 Pathogens

Oidium neolycopersici and *X. euvesicatoria* were used in the RNA-seq experiments as these were the field isolates where efficacy was seen with the MFP. *Pseudomonas syringae* pathovar tomato was the isolate used in the local resistance assay. This pathogen causes tomato bacterial speck, a similar disease to tomato bacterial spot caused by *X. euvesicatoria*. All pathogens were maintained as previously described.

5.2.7.3 Local resistance assays

Tomato inoculations were accomplished using a pressure pump (GAST Manufacturing, Benton Harbor, MI). To inoculate leaves, the procedure of Shine et al., 2015 was used. A leaf was held against a flat surface (ex. Petri plate) to ensure consistence pressure. Treatments of a 10 mM $MgCl_2$ and filtered MFP 8% were used. Samples from the local treated leaves were harvested at 0 and 3 days post inoculation (dpi) of a virulent

strain. Three leaf disks were randomly taken from infected leaves within each group. Samples from each treatment (10 mM $MgCl_2$, BTH, MFP) group were collected and divided into four microcentrifuge tubes each. Samples were homogenized by vortexing in 10 mM $MgCl_2$. DPI 3 samples were diluted 10^3 – or 10^4 – fold. All samples were plated on King's B agar (amended with kanamycin, 50 mg/L and rifampin, 25 mg/L) and incubated at 23° C for two days. All colonies were enumerated.

5.2.7.4 Northern blots

Whole 'Rutgers' tomato plants were sprayed with filtered and unfiltered MFP at an 8% concentrations or DI water, with a small Preval sprayer (Nakoma Products) on whole plants. Three replicate samples were taken at 24 and 48 h after application. Small-scale extraction of RNA from samples was performed using TRIzol reagent (GIBCO/BRL, Gaithersburg, MD) following manufacturer's instructions. Northern blot analysis and synthesis of *PR-1* probe was synthesized as described by Shah et al. (Shah et al. 1999). The RNA gel blot hybridization was performed as described previously (Kachroo et al. 1995).

5.2.7.5 RNA-seq

Xanthomonas euvesicatoria and *O. neolycopersici* inoculum was developed as described previously. Whole 'Rutgers' tomato plants were first sprayed with either water or MFP at 8% concentration. After 24 h tomatoes were inoculated with either *X. euvesicatoria* or *O. neolycopersici* or treated with the respective control. *X. euvesicatoria* inoculum was applied to whole tomato plants by first adjusting the concentration to 1 X 10^6 mL with DI water then spraying with a small Preval sprayer.

Oidium neolycopersici inoculum was applied in the same manner as was described in high tunnel Ch3 Samples for submission to Novogene (Sample Receiving Department, Novogene Corporation Incorporated, 2921 Stockton Blvd., Suite 1810, Sacramento CA 95817) were taken at 24 h post inoculation as described in Northern blot analysis. RNA was prepared as was described for Northern blot analysis. Samples were then prepared for RNA-seq analysis by following requirements described by Novogene (Beijing, China).

5.3 Results

5.3.1 Competition & Hyperparasitisim

No viable microorganisms were found when MFP was plated for 48 h on PDA, water agar, YPD, Lactobacilli MRS agar, PCA, and King's B agar (detection limit =1.5 CFU/mL MFP). Consequently, if viable microorganisms were present, their concentrations would be well below those typically used in spray treatments based on exclusion or competition with pathogens at the leaf surface (Pal and Gardener 2006; Vinale et al. 2008).

5.3.2 Antibiosis/Antimicrobial

5.3.2.1 Bacterial interactions

In the bacterial inhibition assays, at the 8% level, both the filtered and unfiltered MFP showed inhibition of growth of *X. euvesicatoria* (Figure 5.1). X*anthomonas. euvesicatoria* was inoculated into LB at a level of 6.0 Log CFU/mL. After 24 h, *X. euvesicatoria* concentrations in controls reached 8-8.5 Log CFU/mL. Nordox®, as well as

levels of filtered and unfiltered MFP at 4% and lower also showed similar high populations after 24 h incubation. These levels remained relatively unchanged at 48 h. However, both the filtered and unfiltered MFP at 8% had no more than 3.5 Log CFU/mL at 24 h, which again were similar at 48 h. This level represents a loss in population from the starting inoculation level of 6.0 Log CFU/mL. Clearly, MFP at 8% levels can inhibit the growth of *X. euvesicatoria* in liquid media and may also result in cell death. *X. euvesicatoria* is one of the casual pathogens of tomato bacterial spot and was used to inoculate the fields in the 2019 and 2020 trials. The concentration (8%) was the same concentration used in the fields.

Bacterial growth of *P. syringe* was completely inhibited in both the filtered and unfiltered MFP 8% and losses in population also occurred such that populations remaining were below detection (Figure 5.2). Additionally, as opposed to *X. euvesicatoria* complete bacterial inhibition was seen with *P. syringae* at 4% with the unfiltered MFP.

As opposed to the significant inhibition observed with the MFP amended liquid, limited inhibition of *X. euvesicatoria* and *P. syringe* (small zones) was seen when MFP was added with filter discs to agar. Areas directly adjacent to and underneath discs did not have bacterial growth. MFP concentration or filtered status did not appear to impact the size of the inhibition zone.

5.3.2.2 Fungal interactions

5.3.2.2.1 Mycelial colonization

Figure 5.3 shows leaf samples taken from representative leaves from the 2019 Spring high tunnel (Chapter 3). In comparison to the inoculated and non-inoculated control,

both filtered MFP and unfiltered MFP, as well as the copper product have less mycelial colonization. The *B. cinerea* sample appears to have as much mycelial colonization as the inoculated treatment.

Mycelial expansion assays were conducted to examine this effect further (Figure 5.4). *Botrytis cinerea* was used as an alternative to *O. neolycopersici*. All treatments (copper, filtered MPF, unfiltered MFP) reduced mycelial growth as compared to the unamended media control. The copper treatment showed the most efficacy. Both the filtered MFP and unfiltered MFP grouped statistically together ($P>0.05$) ranging from about 45% to 55% inhibition for the length of the experiment.

In the *S. sclerotiorum* assay (Figure 5.5) all three treatments had again shown a reduction in mycelial growth as compared to the unamended PDA control. The unfiltered MFP and filtered MFP have variable efficacy however, the unfiltered MFP grouped statistically ($P>0.05$) with the copper at the two day time point and mostly differed from the filtered MFP.

The assays involving *C. higginsianum* had slightly different control treatments (Fig 5.6). Instead of a copper there was a conventional and an organic standard. However, both these treatments did also contain copper. The conventional and organic standard treatments consistently had the highest ($P<0.05$) percentage of mycelial growth inhibition. The filtered and unfiltered MFP also showed some inhibition of mycelial growth with reductions of approximately 50%, but not as much as the two standards.

With respect to the use of the novel MFP to inhibit *C. higginsianum* (seen in Figure 5.6), as noted both the filtered and unfiltered MFP exhibited some inhibition when

compared to the control (*P*<0.05). However, notably, inhibition was greater for the unfiltered MFP.

Figure 5.7 shows mycelial growth of C. *higginsianum* plated on amended water agar. The control shows similar mycelial colorations through multiple replications. Both the conventional and organic standards not only prevented all mycelial spread, but the plugs appeared blackened over time. In the filtered and unfiltered MFP treated plates, mycelial show orange pigmentation. This orange appearance may be due to sporulation.

5.3.2.2.2 Spore inhibition

Botrytis cinerea spore germination had complete inhibition at the MFP concentration of 12% (Figure 4.8). There was also complete inhibition with the hydrogen peroxide and the copper treatments. At all other concentrations, the spore germination was statistically similar (*P*>0.05) to the water treatment.

Both *C. higginsianum* and *M. oryzae* showed complete spore germination inhibition with MFP at 4% and higher in the spore drop assays (Figures 5.9 and 5.10). At 2% MFP, some inhibition was observed (*P*<0.05) however, at levels lower than 2% no difference from the water control was observed. This same trend is observed in the onion membrane assay (Figure 5.11 and Figure 5.12), with complete spore germination inhibition at concentrations of MFP at 4% and higher. This inhibition indicates that at a minimum of 4% MFP, some component within the MFP can cause complete spore germination inhibition in *C. higginsianum* and *M. oryzae*.

As seen in Figure 5.13 and 5.14, although the lower concentration of MFP complete germination inhibition was not observed, there was abnormal spore morphology in both the *C. higginsianum* and *M. oryzae*. *C. higginsianum* spores can be seen with elongated swollen germ tubes (Figure 5.13). Other abnormal morphology like appressorium development issues are demonstrated in Figure 5.13 as well. In Figure 5.14 some *M. oryzae* appressorium appear to have ruptured. There was no observed abnormal pathogen morphology in the *B. cinerea* experiments.

5.3.3 Plant Defense

5.3.3.1 Northern blots

Both filtered and unfiltered MFP show *PR-1* gene induction in tomato (Figure 5.15). In evaluations of gene induction in tomato, *PR-1* induction was seen at 48 hours post MFP application.

5.3.3.2 Local Resistance assays

In localized resistance in tomato, the MFP statistically differed ($P<0.05$) from the negative control in the level of *P. syringae* found 3 dpi (Figure 5.16).

5.3.3.3 RNA-seq

As seen in Figure 5.17, after the application of 8% MFP there are a number of genes that are both up and down regulated. Figure 5.18 and Figure 5.19 examine the effect of pathogen inoculation on the up/down regulation of genes both individually and in

combination with MFP treatment. Only a limited number of genes with associated annotations were found for both the bacterial spot and powdery mildew pathogens. Notable genes can be seen in Table 5.1.

5.4 Discussion

The MFP appears to have two likely modes of action; antibiosis/antimicrobial interactions with both bacteria and fungi and the induction of plant defense. During this study, viability experiments were conducted to elucidate the possible proprietary MFP's modes of action. The common bioproduct modes of action hyperparasitism, competition, antagonism/antimicrobial, and induction of plant defense (Köhl et al. 2019) were examined.

5.4.1 Competition & Hyperparasitisim

The assays suggest that competition or direct parasitism are unlikely modes for activity of the proprietary MFP. No viable organisms were detected within the MFP as tested. As this product is produced though microbial fermentation and the presence of viable microorganisms may influence disease severity, their absence is an important consideration in the evaluation of MFP efficacy. Although experiments were focused on organisms that were most likely to be present in the MFP based on what is known of the content and fermentation process, it is unlikely other species were present in these products since no viable organisms were above detection limits when grown aerobically on the common bacterial and fungal media. This indicated that any modes of action associated with living organisms is unlikely.

5.4.2 Antibiosis/Antimicrobial

5.4.2.1 Bacterial interactions

In our bacterial assays the MFP did show potential for direct antibacterial effects. Both bacterial strains used (*X. euvesicatoria* and *P. syringae*) showed reduced growth to some extent as field concentration used (8%) in liquid media growth. On the solid media, bacterial growth was reduced only when in direct contact with the MFP impregnated filter paper. One of the possible causes of the difference between solid and liquid media may be the solubility of the compound that produces the antibacterial effect. Compounds such as chitin are non-soluble and have been shown to have direct inhibitory effects on pathogen growth (El Hadrami *et al.*, 2010). Chitin is present in fungal cell wall and is likely present within the MFP since one primary component is yeast cell wall.

5.4.2.2 Fungal interactions

The *in vitro* studies indicated that the MFP slowed the rate of mycelial growth of all examined pathogens. The MFP appeared to influence the pathogens rate of growth to a different degree. This may be to varying sensitivities of the selected pathogens to the MFP. It may also be due to the trophic state the pathogen (biotrophic, hemibiotrophic, necrotrophic). Further research is required to elucidate the exact role based on hemibiotrophic or necrotrophic pathogenicity.

An interesting observation that was made during this study was the difference in coloration of the *C. higginsianum* growth on the MFP plates to the untreated control (Figure

5.7). This coloration can be associated with increased sporulation. This can be caused by hampering the pathogen's growth (Dahlberg and Etten 1982; Su et al. 2012). Starvation or nutritional depletion can lead to spore stimulation (Braun et al. 2011; Dahlberg and Etten 1982; Su et al. 2012; Wulandari et al. 2009). The addition of some compounds that cause fungal stress can also induce sporulation (Masangkay et al. 2000; Shahin and Shepard 1979). The chemical or other factor involved with limiting the mycelial growth may be the same as that causing the increased sporulation.

The MFP was able to inhibit spore germination in the *in vitro* assays with all pathogens. However, in this study, there was some germination variability based on the pathogen. We observed complete *B. cinerea* spore germination inhibition at 12% with the MFP. The *B. cinerea* pathogen was utilized due to its relationship to *O. neolycopersici* and the ability to culture in the laboratory. The non-tomato disease related pathogens examined, C. *higginsianum* and *M. oryzae*, appeared to have a higher sensitivity to the MFP. They showed complete spore germination inhibition as low as 4% concentration, with indications of further sensitivity at 2% concentrations. These indications were observed as abnormal morphology in the some of the germinated spores from *C. higginsianum* and *M. oryzae*. This abnormal formation of germ tube and appressorium may influence the pathogen's ability to penetrate the host. There was no abnormal morphology detected in the *B. cinerea* spore germination assay. However, to confirm that there are no abnormal morphological changes with the *B. cinerea,* it would be helpful to add additional MFP concentrations. This would help to elucidate a sort of dose response and determine if abnormal morphological changes also occur with *B. cinerea*. Fungicides that directly interacted with fungal pathogens may interrupt aspects of the fungal disease life cycle such

as spore germination, host penetration, and colonization of the host. This abnormal morphology can help in understanding the impact of MFP on disease development.

Additional experiments are required to determine if the abnormal morphology seen in certain pathogens may cause a reduction in host penetration. If the MFP impairs the pathogen's ability to develop the penetration structures, it may not have as high an impact on fungi that do not require such appressorium to enter the host. The cause of varying sensitivities should also be investigated. This may play a large role in the application of this product in the field, where rates necessary to reduce pathogen resistance development require definition. The use of further alternative pathogens may allow increased elucidation of the role of these structures.

5.4.3 Plant Defense

Plant defense induction is one other possible mode of action for the MFP. The results indicated that plant defense induction may occur, but at relatively low levels. When examining the MFP's ability to induce local defense, there were statistically significant differences ($P<0.05$) from the untreated control, but the MFP did not statistically group ($P>0.05$) with the positive control. These are preliminary indications that plant defense induction may play a partial role in the efficacy of the MFP.

Similar products such as used in Twamley et al (2019), demonstrated an ability of an MFP to induce known plant defense related genes such as *PR1, PR4, PR5* and *PR9* in wheat. The induction of plant defenses is a less single site-specific mode of efficacy. As this MFP had shown the ability to reduce plant disease severity in both bacterial and fungal systems, plant defense induction may be an additional mode of efficacy.

To further investigate the possibility of plant defense induction northern blots were performed using the *PR-1* gene, an indicator gene for SA mediate plant defense (Ali et al. 2018). Products that induce plant defense genes, such as Actigard®, are shown to induce these genes (Louws et al. 2001; Obradovic et al. 2005). The MFP had demonstrated the ability to induce this gene in tomato systems. However, other non-chemical inducers exist and can confound results. Mechanical damage and insect feeding can also induce plant defense responses. At the 1% concentration the MFP demonstrated the ability to cause damage to more sensitive plants such as *Arabidopsis*. At the concentrations used in these studies, no damage to the plant was observed. Nonetheless, induction due to surface damage cannot be discounted through northern blot. To further investigate the possibility of plant defense induction an RNA-seq analysis was performed. RNA-seq analysis can reveal more detailed information through an examination of increased or decreased gene expression.

RNA-seq analysis revealed that there were some other notable groups of genes associated with plant defense either up or down regulated by the MFP regardless of pathogen inoculation. These genes were associated with general pathogenesis related genes, fatty acid desaturases, and ethylene (transcription factors) pathway related genes. An increase of these general pathogenesis related genes, as opposed to what may normally be up/down regulated due to pathogen infection, may indicate a priming affect by the MFP. However, the identified pathogenesis genes require further investigated to determine their specific roles with the MFP.

Additionally, ethylene related pathways genes were also found to be up-regulated on treatment with the MFP. Ethylene plays a role in mechanisms related to a defense

response to pathogen attack. Induction of ethylene biosynthesis and subsequent intracellular signals can lead to a cascade of transcription factors. Some of the corresponding transcription factors can be involved in the expression of effector genes involved with systemic induced defense responses or mediating different types of induced responses (Broekaert et al. 2006; van Loon et al. 2006; Zhu et al. 2011).

In addition to alterations in expression of ethylene related genes, fatty acid desaturases expression was also altered. Certain fatty acid desaturases can modulate the activation of defense signaling pathways in *Arabidopsis* (Kachroo 2009; Kachroo et al. 2001). In a study by (Li et al. 2011), they demonstrated that in wheat a fatty acid desaturase (TaFAD) was required for powdery mildew resistance. The up and down regulation of these gene groups along with the induction *PR-1* gene found in the Northern blot with a *PR-1* probe, indicates that the application of MFP may induce a plant defense response.

However, these results alone do not completely explain the results seen in high tunnel study and tomato bacterial spot (as described in previous chapters). In the high tunnel study, the use of MFP shows comparable disease severity reduction to the use of copper treatment. It also has statistically higher ($P<0.05$) levels of disease severity reduction than the living organism control, *Bacillus*, which can also induce plant defense. In bacterial spot study, the MFP treatments had shown efficacy in bacterial disease severity reduction as a stand-alone treatment throughout the growing season. This is not typical of plant defense inducers (Louws et al. 2001; Obradovic et al. 2005). Plant defense inducers are most efficacious when used early in the season as a preventative measure. Since their mode of action is a priming of natural plant defenses, once these genes are ‘activated’ the inducers play no further role in plant defense. They are also typically used in combination

with other products such as copper. This type of spray program is demonstrated with our conventional standard spray used in tomato bacterial spot study, this spray has a copper, mancozeb, and BTH based inducer (Actigard®). Consequently, although the MFP may induce plant defense mechanisms, there is likely an additional mode of efficacy that may be pathogen-dependent.

5.4.4 Summary

In summary, the MFP appears to have two likely modes of action. The primary mode of action may be the direct antimicrobial interaction with the pathogens, both bacterial and fungal. The secondary mode of action may be a low level of plant defense induction. The MFP is a natural product and comprised of many components some known but many are unknown. There may be one or many aspects contributing to the observations made in these experiments. In addition, these multiple components may act synergistically to produce these inhibitory effects.

Continued experiments to elucidate the factor(s) contributing to efficacy could begin with the examination of specific components such as chitin as the causative agent. As stated, chitin can both cause inhibition of pathogen growth and plant defense induction. The potential of chitin to be present in the MFP is high given the yeast cell wall content. There would be a benefit to more exploratory assays to narrow down the causal agent. Results from the fungal mycelial and spore inhibitory assays indicate that there may be a compound causing stress to the pathogen or creating an uninhabitable environment for continued growth.

Further examination of the causes of the direct interactions with the pathogens should be a primary focus for a product that is intended for organic production. As this product exhibited some influence on all pathogens tested, it may also affect non-pathogenic microorganisms present. As such, there is speculation that MFP may also have an adverse impact on the host due to a negative effect on non-pathogens and the normal microbiota present (Meena et al. 2020; Sumbula et al. 2021). Additionally, in organic production beneficial microorganisms are often utilized for disease management and soil health. Since this product does not distinguish between beneficial and pathogenic organisms, it may be necessary to perform additional tests to determine compatibility of products prior to extensive usage.

Table 5.1 Genes of Interest based on RNA-seq Data

Gene ID	Up/Down Regulated	Name/function/proposed role
Solyc12g009240.1	↑	Ethylene responsive transcriptional factor 4 gene
Solyc08g080670.2	↑	Osmotin-like protein, acquired resistance and stress responses
Solyc05g018410.2	↑	Enoyl-CoA hydratase, degradation of cis-unsaturated fatty acids
Solyc12g036320.2	↑	Serine/threonine protein kinase B, disease resistance and development regulation
Solyc07g052790.2	↑	A Nbs-Irr, resistance protein
Solyc12g049030.1	↓	Fatty acid desaturase, modulate defense signaling pathway
Solyc12g100250.2	↑	Fatty acid desaturase, modulate defense signaling pathway
Solyc07g052770.2	↑	Tir-nbs-Irr, resistance protein
Solyc11g044400.1	↓	Osmotin and pathogenesis-related protein
Solyc08g078180.1	↓	Pathogenesis-related/ethylene-responsive transcriptional factor
Solyc10g081970.2	↑	Fatty acid hydroxylase, CER1, stem epicuticular wax and pollen fertility
Solyc10g081970.2	↑	HIN1-like protein, disease resistance
Solyc03g00500.1	↑	Ethylene-responsive transcription factor 14, pathogenesis-related
Solyc01g079960.2	↑	Xylanase inhibitor, TAXI-1, increase resistance to *Botrytis cinerea*
Solyc06g065060.2	↑	FAD-binding domain-containing protein
Solyc04g064880.3	↑	Pathogenesis-related protein
Solyc03g119590.1	↑	NIMIN2c protein, control PR-1 gene expression in tobacco
Solyc01g065810.2	↑	NADH-quinone oxidoreductase subunit D
Solyc01g097240.3	↑	Pathogenesis-related protein
Solyc12g044950.2	↑	Omega-6 fatty acid desaturase
Solyc04g040130.1	↑	Omega-6 fatty acid desaturase
Solyc07g007750.3	↑	Defensin protein, antifungal activity and antibacterial activity
Solyc01g096420.3	↑	NADPH-dependent FMN reductase
Solyc12g100260.1	↑	Fatty acid desaturase
Solyc01g086680.3	↑	Glutathione S-transferase (GST), induce systemic resistance
Solyc02g077060.2	↑	Powdery mildew resistance protein (RPW8.2)

Table 5.1. Genes of interest from RNA-seq assays. Shows genes that were either up- or down-regulated with specific connections to plant defense.

Figure 5.1 *Xanthomonas euvescitoria* Growth in Liquid Culture

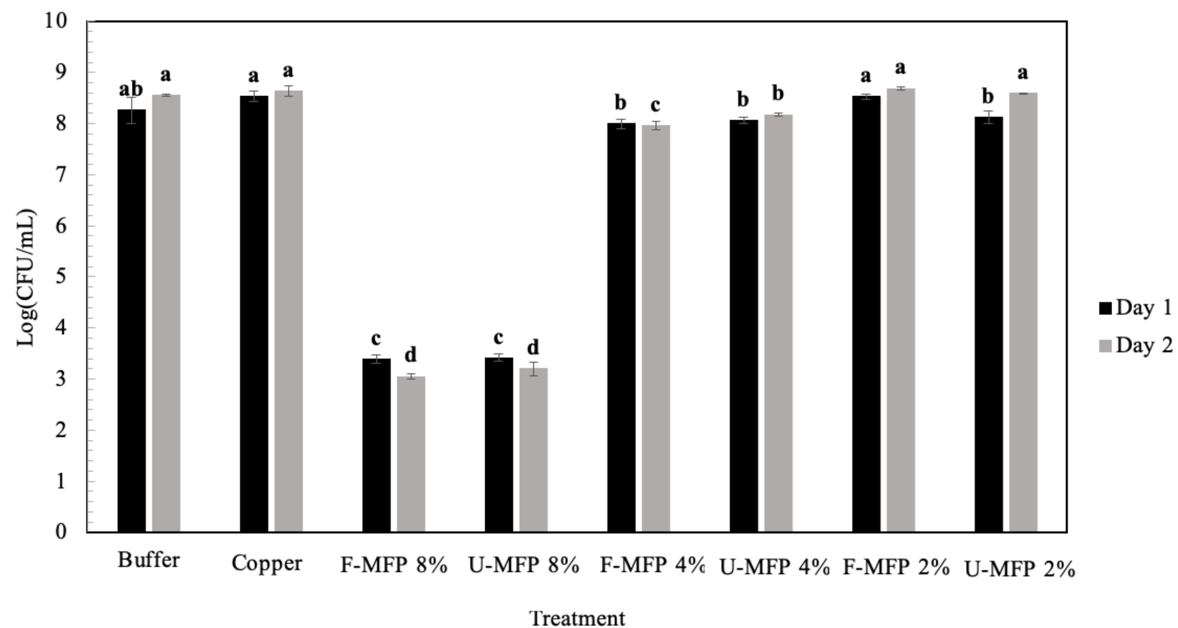


Fig. 5.1. *Xanthomonas euvesicatoria* populations after 1 and 2 days growth in l (LB broth) culture (initial population = Log 6.0 CFU/mL). Treatments of U-MFP (unfiltered microbial fermentation product), F-MFP (filtered microbial fermentation product), copper (Nordox®), and a buffer ($MgCl_2$) were included. Error bars are standard deviation and statistical differences ($P<0.05$) are indicated with letters.

Figure 5.2 *Pseudomonas syringae* Growth in Liquid Culture

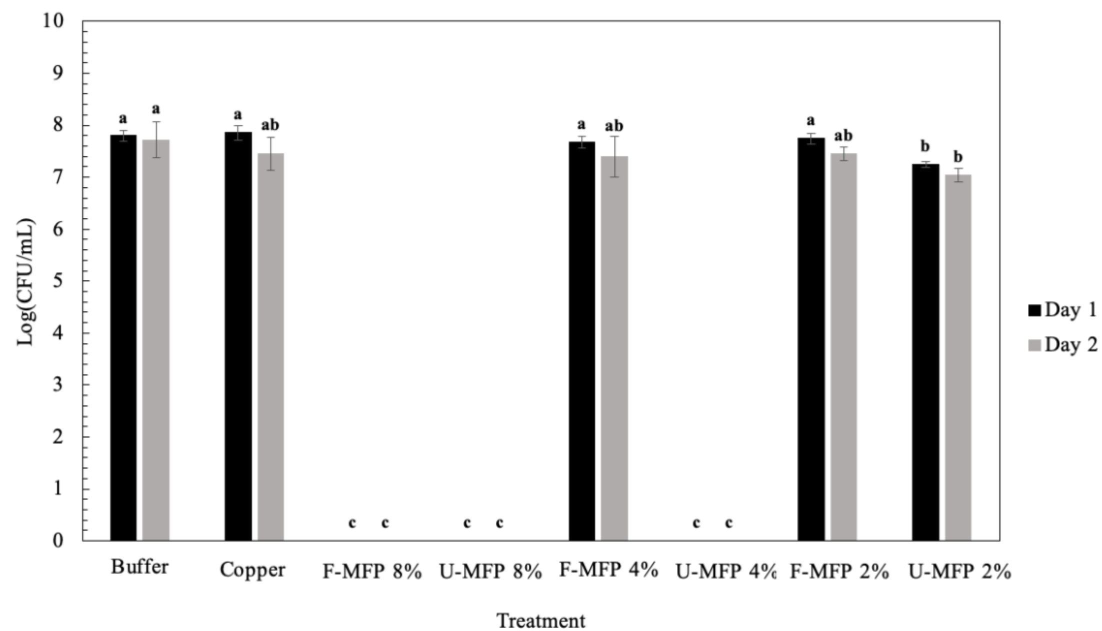


Fig. 5.2. *Pseudomonas syringae* population after 1 and 2 days (initial population = Log 6.0 CFU/mL). Treatments of U-MFP (unfiltered microbial fermentation product), F-MFP (filtered microbial fermentation product), copper (Nordox®), and a buffer ($MgCl_2$) were included. Error bars are standard deviation and statistical differences are indicated with letters (P<0.05).

Figure 5.3 Trypan Stained Leaf Samples from 2019 Spring High Tunnel Trial

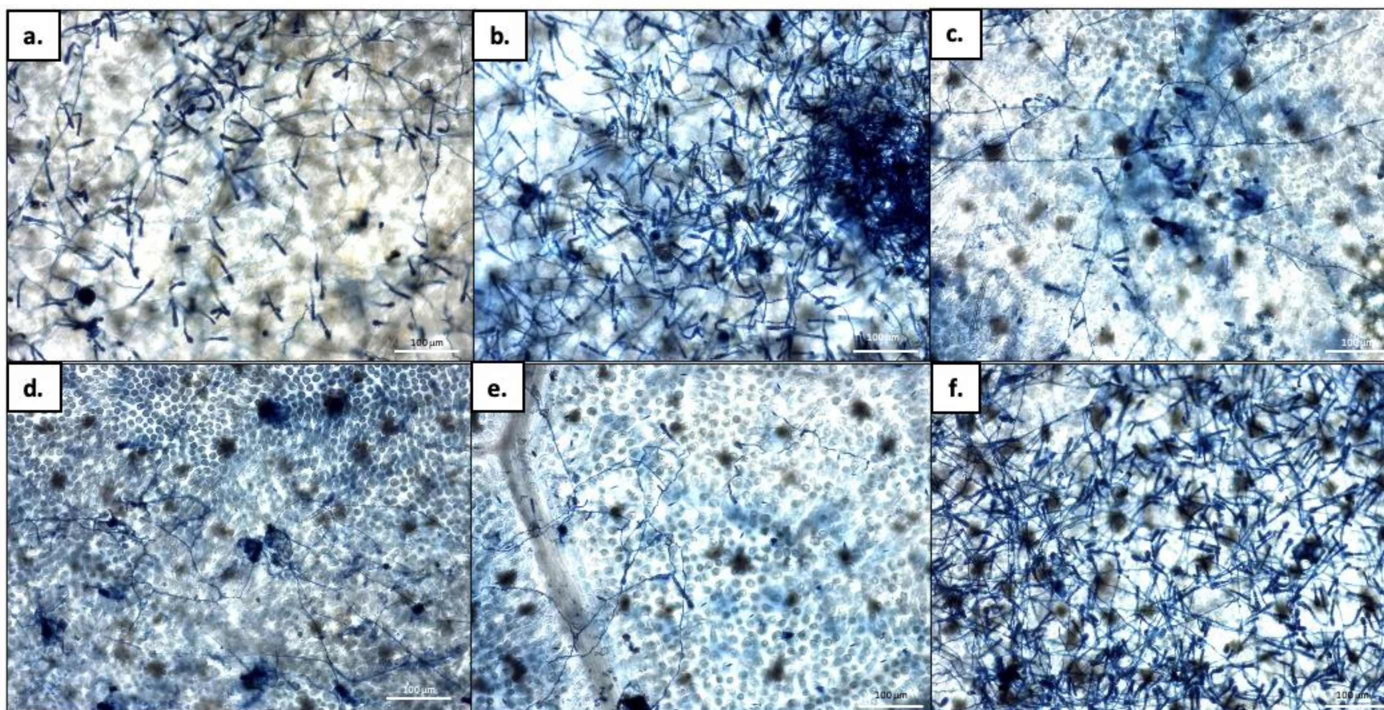


Fig. 5.3. *Oidium neolycopersici* mycelial colonization on tomato leaf from leaf samples collected from the 2019 Spring high tunnel. Leaf samples from the 2019 Spring high tunnel tomato trials, inoculated with *Oidium neolycopersici*. A. Un-inoculated treatment, b. Inoculated treatment, c. Unfiltered MFP 8%, d. Filtered MFP 8%, e. Copper-based product (Nordox®), f. *Bacillus* spp. (Companion®)

Figure 5.4 *Botrytis cinerea* Mycelial Inhibition

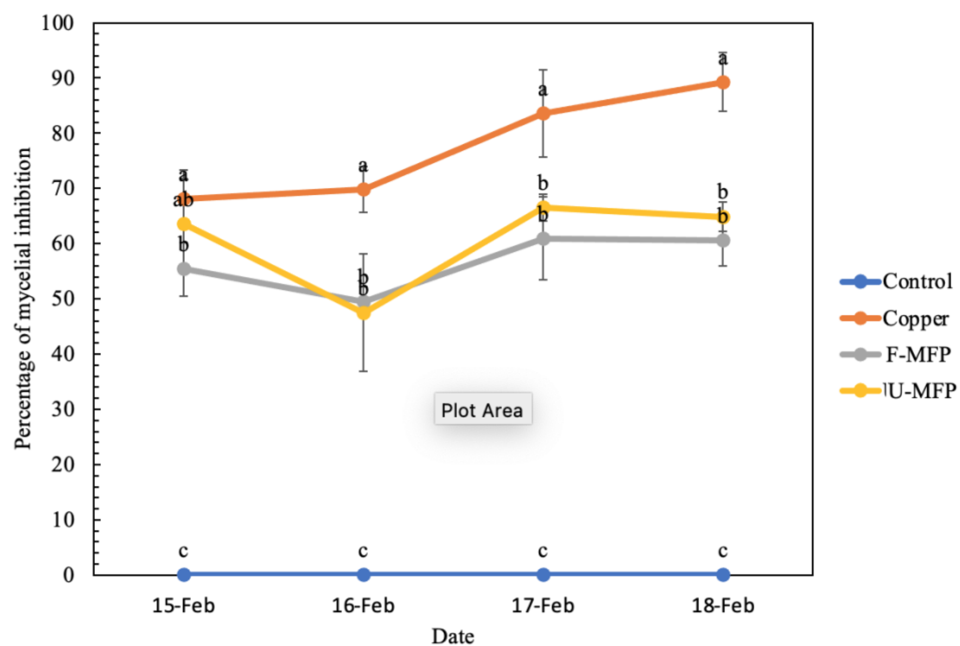


Fig. 5.4. *Botrytis cinerea* mycelial inhibition. Error bars represent standard deviation and statistical differences ($p < 0.05$) are indicated with letters. (n=3, =/- standard deviation). Statical comparison were conducted by individual dates. Calculations were conducted relative to the control unamended plates. Calculations were performed by dividing cm growth in treatment cultures (T) by cm growth in control (C) and multiplying by 100. This number was then subtracted from 100 for percentage of inhibition (100-((T/C)*100)). Treatments were copper (Nordox®), F-MFP (filtered microbial fermentation product), and U-MFP (unfiltered microbial fermentation product). All plates were amended PDA plates.

Figure 5.5 *Sclerotinia sclerotiorum* Mycelial Inhibition

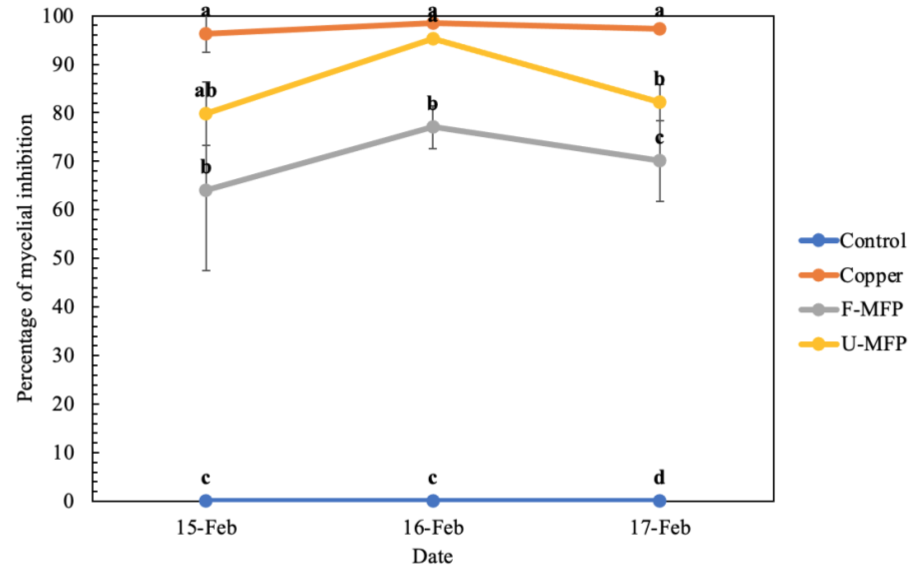


Fig. 5.5. *Sclerotinia sclerotiorum* mycelial inhibition. Error bars represent standard deviation and statistical differences ($P < 0.05$) are indicated with letters. (n=3, =/- standard deviation). Statical comparison were conducted by individual dates. Calculations were done relative to the control unamended plates. Calculations were performed by dividing cm growth in treatment cultures (T) by cm growth in control (C) and multiplying by 100. This number was then subtracted from 100 for percentage of inhibition (100-((T/C)*100)). Treatments were copper (Nordox®), F-MFP (filtered microbial fermentation product), and U-MFP (unfiltered microbial fermentation product). All plates were amended PDA plates.

Figure 5.6 *Colletotrichum higginsianum* Mycelial Inhibition

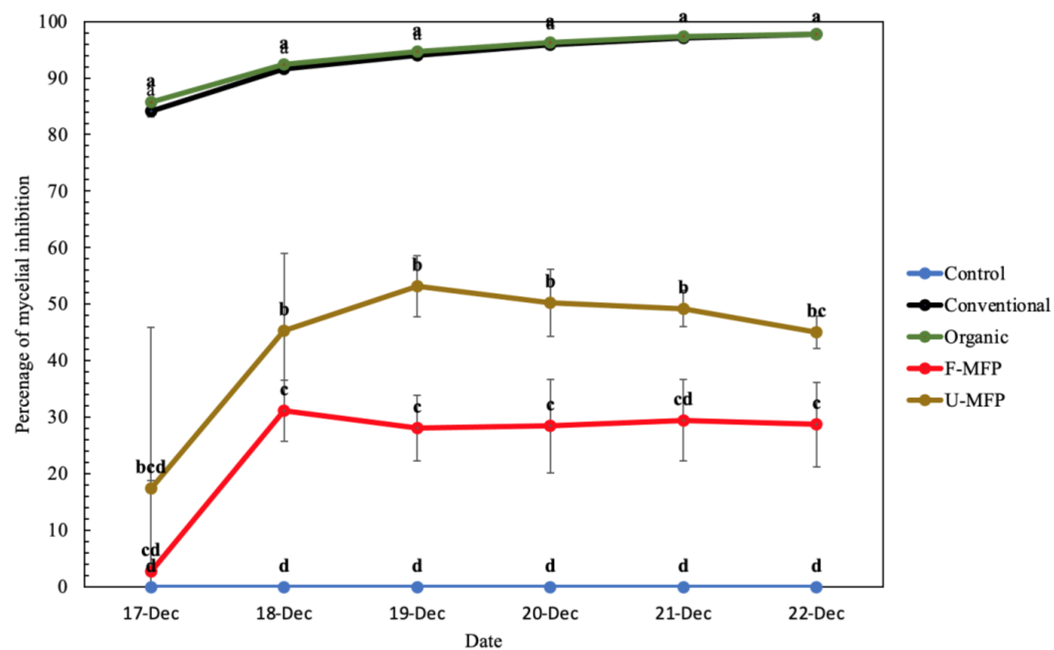


Fig. 5.6. *Colletotrichum higginsianum* mycelial inhibition. Error bars represent standard deviation and statistical differences (P<0.05) are indicated with letters. (n=3, =/- standard deviation). Statical comparison were performed by individual dates. Calculations were conducted relative to the control unamended plates. Calculations were performed by dividing cm growth in treatment cultures (T) by cm growth in control (C) and multiplying by 100. This number was then subtracted from 100 for percentage of inhibition (100-((T/C)*100)). Conventional treatment was comprised of copper (Nordox®), mancozeb (Dithane®), and BTH (Actigard®). The Organic treatment contained copper (Nordox®) and *Bacillus subtilis* GB03 (Companion®). All plates were amended PDA plates.

Figure 5.7 *Colletotrichum higginsianum* Cultures Grown on Ammended Plates

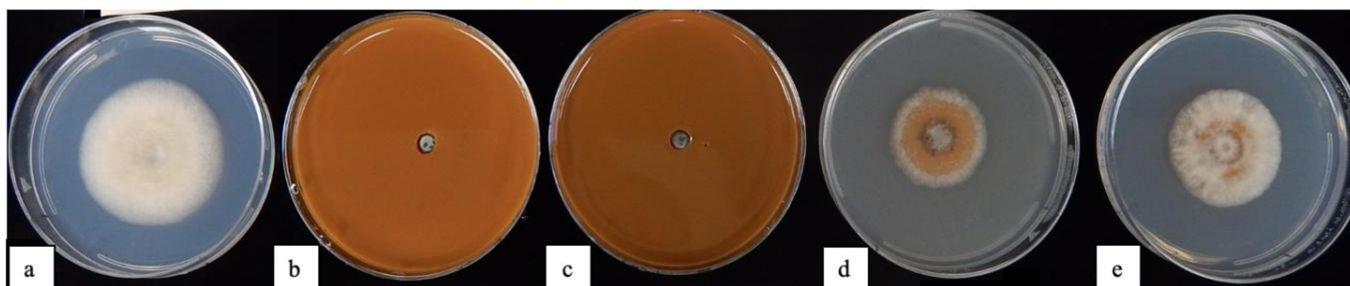


Fig. 5.7. *Colletotrichum higginsianum* cultures grown on amended water agar plates. a. control, b. Organic standard (contained Copper (Nordox®, Brandt, Springfield, IL, 0.6 – 1.1 kg/acre) and *Bacillus* (Companion®, Growth Products, Liberty, MO, 4.2 fl. oz per 100 liters. water), c. Conventional standard ((contained copper (same concentrations as organic), BTH (Actigard®, 0.33-0.75 (oz/acre)), and Mancozeb (Dithane®, Corteva, Wilmington, DE, 0.3-0.7 kg/acre)), d. Unfiltered MFP 8%, e. Filtered MFP 8%. These images were part of a larger experiment, some data of which is not reportable due to proprietary content.

Figure 5.8 *Botrytis cinerea* Conidial Germination

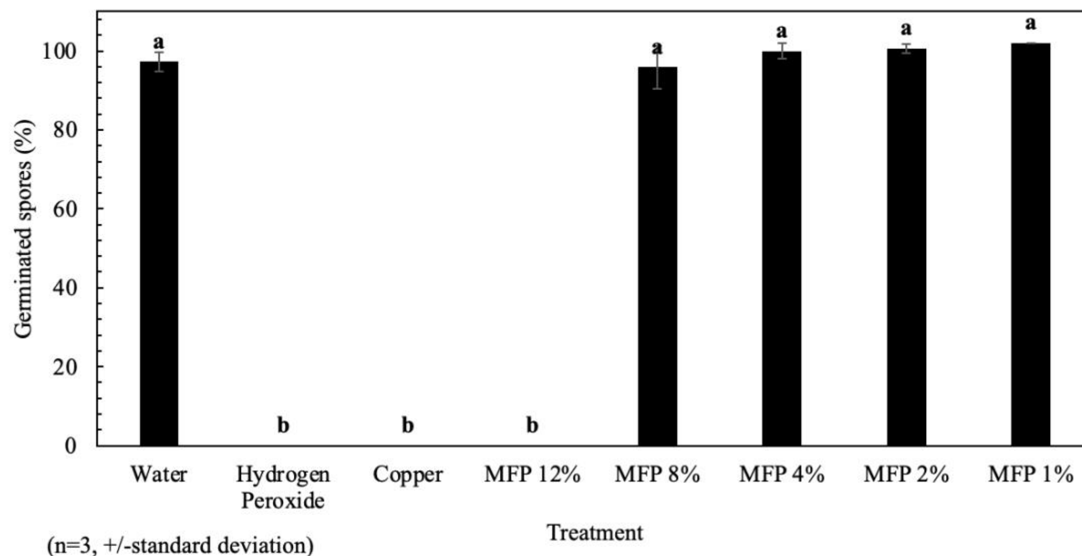


Fig. 5.8. Percentage of *Botrytis cinerea* conidial germination inhibition after 24 h of treatment (spore inhibition assay). Error bars are standard deviation and statical differences ($P < 0.05$) are indicated with letters. n=3. Treatments included Hydrogen peroxide (10%), copper (Nordox®), *Bacillus subtilis* GB03 (Companion®), MFP (filtered microbial fermentation product).

Figure 5.9 *Colletotrichum higginsianum* Conidial Germination

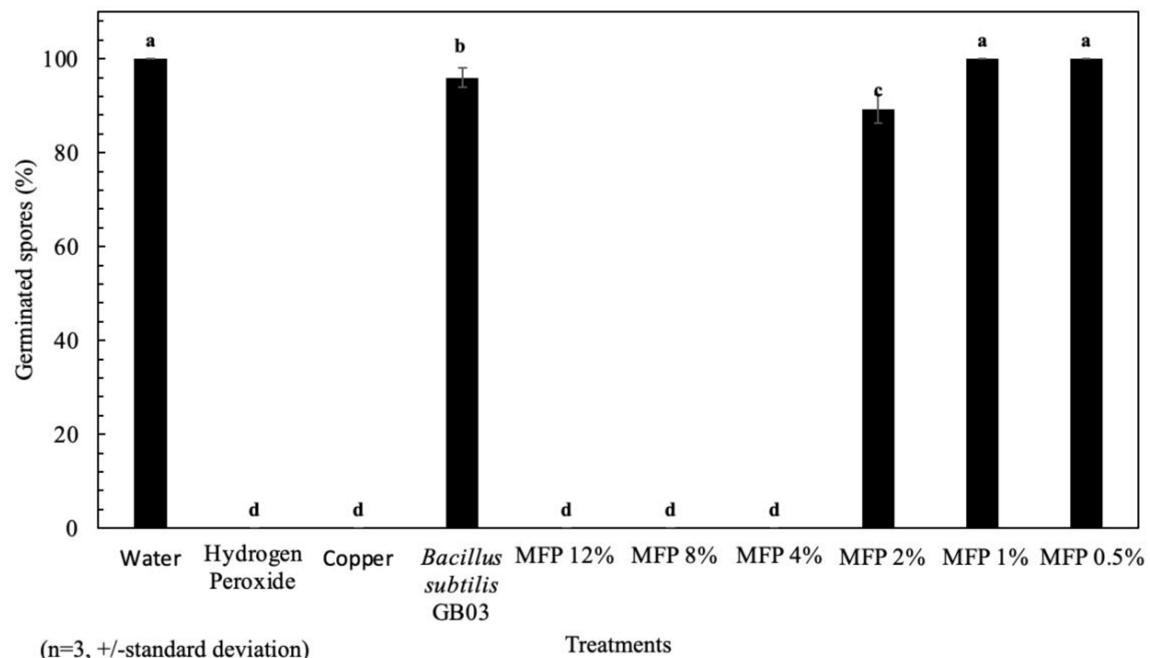


Fig. 5.9. Percentage of *Colletotrichum higginsianum* conidial germination inhibition after 24 h of treatment (spore inhibition assay). Error bars are standard deviation and statical differences ($P < 0.05$) are indicated with letters. n=3. Treatments included Hydrogen peroxide (10%), copper (Nordox®), *Bacillus subtilis* GB03 (Companion®), MFP (filtered microbial fermentation product).

Figure 5.10 *Magnaporthe oryzae* Conidial Germination

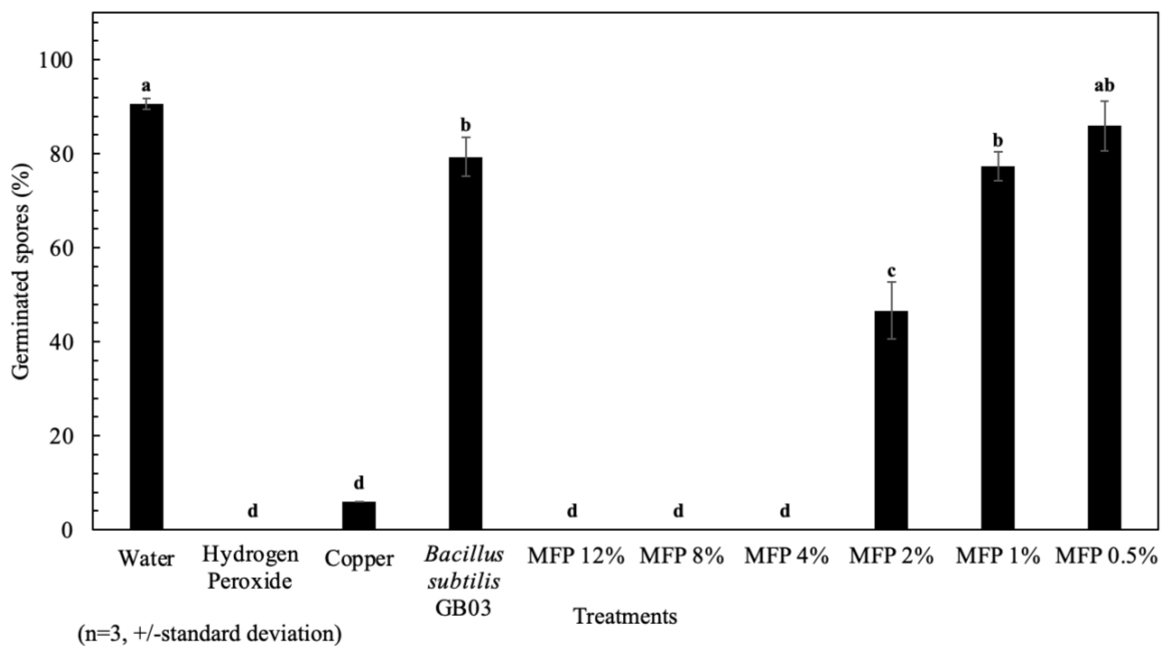
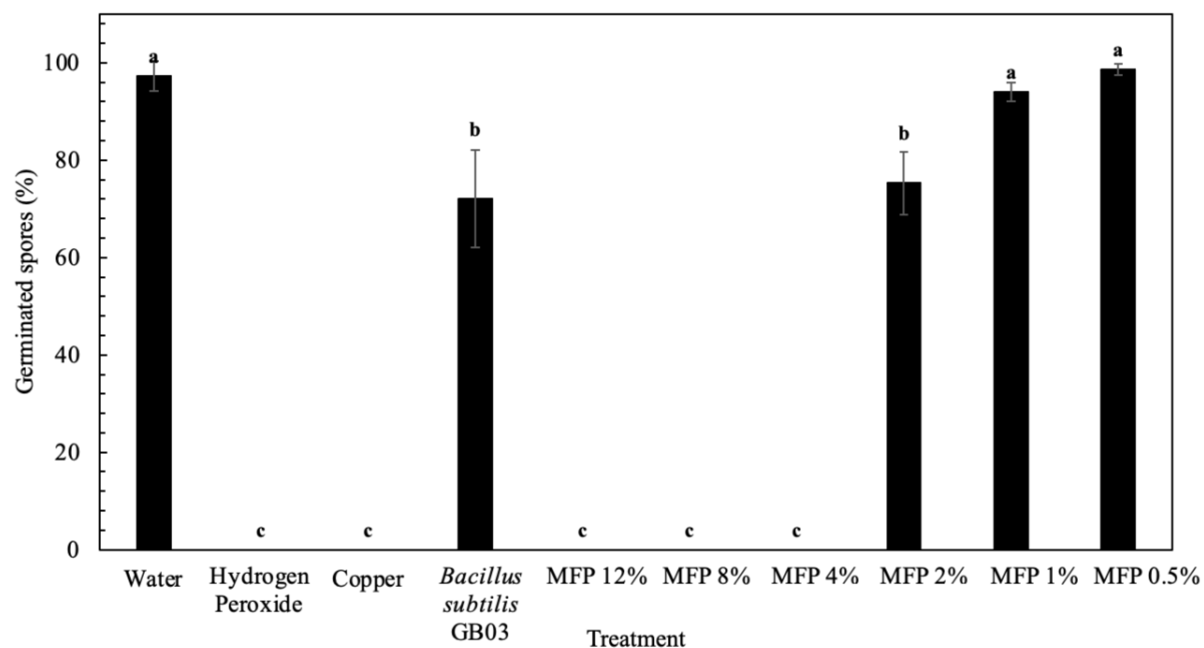


Fig. 5.10. Percentage of *Magnaporthe oryzae* conidial germination inhibition after 24 h of treatment (spore inhibition assay). Error bars are standard deviation and statical differences (P<0.05) are indicated with letters. n=3. Treatments included Hydrogen peroxide (10%), copper (Nordox®), *Bacillus subtilis* GB03 (Companion®), MFP (filtered microbial fermentation product).

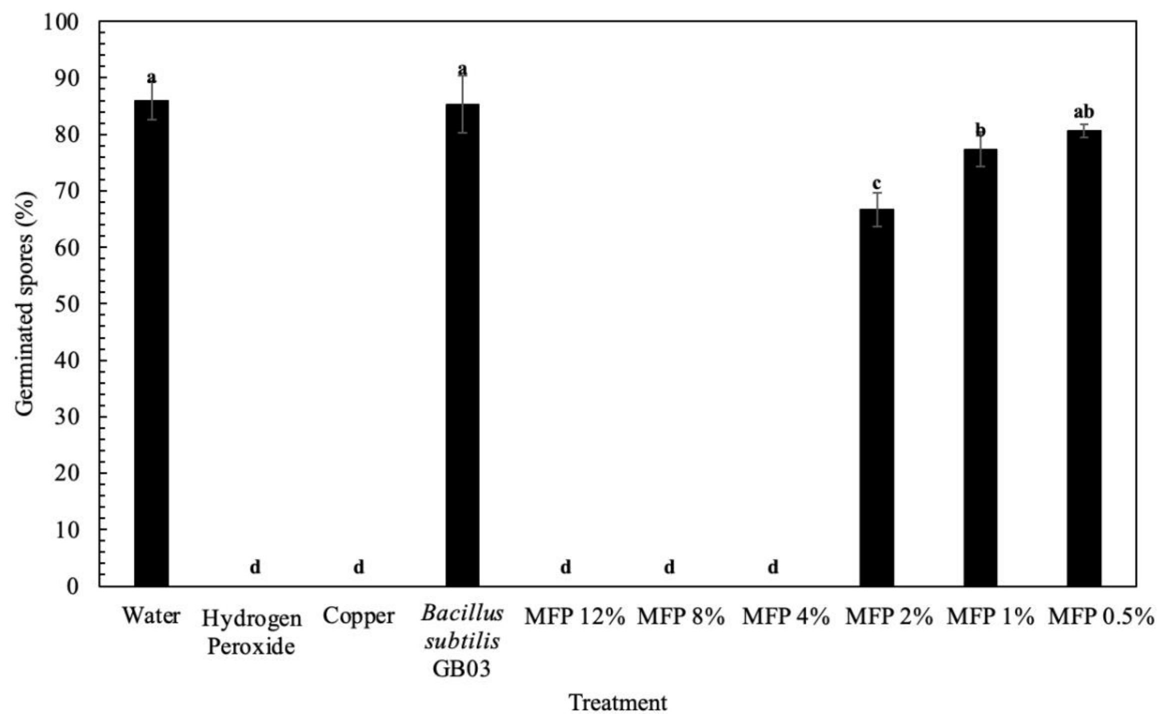
Figure 5.11 *Colletotrichum higginsianum* Condial Germination on Onion Membranes



(n=3, +/-standard deviation)

Fig. 5.11. Percentage of *Colletotrichum higginsianum* conidial germination after 24 h of treatment on onion membranes. Error bars are standard deviation and statical differences ($P<0.05$) are indicated with letters. n=3. Treatments included Hydrogen peroxide (10%), copper (Nordox®), *Bacillus subtilis* GB03 (Companion®), MFP (filtered microbial fermentation product).

Figure 5.12 *Magnaporthe oryzae* Condial Germination on Onion Membranes



(n=3, +/-standard deviation)

Fig. 4.12. Percentage of *Magnaporthe oryzae* conidial germination after 24 h of treatment on onion membranes. Error bars are standard deviation and statical differences ($P<0.05$) are indicated with letters. n=3. Treatments included Hydrogen peroxide (10%), copper (Nordox®), *Bacillus subtilis* GB03 (Companion®), MFP (filtered microbial fermentation product).

Figure 5.13 *Colletotrichum higginsianum* Conidia

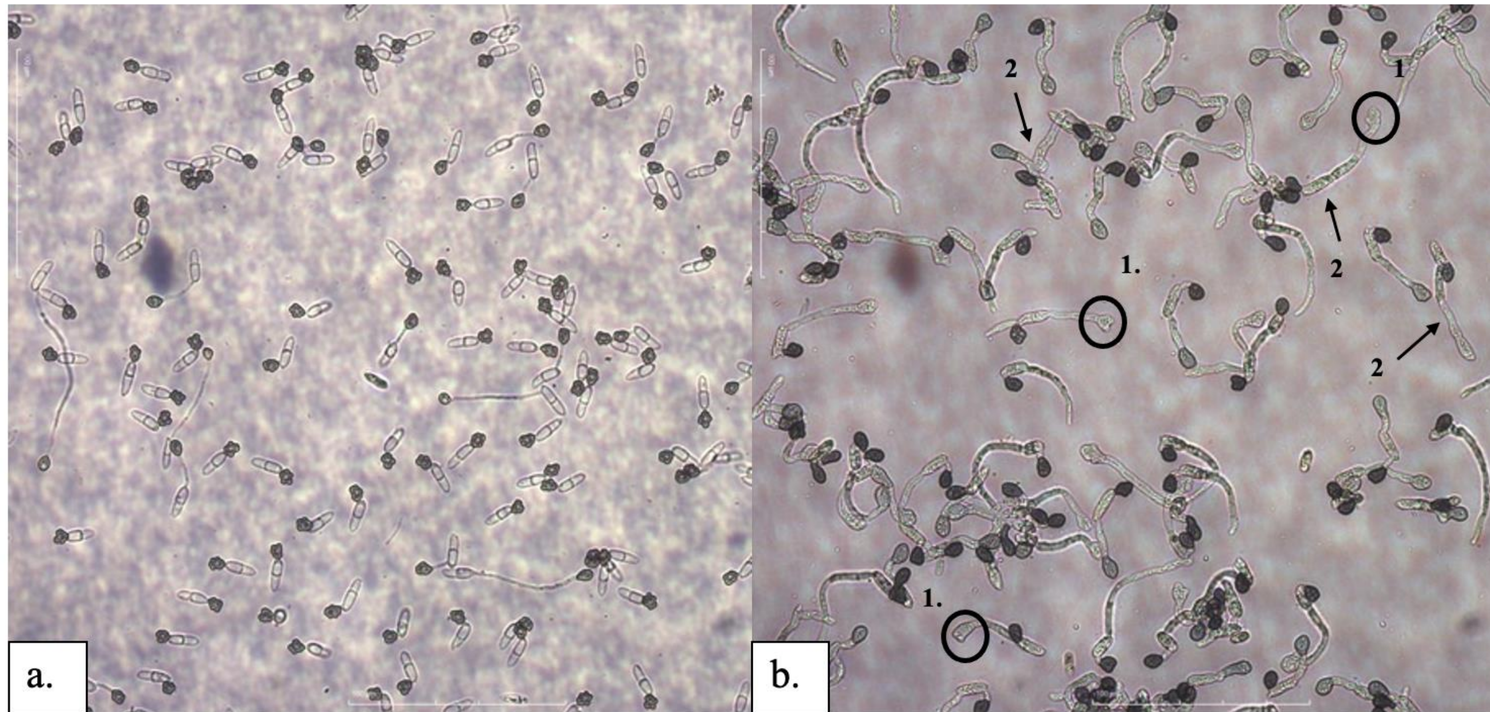


Fig. 5.13. *Colletotrichum higginsianum* conidia with and without 24 h MFP (microbial fermentation product) treatment. a. Water/control, b. filtered MFP 2%, 1. abnormal appressoria, 2. swollen germ tube.

Figure 5.14 *Magnaporthe oryzae* Conidia

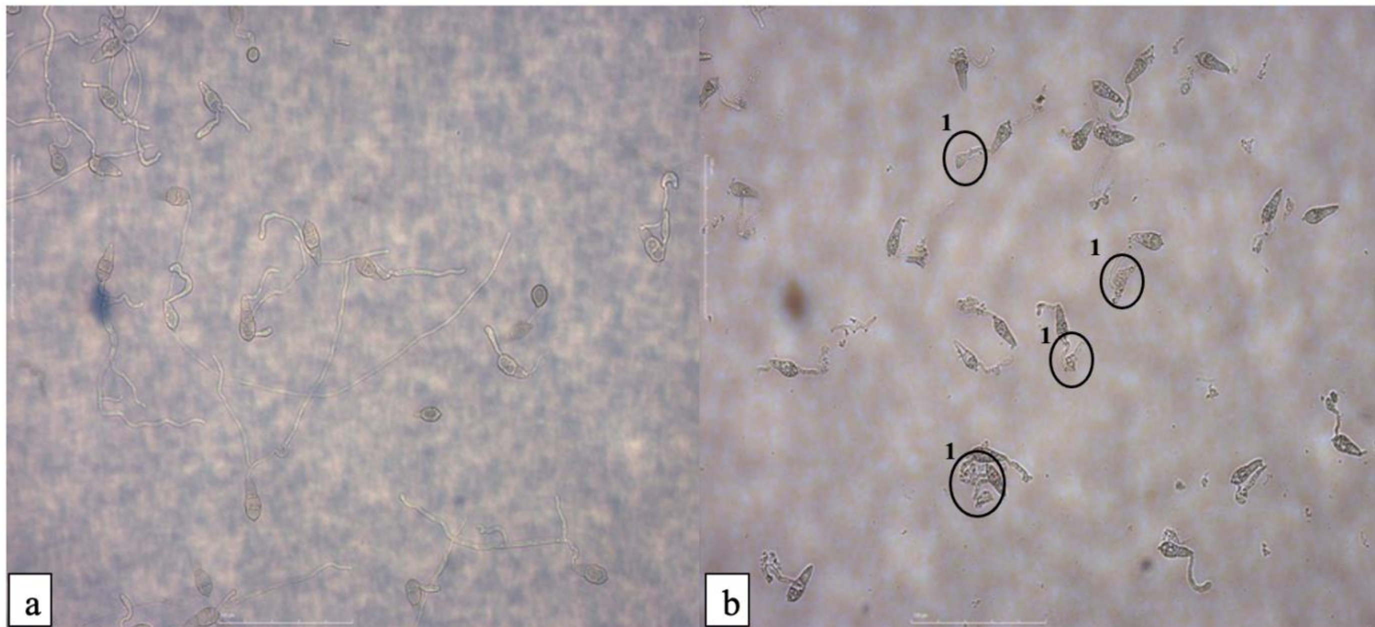


Fig. 5.14. *Magnaporthe oryzae* conidia with and without 24 h MFP treatment (microbial fermentation product). a. Water/control, b. filtered MFP 1%, 1. abnormal appressoria.

Figure 5.15 Northen Blot Anaylsis from Tomato Samples

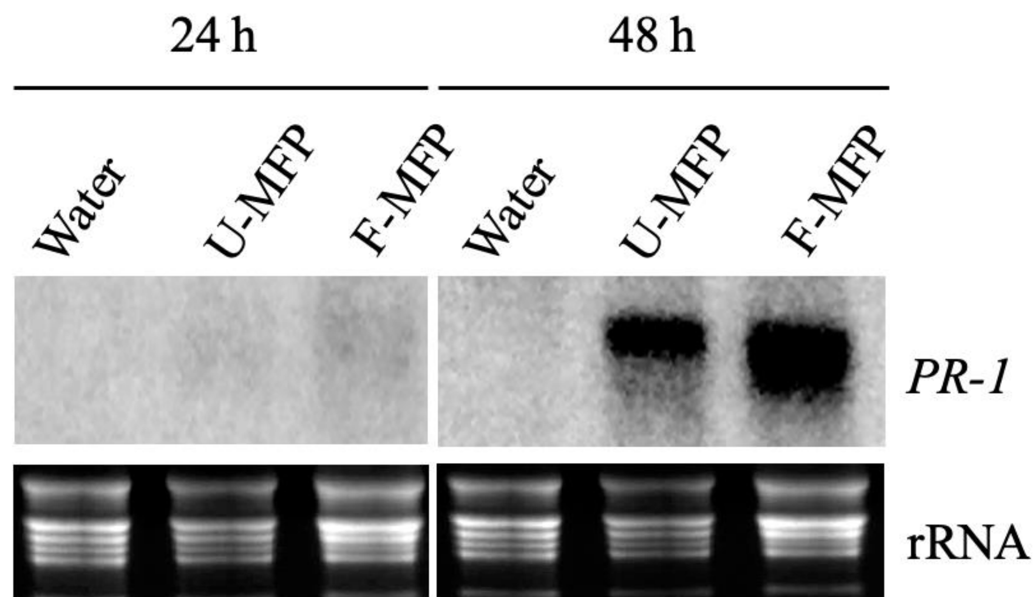


Fig. 5.15. *PR-1* expression on tomato with application of F-MFP and U-MFP (filtered and unfiltered microbial fermentation product) at both 24 h and 48 h post treatment on 'Rutgers' tomatoes. Tomatoes were treated with both F-MFP and U-MFP and samples were taken after 24h and 48 h for RNA preparation. RNA was used for Northern blot analysis as described in methods.

Figure 5.16 Local Resistance Assay

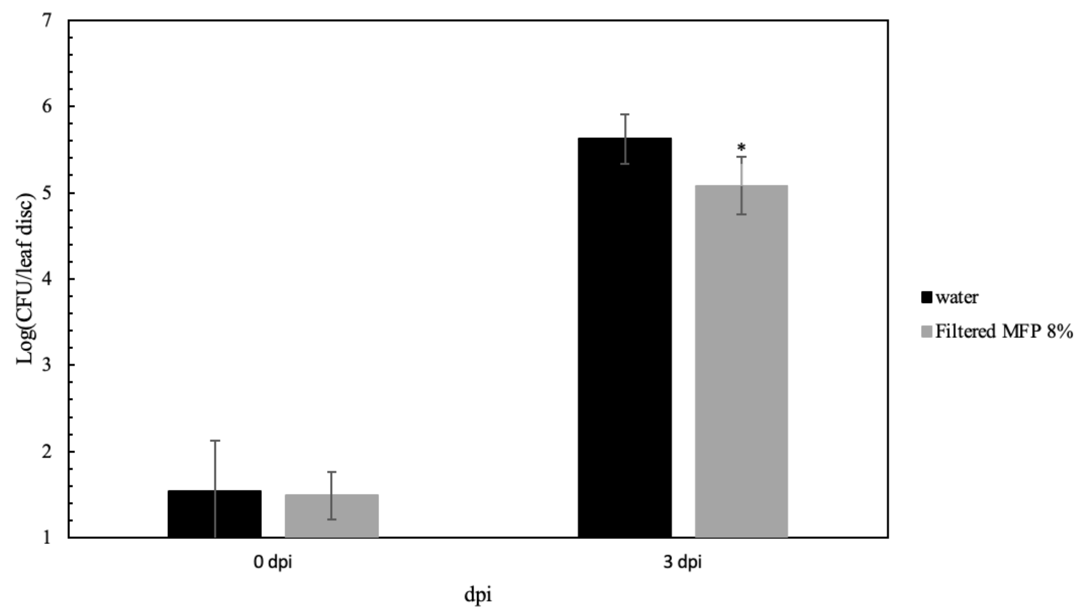


Fig. 5.16. Population of *Pseudomonas syringae* after exogenous application of MFP on 'Rutgers' tomato plants. $MgCl_2$ is the negative control and the treatment application was the MFP (microbial fermentation product) at a 8% final concentration. The 8% concentration corresponds with field application rates. The error bars are standard deviation. The star indicates statistical differences between treatments on the same date. ($P<0.05$)

Figure 5.17 Influence of the Application of MFP on Genetic Expression in Tomato Plants

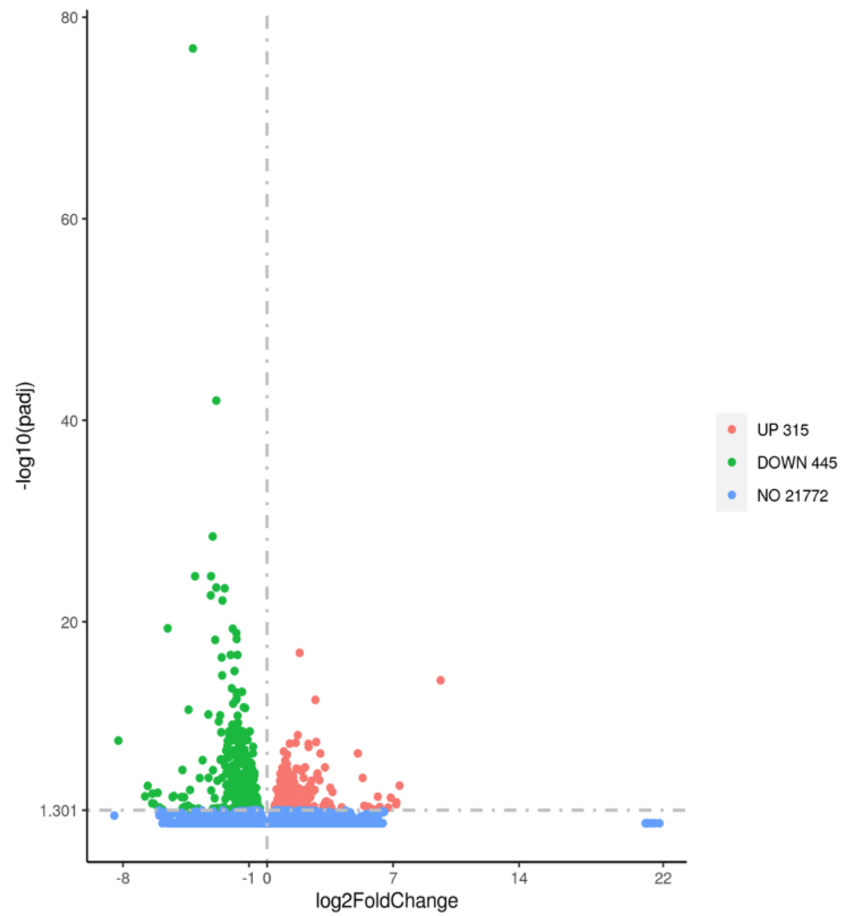


Fig. 5.17. Influence of the application of MFP on genetic expression in tomato plants. Green dots: down regulated genes, Red dots: up regulated genes. Blue dots: genes that were below the fold change threshold of 1.3 log.

Figure 5.18 Tomato Differential Expression with MFP and *Xanthomonas euvescitoria* Inoculation

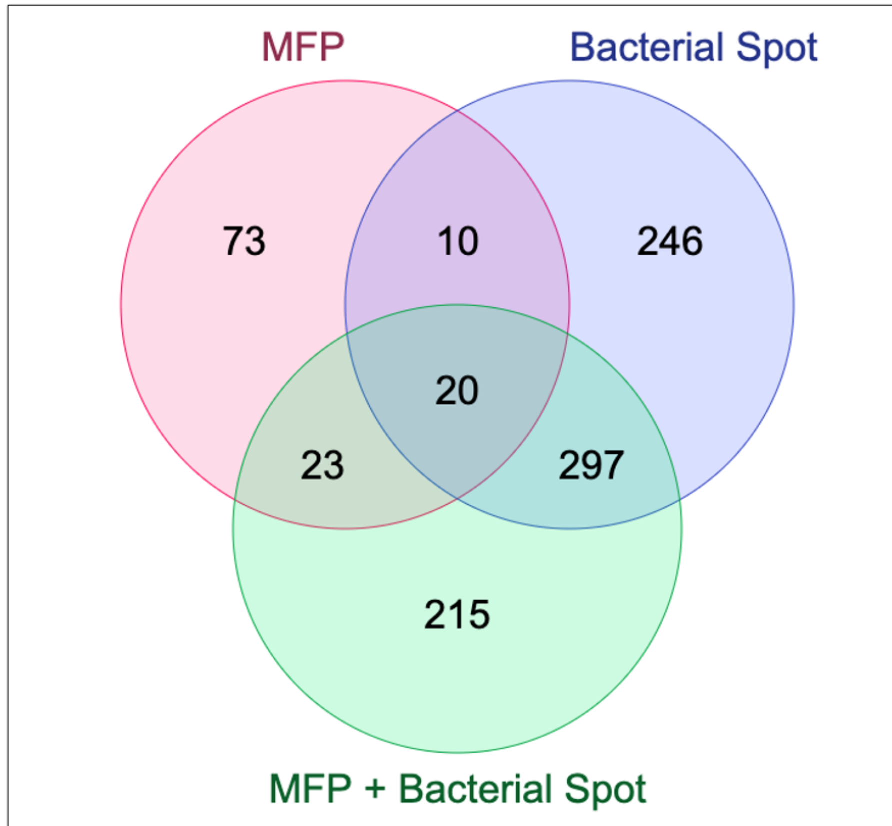


Fig. 5.18. Tomato differential expression with MFP and *Xanthomonas euvesicatoria* inoculation. Intersection indicates up and down regulated genes (above the threshold of 2 fold change) from tomatoes that had been treated with MFP (microbial fermentation product), inoculated with tomato bacterial spot (*Xanthomonas euvesicatoria*), or both treated with the MFP and inoculated with tomato bacterial spot.

Figure 5.19 Tomato Differential Expression with MFP and *Oidium neolycopersici* Inoculation

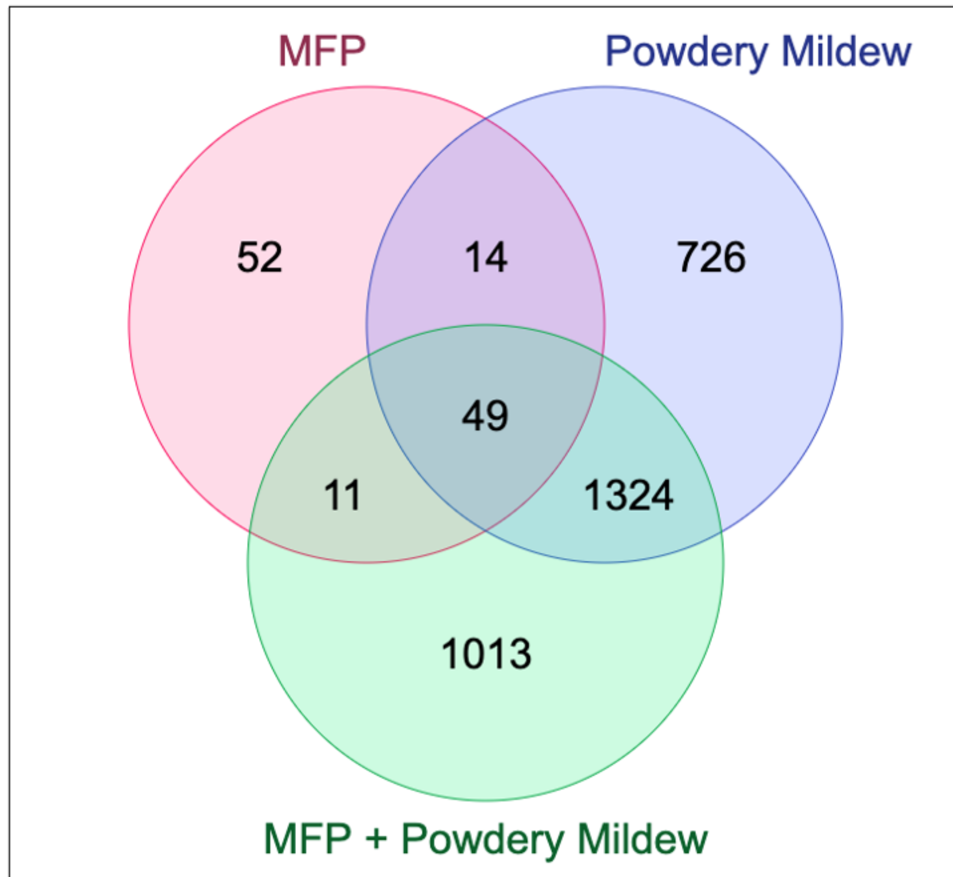


Fig. 5.19. Tomato differential expression with MFP and *Oidium neolycopersici* inoculation. Intersection indicates up and down regulated genes (above the threshold of 2 fold change) from tomatoes that had been treated with MFP (microbial fermentation product), inoculated with tomato powdery mildew (*Oidium neolycopersici*) or both treated with the MFP and inoculated with tomato powdery mildew.

CHAPTER 6. DISSERTATION CONCLUSIONS

Tomatoes are one of the most important vegetable crops grown in the world today. According to the Observatory of Economic Complexity (OEC), in 2019, tomatoes had an estimated global economic value of over 9 billion US dollars (Observatory of Economic Complexity (OEC) 2022; Simoes and Hidalgo 2011). Consequently, production methods and diseases that impact this crop are of considerable importance. This study focused on new methods for disease management in tomato that may be applicable in both field conditions and controlled environments.

Within tomato crops, particular those destined for the fresh market, organic production represents a growing percentage. As such, our initial studies focused on a comparison of spray programs currently in common usage. Two common pathologies were examined, these included tomato early blight and tomato bacterial spot. The spray programs included an examination of newer spray programs and focused on the use of biocontrol methods including the use of either a living microorganism or the use of microbial fermentation products (MFPs). These studies show that in tomato production systems against tomato early blight and tomato bacterial spot our proposed organic and conventional spray programs performed statistically ($P>0.05$) similarly. However, both spray programs contained Nordox® which has cuprous oxide as an active ingredient. Copper-based products are often used in both organic and conventional systems due to their efficacy and suitability for organic production. The overreliance on copper can lead to several environmental issues. Subsequent studies investigated alternatives to copper that could be

made available to both organic and conventional tomato growers to reduce the negative impact of coppers. In the studies report here, use of microbial fermentation products (MFPs) in tomato high tunnels demonstrated similar efficacy in powdery mildew disease reduction as to the use of copper. This indicated that MFP may be used to reduce the overall copper load in agroecosystems. To confirm this result, the MFP was tested in another pathosystem both as an isolated treatment and as a tank-mixture with copper.

The MFP was found to reduce disease severity of tomato bacterial spot in open tomato cropping systems about 20-40% compared to the water-treated control plants. The MFP was also tank-mixed with the copper-based product (Nordox®) to examine the possibility its use in an integrated system to increase efficacy. Tank-mixing these materials did not increase either efficacy when applied alone, or when applied with copper. Indeed, the combination of both MFPs with copper may have resulted in an antagonistic effect. Nonetheless, the ability of MFP to reduce both fungal and bacterial disease in tomato systems indicated it would be a good candidate for further investigation.

Further utility with respect to the use of MFPs requires a greater understanding of the mechanisms by which it may result in in the reduction of tomato disease. Elucidating the possible modes of action will help identify the MFPs appropriate use in the field. Of the four bioproduct modes of action (competition, hyperparasitism, antibiosis/antimicrobial, and plant defense), it was determined that only two were applicable to the MFP studied here: antibiosis and induced resistance.

The primary mode of action appeared to be a direct inhibitory effect against the target pathogens. The growth of the bacterial pathogens, *Xanthomonas euvesicatoria* (isolated from tomato) and *Pseudomonas syringae* pv. *tomato,* were both significantly inhibited in

liquid culture at concentrations below normal field application levels for MFP. In addition, although not at the same high level, the growth of the fungal pathogens *Botrytis cinerea*, *Sclerotinia sclerotiorum, Colletotrichum higginsianum* were also somewhat restricted with the addition of MFP to media.

A secondary mode of action appears to be some induction of plant defense systems. Results of plant defense induction were not as clear with some transitory increase in defense intermediates. Additionally, RNA-seq examination did show some induction of defense related genes was possible with MFP application. These results require further study and for verification and association with plant defense systems. The data from these studies provides a critical starting point starting point for further investigation.

In summary, several bioactive spray programs were compared in both organic and conventional tomato production agroecosystems using both field tests and protected environments. Of these, a novel bioproduct, MFP, shows promise in the reduction of various tomato pathogens. In some tests, it proved as efficacious as products containing copper, which could reduce overreliance on copper for the reduction of tomato disease. While some information has been determined in its mode of action, more will be required for optimization of use in the production of tomatoes.

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VITA

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Educational Background

University of Minnesota - BS, Agroecology (2015)

Professional Positions held

Lead ELISA Lab Technician, Syngenta (Aug. 2016-May 2016)

Research Assistant, Department of Plant Pathology, University of Minnesota (Jun. 2013-Aug. 2015)

Research Assistant, Department of Horticulture, University of Minnesota, (Sept. 2012-May 2013)

ORISE-Oak Ridge Institute for Science and Education Fellow, U.S.A. FDA (Jun. 2012-Aug. 2012)

Scholastic and Professional Honors

Grants

Fealko, E., L. Fann, and L. Araújo. 'Greg Page Community Garden Educational Enhancement Project' The Student Sustainability Council Student Grant. The University Kentucky, April 2021-2022

(Funded $30,255.00)

Student Government Association (SGA) Senators Special Project grant (funded $2,000)

Fealko, E., M. Radhi, L. Fann, L. Araújo, and E. Pfeufer. 'Greg Page Community Garden Educational Enhancement Project' The Food Connection Student Opportunity Grant. The University of Kentucky, May 2020-2021 (Funded $7,500.00)

Leadership Positions

Intern, Public Policy Board, The American Phytopathological Society (APS) 2021-2023

Chair, Graduate Student Committee, APS 2020-2021

Vice-Chair, Graduate Student Committee, APS 2019-2020

Outreach Chair, Midwest Region, National Association of Graduate-Professional Students 2019-2020

Graduate Student Success Team, College of Agriculture, Food and Environment, University of Kentucky 2019-2022

Chief of Staff and Operations, Graduate Student Congress (GSC), University of Kentucky 2019-2020

GSC/Forté Mentorship Program Advisory Committee, University of Kentucky 2019-2020

Committee Coordinator, GSC, University of Kentucky 2018-2019

Fundraising Chair, Association of Plant Pathology Scholars, University of Kentucky 2017-2018

Departmental GSC Representative, University of Kentucky 2017-2018

Awards

University of Kentucky Alumni Association Scholarship 2020

GSC Service Award 2020

Integrated Pest Management (IPM) Travel Scholarship 2019

Top ten 3MT (Three Minute Thesis competition) University of Kentucky 2018

2nd place 3MT, Department of Plant Pathology, University of Kentucky

Professional Publications

Bessin, R., N. Gauthier**, E. Fealko**, R. Rudolph, S. Wright. '2022-23 Vegetable Production Guide for Commercial Growers' University of Kentucky Extension Publication; http://www2.ca.uky.edu/agcomm/pubs/ID/ID36/ID36.pdf

Gauthier, N., and **E. Fealko.** 'Bacterial Spot of Pepper & Tomato' University of Kentucky Extension Publication; PPFS-VG-17, 2021https://plantpathology.ca.uky.edu/files/ppfs-vg-17.pdf

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