THESIS

# THE ROLE OF CHEMICAL CANOPY SPRAYS AND IRRIGATION METHODS ON THE INCIDENCE OF THE PERENNIAL CANKER, *CYTOSPORA PLURIVORA* IN WESTERN COLORADO PEACH ORCHARDS

Submitted by

Sean Wright

Department of Agricultural Biology

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2022

Master's Committee:

Advisor: Jane E. Stewart

Amy Charkowski
Mark Uchanski

ABSTRACT

THE ROLE OF CHEMICAL CANOPY SPRAYS AND IRRIGATION METHODS ON THE INCIDENCE OF THE PERENNIAL CANKER, *CYTOSPORA PLURIVORA* IN WESTERN COLORADO PEACH ORCHARDS

*Cytospora plurivora* is a secondary pathogen that has reached near epidemic levels in peach orchards on the western slope of Colorado. *C. plurivora* is responsible for Cytospora canker disease and is a limiting factor in peach production in the Grand Valley. Peach growers have limited management methods available to combat this disease, which prompted investigation into irrigation practices as well as prophylactic chemical sprays following freeze events. In late 2020, the western slope received a freeze event that caused severe damage to peach shoots, buds and twigs. Freeze damage provides infection courts within tree tissues that *C. plurivora* can infect. This freeze event prompted growers to apply prophylactic chemical sprays of Captan, lime sulfur, and lime sulfur with the addition of NuFilm. An efficacy threshold of three-months post chemical spray was determined for both Captan and lime sulfur treatments. Lime sulfur with the addition of NuFilm showed a loss of efficacy at two-months post spray. Additionally, an investigation into the movement of *C. plurivora* conidia under differing irrigation techniques was conducted. Both drip and micro-sprinkler treatments had positive detections for *C. plurivora* over the course of the study. In these studies, conidia traveled much greater distances than previously shown, traveling up to 135m from the closest canker. Understanding how chemical canopy sprays and different watering practices affect the incidence of Cytospora canker disease will assist in preserving the peach industry on the western slope of Colorado.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my wife Shana for her unwavering love and support through this entire process. Without her patience and positivity, I would not be where I am today. My son Zander has been an amazing motivator and has challenged me to secure a better life for my family. Watching you grow through my time in academia has been amazing.

Dr. Jane Stewart has helped me to grow as a person and a scientist. Thank you for setting me on the right path towards my career and I am forever grateful for the opportunity you provided. In hindsight, I recognize the larger picture that you had planned and slowly opened my eyes to it. Dr. Jorge Ibarra has provided endless hours of mentorship and assistance throughout my degree. His ingenuity and intelligence pulled me through many trials during my studies. The whole Stewart lab has been amazing throughout this journey and have assisted me at various stages through my degree, thank you for everything. A special thanks to everyone in the Stewart lab who has put their time into developing me and has assisted in my research. Dr. Amy Charkowski and Dr. Mark Uchanski, thank you for providing insight on my project and serving as members on my committee. Dr. Luke Tembrock and Frida Zink taught me new skills and served as a sounding board for ideas surrounding the ddPCR project. Thank you both for your time and expertise. Dr. Ann Hess has been vital in my development and understanding with statistics. Your assistance with this project has been a blessing. Thank you!

Many people on the western slope were vital in my field work. I would like to thank Greg Litus, Brad Tonnessen, Emily Dowdy, Jeff Pieper, and Bryan Braddy for making this research possible through your hard work. To all of the growers that allowed me on their land

to conduct these experiments, thank you. I would also like to thank the Colorado Department of Agriculture and the Mesa County for the funding to conduct this research.

TABLE OF CONTENTS

# CHAPTER 1: LITERATURE REVIEW

## 1.1 A history of food production

Humans relationship with food is unlike any other animal on the planet. The advent of agricultural systems revolutionized the way we grow, consume, and live. Access to a reliable food supply was the driver of our current civilization. Cereal crops have been a long-chosen staple of domestication, but new interest is arising in the history of human interactions with fruit crops. The origins of fruit crops are still debated, but domesticated pome fruit production has been observed as early as the Neolithic period 7000 – 1700 BCE in the Mediterranean (Zohary and Spiegel-Roy, 1975). East and Central Asia began domestication of stone fruits including peach, plum, and almonds, during the middle Holocene (Zheng et al., 2014). The advent of these agricultural systems brought with them the ability for pathogens to develop and thrive. The resulting loss of productivity prompted humans to secure food infrastructure through the development of management tools that combat increased disease in food production systems.

Historically, management practices have assisted in facilitating optimal growth conditions for fungal plant pathogens in orchard settings. Irrigation, high density planting, and monoculture have all led to an increase in pathogen pressure (Corredor-Moreno & Saunders, 2020). In orchards, chemical fungicide application is a staple of disease management, but it has led to the development of resistance in fungal pathogens, such as *Venturia inaequalis* in apples and *Monilinia fruticola* in stone fruits (Ma et al., 2003; Pfeufer & Ngugi, 2012). Standardized irrigation practices increased the productivity and yield in fruit bearing orchards, however, the

addition of water also raises ambient humidity and assists in the dissemination of fungal pathogen spores (Grove & Biggs, 2006). As the consumer base grew, so did the desire for high yield outputs, requiring vigorous pruning and high-density plantings to meet demand. Unfortunately, the need to boost productivity through pruning and training also came at a cost, as each cut made infection courts in trees, promoted fungal spread and allowed pathogens to persist in virtually all orchard settings.

Stone fruits are particularly susceptible to many fungal pathogen species that infect the fruit, shoots, and woody tissues of the tree. One of the most common and detrimental fungal pathogens of peaches (*Prunus persica*) is *Cytospora,* the causal agent of Cytospora canker. Without proper investigation into the management practices surrounding this pathogen our long history with peach production may come to an end.

### 1.2 *C. plurivora* and the economics of peach production in Colorado

Peach production has a long and storied history in the state of Colorado. The first peaches were planted in Palisades and Grand Junction, CO in 1882-1883 (Sexton., 1996). Since that time, Colorado's peach industry has grown to be its most profitable fruit crop, bringing in tens of millions of dollars annually (*USDA ERS - Fruit & Tree Nuts*, 2021). Unfortunately, the Colorado peach industry faces increasing hardships due to changing climate, unpredictable frosts, and heavy calcareous soils, all of which increase the susceptibility of trees to pathogens due to a physiological stress response in trees (Miller et al., 2021). Peach crops are susceptible to Cytospora canker infection globally, however Colorado's unique growing conditions cause *C. plurivora* to be the limiting factor in peach production in Colorado (Miller, 2021). The

prevalence of Cytospora canker in the Grand Valley is staggering, with 100% of surveyed orchards having symptomatic trees (LaFantasie et al., 2015). An average of 75% of all trees within an orchard showed symptoms of infection (Mesa County Extension, 2015). These findings were echoed in Ontario, Canada where 98% of the 2000 trees surveyed were symptomatic (Biggs, 1989a). Orchards with Cytospora canker can quickly reach epidemic levels of tree mortality, losing between 3 - 6% of established trees annually (Grove & Biggs, 2006). The pressure of Cytospora canker, mixed with an unprecedented early frost event in the Grand valley, took an economic toll with the annual utilized peach production dropping from 62 to 11.75 million dollars between 2019 and 2020 (Meyer, 2021). The Mesa County Emergency Board for the Farm Service Agency estimated a loss of 80% of the peach crop during this freeze event. This decline is not due solely to Cytospora canker, but is also attributed to severe weather with an early frost occurring in 2020 before tree dormancy, as well as a late spring frost leading to blossom mortality. These two events caused extreme tree damage and mortality, which in turn exacerbated the already epidemic levels of Cytospora canker in western Colorado peach orchards.

### 1.3 *Cytospora*: one fungus = one name

Many Ascomycete fungi live complicated pleomorphic life cycles including organisms which cause Cytospora canker disease. Traditional classification of these fungi split the asexual and sexual morphs into separate taxonomic groups, giving them each unique names. This in turn has caused confusion and ambiguity when referring to these organisms in literature. A push within the scientific community has been to combine the anamorph and teleomorphic stages as one name through the One Fungus = One Name campaign. With the ability to classify

fungi based on molecular tools including polymerase chain reaction (PCR) these relationships can be resolved (Taylor, 2011).

Species within the *Cytospora, Leucostoma, Valsa, Leucocytospora, Valsella, and Valseutypella* have been proposed as one such group to be combined under the One Fungus = One Name campaign. *Cytospora* is the oldest recorded genus for this organism originating from Ehrenb., Sylv. mycol. berol.: 2 (1818): Fr., Syst. Mycol. 2: 540 (1823); designated type species: C. chrysosperma Pers. (1818) (Rossman et al, 2015). This thesis will use the nomenclature from each study referenced however it is important to note that that all associated genera have been collapsed into the genus *Cytospora*.

**1.4 Pathogen Biology**

Canker diseases of tree fruit are predominantly caused by species within the phylum Ascomycota, as well as by a handful of bacterial phyla. Fungal plant pathogens enter host tissues via different mechanisms, such as production of rhizomorphs or haustoria, insect vectoring, or simple infection courts. Infection courts are an extremely common mode of entry for fungal tree pathogens. Damage that leads to infection courts are common in orchard settings and vary wildly in in location, type, and severity. Pruning, cold/freeze events, mechanical damage, and abiotic stressors can initiate the formation of these infection courts.

Once a fungal spore contacts an infection court, germination occurs, and infection begins. Infections occur within the bark and vascular tissue of trees including shoots, branches, and trunks. Common symptoms of early canker infection include discolored bark, sunken areas, gummosis, wilting and localized necrosis. As infection severity progresses, branch flagging,

defoliation, flaking bark, and branch death are common symptoms. Branch death occurs through plugging of the xylem vessels which affects the translocation of water. Tree health and vitality are diminished when little to no water can pass through infected tissues. Younger trees are more susceptible to drought induced mortality than older trees (Agrios, 2005). Cankers eventually develop and within two years fungal fruiting structures may form (Grove & Biggs, 2006). These signs and symptoms are extremely common among plant pathogens, with all occurring during Cytospora canker infection in Colorado peach orchards.

Cytospora canker infections are strongly associated with frost cracking, early and late freeze events, fluctuating winter temperatures, and mechanical damage. *Cytospora* species have a worldwide distribution and infect over 100 species of woody plants, including deciduous trees, conifers, and shrubs (Bergdahl, 2016; Sinclair, 2005; Zhu et al., 2020). Cytospora canker is commonly seen in orchards, including apple (*Malus domestica*), cherry (*Prunus avium*), and peaches (*P. persica*). *Cytospora plurivora* is the most common species causing disease in western Colorado peach orchards (Stewart et al., 2022). *C. plurivora* can live as either saprophyte or necrotroph. Even though *C. plurivora* is characterized as a weak, secondary pathogen, requiring infection courts to gain entry into its host, it is still the main driver in peach mortality on the Western slope of Colorado ( Biggs, 1989a; Miller, 2021).

Cytospora canker can be initiated by both the sexual and asexual (ascospore and conidiospores, respectively) forms of an individual *Cytospora* species, although the sexual ascospore are detected at much lower frequency and quantity than the asexual conidia (Bertrand & English, 1976b). Conidia are the main drivers of new infections and have been

detected in every month of the year, with the abundance of conidia being produced during the summer months (Luepschen, 1969).

As infection progresses, a perennial canker begins to form. During times of tree dormancy, the canker will continue to grow aggressively, and, upon leaving dormancy, the tree's defensive responses activate and slow the progression. Symptoms can change depending on the affected tissues, tree age, and age of the infection. One-year-old shoot growth is typically attributed to new infections and is thought to be considerably more important in disease progression than other woody tissues (Tekauz, 1973). New growth experiences a darkening of the bark as well as necrotic, sunken tissues under the periderm. When newly infected tissue remains on the tree, it can quickly spread down the stem to adjoining scaffolds. Advanced stages of disease exhibit a large black canker with concentric rings. Peach trees commonly exude transparent amber colored gummosis in large amounts at the site of active pathogen growth (Adams, 2005). This defense response is common with tree irritation but can become detrimental if copious amounts are produced (Biggs, 1989a). As disease progresses, the asexual fruiting structure, pycnidia, begin to protrude from bark tissues and can be observed as white pimple-like structures or black raised bumps. During periods of high moisture, pycnidia begin to ooze starchy orange conidiogenous tendrils (cirrus) to progress the infection cycle (Miller et al., 2019).

**1.5 Management**

Traditionally, fungal canker pathogens have been hard to control and have proven impossible to eradicate because of their complicated lifestyles. Currently no Cytospora canker

resistance has been identified in peach and there is currently no method to eradicate *C. plurivora* from infected peach trees. A robust IPM program that includes chemical fungicides, pruning, cultural strategies, and irrigation are all important for management of Cytospora canker. Chemical fungicides are applied through various methods within orchards, including painting over wounded surfaces, backpack sprayers, and air blast canopy sprays. There are no standardized application regimes amongst growers, with chemical type and concentrations varying by operation. Pruning is an essential task in orchards that boosts yields, but each wound introduces a new infection court. Annually up to one-half to two-thirds of one-year-old growth is removed during the late winter pruning cycle (Whiting, 2018).

**1.6 Chemical Control**

On the western slope of Colorado, peach growers use a wide range of chemical treatments. The conventional chemicals Captan and Topsin are commonly used, while organic producers utilize sulfur and copper derivatives (Biggs & El-Koholi, 1994; Miller et al., 2019). Miller et al. (2021) demonstrated that Captan (N-trichloromethylthio-4-cyclohexane-1,2-dicarboximide) mixed with 50% latex is a viable candidate for prophylactic painting of wounds. Likewise, Biggs (1994) found that Captan prevented Cytospora canker development in both chemical amended plates and excised branches when dipped for >30 minutes. These findings suggest that further investigation into the efficacy of Captan as a prophylactic canopy spray is warranted. Although, Captan is highly fungitoxic, Northover (1976) questioned the benefits of Captan as a canopy spray against *Leucostoma* infection, as it was found to have no effect on shoot infection. However, it has been an effective treatment for other fungal diseases in peaches, such as brown rot (*Monilinia fructicola)* and leaf curl (*Taphrina deformans*) during spring applications

(Lalancette et al., 2020). After pruning many producers will seal each wound with protective fungicides mixed with latex to minimize infection. An application of Topsin with 50% latex paint mixture was found to be effective at reducing lesion volume on pruned cuts (Miller et al. 2019; Miller et al. 2021). Preventative trunk paintings of the above-mentioned chemicals also act to minimize sun exposure during the winter months as sun scald is of concern on younger peach trees with thin bark.

Organic producers do not have access to as many reliable chemicals that reduce the severity of Cytospora canker. Lime sulfur and copper are popular treatments currently available for commercial use, however, the efficacy of either treatment has not been demonstrated. Both lime sulfur and copper hydroxide were found to be ineffective at reducing the lesion size once *Cytospora* infection began (Miller et al., 2019). This still begs the question of how effective these treatments would be at stopping initial *Cytospora* colonization when used as a prophylactic canopy spray. It has been suggested that copper derivatives may in fact increase the response to infection because of phytotoxicity, which can cause premature defoliation and stress in trees (Lalancette & McFarland, 2007; Miller et al., 2019).

**1.7 Cultural Controls**

The peach industry employs a suite of cultural controls to decrease the incidence and severity of Cytospora canker. Frost cracking, soil composition and dryer climate all create infection courts or tree stressors which increase susceptibility to *Cytospora* infection. Tree health and vitality are the most important factors which promote healing of wounded sites, which in turn fights infection (Biggs & Grove, 2005). It is thought that conidia are spread through pruning

equipment, poor orchard sanitation, and windblown water (Bertrand & English, 1976b; Biggs, 1989a; Miller et al., 2019). Pruning is necessary in cropping systems as it increases yield, fruit quality, and assists with crop management (Minas et al., 2018). This leaves trees predisposed to *Cytospora* infection through these wound sites. Timing pruning to coincide with lower sporulation from cankers as well as delaying pruning until late spring is associated with faster wound healing and less infection (Biggs & Grove, 2005).

Environmental damages such as sun scald and frost cracking are common in thin-barked trees. Peaches are especially susceptible to these abiotic events, which are notoriously hard to control. The best management option for cold damage is to limit nitrogen fertilization in the later season to induce earlier dormancy (Biggs & Grove, 2005). An extensive breeding program of peach varieties has taken place in recent decades to increase the cold hardiness, however, this has proved challenging as the lower threshold of 90% blossom death occurs between -6$^{o}$C and -3$^{o}$C (Penn State Extension, 2017).

**1.8 Irrigation**

Irrigation practices in orchards vary as wildly as chemical applications. Common irrigation methods include micro sprinklers, drip irrigation, direct root watering, furrow, and impact sprinklers. Previous experiments have shown that water related events, such as irrigation and windblown rain, are the main drivers of conidia dispersal (Bertrand & English, 1976b; Grove & Biggs, 2006). Irrigation mimics these natural events and raises ambient humidity within the orchard. Infected trees were found to exude more conidia between the months of February and June, which is attributed to the periods of high relative humidity

associated with irrigation events (Jones & Leupschen, 1969). During over-the-canopy irrigation, conidia were spread and detected in 100% of collected *Leucostoma* selected media plates out to 100cm from the canker (Grove & Biggs, 2006). Fortunately for the western slope of Colorado, micro sprinklers and drip irrigation are the most common irrigation techniques. Drip irrigation has been shown to promote better soil moisture content at the root zone, whereas micro sprinkler irrigation has been linked to water stress because of intervals between watering (Bryla et al., 2005).

**1.9 Objectives for this Work**

The objectives in this thesis were to 1) assess the chemical efficacy of selected canopy sprays after an early cold frost event and 2) assess the role of irrigation on *C. plurivora* epidemiology within orchards. To address the first objective, an investigation of chemical efficacy was conducted to determine if Captan-M, lime sulfur, and lime sulfur with the addition of NuFilm are effective as prophylactic canopy sprays against infection of *C. plurivora* as a winter spray after a cold frost event. These assessments were conducted through an inoculation study of each spray type in orchard settings. A biodiversity assay was also conducted in conjunction with inoculations to assess changes in fungal communities during treatments. The second objective of this study was to assess the epidemiology of *C. plurivora* under differing irrigation types. This assessment was conducted using multiple peach blocks under drip and micro-sprinkler irrigation.

## Literature Cited


Adams, G. C. (Ed.). (2005). Phylogenetic relationships and morphology of Cytospora species and related teleomorphs (Ascomycota, Diaporthales, Valsaceae) from Eucalyptus. CBS, Centralbureau voor Schimmelcultures.

Anthony, B. M., & Minas, I. S. (2021). Optimizing Peach Tree Canopy Architecture for Efficient Light Use, Increased Productivity and Improved Fruit Quality. Agronomy, 11(10), 1961. https://doi.org/10.3390/agronomy11101961

Bergdahl, A. (2016). Diseases of trees in the Great Plains. RMRS-GTR-335; p. 241. USDA - Forest Service.

Bertrand, R. E., English, H. 1976. Release and dispersal of conidia and ascospores of. Valsa leucostoma. Phytopathology, 66: 987-991.

Biggs, A. R. (1989). Integrated Approach to Controlling Leucostoma Canker of Peach in Ontario. Plant Disease, 73(11), 869. https://doi.org/10.1094/PD-73-0869

Biggs, A. R., & El-Koholi, M. M. (1994). Biggs—Effect of calcium salts on growth, pectic enzyme activity, and colonization of peach twigs by Leucostoma persoonii.pdf. 78(9), 886–890.

Biggs, A. R., & Grove, G. G. (2005). Leucostoma canker of stone fruits. The Plant Health Instructor. https://doi.org/10.1094/PHI-I-2005-1220-01

Bryla, D. R., Dickson, E., Shenk, R., Johnson, R. S., Crisosto, C. H., & Trout, T. J. (2005). Influence of Irrigation Method and Scheduling on Patterns of Soil and Tree Water Status and Its Relation to Yield and Fruit Quality in Peach. HortScience, 40(7), 2118–2124. https://doi.org/10.21273/HORTSCI.40.7.2118

Corredor-Moreno, P., & Saunders, D. G. O. (2020). Expecting the unexpected: Factors influencing the emergence of fungal and oomycete plant pathogens. New Phytologist, 225(1), 118–125. https://doi.org/10.1111/nph.16007

Grove, G. G., & Biggs, A. R. (2006). Production and Dispersal of Conidia of Leucostoma cinctum in Peach and Cherry Orchards Under Irrigation in Eastern Washington. Plant Disease, 90(5), 587–591. https://doi.org/10.1094/PD-90-0587

Lalancette, N., Blaus, L. L., & Engel, P. (2020). Refinement of Peach Cover Spray Programs for Management of Brown Rot at Harvest. Plant Disease, 104(5), 1527–1533. https://doi.org/10.1094/PDIS-04-19-0888-RE

Lalancette, N., & McFarland, K. A. (2007). Phytotoxicity of Copper-Based Bactericides to Peach and Nectarine. Plant Disease, 91(9), 1122–1130. https://doi.org/10.1094/PDIS-91-9-1122

Ma, Z., Yoshimura, M. A., & Michailides, T. J. (2003). Identification and Characterization of BenzimidazoleResistance in Monilinia fructicola from Stone Fruit OrchardsinCalifornia. Applied and Environmental Microbiology, 69(12), 7145–7152. https://doi.org/10.1128/AEM.69.12.7145-7152.2003

Mesa County Extension. (2015). Cytospera Canker (Cytospera spp.)—Mesa County, Colorado. https://www.mesacounty.us/pest/ugvpcd/insects_weeds_diseases/diseases2/information/cytospera-in-peaches/

Meyer, B. (2021). Fruit & nut annual summary (Regional News Release, p. 2). National Agricultural Statistics Service. United States Department of Agriculture.

Miller, S. T. (2021). Management and epidemiology of Cytospora perennial canker, Cytospora plurivora, in western Colorado [Dissertation].

Miller, S.T. Otto, K. L., Sterle, D., Minas, I. S., & Stewart, J. E. (2019). Preventive Fungicidal Control of Cytospora leucostoma in Peach Orchards in Colorado. Plant Disease, 103(6), 1138–1147. https://doi.org/10.1094/PDIS-05-18-0801-RE

Miller, S. T., Sterle, D., Minas, I. S., & Stewart, J. E. (2021). Exploring fungicides and sealants for management of Cytospora plurivora infections in western Colorado peach production systems. *Crop Protection*, *146*, 105654. https://doi.org/10.1016/j.cropro.2021.105654

Minas, I. S., Tanou, G., & Molassiotis, A. (2018). Environmental and orchard bases of peach fruit quality. Scientia Horticulturae, 235, 307–322. https://doi.org/10.1016/j.scienta.2018.01.028

Northover, J. (1976). Protection of peach shoots against species of Leucostoma with Benomyl and Captafol.PDF. Phytopathology®, 66, 1125–1128. https://doi.org/10.1094/Phyto-66-1125

Penn State Extension. (2017). Orchard Frost—Critical Temperatures for Various Fruits. Penn State Extension. https://extension.psu.edu/orchard-frost-critical-temperatures-for-various-fruits

Pfeufer, E. E., & Ngugi, H. K. (2012). Orchard Factors Associated with Resistance and Cross Resistance to Sterol Demethylation Inhibitor Fungicides in Populations of Venturia inaequalis from Pennsylvania. Phytopathology®, 102(3), 272–282. https://doi.org/10.1094/PHYTO-04-11-0117

Rossman, A.Y., Adams, G.C., Cannon, P.F., Castlebury, L.A., Crous, P.W., Gryzenhout, M., Jaklitsch, W.M., Mejia, L.C., Stoykov, D., Udayanga, D., Voglmayr, H., Walker, D.M. Recommendations of generic names in Diaporthales competing for protection or use. IMA Fungus. 2015 Jun;6(1):145-54. doi: 10.5598/imafungus.2015.06.01.09. Epub 2015 Jun 4. PMID: 26203420; PMCID: PMC4500080.

Sexton, J. (2018). History of the Fruit Industry in Mesa County. AES-WCRC. https://aes.colostate.edu/wcrc/stations/orchard-mesa/history-of-the-fruit-industry-in-mesa-county/

Sinclair, W. A., & Lyon, H. H. (2005). *Diseases of trees and shrubs* (2nd ed). Comstock Pub. Associates.

Taylor, J.W. (2011). One Fungus = One Name: DNA and fungal nomenclature twenty years after PCR. IMA Fungus. Dec;2(2):113-20. doi: 10.5598/imafungus.2011.02.02.01.

USDA ERS - Fruit & Tree Nuts. (2021). https://www.ers.usda.gov/topics/crops/fruit-tree-nuts/#periodic

Whiting, D. (2018). Growing Tree Fruit in Colorado Gardens.pdf (No. 771; Colorado Master Gardener GardenNotes). Colorado State University.

Zhu, H., Pan, M., Bezerra, J. D. P., Tian, C., & Fan, X. (2020). Discovery of Cytospora species associated with canker disease of tree hosts from Mount Dongling of China. MycoKeys, 62, 97–121. https://doi.org/10.3897/mycokeys.62.47854

## CHAPTER 2: TESTING EFFICACY OF CANOPY SPRAYS AGAINST THE CYTOSPORA CANKER PATHOGEN, *CYTOSPORA PLURIVORA,* IN WESTERN COLORADO PEACHES

### 2.1 Introduction

The Palisade Peach, grown in Colorado and sold on the fresh peach market, is a popular product, even outside of Colorado, which enables a high premium in sales. In 2019, peaches grown on the western slope of Colorado commanded the highest price per ton of any peach producing region in the United States at $2,100/acre (USDA, 2021). These premiums are due to the anecdotal perception that western Colorado peaches are larger, sweeter, and of better fruit quality than that of other regions. Previous peach seasons have brought 15-40 million dollars of revenue into the Colorado western slope, with cold weather-related events assisting in these price fluctuations. In 2021, the Grand Valley in Colorado profited 25 million in production value from peaches (*USDA ERS - Fruit & Tree Nuts*, 2021). This revenue is extremely important to the vitality of the western slope region in Colorado and as such measures are taken to protect the crop from cold damage events and a fungal disease called Cytospora canker caused by *Cytospora plurivora* that is present in 100% of the orchards in the region.

In Colorado, freeze related events are common and there are three main time periods during the year in which freeze damage are likely to result in peach yield loss. One freeze damage type occurs during the early fall when temperatures drop quickly before trees enter dormancy. This rapid temperature fluctuation causes intracellular ice to form which leads to a shredding of the xylem parenchyma cells (Ashworth & Wisniewski, 1991). This type of freeze event has the potential to kill buds, shoots, and scaffolds. Losses due to early fall cold damage vary depending on geographic location and severity of the cold event. Michigan has

experienced nine separate cold related mortality events since the 1960s (Shane, 2019). Likewise, Tennessee has experienced periods of little to no production 6 out of 11 years between 1989 and 2000 due to cold damage (Logan et al., 2000). In Colorado, between 2000 and 2020, the western slope experienced 7 freeze events in October, with temperatures low enough to cause tree damage (weather.gov). Mesa County's Emergency Board estimates that a freeze event in 2020 caused 80% peach crop loss.

A second type of freeze damage can occur during extreme cold periods while a tree is in dormancy. Extracellular water in the bark freezes, causing water to be drawn from the intercellular spaces, which in turn changes the cellular volume as well as the solutes present (Burke et al., 1976). It is suspected that a dehydration effect occurs, which in turn causes mortality to these tissues. Lastly, the third type of freeze damage that causes yield loss is when low temperatures occur as buds are open and new tissues are expanding in the spring. A drastic drop in temperature to -17.2$^{o}$C during the initial swelling was shown to cause a 90% bud kill (Logan et al., 2000).

Not only do Colorado growers experience large losses because of freeze related injuries, but they also experience extreme loses due to infections by *C. plurivora*. There is no effective way to eradicate *Cytospora* from an infected tree, so preventative measures aimed at decreasing infection rates are the best option (Rosenberger, 1982). *C. plurivora* is typically thought of as a secondary pathogen and requires infection courts to enter the host (Biggs, 1989b). However, in western Colorado, extreme freeze events are a common occurrence which creates infection courts in peach trees through frost cracking. This damage allows fungal spores to germinate, penetrate, and infect woody tissues. *C. plurivora* is the main driver of preach tree

mortality on the western slope of Colorado, likely exacerbated by rapidly fluctuating climatic events, alkaline soils, and water access (Miller et al., 2019).

Growers attempt to manage Cytospora canker while promoting optimal fruit growth conditions. Crop load management, pruning, architecture, and irrigation are all important factors in promoting fruit growth (Minas et al., 2018). However, orchard maintenance and management for optimal fruit growth are at odds when it comes to infection. Pruning is a requirement which boosts production and fruit quality, but it comes at a price as each cut opens the tree to infection from *C. plurivora*. Pruning is typically conducted annually in the late winter before trees leave dormancy, which is thought of as the time of year with lower disease pressures compared to the late fall or spring when temperatures and humidity increase (Pokharel, 2013). However, it has been demonstrated that *C. plurivora* conidiospores are present through all months of the year (Miller et al., 2019, Grove & Biggs 2006). The need to prune for optimal peach production compounds the risk of *C. plurivora* infection, which in turn prompts growers to apply chemicals as part of their integrated pest management strategies.

Fungicides are commonly applied in peaches as part of a robust IPM program. Although many chemicals are applied, none are registered for treatment of Cytospora canker (Pokharel, 2013). Previous studies on the efficacy of several chemicals have been conducted with conflicting results, leaving growers with few chemical options (Grove & Biggs, 2006; Miller et al. 2019; Pokharel, 2013). Miller et al. 2019 demonstrated through laboratory and field trials that chemicals including Captan, Topsin, and lime sulfur can reduce the hyphal growth of *Cytospora*, when applied directly to cut branches. These types of spot applications work for small grower operations; however, canopy spraying is the most effective means of chemical application in

large commercial orchards. Growers commonly apply prophylactic chemical canopy sprays for *C. plurivora* and other fungal pathogens. Yet, the efficacy of these chemical canopy sprays applied as a prophylactic treatment, rather than directly to cut branches, has not been well characterized in Colorado.

The purpose of this study was to investigate if the chemical applications following an early fall freeze event are effective in limiting *Cytospora* infections in conventional and organic orchards. As well as to determine the efficacy threshold of prophylactic canopy sprays following freeze events.

## 2.2 Methods and Materials

**Field sites and chemical applications.** Experiments were conducted in peach orchards on the western slope of Colorado from December 2020 to March 2021 and December 2021 to March 2022. During the 2021 season, chemical treatments began the first week of November after a frost event in October caused significant shoot damage. The types of chemicals and their rates of application were left to the discretion of the growers. During the 2021 season, experiments were conducted in five peach orchards on the western slope of Colorado, of which two were conventionally treated orchards that used Captan 4L (Drexel Chemical Memphis, TN) canopy sprays at different application rates. There were also two organic orchards, one of which applied lime sulfur and the other applied lime sulfur and Badge® SC (Gowan Yuma, AZ) with addition of NuFilm P® (Miller Chemical & Fertilizer Corporation; Pennsylvania, USA). An orchard was also included that did not spray after the freeze event; hereby called the no-spray orchard 2021. The conventional peach orchards were located at the Colorado State University Western

Colorado Research Center - Orchard Mesa (WCRC-OM) in Grand Junction, Colorado and one privately owned orchard located in Palisade, Colorado. Captan 4L was applied at a rate of 1.5 quarts per acre with an air sprayer at WCRC-OM. The private orchard followed the same method of spray delivery and applied at a rate of 3.0 quarts per acre. The Colorado State University Organic Agricultural Research Station – Rogers Mesa (OARS-RM) located in Hotchkiss, Colorado applied a lime sulfur organic canopy spray with the addition of Badge and Nu-film through an air delivery sprayer. Three open nozzles were used for application at a rate of 200 gallons per acre with a dilution rate of 3%. The second organic orchard was a private grower located in Hotchkiss, CO that sprayed and organic treatment of lime sulfur at the mid concentration label rate of 3% with an air delivery sprayer (Table 2-1).

During 2022, growers did not use prophylactic chemical sprays as no cold damage occurred early in the season. Three peach orchards were used in the experiments including two conventional WCRC-OM and a private grower both located in Grand Junction. As well as OARS-RM located in Hotchkiss, CO.

***Cytospora plurivora* inoculation and recovery.** Inoculation trials were conducted monthly in December 2020 through March 2021 and repeated monthly in December 2021 through March 2022. A total of 25 trees were chosen per peach block and 3 inoculations were conducted per tree on randomly selected 1-year old shoots. Shoots were selected based on observable apparent cold damage that occurred in November 2020 during the first experimental season. The observations made for cold damage were based on the external appearance of shoots: discoloration and death, as well as the visible internal damages such as discoloration in the xylem tissues (Figure 2-1). The second season did not experience an early

freeze event in November 2021 and so healthy shoots, 3 per tree, were randomly selected and inoculated.

***Cytospora plurivora* inoculum preparation.** The isolate CP5.1 (*C. plurivora*) was grown on ½ PDA for 14 days prior to inoculation (Miller, et al., 2019). All inoculations were conducted with CP5.1 for the entirety of the experiment. A 5mm agar plug of CP5.1 was applied with the fungal side down to the apical bud of a 1-year-old shoot without wounding. The agar was then covered and secured to the bud with parafilm. Shoots were excised at one-month post-inoculation.

Parafilm was removed from each shoot and examined for symptoms and/or signs of infection. All discoloration, lesions, or deviation from healthy tissues were identified. Measurements were taken from the tip of the apical bud to the end of the visible damage. Twigs were then surface sterilized in a 10% sodium hypochlorite solution for 2 minutes and rinsed with sterile DI water. A 5mm cut was then made at the interface of the healthy and dissed tissue margin (Figure 2-2). The cut sections were then bisected and plated on ½ PDA for seven days. A morphological examination was conducted to verify the presence or absence of *C. plurivora.*

**Biodiversity assay.** Two conventional (BT and OM), two organic (OO and RM), and one no spray orchard (PO) were sampled for fungal diversity monthly from December 2020 through March 2021. These measurements were repeated in 2021 from December 2021 through March 2022 to include two conventional orchards (BT and OM) and one organic orchard (RM). A total

of 25 trees were selected each month per orchard. Three one-year-old shoots were randomly selected and a 12cm sections were cut from the tree and placed in a cooler with cold packs.

Shoots were surface sterilized in a 10% sodium hypochlorite solution for 2 minutes and then rinsed in sterile DI water. The apical bud and 4 auxillary buds were then cut from the shoot and plated on ½ PDA. Cultures were incubated at 25°C for five days and emerging fungal colonies were transfered to new ½ PDA plates. A thorough morphological examination was conducted to sort fungal isolates by morphotype.

**Chelex DNA extraction, PCR and sequencing.** Crude DNA extractions were completed using a sterile pipette tip to scrape mycelium from fresh fungal cultures, which was then added to 100uL of 10% Chelex-100 solution. Samples were then heated to 99$^{o}$C for 35 minutes using the Eppendorf Mastercycler PRO (Enfield, CT) and stored in a -20$^{o}$C freezer.

DNA from each fungal isolate was then sequenced at the internal transcribed spacer region (ITS) with the primers ITS1F and ITS4 (White et al., 1990) by Eurofins (Louisville, KY) to determine fungal isolate genus. A 25 µL PCR reaction was conducted as follows: 15.8 µL molecular grade water, 2.5 µL 10x Standard Taq Reaction Buffer (New England BioLabs, Ipswitch, MA), 0.5 µL dNTPs (Gold Biotechnology, St. Louis, MO), 1 µL of the ITS1F (Gardes & Bruns, 1993) and ITS4 (White et al. 1990) primer set at a concentration of 10 µM, and 0.12 Taq DNA Polymerase (New England BioLabs, Ipswitch, MA) Polymerase Chain Reaction (PCR) thermocycling was conducted with the following conditions: 94$^{o}$C for 5 minutes, 35 cycles at 95$^{o}$C for 30 seconds, 52$^{o}$C for 30 seconds and 72$^{o}$C for 30 seconds. This was followed by a final 8 minute elongation at 72$^{o}$C and a perpetual hold at 4°C.

## 2.3 Statistical Analysis

**Inoculation Trials.** Analysis was carried out through R-studio V4.1.2 "Bird Hippie" with the 'car' and 'emmeans' packages (Lenth, 2022). Chemical applications, orchard, and treatment type were treated as predictor variables. Chemical application had four levels: Captan, lime sulfur, lime sulfur + Nu Film, and no spray. Orchard had five levels: BT, OM, OO, RM, PO. Treatment had two levels: conventional and organic. The dependent variable was the presence or absence of *C. plurivora* during the experiment.

The proportion of infection over time was analyzed to investigate the relationship between chemical efficacy over time within each orchard/treatment. The analysis was divided between conventional sprays and organic sprays for a better comparison between chemical treatment. The proportion of infection was used as a basis for logistic regression between orchard, month, and their interaction as factors. 'emmeans' was then used to perform pairwise comparisons between the treatment type and month. This was conducted for both the 2021 and 2022 seasons. A generalized linear model (GLM) was built using *C*. *plurivora* presence or absence as a response variable, with chemical and year treated as fixed effects, then an ANOVA was conducted. This was done to investigate the random effects, not explained by chemical treatment when compared across years.

**Biodiversity Trials.** Shannon's diversity index was calculated using R Studio V4.1.2 "Bird Hippie" and the packages 'vegan', 'emmeans', and 'car' (Fox & Weisberg, 2019; Oksanen et al., 2022). This analysis was performed to assess the diversity of fungal communities within each orchard over time for both the 2021 and 2022 seasons. A linear model was then built using the

Shannon's diversity index score. 'Emmeans' was used to conduct a pairwise comparison within each orchard and collection time (month).

### 2.4 Results

**Conventional: *C. plurivora* detection 2021 and 2022**. During the 2021 season, *C. plurivora* was detected through reisolation and morphological identification in the conventional orchards BT and OM, both of which were treated with Captan. In January, *C. plurivora* collections were relatively low when compared to the final collection in March. BT showed the largest increase in *C. plurivora* infection on sampled trees starting with a *C. plurivora* detection proportion of 20% (5/25) in January and ending with a at 84% (21/25) in March. Organic orchard OM had an initial *C. plurivora* detection proportion of 44% (11/25) in January and ended with a final 64% (16/25) in March. The *C. plurivora* detection proportion for BT was significantly different between January and March (p-value = 0.0001), as well as from February and March with (p-value = 0.0003). These finding suggests that orchard BT experienced a loss of chemical efficacy three months post spray. In contrast, no significant differences were observed in *C. plurivora* detection proportion in the OM orchard over time (Figure 2-3A). During the 2022 season the *C. plurivora* detection proportion was much lower than that of 2021. Orchard RM had 0% (0/25) *C. plurivora* detection proportion in January, 0% (0/25) in February, and 20% (5/25) in March. No data was collected for orchards OO and the no spray PO during this season (Table 2-2). The 2022 season had both diminished grower participation and detection of *C. plurivora*.

**Organic: *C. plurivora* detection 2021 and 2022.** Trends observed in the conventional sprays were mirrored in the organic and no spray orchards during the 2021 season. Orchard OO

began with a 28% (7/25) proportion of detection of *C. plurivora* in January which increased to 72% (18/25) in March. Orchard RM had a *C. plurivora* detection proportion of 36% (9/25) in January, which increased to 68% (18/25) in March. The *C. plurivora* detection proportion also increased over time in the no spray orchard PO from 40% (10/25) in January to 56% (17/25) in March. The *C. plurivora* detection proportion for orchard OO was significantly different between February and March ($p = 0.017$), and between January and March ($p = 0.0076$), suggesting that efficacy of lime sulfur treatment was at the 3-month post spray time point. Orchard RM, however, observed significantly more *C. plurivora* between the January and February collections ($p = 0.034$). This suggests a loss of efficacy of the lime sulfur with the addition of Nu-Film at the two-month post spray mark (Figure 2-3A).

In the 2022 experimental season, no sprays were applied to any experimental orchard. There were also no significant differences between monthly *C. plurivora* detection within orchards (Figure 2-3B). Orchard BT had the highest *C. plurivora* detection proportion of 16% (4/25) in January while 0% (0/25) infection was detected in March at the end of the experiment. Likewise, orchard OM had a *C. plurivora* detection proportion of 12% (3/25) in January and a 0% (0/25) in March (Table 2-2).

**Conventional: lesion length (cm) 2021 and 2022.** The average lesion length increased during the 2021 season by 13% ranging from 1.88 cm to 2.13 cm in orchard BT whereas orchard OM showed an increase in average lesion length of 132% ranging from 1.09 cm to 2.53 cm between January and March (Table 2-3). While the *C. plurivora* detection proportion increased the largest amount in BT, it did not show a greater propensity for an increase in lesion length as time continued (Figure 2-4A). During the 2022 season orchard BT had an average lesion length

of 2.25cm in January, 1.5cm in February, but no *C. plurivora* was detected in March. Orchard OM followed this trend with the initial average lesion length starting at 0.9 cm and with zero detection in February and March (Figure 2-4B).

**Organic: lesion length (cm) 2021 and 2022.** Average lesion length increased during the 2021 season between the first collection in January and the final collection in March for all three orchards; OO orchard increased in average lesion length starting at 1.37cm and ending at 4.58cm, RM orchard increased from 2.35cm to 3.00cm, and PO orchard increased 1.69cm to 4.63cm respectively. During the 2022 season only orchard RM participated in the study and had zero detections in January and February but ended the experiment with an average lesion length of 2.06cm in March.

**Biodiversity Assessments in 2021 and 2022.** During the 2021 season, increased diversity of fungal taxa occurred over time. Organic orchards experienced the same trends of fungal taxa increasing as the experiment continued, however, the unique taxa were higher in the organic treatments than that of the conventional. Across all four 2021 sampling months, 198 isolates (96 morphotypes), 626 isolates (144 morphotypes), 796 isolates (218 morphotypes), and 995 isolates (235 morphotypes) were collected from BT, OM, OO, and RM, respectively (Table 2-4A; Table 2-4A). During the 2022 season orchard OO did not participate in the study. Across all four 2022 sampling months 1064 isolates (184 morphotypes), 1129 isolates (133 morphotypes), 1279 isolates (122 morphotypes) were collected from orchards BT, OM, and RM (Table 2-4B; Table 2-5B).

OO started with 150 isolates in December and ended March with 241. The total collected during experimentation was a total of 796 isolates (Table 2-4A), representing 218 morphologically distinct taxa (Table 2-5A). Orchard RM began with 146 in December and ended with 258 isolates in December. The total isolate count for the experiment was 995, representing 235 morphologically distinct taxa.

In each orchard, fungal diversity increased over time after spray treatments. Orchard BT had the lowest starting Shannon's Diversity Index (SDI) of 0.246 in December, one-month post spray, which significantly peaked in February at 0.735 ($p$ = 0.0041). Orchard OM had a SDI = 1.205 in January, decreasing to SDI = 0.858 (p = 0.017) in February and showed the highest diversity in March at 1.471 (p = 0.0195). Organic orchard OO had a SDI = 0.656 in January and which increased 1.544 in March. SDI was significantly higher three months post spray efficacy in February ($p$ = 0.0005). The organic orchard RM had a SDI = 1.113 in January and a final of 1.664 reported in March. SDI significantly increased from December to January ($p$ = <0.0001 (Figure 2-5A). The 2022 season had 3 orchards participate in the experiment. The conventional orchard BT had a mean SDI = 0.835 in December and raising to a significant level of SDI = 1.491 (p = < 0.0001) during January. OM had no significant changes in diversity month to month howver showed a significant value between December and March (p = 0.0081) and showed an overall decreasing mean SDI through the collections. Orchard RM had no significant time points during the collections during 2022 (Figure 2-5B).

The composition of fungal communities differed between the conventional and organic treatments during the 2021 field season. *C. plurivora* comprised a higher proportion of the fungal community in the lime sulfer and lime sulfer with Nu-Film when compared to the

conventional Captan sprays (Figure 2-6). The most abundant fungal genera in the organic treatments were *Alternaria, Cytospora*, and *Cladosporium*, while the most abundant genera in the conventional spray orchards were *Alternaria* and *Thyrostroma*.. *Fusarium* spp. were found in both conventional and organic treatments however, a larger proportion of *Fusarium* spp. were recovered in orchard RM sprayed with lime sulfur and Nu-Film. The 2022 no spray season showed differences in fungal communities by orchard. The conventional orchard BT showed a higher propotion of *Cytospora* recovered than that of OM and the organic orchard RM. The order Hypocreales, genus *Fusarium*, and class Dothideomycete were represented at much higher proportions in the no spray organic orchard RM than that of the no spray conventional orchards. *Alternaria* comprises a much larger proportion of fungal genera in orchard OM when compared with BT (Figure 2-6).

The mean Shannon Diversity Index score showed differences between the 2021 and 2022 season. The conventional treatment in orchard BT for the year 2021 had a mean SDI = 0.7 and 2022 SDI = 1.3 (Figure 2-7). The trendline for BT during 2022 had a $R^2 = 0.2$. Orchard OM had a SDI = 1.2 in 2021 and SDI = 0.8 in 2022 with a $R^2 = -0.73$. The organic treatments had an SDI = 1.7 in 2021 and SDI = 0.75 in 2022 and a $R^2 = 0.06$. The organic orchard OO and no spray orchard PO did not participate in the 2022 experiment.

**Weather measurements.** In October of 2021 temperatures reached as low as -12 $^{o}$C and were sustained at abnormally low temperatures for 3 days during this time-period compared to the normal average low temperatures. During the month of October in 2022 no sustained cold event occurred (weather.gov). Year was significant with cold damage acting as a random effect ($p = <2e^{-16}$).

## 2.5 Discussion

**Inoculation Trials.** This study suggests that select chemicals may be effective as a prophylactic chemical canopy sprays applied immediately following early season freeze injury. Previous research has confirmed the efficacy of Captan and lime sulfur treatments when applied to excised branches as chemical dips and prophylactic paint amendments applied to wounds (Biggs & El-Koholi, 1994; Miller et al., 2019). The efficacy of conventional orchard sprays that occurred in November 2020 after a freeze were tested from December to March. Within the BT orchard, there was a significant increase in recovery of *C. plurivora* from February to March, suggesting a loss of efficacy at three months post spray when applying Captan at a rate of 3qts/acre. Orchard OM, on the other hand, applied half the Captan as orchard BT at a rate of 1.5qts/acre. The proportion of *C. plurivora* detection during the month of January for orchard OM (44%) was over double that of orchard BT (20%). This suggests that the rate of application may not have been adequate to limit infection. These findings suggest that prophylactic chemical canopy sprays may be an effective management tool during early cold damage events, especially if reapplied at the effective thresholds demonstrated by this research. This work warrants further investigation into the application rates of Captan as a prophylactic canopy spray against cold damage.

The cold damage experienced at each orchard varied by location and *C. plurivora* infection was more prevalent where the severity of shoot damage was greater. Observationally, damages experienced at orchard BT left no living one-year old shoots, whereas orchard OM showed more shoot resilience (Figure 2-1 A&C). Given the ability of *C. plurivora* to survive and propagate on dead tissue it would explain why this necrotrophic pathogen thrived in orchard

BT. In comparison to 2021, no sprays were applied during the 2022 season because no freeze event occurred. With the lack of chemical application, it would be expected that incidence of *C. plurivora* would be higher. However, this trend was not observed in the 2022 season and little infection was detected, suggesting that cold damage is the main factor of infection level differences between the two experimental seasons (Figure 2-3).

The organic orchard OO applied lime sulfur at the concentration of 3%, which is the mid label rate, and there was a significant difference between *C. plurivora* detection between February and March. This suggests that the efficacy of lime sulfur, when sprayed in late fall after a freeze event, may last for a maximum of three-months post spray. Orchard RM, on the other hand, applied lime sulfur at a dilution rate of 3%, but also added a surfatcant Nu-film. The addition of Nu-film may have reduced efficacy of lime sulfur as there was a significant increase in the detection of *C. plurivora* 2 months post spray, rather than three months as was observed with lime sulfur application alone. These findings question the effectiveness of lime sulfur when combined with Nu-Film as a canopy spray and warrants further investigation into its efficacy. The addition of Nu-Film as a surfactant appears to reduce the efficacy of lime sulfur treatments (Miller et al. 2019). Previous research demonstrated that the addition of Nu-film, when combined 70% latex paint, did not reduce the lesion size in comparison with the control. Findings herein concur with this previous work and suggest that Nu-Film is not beneficial in reducing *C. plurivora* infections levels.

The 2021 and 2022 season showed differences in *C. plurivora* detection as well as in lesion length. During the 2021 season, orchards experienced varying degrees of cold damage as well as prophylactic spray treatments, and *C. plurivora* was detected at significant levels

between time points suggesting after 2 or 3 months that the study reached chemical efficacy thresholds. During the 2022 season, no cold damage, nor chemical canopy sprays occurred, and no significant changes in the detection of *C. plurivora* were observed in the orchards. It would stand to reason that if chemicals were not applied, *C. plurivora* infections would increase or be higher during 2022 compared to 2021. However, this is contrary to what was observed. Even with no chemicals applied during the 2022 season, there was no significant increase in *C. plurivora* detection in the surveyed orchards BT, RM, and OM. This suggests that cold damage is one of the main drivers of *C. plurivora* infection in the western slope peach orchards.

Lesion length within each orchard increased during each month during the 2021 season, however, during in 2022 no significant change in lesion length was detected (Table 2-3). As the temperatures started to warm towards the end of collection in March 2021, an increase in lesion length was detected. This finding is expected as warmer temperatures in February and March are closer to optimal growth conditions and would facilitate growth of *C. plurivora*. Helton demonstrated that a minimum temperature of 3$^{o}$C was necessary for *C. leucostoma* growth, while the optimum temperature was 25$^{o}$C (Helton & Konicek, 1962). Interestingly, in 2022, the conventional and organic sprayed orchards showed little change in growth or detection of *C. plurivora* through the experiment.

When comparing lesion size across the conventional and organic treatment, a trend can be seen in 2021 where overall lesion size was smaller in the conventional Captan treatments than in the organic lime sulfur treatments. This may be due to the effectiveness of Captan in completely inhibiting fungal growth. Captan has been shown to inhibit fungal growth on shoots that were chemically dipped for 15-60 min, though the concentration of Captan on dipped

branches is much higher than that of air sprayed canopies (Biggs & El-Koholi, 1994). Air blast sprayers were shown to lose up to 50% of the spray material in apple orchards to drift (Owen-Smith et al., 2019). Future investigations into the method and concentration of spray delivery for Captan treatment would be warranted.

**Biodiversity.** The general trend observed during the 2021 experiment indicates that fungal communities were affected by the application of fungicidal canopy sprays, and that the communities differed not only by orchard, but also by chemical application. The overall trend for both conventional and organic orchards showed that fungal community diversity increased during the 2021 experiment. This is likely due to a multitude of factors including chemical treatment, as well as warming temperatures as spring approached. There was also a noticeable increase in fungal community diversity and number of isolates between conventional versus organic chemical treatments. The conventional sprayed isolations resulted in far fewer fungal isolates, as well as fewer unique fungal taxa, compared to the organic treatments. In apple orchards, previous research demonstrated higher pathogen pressure in organic orchards than conventional, suggesting that greater numbers of pathogens would likely be associated with higher fungal diversity in orchard systems (Amarante et al., 2008).

During the 2022 season, no discernable pattern occurred between treatments and the number of recovered fungal isolates and fungal diversity were fairly stable. The conventional orchard BT experienced several significant changes in Shannon's diversity through the experiment, however no pattern over time occurred, as diversity increased and decreased sporadically. It is likely that the cold damaged woody tissues in 2021 harbored a greater diversity of saprophytes in orchards RM and OM, increasing the mean diversity. This suggests

that damaged tissues allowed for saprophytes and other fungi to cohabitate on the damaged tissues. Orchard BT showed a lower mean diversity during 2021. This is likely attributed to the complete tissue death experienced in this orchard during the 2021 cold event. BT also experienced a large increase in the proportion of *Cytospora* detected within the fungal community between the 2021 and 2022 growing seasons. This is interesting because the detections of *C. plurivora* during the inoculation trials was unsubstantial during the 2022 season. This means that *Cytospora spp*. are being found in bud tissues without causing damage and disease. This finding suggests that the ecology of *Cytospora* is likely more complex than previously identified and warrants further investigation to resolve its habits as well as taxonomy.

Chemical type was indicative of the incidence of *C. plurivora* detected within orchards. The conventional orchards treated with Captan had much lower levels of *C. plurivora* when compared to the organic lime sulfur orchards. These findings suggest that Captan is more effective in reducing the incidence of *C. plurivora* in western Colorado peach orchards. These results, combined with increased fungal diversity and total collections of fungal taxa over time, suggests that chemical canopy sprays are effective in reducing *C. plurivora* in orchards after cold events.

The organic orchard RM, which applied lime sulfur combined with Nu-Film, experienced a significant change in the composition of fungal communities during the second-month post spray. This would align with the findings in the inoculation trials and suggests that lime sulfur with the addition of Nu-Film reduced the efficacy of lime sulfur by one month. It was demonstrated that the fungal communities increased at month two which also coincided with

an increased recovery of *C. plurivora.* The Captan orchards BT and OM, as well as the organic lime sulfur treatment orchard OO, each experienced a significant change in fungal communities at three-months post spray. Many studies have examined the effectiveness of Captan as a fungicide and sulfur compounds have been shown to be highly toxic to fungi (Biggs & El-Koholi, 1994; S. T. Miller et al., 2019; Northover, 1976).

Determining species composition within the characterized fungal genera was outside of the scope of this study, however, it would be interesting to understand the relationships that these individual organisms have with peach trees. There is potential for these organisms to be harmless endophytes or even pathogens of other crops, for which peach trees to act as a reservoir. For instance, the genus *Pyrenochaeta* includes many plant pathogens which live in soil globally and effect a wide range of annual crops. In tomatoes, members of *Pyrenochaeta* have been shown to cause up to 75% loss of yield (Giotis et al., 2009). A study examining fungal communities that cause lesions in apple and pear found that many of the pathogens could infect grape vines as well (Fourie & Crous, 2022). *Alternaria* is a cosmopolitan genus with over 100 species identified worldwide (Thomma, 2003). *Alternaria* comprised a large percentage of the fungal community in peaches and is a well known pathogen of peach. This genera causes black spot in peaches and other species may play a broader roll in the health of peach trees overall. Fungi in the genus *Fusarium* cause various forms of damping off, wilting, branch lesions, and cankers. *Setosphaera* spp. represented a large percentage of the fungal community associated with the lime sulfur spray and Captan. *Setosphaera* spp. were not detected in the lime sulfur and Nu-Film treatment. The genus *Setosphaera* includes species that cause leaf wilts and blights in maize (Dai et al., 2021). *Pyrenochaeta* were detected at the highest percentage in

the fungal communites in the lime sulfur treatment in orchard OO. Understanding fungal communites associated with peach trees has the potential to inform future management practices in surrounding agricultural lands, especially if they are in proximity to other annual crops and fruit orchards. Also, understanding the fungal and host interactions with peach trees could warrant further investigation into the potential of peaches to be a reservoir for pathogens.

**2.6 Conclusions**

Cold damage is difficult to determine without a quantitative assessment comparing actual cold damage between orchards. This is further complicated by differences between peach variety, with some varieties experiencing acclimatization earlier in the year, which may limit mortality during early freeze events (Minas et al., 2017). This experiment would have benefited from a quantitative analysis of cold damages observed in each orchard. This would have provided valuable insight into the actual effect cold damage played in these results. However, this study suggests that prophylactic chemical canopy sprays may have an important role in the management of Cytospora canker following cold damage events. Canopy sprays of Captan and lime sulfur were shown to lose efficacy three months post spray. It may be beneficial for growers to apply these chemicals on a schedule following cold damage to reduce incidence of *C. plurivora*. It was demonstrated that alpha diversity, as measured with SDI, of fungal communities increased over time after canopy sprays and the same held true for *C. plurivora* detection. Chemical canopy sprays are a wildly used tool that is effective in control of many diseases. These findings suggest that prophylactic sprays are also an effective management strategy for growers. Further research into the rate of application will be

necessary to determine the concentrations necessary to reduce infections within peach orchards.

**FIGURES AND TABLES**

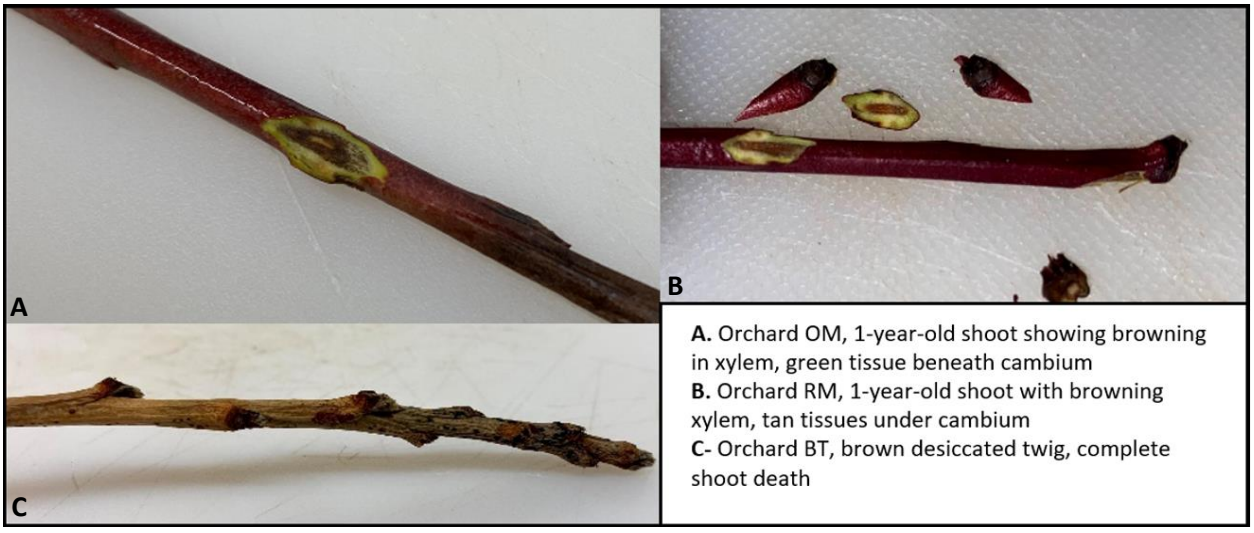


*Figure 2-1. Peach tree damage caused by freeze event in October 2020. A) Orchard OM exhibiting browning in xylem tissues due to freeze injury as well as damage to the apical meristem. B) Organic orchard OM exhibiting oxidative browning. C) Conventional orchard BT exhibiting complete death and desiccation of one-year-old shoot.*

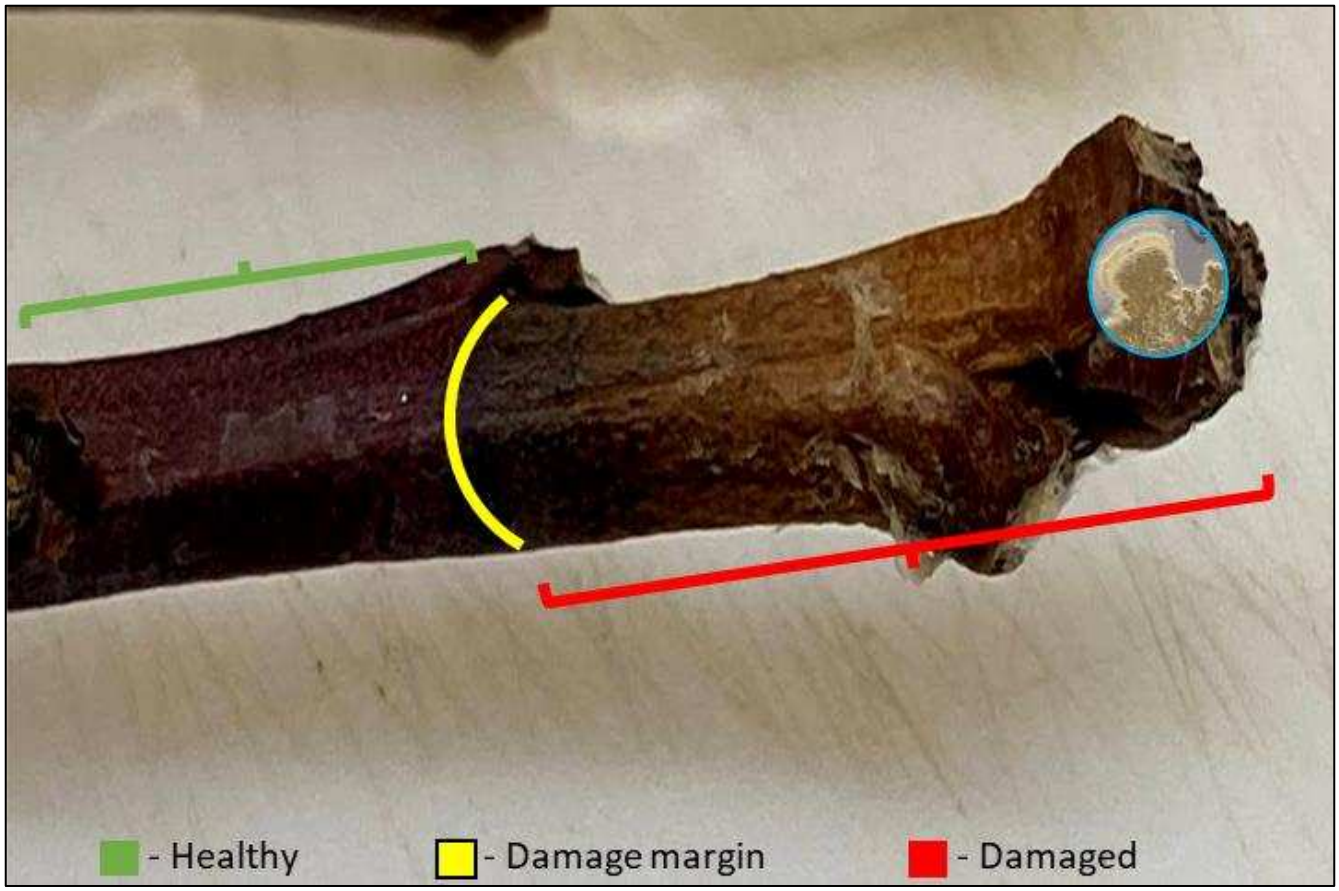


*Figure 2-2. Lesion margin and damage visible and color coded. The green bracket denotes healthy tissues while the yellow follows the damage margin. The red bracket denotes the damaged tissue from a suspected lesion. The blue circle represents the inoculation site with a 5mm agar plug of isolate CP5.1.*

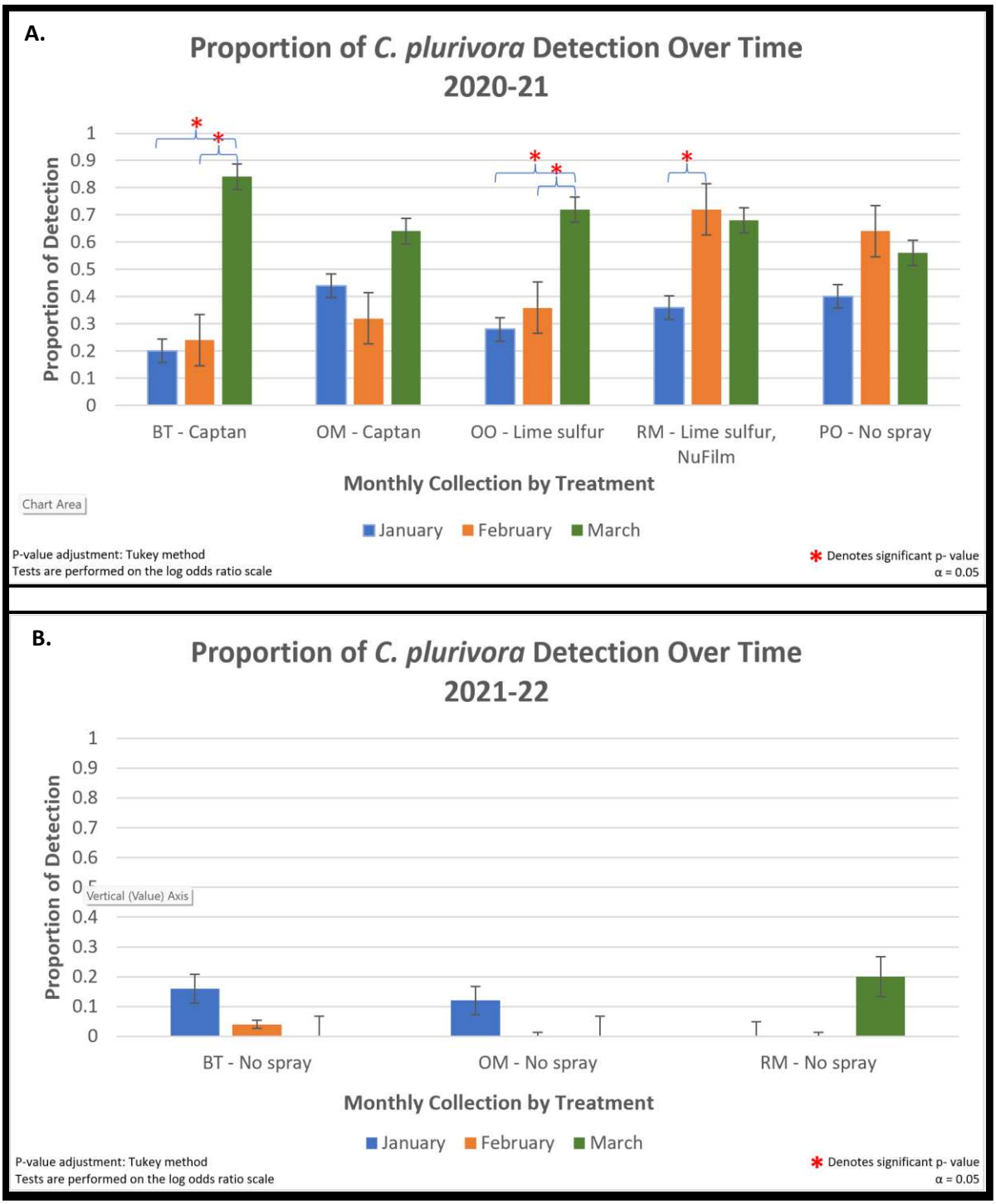


*Figure 2-3. A) Proportion of* Cytospora plurivora *Detection 2020-2021. Peach orchards located in western Colorado: Conventional orchards BT & OM, Organic orchards OO & RM, no spray orchard PO. Significant changes in detection are denoted with red asterisk. B) Proportion of Detection 2021-2022.Conventional orchards BT & OM, Organic orchard RM with no significant changes in detection between time periods. Orchards OO and PO did not participate in 2022.*

*Table 2-1. Chemical spray concentrations by month and year.*

| Chemical Spray Concentration by Year | | | |
|---|---|---|---|
| **Orchard** | **Type** | **2021** | **2022** |
| BT | Conventional | Captan - 3 qts/acre | No spray |
| OM | Conventional | Captan - 1.5 qts/acre | No spray |
| OO | Organic | Lime sulfur - 3% dilution rate | N/A |
| RM | Organic | Lime sulfur, Nu-Film - 3% dilution rate | No spray |
| PO | No spray | No spray | N/A |

*Table 2-2. Positive detections of* Cytospora plurivora *from inoculated peach branches in 2021 and 2022. Positive detections are represented out of 25 totals trees sampled per orchard.*

| **Positive Detections by Month and Year** | | | | | | |
|---|---|---|---|---|---|---|
| | 2020-2021 | | | 2021-2022 | | |
| **Orchard** | January | February | March | January | February | March |
| BT | 5 | 6 | 21 | 4 | 1 | 0 |
| OM | 11 | 8 | 16 | 3 | 0 | 0 |
| OO | 7 | 14 | 18 | No data | | |
| RM | 9 | 18 | 17 | 0 | 0 | 5 |
| PO | 10 | 16 | 14 | No data | | |

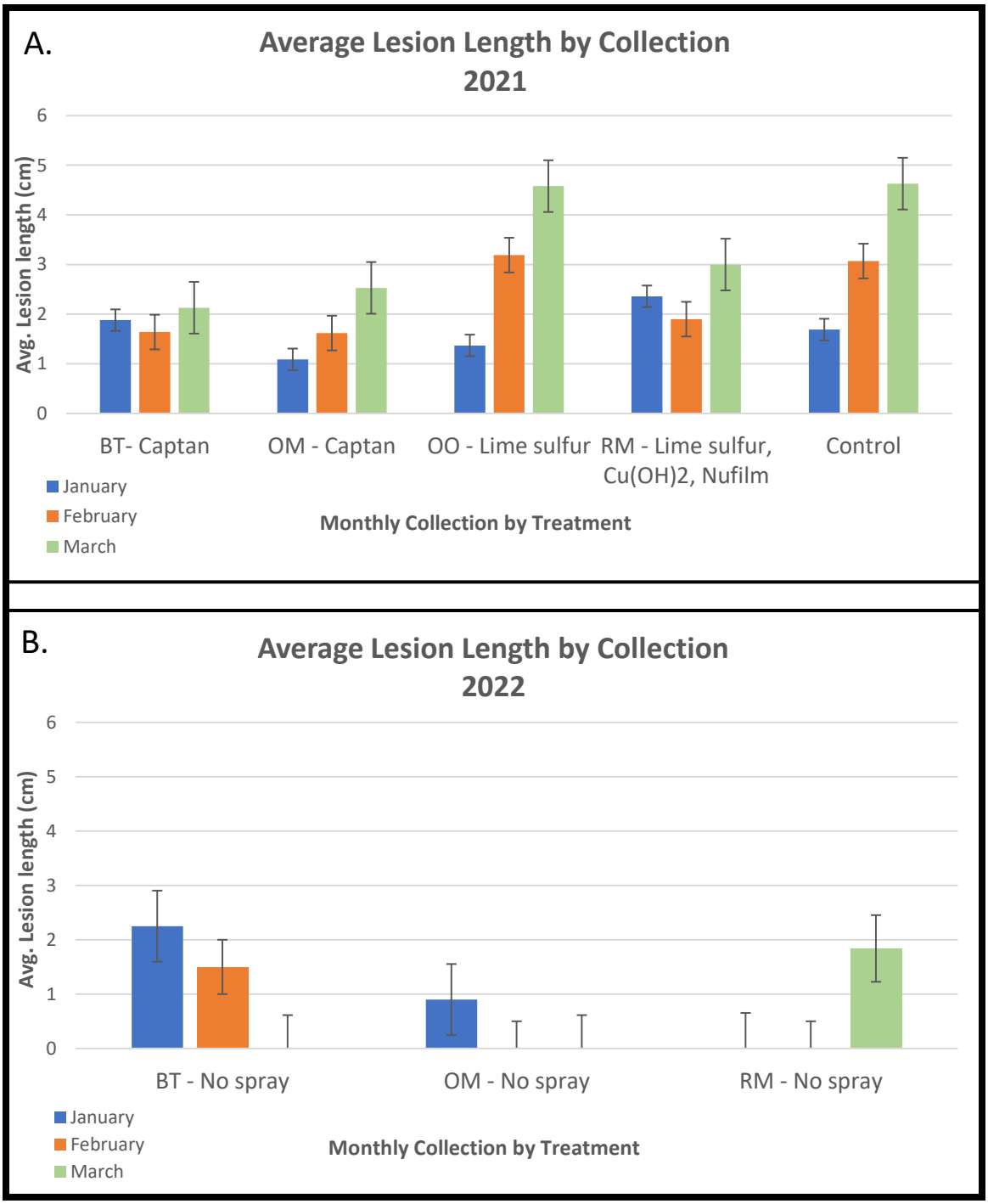


*Figure 2-4. A) Average lesion length in 1-year-old peach branches after inoculation with* Cytospora plurivora *during the months of January, February and March in 2021 (A) and 2022 (B).*

*Table 2-3. Chemical spray, count of peach trees positive or negative for the presence of Cytospora plurivora and average lesion size after inoculation with Cytospora plurivora during January, February and March in 2021 and 2021.*

| Spray, Count, and Lesion Data | | | | |
|---|---|---|---|---|
| **Orchard** | **Collection** | **Pos. Trees** | **Neg. Trees** | **Avg. lesion length (cm)** |
| BT - Captan | Jan. 2021 | 5 | 20 | 1.88 |
| BT - Captan | Feb. 2021 | 6 | 19 | 1.64 |
| BT - Captan | Mar. 2021 | 21 | 4 | 2.13 |
| OM - Captan | Jan. 2021 | 11 | 14 | 1.09 |
| OM - Captan | Feb. 2021 | 8 | 17 | 1.62 |
| OM - Captan | Mar. 2021 | 16 | 9 | 2.53 |
| OO - Lime sulfur | Jan. 2021 | 7 | 18 | 1.37 |
| OO - Lime sulfur | Feb. 2021 | 14 | 25 | 3.19 |
| OO - Lime sulfur | Mar. 2021 | 18 | 7 | 4.58 |
| RM - Lime sulfur, Badge, NuFilm | Jan. 2021 | 9 | 16 | 2.35 |
| RM - Lime sulfur, Badge, NuFilm | Feb. 2021 | 18 | 7 | 1.91 |
| RM - Lime sulfur, Badge, NuFilm | Mar. 2021 | 17 | 8 | 3.00 |
| PO - No spray | Jan. 2021 | 10 | 15 | 1.69 |
| PO - No spray | Feb. 2021 | 16 | 9 | 3.08 |
| PO - No spray | Mar. 2021 | 14 | 11 | 4.63 |
| BT- No spray | Jan. 2022 | 4 | 21 | 2.25 |
| BT- No spray | Feb. 2022 | 1 | 24 | 1.50 |
| BT- No spray | Mar. 2022 | 0 | 25 | 0 |
| OM - No spray | Jan. 2022 | 3 | 22 | 0.90 |
| OM - No spray | Feb. 2022 | 0 | 25 | 0 |
| OM - No spray | Mar. 2022 | 0 | 25 | 0 |
| RM - No spray | Jan. 2022 | 0 | 25 | 0 |
| RM - No spray | Feb. 2022 | 0 | 25 | 0 |
| RM - No spray | Mar. 2022 | 5 | 20 | 2.06 |

*Table 2-4. A) Total fungal isolates collected by orchard and month during 2021 (A) and 2022 (B). Orchard OO did not participate in 2022.*

**A.** **Isolates by orchard and month 2021**

| Time | BT | OM | OO | RM |
|---|---|---|---|---|
| December | 25 | 58 | 150 | 146 |
| January | 51 | 130 | 218 | 289 |
| February | 72 | 148 | 187 | 302 |
| March | 50 | 290 | 241 | 258 |
| Total | 198 | 626 | 796 | 995 |
| | | | TOTAL | 2615 |

**B.** **Isolates by orchard and month 2022**

| Time | BT | OM | OO | RM |
|---|---|---|---|---|
| December | 239 | 304 | N/A | 324 |
| January | 211 | 269 | N/A | 323 |
| February | 317 | 268 | N/A | 302 |
| March | 297 | 288 | N/A | 330 |
| Total | 1064 | 1129 | 0 | 1279 |
| | | | TOTAL | 3472 |

*Table 2-5. A) Total unique fungal morphotype counts in 2021 (A) and 2022 (B)* by month*. Orchard OO did not participate in 2022.*

**A. Morphotype by orchard and month 2021**

| Time | BT | OM | OO | RM |
|---|---|---|---|---|
| December | 17 | 33 | 41 | 49 |
| January | 22 | 23 | 67 | 62 |
| February | 32 | 33 | 47 | 68 |
| March | 25 | 55 | 63 | 56 |
| Orchard Total | 96 | 144 | 218 | 235 |
| | | | Total | 693 |

**B. Morphotype by orchard and month 2022**

| Time | BT | OM | OO | RM |
|---|---|---|---|---|
| December | 36 | 42 | N/A | 33 |
| January | 67 | 27 | N/A | 26 |
| February | 39 | 39 | N/A | 31 |
| March | 42 | 34 | N/A | 32 |
| Orchard Total | 184 | 133 | 0 | 122 |
| | | | Total | 439 |

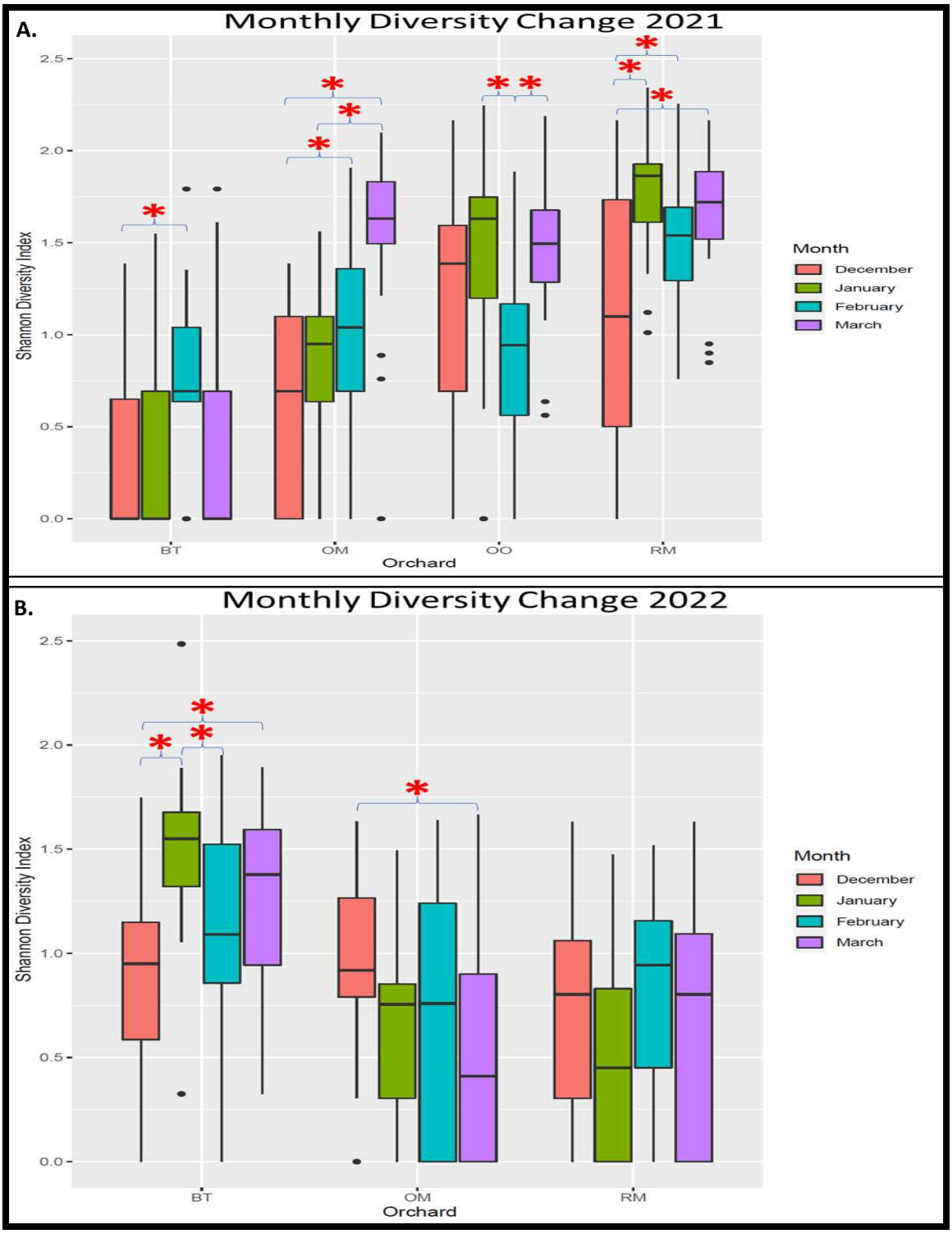


*Figure 2-5. A) Mean Shannon Diversity Index score over months by peach orchard 2021 (A) and 2022 (B). Orchard OO did not participate in 2022. Stars (*) denote significance at P=0.05)*

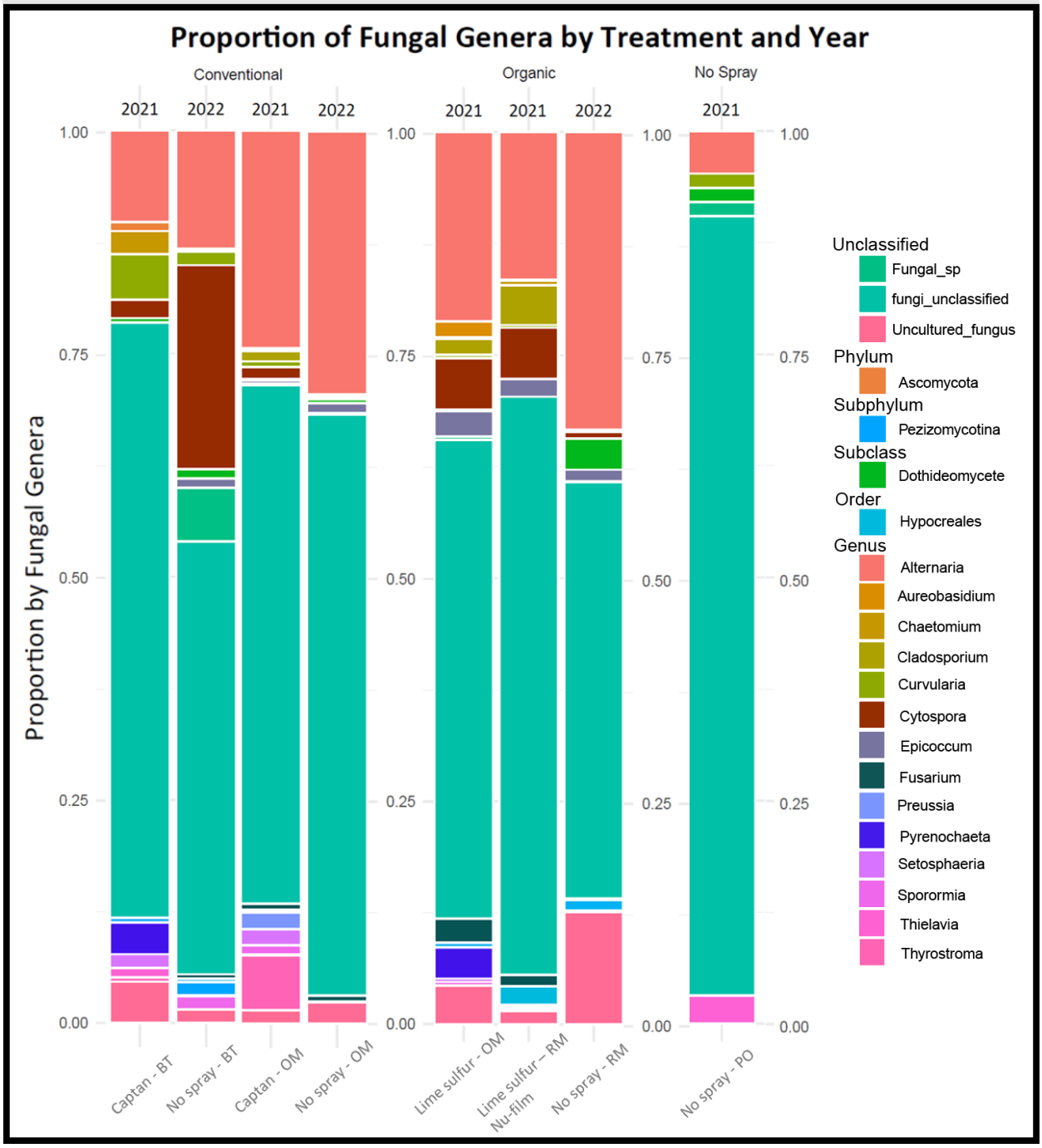


*Figure 2-6. Relative proportions of fungal genera by treatment and year in western Colorado peach orchards 2021 and 2022.*

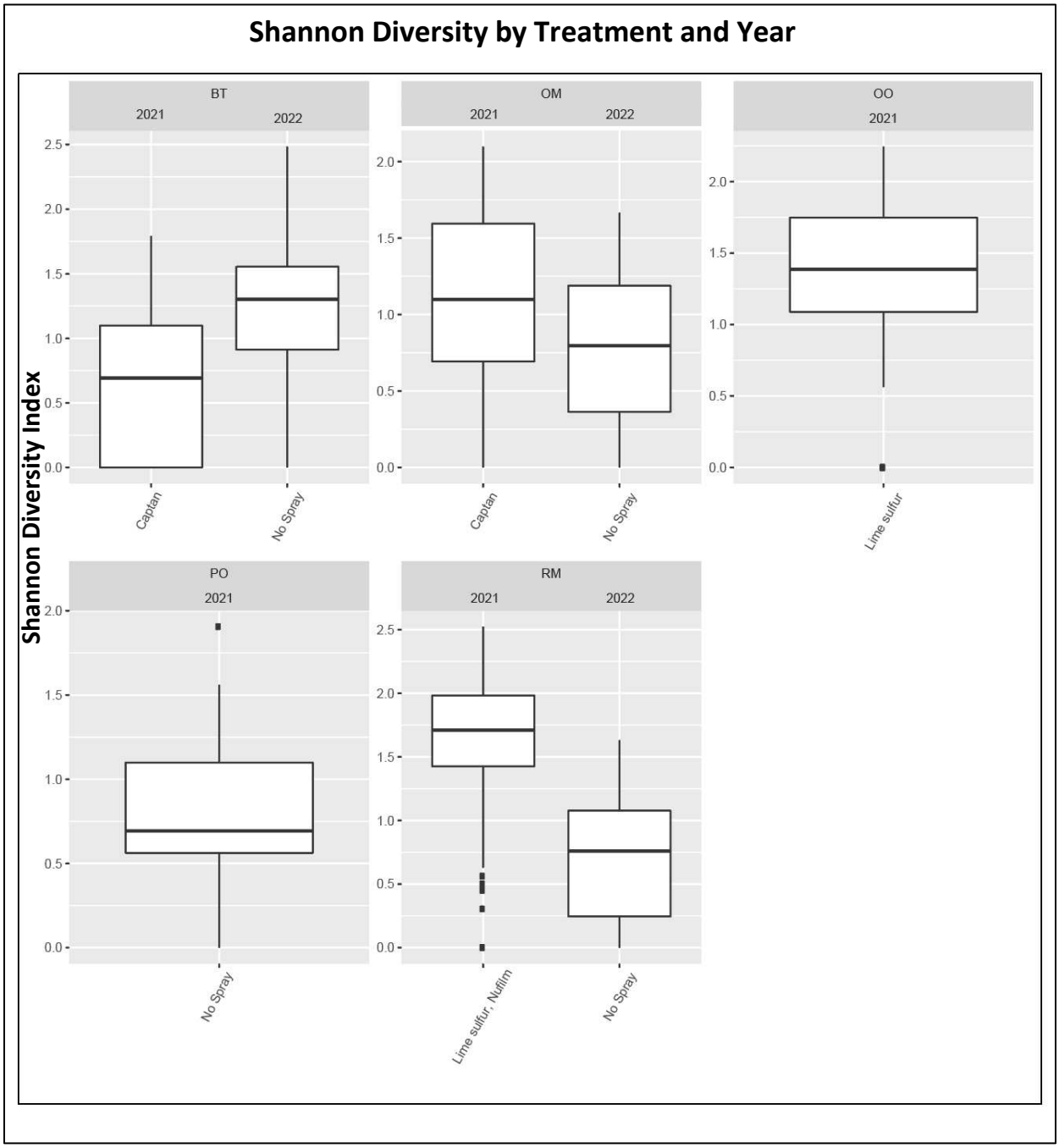


*Figure 2-7. Box plots of the Mean Shannon Diversity Index score* by treatment and year *in western Colorado peach orchards 2021 and 2022*.

## Literature Cited


Amarante, C. V. T. do, Steffens, C. A., Mafra, Á. L., & Albuquerque, J. A. (2008). Yield and fruit quality of apple from conventional and organic production systems. *Pesquisa Agropecuária Brasileira*, *43*(3), 333–340. https://doi.org/10.1590/S0100-204X2008000300007

Ashworth, E. N., & Wisniewski, M. E. (1991). Response of Fruit Tree Tissues to Freezing Temperatures. *HortScience*, *26*(5), 501–504. https://doi.org/10.21273/HORTSCI.26.5.501

Biggs, A. R. (1989). Integrated Approach to Controlling Leucostoma Canker of Peach in Ontario. *Plant Disease*, *73*(11), 869. https://doi.org/10.1094/PD-73-0869

Biggs, A. R., & El-Koholi, M. M. (1994). *Biggs—Effect of calcium salts on growth, pectic enzyme activity, and colonization of peach twigs by Leucostoma persoonii.pdf*. *78*(9), 886–890.

Burke, M. J., Gusta, L. V., Quamme, H. A., Weiser, C. J., & Li, P. H. (1976). Freezing and Injury in Plants. *Annual Review of Plant Physiology*, *27*(1), 507–528. https://doi.org/10.1146/annurev.pp.27.060176.002451

Dai, Y., Gan, L., Lan, C., Lu, X., Yang, X., & Gao, Z. (2021). Genetic Differentiation and Mixed Reproductive Strategies in the Northern Corn Leaf Blight Pathogen Setosphaeria turcica From Sweet Corn in Fujian Province, China. *Frontiers in Microbiology*, *12*, 632575. https://doi.org/10.3389/fmicb.2021.632575

Fourie, H., & Crous, W. (2022). *Fungi associated with die-back symptoms of apple and pear trees, a possible inoculum source of grapevine trunk disease pathogens*. 16.

Fox, J., Weisberg, S. (2019). An {R} Companion to Applied Regression, Third Edition. Thousand Oaks CA: Sage. URL: https://socialsciences.mcmaster.ca/jfox/Books/Companion/

Giotis, C., Markelou, E., Theodoropoulou, A., Toufexi, E., Hodson, R., Shotton, P., Shiel, R., Cooper, J., & Leifert, C. (2009). Effect of soil amendments and biological control agents (BCAs) on soil-borne root diseases caused by Pyrenochaeta lycopersici and Verticillium

albo-atrum in organic greenhouse tomato production systems. *European Journal of Plant Pathology*, *123*(4), 387–400. https://doi.org/10.1007/s10658-008-9376-0

Grove, G. G., & Biggs, A. R. (2006). Production and Dispersal of Conidia of *Leucostoma cinctum* in Peach and Cherry Orchards Under Irrigation in Eastern Washington. *Plant Disease*, *90*(5), 587–591. https://doi.org/10.1094/PD-90-0587

Helton, A. W., & Konicek, D. E. (1962). An optimum environment for the culturing of cytospora isolates from stone fruits. I. Temperature. *Mycopathologia et Mycologia Applicata*, *16*(1), 19–26. https://doi.org/10.1007/BF02136177

Lenth, R.V. (2022). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.7.2. https://CRAN.R-project.org/package=emmeans

Logan, J., Mueller, M. A., & Searcy, M. J. (2000). Microclimates, Peach Bud Phenology, and Freeze Risks in a Topographically Diverse Orchard. *HortTechnology*, *10*(2), 337–340. https://doi.org/10.21273/HORTTECH.10.2.337

Miller, S. T. (2017). *Chemical control of Cytospora leucostoma, a major limiting factor of peach production in western Colorado*. Colorado State University.

Miller, S. T., Otto, K. L., Sterle, D., Minas, I. S., & Stewart, J. E. (2019). Preventive Fungicidal Control of *Cytospora leucostoma* in Peach Orchards in Colorado. *Plant Disease*, *103*(6), 1138–1147. https://doi.org/10.1094/PDIS-05-18-0801-RE

Miller, S. T., Sterle, D., Minas, I. S., & Stewart, J. E. (2021). Exploring fungicides and sealants for management of Cytospora plurivora infections in western Colorado peach production systems. *Crop Protection*, *146*, 105654. https://doi.org/10.1016/j.cropro.2021.105654

Minas, I. S., Sterle, D., & Caspari, H. (2018). *Cold hardiness assessment of peach flower buds using differential thermal analysis (DTA) in western Colorado (dormant season 2016-17)*. 9.

Minas, I. S., Tanou, G., & Molassiotis, A. (2018). Environmental and orchard bases of peach fruit quality. *Scientia Horticulturae*, *235*, 307–322. https://doi.org/10.1016/j.scienta.2018.01.028

Molina, L., Rajchenberg, M., de Errasti, A., Aime, M. C., & Pildain, M. B. (2020). Sapwood-inhabiting mycobiota and Nothofagus tree mortality in Patagonia: Diversity patterns according to tree species, plant compartment and health condition. *Forest Ecology and Management*, *462*, 117997. https://doi.org/10.1016/j.foreco.2020.117997

Northover, J. (1976). Protection of Peach shoots against species of Leucostoma with Benomyl and Captafol.PDF. *Phytopathology®*, *66*, 1125–1128. https://doi.org/10.1094/Phyto-66-1125

Oksanen, J., Blanchet, F.G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P.R., O'Hara, R. B., Simpson, G.L., Solymos, P., Henry, M., Stevens, H., Szoecs, E., Wagner, H. (2020). vegan: Community Ecology Package. R package version 2.5-7. https://CRAN.R-project.org/package=vegan

Owen-Smith, P., Perry, R., Wise, J., Jamil, R. Z. R., Gut, L., Sundin, G., & Grieshop, M. (2019). Spray coverage and pest management efficacy of a solid set canopy delivery system in high density apples. *Pest Management Science*, *75*(11), 3050–3059. https://doi.org/10.1002/ps.5421

Pokharel, R. (2013). *Cytospora Canker in Tree Fruit Crops* (Fact Sheet 2.953; p. 6). Colorado State University.

Rosenberger, D. (1982). *Biology and control of Cytospora fungi in peach plantings* (No. 92; New York's Food and Life Sciences Bulletin). New York Agricultural Experiment Station.

Shane, W. (2019). *Cold damage to peaches*. Michigan State University Extension. https://www.canr.msu.edu/news/cold-damage-to-peaches

Thomma, B. P. H. J. (2003). *Alternaria* spp.: From general saprophyte to specific parasite: *Alternaria*. *Molecular Plant Pathology*, *4*(4), 225–236. https://doi.org/10.1046/j.1364-3703.2003.00173.x

USDA. (2021). *Noncitrus Fruits and Nuts 2021 Summary 05/04/2022*. National Agricultural Statistics Service.

*USDA ERS* (2021). *Fruit & Tree Nuts*. https://www.ers.usda.gov/topics/crops/fruit-tree-nuts/#periodic

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols* (pp. 315–322). Elsevier. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

# CHAPTER 3 – DETECTION AND EPIDEMIOLOGY OF *C. PLURIVORA* UNDER DIFFERENT IRRIGATION PRACTICES

## 3.1 Introduction

Non-citrus fruit tree production is an important staple in U.S. agriculture, valued at over 6 billion dollars in 2021 (USDA, 2022). Pome fruits are the most popular non-citrus crop in the U.S. and comprise approximately half the aggregated utilized production, with peaches (*Prunus persica*) coming in second at 624 million dollars nationally in 2021. The demand for peaches rose 7% from 2020-2021, yet the bearing acreage decreased by 4% during that same time period (USDA, 2021). With consumer demand increasing and the producible acreage shrinking, it is necessary to understand the drivers behind reductions in production. There are a variety of factors that contribute to a decrease in usable acreage, with one main driver being disease.

Peach trees require rigorous management practices because of their susceptibility to diseases as well as the prevalence of diseases within orchards. Many diseases negatively affect peach production through destruction of fruit, leaves, and woody tissues. Peach fruit is susceptible to *Monilinia fruiticola* infection, causal organism of brown rot, as well as *Ventruia carpophila* infection, causal organism of peach scab. These two fruit diseases result in large volumes of crop losses globally (Batra, 1991). Leaves are also susceptible to *Wilsonomyces carpophilus* (Coryneum blight) and *Taphrina deformans* (peach leaf curl). These pathogens are destructive, but can be managed effectively with chemical regiments and sanitation (Layne and Bassi, 2008). When infections occur in woody tissues, the severity of economic losses increase as tree health is adversely affected or trees die. Fungal pathogens *Botryosphaeria dothides*,

*Phomopsis amygdali*, and *Cytospora plurivora* all participate in the decline of peach tree health in the United States. Of these, *Cytospora* is most prevalent in the northern peach growing regions, including *C. plurivora* in Colorado, which significantly reduces the longevity of peach trees (Adams et al., 2002; Stewart et al., 2022). Infection by *C. plurivora* cannot be eradicated once established and intensive management is required to control infection. Understanding the epidemiology of *C. plurivora* is imperative to developing effective management practices.

A fungal spore is the reproductive structure used for the dispersal of fungi. Asexual spores, conidia borne in pycnidia, have been demonstrated to serve as the dominant source of inoculum in walnut orchards with *Cytospora* spp. infections (Luo et al., 2020). Pycnidia can be found embedded in canker material. During the first two years of infection, only conidia have been detected on cankered branches, and the sexual form (release of ascospores from within a perithecium) of *C. leucostoma* was only observed after girdling and dieback of the limb had occurred (Bertrand & English, 1976). Both conidia and ascospores have been detected in every month of the year, although conidia are at much higher quantities and are likely the driver of infection (Bertrand & English, 1976; Grove & Biggs, 2006). Many fungi will not release spores until wetted, either by rain or irrigation, and previous studies have identified water dissemination as the main driver of spore dispersal for *Cytospora* pathogens, including windblown rain and sprinkler irrigation (Barakat, 1995; Meredith, 1973). A previous study detected conidia 76 meters from the nearest Cytospora canker, which was thought to be carried via windblown rain in peaches (Bertrand & English, 1976). These findings are important in further understanding of the dynamics of irrigation in dispersal of spores in orchard settings.

Methods of irrigation play an important role in the incidence of disease, as well as dispersal of pathogen inoculum within orchard settings. Several factors can affect the ability of irrigation to disperse pathogens including irrigation type, watering duration, and leaf wetness duration. Orchards using under-the-canopy impact sprinklers dispersed conidia up to 5.9 meters from the canker source, however, no pattern of spread could be determined (Grove & Biggs, 2006). Reducing the trajectory of irrigation significantly reduced incidence of Botryosphaeria blight in pistachio orchards (Michailides et al., 1993). Likewise, the time spent irrigating is directly correlated to leaf wetness, which stimulated production of spores in fungal pathogens. Increasing the duration of leaf wetness through overhead watering contributed to higher infection rates by the fungal plant pathogens *Colletotrichum*, *Phyllosticra*, and *Alternaria* in citrus crops (Bassimba et al., 2017). Understanding how irrigation affects the distribution of inoculum in an orchard is an important step in recommending effective management practices to peach producers.

The objective of this study was to first identify if *C. plurivora* conidia disperse during micro-sprinkler and drip irrigation; as well as to assess the differences between irrigation type, time, and distances of dispersal of *C. plurivora* in western Colorado peach orchards. Digital droplet PCR (ddPCR) was used to quantify the amount of inoculum present.

### 3.2 Materials and Methods

**Field site design.** Two experimental peach blocks were located at the Western Colorado Research Center - Orchard Mesa (WCRC-OM) Grand Junction, Colorado. Each block consisted of 288 peach trees with a tree spacing of 1.6 m and row spacing of 3.6 m. All trees were trained in a perpendicular V-shape with 2 main scaffolds, similar to current pruning guidelines used in

Colorado orchards. To ensure previous *C. plurivora* infections were not releasing spores, the trunks of each tree was covered by painting on thiophanate-methyl Topsin-M (Nippon Soda Company Tokyo, Japan) amended in 50% latex paint prior to experimentation as described by Miller et al. (2021). Each block was split into two treatments, one drip and one micro sprinkler, enabling replication of treatments. A buffer row was used between the edges and treatments. Block one consisted of 4-year-old peach trees and block 2 was planted with 2-year-old peach trees at the time of experimentation.

***C. plurivora* inoculum preparation.** Two-year old potted peach trees were maintained in the greenhouse at 27$^{o}$C with 12 hours of light per day. Trees were inoculated with *C. plurivora* isolate CP5.1 (Miller, et. al. 2019). This isolate was selected because of its robust formation of pycnidia and large amount of conidia production (Miller et al., 2019). Each tree was inoculated in five locations, six inches apart on the main stem. Pycnidia formation occurred approximately 10 days post inoculation and the amount of pycnidia on each canker was determined using a Meiji EM-50L stereo microscope (Meiji Techno San Jose, California) at 45x magnification. Cankers were then assigned numbers and randomly selected for each treatment and replicate. A spore count was conducted every two weeks to verify the presence of conidia at each collection point (Table 3-1). If zero conidia were detected upon conducting counts, the canker was exchanged for a viable spore producing canker.

**Field trial design.** Six-inch sections containing the CP5.1 induced cankers were zip-tied to eight trees per treatment, with four cankers facing east and four facing west. Spore traps were constructed using a 1.2 m section of 91 cm acrylonitrile butadiene styrene pipe cut in half longitudinally, a funnel, and a ½ gallon jug similar to the construction described in Bertrand and

English (1976) (Figure 3-1). Three spore traps were then placed in an adjacent row at 45$^{o}$, 90$^{o}$, and 135$^{o}$ angles at a distance of 3.6 m from the canker. One spore trap was placed in each successive row at distances of 7.2 m, 10.8 m, and 14.4 m (Figure 3-2).

**Irrigation treatments.** The micro-sprinkler treatments received two weekly irrigations of four hours at a rate of 45 L per minute and the nozzles delivered irrigation water at a rate of 45 L per minute, spaced every 3.2 m within the row. Drip irrigation treatments received two weekly waterings for 4 hours with approximately 2.5 cm of water applied in total. The drip emitters were spaced every foot on the line and water was emitted at a rate of 2 L per hour.

**Irrigation collection.** The night prior to collection, cankers were misted with DI water. This step was conducted to raise the ambient humidity of each canker to stimulate sporulation. The following morning each spore trap was washed with 15ml sterile DI water. This step was used to standardize between the micro-sprinkler and drip irrigation as no water could be collected in the drip irrigation without a wash. The irrigation was then allowed to run for 15 minutes and shut off while water jugs were collected. Irrigation was then started again and run for the remaining 3.5 hours. Each collection week, 195 samples were collected, which consisted of 96 drip irrigation samples split into two replicates, 96 micro sprinkler samples split into two replicates, and 3 non-irrigated control samples. Jugs were collected and total liquid volumes were recorded for each collection. A final maximum volume of up to 200ml was separated after agitating the jugs, and transferred into a falcon tube. Any volume over the 200ml sample volume was discarded from the experiment. The 200ml aliquot was then used for processing. This study spanned an eight-week period from June 7, 2021 to July 27, 2021, with collections made every other week.

**Processing.** Collected samples were kept at 4°C until processing. Tubes were spun down at 5000rpm for 4 minutes in an Eppendorf Centrifuge 5804R (Hamburg, Germany). Randomized microscope-aided visual checks of the supernatant were conducted to assess the efficiency of centrifuging. A vacuum pump (Fisher Scientific G180GDX, Hampton, NH) was then used to remove supernatant to a volume of 5ml. A pipette was then used to remove excess water to a volume of approximately 0.5ml. The remaining pellet was agitated back into solution and transferred to a 2ml microcentrifuge tube. The tubes were then spun down at 15000rpm in a microcentrifuge for three minutes to repalletize the debris and material suspended in water. An Eppendorf Vacfuge plus (Hamburg, Germany) was used to desiccate the samples at 30$^{o}$C for 2.5 hours.

**Extraction.** Two glass beads were added to the 1.5ml tubes containing the debris pellet. The tubes were then submerged in liquid nitrogen and a FastPrep-24™ (MP Biomedical, San Diego, CA) was conducted to facilitate lysing of spores. This step was completed three times. DNA extractions on each sample were then conducted using Qiagen DNeasy PowerSoil Pro Kit (Hilden, Germany). The recommended extraction protocol was followed except that all centrifuge steps were extended to 1.5 minutes. Samples were eluted to volume of 30µl.

**Digital Droplet PCR.** DNA was shredded using QIAshredder columns (Qiagen, Hilden, Germany) prior to ddPCR use. All assays were conducted in 20µl reactions described in Stewart et al. (2022): 10µl ddPCR supermix (No dUTP), 0.7µl of each primer Cyto.Ef1a.24F and Cyto.Ef1a.23R, 0.3µl Cyto.Ef1a.147P, 7.3µl $H_2O$, and 1µl of DNA template. The samples were then loaded in the cassette with 70µl of droplet oil and the cassette was placed in the QX200 Droplet generator (Bio-Rad Laboratories Hercules, CA). Droplets were transferred to a 96 well

plate and sealed using foil in the PX1 plate sealer (Bio-Rad Laboratories Hercules, CA). PCR was conducted at the following parameters: 10 minutes at 95$^{o}$C, followed by 40 cycles of 94$^{o}$C at 30 seconds, 55$^{o}$C for 30 seconds, and 72$^{o}$C for 1 minute, followed by an incubation hold at 98$^{o}$C for 10 minutes, and a 4$^{o}$C infinite hold (Miller, 2021). Droplets were then read on the QX200 Droplet Reader (Bio-Rad Laboratories Hercules, California).

**Controls.** A spore count of *C. plurivora* isolate CP5.1 was conducted, and a serial dilution was created to determine the number of translation elongation factor 1α (TEF1α) gene copies per ml. *TEF1α* is a single copy gene and as such, each individual conidia contains only one copy. This allows for a quantification of the amount of conidia present in a sample. Serial dilutions were as follows: 1000, 500, 100, 50, and 5 copies/ml (Figure 3-3). A positive control was created using isolate CP5.1 which was diluted to 1ng/ml of DNA and used for all ddPCR reactions. Negative controls consisted of 3 spore collectors set at 135M away from the experimental blocks, as well as autoclaved irrigation water, and a twice autoclaved spore suspension at a concentration of 20,000 spores/ml.

**Analysis.** Due to the small number of positive detections, a descriptive analysis was conducted. Comparisons were conducted over time on a biweekly collection basis by analyzing the differences in detections between drip and micro-sprinkler irrigation. Distances that conidia were collected from the canker were also summarized, as well differences between the replicates, which varied slightly.

All ddPCR reactions were assessed using the JavaScript tool 'definetherain', which was used to identify the amplitude cutoff for positive droplets (http://www.definetherain.org.uk) (Jones et. al., 2014). A false positive rate was calculated, and a limit of detection was used to

identify the true positive detections. All reaction wells with >11 droplets were considered a positive detection for *C. plurivora.* This was conducted at a 95% confidence interval.

### 3.3 Results

**Total *C. plurivora* detections.** A total of 18 positive detections of *C. plurivora* were observed during the experiment. Positive detections were defined using the ddPCR assay. In Figure 3-4, the pink line indicates the required amplitude of detection. The cutoff amplitude is defined by each ddPCR 96 well plate reaction. Any blue droplet above the amplitude of 3961 was considered a positive detection of *C. plurivora* and grey droplets below that amplitude are called "rain." All droplets below the threshold amplitude are false positives and were not included in this study (Figure 3-4). Zero positive detections of *C. plurivora* conidia were observed during the first collection of the study while each successive collection had positive detections for *C. plurivora*. The second collection time point had 5 of 18 total positive detections (28%), collection 3 had 7 of 18 total positive detections (39%), and collection 4 had 5 of 18 total positive detections (28%), and1 of 18 positive detections (5%) was made in the control during the second collection time (Table 3-2).

**Positive detection by irrigation type.** Positive detections were found to be the most abundant in the drip irrigation treatment consisting of 61.1% (11:18) total collections. Micro-sprinkler treatments comprised 33.3% (6:18) of the total positive detections. One positive detection was made in the non-irrigated control and made up 5.5% (1:18) of the positive detections (Table 3-2).

**Positive detection by directionality.** Positive detections were also assessed based on which cardinal direction the spore traps faced. During the experiment, 56% (10:18) of positive detections were made in the west facing traps. When assessed by irrigation type, 6 of these positive detections came from the drip irrigation, 3 in micro-sprinkler, and 1 in the control. In the eastern facing spore traps, detections were made in 5 drip irrigation traps and 3 in the micro-sprinkler traps.

**Positive detection by distance.** Positive detections of *C. plurivora* were found at all distances in the study (3.6m, 7.2m, 10.8m, 14.4m, 135m). Of the total collection, 61.1% (11:18) of the positive detections occurred at the three closest spore traps, located at 3.6m from the attached canker at the $45^{o}$, $90^{o}$, and $135^{o}$ positions. The range of positive droplets detected at the 3.6m spore traps varied between 11 to 43 droplets. Two positive detections were collected at each following 7.2m, 10.8m, and 14.4m, equaling 11.1% of the total collections per distance. The 7.2m traps had positive detections between 11 to 18, 10.8m had a range of 17 to 20, and 14.4 had 15 positive droplets. One collection (5.6%) occurred in the control plot located 135m away from the experimental blocks with 15 positive droplets identified in this reaction (Figure 3-5).

**Positive detection by replicate.** When assessing positive detections by replication block, the majority of detections (55.6% or 10:18) were located in the four-year-old tree block Replication 1 (Rep1) which consisted of both Rep1 drip and Rep 1 micros-sprinkler irrigation. *C. plurivora* was detected seven times (36.8% or 7:18) in Replication block 2 (Rep2), which consisted of two-year-old trees. One collection was made in the control over the course of the

experiment during third collection time. During the first, second, and fourth collections, the controls were negative for *C. plurivora*.

### 3.4 Discussion

Collections were made at all distances throughout the study (3.6m, 7.2m, 10.8m, 14.4m) including at one control collection point located 135m away from the experimental blocks. Of these collections, a majority (55.6%) were detected at the closest distance from the Cytospora canker. The radius of micro sprinkler throw is approximately 4.5m depending on pump pressure. Bertrand and English (1976) demonstrated that the highest number of conidia were captured at the closest distance to the canker source and gradually decreased as distance increased. This data agrees with this previous study and demonstrates the most abundant detections of *C. plurivora* were made at the closest spore trap at 3.6m distance. There was also no discernable pattern to the amount of spores collected at each distance as detections ranged between 11 positive drops to as high as 44.

It is important to note that the control spore traps were placed in a grass field, which had no irrigation associated with the site, and were placed roughly 135m m from the nearest peach. A positive detection in the control trap suggests that *C. plurivora* conidia are moving considerably farther than previously identified. Windblown conidia or windblown irrigation water is likely the mode of dissemination. Although *C. plurivora* had higher detections in rain water, it was still detected in both water and air samples using ddPCR in walnut orchards and in air samples, as well as on insects in in Colorado peach orchards (Luo et al., 2020; Miller 2021). This current study identified movement of *C. plurivora* conidia further than previously

demonstrated, however, future research is needed to determine the method of dispersal at these longer distances.

The highest number of collections (61.1%) occurred in the drip irrigation blocks. This finding was surprising as it was expected that more detection would have occurred with the water splash associated with micro-sprinkler irrigation. There was also no irrigation applied directly to the spore trap in the drip irrigation experimental blocks. These findings could be explained by two main mechanisms. One possibility is that the irrigation treatment blocks were too close to each other and windblown water from the micro-sprinkler irrigation blew into the drip irrigation spore traps. The second possibility is that the standardization spray of 15ml of water applied to each canker prior to the collection times raised the ambient humidity around the canker. This led to sporulation, allowing spores to be windblown into the collection traps. With conidia being detected in the control 135m away from the treatments, it is plausible that conidia may have traveled from the micro-sprinkler or drip treatment through either windblown irrigation or wind dispersal. Future experiments would be needed to rule out the possibility of conidia movement from one treatment to the other through greater distances between peach blocks.

Only 33.3% of the positive detections were observed in the micro-sprinkler treatments. This was lower than what was expected given the wide range of previous work showing that water assists in the dissemination of *Cytospora* conidia. It is possible that the methods used in this study altered and decreased the levels of detection. There were multiple steps during the collections process that could have excluded conidia from the final extraction steps. Large volumes of irrigation water were collected in the micro-sprinkler treatments. It was necessary

to subsample these larger volumes and establish a cutoff so that processing was feasible. These subsamples may have contained a fraction of the true spore totals thereby decreasing the ability to detect spores via the ddPCR assay.

There is also a possibility that conidia were lost during the processing steps, specifically during centrifugation. This step was accomplished by spinning the irrigation water at 5000rpm. If conidia remained suspended it could have magnified losses. Previous research indicated that a differential water/sucrose centrifugation techniques produced 185% more spores from the final soil extraction (Ianson & Allen, 2018). This centrifuge technique may have optimized recovery of *C. plurivora* conidia during the experiment.

DNA extraction methods may also have contributed to losses due of the sensitivity of the kit as well as the method of extraction. The final product from centrifugation was a dried debris pellet of ~0.03g. The mass of this soil was approximately 10% of the required amount per instructions on the Qiagen DNeasy Power Soil kit. Many extraction techniques were tested to include Chelex100, CTAB and and Zymo Quick-DNA miniprep (Zymo Research Irvine, CA) however, the power soil kit yielded the best results.

ddPCR is an extremely sensitive and accurate molecular biology technique which is used to quantify DNA (Li et al., 2018). This tool is unparalleled in its ability to quantify amplified DNA, however, limitations still exist. The threshold for detection can become increasingly blurred when small quantifications are being made. The main limitation is the exact threshold of detection between positive and negative droplets (Jones et al., 2014). Currently there is no consensus now how to handle low signal readouts versus “rain” or experimental noise. Previous

work examining circulating tumor DNA highlighted that many studies have used arbitrary amplitudes, random static amplitudes, or statistical approaches to deal with this detection issue, making standardization across studies difficult (Henriksen et al., 2022). The work presented here utilized the current available statistical approach with the 'definetherain' tool. However, with detection at such low copy numbers, it is possible that the threshold may have missed some positive detections. The difference between 1 droplet for the false positive rate would add many data points back into the positive category in this study.

**3.5 Conclusion**

This study demonstrates that *C. plurivora* conidia are capable of movement at much further distances than previously shown. This is supported by findings in the control as well as the drip irrigation spore traps. It is also likely that the sampling techniques were not sufficient to capture all conidia versus the large volumes of water collected. Drip and micro-sprinkler irrigation should be studied further to determine the conidial movements within each treatment. If irrigation is mimicking windblown rain, it very likely that current irrigation practices are exacerbating the spread of *C. plurivora* in western Colorado peach orchards. Although conidia were found to be higher than expected in the drip treatments, it is unlikely that cankers would receive moisture similar to that in commercial orchards. The process of wetting the canker prior to irrigation likely allowed sporulation to occur. It is possible that this would allow for spore dispersal in the drip treatment. When combined with a smaller volume of watering being sampled, detections were likely skewed due to sample collection and processing steps.

Nevertheless, drip irrigation is less likely to wet the canker material during irrigation which is required for the movement of fungal spores to infection courts (Grove & Biggs, 2006). Not only does drip irrigation reduce moisture content on cankered material, but it is also beneficial for growers to reduce their water consumption as water scarcity is increasing. These findings demonstrate that more conidia were detected in drip treatments and that conidia dispersal is greater than previously detected. However, the benefits of drip irrigation in reduction of pathogens and water savings likely outweigh the epidemiological implications of conidia dispersal in orchards.

**FIGURES AND TABLES**


*Figure 3-1. Spore collectors. These spore collectors are comprised of a 3inch PVC pipe cut in half and attached to a t-post with zip ties. The half-gallon jugs were used as a means of collecting the irrigation water.*

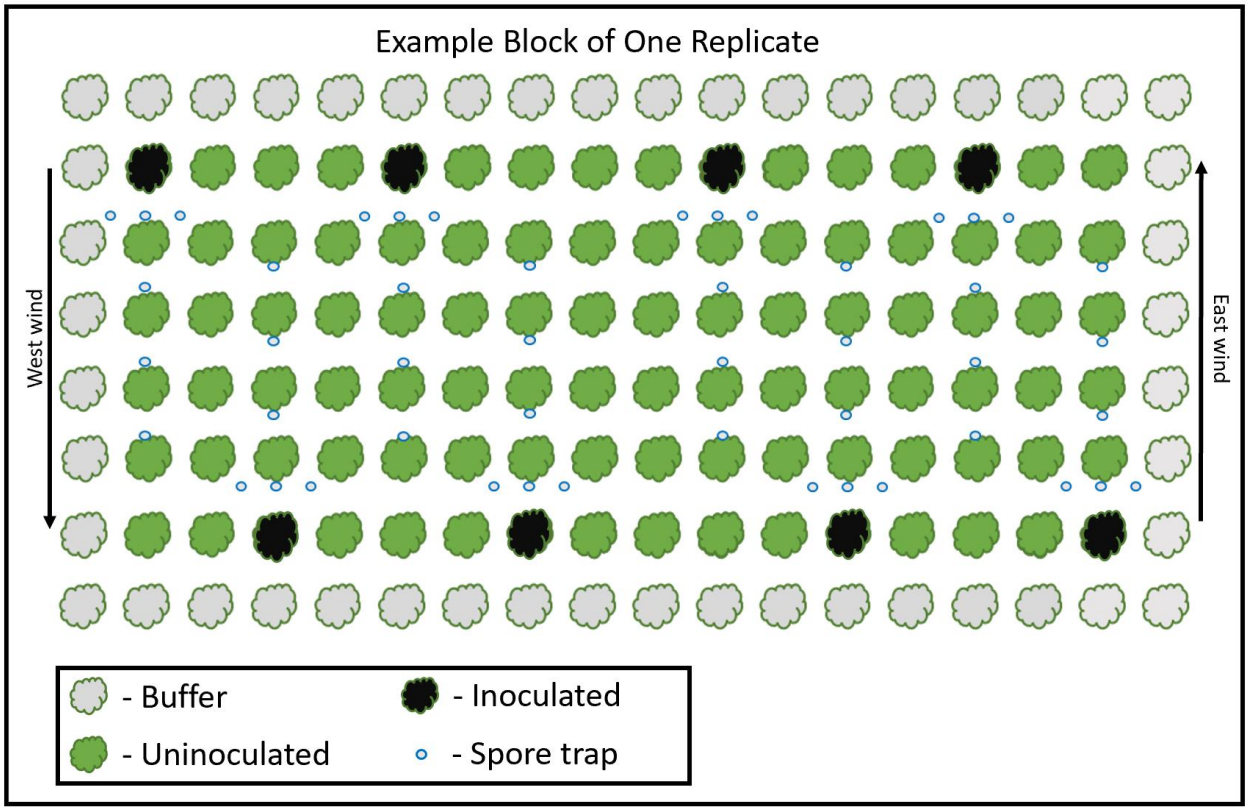


*Figure 3-2. Example block design for one treatment and replicate. Blue circles indicate spore traps located between each row. Distances from cankers were determined by row spacing at 3.6m, 7.2m, 10.8m, 14.4m.*

*Table 3-1. Counts of conidia from a Cytospora plurivora canker which was zip tied to peach trees in a western Colorado peach orchard. Counts were conducted every two weeks. If counts were zero, the canker was exchanged*

| Bimonthly Conida Count | | | |
|---|---|---|---|
| **Canker #** | **27-Jun** | **12-Jul** | **23-Jul** |
| 69 | 141,800 | 86,500 | 15,000 |
| 68 | 66500 | 170,000 | 200,000 |
| 49 | 280000 | 62,500 | 72,500 |
| 59 | 324000 | 43,000 | 100,000 |
| 62 | 228000 | 91,000 | 95,000 |
| 70 | 252000 | 178,000 | 395,000 |
| 58 | 127000 | 48,500 | 32,500 |
| 63 | 181000 | 320,000 | 305,000 |
| 51 | 216000 | 36,000 | 0 |
| 60 | 137000 | 346,000 | 460,000 |
| 65 | 211000 | 22500 | 5,000 |
| 57 | 9600 | 45,000 | 292,500 |
| 42 | 202000 | 98,000 | 0 |
| 64 | 300,000 | 100,000 | 3,000 |
| 44 | 260000 | 5,000 | 0 |
| 47 | 290000 | 77,500 | 100,000 |
| 45 | 329000 | 11,000 | 10,000 |
| 52 | 330000 | 0 | 1,000 |
| 56 | 51500 | 7,500 | 15,000 |
| 48 | 5600 | 26,500 | 0 |
| 66 | 164000 | 16,500 | 12,500 |
| 45 | 130000 | 18,000 | 27,000 |
| 17 | 83500 | 67,000 | 0 |
| 37 | 275000 | 28,000 | 75,500 |
| 67 | 6500 | 6,700 | 0 |
| 55 | 24500 | 2,500 | 0 |
| 46 | 130000 | 0 | 0 |
| 50 | 80000 | 42,000 | 19,000 |
| 53 | 38500 | 39,000 | 35,000 |
| 61 | 13500 | 2,000 | 0 |
| 54 | 9000 | 11,000 | 0 |
| 41 | 1600 | 4,500 | 0 |

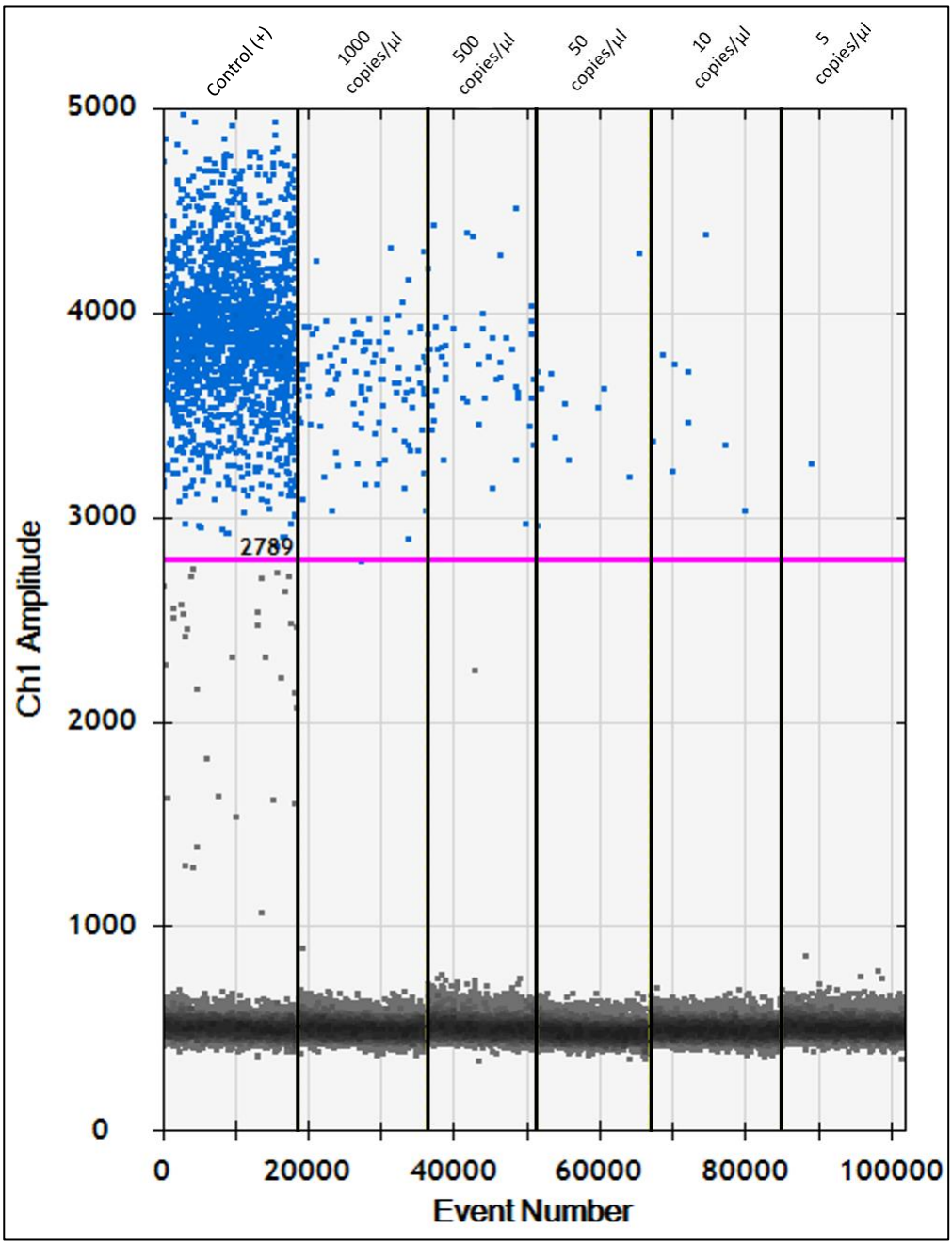


*Figure 3-3. ddPCR serial dilution Cytospora plurivora controls: 1000, 100, 500, 50, 10, 5 copies/µl.*

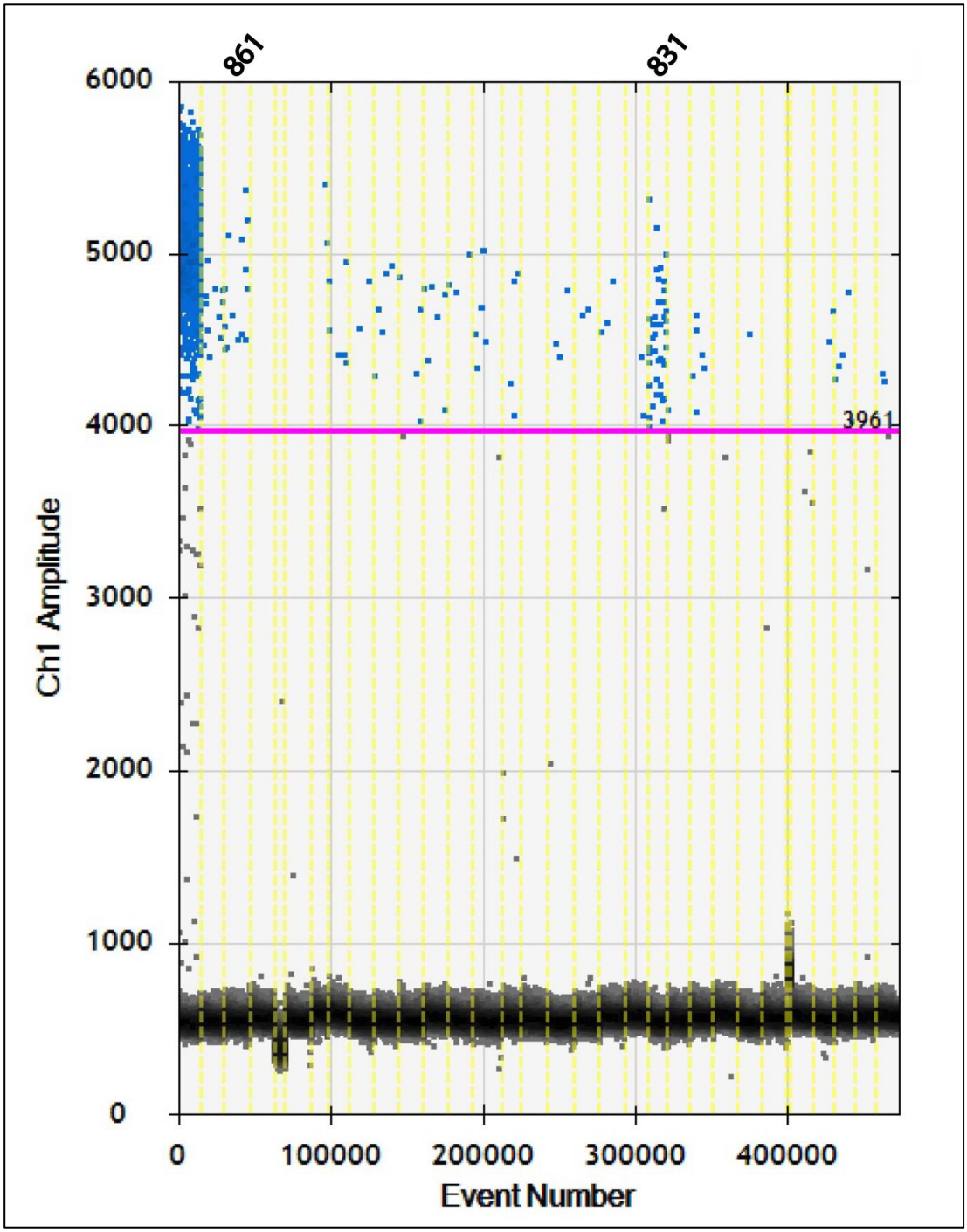


*Figure 3-4. ddPCR reaction including positive and false positive droplets for Cytospora plurivora. The amplitude is defined be each reaction by plate and changes with each run of the ddPCR. The droplets above the amplitude are considered true positive droplets while droplets below the line are "rain" and were disregarded.*

Table 3-2. *Total positive ddPCR detections of Cytospora plurivora in a western Colorado peach orchard*

(A) Collection – Spore trap collection identification. (B) Irrigation type – D = Drip, M = Micro-sprinkler, C = Control. (C) Location – Trap location associated with canker. (D) Rep. – Replicate peach block 1 or 2. (E) Date – Date of collection by week. (F) Positive Droplets – Number of positive droplets in each reaction. (G) Distance – Distance in meters from the canker source.

| A. Collection | B. Irrigation | C. Location | D. Rep. | E. Date | F. Positive Droplets | G. Distance |
|---|---|---|---|---|---|---|
| 511 | D | 1 | 1 | 6/22/2021 | 26 | 3.6 |
| 565 | D | 1 | 2 | 6/22/2021 | 11 | 3.6 |
| 537 | D | 3 | 1 | 6/22/2021 | 26 | 3.6 |
| 515 | D | 5 | 1 | 6/22/2021 | 17 | 10.8 |
| 539 | D | 5 | 1 | 6/22/2021 | 20 | 10.8 |
| 528 | D | 6 | 1 | 6/22/2021 | 15 | 14.4 |
| 841 | D | 1 | 2 | 7/6/2021 | 11 | 3.6 |
| 943 | M | 1 | 2 | 7/6/2021 | 29 | 3.6 |
| 831 | D | 3 | 1 | 7/6/2021 | 44 | 3.6 |
| 861 | D | 3 | 2 | 7/6/2021 | 14 | 3.6 |
| 903 | M | 3 | 1 | 7/6/2021 | 14 | 3.6 |
| 958 | M | 4 | 2 | 7/6/2021 | 11 | 7.2 |
| 989 | C | C | C | 7/6/2021 | 12 | 135 |
| 1370 | M | 2 | 2 | 7/13/2021 | 11 | 3.6 |
| 1221 | D | 3 | 1 | 7/13/2021 | 18 | 3.6 |
| 1317 | M | 3 | 1 | 7/13/2021 | 43 | 3.6 |
| 1198 | D | 4 | 1 | 7/13/2021 | 18 | 7.2 |
| 1380 | M | 6 | 2 | 7/13/2021 | 12 | 14.4 |

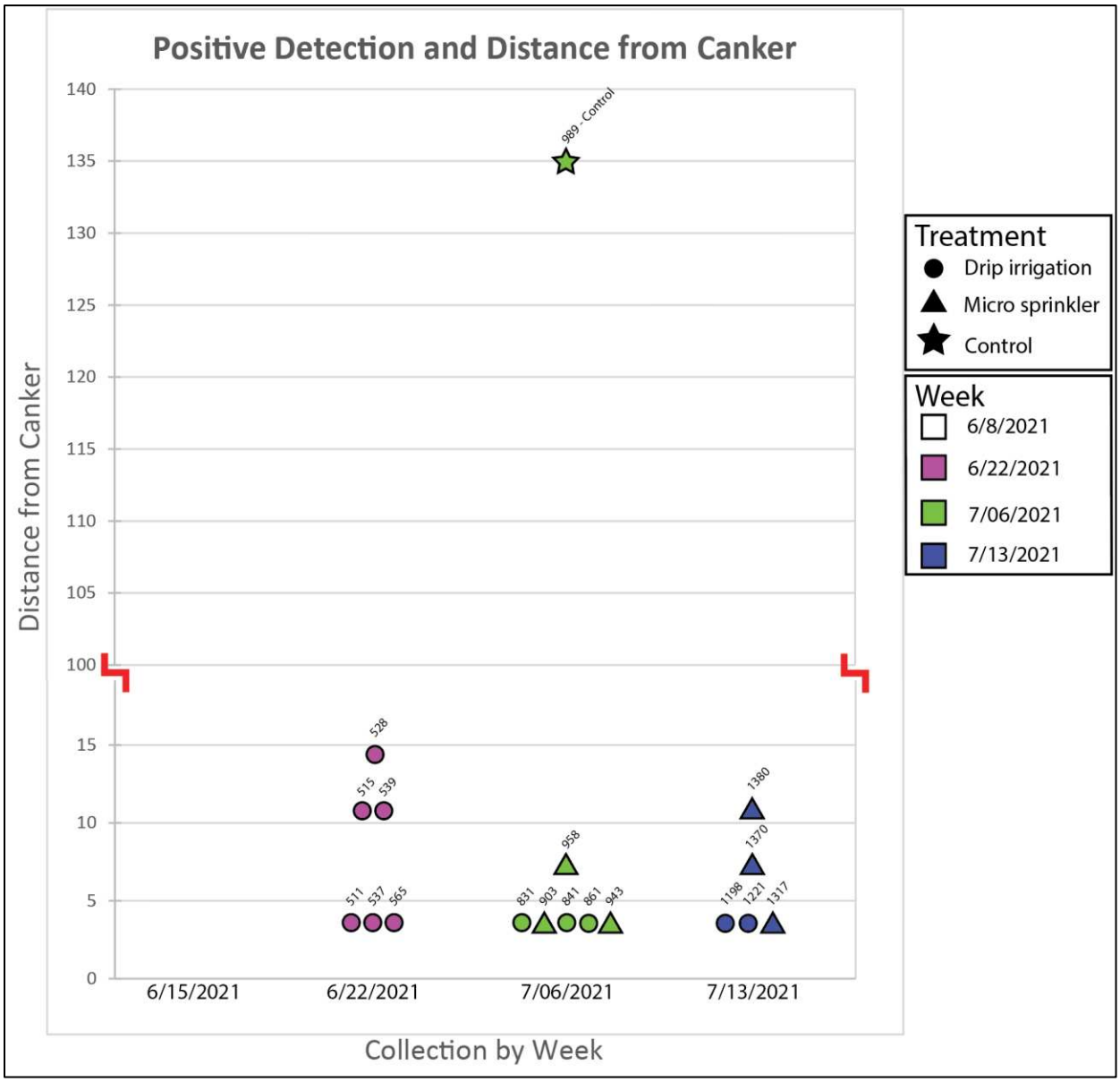


*Figure 3-5. Cytospora plurivora positive detections by collection, distance, and irrigation type*. The red partition located on the Y-axis indicates a broken graph due to distances collected in the controls.

## Literature Cited


Adams, G. C., Surve-Iyer, R. S., & Iezzoni, A. F. (2002). Ribosomal DNA sequence divergence and group I introns within the Leucostoma species L. cinctum, L. persoonii, and L. parapersoonii sp. Nov., ascomycetes that cause Cytospora canker of fruit trees. *Mycologia*, *94*(6), 947–967.

Barakat, R. M. (1995). Factors affecting conidial exudation and survival, and ascospore germination of Leucostoma cincta (R. M. Barakat, D. A. Johnson, & G. G. Grove, Trans.). *Plant Disease*, *v. 79*(12), 1245–1248. PubAg. https://doi.org/10.1094/PD-79-1245

Bassimba, Daniel. D. M., Intrigliolo, D. S., Dalla Marta, A., Orlandini, S., & Vicent, A. (2017). Leaf wetness duration in irrigated citrus orchards in the Mediterranean climate conditions. *Agricultural and Forest Meteorology*, *234–235*, 182–195. https://doi.org/10.1016/j.agrformet.2016.12.025

Batra, L. R. (1991). World species of Monilinia (Fungi): Their ecology, biosystematics and control. *World Species of Monilinia (Fungi): Their Ecology, Biosystematics and Control.*, *No. 16*. https://www.cabdirect.org/cabdirect/abstract/19922316131

Bertrand, P., & English. (1976). Relsease and dispersal of conidia and ascospores of Valsa leucostoma. *Phytopathology®*, *66*, 987–991.

Grove, G. G., & Biggs, A. R. (2006). Production and Dispersal of Conidia of *Leucostoma cinctum* in Peach and Cherry Orchards Under Irrigation in Eastern Washington. *Plant Disease*, *90*(5), 587–591. https://doi.org/10.1094/PD-90-0587

Henriksen, T. V., Drue, S. O., Frydendahl, A., Demuth, C., Rasmussen, M. H., Reinert, T., Pedersen, J. S., & Andersen, C. L. (2022). Error Characterization and Statistical Modeling Improves Circulating Tumor DNA Detection by Droplet Digital PCR. *Clinical Chemistry*, *68*(5), 657–667. https://doi.org/10.1093/clinchem/hvab274

Ianson, D. C., & Allen, M. F. (2018). The Effects of Soil Texture on Extraction of Vesicular-Arbuscular Mycorrhizal Fungal Spores from Arid Sites. *Mycologia*. https://www.tandfonline.com/doi/abs/10.1080/00275514.1986.12025227

Jones, M., Williams, J., Gärtner, K., Phillips, R., Hurst, J., & Frater, J. (2014). Low copy target detection by Droplet Digital PCR through application of a novel open access bioinformatic pipeline, 'definetherain.' *Journal of Virological Methods*, *202*, 46–53. https://doi.org/10.1016/j.jviromet.2014.02.020

Lenth, R.V. (2022). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.7.2. https://CRAN.R-project.org/package=emmeans

Li, H., Bai, R., Zhao, Z., Tao, L., Ma, M., Ji, Z., Jian, M., Ding, Z., Dai, X., Bao, F., & Liu, A. (2018). Application of droplet digital PCR to detect the pathogens of infectious diseases. *Bioscience Reports*, *38*(6), BSR20181170. https://doi.org/10.1042/BSR20181170

Luo, Y., Niederholzer, F. J. A., Felts, D. G., Puckett, R. D., & Michailides, T. J. (2020). Inoculum quantification of canker-causing pathogens in prune and walnut orchards using real-time PCR. *Journal of Applied Microbiology*, *129*(5), 1337–1348. https://doi.org/10.1111/jam.14702

Meredith, D. S. (1973). Significance of Spore Release and Dispersal Mechanisms in Plant Disease Epidemiology. *Annual Review of Phytopathology*, *11*(1), 313–342. https://doi.org/10.1146/annurev.py.11.090173.001525

Michailides, T., Morgan, D., Olson, W., & Grant, J. (1993). Adjusting sprinkler angle reduces spread of disease in pistachio. *California Agriculture*, *47*(5), 16–20. https://doi.org/10.3733/ca.v047n05p16

Miller, S. T., Sterle, D., Minas, I. S., & Stewart, J. E. (2021). Exploring fungicides and sealants for management of Cytospora plurivora infections in western Colorado peach production systems. *Crop Protection*, *146*, 105654. https://doi.org/10.1016/j.cropro.2021.105654

Stewart, J. E., Miller, S. T., Zink, F. A., Caballero, J. I., & Tembrock, L. R. (2022). Genetic and Phenotypic Characterization of the Fungal Pathogen *Cytospora plurivora* from Western Colorado Peach Orchards and the Development of a ddPCR Assay for Detection and Quantification. *Phytopathology®*, *112*(4), 917–928. https://doi.org/10.1094/PHYTO-05-21-0210-R

USDA. (2021). *Noncitrus Fruits and Nuts 2021 Summary 05/04/2022*. National Agricultural Statistics Service.