DISSERTATION

MANAGEMENT AND EPIDEMIOLOGY OF CYTOSPORA PERENNIAL CANKER, *CYTOSPORA PLURIVORA*, IN WESTERN COLORADO

Submitted by

Stephan T. Miller

Department of Agricultural Biology

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Doctoral Committee:

Advisor: Jane Stewart

Troy Ocheltree
Andrew Norton
Ioannis Minas
Mark Uchanski

ABSTRACT

MANAGEMENT AND EPIDEMIOLOGY OF CYTOSPORA PERENNIAL CANKER, *CYTOSPORA PLURIVORA*, IN WESTERN COLORADO

Cytospora canker is a ubiquitous disease in deciduous fruit tree systems in western Colorado. The research conducted herein, explores the host-pathogen-environmental framework which has enabled *Cytospora plurivora* to thrive and become a threat to peach production in the region. My research also focuses on management strategies, both cultural and chemical, which can help mitigate pathogen infections.

Chapter II, published in the *Journal of Crop Protection*, presents preventative control of *C. plurivora* through wound protection of pruned shoots. Several fungicides and sealants were evaluated either *in vitro* or in field trials, to explore antifungal activity. Fungicides which have been shown effective, were also evaluated for half maximum effective concentration rates (EC_{50}) to better understand pathogen dose sensitivity. Chapter III explores the susceptibility of thirteen peach cultivars to *C. plurivora* infection under different abiotic conditions. Abiotic stressors such as water deficit and high-pH can be major limiting factors to tree fruit production and can increase tree susceptibility and pathogen severity. My research shows increased severity of *C. plurivora* infection and decreased plant water potentials when trees experienced increased soil pH and irrigation deficits. Chapter IV provides a detailed analysis of the epidemiology of *C. plurivora* in the field. This study estimates spore production rates and lesion infection rates over a 12-month period. Further, it evaluates possible dissemination mechanisms of *C. plurivora*, reporting detection of *C. plurivora* spores in aerial and on insect samples, although at low concentrations.

The results presented herein help inform management strategies by elucidating field patterns of *C. plurivora* and identifying effective cultural and chemical control measures.

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Finally, I wish to thank my family. Without the encouragement of Jessica and my parents, I would not have been able to complete this work. This dissertation was a family team effort.

DEDICATION

Dedicado a mi inolvidable padre, Tomás Carlos Miller Sanabria, intrépido, fiel, noble protagonista de la clase obrera (1962-2021).

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INTRODUCTION

Peach production occurs across the globe, and as of September 2020, had a total world-wide production of 21.0 million metric tons per year. Top producing regions include China with an estimated 14.5 million metric tons per year, the European Union with 3.5 million metric tons per year, Turkey with 870,000 metric tons per year, and the United States with 691,000 metric tons per year (USDA FAS 2020). Recent reports indicate that production amounts for 2020 decreased by a record 1.1 million metric tons due to adverse weather conditions in China and the European Union (USDA FAS 2020). On a global scale, adverse weather conditions, primarily freeze damage occurring in the spring that damages fruit set, is the major limiting factor to peach production (Rodrigo 2000). In 2020, Chinese peach production dropped by 500,000 metric tons due to an April snow event which damaged fruit set and occurred in most peach production provinces within the country (USDA FAS 2020). This type of damage occurs intermittently in nearly all peach producing regions across the globe. In 2020, in western Colorado, a severe spring freeze caused a drastic drop in total peach production for the state. Due to this adverse weather event, production amounts plummeted from 14,300 tons in 2019 to 3,000 tons in 2020 (USDA NASS 2020). Similar incidences also occurred in Michigan and in New Jersey, although not to the extent seen in Colorado. In New Jersey, production rates decreased from 19,500 tons to 11,500 tons from 2019 to 2020 (USDA NASS 2020).

While freeze damage is immediately detrimental to annual fruit production, it is also one of the primary instigators of *Cytospora* fungal infections that occur in peach production regions. Freeze damage can cause injuries on various peach tree tissues including buds, twigs, branches,

and trunks. Given that *Cytospora* requires wounds as modes of infection, after an atypical spring freeze, the amount of wounded tissue available to *Cytospora* to cause infections increases. Infections can be found near buds killed by cold damage or on cracked tree tissues (Biggs 2005). Along with the immediate yield repercussions of bud freeze damage, *Cytospora* fungal infections raise concerns for long-term tree health. This is due to the necrotrophic lifestyle of the pathogen as it causes eventual tree death through toxin production and tissue degradation (Yin et al. 2015). Thus, the longevity of an orchard is greatly reduced if infections are not managed, as trees within the orchard will have shorter life spans.

In western Colorado, *Cytospora plurivora* has become a ubiquitous pathogen found in nearly all orchards. In 2015, 100% of orchards evaluated, in preliminary surveys in western Colorado, were shown to have some level of *C. plurivora* infections. The rates varied from 33-100% per orchard and the disease is estimated to account for 15-20% of losses in Colorado peach production (Pokharel & Larsen 2009). Integrated pest management programs are essential in regions where Cytospora canker is common.

Species of *Cytospora* in western Colorado. Through molecular analyses, Stewart et al. (2021) showed the causal agent of Cytospora canker in western Colorado was *C. plurivora* (D.P. Lawrence., L.A. Holland & Trouillas, sp. nov.; Lawrence et al. 2018). Previous studies conducted in western Colorado had referred to *C. plurivora* isolates as *C. leucostoma* due to historic classifications of the species. Surve-Iyer et al. (1995) had established three distinct phenetic groups (PG 1, PG 2, PG 3) of *C. leucostoma* based on isozyme markers and vegetative compatibility testing. Isolates belonging to PG 1 were geographically distributed throughout the United States while isolates belonging to PG 2 and PG 3 were distributed only in Michigan and California. Adams et al. (2002) further analyzed these phenetic groupings through ITS sequences and isozyme

markers and found PG 2 and PG 3 to belong to the species: *Leucocytospora paraleucostoma (Telemorph: Leucostoma parapersoonii* Adams, Surve- Iyer and Iezzoni*).* Currently, no direct comparisons have been made between the historic phenetic group species descriptions with the recently described species by Lawrence et al. (2018), though this was explored by Stewart et al. (2021), but further work is needed. Thus far*, C. plurivora* appears to be genetically distinct from isolates of *C. leucostoma* used in Adams et al. (2002).

***Cytospora* Biology.** *Cytospora* species*,* common to peach production systems, have been shown to have both necrotrophic and saprophytic lifestyles. Research highlights the role of cellulase as likely supporting the saprophytic stage of *Cytospora* due to the decomposition of cellulose in wood (Gairola & Powell 1971). Researchers have further analyzed *Valsa mali* genomes, *Cytospora mali*, and have found genes associated with *V. mali* to be similar to other known necrotrophic pathogens. Identified genes included those involved in plant cell wall degradation, proteolysis, and secondary metabolites, all of which are common in necrotrophic pathogens (Yin et al. 2015).

Traditionally, *Cytospora* pathogens have been reported as secondary pathogens due to their need for a wound as a mode of entry (Biggs 1989). Exposed, wounded tree tissues are common in peach production systems given the mandatory pruning cultural practices conducted by growers. Along with cold damage and adverse weather events, *Cytospora* have various potential infection courts throughout the year. Once an infection occurs, the fungus is able to degrade tree tissues through toxin production and rapid mycelial growth. Eventually this degradation leads to the constricting and girdling of the tree vascular tissues causing die back (Wang et al. 2016). The loss of complete scaffolds and/or entire trees then occurs depending on the location of the canker on the tree. Once tree death occurs through constriction of the vascular tissues, *Cytospora* species are

able to persist in non-living tissues as saprophytes. Thus, sanitary cultural practices are essential to maintaining low field inoculum levels.

Mature *Cytospora* cankers, whether on living or non-living tissues, will develop fruiting bodies. While fruiting bodies can occur on live or dead tissues, it has been reported that the teleomorph, or sexual stage of the fungus, rarely occurs on live trees (Kern 1955; Wensley 1964; Adams et al. 2002). Growers typically prune or remove mature cankers and since the teleomorphic stage may take up to two years to form, the anamorphic stage is more common (Bertrand & English 1976). For these reasons, the sexual fruiting bodies are more likely to be found in non-living tree tissues after scaffold death or pruning by growers. However, pycnidia, asexual fruiting bodies, can be identified as dark pimple-like protrusions on the surface of fungal cankers. They can form conidial spore chains (cirrus) which release masses of spores under moist conditions (Stewart et al. 2019). Grove and Biggs (2006) reported sporulation from fruiting bodies in peach orchards to occur throughout the year, with the highest concentrations occurring in times of increased humidity and decreased temperature. Known literature has attributed the primary method of spore dispersal to be water dissemination, through wind-blown water, rain events and/or sprinkler irrigation systems (Barakat et al. 1995; Grove & Biggs 2006; Luepschen et al. 1969).

Fungicidal Control of *C. plurivora*. Given the biology of *C. plurivora,* management strategies focus on preventive measures that shield exposed, wounded cambial and vascular tissues from infections. Canopy applications of fungicides have yielded mixed results, whereas targeted applications yield the most effective results. Previous studies found that Captan was ineffective when applied in canopy field applications (Northover 1976) while more recent studies have shown that targeted preventive field applications are significantly effective in preventing infections (Miller et al. 2019; 2021). Along with Captan, several fungicides have been evaluated as effective

for control of *Cytospora* species, as targeted preventative applications in *in-vitro* or field trials. The two most effective fungicides, well-documented in literature to reduce pathogen growth, include thiophanate-methyl and Captan (Biggs et al. 1994; Froelich & Schnabel 2019; Miller et al. 2019; 2021). These fungicides should be used in rotation given the high risk of fungicide resistance to thiophanate-methyl (FRAC 2019). Copper based fungicides have also been shown to be effective *in-vitro* on pathogen growth. Effectiveness has also been observed with Lime Sulfur, but incidences of phytotoxicity have been reported with both fungicides in field applications, thus application timing and rates should be further investigated specific to peach production systems (Lalancette & McFarland 2007; Miller et al. 2019; Northover 1976; Tate et al. 2000).

Since *Cytospora* spp. are both necrotrophic and saprophytic, orchard sanitation is essential to reducing inoculum pressure. If pruned tissues are not removed from orchards, increased incidences of the teleomorphic stage are likely to occur (Bertrand & English 1976). The presence of the sexual reproductive stage of the pathogen inevitably raises concerns for increased genetic diversity and evolved fungicide resistance. Thiophanate-methyl is a high-risk fungicide due to its targeted mode of action (FRAC Code List 2021). Evolved resistance to methyl benzimidazole carbamates has been reported by various fungal species including *Botrytis cinerea*, *Cochliobolus heterostrophus*, *Helmintosporium solani*, *Monilinia fructicola*, *Colletotrichum* spp., *Penicillium* spp., *Sclerotinia sclerotiorum*, and *Venturia* spp. (Chung et al. 2010; Ma & Michailides 2005).

Cultural Control of *C. plurivora.* Cultural control methods for *C. plurivora* should be focused on wound prevention and overall tree vigor/ health. The most common sources of wounds in peach orchards are from cultural pruning practices, insect damage, and abiotic damage (primarily cold damage). In peach production systems, trees are pruned for increased aeration, light interception, optimized fruit quality, and crop load management (Minas et al. 2018). Given this essential

practice, wounds should be targeted and covered with fungicides and/or sealants to prevent fungal infections. Another common source of wound damage is from insect borers. Swift (1986) established a relationship between *Cytospora* infected borer wounds and increased incidence levels of the lesser peach tree borer (LPTB), suggesting that *Cytospora* infections could alter wounds to provide a more suitable environment for LPTB egg deposition and larval entry. Thus, it is important to control insect borers in both the initial non-bearing years, and also in subsequent years as feeding sites, egg deposition sites, and larval entry sites create increased numbers of infection courts. Abiotic factors including cold damage, saline irrigation water/ soils, and water deficit all can physiologically stress trees. Management of these stressors is vital for tree health and continual market success. Layne and Tan (1984) reported that proper irrigation of peach trees, increases tolerance to cold damage and also decreases incidence of Cytospora canker infections. Similarly, research has shown that salinity from irrigation water and soils may cause osmotic adjustments in plants, mineral deficiencies, reduction of carbon dioxide availability (stomatal blockage), photosynthesis prohibition, cell division prohibition (through interaction of salts with plant cellular components), and ion toxicity (Bahmani et al. 2015; Navarro et al. 2007). Thus, it is important to culturally manage such abiotic factors by understanding plant hardiness zones and the make-up/consistency of irrigation water sources and soils.

The objectives of the research presented herein are to explore both chemical and cultural control methods for *C. plurivora*. Further, this research provides an exploration of the epidemiology of the fungal pathogen in western Colorado. More specifically the objectives are to:

1. Test preventive fungicidal/ sealant combinations both in-vitro and in-field trials for shielding tree wounds to *C. plurivora* infections,

2. Evaluate the relationship between peach cultivars and *C. plurivora* under abiotic stress conditions to better understand the host-pathogen interactions and to elucidate differences in susceptibility/ tolerance across peach cultivars, and
3. Elucidate epidemiological patterns of *C. plurivora* specific to western Colorado by monitoring field sporulation, growth patterns, and investigating potential pathogen vector and introduction mechanisms.

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CHAPTER II**. Exploring fungicides and sealants for management of *Cytospora plurivora* infections in western Colorado peach production systems**[1]

SUMMARY

Cytospora plurivora, in western Colorado, is a limiting factor to peach production. The pathogen requires a wound, and entry points can be ubiquitous on peach trees depending on the season because of the need for tree cultural management (eg. dormant and/or summer pruning) and annual winter damage. Our study is focused on preventive control of the pathogen through wound protection. Fungicides and sealants were either evaluated *in vitro*, for antifungal activity, or selected from the present and previous trials to be evaluated in the field. Ten fungicide/sealant combinations were evaluated in the field for pathogen control during spring of 2018 and 2019 in western Colorado. Treatments included combinations of thiophanate-methyl, lime sulfur, VitiSeal (alone and in combination with other chemicals), with sealants (latex paint, paraffinic oil, and Nu-film 17). The fungicides/sealants that were most effective in reducing *C. plurivora* pathogen viability in branches and canker sizes was thiophanate-methyl alone, combined with VitiSeal, and combined with latex paint at 50 and 70% and VitiSeal combined with lime sulfur. The sealants Nu-film 17 and paraffinic oil, when combined with lime sulfur, were ineffective in all trials. Further, fungicides which showed evidence of efficacy in these studies and in previous studies were analyzed for their half maximum effective concentration rates (EC_{50}) for an understanding of pathogen dose sensitivity.

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INTRODUCTION

The stone fruit pathogen, newly classified as *Cytospora plurivora* D.P. Lawr., L.A. Holland & Trouillas, sp. nov., (historically known as *C. leucostoma*) has been documented as one of the primary diseases in peach production systems (Grove & Biggs 2006), particularly in western Colorado where fluctuating winter temperatures, spring frosts, and heavy calcareous soils can cause physiological stress in trees, leading to increased pathogen susceptibility (Sharp & Cooley 2004). Previously, isolates collected from Colorado were referred to as *C. leucostoma*, but recent phylogenetic studies demonstrated that these isolates belong to *C. plurivora* (Stewart et al. 2021).

Cytospora plurivora requires wounded tissue for establishment (Wilson et al. 1984) thus current management strategies in western Colorado are focused on preventive measures which shield exposed, wounded cambial and vascular tissues from infections (Miller et al. 2019). Previous studies by Miller et al. (2019), found evidence of fungicidal efficacy for preventive control of *C. plurivora* in both *in vitro* and field trials, but this research included a limited number of effective fungicides and sealants. The most effective chemical identified for in field use was thiophanate-methyl. Thiophanate-methyl belongs to the chemical group of Benzimidazoles. The specific target cite is the inhibition of ß-tubulin polymerization that interferes with spore germination, germ tube elongation, cellular multiplication, and mycelial growth of fungi (Davidse 1988; FRAC 2006; Hollomon et al. 1998). Given the targeted mode of action, Benzimidazoles have shown to be high risk for evolved resistance in fungal pathogens found in stone fruit and curcurbit crops and thus should not be used as the only chemical treatment. Evolved resistance has been reported in populations of *Monilinia fructicola* and *Podosphaera xanthii* (Ma et al. 2003; McGrath 2001). The fungicide captan was also documented as effective in inhibiting *Cytospora* species (Biggs et al. 1994; Miller et al. 2019). Captan belongs to the phthalimides chemical group

and has traditionally been considered a low-risk fungicide by the Fungicide Resistance Action Committee (FRAC) due to its multi-site activity mode of action. Both thiophanate-methyl and captan are admissible solely for conventional production systems.

Fungicides and sealants, or in combination, can be used to deter infections by *C. plurivora* as the toxicity of the fungicide kills spores and the barrier created by the sealant protects vulnerable wounded tissue from spores. Previously, only a limited number of fungicides have proven effective for preventive strategies in conventional and organic peach orchards, and fungicide combinations with sealants have not been explored at depth in field trials. Thus, the objectives of this research were to explore novel fungicidal-sealant combinations as preventive measures for control of *C. plurivora*, first *in vitro* (for fungicides) and then in field trials (fungicides and sealants). Further, half maximum effective concentration rates (EC_{50}) were estimated for copper hydroxide, captan, and lime sulfur currently used by growers, and VitiSeal as a potential effective product for *C. plurivora* control.

MATERIALS AND METHODS

Isolation of *C. plurivora.* Canker margins, no larger than 1 cm^3, were collected from experimental and commercial orchards in Grand Junction, western Colorado. Samples were sterilized with 10% sodium hypochlorite (Clorox^{tm}; Oakland, CA, 94612, USA) for 5 minutes, rinsed with sterile distilled water, and placed on half-strength potato dextrose agar [PDA: 4.0 g potato starch (BD Difco^{TM}), 19.5 g dextrose (BD Difco^{TM}), 7.5 g agar per liter (BD Difco^{TM})]. Plates were grown at 25°C for five days. Hyphal tips were then transferred to Leonian's agar plates [LA: 6.25 g peptone (BD Difco^{TM}), 6.25 g maltose (Oxchem^{TM}), 6.25 g malt extract (BD Bacto^{TM}), 1.25 g KH_2PO_4 (Fisher Scientific), 0.625 g $MgSO_4 \cdot 7H_2O$ (MilliporeSigma), and 20.0 g agar per

liter (BD Difco™); (Booth 1971)]. Two isolates were selected for the chemical toxicity trials CP 92.1 and CP72.1. Four isolates were selected for the (EC_{50}) in *vitro* trials: CP 56.2, CP 92.1, CP 72.1, and 5.1. For field trials, spore suspensions (10^5 spores/ml) were prepared from isolate CP5.1, an isolate that had previously been shown to vigorously produce pycnidia and conidia (Miller et al. 2019).

***In-Vitro* Toxicity of Fungicides and Sealants and EC_{50} Trials.** Prior to selecting treatments for field trials and prior to conducting EC_{50} trials, *in vitro* trials were conducted to investigate efficacy of fungicides and sealants not previously evaluated. VitiSeal (VitiSeal™ RTU; VitiSeal™ International, LLC; San Diego, California, USA) and copper octanoate (Cueva®, Certis, LLC; Columbia, MD, USA) were evaluated and amended in autoclaved, cooled half strength PDA medium. The VitiSeal was amended at a rate of 2% while the Cu-octanoate was amended at a rate of 10%. Captan (Loveland Products; Greeley, Colorado, 80632, USA) was used as a control, since it had previously been shown to inhibit colony growth (Miller et al. 2019). Agar plugs, 4mm diameter, were taken from the edge of three-day old *C. plurivora* cultures. Chemically amended plates were inoculated in the center and then incubated at 25°C for seven days. Colony diameters of each sample were measured and fungal growth area (Πr^2) was determined. Isolates CP 92.1 and CP 72.1 were evaluated with 5 replicates per isolate per fungicide/sealant. VitiSeal was then selected to be evaluated further in field trials. Chemicals that were effective against *C. plurivora* both *in vitro* trials and field trials were further tested for half maximum effective concentration rates.

Fungicides and sealants evaluated for half maximum effective concentration (EC_{50}) rates included captan (Loveland Products; Greeley, Colorado, 80632, USA), lime sulfur (BSP lime sulfur; Ag Formulators; Fresno, CA, 93725, USA), VitiSeal (VitiSeal™ Concentrate; VitiSeal™

International, LLC; San Diego, California, USA), and copper hydroxide (Nucop-WP; Agri Star; Ankeny, IA, 50021, USA). Methods were conducted as described in Froelich & Schnabel (2019). To determine EC_{50} values, captan, lime sulfur, copper hydroxide, and VitiSeal were investigated at the following rates respectively, (0.01, 0.05, 0.10, 0.50, 1.50, and 3.0 µl/ml ai), (0.05, 0.125, 0.50, 1.0, 8.0, 35.0 µl/ml ai), (0.01, 0.05, 0.10, 0.50, 1.0, 3.0, 5.0 µg/ml ai), (0.05, 0.10, 0.50, 1, 15, 50 µg/ml ai). Four total isolates (CP 5.1, CP 56.2, CP 72.1, and CP 92.1) were evaluated with 3 replicates per isolate per dose for each chemical treatment, including positive controls with inoculations on non-amended media.

In Field Fungicidal Efficacy Trials. Field application studies were conducted at the Colorado State University's Experimental Orchard in Western Colorado Research Center-Orchard Mesa (WCRC-OM) in Grand Junction, CO, in the spring (March through June) of 2018 and 2019. Trees in the field trials were planted in the spring of 2013 and consisted of 'Cresthaven' peach scions grafted on 'Viking' rootstocks (planting density: 1784 trees per ha) and trained to a perpendicular-V. Prior to fungicidal treatments and spore inoculations, one-year old shoots were wounded with a hand-pruning shear at 45°. Treatments included combinations of fungicides/sealants shown to be effective in previous studies and were applied at recommended label mid-rates (Miller et al. 2019). New treatment solutions included VitiSeal combined with thiophanate-methyl (total amount of ai in solution: 10% VitiSeal, 0.053% thiophanate-methyl), 50% latex paint (Latex paint; Rust-oleum^{tm}; Vernon Hills, Illinois, USA) combined with thiophanate-methyl (total volume of ai in solution: 50% latex paint, 0.053% thiophanate-methyl), 70% latex combined with thiophanate-methyl (total volume of ai in solution: 70% latex paint, 0.053% thiophanate-methyl), paraffinic oil (JMS Stylet-Oil, Flower Farms Inc.; Florida, USA) combined with lime sulfur (total volume of ai in solution: 90% paraffinic oil, 3% Calcium Polysulfide), VitiSeal combined with lime sulfur (total

volume of ai in solution: 10% VitiSeal™; 3% calcium polysulfide), Nu-film (Nu-Film® 17, Miller Chemical & Fertilizer Corporation; Pennsylvania, USA) combined with lime sulfur (total volume of ai in solution: 90% Nu-Film ®, 3% Calcium Polysulfide), and 70% latex combined with lime sulfur (total volume of ai in solution: 70% latex paint, 3% Calcium Polysulfide). Fungicides/sealants were applied the same day of wounding as label mid-rates (Table 2.1) using hand-spray bottles and sprayed until runoff or approximately 3 ml per branch. All treatments were randomized within a tree to minimize directional effects of solar radiation and temperature. Twenty total trees were evaluated in a complete block design, with each tree (block) containing the full number of treatment combinations, including non-chemical but inoculated positive controls and non-chemical and non-inoculation negative controls. Within each tree (block) ten branches were inoculated by fungicide treatment. Studies were conducted in 2018 and repeated in 2019.

Inoculum was prepared from fungal cultures as described in Miller et al. (2019). Two-month-old cultures with well-formed pycnidia were immersed in 20 to 40 ml distilled water for 3-5 min while being agitated. Pycnidia were erupted with sterilized tweezers. The concentration of the spore suspension was verified and adjusted to 10^5 spores/ml using a hemocytometer. Mycelial and empty pycnidia debris were filtered out from the spore suspension with a cheesecloth filter. Shoots were inoculated 1-day post fungicide/sealant treatment with 100µl of the 10^5 spores/ml spore suspension. The suspension was applied individually on each wound with a pipette and wrapped in Parafilm (American National Can; Chicago, IL, 60631, USA). Spores were allowed to develop for 90 days. After this period of lesion expansion, canker sizes were measured using a digital caliper, and volume of the decayed tissue (mm^3) was calculated based on branch diameter and canker length. Wound reaction lesions of the host were subtracted from all measurements. Further,

canker margins, no larger than 1 cm^3, from every artificially inoculated branch were removed and placed on half-strength potato dextrose agar to confirm viability of the pathogen. Plates were grown in an incubator at 25°C and morphological verified as *C. plurivora*. DNA was extracted from lyophilized mycelia using ZR Fungal/Bacterial DNA MiniPreps (Zymo Research Corporation, Irvine, CA). Extractions were performed following manufacturer's protocols with some modifications, including addition of 25-35 mg of lyophilized material, and 4-5 3 mm glass beads, to the cell lysis tube. DNA was quantified using a Nanodrop 2000 (ThermoFischer, Inc., Waltham, MA). DNA was then sequenced at the internal transcribed spacer region (ITS) to verify species identification (primers ITS1/ITS4: White et al. 1990). Amplification reaction mixtures (total 25 µL) contained 20-40 ng of template DNA (or no DNA template for negative control), 2.5 µL 10x Standard *Taq* Reaction Buffer (New England BioLabs Inc., Ipswich, MA), 0.5 µL 10mM dNTPs (Roche Applied Science, Penzberg, Germany), 1 µL each of 10 µM primer and 0.125 µL *Taq* DNA Polymerase (New England BioLabs Inc.) Amplifications were performed using the following PCR thermocycling conditions: 94°C for 1 min, 35 cycles at 95°C for 30 s, or 58°C for 30 s, and 72°C for 45 s, and final extension step at 72°C for 10 min followed by an infinite hold at 4°C. All PCR amplification was run using an MJ PTC-200 thermocycler (Bio-Rad Laboratories Inc., Hercules, CA). PCR products were visualized by running a 1.5% agarose gel with 0.5X TBE buffer and stained with GelRed (Biotium, Fremont, CA) and using UV light to confirm amplification. Successful PCR products were purified using ExoSAP-IT® PCR Product Cleanup (Affymetrix, Santa Clara, CA) following manufactures instructions and were Sanger sequenced at Eurofins (MWG Operon USA, Louisville, KY). The percent of viable isolations from each treatment was calculated.

Statistical Analysis. RStudio was used for statistical analyses. Packages used included drc, lme4, lmertest, pbkrtest, and lsmeans (Bates et al. 2015; Halekoh and Højsgaard 2014; Kuznetsova et al. 2017; Lenth 2016; R Core Team 2019; Ritz et al. 2019). Summary statistics and graphical depictions were created using the plyr package (Wickham 2011) and the ggplot2 (Wickham 2009) package. For the EC_{50} trials, the drc package in R was used to calculate EC_{50} values using non-linear regression. For the field trials and other *in vitro* trials, mixed models were created. For the *in vitro* trials, a continuous response variable of fungal growth area (mm^2), with a fixed fungicide/sealant treatment predictor variable, and a random effect predictor variable of isolate were assigned in the mixed model. For the lesion volume field trial, a mixed model was built based on a randomized complete block design. The continuous response variable was lesion volume (mm^3), and the predictor variables included fungicide/sealant treatment as a fixed effect, with two blocking variables of tree and year as random effects. To correct for assumptions of normality and equal variances, the data was square root transformed. A linear model analysis of variance (ANOVA) was run on the mixed model, followed by Tukey adjusted pairwise comparisons ($\alpha = 0.05$) (Tukey's HSD adjusted p-values are $P = 0.05$). For the spore viability analysis, Pearson's chi-square test was conducted using RStudio. Deviation in the chi-square value from the expected null hypothesis indicate that response is dependent of treatment. Further, Fisher's Exact Test for contingency tables was used to compare each of the active treatments to the positive control for evidence of efficacy. Tests were carried out using RStudio, $P \leq 0.01$ was considered significant. Results are presented by percent relative frequency of tabulated presence/absence data. Percent relative frequency was calculated by multiplying the relative frequency of viable fungal isolations by 100.

RESULTS

In-Vitro **Toxicity of Fungicides/Sealants and EC_{50} trials.** When evaluating new treatments *in vitro* (Table 2.1), copper octanoate and VitiSeal reduced *C. plurivora* growth by 100% relative to the inoculated but no chemical positive control. Both the fungicide and sealant were effective *in vitro* when compared to the captan control ($P > 0.99$).

When fungicides were evaluated in the EC_{50} trials (Table 2.2), the following lack of fit values for the curves were calculated. The lack of fit test for the dose response curve of captan yielded values of $P = 0.48$, $P = 0.89$, $P = 0.98$, $P = 0.42$ for isolates CP5.1, CP56, CP72, and CP92, respectively. Copper hydroxide yielded values of $P = 0.76$, $P = 0.16$, $P > 0.99$, $P = 0.15$, respectively. The lack of fit test for lime sulfur yielded the following values, for isolates CP5.1, CP56, CP72, and CP92, $P = 0.27$, $P = 0.73$, $P > 0.34$, $P = 0.05$. Finally, the lack of fit test for the curve of VitiSeal, and the respected isolate values, were calculated as $P = 0.23$, $P = 0.03$, $P > 0.88$, $P = 0.54$.

Table 2.1. Chemicals used at label mid-rates to test fungal growth inhibition of *Cytospora plurivora*. Combinations of fungicides/sealants used in field trials were mixed as percentages of the total volume.

Registration use	Active compound or ingredient(s)	Product commercial name	Recommended product label rate [per 757 L (200 gal.) or %]	Label rate used	% Active compound or ingredient in working solution
Conventional	Captan	Captan	2.8 - 3.8 L	3.3 L	0.213%[ce]
Conventional	Acrylic copolymer, limestone, water, titanium dioxide, nepheline syenite pigment, cinnamon oil	VitiSeal™	75.7 L	75.7 L	10%[de]
Conventional	Thiophanate-methyl	Topsin-M	450 - 680 g	570 g	0.053%[d]
Conventional	Latex	Latex Paint	N/A	378L or 530L[b]	50% or 70 %[d]
OMRI[a]	Copper octanoate	Cueva	12.1 – 49.9	31 L	10%[c]
OMRI	Paraffinic oil	JMS Stylet-Oil	N/A	681 L	90%[d]
OMRI	Terpene Polymers, mineral oil, and alkyl amine ethoxylated	Nu-Film 17	N/A	681 L	90%[de]
OMRI	29% Calcium polysulfide	BSP Lime Sulfur	75 - 91 L	83 L	3%[d]

[a]OMRI (The Organic Materials Review Institute): Treatments labeled OMRI are certified for use in organic production systems by the USDA National Organic Program.
[b] Latex was either applied at 50% (378L / 757L) or at 70% (530L / 757L).
[c] Active ingredient dose used only for *in vitro* trials (not EC_{50} or field trials).
[d] Active ingredient dose used only for field trials.
[e] Multiple active ingredient doses also used to develop EC_{50} curves (see methods).

Table 2.2. Half maximum effective concentration rates (EC_{50}) of fungicides and sealants to four isolates of *Cytospora plurivora*.

	EC_{50} values (µg/ml)			
Isolate ID	Captan	Copper hydroxide	Lime sulfur	VitiSeal
CP 5.1	0.023	0.025	0.210	0.552
CP 56	0.035	<0.01	0.034	0.171
CP 72	0.041	<0.01	0.144	0.330
CP 92	0.049	0.013	< 0.01	0.038

Fungicidal Efficacy of Trials in the Field. Ten treatments were investigated in field trials as preventive measures for control of *C. plurivora*. Thiophanate-methyl, VitiSeal, VitiSeal combined with thiophanate-methyl, 70% latex paint ($P < 0.0022$), 50% latex paint combined with thiophanate-methyl, 70% latex paint combined with thiophanate-methyl, and VitiSeal combined with lime sulfur all significantly reduced necrotic tissue sizes as compared to the positive control (Figure 2.1a; $P < 0.0001$). However, Nu-film combined with lime sulfur and 70% latex paint combined with lime sulfur did not yield decreased lesion sizes (Figure 2.1a; $P > 0.99$; $P = 0.09$, respectively). The paraffinic oil combined with lime sulfur treatment yielded the largest necrotic tissue sizes, significantly larger than the positive control ($P < 0.0001$). The treatments thiophanate-methyl, VitiSeal combined with thiophanate-methyl, 50% latex paint combined with thiophanate-methyl, and 70% latex paint combined with thiophanate-methyl did not differ significantly when compared to the negative control (Figure 2.1a; $P = 0.99$; $P > 0.99$; $P = 0.95$; $P = 0.23$; respectively).

In the percent viability trials, response of the percent viable spores among chemical treatments varied significantly as indicated by Pearson's chi-square ($P < 2.2e\text{-}16$). The treatments with the lowest percentage of re-isolated infections included thiophanate-methyl (7.0%), 70% latex paint

combined with thiophanate-methyl (6.9%), VitiSeal combined with thiophanate-methyl (9.7%), VitiSeal combined with lime sulfur (10.5%), 50% latex paint combined with thiophanate-methyl (13.3%), and VitiSeal alone (43.1%) (Figure 2.1b). Treatments with high percentages of viable spores included the 70% latex paint combined with lime sulfur (85.4%), Nu-Film combined with lime sulfur (96.5%), 70% latex paint (89.7%), and paraffinic oil combined with lime sulfur (100%) (Figure 2.1b). When treatments were compared to the inoculated but non-treated positive control, through Fisher's Exact Test (FET), thiophanate-methyl, VitiSeal, VitiSeal & thiophanate-methyl, 50% latex paint & thiophanate-methyl, 70% latex paint & thiophanate-methyl, VitiSeal & lime sulfur all differed significantly (Figure 2.1b; $P < 0.0001$). Treatments 70% latex paint, paraffinic oil & lime sulfur, Nu- film 17 & lime sulfur, and 70% latex paint & lime sulfur did not differ statistically from the positive control (Figure 2.1b; $P > 0.01$).

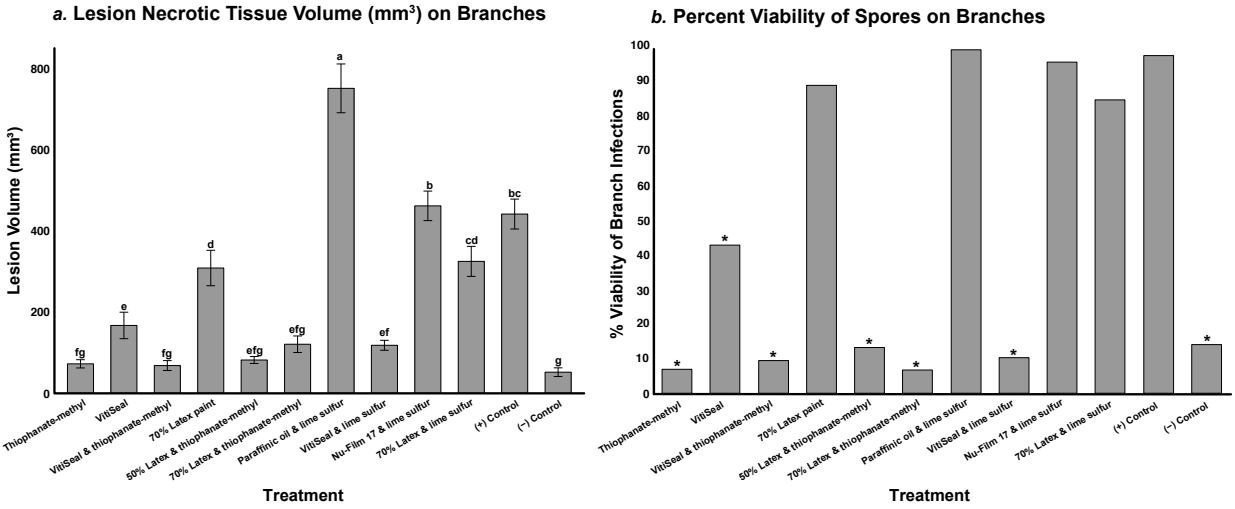

Figure 2.1. Effects of fungicide and sealant treatments on *Cytospora plurivora* inoculations (100 µl of 1 x 10^5 spores / ml) based on: **a)** necrotic tissue volume (mm^3) or **b)** percent viability of spores. Inoculations were made on branches 24 hours post chemical treatment. The positive control consists of inoculations on wounded branches with no chemical applications, the negative control consists of branch wounding with no chemical application and no pathogen inoculation. In Figure 2.1a, means labeled with the same letters are not significantly different at $P = 0.05$ according to Tukey's test. In Figure 2.1b, starred relative frequencies were significantly different when compared to the positive control $P = 0.01$ according to Fisher's Exact Test for contingency tables.

DISCUSSION

Due to essential maintenance practices of pruning in peach production systems, *C. plurivora* has an abundance of potential infection courts. Lawrence et al. (2018) reported finding 92% of infections from *Cytospora* spp. to be found on pruning cuts in a French prune orchard. Fungicides and sealants can be used to deter infections from occurring on pruning wounds, as they can act together with toxicity and barrier factors. *In vitro* trials were conducted to explore toxicity of potentially new fungicides to *C. plurivora*, and field trials were used to verify novel fungicide/sealant combination efficacy over two years.

VitiSeal and copper octanoate were investigated *in vitro* prior to selecting fungicides for field trials. Although VitiSeal is marketed as a sealant due to its waterborne acrylic co-polymer ingredient (weight percent: 0-5%), it also contains cinnamon oil (weight percent: <1%) which has previously shown to have antifungal activity when evaluated against *Aspergillus flavus* and *Penicillium expansum* both *in vitro* and *in vivo* (Xing et al. 2010). Copper octanoate is commonly applied by growers in western Colorado as a copper soap fungicide, and previous literature evaluating this active ingredient on *Cytospora* species is sparse. Previous researchers have shown evidence of toxicity of copper octanoate to *Colletotrichum* species complexes (Oliver 2016). Thus, interest in whether or not label mid-rate grower applications are even toxic to *Cytospora* species were evaluated in the lab on fungal cultures to ensure a direct comparison without other environmental factors. Both copper octanoate and VitiSeal inhibited fungal growth of *C. plurivora* by 100% and even though copper octanoate showed evidence of efficacy, it was not selected for field analysis due to the previous evidence of phytotoxicity of copper-based fungicides to peach trees (Lalancette and McFarland 2007; Miller et al. 2019; Northover 1976). Given the antifungal efficacy of VitiSeal *in vitro* and at growers' requests, it was selected for further investigation in

field trials.

In the field trials, thiophanate-methyl (Topsin-M), VitiSeal, 70% Latex paint, VitiSeal combined with thiophanate-methyl, 50% latex combined with thiophanate-methyl, 70% latex combined with thiophanate-methyl, and VitiSeal combined with lime sulfur were all effective, resulting in slower fungal growth when compared to the positive control. These results validate previous research conducted in cherry orchards evaluating the efficacy of VitiSeal. Researchers found significant reduction in cherry tree infections to *C. leucostoma*, *Calosphaeria pulchella*, and *Eutypa lata* (Abramians 2016). The efficacy of thiophanate-methyl has also been reported *in vitro* and on excised branches by Biggs et al. (1994) and more recently by Froelich & Schnabel (2019) and Miller et al. (2019).

Six of these treatments, except latex alone, also significantly lowered the percent viability of inoculated spores, indicating toxicity to spores. Smaller lesion sizes or no lesions at all, may still constitute an infection, if spores are viable. Given the necrotrophic lifestyle of the pathogen, this could lead to eventual tree death through tissue degradation and girdling of the vascular tissues. This is especially true when considering that *Cytospora* species have been reported as toxin producing necrotrophs (Yin et al. 2015). This was observed when examining the results from 70% latex paint treatment in this study. When 70% latex was applied without fungicides, reduced lesion sizes were observed, but when tissue was isolated from the branches, 85.4% of branches contained viable spores. Thus, latex treatments without fungicides are not effective because once there is cracking of the latex cover, new infections can occur. Further, Biggs & Peterson (1990) showed that latex paints can inhibit the development of suberin and lignin in peach tree prune wounds, thereby questioning the long-term effectiveness of latex as a sealant. This is especially true if spores are still viable, as shown herein.

The treatment of paraffinic oil combined with lime sulfur was also ineffective. Unfortunately for organic growers who do not have many options for chemical controls, lesions sizes were significantly larger than the positive control suggesting a potential phytotoxic interaction between the lime sulfur, paraffinic oil, and tree tissues. Previous studies have reported potential phytotoxicity of lime sulfur to foliage of apple trees, but this damage was not observed across all treatments with lime sulfur in the present research (Tate et al. 2000). Effective chemical controls for organic growers should be a focus for future research.

CONCLUSION

This research expands on previous studies by exploring novel fungicide and sealant combinations. Previous recommendations of viable options for preventive *C. plurivora* control were made based on efficacy data of thiophanate-methyl, captan, 50% latex and lime sulfur (Miller et al. 2019). Considering the FRAC guidelines in avoiding evolved fungicide resistance, this previously study was limited. Therefore, this current study provides more efficacy data on a broader range of fungicides and sealants for growers. This research validates the efficacy of thiophanate-methyl alone and in combinations with latex covers, not only by evaluating lesion sizes in field trials, but by also evaluating the percent viability of spores on branches after chemical exposure. In commercial production systems, it may not always be appropriate or possible to cover the multitude of tree wounds directly with sealant combinations given time and economic constraints. Nonetheless, thiophanate methyl has shown to be effective alone and in different sealant combinations. Thiophanate methyl and captan, which has also shown to be effective, can both be applied through conventional means (e.g. air blast sprayers) (Miller et al. 2019).

The study provides data that suggests ineffectiveness of latex when applied as a preventive

wound application without the addition of a fungicide, despite reduction of lesion sizes observed in previous research. Further, this research provides EC_{50} values of effective fungicides/sealants, providing sensitivity ranges for *C. plurivora* isolates in western Colorado. As an alternative to latex as a wound sealant, thiophanate-methyl combined with VitiSeal and lime sulfur combined with VitiSeal are viable options for preventive control in conventional production systems. Fungicide combinations with OMRI approved sealants such as paraffinic oil and Nu-film, did not prevent fungal infections thus no novel recommendations can be made for organic production systems from this study. Nonetheless, organic production systems can apply lime sulfur alone and lime sulfur in combination with kaolin clay as preventive measures for *C. plurivora* as previously reported (Miller et al. 2019). Future trials should investigate an extensive list of effective fungicides/sealants for organic production systems, given the limited options currently available to growers. Application timings and duration of efficacy of fungicides applied on wounded tissues are also important next steps. OMRI approved fungicides, including copper octanoate, copper oxychoride, and copper hydroxide are effective against *C. plurivora in vitro*. These copper-based fungicides should be further investigated for their phytotoxicity to tree fruit in field applications, as limited studies have shown evidence of phytotoxicity. Overall, this work has generated more potential options for peach growers managing canker pathogens.

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CHAPTER III. **Water deficit and high pH soil as stress induced factors on thirteen peach cultivars when inoculated with *Cytospora plurivora***

SUMMARY

Understanding the host- pathogen- environmental interactions in a pathosystem is essential for management of diseases and for avoidance of diminished crop yields. Abiotic stressors such as cold damage, water deficit, and high pH soils can all be major limiting factors to deciduous tree fruit production. Along with decreased yields, these abiotic factors have direct implications for disease severity within orchards. *Cytospora plurivora* is a ubiquitous fungal canker pathogen in western Colorado and is a major focus for integrated pest management strategies due to the pathogen's necrotrophic lifestyle. The focus of this research was to evaluate both biotic and abiotic stress factors influencing peach tree health. The tree cultivars Glohaven®, Glowingstar®, Blushingstar®, Starfire®, Newhaven®, Flamin' Fury PF® 19-007, Flamin' Fury® PF 23, Flamin' Fury PF® 24, Redhaven®, O'Henry®, Angelus®, Suncrest®, and Cresthaven® on the root stock 'Lovell' were evaluated in both greenhouse and field conditions in the summer of 2018 and 2020 in western Colorado. Under deficit-irrigation, *C. plurivora* infections were significantly larger in both greenhouse and in field trials when compared to the full-irrigation controls. In controlled greenhouse conditions, a positive correlation between lesion size and water potential by treatment was evident, but no trend of cultivar tolerance was observed. Further, increase of irrigation water pH, through additions of sodium carbonate and bicarbonate, in greenhouse trials resulted in decreased leaf water potentials and increased pathogen necrotic tissue volumes (mm^3). In field trials, there was no positive relationship between lesion size and water potential. Trees displaying

the most negative water potentials also had the smallest lesions sizes, suggesting that other abiotic factors may be shielding water stressed trees from increased pathogen aggression.

INTRODUCTION

Management of abiotic stressors, such as water deficit, high pH, and cold damage, is vital for agricultural crop success. Abiotic stressors are considered the primary limiting factors of agricultural production, affecting 70% of agricultural crops around the world (Khan et al. 2015; Kaur et al. 2008; Mantri et al. 2012). In the western United States, multi-decal droughts are occurring with little evidence to suggest increases in precipitation in the future (Udall & Overpeck 2017). Linked to water deficit, high pH and soil salinization is also projected to cause 30%-50% reduction in agricultural land by 2050 (Khan et al. 2015; Wang et al. 2003). Further, cold damage is considered the major limiting factor to deciduous tree fruit production on a global scale. Adverse weather conditions, primarily freeze damage occurring in the spring, can damage fruit set resulting in massive reductions in crop yield (Rodrigo 2000). In 2020, Chinese peach production dropped by 500,000 metric tons due to an April snow which decimated fruit yields (USDA FAS 2020). Within the United States, Colorado, Michigan, and New Jersey, substantial losses due to spring freezes occurred in 2020 (USDA NASS 2020). Colorado saw the most drastic reduction in fruit yields, with production amounts plummeting from 14,300 tons in 2019 to only 3,000 tons in 2020 (USDA NASS 2020). Along with immediate reduction in fruit totals, cold damage of tree tissues can increase incidence of Cytospora canker, raising concerns for overall tree health and lifespan. Interactions among different abiotic stressors can also occur; increased irrigation has been shown to be linked to increasing cold tolerance and reducing canker severity in peach (Layne & Tan

1984). Thus, in order to manage limiting factors in peach production of both abiotic and biotic stress, it is important to explore host-pathogen-environment relationships.

Orchard water dynamics, including availability and quality, have direct implications for peach fruit production. For Colorado, and many other western regions including Utah, Wyoming, Arizona, California, Nevada, Northern Mexico, and New Mexico, the Colorado River Basin is a major irrigation and water source (Udall & Overpeck 2017). The Colorado River has seen an unprecedented drought over the last 15 years with a decrease in water levels of 19% when compared to water status levels from 1906-1999. Yet, there is no current evidence to suggest possible future increases in precipitation (Udall & Overpeck 2017). While water deficit irrigation strategies are sometimes employed with minimal differences in quality and/or quantity of harvest yield, it is not well known how sustainable these practices may be long-term considering potential soil salinization risks (Aragüés et al. 2014). Further, increased Cytospora canker development has been correlated to water deficits and reduced levels of bark moisture in French prune trees (*Prunus domestica* (L.) 'French') (Betrand et al. 1976). Bertrand et al. (1976) found that water stress for one fall season significantly increased Cytospora canker activity in the following seven months, and the authors surmised that drought and mismanaging of irrigation were likely linked with *Cytospora* infections.

Deficit irrigation has also been linked to increased soil pH and salinization (Kaman et al. 2006), which can cause physiological complications for peach fruit production. Peach trees are categorized as sensitive to soluble salts with possible yield and growth reductions, depending on the soil and irrigation water salt concentrations (Aragüés et al. 2014; Hoffman 1989; Maas & Hoffman 1977). Western Colorado, as well as other western states, have characteristically high soil pH which can lead to tree growth limitations due to nutrient deficiencies in Zn, Fe, or Mn

(Catlett et al. 2002; Fernandez et al. 2008). These deficiencies can lead to a decrease in leaf chlorophyll concentration, in plant fresh and in dry weight per leaf area and can cause a delay in fruit ripeness by two weeks (Morales et al. 1998; Sanz et al. 1997). In Colorado, high pH can be a major factor in heavily irrigated areas; in fact, almost 1 million acres have been estimated to have been impacted by excess salts (Cardon 1998). The Green River Formation, within Delta and Mesa counties where peach production occurs, is known to be a world class source of sodium carbonate and sodium bicarbonate (Dyni 1996). Thus, given the risk of high pH in soils in western Colorado, it is essential for cultural practices to include salt mitigation in irrigation waters and in soils to prevent physiological damage to trees.

Evidence of tolerance to *Cytospora* spp. across different peach cultivars has been demonstrated. Froelich & Schnabel (2019) reported varying lesion sizes on four tree cultivars on inoculated, excised branches. While excised branches may be a preliminary indicator of differences among cultivar tissues, water transport in live plants has been attributed as a primary factor in differences in tolerance among cultivars. Cytospora canker causes differences in xylem function between live inoculated and non-inoculated plants (Hampson & Sinclair 1973). However, Hampson & Sinclair (1973) were unable to identify differences of xylem function among five different cultivars. More recently, Chang et al. (1989 & 1991) attributed cultivar differences to water transport maintenance through cankered tissues areas; Identified differences included necrotic lesion length with progeny from the Russian germplasm 'Yennoh' exhibiting the smallest canker sizes. These results suggest that variation in peach cultivar tolerance to *Cytospora* spp. infections occurs. Thus the objectives of this study were to evaluate thirteen peach cultivars, commonly planted in western Colorado, for susceptibility to *C. plurivora* under drought and high pH conditions in greenhouse and in field conditions.

MATERIALS AND METHODS

Greenhouse Cultivar Susceptibility Trials. Thirteen, two-year-old, *Prunus persica* scions grafted on 'Lovell' rootstocks were selected from two different breeding programs. Selected scions included Glohaven®, Glowingstar®, Blushingstar®, Starfire®, Newhaven®, Flamin' Fury PF® 19-007, Flamin' Fury® PF 23, Flamin' Fury PF® 24, Redhaven®, O'Henry®, Angelus®, Suncrest®, and Cresthaven®. All selected cultivars, 5 replicates per each, received heading cuts and were planted in 56.8-liter containers with potting mix (Pro-mix BX, Quakertown, PA, USA). Trees were grown for two months (May – July 2018) and watered at full pot capacity in a shade house at Colorado State University, Fort Collins, Colorado. Trees were then transferred to a greenhouse to control for precipitation in water deficit treatments.

Trees were placed into three treatment groups: 1. Control (100% pot capacity, soil pH 7.0), 2. Deficit-Irrigation (60% pot capacity, soil pH 7.0), and 3. High-pH (100% pot capacity, soil pH 9.0). A total of 65 trees per treatment group were completely randomized, consisting of 13 cultivars and 5 tree repetitions per cultivar. To determine pot capacity, all trees were watered to full saturation and allowed to drain for one hour. After sufficient draining, trees were weighed with a mail scale (Dymo® 400) to determine 100% pot capacity. Trees subjected to deficit-irrigation were watered once a week for approximately two months at 60% of their full pot capacity during watering times while all other trees received 100% pot capacity. For the high pH treatment, soil pH was adjusted to a pH of 9.0 by adding 0.10g sodium carbonate and 1.0g sodium bicarbonate per liter of water to irrigation water. Water pH was confirmed with a pH gauge (Ecotestr pH1, Oakton instruments, Vernon Hill, IL) prior to watering once a week for two months. For pH analysis, soil was collected two months after continual application of pH treatments. Soil was collected from the rhizosphere of each 56.8 liter pot and samples were sieved and weighed (2.0 g

of soil in 50 mL conical tubes). Each tube was then prepared with 5mL distilled water, and soil slurries were mixed for 15 minutes. Slurries were placed in 50ml conical tubes and pH levels were recorded with a pH probe (AB150 Fisherbrand™ Accumet™, Fisher Scientific Inc., Waltham, MA, USA).

Field Cultivar Susceptibility Trials. Twelve of the thirteen, three-year-old *Prunus persica* scions, were transported to Colorado State Universities' Rogers Mesa Organic Agriculture Research Station (Hotchkiss, Colorado) in the fall of 2018. Experiments were conducted from June 2020 – August 2020. Trees, five replicates per cultivar, were placed into two treatment groups: 1. Full Irrigation and 2. Irrigation deficit. A total of 65 trees per treatment group were completely randomized, consisting of 12 cultivars and 5 tree repetitions per cultivar in three rows. Tree trunk diameter was measured 15 cm above the graft union prior to applying irrigation treatments to ensure uniformity across trees within cultivars and rows.

Given the clay loam soil profile of the planted area, 2.1 acre/inches of water per irrigation period was applied as the full irrigation control to reach field capacity, and to fulfill the estimated readily available water (RAW) requirement of the trees. The RAW requirement of the trees, in the clay loam soil profile, was estimated through irrigation recommendations made by Washington State University (Smith et al. n.d.). For the deficit irrigation treatment, 1.26 acre/in was applied to repeat the 60% water application done in the greenhouse trials. Total size of each row was determined to be 0.076 acre using Google maps (Google n.d.). Given that there are 102,790 liters of water in 1 acre/inch, for an application of 2.1 acre/inch, a total of 215,859 liters would have to be applied. Similarly, for an application of 1.26 acre/inch of water, a total of 129,515 liters would have to be applied. These totals were adjusted for the 0.076 acre planted area of each row: 16,405 liters for the full irrigation row and 9,843 liters for the deficit irrigation row. Given there were 33

sprinkler heads per row, with a total output of 1,187 liters/ hour per row, sprinklers were run for 13.8 hours for the full irrigation row and 8.3 hours for the deficit irrigation row. At these rates, the volume of water to reach 2.1 acre/inch and 1.26 acre/inch at an area of 0.076 acres per treatment row was calculated. Irrigation occurred once per week throughout the 2020 growing season. Pre-dawn water potential (PWP) measurements were taken to compare plant water status in order to validate the irrigation treatment differences.

Plant Water Potentials. For the greenhouse trials, leaf water potential (LWP) measurements were taken on all trees at solar midday two months post establishment of stress treatments. Climatic conditions during leaf collection included no precipitation and an average temperature of 28.3°C. Leaves were cut from trees and pressure readings were taken using a Scholander Pressure Chamber (Model 1000; PMS Instrument Company; Albany, Oregon). A magnifying glass was used to determine when the compressed nitrogen had provided sufficient gas to force the internal leaf water to the cut edge of the leaf. Pressure values are represented as negative megapascals.

For the field trials, pre-dawn water potentials (PWP) were assessed prior to sunrise between 0300HR and 0600HR daily for seven days to follow the soil water curve for an entire irrigation period. A minimum of 15 trees were evaluated within the full irrigation row to ensure minimal water stress, while a minimum of 35 trees were evaluated in the irrigation deficit row. This sampling method ensured that irrigation treatments were affecting PWP across treatments. PWPs were taken daily from the most hydrated day after watering, to the least hydrated day, one day before the next watering period. PWPs were also measured during the weeks of inoculation and branch harvest to assess the water status of the trees during these periods. To ensure a proper insertion into the chamber seal, a razor blade was used to make a clean cut of the leaf base away from the petiole.

Fungal Inoculations (Greenhouse and Field). Cultures of *Cytospora plurivora* isolate CP5.1, used in previous studies (Miller et al. 2019; 2021), were grown on ½ PDA for four days prior to inoculation. For inoculations, the outer bark of the branch was removed with 4 mm corer to wound the tissue. Hyphal agar disks (4 mm in diameter), taken from four-day-old cultures, were placed on each branch wound and wrapped with Parafilm (American National Can; Chicago, IL, 60631, USA). Branches were harvested 8 days post inoculations. Canker sizes were measured using a digital caliper, and volume of the decayed tissue was calculated based on branch diameter and canker length. Koch's postulates were satisfied by the plating of symptomatic plant tissue on ½ PDA media and confirming *C. plurivora.*

Statistical Analysis. Rstudio was used for statistical analyses and packages used included lme4, lmertest, pbkrtest, and emmeans (Bates et al. 2015, Halekoh & Højsgaard 2014, Kuznetsova et al. 2017, Lenth 2016, R Core Team 2017). Summary statistics and graphical depictions were created using the plyr and the ggplot2 package, respectively (Wickham 2011; Wickham 2009). In greenhouse and field trials, separate one-way ANOVA models were built for the multiple response variables of water potential (-Mpa) and/or soil pH. A two-way ANOVA was also used to evaluate differences in lesion size across treatments (pH, water deficit, and no stress) for greenhouse and field trials (Table 3.1a; Table 3.1b, respectively). Treatment and cultivar were used as fixed predictor variables, with an interaction term included between treatment and cultivar. In field trials, a two-way ANOVA was also used to evaluate differences of trunk diameter, prior to irrigation treatments, with cultivar and row location as fixed predictor variables, and with an interaction between cultivar and row location (Table 3.1c). In both the greenhouse and field trials, linear regressions were evaluated in Excel (2020) to determine the relationship between lesion volume

and plant water potential values. To correct for assumptions of normality and equal variances, greenhouse trial data was square root transformed.

Table 3.1. Two-way ANOVA tables.

a. Greenhouse trials; Response = lesion volume.

Predictors	*df*	Sum of Squares	Mean Squares	*F*	*p*
Treatment	2	3801701	1900850	95.98	< 2E-16
Cultivar	12	438818	36568	1.85	0.046
Treatment:Cultivar	24	386250	16094	0.81	0.716
Residuals	143	2832077	19805		

b. Field trials; Response = lesion volume.

Predictors	*df*	Sum of Squares	Mean Squares	*F*	*p*
Treatment	1	1628562	1628562	29.60	2.808E-7
Cultivar	12	1129384	94115	1.71	0.072
Treatment:Cultivar	11	697333	63394	1.15	0.327
Residuals	121	6655687	55006		

c. Field trials; Response = trunk diameter.

Predictors	*df*	Sum of Squares	Mean Squares	*F*	*p*
Cultivar	12	248.36	20.697	0.99	0.467
Row	1	49.98	49.977	2.39	0.126
Cultivar:Row	12	196.51	16.376	0.78	0.665
Residuals	70	1463.89	20.913		

RESULTS

Greenhouse Cultivar Susceptibility Trials. Differences in leaf water potential (LWP; -Mpa) and soil pH were evaluated across cultivars and treatments to ensure treatment stress was present (Figure 3.1a & b). Further differences in overall lesion volume were also evaluated (Figure 3.1c). When grouping all cultivars by each of the three treatments, differences in tree leaf water potential (LWP) were found across the three treatment groups ($P < 0.0001$) (Figure 3.1a). Trees within the high-pH and deficit-irrigation treatments had significantly more negative LWPs when compared to control trees ($P < 0.0001$; $P < 0.0001$, respectively) (Figure 3.1a), but did not differ between the two stress treatments ($P = 0.5761$) (Figure 3.1a). When evaluating soil pH differences between treatments, the high-pH treatment was significantly higher, as expected, than the control and the deficit-irrigation treatments ($P < 0.0001$; $P < 0.0001$, respectively) (Figure 3.1b), but no difference was observed between the control and irrigation deficit treatments ($P = 0.884$) (Figure 3.1b). Differences in lesion volume did occur ($P < 2E\text{-}16$) (Figure 3.1c). The largest lesion volumes were produced in the deficit-irrigation treatment, whereas the smallest lesion volumes were observed in the control treatment (Figure 3.1c). The treatment by cultivar interaction, in the model with lesion volume as the response, yielded no significance ($P = 0.716$) (Table 3.1a). The cultivar predictor variable in the model yielded a significant value ($P = 0.046$) (Table 3.1a). When further exploring this significance by cultivar, only O'Henry and FF23 differed significantly from one another ($P = 0.026$) while all other comparisons were non-significant.

When trees were subjected to deficit-irrigation (60% pot capacity, pH 7) and then inoculated with *C. plurivora*, no differences in lesion volume across cultivars were found ($P = 0.7819$) (Figure 3.2). When trees were grown under high-pH conditions (100% pot capacity, pH 9) and compared across cultivars, only Glowingstar® and FF23® showed significant differences between one

another, with Glowingstar® developing larger lesions than the FF23® cultivar (P = 0.0376) (Figure 3.3). When volume sizes on cultivars were compared only within the control treatment (100% pot capacity, pH 7), no significant difference among cultivars was observed (P = 0.3558) (Figure 3.2 & 3.3).

To explore the relationship between the leaf water potential variable and lesion volume variable, a linear regression was evaluated (R^2 = 0.173; P = 1.36E-08) (Figure 3.4a). Trees with the largest lesion volume belonged to the deficit-irrigation treatment (60% pot capacity, pH 7 treatment), while trees with the smallest volume and least negative water potentials belonged to the control treatment (100% pot capacity, pH 7) (Figure 3.4a). This regression trend highlighted that trees with more negative leaf water potentials also had larger lesion volumes. However, within treatments (Figure 3.4b), no clustering/ grouping by cultivar was observed. Cultivars were highly variable in both leaf water potential and lesion volume values across treatments (Figure 3.4b).

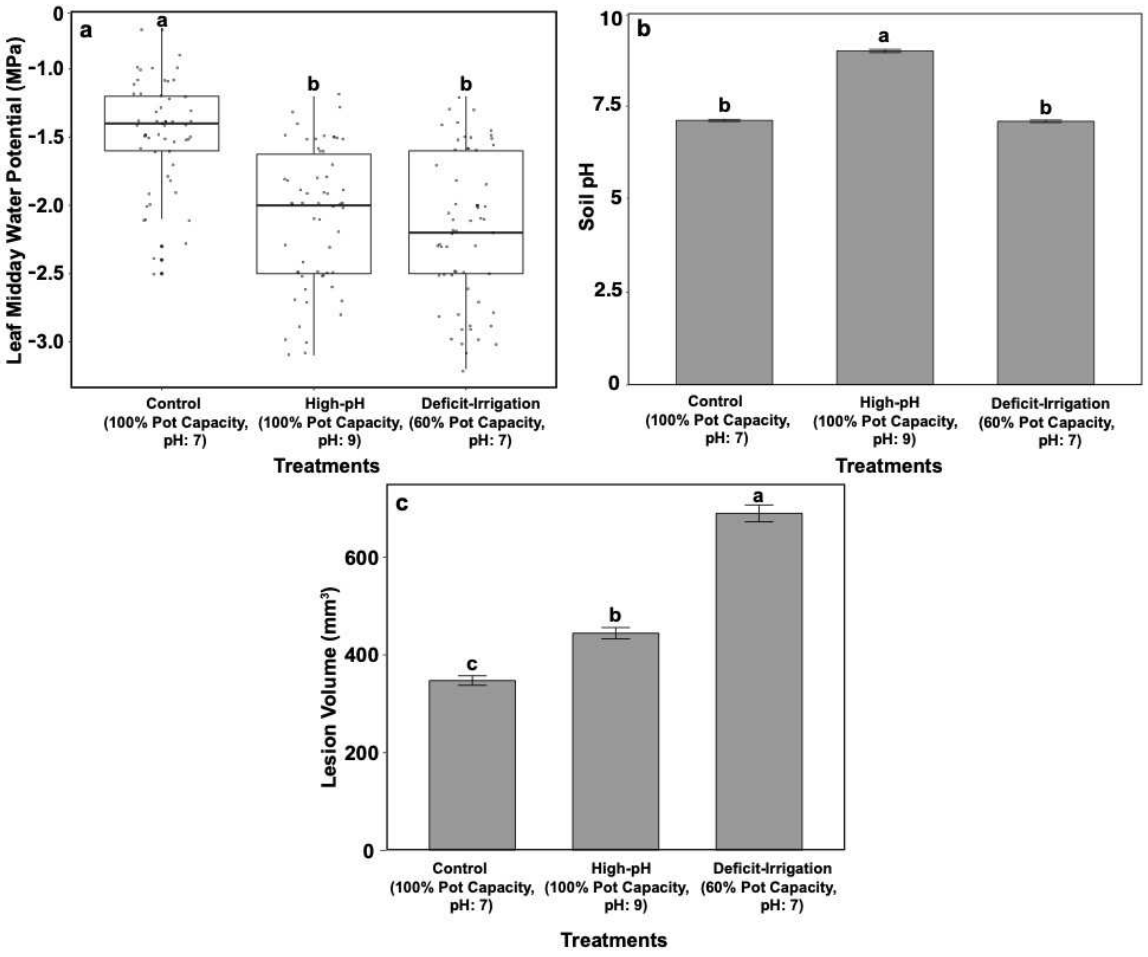

Figure 3.1. Greenhouse trials: Treatments are compared in figures a, b, and c include: 1) Control **(**100% pot capacity, pH of 7), 2) High-pH (100% pot capacity, pH of 9), and 3) Deficit-Irrigation (60% pot capacity, pH of 7). **A)** Midday leaf water potentials (Mpa) across treatments. **B)** soil pH across treatments. **C)** Lesion volume (mm^3) after *C. plurivora* inoculation. Means labeled with the same letters are not significantly different at $P = 0.05$ according to Tukey's test.

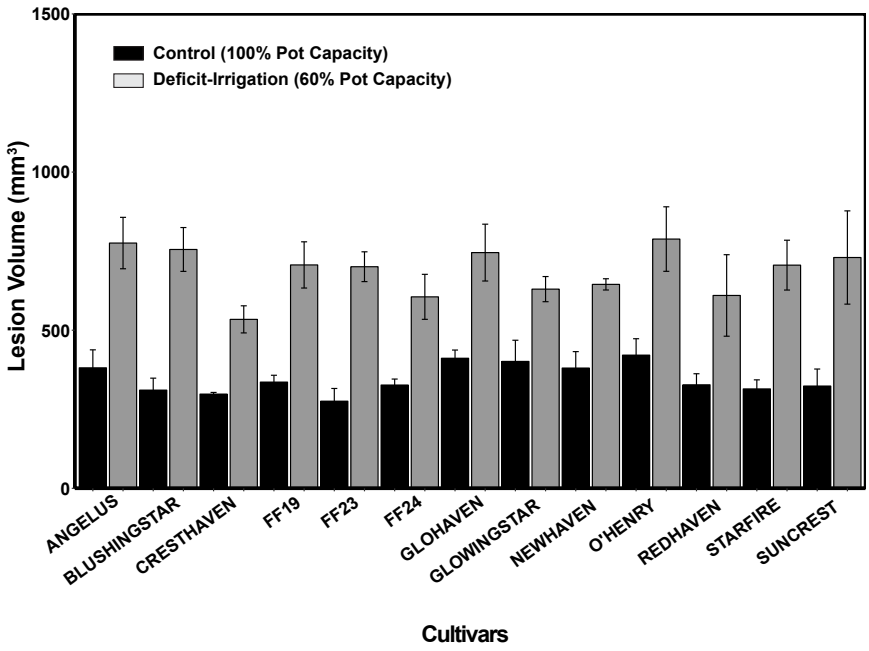

Figure 3.2. Greenhouse trials: Tree necrotic tissue volume (mm^3) in response to *C. plurivora* inoculations under two treatment conditions: 1. Control (Black bars; 100% pot capacity) and 2. Deficit-Irrigation (Gray bars; 60% pot capacity). Branch inoculations were made after two months of weekly watering treatments based on pot capacity.

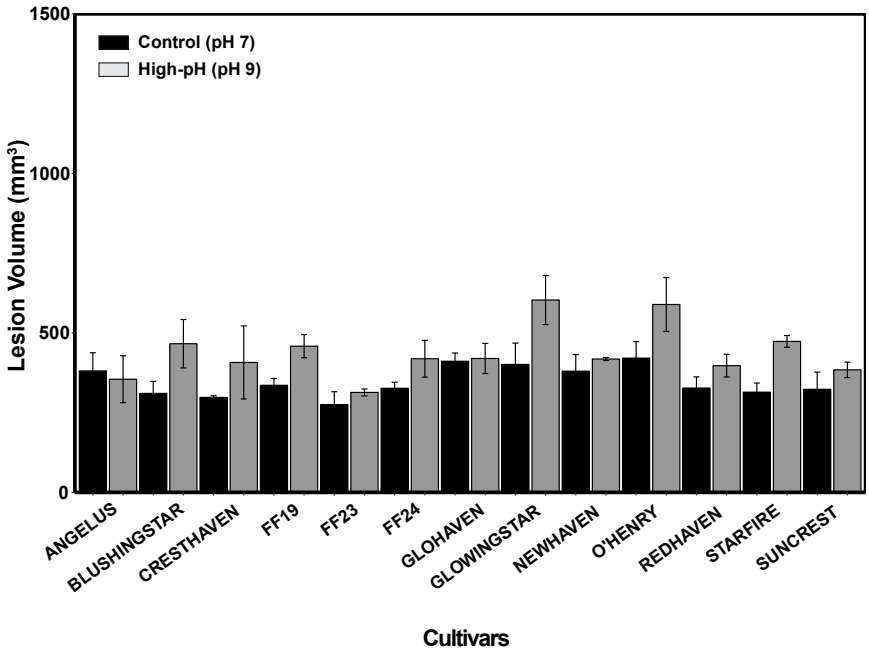

Figure 3.3. Greenhouse trials: Tree necrotic tissue volume (mm^3) in response to *C. plurivora* inoculations under two treatment conditions: 1. Control (Black bars; pH of 7) and 2. High-pH (Gray bars; pH of 9). Soil pH adjusted to a pH of 9.0, were made through irrigation water, with sodium carbonate and sodium bicarbonate. Branch inoculations occurred after two months of continuous watering treatments.

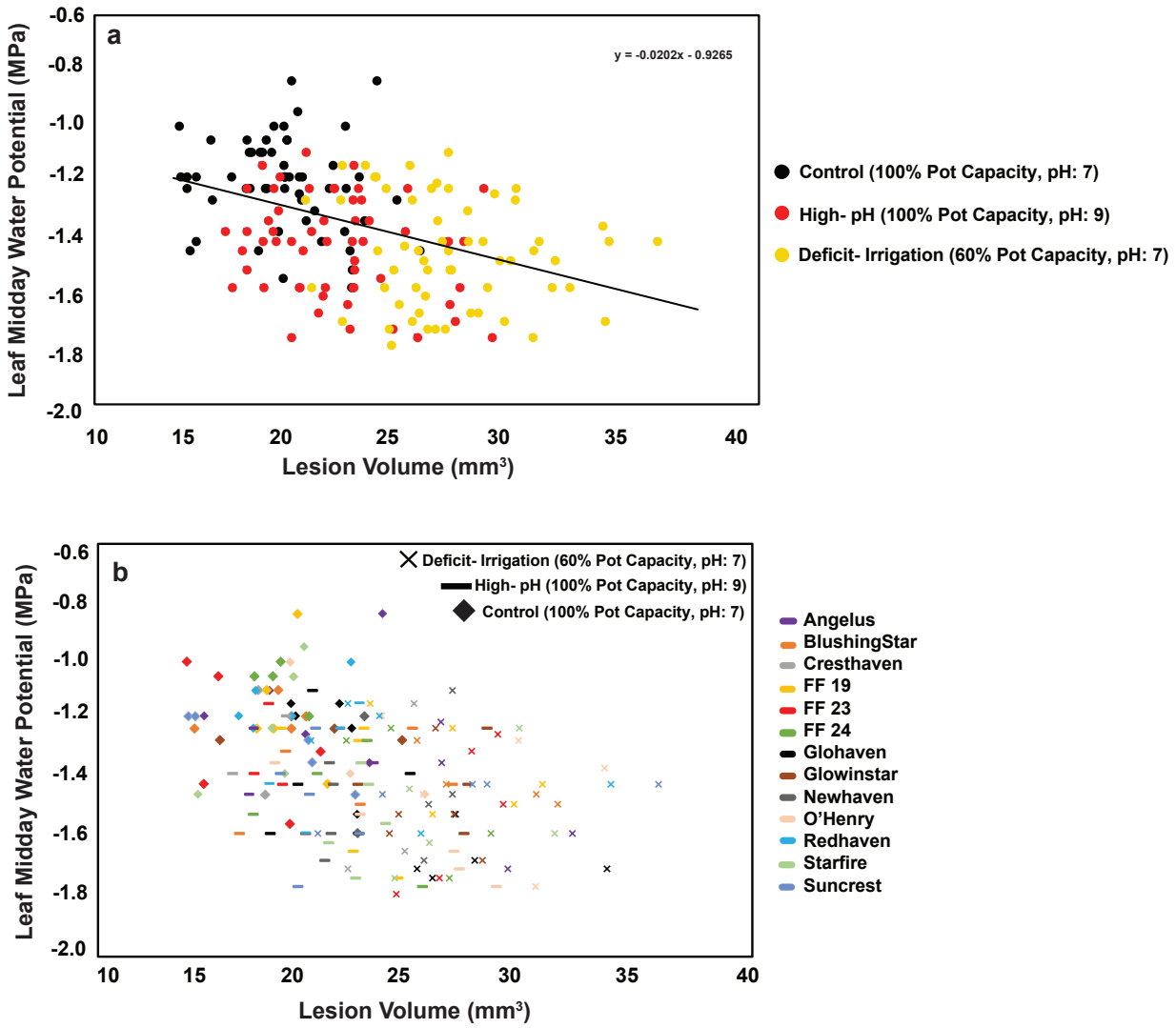

Figure 3.4. Greenhouse trials: Relationship (R^2 = 0.1732; *P* = 1.36E-08) between midday leaf water potentials and lesion volume in response to *C. plurivora* inoculations on peach trees under different irrigation and pH treatments. **a)** includes three treatments ordered by color: 1) Control (Black; 100% pot capacity, pH 7), 2) High-pH (Red; 100% pot capacity, pH 9), and 3) Deficit-Irrigation (Yellow; 60% pot capacity, pH of 7). **b)** Same data is shown, but treatment is characterized by shape, and cultivar scion tissue is denoted with colors.

Field Susceptibility Trials. Prior to applying irrigation treatments, trees showed no statistical difference in trunk diameter when evaluated by cultivar and there was no row effect by cultivar (P = 0.467; P = 0.665; respectively) (Table 3.1c; Figure 3.5). After irrigation treatment applications, trees in the full-irrigation and deficit-irrigation treatments were evaluated for lesion size differences. No lesion volume differences were observed among the twelve cultivars within either the full-irrigation or within deficit-irrigation treatments (P = 0.0966 and P = 0.5557, respectively) (Figure 3.6). When combining all cultivars, there was a significantly larger lesion volume in the deficit-irrigation treatment with an average lesion size of 808.85 mm^3, compared to 577.15 mm^3 in the full-irrigation treatment (P = 2.808E-07; Figure 3.7). The treatment by cultivar interaction, in the model with lesion volume as a response, yielded no significance P = 0.32746. To assess differences in tree water status and soil water potential across treatments, pre-dawn water potentials (PWP) were measured to capture an entire irrigation dry down period (Figure 3.8). Trees receiving full-irrigation, on average, had significantly lower PWP's (-MPa) at every measurement time point compared to trees subjected to the deficit-irrigation treatment ($P < 0.0001$) (Figure 3.8). After watering, PWP was less negative, but after four days (96 hours) trees began to display more negative PWPs. Prior to irrigation on 7/17/2020, trees were most stressed, and the soil water status was lowest. No significant differences were observed between cultivar type within the irrigation-deficit treatment and PWP measurement date (Figure 3.8). Dates evaluated included 7/11/2020, 7/12/2020, 7/13/2020, 7/14/2020, 7/15/2020, 7/16/2020, 7/17/2020, 7/27/2020, and 7/28/2020. Comparisons across cultivars within each date yielded the following values (P = 0.217; P = 0.4304; P = 0.9982; P = 0.9946; P = 0.9131; P = 0.7142; P = 0.6685; P = 0.948; P = 0.1728, respectively).

A linear regression explored the relationship between PWP and lesion volume variables, and no correlation was identified ($R^2 = 0.013$, $P = 0.386$) (Figure 3.9). Interestingly, some trees in the irrigation deficit treatment, displaying the most negative water potential values, had the smallest lesion sizes (Blushingstar®, Redhaven®, Glowingstar®, and Starfire®, respectively) (Figure 3.9). On average, trees with the largest lesion volumes received the irrigation-deficit treatment. Within treatments, cultivars did not cluster together and were highly variable in both PWP and lesion volume values (Figure 3.9).

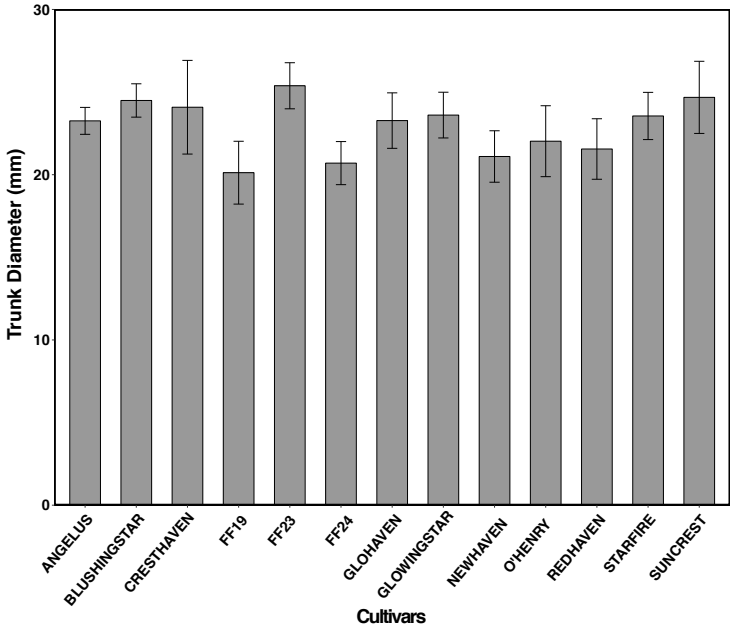

Figure 3.5. Field trials: Tree trunk diameter (mm) of all planted trees grouped by cultivar. Measurements were taken prior to applying deficit-irrigation and full-irrigation treatments. All measurements were taken 15 cm above the graft union. Trees were planted in the fall of 2018 and trunk diameters were measured spring 2020. Means are not significantly different between trees at $P = 0.05$ according to Tukey's test.

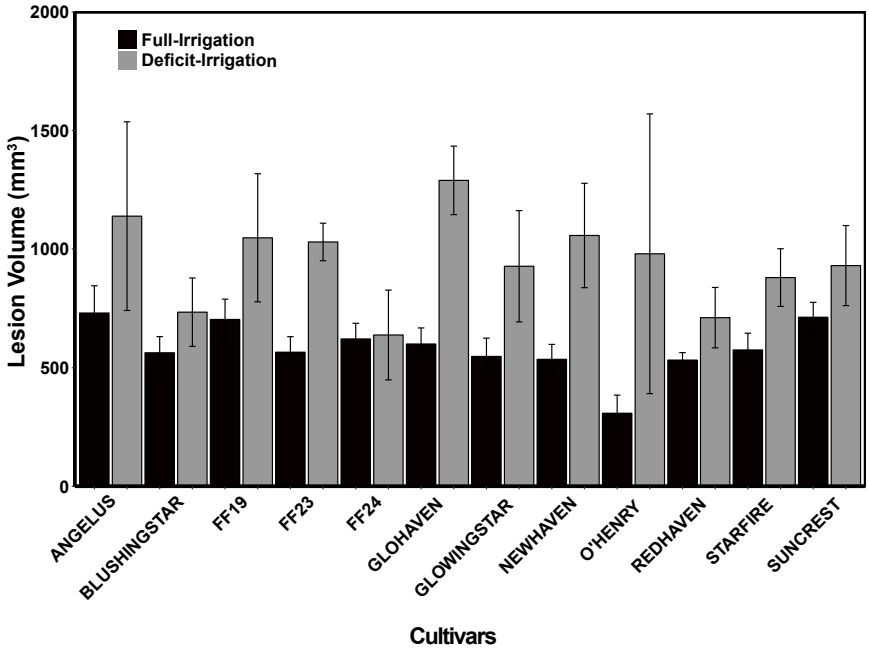

Figure 3.6. Field trials: Tree necrotic tissue volume (mm^3) in response to *C. plurivora* inoculations under two treatment conditions: 1. Control (Black bars; Full irrigation) and 2. Deficit Irrigation (Gray bars; Deficit irrigation). Branch inoculations were made after three months of irrigation treatments which were based on the readily available water of the planted area's soil profile.

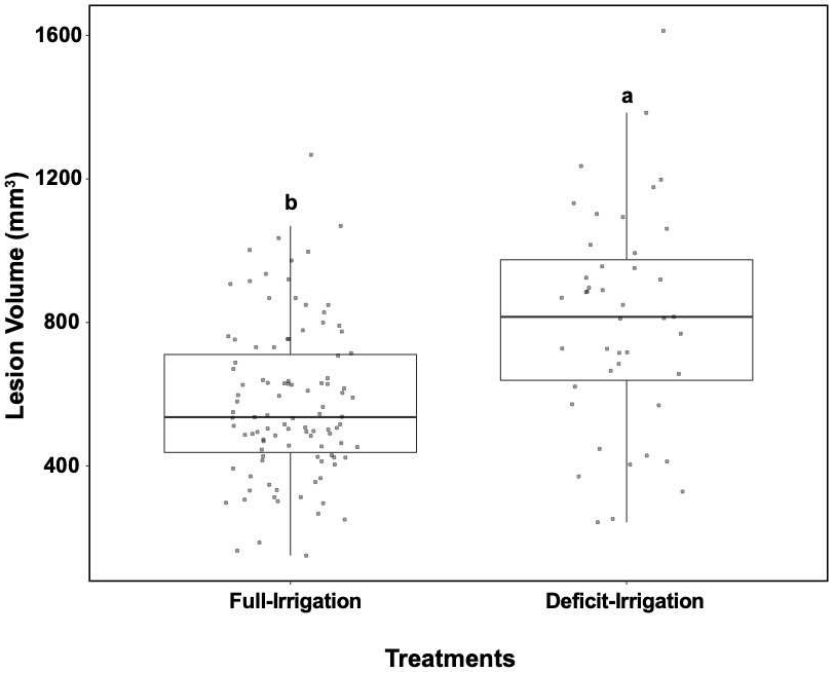

Figure 3.7. Field trials: Effects of full-irrigation/ deficit-irrigation treatments based on *Cytospora plurivora* lesion size on peach trees. Response calculated is the necrotic tissue volume (mm^3). Trees were watered at the given treatments from June 2020 – August 2020. Different letters represent significantly different means (Tukey's test, $P = 0.05$).

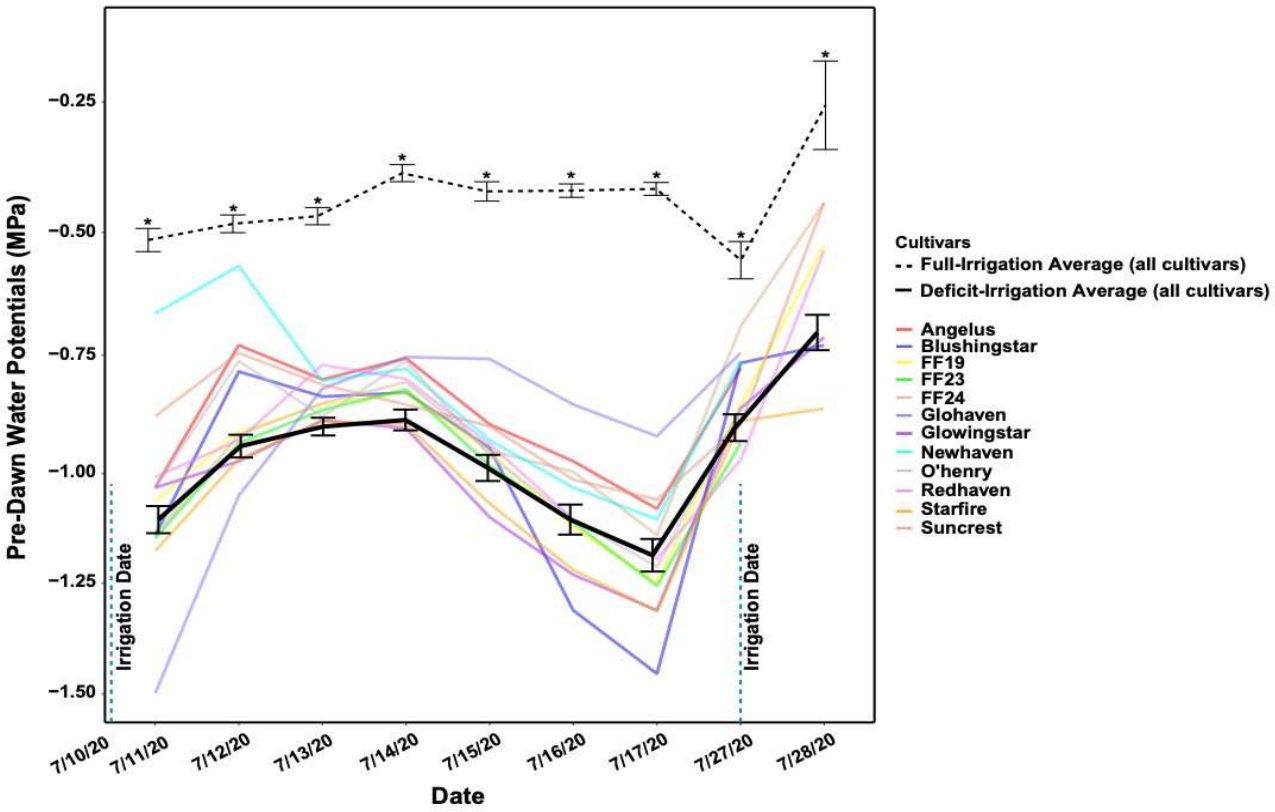

Figure 3.8. Predawn water potentials (PWP) of peach cultivars in one full-irrigation row and one deficit-irrigation row at 60% RAW according to the soil profile. The average PWP values for the full-irrigation treatment are denoted by a dotted line while the average values for the deficit-irrigation treatment are represented by a solid black line. Colored lines represent PWP values for cultivars within the deficit-irrigation treatment. Bars show standard errors and dotted- vertical lines at 7/10/20 and 7/27/20 represent irrigation dates. Starred full-irrigation averages are significantly different than counterpart deficit-irrigation averages at $P = 0.05$ according to Tukey's test.

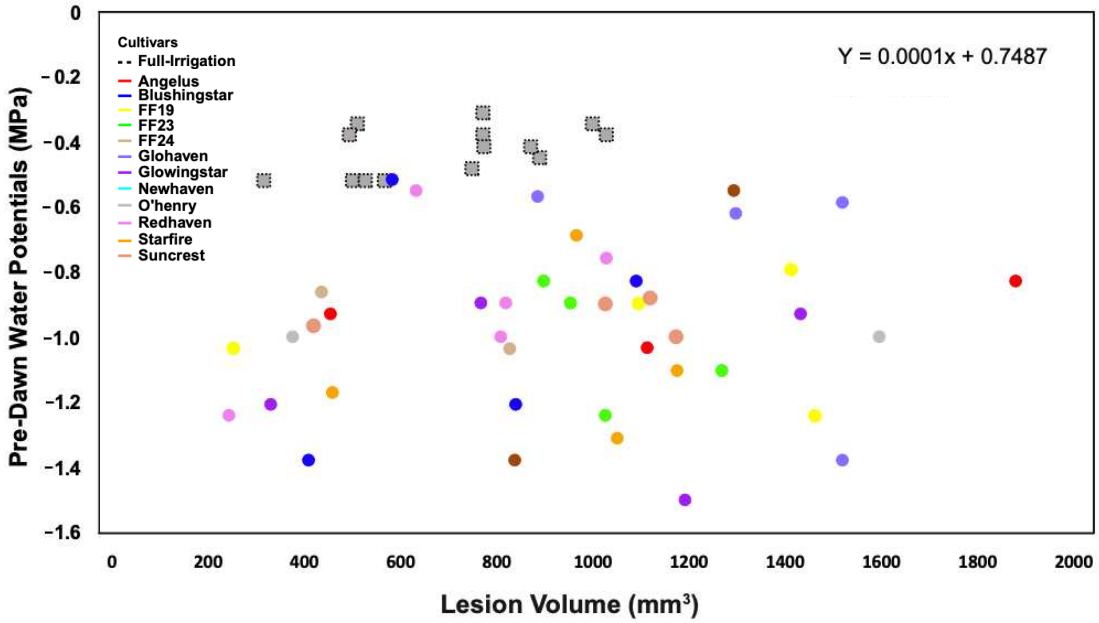

Figure 3.9. Field trials: No relationship was observed ($R^2 = 0.013$; $P = 0.386$) between predawn water potential (PWP) and lesion volume (mm^3) in response to *C. plurivora* inoculations on peach trees under full-irrigation and deficit-irrigation. Treatments are characterized by shape: The full-irrigation treatment is denoted by gray-dotted squares and the deficit-irrigation treatment by circles. Cultivar scion tissue is denoted with colors.

DISCUSSION

In the greenhouse and field trials, there was a clear overall treatment effect whereby trees under irrigation deficits had significantly larger lesion volumes after inoculation with *Cytospora plurivora*, suggesting that drought stress enhances peach susceptibility. Previous studies have documented similar drought stress effects on plants inoculated with *Cytospora* species (Guyon et al. 1996; McIntyre et al., 1996; Kepley and Jacobi, 2000). More specifically in peach systems, researchers have correlated increased *C. leucostoma* growth on trees under water deficit and with reduced levels of bark moisture (Betrand et al. 1976). Further, increased irrigation rates for peach trees have been reported to decrease Cytospora canker severity and to increase cold tolerance in peach (Layne & Tan 1984). Presently, there have been no studies evaluating the virulence of the newly described *C. plurivora* species on drought-stressed trees.

Overall, comparisons among tree cultivars inoculated with *C. plurivora* yielded few differences in lesion size in both the greenhouse and field trials. Only O'Henry differed statistically from FF23 in greenhouse trials (Table 3.1b). Further, no clustering by cultivar occurred in the regression analyses comparing water potentials and lesion sizes for any treatment. As a control to test differences among scions, all trees were grafted onto 'Lovell' rootstock. Our results suggest that susceptibility to *C. plurivora* under water deficit may not be driven by scion tissue, however, there may be an effect of the rootstock influencing the physiological response of the scion. It is well known that the rootstock plays a major role in the physiological signaling within drought-stressed plants. The regulation of stomatal behaviors has been linked to abscisic acid (ABA) production in the roots of drought-stressed plants (Mansfield et al. 1990). In grapevine systems, manipulation of root stock derived ABA, through partial root deficit (PRD) irrigation, can be used to control canopy water use in grapevines plants (Dry et al. 1995, Loveys et al. 1998). Rootstock

may have a strong influence on scion water potential and/or *Cytospora* susceptibility; Nonetheless, despite not observing statistical differences among cultivars, the cultivar FF24® showed similar levels of tolerance under altered water deficit in field trials when compared to full irrigation. Flamin' Fury® (PF-24C) (FF24) has been bred and marketed as a cold hardy cultivar, thus it may be that qualities that contribute to cold hardiness may also play a role in the physiological adjustments within the plant's self-defense response (Maughan et al. 2016). When analyzing immediate response to shoot wounding, Biggs and Miles (1988) and Wensley (1966) found the rate of callus formation and suberized phellem served as resistance barriers to infections in peaches. Thus, FF24® may form such structures faster than other scion types in response to wounding, freeze wounds or other wound types exposing underlying tissues.

While the greenhouse trials confirm known literature suggesting a correlation between lesion size and water potential, the field trials did not display the same trend. Interestingly, a selection of water deficit trees displayed the most negative water potentials but had the smallest lesion sizes. There was no linear relationship between water potential and lesion size in field trials despite there being significant differences overall between the lesion sizes due to drought treatment. It is likely that other abiotic and/or biotic variables may be obscuring the relationship. One hypothesis is that there may be an influence of the microbiome present in the field trials. Both fungal and bacterial biocontrol agents have been reported to be effective against Cytospora canker. Yi and Chi (2011) reported significant inhibition of Cytospora canker in poplar stands in China by the cosmopolitan fungi *Trichoderma longibrachiatum.* Hyphal growth of *Cytospora chrysosperma* was significantly inhibited in both in vitro and field trials when exposed to *T. longibrachiatum*. Similarly, researchers reported the ability of *Fusarium* sp. to limit *Cytospora* sp. in poplar trees (Xiang et al. 1991). In apple production systems, Gao et al. (2000) reported isolates of *T. harzianum* that

inhibited *Valsa mali* growth. Further, Zhang et al. (2015) found that the bacterium *Bacillus amyloliquefaciens* significantly inhibited the development of *V. mali* in apple orchards in China. Species of *Trichoderma*, *Fusarium,* and *Bacillus* are ubiquitous in the environment and may have caused antagonism in my study. Future research trials should isolate fungi from tree tissues to evaluate the microbiome present within orchards in western Colorado.

Another hypothesis for the lack of correlation between tree water potential and lesion size in field trials may be the effect of nutrient availability and tree position within an orchard. Nutrient deficiencies may be present unevenly within an orchard row causing physiological advantages or complications despite the watering treatment of a row. Tree growth limitations are common when Zn, Fe, or Mn deficiencies are present causing decreases in leaf chlorophyll concentration, in plant fresh and dry weight per leaf area, and can also cause a delay in peach fruit ripeness by two weeks (Fernandez et al. 2008; Morales et al. 1998; Sanz et al. 1997). Further, differences in microclimates within an orchard may be more or less conducive to *C. plurivora* growth. These differences can be caused by topographical differences within an orchard, location of sprinkler or drip irrigation systems, and/or variation in the surrounding vegetative growth near trees. Such abiotic and biotic variables are potentially influencing field trials and should be investigated in future studies.

In western Colorado, low quality water sources with high pH are common. Despite maintaining full irrigation schedules in controlled greenhouse conditions, this study documented that irrigation water amended with sodium carbonate and sodium bicarbonate decreases leaf water potential and increases *C. plurivora* lesion sizes. Water stress is a predictor of tree health and measurable through leaf and stem water potential due to anisohydric tendencies of peach trees (Remorini & Massai 2003; Bertrand et al. 1976; Marsal & Girona 1997). This study confirms previous literature which has documented that increased salts in soil can have a direct effect on the water potential

of anisohydric plants due to changes in root hydraulic resistance altering the flow of water within the plants (Navarro et al. 2007). Other effects of salinity from irrigation water may include mineral deficiency, stomatal blockage, photosynthesis prohibition, cell division prohibition through interaction of salts with plant cellular components, and ion toxicity (Bahmani et al. 2015). When overall treatment effects were compared in the greenhouse trials, trees with increased pH had significantly larger lesion sizes, similar to other studies which have shown that increasing salinity in soils can increase disease severity. Severity of *Phytophthora parasitica* infections to tomato plants was shown to increase with high salinity levels (Snapp & Van Bruggen 1991). More specifically, in peach systems, studies have shown decreased fruit production totals in response to increased soil pH and salt concentrations (Bernstein 1980; Hoffman et al. 1989). Correlations between lesion size and water potential were evident in the greenhouse high-pH treatment, suggesting a direct effect from altered pH levels on tree stress. For growers with high pH irrigation waters, sulfur burners may be a viable option for amending irrigation water (Gale et al. 2001) by neutralizing carbonates (CO_3^{2-}) and bicarbonates (HCO_3^-) present through the production of sulfur dioxide gas (SO_2). SO_2 is dissolved in the irrigation water forming sulfuric acid (H_2SO_4), which in turn, neutralizes carbonates and bicarbonates (Ezlit et al. 2010). Sulfur burners have been shown to be efficient in acidifying stream irrigation water and removing all bicarbonates. Recent studies showed irrigation with sulfur burner blended water resulted in decreased soil salinity and improved yields and quality of cotton harvests (Ganjegunte et al. 2018). Sulfur burners may be a practical solution for areas with relatively high pH of irrigation water but should be further investigated as there is little research on the actual long term soil effect and physiological effect specific to peach trees.

CONCLUSION

It is evident that drought stress enhances peach susceptibility to the recently described *Cytospora* pathogen, *C. plurivora*. Our study documents that along with drought stress, high pH also increases tree susceptibility to *C. plurivora* and also decreases overall peach tree water potential. Under more controlled greenhouse conditions, there was a positive correlation between lesion size and leaf water potential by treatment further emphasizing the susceptibility of peach trees under pH and deficit irrigation stress treatments. Thus, cultural practices should ensure that irrigation water quality is acceptable and evenly distributed within fields. Growers should avoid applying irrigation water with high pH and/or concentrations of soluble salts. Further, soils should be tested regularly in areas with high risk of saline soils. Though differences among cultivars were slight, cultivar FF24® showed higher tolerance to *C. plurivora* inoculation in all trials and should be further explored as a hardy scion tissue option for growers. Further exploration into differences among a suite of rootstock may prove useful for identifying sources of tolerance against *C. plurivora*. Additionally, future trials should also explore variables which may be obscuring the correlation between water potential and lesion size in field trials. Evaluating the microbiome within peach orchards may yield potential bio-control agents which are serving as antagonists to *C. plurivora*.

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CHAPTER IV. **Epidemiology of *Cytospora plurivora* in western Colorado**

SUMMARY

Elucidating the epidemiology of the canker pathogen *Cytospora plurivora* is essential to management. This pathogen is thriving in western Colorado as field surveys have shown it is ubiquitous and present in all peach orchards evaluated. The focus of this work was to outline the annual lifecycle and movement of pathogen inoculum. Sporulation rates throughout the year and tree susceptibility to infection were evaluated. Further, dissemination methods were investigated with a molecular droplet digital PCR technique to better understand how inoculum is being introduced and spread within and across orchards. Five mature cankers were analyzed for 12 months and found to produce spores throughout the year despite seasonal temperature fluctuations. Further, peach tree prune wounds were investigated for their susceptibility to infections bi-weekly throughout the 12-month study. Infections occurred throughout the year, with lowest severity occurring during the dormant season, particularly in December and January. Water dissemination has been documented as the primary dispersal mechanism of *Cyotspora* species. The advent of new molecular techniques allows for highly sensitive detection of target DNA molecules, allowing for analysis of potential other dissemination methods. In the following research, positive amplification of *C. plurivora* DNA was found on borer insect species and aerial samples, but in low concentrations. These findings suggest that dissemination may occur aerially but in low concentrations, and that borers may disseminate spores but are not primary vectors of *C. plurivora*. Finally, 330 peach trees from nurseries were evaluated for *C. plurivora* presence both symptomatically and asymptomatically. None of the trees contained symptomatic *Cytospora* infections and asymptomatic tissue sampling yielded no *Cytospora* species. This research will

enhance management by elucidating patterns of *C. plurivora* in western Colorado, allowing for improved and targeted management practices.

INTRODUCTION

Canker pathogens in deciduous tree production systems can cause major losses in fruit production totals. Ascomycete fungi, belonging to *Botryosphaeriaceae* and *Diaporthaceae*, have historically been reported in deciduous production systems including nut, pome fruit, stone fruit, and grapevine (Phillips et al. 2013; Wang et al. 2011; Lawrence et al. 2018; Urbez-Torres et al. 2006; Luo et al. 2020). Thirteen fungal species within the *Botryosphaeriaceae* family have been reported as casual agents of Botryosphaeriaceae canker (Urbez-Torres & Gubler 2011; Urbez-Torres et al. 2006). In grapevine systems in California, Botryosphaeriaceae canker results in losses over $260 million annually (Siebert 2001; Urbez-Torres & Gubler 2011). Within the *Diaporthaceae* family, the most common fungal diseases include Cytospora canker and Phomopsis canker. Incidence of Botryosphaeriaceae and Diaporthaceae canker are reported extensively in all major deciduous tree production areas in the world including the United States, China, and the European Union (Luo et al. 2020; Tian et al. 2018; Moral et al. 2019).

In tree production systems, due to the extensive pruning required for tree architectural training, *Botryosphaeriaceae* and *Diaporthaceae* are able to thrive by infecting the wounded tissue. During pruning, the risk of infection is increased especially if rain events coincide with pruning. Lawrence et al. (2018) showed that 92% of Cytospora canker infections in California French prune orchards occurred on pruning wounds. Similarly, *Botryosphaeriaceae* species primarily infect through prune wounds, and many studies have evaluated seasonal risk of infections, especially in vineyards (Petzoldt et al. 1981; Urbez-Torres et al. 2010).

For both the *Botryosphaeriaceae* and *Diaporthaceae*, spores are thought to be primarily disseminated through rain splash or wind-rain events. Urbez-Torres et al. (2010) reported increased concentrations of *Botryosphaeriaceae* spp. spores during rain events and irrigation events. Researchers found that during the dormant season, spore concentration is also highest during rain events, thus they recommend growers prune in the latter portions of the dormant period when the risk of a precipitation event is lower (Urbez-Torees et al. 2010). Grove and Biggs (2006) found increased concentrations of *Cytospora* spp. spores during in warmer temperatures and in seasons with increased precipitation. Though, rain splash is a known mechanism of dispersal for *Cytospora* species, few studies have reported evidence of non-water borne release of conidia (Bertrand & English 1976). Recent studies, however, have challenged these claims. Luo et al. (2010) reported the presence of *Cytospora* spp. spores from aerially collected samples within walnut orchards in California, and previous researchers have correlated high incidence of insect borer wounds with Cytospora cankers suggesting that borer insects may be serving as vectors (Swift 1986).

In the western Colorado where peach production occurs, climate conditions are much drier compared to other peach growing regions. Essential to well-coordinated, integrated pest management strategies for *C. plurivora* is the knowledge of pathogen introduction, epidemiology and infection processes specific to the environmental conditions within a growing area, especially in the unique climatic conditions that exist in western Colorado. The host, pathogen and environmental framework of *C. plurivora,* which has led to the pathogen's success across western Colorado, is unknown. Thus, it is important to evaluate orchards in western Colorado to determine if *C. plurivora* is able to spread aerially as reported by Luo et al. (2010). Along with water, aerial, and insect dissemination, it is possible that introductions of *C. plurivora* are also occurring through

nursery stock. Infections may be originating in nurseries and are spread when young trees are transplanted into existing orchards or when new orchards are established. Thus, the objectives of this research are to elucidate the life cycle and movement of pathogen inoculum within and across orchards. The primary objectives of this study were to **a)** monitor *C. plurivora* spore production and tree infection rates throughout the year in Grand Junction, Colorado and **b)** evaluate potential dissemination mechanisms through orchards via aerial and insect borer collections, and nursery sampling with the use of molecular marker for *C. plurivora* detection.

MATERIALS AND METHODS

Field inoculations of *C. plurivora.* To determine peach tree susceptibility to *C. plurivora* throughout a twelve-month period, trees were inoculated bi-weekly for twelve months. Studies were conducted in an experimental orchard at the Western Colorado Research Center (WCRC) in Grand Junction, CO, from June 2016 through May 2017. The block was planted in 2013 with 'Cresthaven' peach scions grafted on 'Vicking' rootstock and irrigated with a micro sprinkler system. For a given inoculation time point, five trees with five branches per tree were inoculated. Negative controls included trees that were wounded, but not inoculated. Pruning wounds were cut with standard hand-pruning shears at 45° to assist in precipitation runoff. Following prune wounds, tree branches were inoculated the same day with a 10^5 spores/ml suspension via methods described in Miller et al. (2019). Shoot infections were cut 90 days post inoculation. Necrotic tissue was measured using a digital caliper, and volumes were calculated based on branch diameter and canker length. Koch's postulates were satisfied by plating symptomatic plant tissue on half-strength potato dextrose agar [PDA: 4.0 g potato starch (BD Difco™) media and confirming *C.*

plurivora presence via fungal morphology. Linear regressions were evaluated in Excel (2020) to determine the relationship between lesion volume and temperature/ % relative humidity.

Long-term growth of cankers was also investigated. Inoculations were made November 2017 on trees from hyphal agar disks (4mm in diameter) taken from three-day old cultures. A core borer, sterilized in between trees with a propane torch, was used to imprint the circular 4mm indentation on the outer bark of the tree wood. The outer layer of tissue was peeled back, and the hyphal core was placed on the wounded area. Wounds were then wrapped with Parafilm (American National Can; Chicago, IL, 60631, USA). Two inoculations were made on each tree, one per scaffold. A total of 93 trees were inoculated resulting in 186 total cankers. Canker areas were evaluated twenty months after inoculations. Canker diameters of each sample were measured and fungal growth area (Πr^2) was determined.

Field investigations of *C. plurivora* spore production. To assess year-long spore production by *C. plurivora* cankers, studies were completed at the WCRC from May 2016- May 2017. The orchard was planted with 'Sierra Rich' peach scions irrigated by micro sprinklers. Five trees, containing one *C. plurivora* canker per tree, were selected. Cankers were assessed for spore production twice a month, once every two weeks. Using a funnel and a laboratory wash bottle, cankers were washed with 10 ml of water and effluent was collected in 15ml tubes. Effluent was then centrifuged and concentrated to 5ml. Spore concentration (per ml) was estimated by first agitating the 5 ml effluent and then counting spores via a hemacytometer. The estimated number of spores was extrapolated to determine the number of spores in the original 10ml sample. Linear regressions were evaluated in Excel (2020) to determine the relationship between spores per ml and temperature/ % relative humidity.

Testing for presence of *C. plurivora* on peach nursery stock. Twenty-two different scion/

rootstock combinations from three different nurseries within the United States were evaluated for the presence of *Cytospora* sp. in two stages: 1. Asymptomatically, upon arrival from nursery (pre-potting), and 2. Symptomatically, after trees were potted. In stage 2, trees were assessed for visual symptoms over 3 months (Table 4.1). A total of twenty-five tree replicates of each scion/rootstock combination were evaluated, 10 were sampled directly after shipment and 15 were sampled once potted and drought stressed over 3 months. Upon arrival from the nurseries, sampling for *Cytospora* spp. was conducted by removing three small tissue samples, no larger than 1 cm^3, from the branches and the mainstem of 10 trees per cultivar/rootstock. Samples were sterilized with 10% sodium hypochlorite (Clorox^{tm}; Oakland, CA, 94612, USA) for 5 minutes, rinsed with sterile distilled water, and placed on half-strength potato dextrose agar [PDA: 4.0 g potato starch (BD Difco^{TM}), 19.5 g dextrose (BD Difco^{TM}), 7.5 g agar per liter (BD Difco^{TM})]. Plates were grown in an incubator at 25°C, and subcultures were re-plated on half-strength PDA, via hyphal tips from all samples which appeared morphologically similar to *Cytospora.*

The other set of 15 trees were planted in 56.8-liter containers and placed in an outdoor shade house at Colorado State University, Fort Collins. Potted trees were watered to full capacity 1 time per week April-September. Trees were surveyed once a week for four months to assess for *Cytospora* signs or symptoms, including fruiting bodies, discoloration, gummosis, and/or dieback. No signs were evident throughout the observation period, but discoloration and die-back symptoms were observed on some trees. A total of three hundred and sixty-one symptomatic isolations were made over the 4 months. For plating of symptomatic tissues, pieces no larger than 1 cm^3 were sterilized with 10% sodium hypochlorite (Clorox^{tm}; Oakland, CA, 94612, USA) for 5 minutes, rinsed with sterile distilled water, and placed on half-strength PDA. Plates were grown in an incubator at 25°C, and subcultures were re-plated on half-strength PDA, via hyphal tips from all

samples which appeared morphologically similar to *Cytospora.*

For cultures processed for DNA sequencing, single hyphal tipped cultures were grown in 250 mL flasks containing 50mL sterilized modified V8 juice medium (Leuchtmann 1994). Samples were grown for 7 days on a rotary shaker at 100 rpm. Mycelium was lyophilized and DNA extractions were made using ZR Fungal/Bacterial DNA MiniPreps (Zymo Research Corporation, Irvine,CA), with modifications including a total of 25-35 mg of lyophilized material was added, along with 4-5 3 mm glass beads, to the Bead Lysis Tube. DNA was quantified using a Nanodrop 2000 (ThermoFischer, Inc., Waltham, MA).

The internal transcribed spacer region (ITS) was amplified from all samples. Amplification reaction mixtures (total 25 µL) contained 20-40 ng of template DNA (or no DNA template for negative control), 2.5 µL 10x Standard *Taq* Reaction Buffer (New England BioLabs Inc., Ipswich, MA), 0.5 µL 10mM dNTPs (Roche Applied Science, Penzberg, Germany), 1 µL each of 10 µM primer (Table 4.2) and 0.125 µL *Taq* DNA Polymerase (New England BioLabs Inc.) Amplifications were conducted using the following PCR thermocycling settings: 94°C for 1 min, 35 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 45 s, and final extension step at 72°C for 10 min followed by an indefinite hold at 4°C. PCR amplifications were conducted using a MJ PTC-200 thermocycler (Bio-Rad Laboratories Inc., Hercules, CA). PCR products were run on 1.5% agarose gels with 0.5X TBE buffer and stained with GelRed (Biotium, Fremont, CA). Bands were visualized using UV light to confirm amplification. PCR products were purified using ExoSAP-IT® PCR Product Cleanup (Affymetrix, Santa Clara, CA) following manufactures instructions and were Sanger sequenced at Eurofins (MWG Operon USA, Louisville, KY). Forward and reverse reads from electropherograms were analyzed and edited using Geneious Prime 2019.2.1 (https://www.geneious.com). Sequences were then compared to the National

Center for Biotechnology Information (NCBI) database.

Table 4.1. Peach scion/ rootstock combinations evaluated from nurseries.

Scion/ Rootstock	Nursery	# of Trees
Cresthaven / Krymsk 1	Nursery 1	25
Blushingstar FA18 / Krymsk 1	Nursery 1	25
Reliance / Krymsk 1	Nursery 1	25
Contender / Krymsk 86	Nursery 1	25
Blazingstar / Krymsk 86	Nursery 1	25
PF Lucky 13 / Krymsk 86	Nursery 1	25
Coralstar / Krymsk 86	Nursery 1	25
All-star / Krymsk 86	Nursery 1	25
O'Henry / Krymsk 86	Nursery 1	25
Glohaven / Krymsk 86	Nursery 1	25
Redhaven / Krymsk 86	Nursery 1	25
Flamin' Fury PF17 / Krymsk 86	Nursery 1	25
Glowingstar / Krymsk 86	Nursery 1	25
Flamin' Fury PF 28-007 / Krymsk 86	Nursery 1	25
Angelus / Krymsk 8	Nursery 1	25
Cresthaven / Halford	Nursery 2	25
Glohaven / Halford	Nursery 2	25
Glowingstar / Halford	Nursery 2	25
Starfire / Halford	Nursery 2	25
Redhaven / Halford	Nursery 2	25
Suncrest / Halford	Nursery 2	25
Flamin' Fury PF23 / Lovell	Nursery 3	25

Aerial field spore collections and Chelex DNA extractions. Six orchards were chosen for collection of aerial samples in western Colorado. Three orchards were in organic production systems while the other three were in conventional production systems. Five mature cankers were flagged per orchard and five aerial collections per location were made weekly for ten weeks from 6/6/2019 – 8/13/2019. Collections were made using a rotary vane sampling pump (Zefon International, Ocala, FL, USA) positioned 0.5 meters away from each canker. The pump suction operated at 20 liters per minute, extracting ambient air onto a half-strength potato dextrose agar plate. The pump was run for 5 minutes per each location within an orchard, and a separate agar plate was used for each aerial sample collected. After each collection, the agar plate was sealed with Parafilm (American National Can; Chicago, IL, 60631, USA). Plates were then washed with sterile distilled water within 5 hours of collection, and the plate effluent was collected in a 15 ml tube. Collection tubes were stored at 2.7°C until processed. As a storage control, a 10^5 spores/ml spore suspension of *C. plurivora* was also stored alongside the field collections as a control to ensure that spores did not degrade during the storage period.

Before DNA extractions, collections were pooled by combining the five tubes collected within a specific orchard and collection day. The five, 15ml tubes were centrifuged at 5,000 rpm for five minutes and all but 1ml of supernate was removed. Each tube was then vortexed for 10 seconds and liquid from each of the 5 tubes was combined in a single 15ml tube of pooled effluent. The pooled effluent was then centrifuged at 5,000 rpm for five minutes, and again concentrated to 1ml by removing the supernate. The 1ml sample was vortexed and transferred to a 2ml tube. To dry the sample, the 2ml tube was placed in a Vacufuge Concentrator (Vacufuge Plus, Eppendorf, Hamburg, Germany) at 45°C for 3 hours. Once dry, DNA extractions were conducted by rehydrating with 75uL of 5% Chelex-100 (Chelex 100, Bio-Rad, Hercules, CA, USA). Samples

were then heated on a PCR block (VWR Digital Heat block, Radnor, PA, USA) at 100°C for twenty minutes. Samples were then frozen prior molecular analyses.

Insect field collections and chelex DNA extractions. Insects were collected from the same six orchards described in the aerial sampling. The three borer insects which were targeted for collections included the peach twig borer (PTB) (*Anarsia lineatella*), the metallic wood borer (MWB) (*Chrysobothris mali*), and the greater peach tree borer (GPTB) (*Synanthedon exitiosa*). Sticky traps (Scentry wing traps GL/SC-1309-00; Billings, MO, USA) were used to trap PTBs and GPTBs with species-selective lures (Trece Pherocon, Trece Incorporated, Adair, OK, USA). The MWBs were targeted with purple colored sticky traps (VivaTrap!, Westminister, CO, USA). Within each orchard, three traps (one for each borer type) were set in each of the four corners and in the center of the orchard. Thus, fifteen traps were placed in each orchard and traps were replaced weekly or as needed when insects were found. Collections were made once a week from June to August 2019, for a total of ten weeks. Insects were placed individually into 1.5 ml tubes containing 200 proof ethanol and frozen immediately after capture.

Insect collections were pooled by trap location within an orchard, orchard, and collection date. All species were kept separate. Prior to pooling, each individually collected insect, in its own 1.5ml tube, was vortexed for 20 seconds. The insect was then removed, and the individual sample was centrifuged at 13,000 rpm for 2 minutes. The effluent from collection tubes from the same orchard, same trap location within an orchard, and the same date were then combined into 15ml tubes. The effluent in the 15ml tubes was then concentrated and processed through the vacufuge and Chelex DNA extraction method as described above in the "Aerial field spore collections and Chelex DNA extractions" section.

Molecular detection of *C. plurivora* using a droplet digital PCR assay. DNA from the aerial

and insect collections were first shredded using QIAshredder columns (Qiagen, Hilden, Germany) before being processed. Methods for droplet digital (ddPCR) were conducted as in Stewart et al. (2021). The assay is species-specific to *C. plurivora* DNA and was developed by Stewart et al. (2021). The forward primer, reverse primer, and probe used were the following, respectively: "(5'-CAAGTATCTCGACCGTGA-3')", "(5'-TGGACCTATTTGGCAGAG-3')", and "(5'-FAM-CATCAGACCTTCGTCCAGG-BHQ1-3')" (Stewart et al. 2021). The PCR reaction mix included 10 µL 2x ddPCR SuperMix for Probes (No dUTP) (Bio-Rad Laboratories Inc.), 350nM of each primer, 150nM of probe, 1 µL of DNA, and water to bring the volume to 20 µL. A QX200 Droplet Generator (Bio-Rad Laboratories Inc.) was used to emulsify the 20 µL reaction with 70 µL of Droplet Generation Oil for Probes. A 96 well plate (Eppendorf AG, Hamburg, Germany) was used to contain the droplets. The plate was sealed with foil using a PX1 plate sealer (Bio-Rad Laboratories, Inc.). PCR parameters utilized included: 95°C for 10 min; 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 60 s; followed by an incubation at 98°C for 10 min and an infinite hold at 4°C. After thermocycling (Bio-Rad C100), droplets were read on a QX200 Droplet Reader (Bio-Rad Laboratories, Inc.). Results were analyzed using the BioRad Analysis Pro Software (Bio-Rad Laboratories, Inc.).

RESULTS

Field inoculations of *C. plurivora.* Throughout the 12-month period, lesion volumes fluctuated but growth occurred during the entire year. Lesions grew largest when temperatures were above 10°C during the months of March, April, May, June, July, August, and September (Figure 4.1a). When monthly averaged temperature dropped below 10°C, lesion volumes decreased (Figure 4.1a; Figure 4.1b). There was significant relationship between volume size and

temperature (P = 1.794E-32; Figure 4.1b), and an inverse relationship between temperature and percent relative humidity (R^2 = 0.7383; P = 7.599E-08)(Figure 4.1a). December and January yielded the lowest temperatures with averages of -1.11°C and -0.73°C, respectively. Lesion volumes for the first week of December and the first week of January were the lowest for the entire 12-month study period at 15.93mm^3 and 11.06mm^3, respectively (Figure 4.1a). For the extended growth investigations, after twenty months of growth, the average canker area was 960.01 mm^2 (diameter of 35 mm) with a standard error of 58.99 mm^2 (Figure 4.2). Flagging was not present on any of the inoculated scaffolds after this period.

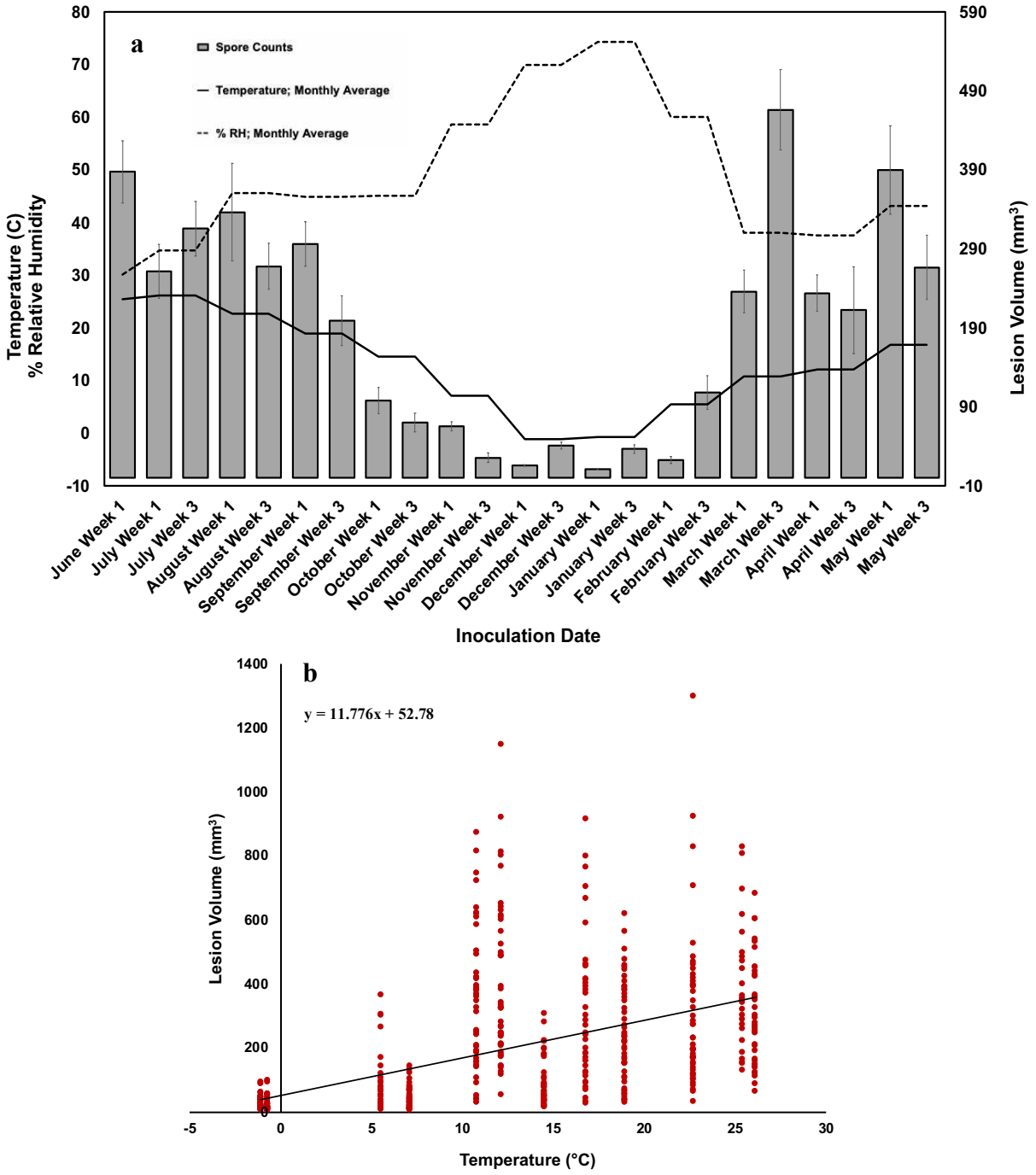


Figure 4.1. Trees inoculated twice per month at the Western Colorado Research Center (WCRC) over a twelve-month period: **a)** Bars represent necrotic tissue volume (mm^3) from *C. plurivora* inoculations. Lesion measurements were made on shoots 90 days after each inoculation time point. Overlayed on the Figure is temperature °C (solid line) and percent relative humidity (dotted line); **b)** Relationship between average temperature °C and lesion volume (mm^3) response of peach trees to *C. plurivora* infection throughout the 12-month study period (P = 1.794E-32).

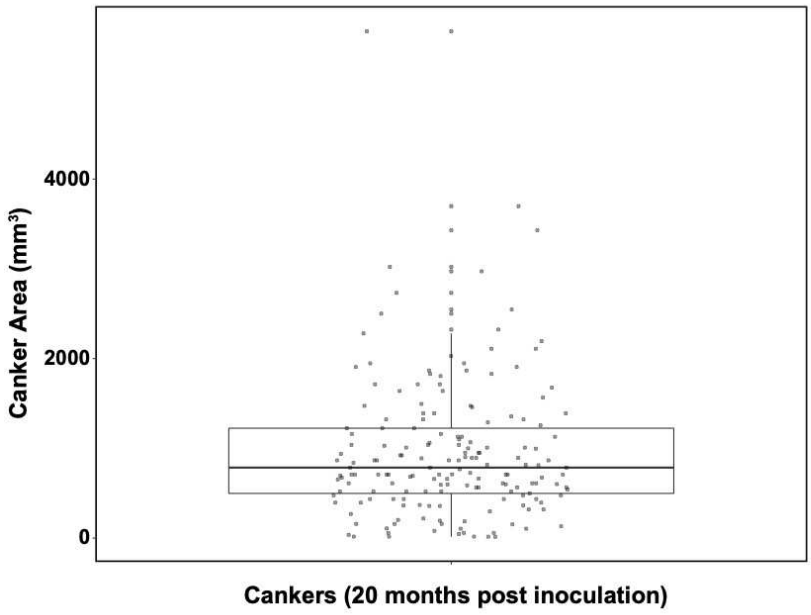
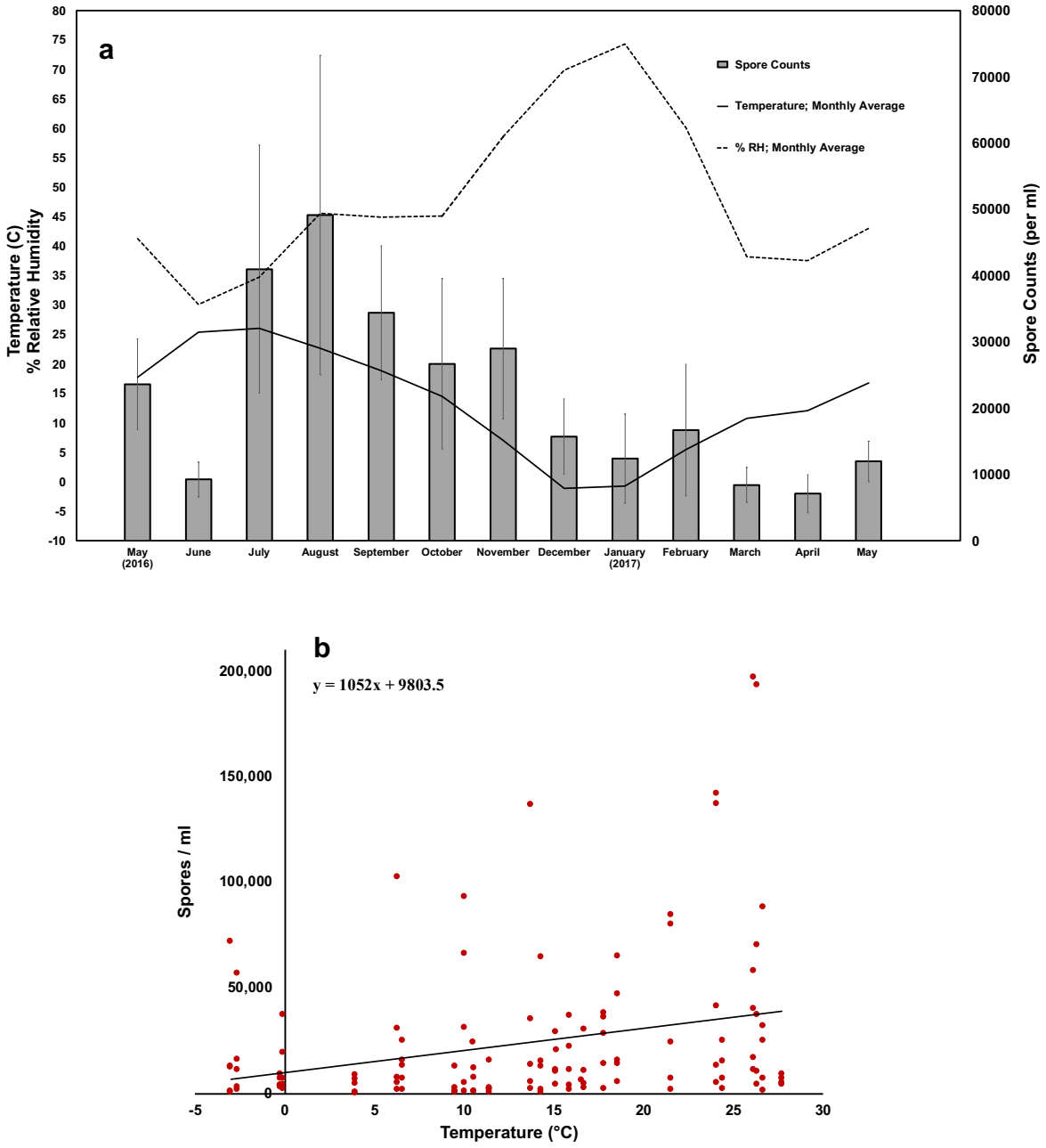


Figure 4.2. Canker area (mm^2) twenty-months after inoculation with *Cytospora plurivora* on 'Cresthaven' peach scions grafted on 'Vicking' rootstock trees in Grand Junction, Colorado. A total of 93 trees were inoculated, with a total of 186 cankers. Canker diameters (Πr^2) of each sample were determined.

Field investigations of *C. plurivora* spore production. Spores were observed in collections from all cankers throughout the 12-month period from May 2016 to May 2017 (Figure 4.3a). Spore count averages ranged from 7,100 spores/ml to 49,140 spores/ml throughout the year. When temperatures reached the minimum average temperature of -0.733°C in January 2017, an average of 12,393 spores/ml were still produced. There was significant relationship between spore counts per ml and temperature (P = 0.00250081; Figure 4.3b). The highest recorded spore production values occurred when temperatures were highest (Figure 4.3b). Further, highest spore count averages were recorded when both temperature and % relative humidity were highest in the months of July (26.05°C; 34.63% RH) and August (22.70°C; 45.60% RH) (41,000 spores/ml and 49,140 spores/ml, respectively) (Figure 4.3a). Lowest spore counts were recorded in the months of March (10.77°C; 38.19% RH) and April (12.11°C; 37.56% RH) with 8,415 spores/ml and 7,100 spores/ml, respectively.



Figures 4.3. Tree canker effluent collected and evaluated from five trees at the Western Colorado Research Center from May 2016 – May 2017: **a)** Bars represent averaged counts (spores/ml) from *C. plurivora* cankers. Overlayed on the Figure is average monthly temperature °C (solid line) and average monthly percent relative humidity (dotted); **b)** Relationship between spore counts (spores/ml) and temperature (°C) throughout the 12-month study period (P = 0.0025).

Testing for presence of *C. plurivora* on peach nursery stock. As mentioned, trees from nurseries were evaluated in two steps: 1. upon arrival from nursery (pre-potting), and 2. after trees were potted and evaluated for symptoms. In the first phase, 562 plates were evaluated for *Cytospora* growth. No evidence of *Cytospora* spp. could be confirmed. In the second phase over the four months, 361 symptomatic tissues were plated. Symptoms of interest included gummosis or twig die back. No *Cytospora* signs such as fruiting bodies were observed at any point and all 361 plates yielded no evidence of *Cytospora* spp. growth. Of all the plates evaluated, seventeen isolates were morphologically of interest and BLAST identities are listed in Table 4.2.

Table 4.2. BLAST identity of isolates extracted from nursery tree samples.

Sample	Blast identity	Blast identity; e-value	Cultivar
1	*Botrytis cinerea*	99%; 0.0	Redhaven
2	*Epicoccum nigrum*	100%; 0.0	Suncrest
4	*Rhizoctonia* sp.	99%; 0.0	Glohaven
8	*Phoma* sp.	100%; 0.0	Glowingstar
9	*Phoma* sp.	100%; 0.0	Flamin' Fury PF23
10	*Botrytis cinerea*	100%; 0.0	Glowingstar
11	*Alternaria infectoria*	99%; 0.0	Flamin' Fury PF23
12	*Alternaria* sp.	99%; 0.0	Flamin' Fury PF23
13	*Alternaria arborescens*	100%; 0.0	Flamin' Fury PF23
15	*Epicoccum nigrum*	100%; 0.0	Suncrest
16	*Rhizoctonia alpina*	100%; 0.0	Glohaven
17	*Alternaria* sp.	100% 0.0	Crest Haven

Insect and aerial collections with ddPCR results. Throughout the season a total of 2,628 PTBs, 119 GPTBs, and 170 MWBs were collected. Of these collections, organic production system locations had the highest concentrations of PTBs and MWBs, with 2,405 PTBs and 159 MWBs while locations in conventional systems only had 223 PTBs and 11 MWBs. Given the effective mating disruption pheromone control method for the GPTB, all locations using such disruptors had 0 collection counts of GPTBs. GPTBs were only trapped in one location where mating disruption pheromones were not utilized due to small orchard size. One-hundred and sixty-nine total ddPCR reactions were run after samples were pooled by insect species, date, orchard, and location within an orchard. Only 27 total samples yielded amplification of target DNA, but in low concentrations. Of the 169 total ddPCR reactions run, 119 reactions consisted of PTB samples, 31 consisted of MWB samples, and 19 consisted of GPTB samples. For the pooled PTB samples, 17.6% (21 out of the 119 total reactions) yielded positive amplification of target DNA (Figure 4.4). Number of pooled PTB insects per reaction ranged from 6 to 10. Concentration of PTB target amplification/ul ranged from 0.0603 copies/ul to 0.484 copies/ul with an average of 0.152 copies/ul (Figure 4.4). For the pooled MWB samples, 12.9% (4 out of 31 total reactions) yielded positive amplification of target DNA. The highest sample yielded 0.214 copies/ul and the lowest sample yielded 0.0705 copies/ ul (Figure 4.5). Number of pooled MWB insects per reaction ranged from 2 to 17. For the pooled GPTB samples, 10.5% (2 out of 19 total reactions) yielded positive target DNA amplification with values of 0.088 and 0.084 copies/ul (Figure 4.5). Number of pooled GPTBs per reaction ranged from 2 to 18.

For the aerial field samples, a total of 64 samples were tested with ddPCR assays after pooling the collection by date and orchard location. Of the 64 total samples, 42.1% (27 out of 64 total reactions) yielded positive target amplification, but again in low concentrations (Figure 4.6). The

highest concentration of target DNA yielded 0.711 copies/ul while the lowest concentration yielded 0.0605 copies/ul (Figure 4.6).

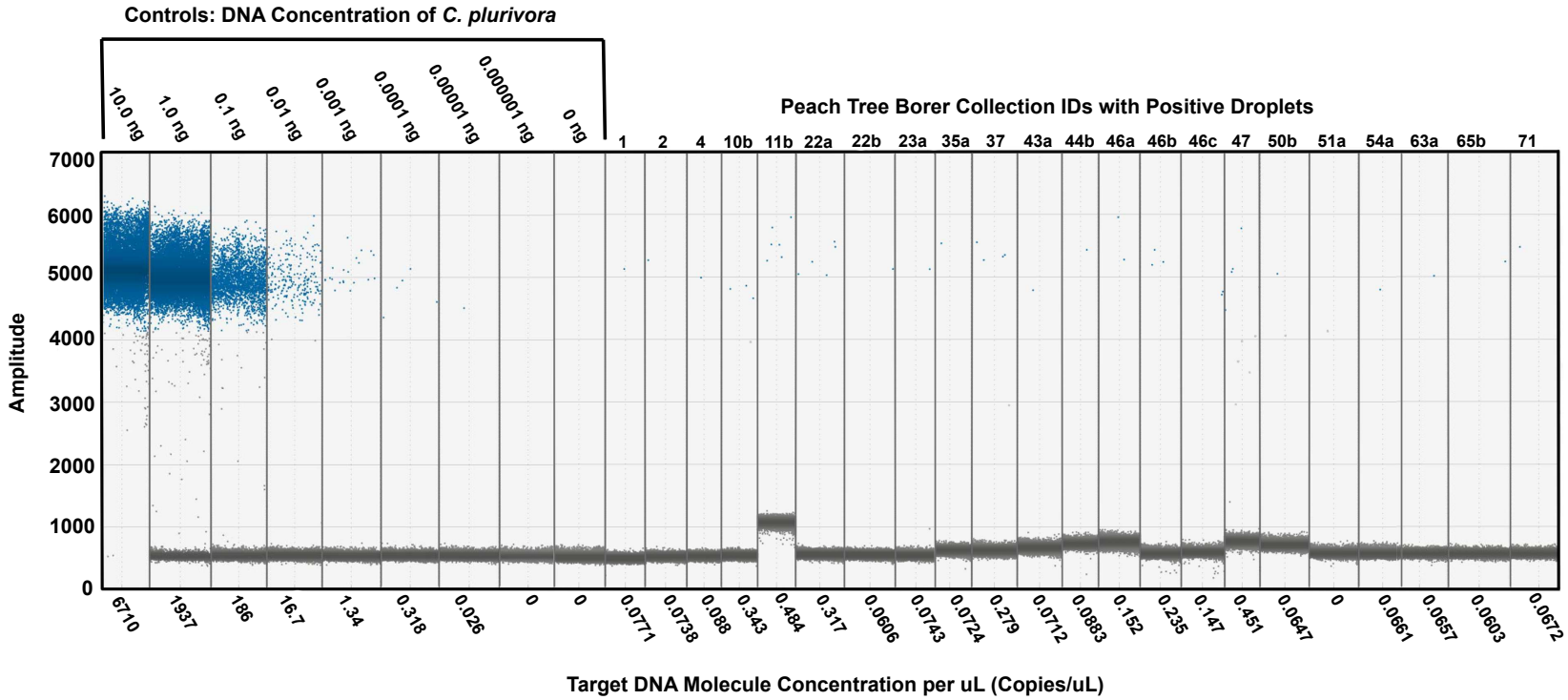


Figure 4.4. Peach twig borer (*Anarsia lineatella*) samples which yielded positive amplification using ddPCR. Blue dots are positive droplets, whereas grey dots are negative for detection. Positive controls (bracketed) include a serial dilution of *C. plurivora* DNA concentrations. Values are represented on the X-axis as number of target concentration (copies/ul).

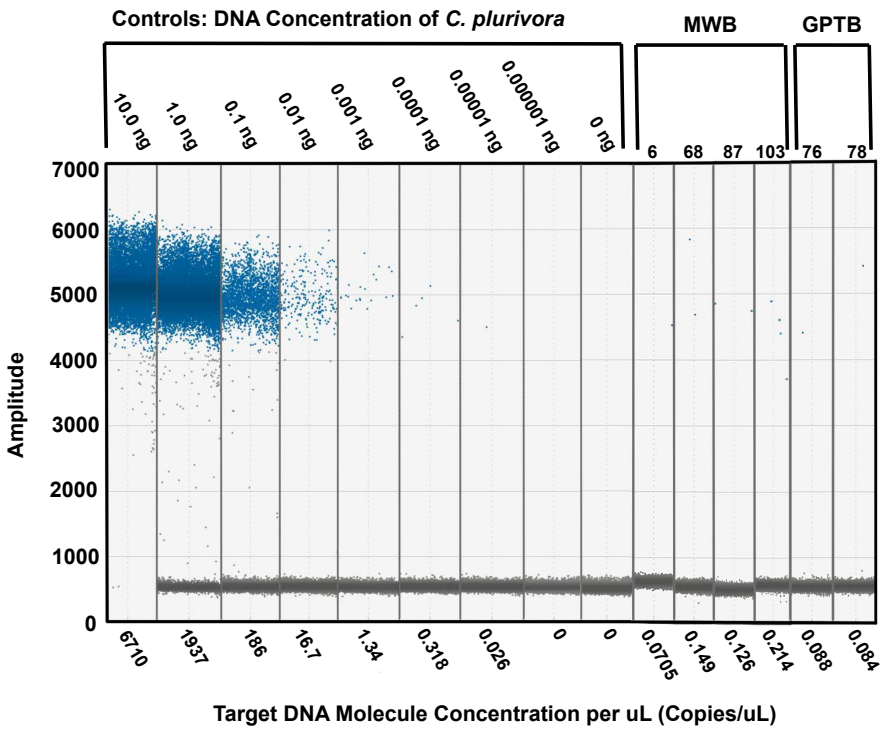


Figure 4.5. Metallic wood borer (*Chrysobothris mali*) and greater peach tree borer (*Synanthedon exitiosa*) samples which yielded positive amplification using ddPCR. Blue dots are positive droplets, whereas grey dots are negative for detection. Positive controls (bracketed) include a serial dilution of *C. plurivora* DNA concentrations. Values are represented on the X-axis as number of target concentration (Copies/ul).

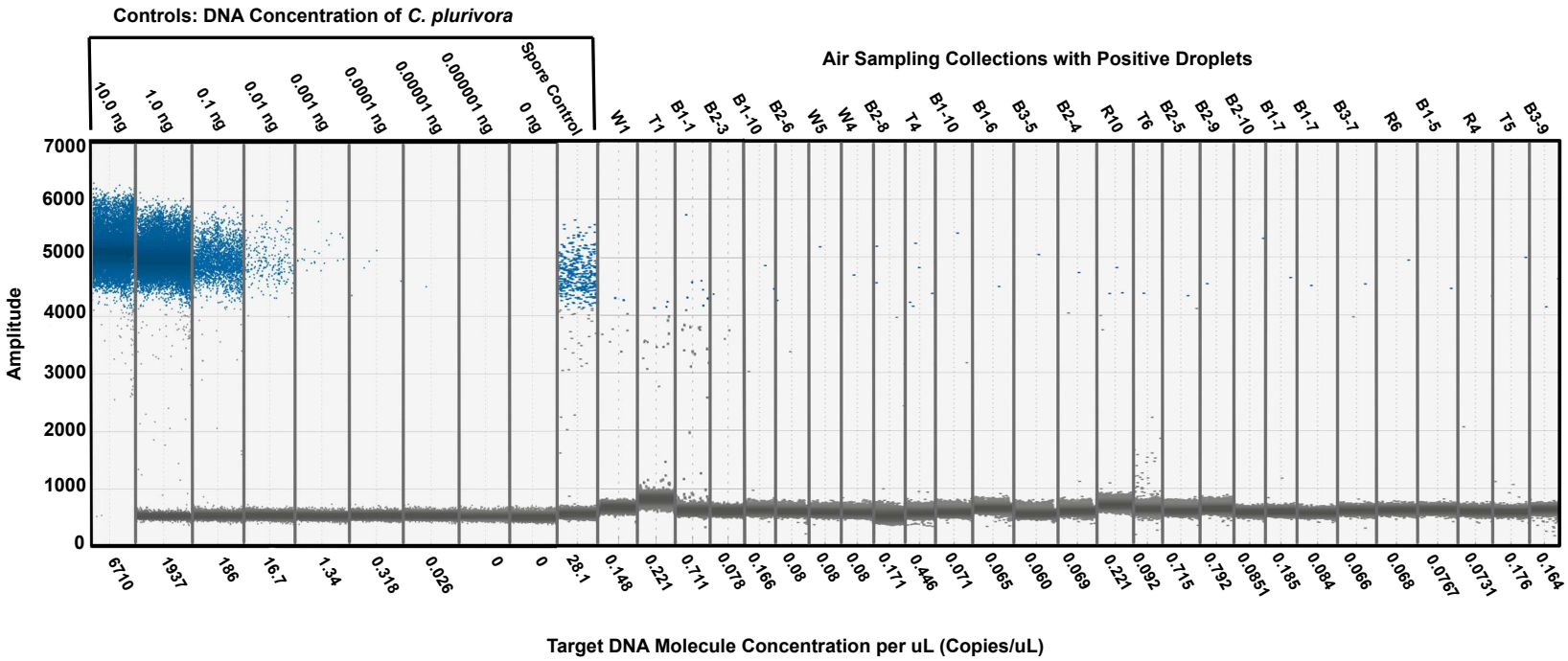


Figure 4.6. Aerial samples which yielded positive amplification using ddPCR. Blue dots are positive droplets, whereas grey dots are negative for detection. Positive controls (bracketed) include a serial dilution of *C. plurivora* DNA concentrations. The spore control consists of a spore suspension which was stored the same time as field collections to ensure spore and DNA degradation did not occur. Values are represented on the X-axis as number of target concentration (copies/ul).

DISCUSSION

Through the year-long study, it is evident that spore production and infections of *C. plurivora* are capable all twelve months in peach orchards; Artificial inoculations and spore production by cankers were viable every single month. In terms of spore production, similar results have been documented in Washington State for *Cytospora* spp., with the highest concentrations occurring in spring and summer months (Grove and Biggs 2006). In China, investigations of *Cytospora mali* in apple orchards also reported year-round spore production (Wang et al. 2016). These reports, and the presented evidence, suggest that peach trees are vulnerable to infections throughout the year despite differences in monthly field inoculum loads. Differences in spore loads and in monthly pathogen infection rates are primarily driven by seasonal temperatures with favorable temperatures aligning with the irrigation season. As temperature increases, canker lesion size and spore production increases, both variables had a significant relationship to temperature. Given the day- and night- temperature fluctuations, there is no month in western Colorado that is either too hot or cold for growth or spore production to completely halt. Laboratory growth assays have shown that *C. plurivora* slows and nearly halts in growth at around 10°C and 37°C (Stewart et al. 2021), but studies on other *Cytospora* species have shown capable growth in the laboratory at temperatures as low as 3°C (Helton and Konicek 1962). Further, in field trials it was evident that canker lesion growth is extremely variable over a twenty-month period, and thus it is difficult to estimate the age of a mature canker. Differences in day and night temperatures allow for continual growth thus, trees are vulnerable year-round, despite months when temporary temperature extremes may be comparable to extreme temperatures tested in laboratory growth trials.

Though spore production and infections occurred throughout the study period, lesion size and spore counts were at a minimum during the dormant season. Similarly, studies conducted in

grapevines in California found that vines pruned during the dormant seasons were less susceptible to Botryosphaeria canker and *Eutypa lata* though infections were still able to occur (Urbez-Torres et al. 2011; Munkvold & Marois 1995). Similarly, our data also suggests the risk of infection is lowest during the dormant season, but there are times in the winter months when spore production is still high, despite colder averages. For existing stem cankers, for which growers are unwilling to remove, we suggest using fungicide/ sealant combinations as covers for the cankers to decrease the inoculum during pruning. Previous studies have shown suppressive canker covers such as latex and captan can reduce spore dissemination on mature cankers for a limited time (Miller 2017). Treating wounded and/or pruned tissues preventively with fungicides will decrease the likelihood of infection occurring during pruning.

The results of this study show that water-rinses from cankers yielded much higher spore concentrations than other potential dissemination mechanisms, but that aerial and insect dissemination are possible. Even though spores may be moved aerially, water dissemination such as rainwater/splash or irrigation splash are likely the primary dispersal method. A similar study conducted by Luo et al. (2020) also reported the importance of rainwater for spread of *Cytospora* spp. spores in walnut orchards in California. Using a real-time species-specific PCR assay, researchers found higher rates of spores in water samples when compared to aerial samples, but they were also able to detect *Cytospora* sp. spores in air collections. Our results confirm previous work that has shown that *Cytospora* spores are primarily disseminated through water (Grove & Biggs 2006), and Bertrand & English (1976), but these studies also reported no evidence of aerial dissemination. In contrast, however, with the use of molecular tools, we provide evidence that spores are disseminated aerially in western Colorado, albeit in low concentrations. New highly

sensitive molecular tools such as real-time PCR and ddPCR enable detection of dispersal methods not previously characterized.

We also detected *C. plurivora* in insect washes of the peach twig borer (PTB) (*Anarsia lineatella*), the metallic wood borer (MWB) (*Chrysobothris mali*), and the greater peach tree borer (GPTB) (*Synanthedon exitiosa*), though again at low target amplification rates. Our data suggest that these insects are not vectors, but may be able to disseminate spores, as they feed and breed within infected trees. Swift (1986) established a relationship between Cytospora cankers and high incidences of the lesser peach tree borer (LPTB) suggesting that *Cytospora* infections could provide a more suitable environment for LPTB egg deposition and larval entry. It is likely that borers are capable of dispersing spores, thus causing new infections but are not true vectors nor are a strong source spore movement.

Another hypothesis for the introduction and/movement of *Cytospora* spp. into orchards in western Colorado is through asymptomatic nursery stock. Recent studies evaluating the diversity of latent pathogens and endophytes in Uruguay reported that *Cytospora* species may potentially function as endophytes (Sessa et al. 2018). Further, Adams et al. (2006) suggested that *Cytospora* spp. may be latent pathogens whereby asymptomatic, endophytic infections predominantly exist in the xylem of *Eucalyptus* spp. trees in Uruguay and South Africa (Smith et al. 1996; Bettucci & Saravay 1993). Studies by Ke et al. (2013) have shown that *C. mali* can be present in host tissues far beyond canker margins and can exist in xylem tissues for extended periods of time. *Cytospora* could be surviving within tissues of young trees from nurseries then becoming symptomatic and spreading only after trees are planted within fields. Nonetheless, after intensive sampling of 330 peach trees from 3 different nurseries, we could not detect *Cytospora* infections either symptomatically or asymptomatically. However, it is possible that the latency period is longer and

more sustained than we accounted for in the sampling time frame, if *Cytospora* species are functioning as endophytes. It may require more sustained field stress for symptoms to be expressed on trees. Future studies should evaluate trees through an entire dormancy period as abiotic stressors. It is also possible that introductions are not occurring from nurseries, but more studies should be conducted. Our research shows that there is no evidence of introduction from nurseries.

CONCLUSION

This research provides an outline of *C. plurivora* sporulation and tree susceptibility throughout the year in western Colorado. Given that incidences of high sporulation were found year-round and that infections were capable of occurring throughout the year, it is recommended that growers prune during dormant seasons. Temperature was correlated positively with pathogen infection, canker lesion size and sporulation. Thus, conducting cultural practices such as pruning during times of decreased temperatures is suggested. This mirrors recommendations made in China to reduce the risk of *C. mali* infections, which suggest pruning in the latter portions of the dormant season (Wang et al. 2016). Such cultural practices, in combination with preventive and suppressive fungicidal applications (Biggs et al. 1994; Froelich & Schnabel 2019; Miller 2017; Miller et al. 2019; 2021), will reduce the risk of infection.

This study further provides evidence that dissemination of *C. plurivora* beyond rain-splash is possible, though spore counts from aerial and insect samples was low when compared to the high concentrations of spore counts which were found from canker-wash effluent. However, we did not capture spores during rain events directly. Future studies should investigate spore production during rain events with the use of the highly sensitive ddPCR marker. Aerial collections during rain events would likely yield higher *C. plurivora* DNA concentrations given the increased chance

of water splash, and such events may be a heightened time-period for spore dispersal and infection. Further, studies should also evaluate differences among irrigation systems and microclimates within orchards during irrigation and growing seasons. Finally, a larger variation of insects can be investigated as the results herein have shown dissemination by insects possible, although only borers were investigated.

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CHAPTER V. **Conclusions and Recommendations**

CONCLUSION

This dissertation offers a two-fold analysis of the *Cytospora plurivora* pathosystem in western Colorado by exploring both management and epidemiology. It presents the host-pathogen-environmental framework which has led to the success of *C. plurivora* in western Colorado, and it identifies necessary cultural and chemical control methods which can mitigate future economic losses in fruit production.

***C. plurivora* management** This research has analyzed management options for *C. plurivora* from chemical and cultural control perspectives. In terms of chemical control, preventive applications of fungicides and sealants are most successful (Miller 2017; Miller et al. 2019; 2021). Thiophanate-methyl alone and in combinations with latex were validated as most effective, not only by evaluating lesion sizes in field trials, but by also evaluating the percent viability of spores on branches after chemical exposure (Chapter II). Further, results showed the ineffectiveness of latex sealants when applied without fungicides. Previous research failed to take into consideration spore viability, and considered latex treatments effective, as latex reduces lesion size, but results herein show latex does not decrease spore viability (Chapter II). Interestingly, VitiSeal was shown to serve as a potential alternative to latex as a wound sealant treatment since thiophanate-methyl combined with VitiSeal and lime sulfur combined with VitiSeal were effective in reducing lesion sizes and spore viability (Chapter II).

In terms of cultural control methods, tree vigor should be maintained by reducing field stress. Adequate water distribution and quality should be a priority. Avoiding poor water sources and/or tree water deficit reduces the possibility of increased *C. plurivora* infections. Trees experiencing heightened pH stress and water stress, had increased lesion sizes by the pathogen in trials when

compared to trees not experiencing such stressors (Chapter III). Poor quality irrigation sources can contribute to increased salinity and sodicity in soils, thus growers should regularly monitor pH levels in both irrigation water and soils. Further, pruning of trees should be conducted during the dormant season as sporulation and pathogen lesion sizes have shown to be lowest during these times (Chapter IV), which is also the recommended treatment for *C. mali* on apple (Wang et al. 2016). Often times, in field scenarios, growers are unwilling to remove cankers which are located on the main stem due to economic constraints. The research presented has shown that such cankers may still sporulate during the coldest months of the year, risking infection even during dormant pruning periods (Chapter IV). Thus, in such circumstances, growers may suppressively apply fungicides as described in Miller (2017). While Miller (2017) found mixed results on suppressive measures, there was a trend of decreased sporulation after the first month of latex and thiophanate methyl applications as well as latex and captan applications when compared to non-treated cankers.

***C. plurivora* epidemiology.** The research presented herein provides an overall perspective of how the *C. plurivora* life cycle fluctuates throughout the year. It is evident that the pathogen can grow year-round, producing asexual spores and capable of causing infections in the most extreme seasonal temperatures (Chapter IV). During the dormant season, pathogen growth rates and sporulation rates were decreased, but not disabled by temperature, as typically reported in laboratory assays (Chapter IV). Nonetheless, temperature was shown to have a positive relationship with sporulation and lesion size and an inverse relationship with percent relative humidity (Chapter IV). Further, it is evident that several host-environmental conditions have direct implications for tree susceptibility to *C. plurivora* infection. Drought stress and high pH were shown to directly influence the severity of infections. When trees are subjected to either stress

condition, their overall water potential decreases and pathogen fitness increases (Chapter III). These results fit well with the traditional description of Cytospora canker species in fruit systems as weak or secondary pathogens (Biggs 1989).

Historically, Cytospora canker spores have been thought to primarily be vectored through water dissemination with no evidence of spores capable of being produced aerially (Bertrand & English 1976; Grove & Biggs 2006). New, highly sensitive molecular tools such as ddPCR now allow for increased detection of specific target DNA. The results herein have shown that *C. plurivora* DNA can be detected aerially, albeit in low concentrations (Chapter IV). Thus, aerial dissemination is likely not the primary method of dispersal but is possible. Similarly, recent studies have also determined that it is possible to detect *Cytospora* spp. spores aerially (Luo et al. 2020). Further, *C. plurivora* was detected on peach twig borers, metallic wood borers, and greater peach tree borers in field trials (Chapter IV). As detection was in low concentrations on insects, it is likely that borers do not vector *C. plurivora* spores, but dissemination may be possible. Nonetheless, in accordance with known literature, the studies presented herein confirm that spore concentrations are highest when collected as water effluent from cankers, suggesting water dissemination to be the primary method of dispersal (Chapter IV).

Future Research. The presented studies have raised new research questions which should be explored. It is evident that an analysis of rootstock with cultivar held constant, should be conducted. Understanding the influence of rootstock on tolerance to pH stress, water deficit stress, and cold damage is essential to developing vigorous cultivars which may show decreased severity of Cytospora infections. Given the high pH soils of western Colorado and the presented relationship between pH stress and *C. plurivora* infection severity, high pH tolerant rootstock/ scion combinations should be incorporated in breeding programs. Further, a suite of different

rootstocks should be evaluated for sensitivity to water deficit after inoculated with *C. plurivora*. Such studies can help solidify cultural control methods in areas of increased pathogen presence. Regarding research of chemical controls, future studies should evaluate chemistries available for organic production systems. With the exception of lime sulfur, the majority of effective fungicides, including the ones presented herein, are only registered for conventional production systems. Further, known literature has shown biocontrol agents as potentially effective against *Cytospora* spp. and should be explored in more detail for *C. plurivora* (Gao et al. 2000; Yi & Chi 2011; Xiang et al. 1991; Zhang et al. 2015).

Given the low detection of *C. plurivora* DNA through aerial and insect samples, future studies should further evaluate these disseminations methods. Further, collections made during precipitation events should also be explored with the recently developed ddPCR primers as a comparison to the results reported herein (Stewart et al. 2021). The question of whether different seasons or different irrigation techniques spread *C. plurivora* at different densities should be studied, especially since collection of water effluent yields high spore concentrations (Chapter IV). By using molecular assays, field inoculum load can be more accurately detected under different conditions. Further, within an orchard, different microclimates may be influencing spore production on a daily basis. Such microclimate differences may be influenced not only by irrigation mechanisms, but by surrounding vegetative growth, mulch density, or geographical location of trees. Finally, a larger sampling of different insects may yield species which are able to disseminate larger concentrations of spores. Thus, future studies should evaluate insects which are at high densities within orchards, as different factors may enable certain species to carry higher concentrations of *C. plurivora*.

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