Title
Biology of Fungal Canker Diseases of Sweet Cherry in California

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Publication Date
2023

Peer reviewed|Thesis/dissertation

Biology of Fungal Canker Diseases of Sweet Cherry in California

By

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MASTER'S DEGREE - THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Plant Pathology

in the

COLLEGE OF AGRICULTURE AND ENVIRONMENTAL SCIENCES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2023

Acknowledgements

First and foremost, I would like to express my gratitude to my major professor, Dr. Florent Trouillas, for the opportunity in doing research under his guidance. The knowledge that you impart, your patience, and the examples you set as a professional were invaluable in developing me into a well-rounded plant pathologist. I also thank our project scientists, Dr. Renaud Travadon and Dr. Daniel Lawrence for their expertise, advice, and friendship. I am greatly appreciative of the help and mentorship of these members of Trouillas lab as they were integral to the completion of my research. Many thanks to my thesis committee, Themis Michailides and Akif Eskalen for their expertise and feedback. Additionally, this endeavor would not be possible without the generous support from the California Cherry Board, who funded my research.

I have not always been interested in plant pathology. I extend my appreciation to Dr. Richard Bostock and Dr. Robert Gilbertson for introducing to me and igniting a passion in me to the fascinating field of study. Additionally, I have learned numerous technical research skills from the labs I have worked in before graduate school. In this regard, I thank Dr. Mike Davis, Dr. Hung Doan, Dr. Richard Bostock, and Dr. Johan Leveau for the opportunity.

Lastly, I could not have undertaken this journey without the unrelenting support and encouragement from my family and friends. With my deepest sincerity, I extend my love and appreciation to my fiancée, Katie for her boundless patience and support that have allowed me to persevere through difficult times. Thank you.

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Abstract: Biology of Fungal Canker Diseases of Sweet Cherry in California

Calosphaeria canker caused by *Calosphaeria pulchella*, Cytospora or Leucostoma canker caused by *Cytospora sorbicola*, and Eutypa dieback caused by *Eutypa lata* are the main canker diseases of sweet cherry in California commercial orchards and a limiting factor to orchard productivity and longevity. It was understood that pruning wounds serve as the primary avenue of infection for these fungal canker pathogens; however, very little is known about the other infection courts of *Cal. pulchella*, *Cyt. sorbicola* and *E. lata*. Thus, this present study was undertaken to identify common infection courts other than pruning wounds for fungal canker pathogens affecting sweet cherry. This was determined by assessing the field incidence of canker pathogens in the wood below pruning wounds, in buds, fruiting spurs and unpruned apical shoots exhibiting dieback symptoms. This work was supplemented by assessing the susceptibility of naturally occurring wounds such as leaf scars, bud scale scars, and harvest wounds on fruiting spurs to the three major canker pathogens of sweet cherry. Additionally, little is known about the effects of temperature and season on the biology of these three pathogens (except for *E. lata*). Therefore, the second objective of this study were to ascertain their optimal temperatures for spore germination and mycelial growth *in vitro* among temperatures 5, 10, 15, 20, 25, 30, 35, and 40°C. The third objective of this study supplements the second objective by comparing the susceptibility of pruning wounds to infection by *Cal. pulchella*, *Cyt. sorbicola* and *E. lata* made in summer and winter.

Our survey revealed disease incidences of *Cal. pulchella* and *Cyt. sorbicola* to be as high as 20% and 35%, respectively, in shoot tips and up to 19.4% and 26.7%, respectively, in declining spurs. Additionally, harvest wounds of fruiting spurs were shown to be highly susceptible to canker infection by the three pathogens with 60% recovery for *Cyt. sorbicola*, and

42.5% for *Cal. pulchella* and *E. lata*. The optimal temperatures for spore germination were estimated to be 29.15°C for *Cal. pulchella*, 26.95°C for *Cyt. sorbicola*, and 22.85°C for *E. lata*; and optimal temperatures for mycelial growth were estimated to be 27.2°C, 23.85°C and 22.5°C, respectively. In field experiments, average recovery of *Cal. pulchella* was significantly greater from pruning wounds made in summer than that of winter with 84.6% and 10.7% recovery, respectively. The average recovery of *E. lata* from pruning wounds made in winter was 92.8%, which was significantly greater than that of summer with 80% recovery. Overall, our findings demonstrated that summer pruning of sweet cherry in California poses greater risks of infection by *Cal. pulchella*. In contrast, winter pruning poses greater risks of infection by *E. lata*. This study represents new knowledge on the seasonal susceptibility of pruning wounds of sweet cherry to canker infection by *Cal. pulchella* and *Cyt. sorbicola*. Additionally, this study provided new information on additional infection courts of major canker pathogens of sweet cherry in California. Since distinct cherry canker pathogens vary in their seasonal infectivity, synchronizing maintenance pruning with low periods of inoculum pressure will be critical for canker disease management in sweet cherry orchards.

Introduction Chapter: Fungal canker diseases of sweet cherry

The United States is among the world's leading sweet cherry (*Prunus Avium* L.) producers with nearly 370 kilotons produced in 2021 and only second to Turkey (USDA-NASS 2022, FAOSTAT 2020). Within the United States, California is the second sweet cherry producer after Washington State (USDA-NASS 2021). In California, Sweet cherry orchards are grown across 14 California counties totaling 40,649 harvested acreage with more than 112 kilotons produced and a total annual crop value of over $378 million. Leading counties by gross value of agricultural cherry production include San Joaquin, Fresno, and Kern. Furthermore, cherry production and the harvested acreage have nearly doubled in the past 20 years (CDFA 2022). Despite the growth of the sweet cherry industry in California, canker and dieback diseases are a major threat to cherry production as they can affect tree health, longevity, and yield.

Canker diseases are caused by fungal pathogens in the Ascomycota and commonly affect tree branches and trunks, manifesting as localized necroses of vascular tissue (Sinclair and Lyon 2005). Wood necrosis and cankers are often accompanied by branch dieback due to constriction of vascular tissues. Canker diseases generally initiate at pruning wounds and infect the wood of branches, causing wood discoloration and sunken lesions on the bark due to desiccated, dead tissues. The infected areas often exhibit gummosis, meaning sap (gumgalls) development on the bark surface. Infection of large size scaffolds and trunks may occur, eventually killing entire trees.

A previous study identified Calosphaeria canker caused by *Calosphaeria pulchella*, Cytospora or Leucostoma canker caused by *Cytospora sorbicola*, and Eutypa dieback caused by *Eutypa lata* as the main canker diseases of sweet cherry in California commercial orchards

(Trouillas *et al*. 2012). *Calosphaeria pulchella* (Anamorph: *Calosphaeriophora pulchella* Réblová, L. Mostert, W. Gams & Crous, sp. nov, Calosphaeriales, Réblová *et al.* 2004) was first reported in 2010 as the causal agent of Calosphaeria canker of sweet cherry in California and it was shown to occur on diseased cherry trees in France, Italy and South Australia, (Trouillas *et al.* 2010). More recently, *Cal. pulchella* was reported on sweet cherry in Spain (Berbegal *et al*. 2014) and in Chile (Auger *et al*. 2021). In addition to cherries, *Cal. pulchella* was also shown to cause cankers on other *Prunus* spp. such as peaches, nectarines, and almonds (Bergebal and Armengol 2018, Arzanlou and Dokhanchi 2013). *Calosphaeria pulchella* commonly produces perithecia (sexual fruiting bodies) beneath the periderm of dead and diseased branches and ascospores are released from perithecia following wetting from rain or sprinkler irrigation (Trouillas *et al.* 2012). Ascospores serve as the primary inoculum and are primarily dispersed via rain and wind to infect open wounds. Asexual fruiting bodies of *Cal. pulchella* have not been observed in nature although conidia readily form on colonies growing on a Potato-Dextrose-Agar (PDA) medium. The fungus invades first the heartwood and eventually colonize all vascular tissues including the sapwood causing wood discoloration. Once a canker has developed, *Cal. pulchella* rapidly produces distinct perithecia under the bark of diseased branches, which can help with field diagnosis of Calosphaeria canker. The dark, flask-shaped perithecia with long cylindrical necks are arranged ununited in disk-shaped, circinate groups with converging necks (Fig. 1a). The ascospores (Fig. 1c) are released through the ostioles at the end of perithecial necks; they are hyaline and allantoidal (Réblová *et al.* 2004). *Cal. pulchella* colonies grown on potato dextrose agar (PDA) at room temperature (23-24°C) exhibit a pink to red color with white margin as early as 3-4 days old. The colony takes on a darker pink to red pigment with little

aerial mycelium as the colony ages. Only asexual spores in the form of conidia are observed in culture.

Cytospora canker, also known as Leucostoma canker or perennial canker, is caused by species of *Cytospora* and can affect stone fruits, pome fruits and nut crops, and is especially devastating on plum and prune cultivars as well as sweet cherry in California. While many *Cytospora* spp. are known to cause canker diseases, *Cytospora sorbicola* (Norphanphoun, Bulgakov, T.C. Wen & K.D. Hyde) was recently described as the main causal agents of Cytospora canker of sweet cherry and other stone fruits in California (Lawrence *et al.* 2018). Furthermore, some isolates of *Cyt. sorbicola* appeared to be host-specialized with cherry in California based on multi-loci phylogenetic analyses (Lawrence *et al.* 2018). Cytospora canker pathogens mainly disseminate via conidia (asexual spores), although the sexual morph can also occur in nature (Pan *et al.* 2021). Much like with Calosphaeria canker, pruning wounds serve as the main entry point for infectious spores, which are disseminated via rain splash and wind. Cytospora cankers exhibit irregular-shaped or wedge-shaped vascular discolorations of the xylem tissue. Pycnidia (asexual fruiting bodies) often develop beneath the periderm of dead, infected branches, producing a characteristic “pimpled” appearance of the bark surface (Fig. 2a). Mature pycnidia eventually ruptures the bark to release dark cirri (Fig. 2b) and tendrils that contain hyaline, allantoid conidia (Fig. 2d) (Adams *et al.* 2006, Lawrence *et al.* 2018). *Cytospora sorbicola* colonies grown on PDA at room temperature (23-24°C) exhibit a smoke-grey center with pale-olivaceous, non-uniform, lobate margins. Pycnidia form in culture in a random pattern. Cytospora canker can cause up to 5% tree mortality in cherry orchards per year and if not managed, it can have a compounding effect on the orchard survivability due to canker’s ability to persist in fields and the pathogen to produce a large amount of inoculum (Grove and Biggs

2006). In Colorado, Pokharel and Larsen (2008) reported that Cytospora canker caused up to 30% tree loss in sweet cherry orchards.

Eutypa lata (Persoon: Fries) Tulasne and C. Tulasne (synonym = *E. armeniacae* Hansf. & M. V. Carter) is the causal agent of Eutypa dieback of sweet cherry grapevine and apricot (Carter, 1991; Munkvold and Marois 1991, 1994). Over 80 plant species have been reported globally as hosts for *E. lata*. Main hosts in California include almond, apple, pear, oleander, sweet cherry and willow (Trouillas *et al*. 2010). Oleander and willow also were shown as important reservoirs of inoculum of *E. lata.* On the other hand, perithecia of *E. lata* were rarely observed on cherry trees in California (Trouillas *et al*. 2010; Gubler *et al*. 2005). *Eutypa lata* was long considered to be the primary causal agent of canker diseases of sweet cherry in California. However, a recent survey of cherry orchards revealed a low incidence in cankers as compared to *C. pulchella* and *C. sorbicola* (Trouillas *et al*. 2012). Perithecia of *E. lata* are immersed in a stroma and produce allantoid, hyaline ascospores when exposed to wetness. Ascospores serve the main inoculum for Eutypa dieback (Munkvold and Marois 1994, Rolshausen 2005; Trouillas and Gubler 2010). They are dispersed by wind and rain to infect primarily pruning wounds (Moller and Carter 1964, Ramos *et al*. 1975). *Eutypa lata* infects the sapwood of open wounds and eventually spreads to the cambium and phloem, often forming wedge-shaped discolorations as seen in cross-sections of diseased wood (Munkvold *et al.* 1993, Trouillas and Gubler 2010). On PDA, *Eutypa lata* grows as white, cottony colonies, which turn grey to black with time and produce black, round pycnidia that contain filiform conidia.

Although canker diseases pose a major threat to the cherry industry in California, little is known about their biology and disease cycle, including infection pathways. While infections often develop at pruning wounds, canker initiating from shoot tips and lateral spurs in branches

have been observed in the field in the absence of clear mechanical wounding and injuries. These preliminary observations have suggested that alternative infection courts other than pruning wounds may play a role in canker diseases' biology.

In addition, no information is available about the seasonal susceptibility in sweet cherry of pruning wounds to the various canker pathogens. However, in previous field experiments, we have failed repeatedly to infect pruning wounds following winter pruning and artificial inoculations with ascospore suspensions of *C. pulchella*. Accordingly, we hypothesized that this pathogen may favor warm temperatures and summer pruning for successful infection of pruning wounds.

The objectives of this study were (1) to investigate the main infection pathways for fungal canker diseases of sweet cherry caused by *Calosphaeria pulchella, Cytospora sorbicola, and Eutypa lata*; (2) to investigate the effect of temperature on spore germination and mycelium growth rate, and (3) to determine the seasonal susceptibility of pruning wounds to canker pathogens.

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Figures and Tables (Introduction):

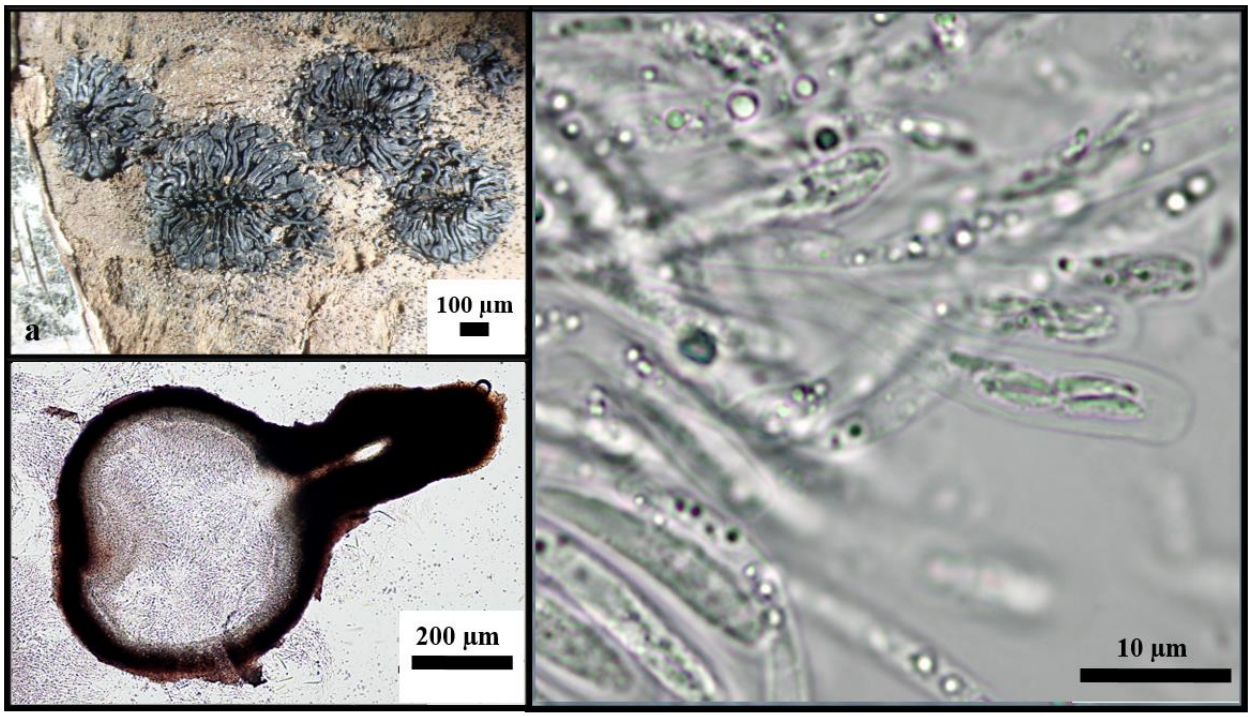

Figure 1: *Calosphaeria pulchella* sexual fruiting bodies on cherry. (a) Perithecia found beneath the periderm of dead, infected cherry branch; **(b)** a section parallel to ostiole with asci lining the hymenium layer of interior wall of perithecium; **(c)** ascospores enclosed in asci.

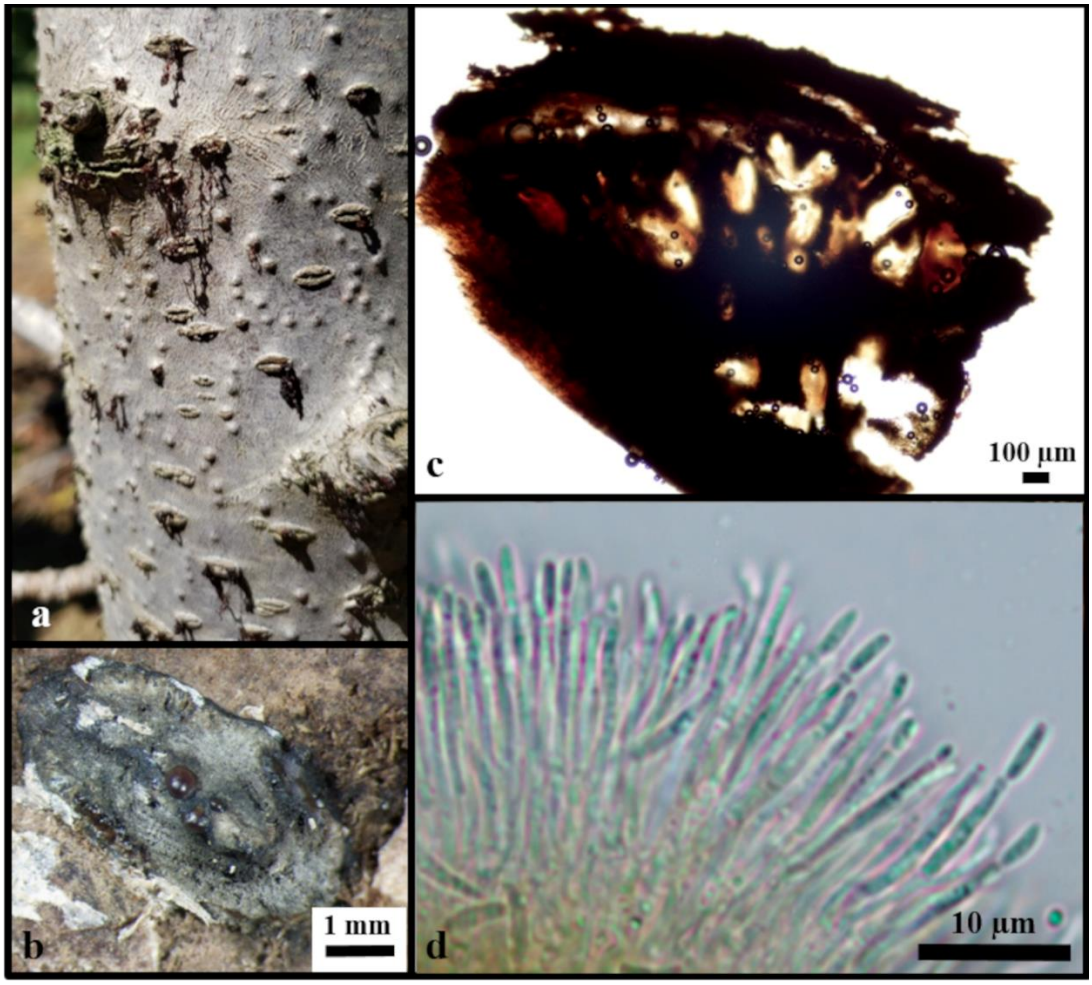

Figure 2: ***Cytospora sorbicola*** **asexual fruiting bodies on cherry. (a)** cirri accumulation originating from pycnidia that formed beneath the periderm of dead, infected cherry branch; **(b)** pycnidium oozing mucilage (cirri) after soaked with water; **(c)** longitudinal section of pycnidium reveals multiple spore-producing chambers within; and **(d)** asexual spores produced on the tip of conidiogenous hyphae on hymenium layer within a chamber of the pycnidium.

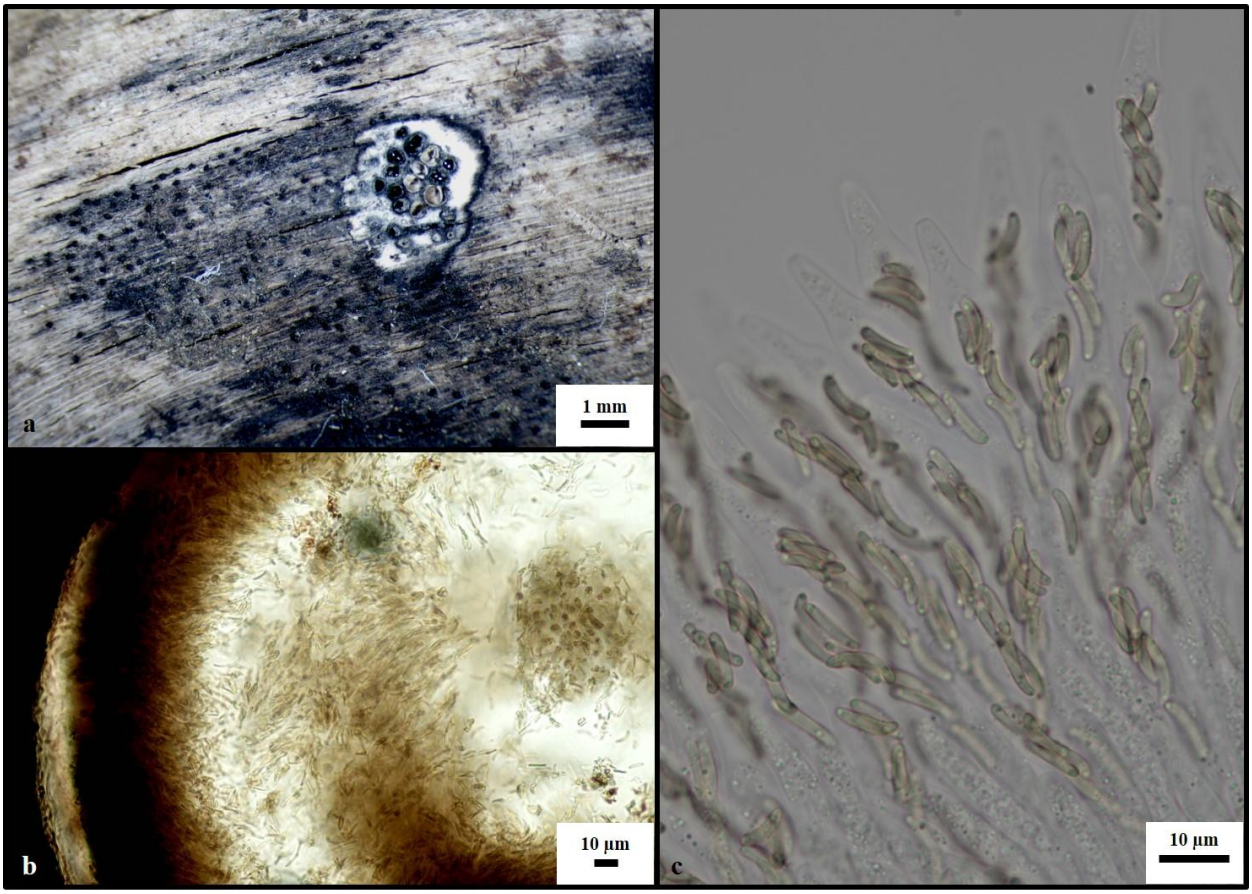

Figure 3: Sexual fruiting bodies of *Eutypa lata* on oleander. (a) Ostiole of perithecia visible on wood surface and the interior revealed after slicing away wood surface; **(b)** layers of spore-producing asci in the hymenium layer lining the interior of perithecium cross-section; and **(c)** ascospores enclosed in asci.

Chapter I: Investigation the infection pathways for fungal canker diseases of sweet cherry in California

Introduction:

Calosphaeria canker caused by *Calosphaeria pulchella*, followed by Cytospora canker caused by *Cytospora sorbicola* and Eutypa dieback caused by *Eutypa lata*, were recognized as the most common fungal canker diseases of sweet cherry in California (Trouillas *et al.* 2012). Previous studies also determined that pruning wounds serve as the main infection courts for these pathogens (Biggs 1989; Trouillas *et al*. 2012; Trouillas and Gubler 2010). Thus, management strategies for fungal canker diseases of sweet cherry have primarily focused on preventing pruning wound infections. However, we hypothesize that infection sites other than pruning wounds most likely occur in cherry trees. In peach trees for example, leaf scars, winter-killed buds, and buds during bud break have been shown to serve as additional infection courts for *Cytospora* pathogens (Rosenberger 1982; Royse and Ries 1978; Tekauz and Patrick 1973; and 1974; Wilcox 1995) and *Phomopsis* sp. (Uddin and Stevenson 1998; Rddin *et al.* 1997). Furthermore, Cytospora canker infections may initiate on senescing fruiting spurs, shaded twigs, and senescing buds suggesting that latent infections in asymptomatic shoots also result in disease symptoms (Adams 2016).

Canker diseases caused by several species of Botryosphaeriaceae are capable of direct infection through lenticels and stomata of healthy plants like in apple (Kim *et al.* 1999) or pistachio trees (Luo *et al*. 2019). *Neonectria ditissima*, the causal agent of European canker of apples, can cause infection by entry through leaf scars, bud scale scars, and harvest wounds resulting from apple picking (Alves and Nunes 2017; Amponsah *et al*. 2015; Crowdy 1952,

Marsh 1939; Wiltshire 1921). *Pseudomonas syringae* pv. *syringae*, the causal agent of bacterial canker of sweet cherry infects trees through leaf scars and at the base of fallen bud scales (Cameron 1962; Cao *et al*. 2013).

In California cherry, no studies were conducted to determine other infection pathways for *Cal*. *pulchella*, *Cyt*. *sorbicola*, and *E. lata*. However, preliminary surveys of sweet cherry orchards have suggested that additional infection courts are likely to occur for cherry canker diseases. Indeed, symptoms of shoot dieback, dead spurs and cankers in branches were observed in orchards without any apparent pruning wounds or other mechanical wounds. Initial sampling of the discolored wood beneath dead spurs exhibiting gummosis commonly detected fungal canker pathogens. Here we hypothesized that naturally occurring wounds such as those from abscission of leaves during senescence, scars formed from fallen bud scales (cataphyll) during bloom, or bud scars at bud break could serve as additional entry points for fungal canker pathogens. Because cherry fruits are delicate and susceptible to damage, fresh-market cherries are commonly harvested manually by skilled workers rather than by mechanical shaker harvesting (Seavert *et al*. 2008; Zhou *et al*. 2016). Nevertheless, manual harvesting often leads to substantial damages in trees such as breaking of fruiting spurs as suggested by the many spurs and leaf debris found on the grounds of orchards following harvest. Therefore, we further hypothesize that wounds caused by fruit harvesting can serve as additional infection routes for canker pathogens, in addition to pruning wounds. Furthermore, canker-causing pathogens often can colonize healthy plant tissues including buds and shoots, without causing any symptoms. These events are known as latent infections. Symptoms eventually become apparent when the host plant is subjected to biotic or abiotic stresses (Schoeneweiss 1981, Slippers and Wingfield 2007, Smith *et alet al*. 1994 and Luo *et al*. 2019). It is understood that symptom expression from

latent infections are triggered by unfavorable growth conditions for the host such as extreme heat, drought, water stress, frost damage, transplantation, infection by other pathogens, or insect damage (Luo *et al.* 2019 and Slippers and Wingfield 2007). In general, latent infections occur before or after bud break after sufficient moisture from rain has accumulated to create conditions conducive for sporulation and spore release during the fall, winter or springtime (Luo *et al.* 2019). It is then probable that latent infections evolve to cause disease following stress, leading to canker formation and development, and eventually dieback.

This study aims to identify common infection pathways other than pruning wounds for fungal canker pathogens affecting sweet cherry trees. This will be determined by assessing and comparing the incidence in the field of prominent canker pathogens in the wood below pruning wounds, in buds, fruiting spurs and from unpruned apical shoots showing dieback symptoms. This work will be complemented by assessing the susceptibility of naturally occurring wounds such as leaf scars, bud scars, bud scale scars, and harvest wounds on fruiting spurs to the three major canker pathogens of sweet cherry.

Materials and Methods:

Field surveys. A survey of the incidence of common fungal canker pathogens, such as *Cal. pulchella*, *Cyt. sorbicola*, *E. lata*, of sweet cherry in California orchards was conducted across three different counties (Yolo, San Joaquin, and Stanislaus). Wood tissues were sampled for each of the following tree parts: shoot tips exhibiting dieback symptoms and with no apparent pruning wounds; fruiting spurs exhibiting dieback symptoms and with no apparent pruning wounds, branches between 1 – 3 cm diameter exhibiting canker symptoms originating from pruning wound; and buds (before bud break) with no apparent symptoms. Shoot tips (n=20),

spurs (n=31) and branches (n=27) samples were collected from 1 experimental orchard in Yolo County (Yolo 1) between June and November of 2019. The same wood-types were collected from 3 different commercial orchards in San Joaquin county (San Joaquin 1 and 2 during September; San Joaquin 3 during November 2019) and 1 commercial orchard in Stanislaus county (Stanislaus 1 during November 2019). Twenty of each wood-type were sampled from each orchard. Bud samples (n=100 per orchard) were collected at random from two different orchards in Yolo 1 (1a and 1b) during January 2020 and another orchard (Yolo 2) during February 2020; two commercial orchards in Stanislaus County (Stanislaus 1 and 2) during February 2020; and from 3 orchards in San Joaquin County (San Joaquin 1, 2 and 3) during February 2020.

Fungal isolations and identification. All field samples were either processed on the same day of collection, or between 2 to 7 days following storage at 4°C in a cold room. All isolations were plated on potato dextrose agar (PDA; Sigma-Aldrich®, St. Louis, MO) amended with 1 ppm tetracycline (PDA-Tet). For each branch sample, the exposed layer of pruning wound and surrounding bark were removed and surface disinfected by brief flaming. Ten wood fragments of 4 mm × 4 mm × 4 mm were cut from the margin between healthy and infected tissue (discolored wood) and plated. Shoot tip and fruiting spur samples were treated in a similar manner where the bark was removed and smaller pieces of 3 mm × 3 mm × 3 mm fragments were taken from the margin of necrotic tissues. These fragments were placed in small cassettes and surface-sterilized by submerging in a 10% dilution of commercial sodium hypochlorite (bleach; Chlorox® Concentrated 8.25%, Oakland, CA) for 60 seconds followed by two rounds of 60 seconds soaking in sterile deionized (DI) water and allowed to dry over clean paper towel before plating. Bud samples were surface sterilized with bleach using the same method and then

plated directly. All isolation plates were incubated in clear crisper boxes (30 cm × 24 cm × 12 cm) at the laboratory ambient temperature (22-24°C) and natural photoperiod. Cultures were checked every two days for growth of prospective canker-pathogen colonies, which were then transferred to fresh PDA-Tet Petri dishes and allowed for growth as pure cultures. The identity of isolates was determined by colony morphology. Molecular methods were used to supplement identification of unknown isolates. This was done first by extracting total genomic DNA from mycelium of 2-week-old cultures using the Dneasy® Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Polymerase chain reaction (PCR) amplification of rDNA between the small and large subunit rRNA gene loci, including the internal transcribed spacers (ITS) regions 1, 2, and 5.8S rDNA, utilized AccuPower™ PCR Premix (Bioneer, Alameda, CA) and primers ITS-5 and ITS-4 (White *et al*. 1990) with slight modification to thermocycler program (initialization at 95°C for 5 min; 35 cycles of denaturing at 95°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min; and final extension at 72°C for 15 min). PCR products were visualized on 1.5% agarose gel (110 V and 400 mA for 35 min) using GelRed® stain (Biotium, Fremont, CA) to confirm size and presence of DNA. PCR products were purified using ExoSAP-IT™ PCR Cleanup (Applied Biosystems; Thermo Fischer Scientific, Waltham, MA). Sequencing was done bi-directionally on an ABI Prism®3730 Genetic Analyzer (Applied Biosystems) by the College of Biological Sciences UCDNA Sequencing Facility at the University of California, Davis. Consensus of the forward and reverse nucleotide sequences were constructed using BioEdit v. 7.2.5 (Hall 1999) and compared with reference sequences from the NCBI database using the BLASTn function. The incidence of canker pathogens was determined by the percentage of samples (per sample type) with recovery of the pathogen out of total samples processed for each orchard and sample type.

Preparation of inoculum. *Cal. pulchella* ascospores were collected from fruiting bodies (perithecia) found under the periderm of dead, infected sweet cherry scaffold branches. These branches were collected and stored at room temperature. Small pieces of dead wood containing perithecia were cut from branches and affixed to a plastic Petri lid with petroleum jelly (Vaseline® 100%, Greenwich, CT) and submerged in deionized water for 15 to 30 minutes. Water was then poured out and perithecia were gently blotted dry with Kimwipes™ paper sheets (Kimwipes; Kimtech Science® Kimberly-Clark Professional®, Roswell, GA). The lid with perithecia was placed back over a new, sterile Petri dish to allow for spore discharge. At least 2 hours were allowed for ascospores to fully discharge onto the dish bottom. To collect ascospores, 200 ul of deionized water were pipetted up and down repeatedly to dislodge and collect the recently discharged ascospores. Spore suspension concentration was estimated using a standard hemocytometer and adjusted with sterile DI water to 10^6 spores·mL^{-1}. Spores were stored at 4°C until ready for use.

The conidia of *Cyt. sorbicola* were obtained from pycnidia found on dead, infected cherry wood. Conidia exuded from pycnidia in the form of mucilage after exposure to wetness via an ostiole. Overtime, this mucilage may accumulate and dry into tendril-like masses (cirri). These cirri were collected and fully dissolved by vortexing in sterile DI water to obtain a conidial suspension. Spore inoculum concentration was adjusted to 10^6 spores·mL^{-1} using the above methods.

The ascospores of *E. lata* were harvested from perithecia growing on dead, infected oleander (*Nerium oleander*) wood. To collect ascospores, a thin razor blade was used to cut tangentially along the wood surface to remove thin layers of wood to expose the internal

perithecial cavity. Sterile DI water was added over these openings to rehydrate and produce a dark, gel-like matrix containing an abundance of ascospores. With the aid of a dissecting microscope and a dissecting needle, the spore matrix was carefully collected in 1.5 ml Eppendorf tubes containing sterile DI water and vortexed for 2 minutes to dislodge and evenly suspend ascospores. Spore inoculum concentration was adjusted to 10^6 spores·mL^{-1} using above methods.

Each spore suspension was then transferred to a 100 ml fine mist spray bottle that outputs about 0.13 ml or 1.3×10^5 spores per spray. Spray bottles were then used to apply inoculum onto the various plant wounds in field experiments by spraying until runoff (~2 to 4 sprays).

Field inoculations and re-isolation. Wounds occurring through natural processes such as leaf drop (leaf senescence), bud break (new shoot emergence and bud scales falling) and mechanical process due to harvest (fruit peduncle scars and broken fruit spurs) were simulated and inoculated to determine their susceptibility to infection by fungal canker pathogens. A total of 4 treatments—*Cal. pulchella* ascospores, *Cyt. sorbicola* conidia, *E. lata* ascospores, and sterile DI water as control—were spray-inoculated directly onto each potential infection sites.

Leaf scars (Fig. 1A) were inoculated in December 2019, 2020, and 2021 when leaves naturally senesced. Senescing leaves were removed by light, but not forceful pulling. Each treatment was inoculated onto all leaf scars from each of 10 bud clusters or spurs. Bud scars (Fig. 1B and 1C) were inoculated in March 2020 and 2021when the bud scales fell off due to flower bloom. For each treatment, 10 flower bud clusters were inoculated and repeated over 10 trees. Fruiting spur scars were inoculated in July 2020, June 2021, and June 2022 following harvest. In the first trial in July 2020, each treatment was applied to 12 fruiting spur wounds that were created by manual harvest of whole cherry clusters by pulling the peduncles. The second and third trials of fruit scar inoculations in June 2021 and 2022, respectively, were conducted onto 2

distinct types of mechanical wounds (peduncle and spur damage) that are commonly caused during manual fruit harvesting. “Peduncle” wounds (Fig. 1D) were created by harvesting fruit clusters by pulling from the peduncles (fruit stems), which occasionally caused tearing along the fruiting spur. “Fruiting spur” wounds (Fig. 1E) were created by harvesting fruit clusters by pulling the attached spur, which caused either partial or full breakage of the spur. Each treatment was applied on 5 wounds per wound-type and repeated over 4 trees.

After 3 months following inoculations of each potential infection site, wood samples were collected for re-isolation to determine the rate of infection of each pathogen at each site. For each leaf and bud scar sample, the bark was removed and wood cross-sections above, below, and on the inoculated wound were taken. Ten 3 mm × 3 mm × 3 mm wood fragments of each section were surface sterilized with 0.875% sodium hypochlorite solution as described above and plated onto PDA-Tet. The bark was similarly stripped from fruit spur samples, and discolored tissues were surface-sterilized and plated as described above. Every two days, prospective fungal colonies of canker pathogens were transferred to fresh PDA-Tet. Growing fungal colonies were identified using colony morphology. The rate of infection or recovery of each pathogen on each type of tissue was determined by the percentage of samples that had at least one colony of the pathogen isolated out of the total number of samples for that wood type.

Results:

Field survey of symptomatic tissue (pruning wounds, shoot tips, and spurs). Both *Cal. pulchella* and *Cyt. sorbicola* were present in all five orchards sampled. *E. lata* was only isolated from Yolo (1), San Joaquin (3) and Stanislaus (1) Counties. Major canker pathogens (*Cal. pulchella*, *Cyt. sorbicola*, and *E. lata*) were found at the highest proportion compared to

other canker pathogens (species of *Diaporthe*, *Botryosphaeria*, and *Fusarium*) in all counties and all wood types, except for *E. lata* which had low and no occurrence in spurs and shoot tips, respectively. *Cal. pulchella* was the most common fungal canker pathogen isolated from branch samples below pruning wounds and was found in 25.9% (n = 27) of branch samples in Yolo, 30% (n = 60) in San Joaquin, and 25% (n = 20) in Stanislaus. *Cyt. sorbicola* was found in 18.5%, 15%, and 20% in branch samples below pruning wounds in each respective county. *E. lata* was found in 3.7%, 5%, and 20%, respectively, in branch samples below pruning wounds.

Cyt. sorbicola was the most common pathogen isolated from shoot tips showing dieback and declining spurs with the exception of Yolo, where *Cal. pulchella* was the main pathogen isolated from shoot tips. *Cal. pulchella* was found in 20% (n = 20) of shoot tips in Yolo, 6.7% (n=60) in San Joaquin, and 5% (n = 20) in Stanislaus. *Cyt, sorbicola* was found in 15%, 13.3% and 35%, respectively, in shoot tips. *E. lata* was not isolated from any shoot tip samples.

Cyt. sorbicola, followed by *Cal. pulchella*, was the most frequently isolated pathogen from declining spurs across all counties. *Cal. pulchella* was found in 19.4% (n = 31) of declining spurs in Yolo, 6.7% (n = 60) in San Joaquin and 10% (n = 20) in Stanislaus Counties. *Cyt. sorbicola* was found in 22.6%, 26.7% and 20%, respectively, in declining spurs. *E. lata* was only found in 3.2% of declining spurs in Yolo County.

The overall incidence of fungal canker pathogens was as follow: *Cal. pulchella* was found in 28% of symptomatic branches below pruning wounds, 10.8% of declining spurs and from 9% of shoot tips showing dieback; *Cyt. sorbicola* was found in 16.8% of branches below pruning wounds, 24.3% of declining spurs and from 18% of declining shoot tips; *E. lata* was found in 7.5% of declining branches below pruning wounds, and less than 1% from declining spurs and was not isolated from any shoot tips.

From Yolo County (1) (Fig. 2A), *Cal. Pulchella* was found in 19% of spurs, 20% of shoot tips and 27% of branches below pruning wounds; *Cyt. Sorbicola* was found in 23% of spurs, 15% of tips and 18% of branches below pruning wounds; *E. lata* was found in 3% of spurs and 4% of branches below pruning wounds. Additionally, other canker-causing fungi were found in Yolo 1 samples such as *Botryosphaeria* spp., which were isolated from all wood types with 6% of spurs, 10% from shoot tips and 7% from branches below pruning wounds; and *Diaporthe* spp. were found in 13% of spurs and 26% of branches below pruning wounds.

From San Joaquin County (1) (Fig. 2B), *Cal. pulchella* was present in 10% of spurs, 20% of shoot tips and 20% of branches; and *Cyt. sorbicola* was found in 65% of spurs, 25% of tips and 20% of branches. *E. lata* was not found in this orchard. Other canker-causing fungi were found in San Joaquin 1 samples such as *Botryosphaeria* spp. found in 5% of both shoot tips and branches while *Diaporthe* spp. were found in 5% of spurs and 10% of shoot tips.

From San Joaquin County (2) (Fig. 2C), *Cal. pulchella* was only present in 27% of branches below pruning wounds; and *Cyt. sorbicola* were found in 5% of spurs, 15% of tips and 14% of branches below pruning wounds. *E. lata* was not found in this orchard. *Botryosphaeria* spp. were found in 5% of both spurs and shoot tips and 18% of branches below pruning wounds while *Diaporthe* spp. were found in 20% of spurs and 5% of both shoot tips and branches below pruning wounds.

From San Joaquin County (3) (Fig. 2D), *Cal. pulchella* was present in 10% of spurs and 40% of branches below pruning wounds; *Cyt. sorbicola* was found in 10% of spurs and 10% of branches below pruning wounds; and *E. lata* was found in 15% of branches below pruning wounds. *Botryosphaeria* spp. were found 5% of branches below pruning wounds while *Diaporthe* spp. were found in 5% of each of all wood types.

From Stanislaus County (1) (Fig. 2E), *Cal. pulchella* was present in 20% of spurs, 5% of tips and 25% of branches below pruning wounds; and *Cyt. sorbicola* was found in 20% of spurs, 35% of tips and 20% of branches below pruning wounds. *E. lata* was found in 20% of branches below pruning wounds. *Fusarium* sp. was found in 5% of spurs.

Field survey of asymptomatic buds. Among the major fungal canker pathogens in California, only *Cyt. sorbicola* was found in asymptomatic buds and present in 5 out of 8 orchards sampled across all three counties (Table 1). The most notable result was *Cyt. sorbicola* being found in the highest incidence of 50% of buds in Yolo 1A, but less than 3% incidence in Yolo Co. (1B), Stanislaus 1, San Joaquin 1, and San Joaquin 2. No fungal canker pathogens were found in buds in Yolo Co. (2), Stanislaus Co. (2, and San Joaquin Co. 3. Other fungal canker pathogens such as *Botryosphaeria* spp., *Diaporthe* spp., and *Fusarium* spp. were isolated at low rates of below 5% of buds sampled from each orchard except for those from Yolo 1A and 1B where *Botryosphaeria* sp. and *Diaporthe* sp., respectively, were found in 7% of buds.

Field inoculation of leaf scars. *Cyt. sorbicola* was recovered from 5% of *Cytospora*-treated leaf scars (n = 20) based on data averaged from 2 trials (Fig. 3A). Neither *Cal. pulchella* nor *E. lata* were recovered from the respectively treated leaf scar tissue samples. From leaf scar tissue treated with *Cal. pulchella*, *E. lata* and H2O control, numerous non-canker related fungi were isolated including those belonging to *Alternaria*, *Penicillium*, *Rhizopus*, *Cladosporium*, *Aspergillus*, and some yeasts (Fig. 2A).

Field inoculations of bud scars. *Cal. pulchella* was recovered from 5% of *Calosphaeria*-treated bud scars (n = 20) based on data averaged from 2 trials (Fig. 3A). Neither *Cyt. sorbicola* nor *E. lata* were recovered from the treated bud scars. From bud scars treated with *Cyt. sorbicola*, *E. lata* and H2O control, numerous non-canker related fungi were isolated

including those belonging to *Alternaria*, *Penicillium*, *Rhizopus*, *Cladosporium*, *Aspergillus*, and some yeasts.

Field inoculations of fruit scars. *Cyt. sorbicola* was recovered from 33% of *Cytospora*-treated fruit scars (*n* = 19) based on data from trial 1. Neither *Cal. pulchella*, nor *E. lata* were recovered from the treated fruit scars in trial 1. *Cyt. sorbicola* was recovered from 11.1% of water-treated controls (*n* = 18). On the second trial (Fig. 3A and 3B) where wounds on fruiting spurs were intentionally made, recovery from pathogen-treated samples were consistently higher. Based on the total average of fruit scar inoculations, *Cyt. sorbicola* (n = 40) was recovered from 60% of fruit scars, and *Cal. pulchella* (n = 40) and *E. lata* (n = 40) were both recovered from 42.5% of fruit scars. Based on the average of individual wound types on the fruiting spur, *Cal. pulchella* was recovered from 50% of broken spur wounds (*n* = 20) and 35% of peduncle removal wounds (*n* = 20). *Cyt. sorbicola* was recovered from 75% of broken spur wounds (*n* = 20) and 45% of peduncle wounds (*n* = 20). *E. lata* was recovered from 70% of broken spur wounds (*n* = 20) and 15% of peduncle removal wounds (*n* = 20). No canker pathogens were recovered from water-treated controls. Instead, numerous non-canker related fungi were isolated including those belonging to *Alternaria*, *Penicillium*, *Rhizopus*, *Cladosporium*, *Aspergillus*, and some yeasts.

Discussion:

This is the first study to determine the infection courts of *Calosphaeria pulchella*, *Cytospora sorbicola*, and *Eutypa lata* in sweet cherry. Our surveys of California cherry orchards identified *Cal. pulchella* as the main fungal canker pathogen affecting pruning wounds, followed by *Cyt. sorbicola*, and *E. lata*. These pathogens were present in all three counties and represent

the highest incidence of canker infection of pruning wounds of cherry in each county, with the exception of *E. lata*. These results are consistent with Trouillas *et al.* (2012), who reported *Cal. pulchella* as the most common fungal canker pathogen found in 95% of sampled sweet cherry orchards (n=20) in California. The occurrence of *E. lata* at pruning wounds appeared less common than previously reported (Trouillas *et al.* 2012). In the past, branch dieback and cankers in sweet cherry trees were largely associated with *E. lata*, with particularly high incidence of the pathogen in the Central Valley of California (Munkvold and Marois 1991; and 1994). Because of the high susceptibility of pruning wounds to *Eutypa lata* during the dormant season, recommendations were made in favor of summer pruning. Currently, it is common for growers to prune sweet cherry trees following harvest in early to mid-summer as well as during early fall, before the rainy season. The shift from winter pruning to summer pruning likely is responsible for a lower occurrence of *E. lata* below pruning wounds as reflected in our survey. Furthermore, it is probable that the past decade of drought conditions in California had reduced the occurrence of perithecia of *E. lata*, ultimately reducing available inoculum in cherry orchards (personal communications with Florent Trouillas 2022).

Successful isolation of major fungal canker pathogens (*Cal. pulchella, Cyt. sorbicola,* and *E. lata*) from spurs and shoot tips that exhibited dieback symptoms and with no apparent mechanical damage suggests that alternative infection courts to pruning wounds are common in cherry trees. Both *Cyt. sorbicola* and *Cal. pulchella* were largely present in declining fruiting spurs and shoot tips showing dieback sampled across all three counties, whereas *E. lata* was only rarely isolated from these tissue types. The isolation of *Cyt. sorbicola* from asymptomatic cherry buds suggests a potential for this pathogen to develop latent infections of canker diseases. This finding is consistent with Luo *et al*. (2019; and 2021) who supported the latent phase of various

canker-causing pathogens, including *Cytospora* spp., of stone fruits and nut crops after *Cytospora* spp. were detected commonly from asymptomatic buds and shoots of almond, plum, pistachio, and walnut. Worrall *et al*. (2010) had detected *Valsa melanodiscus* (anamorph *Cytospora umbrina*) in asymptomatic buds and healthy vascular tissue of mountain alder in Wyoming. It was further suggested that canker symptoms form on latently infected mountain alder during warm weather conditions (Worrall *et al*. 2010). Although not specific to buds, Schoeneweiss (1983) demonstrated that *Cyt. kunzei* inoculated on blue spruce are able to colonize the host with no canker formation. Symptoms of Cytospora canker only manifested when the host plant undergoes stress (Schoeneweiss 1983). Amponsah *et al*. (2015) recovered *Neonectria ditissima* from 22.8% of asymptomatic bud scale scars and shoots emerging from those buds 18 months after inoculations, demonstrating the latent ability of this canker pathogen in apple trees. A survey by Ntahimpera *et al*. (2002) had detected *Botryosphaeria dothidea* in asymptomatic pistachio buds in California with incidence of up to 60%. The high incidence of *Cyt. sorbicola* found in buds in Yolo 1A orchard might be explained by the high abundance of Cytospora fruiting bodies and inoculum sources in this orchard. Orchards with relatively low amounts of fungal fruiting bodies (Yolo County orchard 2 and commercial orchards from Stanislaus and San Joaquin County) showed a low occurrence of latent infection in buds and diseased tissues overall. Interestingly, there was no recovery of *Cal. pulchella* nor *E. lata* from dormant buds in all orchards sampled. *Cal. pulchella* and *Cyt. sorbicola* were particularly common in symptomatic spurs, shoot tips, and in branches below pruning wounds. Other canker-causing pathogens, such as *Botryosphaeria* spp., *Diaporthe* spp., and *Fusarium* spp, were detected in asymptomatic cherry buds in this study. These findings are consistent with Luo *et al*. (2019), who detected canker-causing pathogens belonging to *Diaporthe*, *Botryosphaeria*,

Diplodia, *Lasiodiplodia*, and *Neofusicoccum* from asymptomatic buds and shoots of almond, prune, pistachio, and walnut. The same canker-causing pathogens were also detected from symptomatic shoot tips, declining spurs, and branches below pruning wounds in this study. However, we did not include them in our field inoculations as they occurred at low incidence in cankers of sweet cherry and are not considered to be a major threat to the cherry industry (Trouillas *et al*. 2012).

Additional infection courts of major fungal canker pathogens of sweet cherry were determined after spray-inoculating naturally occurring wounds or openings such as leaf scars (due to leaf senescence), bud scars (due to flower bloom) and wounds resulting from harvest (peduncle removal and broken and damaged fruiting spurs due to fruit removal) with spore suspensions. Our results indicate that leaf scar and bud scar can serve as entry sites for *Cal. pulchella* and *Cyt. sorbicola* although recovery of these fungal pathogens following inoculation of these tissues was low overall. Our findings are in contrast with Alves and Nunes (2017), who demonstrated the ability of *Neonectria ditissima* to infect 24.3% of inoculated leaf scars in apple trees. Tekauz and Patrick (1974) had demonstrated that leaf scars and buds of peach may serve as infection courts for *Leucostoma cincta*. The infection rate of *L. cincta* from simulated leaf scars and buds were 16% and 3%, respectively (Tekauz and Patrick 1974). Uddin and Stevenson (1997) reported that a *Phomopsis* sp. was able to cause canker infection in all inoculated wounds of peach in Georgia, which included wounded dormant buds, breaking buds, and simulated bloom and leaf scars. *Pseudomonas mors-prunorum*, a causal agent of bacterial canker of stone fruits, inoculated on simulated leaf scars of sweet cherry resulted in up to 79.6% infection rate by the pathogen (Crosse 1951; and 1956).

Following harvesting of the fruits, all three major fungal canker pathogens were able to infect wounds resulting from the breakage of fruiting spurs and detachment of fruit peduncles. Nevertheless, wounds at the base of fruit peduncles resulted in lower infection rates when compared with wounds made from the partial or complete breakage of the spur. Peduncle removal mainly exposes the inner bark tissues, while a broken spur usually exposes the sapwood, heartwood, and pith. This difference could explain the higher infection rates of broken spurs as fungal canker pathogen require woody tissue for infection. Sallato *et al*. (2021) stated that the hand removal of spurs in sweet cherry may increase the risk of infection by *Pseudomonas syringae* pv. *syringae*, a causal agent of bacterial canker. Accordingly, limiting harvest damage of fruiting spur during harvest should reduce the risk of infection of cherry trees by fungal canker pathogens.

In conclusion, this is the first study to prove that wounds on spurs caused by manual fruit harvesting can serve as an additional and substantial infection court for major fungal canker pathogens of sweet cherry, namely *Calosphaeria pulchella*, *Cytospora sorbicola*, and *Eutypa lata*. The high incidence of fungal canker pathogens below pruning wounds confirm that pruning wounds also act as the primary entry point for canker infection. Also less susceptible, leaf and bud scars could lead to significant infections in orchards due to the large amount of such wounds produced each year during leaf fall in the fall and bud break in the spring, respectively. The infection of such wounds also could explain the decline of shoot tips with no apparent pruning or other mechanical injury. Finally, the susceptibility of other potential infection courts such as lenticel openings and insect-caused wounds should be explored in future research to expand our understanding of infection courts of canker pathogens. Additionally, the efficacy of wound

protectants, chemical and biological controls to protect the newly determined infection sites should be investigated.

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Figures and Tables (Chapter 1):

Figure 1: Examples of infection courts utilized in this study includes the following wounds: (A) leaf drop (leaf senescence), (B) bud break during new shoot emergence, (C) bud scales falling during flower bloom, (D) harvest by pulling fruit cluster peduncles, and (E) harvest by pulling fruit cluster spurs.

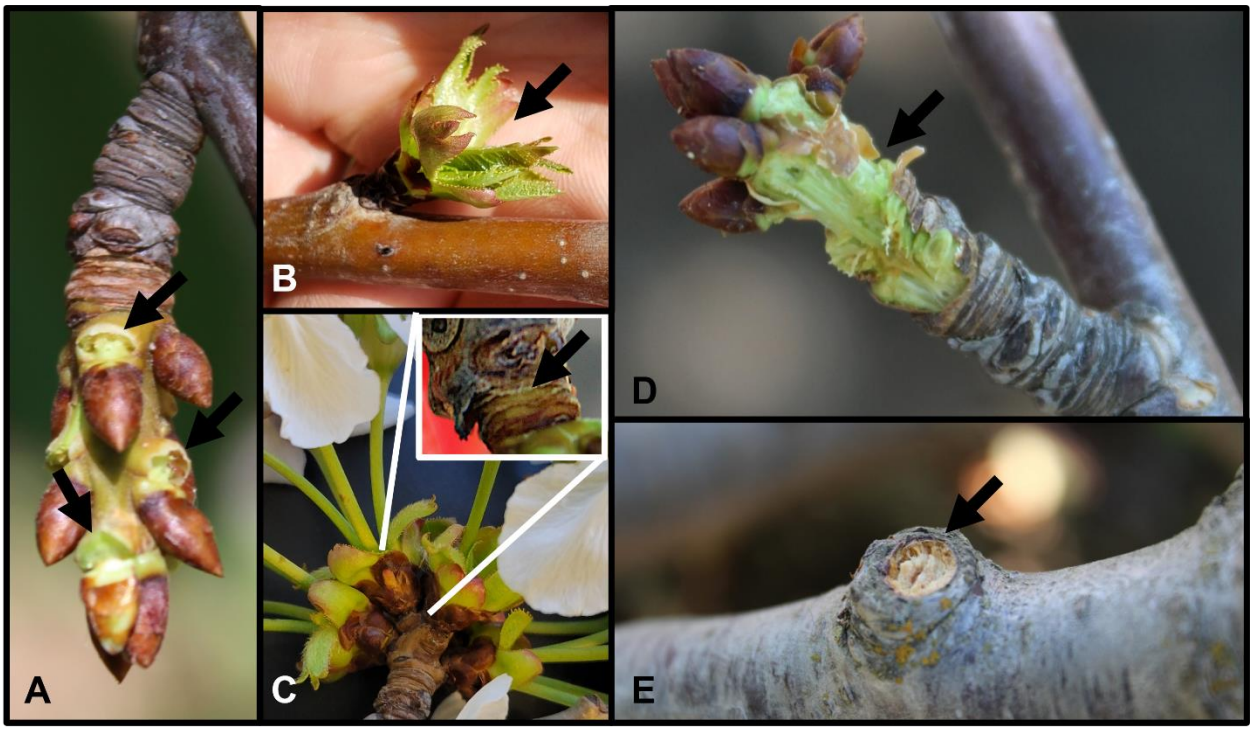


Figure 2: Survey of fungal canker pathogens in cherry spur, shoot tips and branches below pruning wounds exhibiting canker and dieback symptoms. Incidence of pathogen is reflected as percentage of recovery out of all samples (n) of that wood-type. Non-pathogenic fungi refer to non-canker forming fungi. Survey data represents samples from 5 orchards across three different California counties: **(A)** Field 1 of Yolo County; **(B)** field 1, **(C)** field 2, and **(D)** field 3 of San Joaquin County; and **(E)** field 1 of Stanislaus County.

Fig. 2A

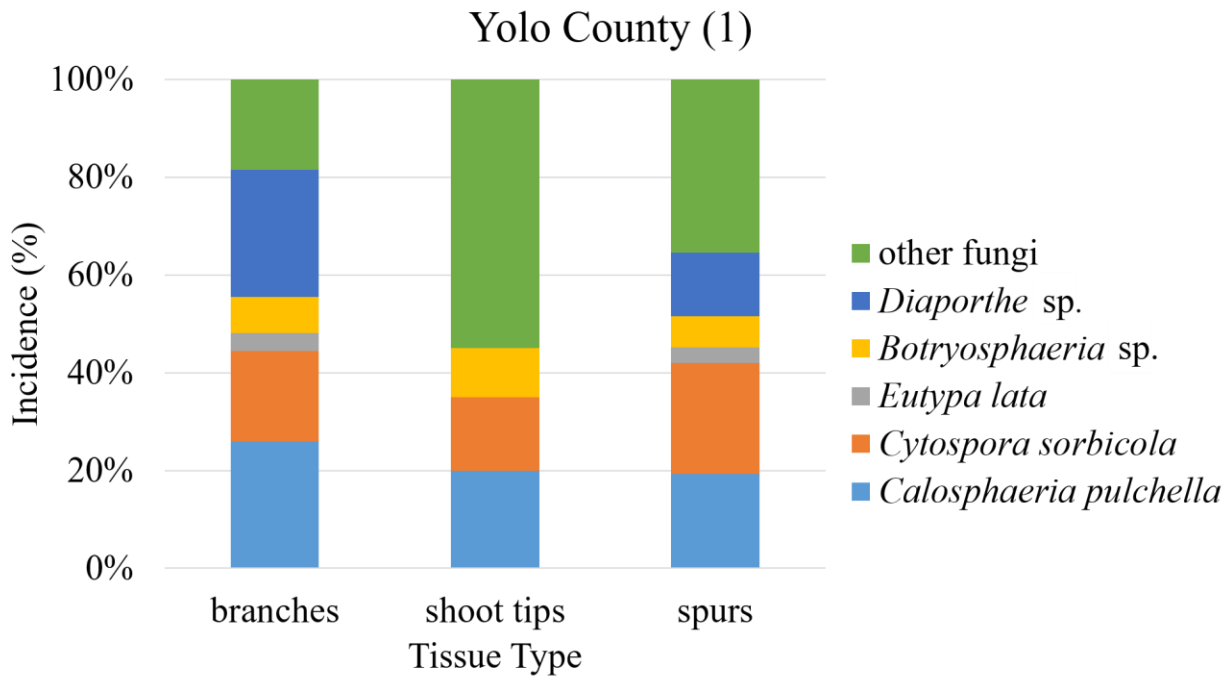


Fig 2B

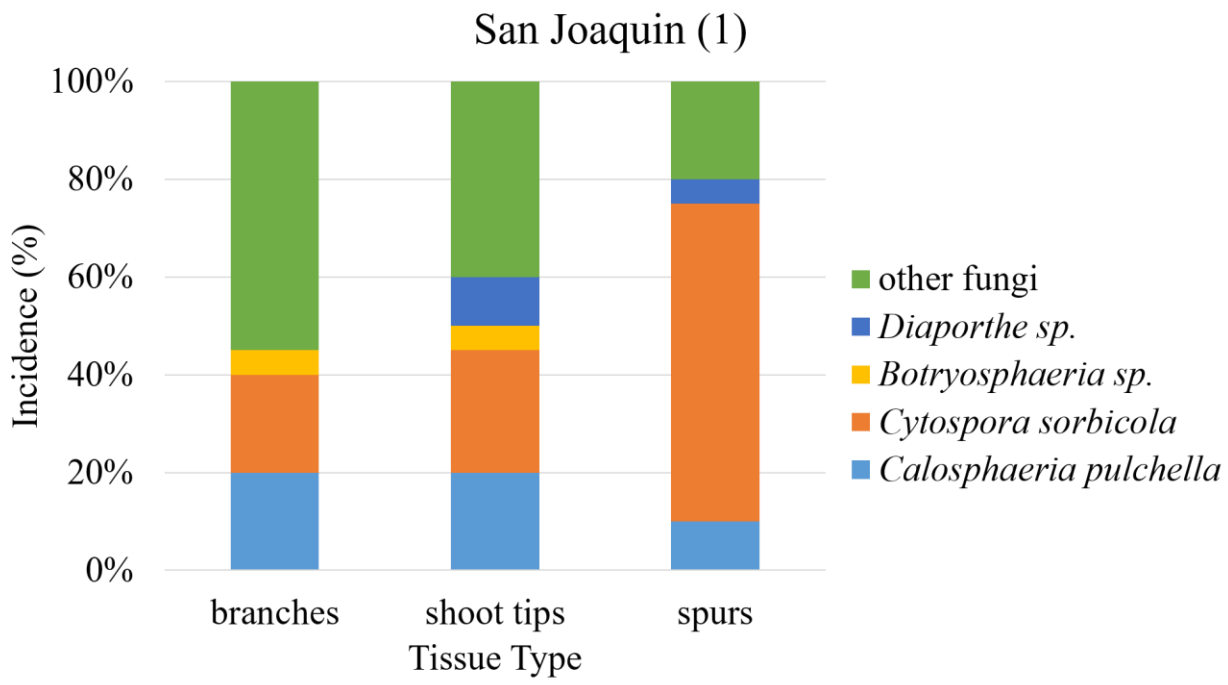

San Joaquin (1)
100%
80%
60%
40%
20%
0%
Incidence (%)
branches
shoot tips
spurs
Tissue Type
other fungi
Diaporthe sp.
Botryosphaeria sp.
Cytospora sorbicola
Calosphaeria pulchella

Fig. 2C

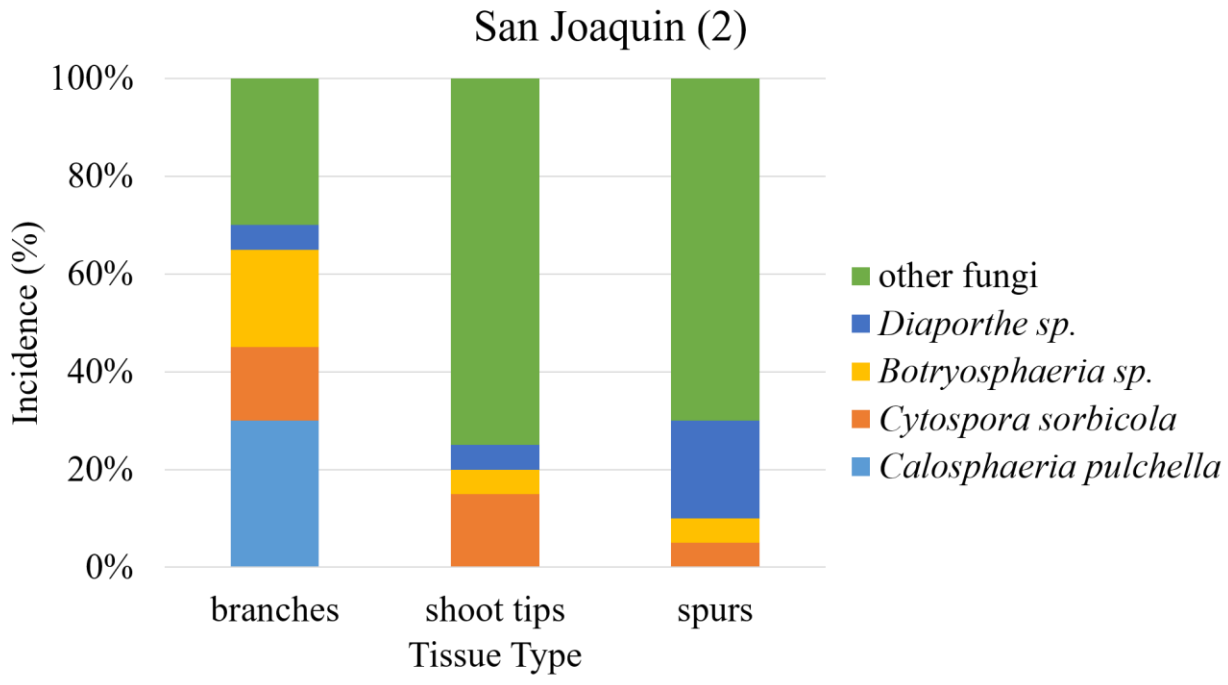

San Joaquin (2)
100%
80%
60%
40%
20%
0%
Incidence (%)
branches
shoot tips
spurs
Tissue Type
other fungi
Diaporthe sp.
Botryosphaeria sp.
Cytospora sorbicola
Calosphaeria pulchella

Fig. 2D

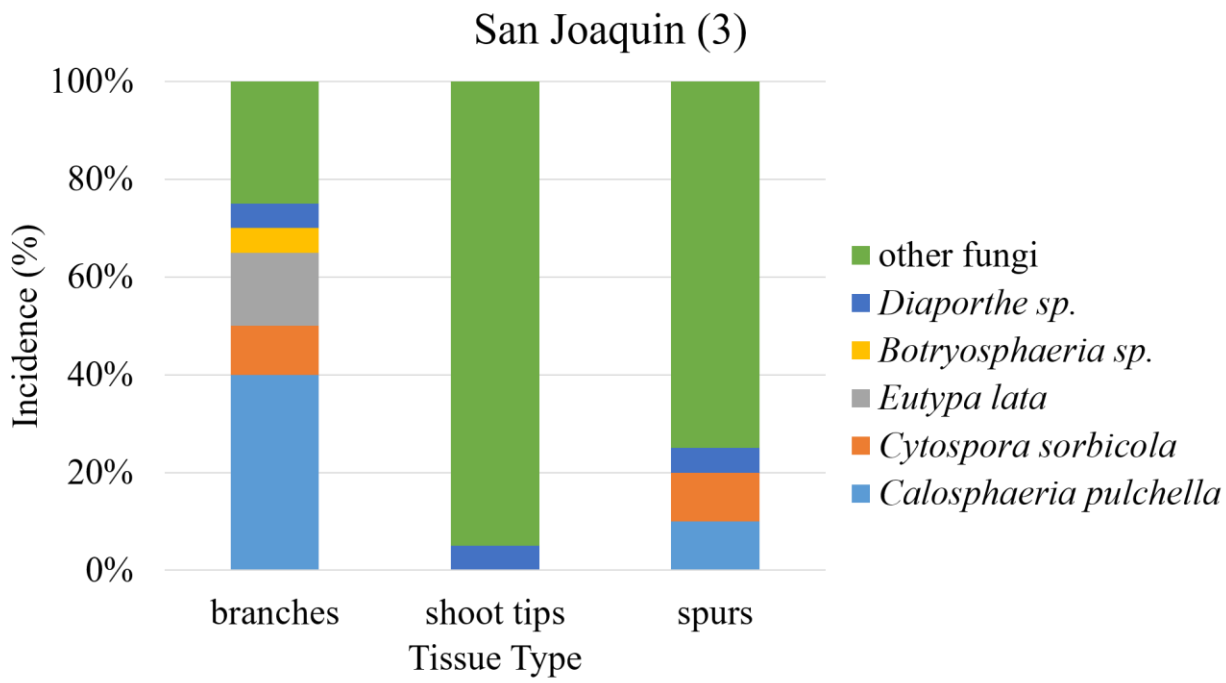

San Joaquin (3)
100%
80%
60%
40%
20%
0%
Incidence (%)
branches
shoot tips
spurs
Tissue Type
other fungi
Diaporthe sp.
Botryosphaeria sp.
Eutypa lata
Cytospora sorbicola
Calosphaeria pulchella

Fig. 2E

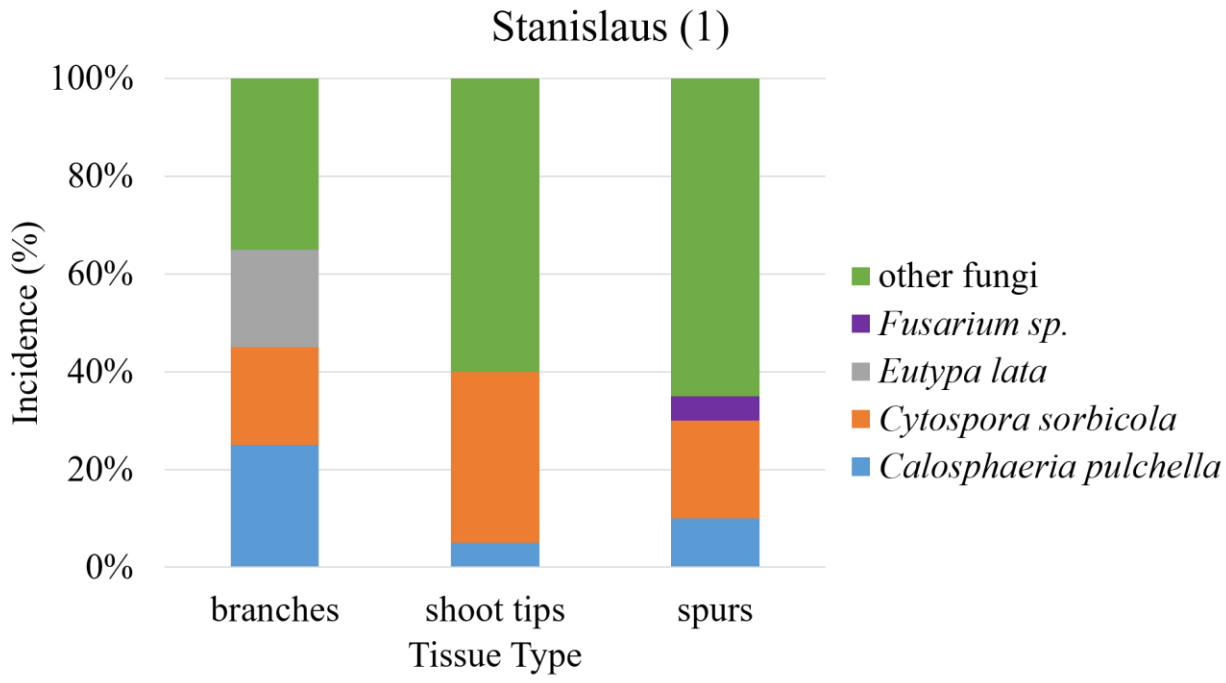

Stanislaus (1)
100%
80%
60%
40%
20%
0%
Incidence (%)
branches
shoot tips
spurs
Tissue Type
other fungi
Fusarium sp.
Eutypa lata
Cytospora sorbicola
Calosphaeria pulchella

Table 1: Survey of fungal canker pathogens in asymptomatic cherry buds. Asymptomatic buds (N=100) were collected at random from 8 different orchard spanning across three counties. The incidence of canker pathogen is reflected as a value out of 100. Lesser California fungal canker pathogens were detected and noted from survey.

County/ Orchard Designation	**Date Sampled**	**Fungal isolation**	**Incidence % (out of 100 buds)**
Yolo 1A	1/22/2020	*Cytospora sorbicola*	50
		Diaporthe sp.	4
		Botryosphaeria sp.	7
		Non-canker-forming fungi	39
Yolo 1b	1/30/2020	*Cytospora sorbicola*	2
		Diaporthe sp.	7
		Botryosphaeria sp.	4
		Fusarium sp.	2
		Non-canker-forming fungi	85
Yolo 2	2/4/2020	*Diaporthe* sp.	2
		Botryosphaeria sp.	5
		Non-canker-forming fungi	93
Stanislaus 1	2/8/2020	*Cytospora sorbicola*	2

		Diaporthe sp.	1
		Non-canker-forming fungi	97
Stanislaus 2	2/8/2020	*Botryosphaeria* sp.	3
		Fusarium sp.	1
		Non-canker-forming fungi	96
San Joaquin 1	2/13/2020	*Cytospora sorbicola*	1
		Diaporthe sp.	1
		Botryosphaeria sp.	1
		Fusarium sp.	2
		Non-canker-forming fungi	95
San Joaquin 2	2/13/2020	*Cytospora sorbicola*	1
		Diaporthe sp.	2
		Botryosphaeria sp.	1
		Non-canker-forming fungi	96
San Joaquin 3	2/13/2020	Non-canker-forming fungi	100

Figure 3: Recovery of *Calosphaeria pulchella, Cytospora sorbicola,* and *Eutypa lata* from inoculated wounds due to natural leaf senescence, bud break and mechanically induced wounds of fruiting spurs of sweet cherry. (A) Comparison of pathogen recovery among leaf, bud, and spur wounds; **(B)** Comparison of recovery from two distinct types of spur wounds (peduncle and spur).

Fig. 3A

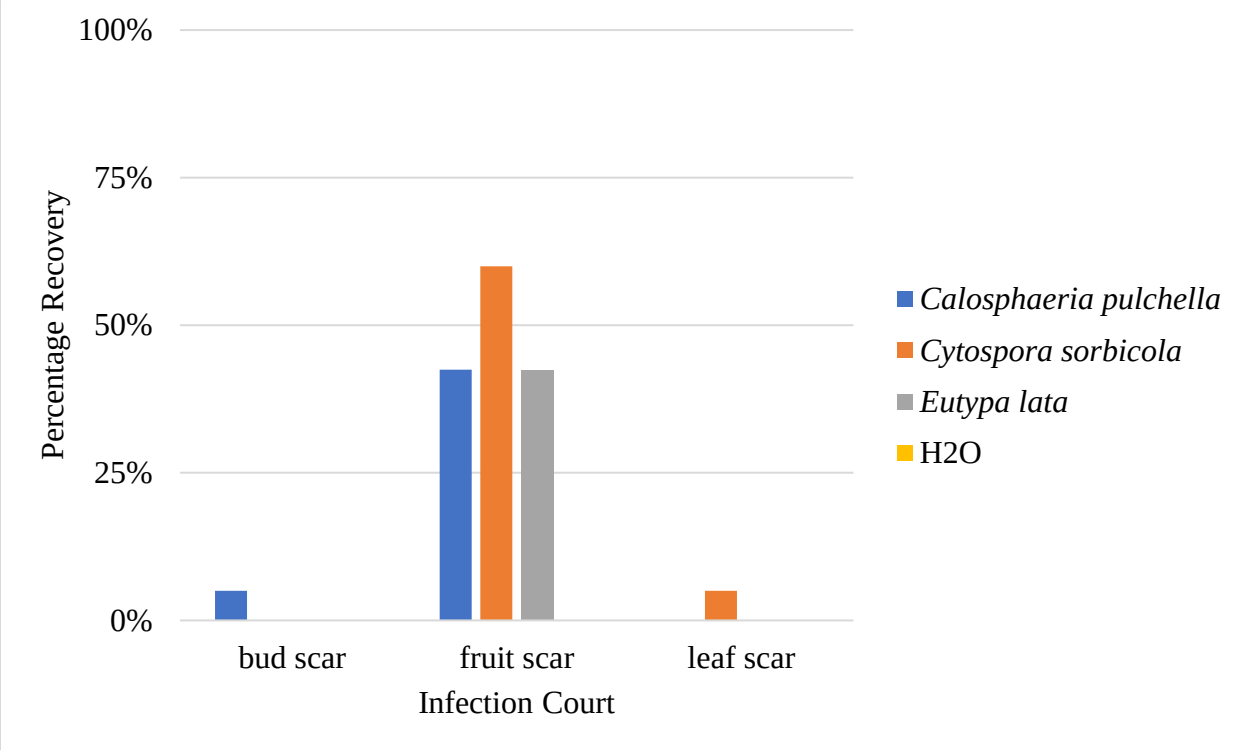
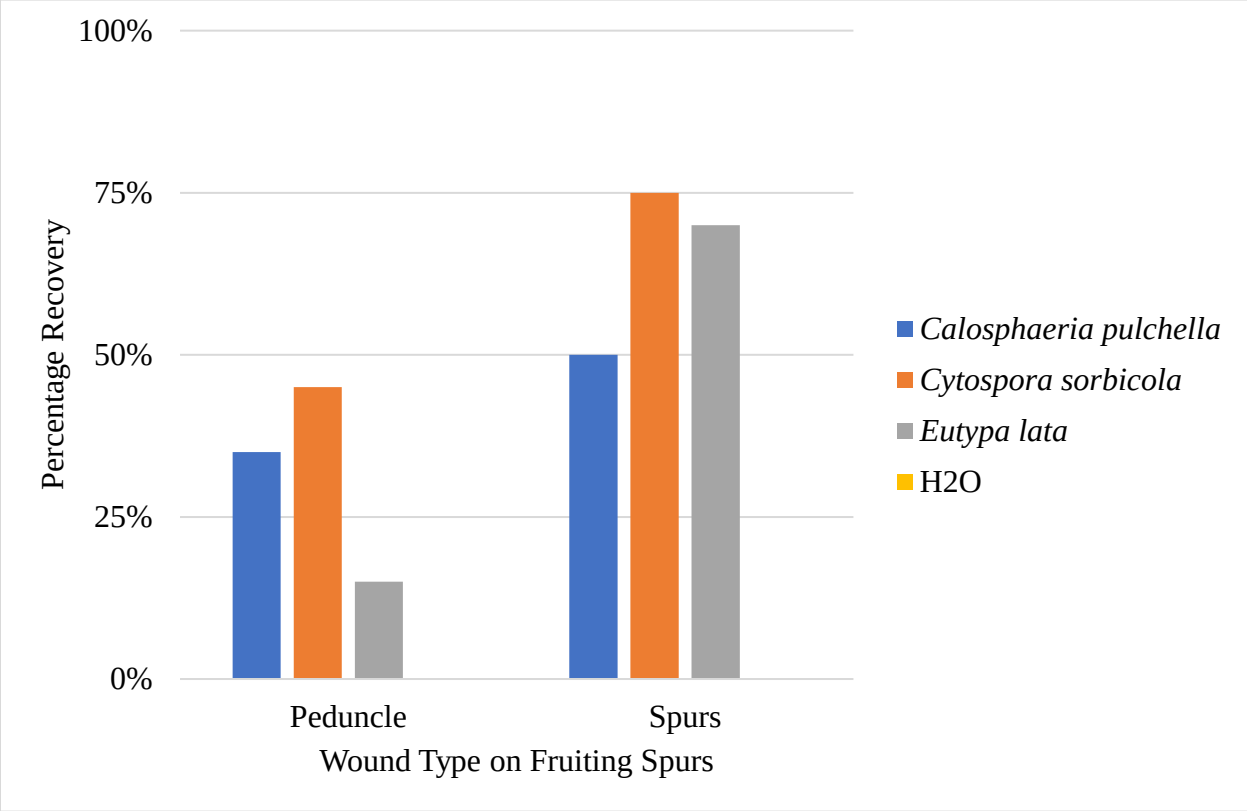


Fig. 3B


100%
75%
50%
25%
0%
Percentage Recovery
Peduncle
Spurs
Wound Type on Fruiting Spurs
Calosphaeria pulchella
Cytospora sorbicola
Eutypa lata
H2O

Chapter II: Effect of temperature on spore germination and mycelial growth of *Calosphaeria pulchella, Cytospora sorbicola* and *Eutypa lata* associated with sweet cherry canker diseases.

Introduction:

Calosphaeria canker caused by *Cal. pulchella*, *Cytospora* canker caused *Cyt. sorbicola*, and *Eutypa* dieback caused by *E. lata* are the main fungal canker diseases occurring in California cherry orchards (Trouillas *et al.* 2012). These canker pathogens constitute a major threat to the cherry industry and reduce the productivity and longevity of the orchard. *Calosphaeria pulchella* (Anamorph: *Calosphaeriophora pulchella* (Réblová, L. Mostert, W. Gams & Crous, sp. nov) is the causal agent of Calosphaeria canker and belongs to the order of Calosphaeriales (Réblová *et al.* 2004). *Cal. pulchella* causing canker diseases of sweet cherry was first reported in California in 2010, but was also shown to occur on cherry in South Australia, France, Italy (Trouillas *et al*. 2010a), Spain (Berbegal *et al*. 2014), and Chile (Auger *et al*. 2021). In addition to cherries, *Cal. pulchella* was also shown to cause cankers on other *Prunus* spp. such as peaches, nectarines, and almonds (Arzanlou and Dokhanchi 2013, Berbegal and Armengol 2018). When wetted, *Cal. pulchella* perithecia (sexual fruiting bodies) found beneath the periderm of diseased branches produce and release ascospores, the main infectious propagule. The occurrence of conidia (asexual spores) in *Cal. pulchella* have not been observed in nature; however, these are produced in abundance in culture. Early spore trapping studies by Trouillas *et al*. (2012) in California showed that fall, winter and spring rainfalls can trigger the release of ascospores of *Calosphaeria pulchella*. Additionally, it was understood that the use of sprinkler irrigation is a major contributor to the dispersal of the spore inoculum of Calosphaeria canker (Trouillas *et al*. 2012), Cytospora canker

(Biggs and Grove 2005; Grove and Biggs 2006) and Eutypa dieback (Munkvold and Marois 1994; Petzoldt *et al*. 1983). Overall, drip-irrigated cherry orchards produced significantly fewer cankers than those that were sprinkler-irrigated in California (Gubler *et al*. 2014).

Cyt. sorbicola (Norphanphoun, Bulgakov, T.C. Wen & K.D. Hyde, sp. Nov.) was recently reported as the main causal agent of Cytospora canker of sweet cherry in California and was reported to also cause canker diseases in olive, plum, prune, peach, almond in California (Lawrence *et al*. 2018), and sour cherry in Russia (Norphanphoun *et al*. 2017). Conidia produced in pycnidia are the primary inoculum for *Cyt. sorbicola* infection in sweet cherry in California. Pycnidia occur beneath the periderm of diseased cherry branches and release conidia in the form of mucilage when pycnidia are wetted by rain. Dispersal of conidia are facilitated by rain splashing. The sexual morph or perithecial stage of *Cyt. sorbicola* has been sporadically observed in sweet cherry in California (Florent Trouillas, personal communication) but its distribution and contribution to disease spread is unknown. *Cytospora* spp. were reported to release spores year-round in Yolo County prune orchards when rain occurs, with the highest amounts during fall rains and fewest in mid-and late-winter (Bertrand and English 1976). *Cytospora cincta*conidia are present throughout the year with the highest quantity occurring in spring and summer in both cherry and peach orchards in eastern Washington (Grove and Biggs 2005).

Eutypa lata (Pers. Fr.) Tul. & C. Tul. (Syn. *E. armeniacae* Hansf. & Carter) causing Eutypa dieback is widespread in cherry, grapevine, and apricot in California and produces ascospores as the main source of disease inoculum. Perithecia of *E. lata* are embedded in the bark or wood of its plant host, including cherry, grapevine, big leaf maple, apricot, apple, pear, oleander, and willow (Trouillas and Gubler 2010). *Eutypa lata* perithecia found in dead, infected apricot wood are able to discharge ascospores within 5-10 min of immersion in water (Carter 1957). Moreover, spore

release of *E. lata* is influenced mainly by temperature, rainfall and high relative humidity (van Niekerk *et al*. 2010). *Eutypa lata* ascospores are released during and shortly after rain events with the greatest amounts occurring in fall to late-winter in apricot orchards in Solano County, California (Ramos *et al.* 1975). Ascospores can be carried by wind over great distances and up to 50-60 km from their source (Petzoldt *et al*. 1983).

Temperature is known to play a critical role in spore germination of most fungal pathogens and the effect of temperature on spore germination and mycelial growth has been widely studied to determine conditions required for fungal infection and disease development. Several studies have investigated the effect of temperature on spore germination and mycelial growth of important canker pathogens, including the *Botryosphaeriaceae, E. lata,* and *Cytospora* species (Amborabé *et al*. 2005; Bakarat 1995; Bakarat *et al*. 2005; Carter 1957; Helton and Konicek 1962; Luo *et al*. 2022; Trese 1980; Úrbez-Torres *et al*. 2006; Úrbez-Torres *et al*. 2010; Wang *et al*. 2016; Zang and Huang 2007). Temperature also was shown to play a major role in the pathogenicity and virulence of *Cytospora* species and *E. lata* causing cankers (Barakat and Johnson 1997; Chen *et al*. 2016; Luo *et al* 2022; Scharpf 1983; Treshow *et al*. 1958; Wensley 1964; and Worrall *et al*. 2010; Gubler *et al*. 2005; Lardner *et al*. 2005; Munkvold and Marois 1994; Sosnowski *et al*. 2007; Trese 1980). In almond and prune, Luo *et al*. 2022 indicated that optimal temperatures for colony growth differed significantly among different canker pathogens. The same authors showed also that canker development and growth within twigs was strongly influenced by temperature (Luo *et al*. 2022). Úrbez-Torres *et al*. (2006) indicated that climatic conditions, and temperature, in particular, can influence the geographical distribution of fungal canker pathogens in the Botryosphaeriaceae.

Although the effect of temperature on spore germination and mycelial growth of several *Cytospora* spp. was studied previously (Bakarat *et al*. 2005, Kamiri and Laemmlen 1981, Scharpf

1983, Zhang and Huang 2007), the influence of temperature on *Cyt. sorbicola* is unknown. Similarly, no information is available regarding the effects of temperature on ascospore germination and mycelial growth for *E. lata* isolates originating from cherry cankers in California, although these were investigated in Australia and France, respectively (Carter 1957; Amborabé *et al*. 2005). Furthermore, despite the common occurrence of Calosphaeria canker in sweet cherry orchards, little is known about the effect of temperature on spore germination and growth of *Cal. pulchella.* Yet, our laboratory has failed repeatedly to infect pruning wounds of sweet cherry trees following winter pruning and ascospore inoculations, suggesting that *Cal. pulchella* most likely requires warm temperatures for infection.

Sweet cherry in California are typically pruned when they are dormant in winter, or after harvest during summer. Understanding the effects of temperature on spore germination and mycelial growth of major fungal canker pathogens in California is required to better elucidate the impact of seasonal temperatures on the infection rates and successful colonization of pruning wounds. Ultimately, this knowledge will improve the development of more efficient management guidelines for major canker diseases of cherry in California. Thus, the objective of this chapter was to identify the optimal and critical temperatures for mycelial growth and spore germination of *Cal. pulchella*, *Cyt. sorbicola*, and *E. lata* associated with cherry canker diseases.

Materials and Methods:

Maintenance of cultures for mycelial growth studies. Isolates of *Cal. pulchella* (RTFT004, RTFT009, RTFT015 and Wint960) and *Cyt. sorbicola* (RTFT010, RTFT016 and CCK013) were obtained from diseased cherry wood exhibiting canker symptoms in Yolo County, except for *Cal. pulchella* Wint960, which was from Solano County. Isolates of *E. lata* (Sum9-7)

originated from cherry in Yolo County and isolates (B4-2-1 and P5-3-1) originated from cherry in the Central Valley. Cultures were grown and maintained on potato dextrose agar (PDA; Sigma-Aldrich®, St. Louis, MO) amended with tetracycline (PDA-Tet; PDA; Sigma-Aldrich®, St. Louis, MO) at a 1 mg/Lconcentration at the laboratory ambient temperature (22-24°C) and natural photoperiod. Each isolate was saved into culture collection by cutting and placing 4 mm × 4 mm × 4 mm cubes of agar from the margin of the colony into sterile deionized water (DI) vials and storing them at 4°C. Fresh cultures were prepared from this collection for each experiment in this study.

Preparation of spores for germination studies. *Cal. pulchella* ascospores were collected from perithecia found under the periderm of dead, infected sweet cherry branches from a UC Davis experimental orchard located in Yolo County. Pieces of wood containing *Cal. pulchella* perithecia were cut from branches and affixed to a plastic Petri lid with petroleum jelly (Vaseline® 100%, Greenwich, CT) and submerged in deionized (DI) water for 15 to 30 minutes (min) to fully saturate the wood and fruiting bodies. Water was then poured out and perithecia were gently blotted dry with Kimwipes™ paper sheets (Kimwipes; Kimtech Science® Kimberly-Clark Professional®, Roswell, GA). The lid with perithecia was placed over a new, sterile Petri dish bottom. At least 2 hours (h) were allowed for ascospores to fully discharge onto the dish bottom. DI water was pipetted up and down repeatedly to dislodge and collect the recently discharged spores. *Cal. pulchella* conidia were obtained from one-week-old representative cultures by pipetting up and down 500 µl of sterile deionized water over the colony to wash and draw up spores.

Cyt. sorbicola conidia were obtained from conidial cirri found on infected sweet cherry branches from a UC Davis experimental orchard located in Yolo County and from San Joaquin

County. Additional conidia exuded from pycnidia in the form of mucilage after exposure of pycnidia to sterile water. Tendril-like masses (cirri) were collected and fully dissolved by vortexing in sterile DI water to obtain a conidia suspension.

Eutypa lata ascospores were collected from perithecia growing on dead, infected oleander (*Nerium oleander*) wood from Yolo County. Ascospores were obtained as described by Trouillas *et al*. (2010b). A thin, flame-sterilized razor blade was used to cut tangentially along the wood surface, where ostioles were visible, to remove thin layers of wood and expose the internal perithecia cavity. Sterile DI water was added over these openings to hydrate the hymenium and produce the typical, dark, gel-like matrix containing the ascospores. With the aid of a dissecting microscope and a flame-sterilized dissecting needle, the spore matrix was carefully collected and transferred into 1.5 mL Eppendorf tubes containing sterile DI water. The resulting spore suspension was vortexed for 2 min to dislodge and evenly suspend ascospores.

Each spore suspension concentration was estimated using a hemocytometer and adjusted with sterile DI water to 5×10^4 spores·mL^{-1}. Spores were stored at 4°C to prevent germination until ready for use. All spore suspensions were used on the day of preparation.

Germination of spores under different incubation temperatures. The effect of temperature on *Cal. pulchella* ascospores germination was studied *in vitro*. A 10 µL suspension of ascospores was pipetted onto the surface of 2% water agar using three Petri plate replicates for each temperature 5, 10, 15, 20, 25, 30, 35, and 40°C. The same was done for *Cal. pulchella* conidia, *Cyt. sorbicola* conidia and *E. lata* ascospores. Spore germination at the different temperatures was assessed after 12, 24 and 36 h incubation directly on the Petri dish at 200× magnification under a compound microscope. A spore was considered germinated when its germ tube length had reached or exceeded the length of the spore itself. Germination rates were calculated as the number of

spores germinated out of 100 randomly counted spores for each repetition and temperature. The average values from 2 replication trials of each spore type were used as final germination rates. A trend line was created for each set of data points using a polynomial curve fit (described in the results) and used for calculating an estimated optimal germination temperature for the spores of each pathogen. In addition, *Cal. pulchella* conidia and ascospores germination rates were evaluated after 12, 24, 48, 72, 96, 120, and 144 h of incubation at 5, 10, and 15°C.

Mycelial growth under various incubation temperatures. The effect of temperatures on mycelial growth was studied using 4 representative isolates of *Cal. pulchella* RTFT004, RTFT009, RTFT015 and Wint960; 3 representative isolates of *Cyt. sorbicola* RTFT010, RTFT016 and CCK013; and 3 representative isolates of *E. lata* Sum9-7, DixAl-24 and Bx1-10. From the margins of 7-day-old colonies, 5 mm diameter agar plugs were transferred using a flame-sterilized cork borer to the center of potato dextrose agar (PDA) Petri plates using 3 replicates per isolate for each incubation temperature. Each *Cal. pulchella* isolate was incubated at temperatures of 5, 10, 15, 20, 25, 30, 35, and 40°C for 12 days. Each *Cyt. sorbicola* and *E. lata* isolate was incubated at the same temperatures for 8 and 7 days, respectively. The diameters of each colony were measured at two orientations perpendicular to each other. To account for the initial diameter of the agar plug, 5 mm were subtracted from each measurement. Measurements were averaged among all isolates for each pathogen to quantify the mycelial growth rate. Similarly to the temperature study on spore germination, a trend line was created for each set of data points using a polynomial curve fit (described in the results) to estimate the optimal temperature for the mycelial growth of each pathogen.

Results:

***In-vitro* temperature study in spore germination.** After 36 h of incubation, *Cal. pulchella* ascospores exhibited the highest germination rate at 30°C while no germination was observed at 40°C and 15°C and below temperatures (Figure 1a). Similarly, the maximal germination rate of conidia was 30°C after 36 h of incubation. Less than 5% germination was observed for *Cal. pulchella* conidia at 15°C after 36 h of incubation while no germination was observed at 10°C or below temperatures (Figure 1b). *Cyt. sorbicola* conidia had the highest germination rate at 25°C. No germination was observed at 10°C and below, nor was it observed at 35°C and above (Figure 1c). *Eutypa lata* ascospores had the highest germination rate at 25°C. No germination was observed at 5°C, and 35°C and above (Figure 1d). Plates of *Cyt. sorbicola* conidia and *E. lata* ascospores that were incubated at 35°C and 40°C were further incubated at ambient temperature (22-24°C) for 1 week after which no spore germination was observed for either pathogen. The spores of all three pathogens that were incubated at 5°C were able to germinate after an additional 36 h of incubation at ambient temperature. The following polynomial equations (based on the fitted curve at 36 h) were used to estimate the optimal temperature for spore germination for each pathogen: $y=0.0681x^6-1.4628x^5+11.043x^4-34.915x^3+44.46x^2-13.517x-5.6825$ ($R^2=1.000$) for *Cal. pulchella*, $y=0.0801x^6-1.7269x^5+13.806x^4-52.831x^3+108.81x^2-114.85x+46.708$ ($R^2=1.000$) for *Cyt. sorbicola*, and $y=0.1126x^6-2.9377x^5+30.839x^4-166.75x^3+477.18x^2-627.33x+288.85$ ($R^2=0.999$) for *E. lata*. The polynomial equation fitted on *Cal. pulchella* conidia germination after 36 h incubation was expressed as $y=-0.0513x^6+1.3131x^5-12.981x^4+59.715x^3-119.14x^2+84.637x-10$ ($R^2=0.980$). Accordingly, the estimated optimal temperature for spore germination was 29.15°C for ascospores and 29.55°C for conidia of *Cal. pulchella*, 26.95°C for *Cyt. sorbicola* conidia and 22.85°C for *E. lata* ascospores (Figure 1e).

The ability of *Cal. pulchella* to germinate at lower temperatures also was assessed (Figure 2). Ascospores and conidia of *Cal. pulchella* did not germinate after 144 h of incubation at 5°C. At 10°C, a 1.9% germination rate of ascospores was observed after 72 h of incubation while the conidia germination rate was 1.7% after 48 h of incubation. Ascospores and conidia reached 50% germination between 96 and 120 h of incubation at 10°C. After 48 h of incubation at 15°C, germination rates for ascospores and conidia were 19.8% and 40.8%, respectively. Both spore types had reached 50% germination after 72 h of incubation at 15°C.

***In-vitro* temperature study in mycelial growth.** After incubation at 5 to 40°C at 5-degree increments for 12, 8, and 7 days for *Cal. pulchella*, *Cyt. sorbicola* and *E. lata*, respectively, mycelial growth rates were determined from the average colony diameter at the different time points (Figure 3). It should be noted however that the colony growth of *Cyt. sorbicola* isolates used in this study were highly lobate with irregular margins, which made it difficult to accurately measure colony diameters. *Cal. pulchella*, *Cyt. sorbicola* and *E. lata* colonies all produced the highest diameter of growth when incubated at 25°C compared to other incubation temperatures. The colony diameters of each pathogen respectively reached 75 mm, 59 mm, and 70 mm and respectively after 12, 8, and 7 days of incubation. No colony growth was observed on *Cal. pulchella* plates incubated at 5°C and 40°C. While both *Cyt. sorbicola* and *E. lata* colonies were able to grow at 5°C at a slow rate, no growth was observed for both pathogens incubated at 35°C and higher. The following polynomial equations were fitted over each set of data points to estimate the optimal temperature for mycelial growth for each pathogen: $y=0.1345x^6-3.2801x^5+31.142x^4-147.71x^3+369.19x^2-438.67x+189.61$ ($R^2=0.999$) for *Cal. pulchella*, $y=0.1161x^5-1.9669x^4+10.027x^3-14.542x^2+6.0252x+2.125$ ($R^2=0.990$) for *Cyt. sorbicola*, and $y=0.1035x^5-1.5921x^4+6.3807x^3-1.1606x^2-8.9486x+8.2187$ ($R^2=0.9505$) for *E. lata*. As with spore germination,

Cal. pulchella had the highest optimal temperature for mycelial growth (27.20°C), followed by *Cyt. sorbicola* (23.85°C) and *E. lata* (22.50°C).

Discussion:

The effect of temperature on mycelial growth and spore germination of *Cal. pulchella*, *Cyt. sorbicola* and *E. lata* causing canker diseases of sweet cherries in California was investigated. This study revealed that the optimal temperature for spore germination was 29.15°C for *Cal. pulchella* ascospores, 31.05°C for *Cal. pulchella* conidia, 26.95°C for *Cyt. sorbicola* conidia, and 22.85°C for *E. lata* ascospores. Additionally, it was found that the optimal temperature for mycelial growth was 27.20°C for *Cal. pulchella*, 23.85°C for *Cyt. sorbicola* and 22.85°C for *E. lata*. The results of our temperature study for *Cal. pulchella* and *Cyt. sorbicola* spore germination and mycelial growth represent new findings for California.

Our study indicates that *Cal. pulchella* is well adapted to the warm temperatures of California. Average high temperatures in California range on average from 27 to 34°C during the summer, which closely overlap with the optimal spore germination and growth temperatures for *Cal. pulchella* (San Joaquin County, NOAA 2022). In contrast, cold temperatures appear inhibitive of *Cal. pulchella* spore germination and mycelial growth. Indeed, as determined in the present study, the germination rate for both the conidia and ascospores of *Cal. pulchella* is relatively low to null at temperatures <15°C. California's average high temperatures during December and January are around 13°C (San Joaquin County, NOAA 2022). Thus, germination of *Cal. pulchella* spores during the winter season is unlikely. Although conditions of mild temperatures (15°C) may occur during winter in California, these events are generally short-lasting during daytime with temperatures significantly dropping at nighttime. Accordingly, winter temperatures in California,

including mild winter temperatures, are unlikely to contribute to spore germination and infection of cherry trees. While *Cal. pulchella* conidia were able to germinate at temperatures as high as 40°C, there is no evidence that the asexual stage (conidia) of *Cal. pulchella* occurs in nature, nor it plays a role in the disease cycle. *Cal. pulchella* ascospores have been the only spore type observed in the field and are associated with perithecia below the bark of cankered branches or trunk of sweet cherry trees. Although conidia of *Cal. pulchella* were not found in nature, these are readily produced in culture on PDA. Thus, we included both *Cal. pulchella* ascospores and conidia in this study.

Eutypa lata had the lowest optimal temperatures for spore germination and mycelial growth amongst the other fungal canker pathogens that were studied. Results of our study were consistent with those of Carter (1957), who reported optimal temperatures for *E. lata* between 22 and 25°C as well as no spore germination at 35°C and above. Furthermore, our results are consistent with Amborabé *et al*. (2005), who found the optimal temperatures for *E. lata* mycelial growth to be between 20 and 25°C, while temperatures below 15°C and above 30°C inhibited fungal growth. Trese *et al*. (1980) demonstrated that ascospores and mycelium of *E. armeniacae* were able to survive temperatures as low as -20°C, retaining rates of viability of up to 65% after exposure for 14 days. The ability of *E. lata* to germinate and grow under temperatures as low as 10°C indicates that it is well adapted to temperate climates and regions with mild to cold winter temperatures. Previous studies have indicated that the pathogenicity and virulence of *E. lata* is less influenced by low temperatures. Chapuis *et al*. (2007) demonstrated the ability of *E. lata* to cause infection on pruning wounds of grapevine cane segments after inoculation with ascospores and incubation at 5°C. Sosnowski *et al*. (2007) revealed that increased temperature in spring correlated with decreases in disease incidence and prevalence. Additionally, Lardner *et al*. (2005) reported that *E.*

lata toxin production and grapevine foliar symptoms are influenced by rainfall as well as low temperatures.

The results of this study on the effect of temperature on conidial germination and mycelial growth of *Cyt. sorbicola* are consistent with those from previous studies. Wang *et al*. (2016) demonstrated that the conidia of *V. mali*, which causes Cytospora canker of apple, germinated most rapidly at 25 and 30°C. Similarly, Zang and Huang (2007) reported that the optimal temperature for conidia germination of *V. mali* was between 25 and 28°C. *Cytospora kunzei*, a pathogen of blue spruce, achieved maximal conidial germination and mycelial growth at temperatures of 24 and 27°C, respectively (Kamiri and Laemmlen, 1981). Mycelium of *Cytospora abietis*, a pathogen of white fir, grew optimally between 25 and 30°C (Scharpf, 1983). Both Luo *et al*. (2022) and Helton and Konicek (1962) also indicated that *Cyt. leucostoma* colonies grew optimally at 25°C, which is slightly higher than the results from the present study. In addition, Luo *et al*. (2022) demonstrated that canker expansion on shoots of almond and prune inoculated with *Cyt. leucostoma* was highest at 25 and 30°C. Bakarat (1995) and Bakarat *et al*. (2005) reported that the optimal temperature for conidial germination for *Cyt. cincta* was 28°C. The optimal temperature for colony growth of *Cyt. quercinum* and *Cyt. vinacea* isolated from oak was determined to be 20.1 and 20.8°C, respectively (Pan *et al*. 2021). The broad range of temperatures at which *Cyt. sorbicola* was able to grow suggests it is well adapted to different seasonal conditions and climates. Rozsnyay (1986) found that *Cyt. cincta* can successfully infect apricot trees during all months of the year in Hungary with the optimal temperature for inoculation below 10°C and optimal temperature for canker expansion at -5 to -8°C. Bakarat *et al*. (1995) reported that dried cirri on sweet cherry containing conidia of *Cyt. cincta* remained highly viable for 30 days in the field under the summer conditions of Prosser, WA with maximum temperatures reaching 32.6°C.

Accordingly, one can hypothesize that *Cyt. sorbicola* can successfully infect cherry across all seasons of the year.

Our results on the effects of temperature on spore germination and mycelial growth of *Cal. pulchella*, *Cyt. sorbicola* and *E. lata* provided new insight into canker disease biology. The following chapter will investigate the seasonal susceptibility of pruning wounds of sweet cherry to these pathogens and will be greatly complementary to this study.

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Figures and Tables (Chapter 2):

Figure 1. Average spore germination rates of each pathogen after incubation for 12, 24, and 36 hours at temperatures ranging from 5 to 40°C at 5-degree increments. (a) *Calosphaeria pulchella* ascospores and (b) conidia, (c) *Cytospora sorbicola* conidia, and (d) *Eutypa lata* ascospores. (e) A visual comparison of optimal temperature for germination was made.

Fig. 1a.

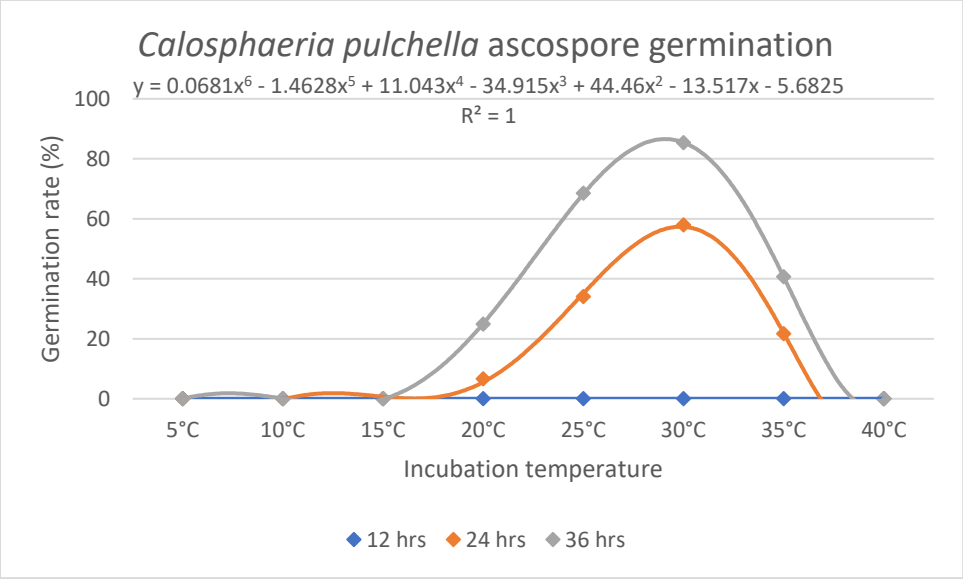


Fig. 1b.

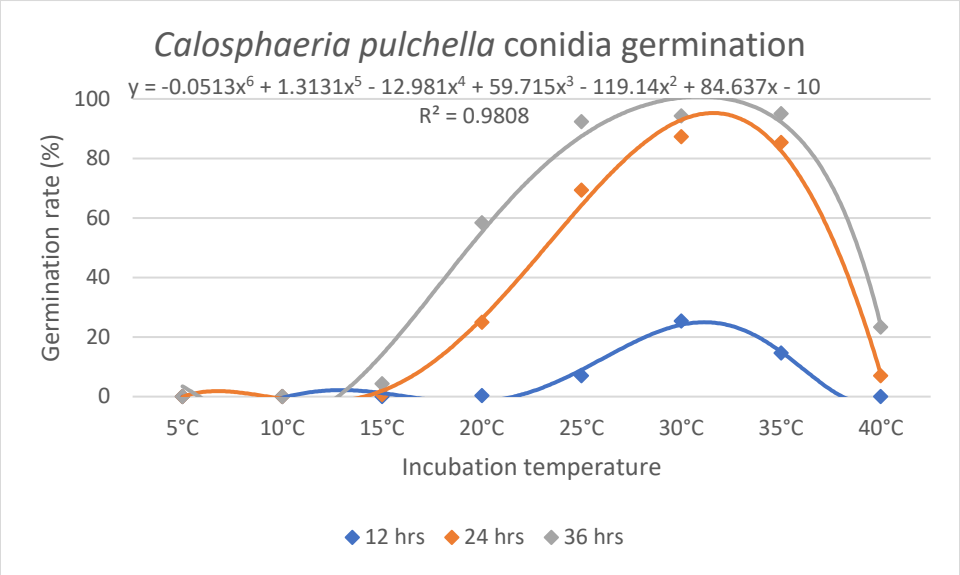

Calosphaeria pulchella conidia germination
y = -0.0513x⁶ + 1.3131x⁵ - 12.981x⁴ + 59.715x³ - 119.14x² + 84.637x - 10
R² = 0.9808
Germination rate (%)
100
80
60
40
20
0
5°C
10°C
15°C
20°C
25°C
30°C
35°C
40°C
Incubation temperature
12 hrs
24 hrs
36 hrs

Fig. 1c.

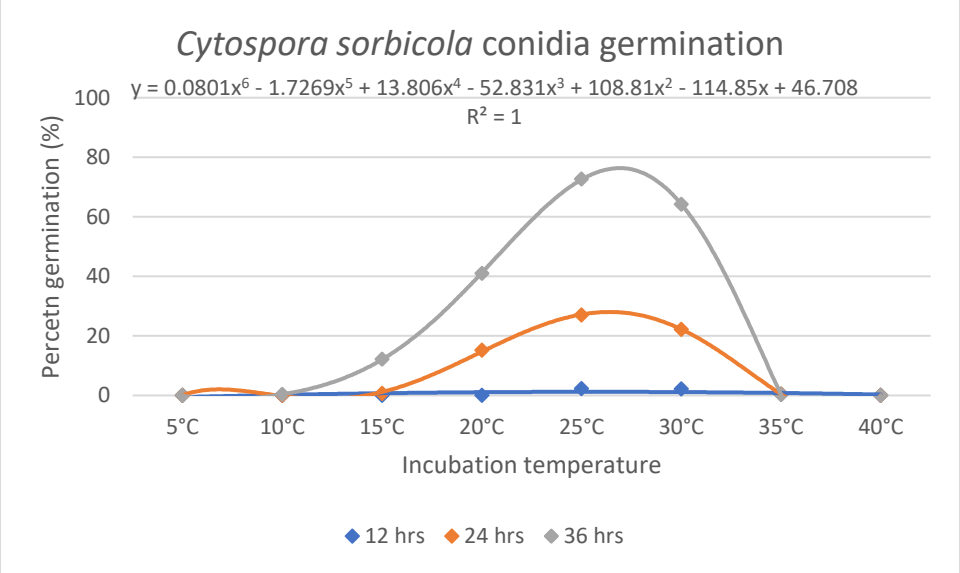

Cytospora sorbicola conidia germination
y = 0.0801x⁶ - 1.7269x⁵ + 13.806x⁴ - 52.831x³ + 108.81x² - 114.85x + 46.708
R² = 1
Percetn germination (%)
100
80
60
40
20
0
5°C
10°C
15°C
20°C
25°C
30°C
35°C
40°C
Incubation temperature
12 hrs
24 hrs
36 hrs

Fig. 1d.

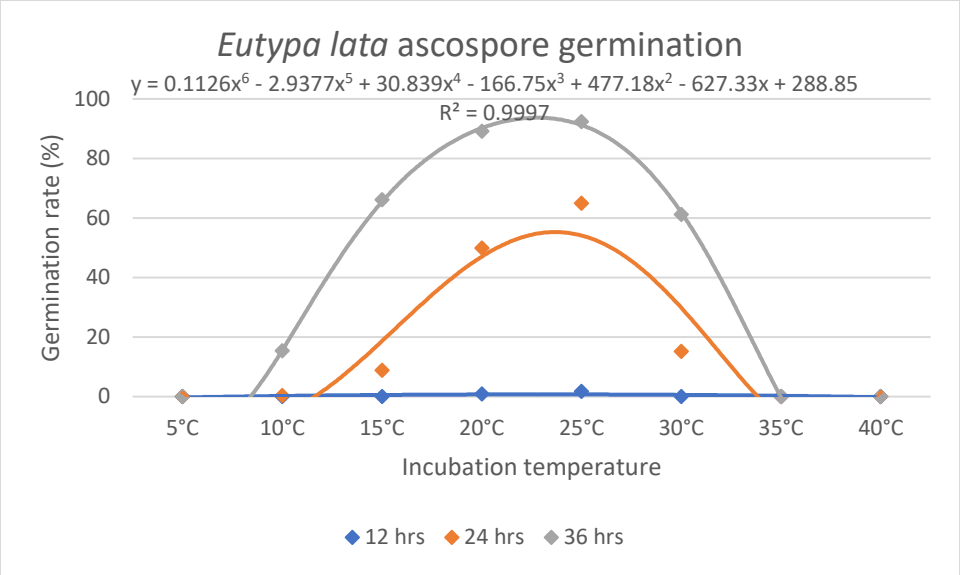

Eutypa lata ascospore germination
y = 0.1126x⁶ - 2.9377x⁵ + 30.839x⁴ - 166.75x³ + 477.18x² - 627.33x + 288.85
R² = 0.9997
Germination rate (%)
100
80
60
40
20
0
5°C
10°C
15°C
20°C
25°C
30°C
35°C
40°C
Incubation temperature
12 hrs
24 hrs
36 hrs

Fig. 1e.

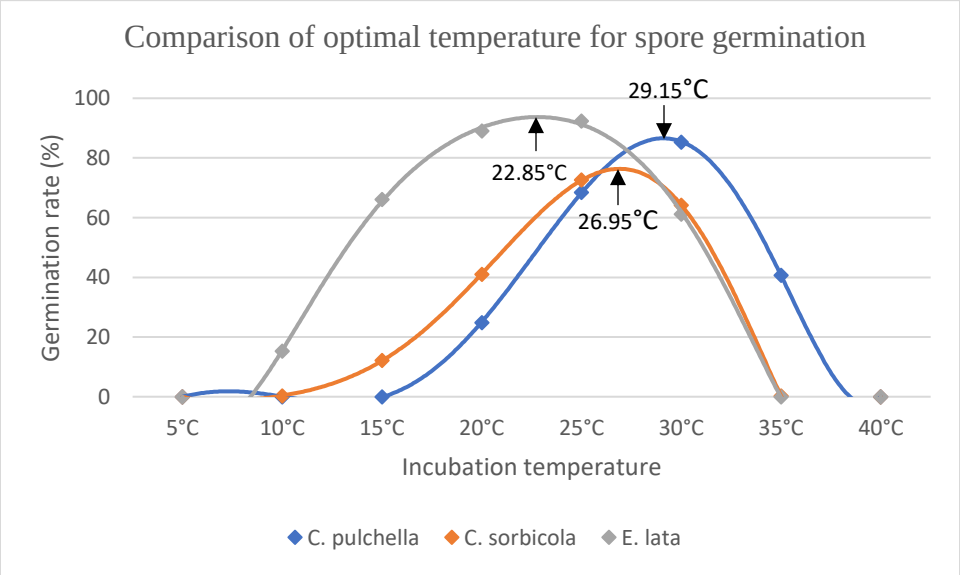

Comparison of optimal temperature for spore germination
Germination rate (%)
100
80
60
40
20
0
29.15°C
22.85°C
26.95°C
5°C
10°C
15°C
20°C
25°C
30°C
35°C
40°C
Incubation temperature
C. pulchella
C. sorbicola
E. lata

Figure 2. *Calosphaeria pulchella* (a) ascospore and (b) conidia germination over 144 hrs. (six days) in constant incubation temperatures 5, 10, and 15°C. Values represent an average of two repeated trials.

Fig. 2a.

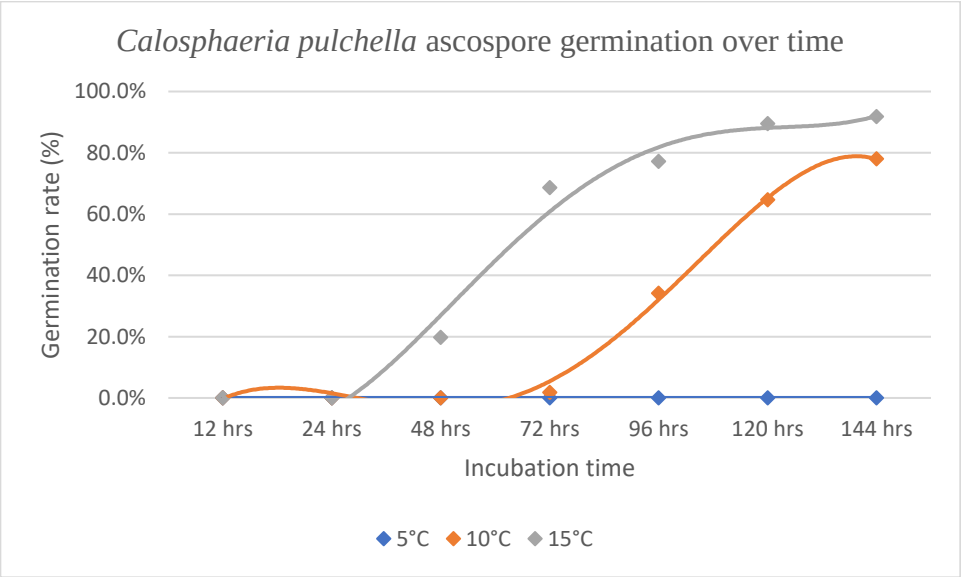


Fig. 2b.

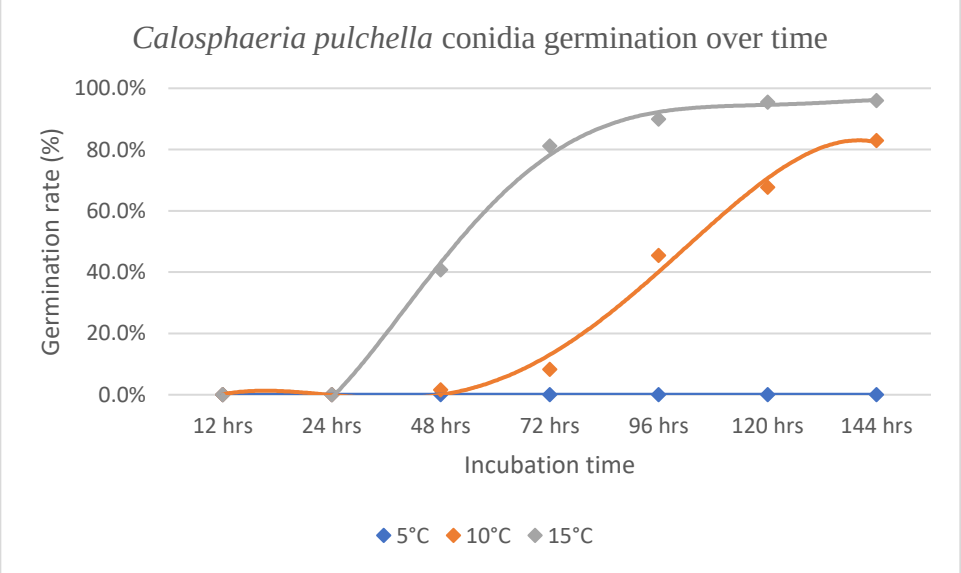


Figure 3. Average colony growth (mm) of (a) *Caloshaeria pulchella*, (b) *Cytospora sorbicola* and (c) *Eutypa lata* colonies after incubation for 12, 8, and 7 days, respectively, at temperatures ranging from 5 to 40°C at 5-degree increments.

Fig. 3a.

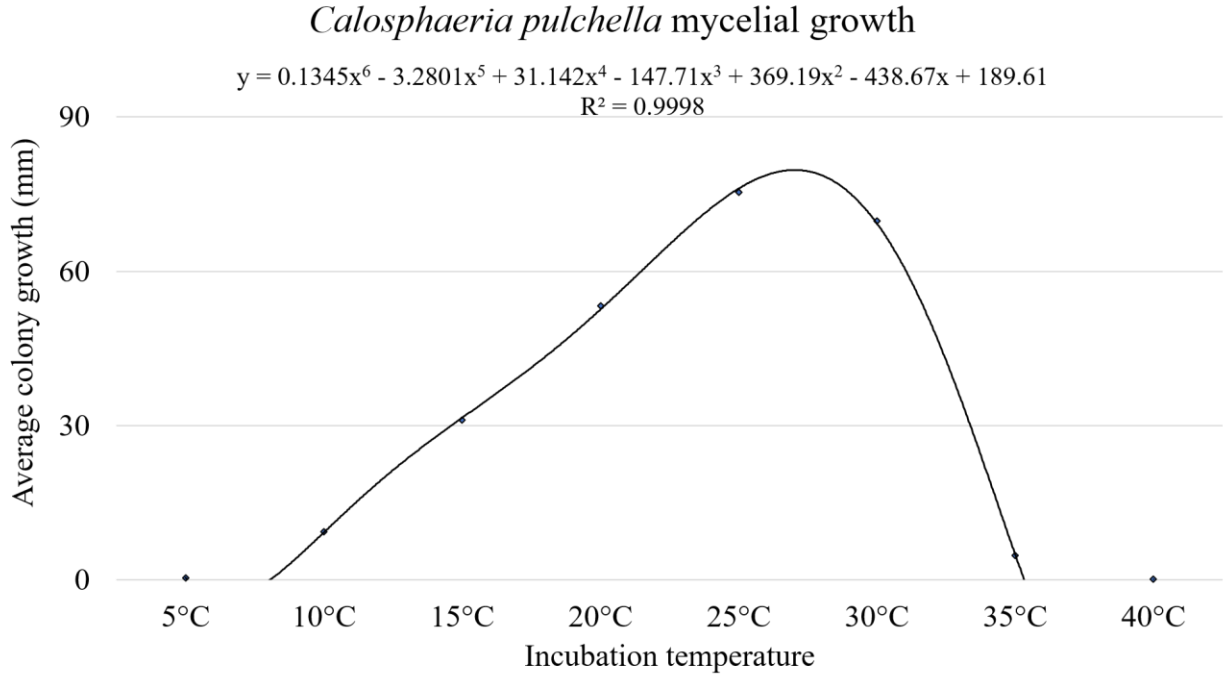


Fig. 3b.

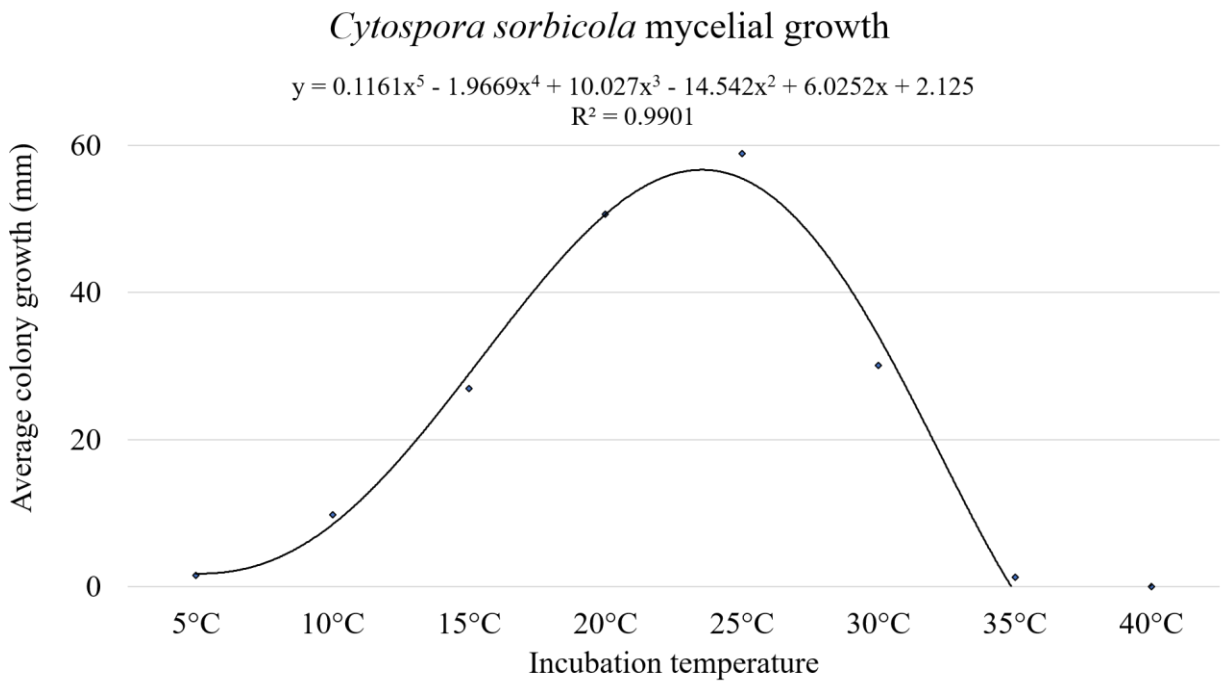
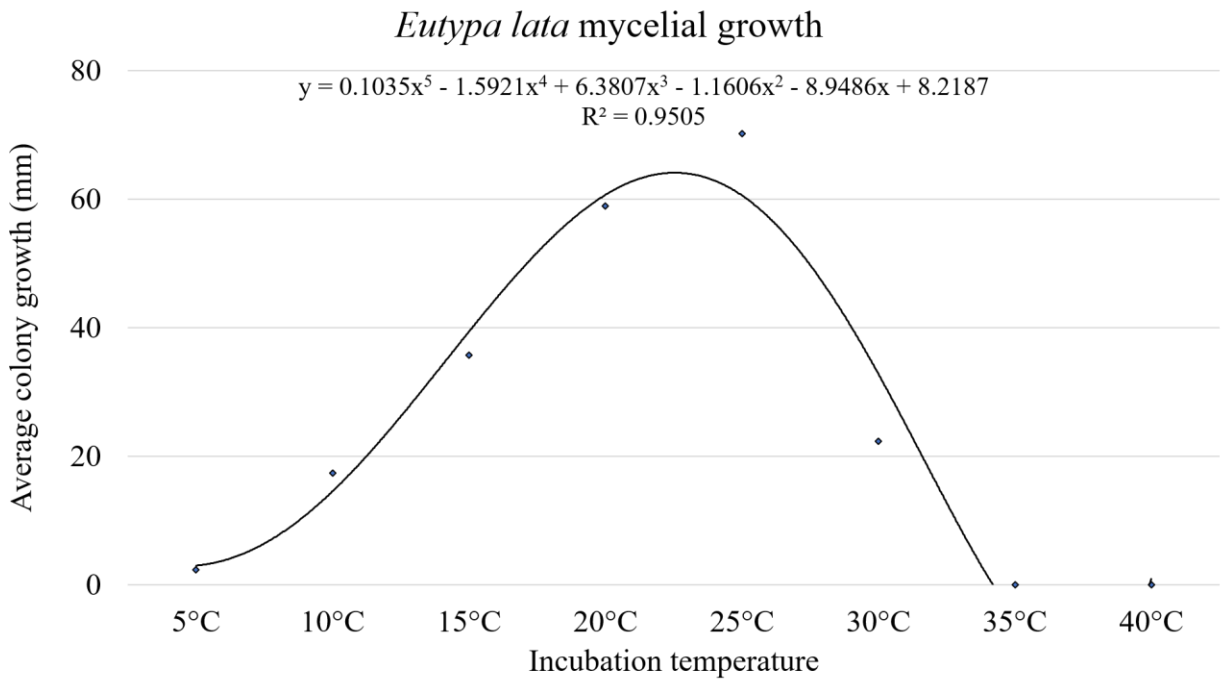

Cytospora sorbicola mycelial growth
y = 0.1161x⁵ - 1.9669x⁴ + 10.027x³ - 14.542x² + 6.0252x + 2.125
R² = 0.9901
Average colony growth (mm)
60
40
20
0
5°C
10°C
15°C
20°C
25°C
30°C
35°C
40°C
Incubation temperature

Fig. 3c.


Eutypa lata mycelial growth
y = 0.1035x⁵ - 1.5921x⁴ + 6.3807x³ - 1.1606x² - 8.9486x + 8.2187
R² = 0.9505
Average colony growth (mm)
80
60
40
20
0
5°C
10°C
15°C
20°C
25°C
30°C
35°C
40°C
Incubation temperature

Chapter III: Seasonal susceptibility of pruning wounds to *Calosphaeria pulchella, Cytospora sorbicola* and *Eutypa lata* in sweet cherry.

Introduction:

Canker diseases constitute a major limiting factor to productivity in cherry orchards in California. *Calosphaeria pulchella*, the causal agent of Calosphaeria canker, is the most common and prevalent fungal canker pathogen affecting sweet cherry in California (Trouillas *et al*. 2012). Ascospores produced by the perithecia of *Cal. pulchella* are the main infectious propagule for this disease. While conidia of *Cal. pulchella* are produced in abundance in culture medium, their role as infectious propagules in the field has not been demonstrated. *Cal. pulchella* causes canker disease on other *Prunus* spp. such as peach (Adaskaveg *et al*. 1993), nectarine (Trouillas *et al*. 2012), and almond (Arzanlou and Dokhanchi 2013). *Cytospora sorbicola*, the main causal agent of Cytospora canker of sweet cherry in California, produces conidia from pycnidia which are the main inoculum for this disease (Lawrence *et al*. 2018). *Cyt. sorbicola* was reported to cause canker disease on apple (Hanifeh *et al*. 2022), olive, plum, peach, almond (Lawrence *et al*. 2018), and sour cherry (Norphanphoun *et al*. 2017). *Eutypa lata*, the causal agent of Eutypa dieback of sweet cherry, releases ascospores from perithecia, which serve as the main inoculum for this disease. *E. lata* is not host-specific and its host-range in California includes mainly grapevine, almond, apricot, apple, pear, oleander, and willow (Trouillas and Gubler 2010).

Cal. pulchella, Cyt. sorbicola, and E. lata typically initiate infection at pruning wounds, leading to vascular necroses, cankers and sunken lesions of the bark of infected branches (Sinclair and Lyon 2005, Trouillas *et al*. 2012). Infection generally leads to dieback of branches

and entire scaffolds, which significantly reduces the productivity and longevity of orchards. Previous studies have investigated the seasonal dynamic of spore release of main canker pathogens in California (Bertrand and English 1976, Ramos *et al*. 1975, Trouillas *et al*. 2012). *Cal. pulchella* ascospores were detected in cherry orchards mainly during fall, winter, and spring rainfalls (Trouillas *et al*. 2012). *Cytospora leucostoma* releases conidia year-round in prune orchards when rain occurs, with the highest amounts of spore released during fall rains, and fewest during mid-and late-winter rains (Bertrand and English 1976). *Eutypa lata* ascospores are released during rainfalls with the greatest amount occurring in fall to mid-winter in California (Ramos *et al*. 1975). Previous studies have suggested that the rate of canker development caused by *Leucostoma cincta* (syn. *Cytospora cincta*) (Barakat and Johnson 1997, Rozsnyay 1986) and *Cyt. leucostoma* (Luo *et al*. 2022) in pruning wounds is influenced by seasonal temperatures. Seasonal temperatures also seem to play a major role in the aggressiveness of *E. lata* within grapevines (Gubler *et al*. 2005, Lardner *et al*. 2005, Munkvold and Marois 1994, Sosnowski *et al*. 2007, Trese 1980).

The abundance, in orchards, of high spore inocula of canker pathogens during the fall and winter months in California often coincides with pruning of sweet cherry trees, thus exposing trees to fungal infection. In California sweet cherry, winter pruning has been associated with the highest infection rates of Eutypa dieback (Munkvold and Marois 1994). Summer or post-harvest pruning has been broadly adopted in recent years by cherry growers in California in order to reduce the risk of pruning wound infection. Nevertheless, high ascospore inoculum of *Cal. pulchella* was detected during summer months in cherry orchards that use sprinkler irrigation (Trouillas *et al*. 2012). This suggests that summer pruning also could lead to substantial infection of cherry trees.

Despite the common occurrence of Calosphaeria canker in sweet cherry orchards, disease biology is not well understood. In particular, little information is available on the seasonal susceptibility of pruning wounds to *Cal. pulchella* in California. In previous experiments, our laboratory has failed repeatedly to infect pruning wounds of sweet cherry trees following winter pruning and artificial inoculations with ascospore suspensions of *Cal. pulchella*. This suggested that *Cal. pulchella* most likely requires warm temperatures for infection of pruning wounds in cherry, such as those occurring during summer in California. Similarly, the seasonal susceptibility of pruning wounds to *Cyt. sorbicola* and *E. lata* in California sweet cherry remain poorly understood.

Therefore, the objective of this chapter was to compare the susceptibility of summer and winter pruning wounds in sweet cherry to *Cal. pulchella, Cyt. sorbicola,* and *E. lata* in field trials. Understanding the seasonal effects on the susceptibility of pruning wounds to *Cal. pulchella, Cyt. sorbicola* and *E. lata* will allow us to improve management guidelines and minimize the risks of infection of pruning wounds by fungal canker diseases.

Materials and Methods:

Preparation of spore inoculum and culture maintenance. *Cal. pulchella* ascospores were collected from perithecia found under the periderm of dead, infected sweet cherry branches from a UC Davis experimental orchard located in Yolo County. Pieces of wood containing *Cal. pulchella* perithecia were cut from branches and affixed to a plastic Petri lid with petroleum jelly (Vaseline® 100%, Greenwich, CT) and submerged in deionized (DI) water for 15 to 30 minutes (min) to fully saturate the wood and fruiting bodies. Water was then poured out and perithecia were gently blotted dry with Kimwipes™ paper sheets (Kimwipes; Kimtech Science® Kimberly-

Clark Professional®, Roswell, GA). The lid with perithecia was then placed over a new, sterile Petri dish bottom. At least 2 hours (h) were allowed for ascospores to fully discharge onto the Petri plate bottom. DI water was pipetted up and down repeatedly to dislodge and collect the recently discharged spores.

A *Cal. pulchella* isolate RTFT004 was obtained from diseased cherry wood exhibiting canker symptoms in Yolo County, California. Cultures were grown and maintained on potato dextrose agar (PDA; Sigma-Aldrich®, St. Louis, MO) amended with tetracycline (PDA-Tet; PDA; Sigma-Aldrich®, St. Louis, MO) at 1 mg/L concentration at the laboratory ambient temperature (22-24°C) and natural photoperiod. RTFT004 was saved into long-term storage by cutting and placing 4 mm × 4 mm × 4 mm cubes of agar from the margin of the colony, where growth is most active, into sterile deionized water (DI) vials and storing them at 4°C. Fresh cultures were prepared from this collection for each experiment in this study. Conidia were obtained from a one-week-old RTFT004 culture by pipetting up and down 500 µl of sterile deionized water over the colony to wash and draw up spores.

Cyt. sorbicola conidia were obtained from conidia found on dead infected sweet cherry branches from a UC Davis experimental orchard located in Yolo County and from San Joaquin County. Conidia were exuded from pycnidia in the form of mucilage after exposure to sufficient wetness. Fresh conidial cirri were collected in the field and fully dissolved by vortexing in sterile DI water to obtain a conidia suspension.

Eutypa lata ascospores were collected from perithecia growing on dead, infected oleander (*Nerium oleander*) wood from Yolo County. The ostioles of *E. lata* perithecia were observed on the wood surface. To access ascospores, a thin, flame-sterilized razor blade was used to cut tangentially along the wood surface where ostioles were visible to remove thin layers

of wood and expose the internal perithecia cavity. Sterile DI water was added over these openings to rehydrate and produce a dark, gel-like matrix containing an abundance of ascospores. With the aid of a dissecting microscope and a flame-sterilized dissecting needle, the spore matrix was carefully collected in 1.5 mL Eppendorf tubes containing sterile DI water and vortexed for 2 min to dislodge and evenly suspend ascospores.

Spore suspension concentration was estimated using a standard hemocytometer and adjusted with sterile DI water to 1×10^4 and 1×10^6 spores·mL^{-1}. Spores were stored at 4°C to prevent germination until ready for use. All spore suspensions were used on the day of preparation.

Field inoculation and re-isolation. The infection rate of pruning wounds by *Cal. pulchella, Cyt. sorbicola, and E. lata* in sweet cherry trees was assessed and compared following summer (June-July) and winter (January) pruning and inoculation of spore suspensions. For each treatment, both old branches (3- to 5-year-old) and young branches (1- to 2-year-old) were inoculated. For field inoculations, we used *Cal. pulchella* ascospores and conidia, *Cyt. sorbicola* conidia, and *E. lata* ascospores as inoculum. For each pruning time (summer and winter), a flat cut was made on each selected branch with pruning shears and wounds were sprayed with DI water to mimic rain before inoculating with 100 µL spore suspensions in water at concentrations of 1×10^4 spores/mL. For each treatment, infection rates (= recovery rate from wounds) between summer and winter pruning trials were compared.

In July and January 2019 trials in Orchard 1, only *Cal. pulchella* spores were used for inoculations. Three of each of the old (3-to-5-year-old) and young (1-to-2-year-old) branches per cherry tree were inoculated with each spore type, and 4 tree replicates were used. The experiment was conducted in 30-year-old cherry trees of the Bing cultivar maintained at the UC

Davis Plant Pathology Research Field Station in Davis, CA. All trials in 2021 – 2022 in Orchards 2 and 3 were conducted using 1 branch per treatment and per tree with a total of 12 tree replicates and included all three pathogens. Sterile water was used as a negative control to test for the presence of local inoculum. Two different concentrations of *Cal. pulchella* ascospores and conidia at 1×10^4 and 1×10^6 spore/mL were used for trials in May and January 2022 in Orchard 1; and June 2021 and January 2022 in Orchard 3. Tree age, pruning dates, and pathogens used in each orchard were organized in **Table 1**.

Summer trials were collected after three months post-inoculation, whereas winter trials were collected after 4-5 months post-inoculation. A flame-sterilized knife was used to remove the exposed bark layer and approximately 0.5 cm of dried wood below the cut surface was removed. The remaining branch segment was then surface sterilized by brief flaming. Ten wood fragments of 3 mm × 3 mm × 3 mm were cut from the margin between discolored and clean tissues for each branch sample and plated onto PDA-Tet. Isolations were incubated at the laboratory ambient temperature (22-24°C) and natural photoperiod. Plates were examined every 2 days for prospective colonies of *Cal. pulchella, Cyt. sorbicola, and E. lata* from which we transferred hyphal tips to fresh PDA-Tet and allowed to grow for 12 days as pure culture. Pathogen identity was determined based on colony morphology. The infection rate was assessed as the % recovery of the pathogen re-isolated from each branch samples out of the total number of branches inoculated per treatment. We defined a positive pathogen recovery from a treatment when the pathogen was isolated from at least one of the ten diseased wood pieces in a plate. The % recovery for each comparison consisted of averages of data from all orchards used in this study. To determine statistical significance of incidence between summer and winter pruning susceptibility, confidence intervals (CI) for recovery differences in proportions were calculated

as: CI = $p_1 – p_2 \pm 1.96 \times \sqrt{[(p_1 \times q_1/(n_1\text{-}1)) + (p_2 \times q_2/(n_2\text{-}1))]}$ where p_1 and p_2 were proportions of recovery from each pruning time, q_1 and $q_2 = 1 – p$, n_1 and n_2 were the sample sizes of each treatment, 1.96 corresponded to the z-score of the 95% confidence level, and degrees of freedom (df) = $(n_1 – 1) + (n_2\text{-}1)$.

Results:

In field trials, the susceptibility of pruning wounds to *Cal. pulchella*, *Cyt. sorbicola*, and *E. lata* made during summer (July 2019, June 2021, and twice in May 2022) and winter (January 2019, 2021, and twice 2022) was compared (Figure 1). The average recovery of *Cal. pulchella* from summer-inoculated branches was 84.6%, which was significantly greater than that of winter-inoculated branches with 10.7% recovery (CI = [0.682, 0.796], P-value = 0.05, df = 545). The average recovery of *Cyt. sorbicola* from summer and winter-inoculated branches was 78.5% and 66.7%, respectively, with no significant difference between the two pruning times (CI = [-0.033, 0.269], P-value = 0.05, df = 132). The average recovery of *E. lata* from summer-inoculated branches was 80%, which was significantly less than that of winter-inoculated wounds with 92.8% recovery (CI = [0.012, 0.244], P-value = 0.05, df = 132). No pathogens were recovered from the water-inoculated control branches.

In addition, we compared the pruning wound susceptibility to canker pathogens between old (3-to-5-year-old) and young (1-to-2-year-old) cherry branches during summer (Figure 2a) and winter (Figure 2b). The recovery of *Cyt. sorbicola* from old branches inoculated during summer was significantly greater than that of young branches, with 90.6% and 65.6% recovery, respectively (CI = [0.058, 0.448], P-value = 0.05, df = 63). There was no significant difference between the recoveries of *Cyt. sorbicola* old and young branches inoculated in winter (recovery

[old, young] = 64.7%, 68.6%, CI = [-0.265, 0.187], P-value = 0.05, df = 67) nor between the recoveries of *Cal. pulchella* old and young branches inoculated in summer (recovery [old, young] = 88%, 81.2%, CI = [-0.019, 0.155], P-value = 0.05, df = 264) and winter (recovery [old, young] = 9.9%, 11.4%, CI = [-0.057, 0.087], P-value = 0.05, df = 279). No significant difference also was found between the recoveries of *E. lata* old and young branches inoculated in summer (recovery [old, young] = 84.8%, 75%, CI = [-0.108, 0.288], P-value = 0.05, df = 63) and winter (recovery [old, young] = 94.3%, 91.2%, CI = [-0.093, 0.155], P-value = 0.05, df = 67).

We also compared the infection rate between the ascospores and conidia of *Cal. pulchella* during summer and winter (Figure 3). The recovery of *Cal. pulchella* from ascospore-inoculated branches was significantly less than that of conidia-inoculated branches during the winter, with 6.5% and 14.7% recovery, respectively (CI = [0.011, 0.153], P-value = 0.05, df = 279). There was no significant difference between recoveries from ascospores and conidia-inoculated branches during summer, with 82.8% and 86.4% recovery, respectively (CI = [-0.051, 0.123], P-value = 0.05, df = 264).

Lastly, we compared the infection rate between two different spore concentrations (1×10^4 and 1×10^6 spores/mL) of *Cal. pulchella*. Ascospores and conidia of different concentrations were tested in pruning wounds made during summer (Figure 4a, 2021 in Orchard 3 and 2022 in Orchard 1) and winter (Figure 4b, 2022 in Orchard 1 and Orchard 3). The recovery of *Cal. pulchella* form pruning wounds inoculated with 10^6 ascospores/mL was significantly greater than that of 10^4 ascospores/mL inoculations during winter, with 14% and 0% recovery, respectively (CI = [0.043, 0.237], P-value = 0.05, df = 98). Similarly, recovery from 10^6 conidia/mL inoculations was significantly greater than that of 10^4 conidia/mL inoculations during winter,

with 20.4% and 0% recovery, respectively (CI = [0.090, 0.318], P-value = 0.05, df = 97). There was no significant different between the infection rates of the two spore concentrations inoculated in summer for both ascospores (recovery [10^4, 10^6] = 78.6%, 92.5%, CI = [-0.011, 0.289], P-value = 0.05, df = 80) and conidia (recovery [10^4, 10^6] = 85.7%, 95%, CI = [-0.034, 0.220], P-value = 0.05, df = 80).

Discussion:

The seasonal susceptibility of sweet cherry pruning wounds to *Cal. pulchella*, *Cyt. sorbicola* and *E. lata* following summer and winter pruning was compared in field trials. Overall, our study indicates that pruning wounds made during summer were significantly more susceptible to *Cal. pulchella*. In other words, summer pruning of sweet cherry trees in California poses greater risks of infection by *Cal. pulchella,* while winter pruning may allow to avoid the disease. These results are consistent with that of our in vitro temperature studies in Chapter 2, indicating that warm temperatures favor *Cal. pulchella* spore germination and mycelial growth. *Calosphaeria pulchella* seems well adapted to warm temperatures, and infection of cherry orchards in California is most likely the result of broadly adopted summer pruning. On the other hand, infection of sweet cherry trees at pruning wounds by *Cal. pulchella* was limited when pruning occurred during the low winter temperatures of California. These results represent new findings and suggest that winter pruning could be part of an integrated disease management strategy against Calosphaeria canker of sweet cherry in California. The results of this study on the seasonal susceptibility of pruning wounds to *Cal. pulchella* infection contrasts with Berbegal and Armengol (2018), who investigated the seasonal susceptibility of pruning wounds to *Cal. pulchella* in the Alicante Province of Spain and found no significant difference between summer

and winter pruning. However, this study by Berbegal and Armengol (2018) used pruning shears to transmit inoculum from infected to non-infected branches as well as mycelial agar plugs to inoculate wounds. In contrast, we used ascospores from fruiting bodies found on infected cherry wood and conidia from cultures to inoculate pruning wounds. Also, according to the National Centers for Environmental Information, Yolo County of California generally experiences hotter summers, cooler winters, and significantly more rainfall than the Alicante region, which could explain the differences between our results and those of Berbegal and Armengol (2018). There was no significant difference between summer and winter pruning wound susceptibility to *Cyt. sorbicola*. In other words, *Cyt. sorbicola* was consistently infectious to pruning wounds following both summer and winter pruning. Pruning of cherry in summer and in winter may pose equal risks to *Cyt. sorbicola* infection if inoculum is present, although the seasonal abundance of *Cyt. sorbicola* conidia in sweet cherry orchards has not been studied. The results of this present study are consistent with Rozsnyay (1977) and Rozsnyay and Klement (1973), who inoculated wounds on apricot branches and trunks each month of the year with *Cyt. cincta*, and found that the trees were susceptible to infection by *Cyt. cincta* during all months of the year in Hungary. Bakarat and Johnson (1997) demonstrated that infection of pruning wounds by *Cyt. cincta* can occur during all months of the year in sweet cherry in Washington State, although differences were found in the susceptibility of pruning wounds of sweet cherry to Cytospora canker according to the month of pruning . Indeed, greatest incidence of infection occurred from wounds made in the spring and summer months (Bakarat and Johnson 1997). Chen *et al*. (2016) and Wang *et al*. (2016) both showed that pruning wounds of apple made during winter were most susceptible to infection by *Valsa mali* (anamorph *Cyt. sacculus*) in China, although infection can occur at different times of the year. Wang *et al*. (2016) suggested that pruning in

early winter most likely promote severe infections of apple trees by *V. mali* and could play an important role in the spread of the disease. Pruning wounds of peach trees were shown to be less susceptible to *Cyt. leucostoma* infection when made in spring and summer compared to those occurring in winter in Colorado (Luepschen and Rohrbach 1969). Overall, pruning wounds made during the dormant periods of fruit trees were at a higher risk for infection by *Cytospora spp.* in Colorado (Pokharel 2012). However, the French prune pathogen, *Leucostoma cincta* (syn. *Cytospora cincta*), was considered to be primarily a summer disease in California (Bertrand and English 1976), with the lowest infection rate occurring in winter months. Our results on the seasonal susceptibility of pruning wounds to *E. lata* indicated that pruning of cherry trees during winter poses a greater risk to infection by this pathogen, as opposed to summer pruning. The results of this study are consistent with Munkvold and Marois (1994), who concluded that pruning wounds of sweet cherry made in late December were significantly more susceptible to *E. lata* infection than those made in late June in California. Furthermore, they found that pruning wounds made in December have a longer duration of susceptibility to *E. lata* infection than pruning wounds made in June (Munkvold and Marois 1994). Also consistent with this present study, other authors have found that pruning wounds made during winter are more susceptible to infection by *E. lata* in grapevine in California (Munkvold and Marois 1995, Petzoldt *et al*. 1981, 1982), Michigan (Trese *et al*. 1982), and France (Chapuis *et al*. 1998, Lecompt and Bailey 2011); and in apricot in California (Ramos *et al*. 1975) and South Australia (Carter and Moller 1967). Furthermore, pruning wounds of grapevine were shown to be less susceptible to infection by *E. lata* under increasing temperature conditions in California (Munkvold and Marois 1995, Trese *et al* 1980) and France (Chapuis *et al*. 1998). Chapuis *et al*. (1998) indicated that lower infection rates in pruning wounds of grapevine during warmer temperatures maybe due to an

increase in microflora diversity and natural competitor organisms on the wound surface during warm weather conditions. It was further suggested that pruning wound susceptibility could vary at the regional scale due to the diversity of putative abiotic, biotic, and environmental factors interfering with the infection process of *E. lata* (Chapuis *et al*. 1998).

The susceptibility of pruning wounds on young (1-to-2-year-old) and old (3-to-5-year-old) cherry branches to fungal canker pathogens was compared. Our results indicated that pruning wounds made on older branches were more susceptible than those from younger branches to infection by *Cyt. Sorbicola* during summer. Branch age, however, did not play a role in pruning wound susceptibility to infection by *Cyt. sorbicola* during winter and *Cal. pulchella* or *E. lata* in both summer and winter. These results are consistent with Chapuis *et al*. (1998) Munkvold and Marois (1995), Trese *et al*. (1982), who showed that the wood age of grapevine does not affect pruning wound susceptibility to infection by *E. lata* among 1-, 2- and 3-year-old canes. On the other hand, Moller and Kasimatis (1980) showed that grapevine pruning wounds on 1-year-old cane were less susceptible to *E. lata* infection than pruning wounds on older wood. It was also found that larger pruning wounds of apricot were significantly more susceptible to infection by *E. lata* than small pruning wounds under the conditions of South Australia (Carter and Moller 1967). Although not specific to pruning wounds, Rozsnyay Z. D. (1977) concluded that young apricot trees were more susceptible to *Cyt. cincta* infections than older trees. Additionally, Hildebrand (1947) reported that older peach trees were generally less susceptible to Cytospora canker than younger trees in New York State. Wensley (1966) found a higher incidence of *Cytospora* spp. on wounds of 1- and 2-year-old branches than older wood on peach trees in Ontario, Canada. Worrall *et al*. (2010) reported that stem age of *Alnus* does not play a role in susceptibility to natural infection by *Cyt. umbrina* in the Southern Rocky Mountains.

The pruning wound susceptibility to infection by ascospores and conidia of *Cal. pulchella* was compared during summer and winter trials. Our results from one of the orchards indicated that pruning wounds were more susceptible to conidia infection than ascospores during winter. Pruning wounds were equally susceptible to both spore types during summer for all orchards. Although both spore types were demonstrated to be equally infectious during summer, the conidia of *Cal. pulchella* and its asexual fruiting structures have not been observed in the field. Thus, the role *Cal. pulchella* conidia remains unknown in the disease cycle. Pokharel (2013) and Biggs and Grove (2005) have indicated that conidia of *Cyt. leucostoma* and *Cyt. cincta* are the main inoculum for infection of wounds on stone fruit trees while ascospores do not play any role in the disease cycle. Gubler *et al*. (2005) have concluded that the ascospores of *E. lata* are the main inoculum for infection of wounds while conidia play no role in causing disease.

Lastly, the infection rate of two different concentrations (1×10^4 and 1×10^6 spores/mL) of *Cal. pulchella* spores were compared on pruning wounds. Inoculations with 1×10^6 spores/mL achieved higher success in infection of pruning wounds made during winter. Previous studies had found that inoculation of pruning wounds with higher ascospore concentrations resulted in higher incidence of recovery of *E. lata* than inoculations with lower ascospore concentrations in almond, peach and prune in South Australia (Carter and Moller 1971) and in grapevine in California (Petzholdt *et al*. 1981). Furthermore, Carter and Moller (1971) had concluded that the amount of ascospores of *E. lata* that are naturally deposited on a pruning wound rarely exceeds 10, and that this amount is sufficient for infection in almond, peach, and prune in South Australia.

In California, cherry trees were traditionally pruned during the dormant season (winter). However, following the identification of Eutypa dieback as a major canker disease of sweet

cherries, pruning during the winter season was discouraged in favor of summer pruning (Munkvold and Marois 1991; and 1994). Currently, a typical time for pruning sweet cherries is after harvest in early to mid-summer (June – July) or in the early fall (October) when temperatures remain warm. The shift from winter to summer pruning likely has favored the emergence of Calosphaeria canker as a major canker disease in California cherry orchards during the past two decades. A previous survey of cherry orchards in California also found *E. lata* to be of low incidence relative to *Cal. pulchella*, particularly in regions of the southern San Joaquin Valley where rainfalls are low (Trouillas *et al*. 2012). This work indicates that pruning of sweet cherry trees under cold winter temperatures can significantly reduce the risks of infection by *Cal. pulchella*. However, winter pruning will not prevent infection by *E. lata* or *Cyt. sorbicola*. Nevertheless, winter pruning may be considered as part of an integrated management strategy for Calosphaeria canker, especially in cherry orchards with abundant inoculum sources of *Cal. pulchella*. Similarly, winter pruning may be considered as a management strategy in the southern part of the state (another important sweet cherry production for California), where the inoculum sources of *E. lata* are absent. A survey of vineyards across 21 California counties by Úrbez-Torres *et al*. (2006) reported little to no detection of *E. lata* from grapevine cankers in southern counties including Santa Barbara, Fresno, Kern, Tulare, and Riverside County. Similarly, Trouillas and Gubler (2010) reported no occurrence of perithecia of *E. lata* from a great diversity of plant hosts surveyed in southern counties such as Madera, Fresno, Kings, and Tulare Counties. Furthermore, Ramos *et al*. (1975) found that the occurrence of *E. armeniacae* (syn. *E. lata*) perithecia in apricot orchards in California was most abundant in counties with the highest annual rainfall, whereas counties with annual rainfall below 330 mm resulted in no detectable perithecia. The correlation of perithecia occurrence to the amount of rainfall explains the absence

of *E. lata* fruiting bodies in the southern counties in California as these counties have warmer and drier climates compared to northern counties. Therefore, winter pruning in southern California counties could potentially eliminate the risks of infection of sweet cherry trees by most major fungal canker pathogens, with the exception of *Cytospora*. A spore trapping study by Bertrand and English (1976) in California demonstrated that increased rain duration and temperature between rain events correlated to higher spore counts of *Cytospora leucostoma* found in prune orchards. Thus, the risk of *Cytospora* infections of sweet cherries may be mitigated by adjusting the pruning times to colder months of the year with the lowest precipitation.

The scope of our seasonal susceptibility trials was limited to pruning times in June or July for summer and December or January for winter. As such, the susceptibility of pruning wounds to *Cal. pulchella* relative to canker pathogens at times such as late summer, fall, late winter, or spring should be tested in order to fully understand the optimal time for pruning. Investigating the susceptibility of pruning wounds during each month of the year to all major canker pathogens would be complementary to the present study.

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Figures and Tables (Chapter 3):

Table 1. Pruning dates of sweet cherry for each season in a 30-year-old Bing with unknown rootstock (Orchard-1), 11-year-old Bing/Colt (Orchard-2), and 4-year-old Sweetheart/Krymsk5 (Orchard-3) cherry orchard.

Orchard #	Tree age (years-old)	Canker pathogens inoculated	Season	Pruning dates
1	30	*Cal. pulchella*	Summer	7/21/2019
		Cal. pulchella	Winter	1/4/2019
	30	*Cal. pulchella, Cyt. sorbicola, E. lata*	Summer	5/26/2022
		Cal. pulchella, Cyt. sorbicola, E. lata	Winter	1/20/2022
2	11	*Cal. pulchella, Cyt. sorbicola, E. lata*	Summer	5/26/2022
		Cal. pulchella, Cyt. sorbicola, E. lata	Winter	1/22/2021
3	4	*Cal. pulchella, Cyt. sorbicola, E. lata*	Summer	6/10/2021
		Cal. pulchella, Cyt. sorbicola, E. lata	Winter	1/19/2022

Figure 1. Seasonal pruning wound susceptibility to *Calosphaeria pulchella*, *Cytospora sorbicola* and *Eutypa lata*. Sterile H2O was inoculated as negative control to test for natural local inoculum in the orchard. Percentage recovery was calculated using combined average values of all 3 orchards. Average recovery of each pathogen was compared between summer and winter-inoculated pruning wounds on sweet cherry. (*$P < 0.05$)

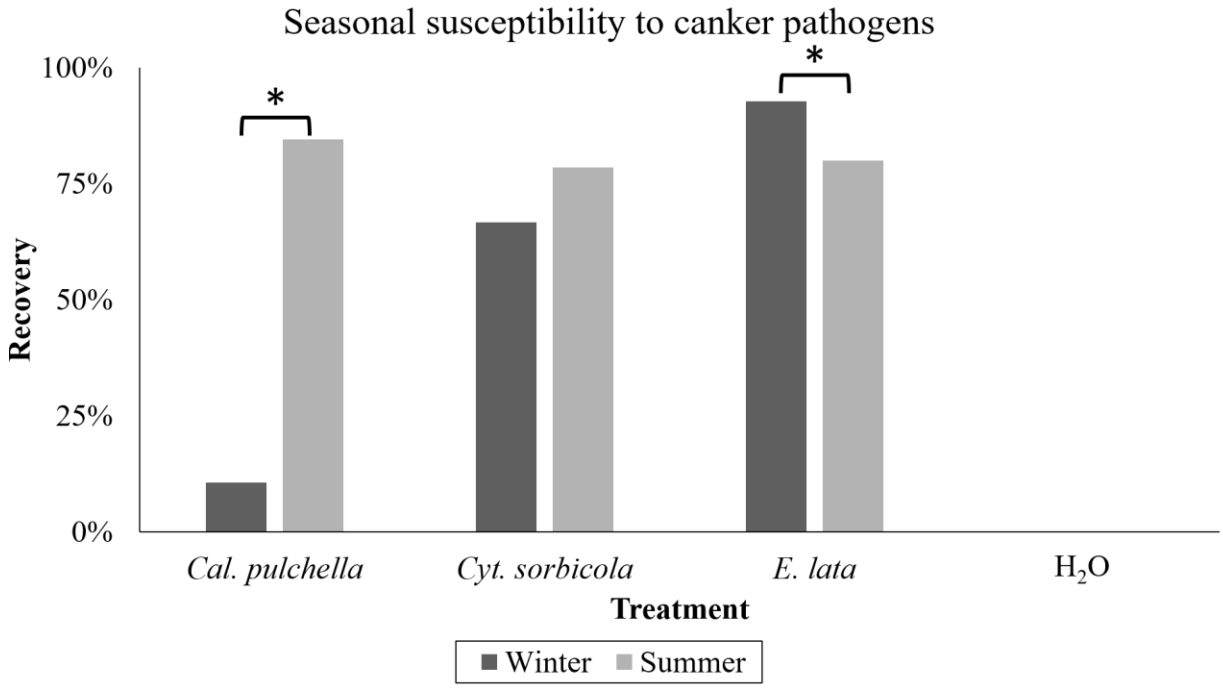


Figure 2. Pruning wound susceptibility of wood age to *Calosphaeria pulchella*, *Cytospora sorbicola*, and *Eutypa lata* during (a) summer and in (b) winter. Average recoveries were compared between inoculated old and young branches, where wood age is 3-to-5-years-old and 1-to-2-years-old, respectively. (*$P < 0.05$)

Figure 2a.

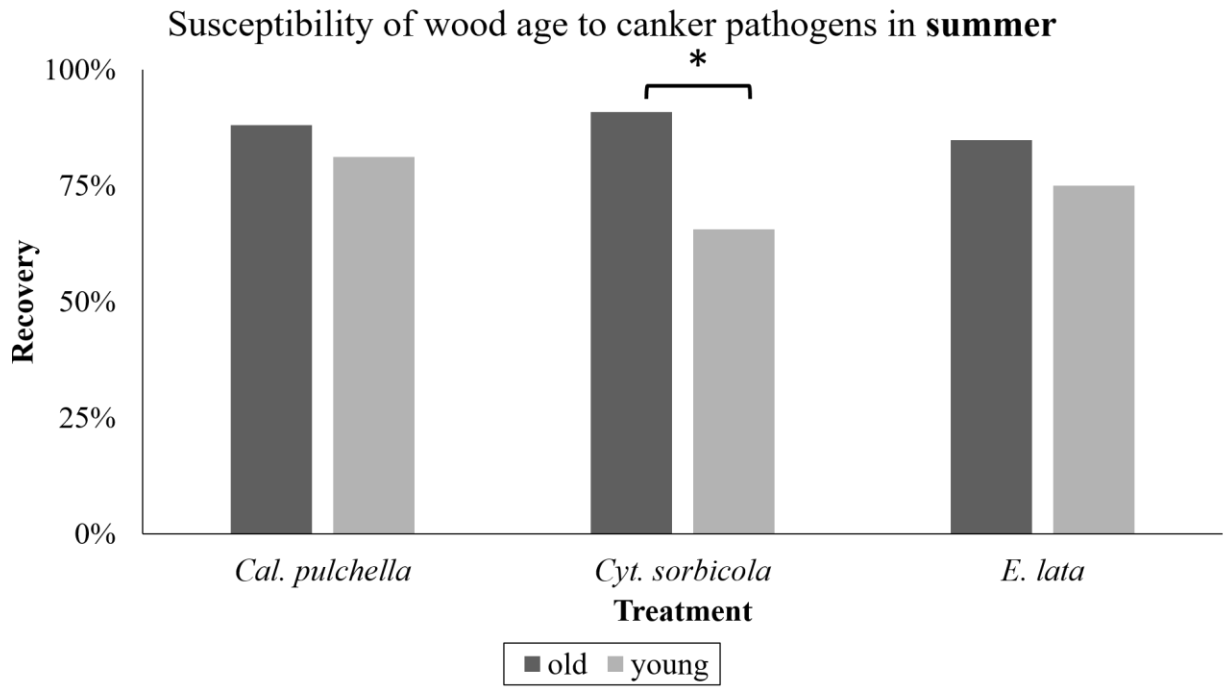


Figure 2b.

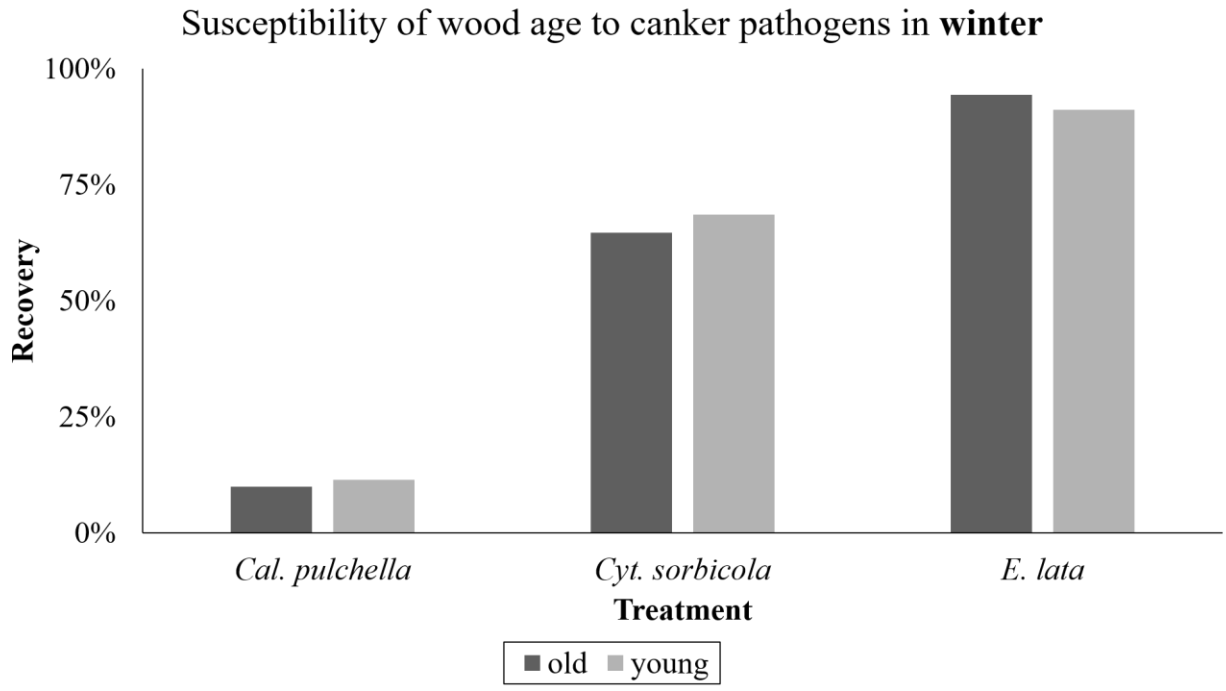


Figure 3. Pruning wound susceptibility between ascospores and conidia of *Calosphaeria pulchella* were compared. These trials were performed both in summer and in winter. (*$P < 0.05$)

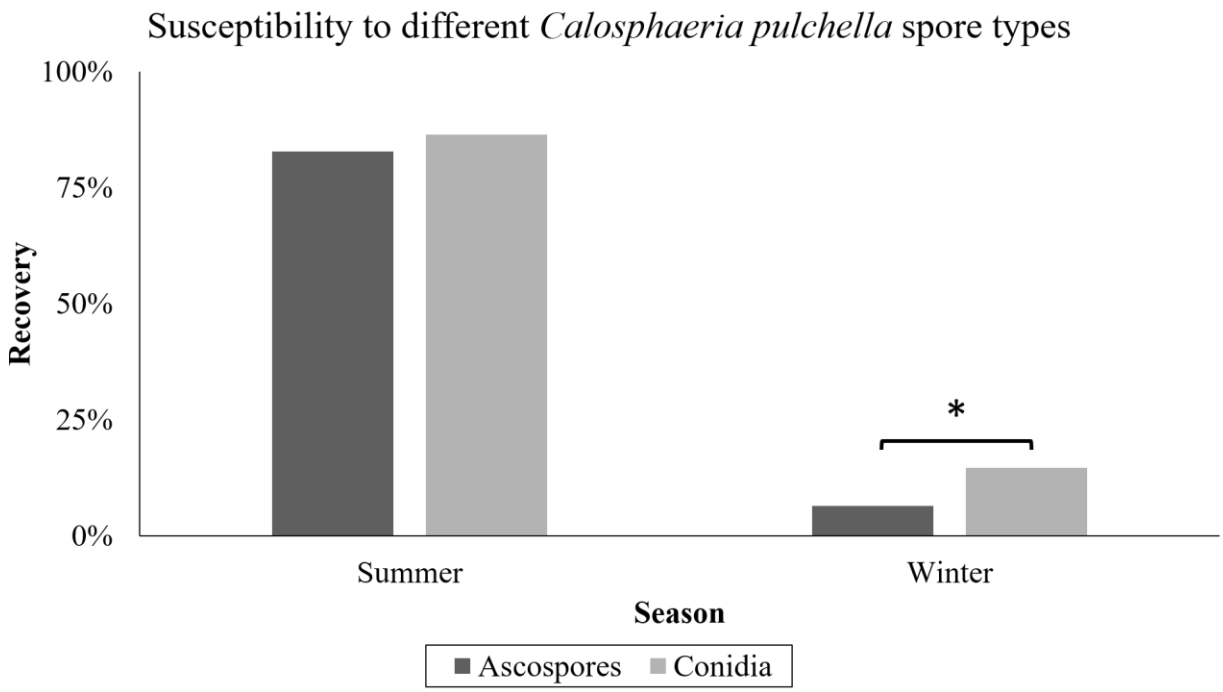


Figure 4. Pruning wound susceptibility to *Calosphaeria pulchella* in two different spore inoculum concentrations, 1×10^4 and 1×10^6 spores per ml, done in (a) summer and in (b) winter. Two different spore types, ascospores and conidia, were used in comparison between the two inoculum concentrations. Average recoveries from each type of spore inoculations were used to assess their pathogenicity. (*$P < 0.05$)

Figure 4a.

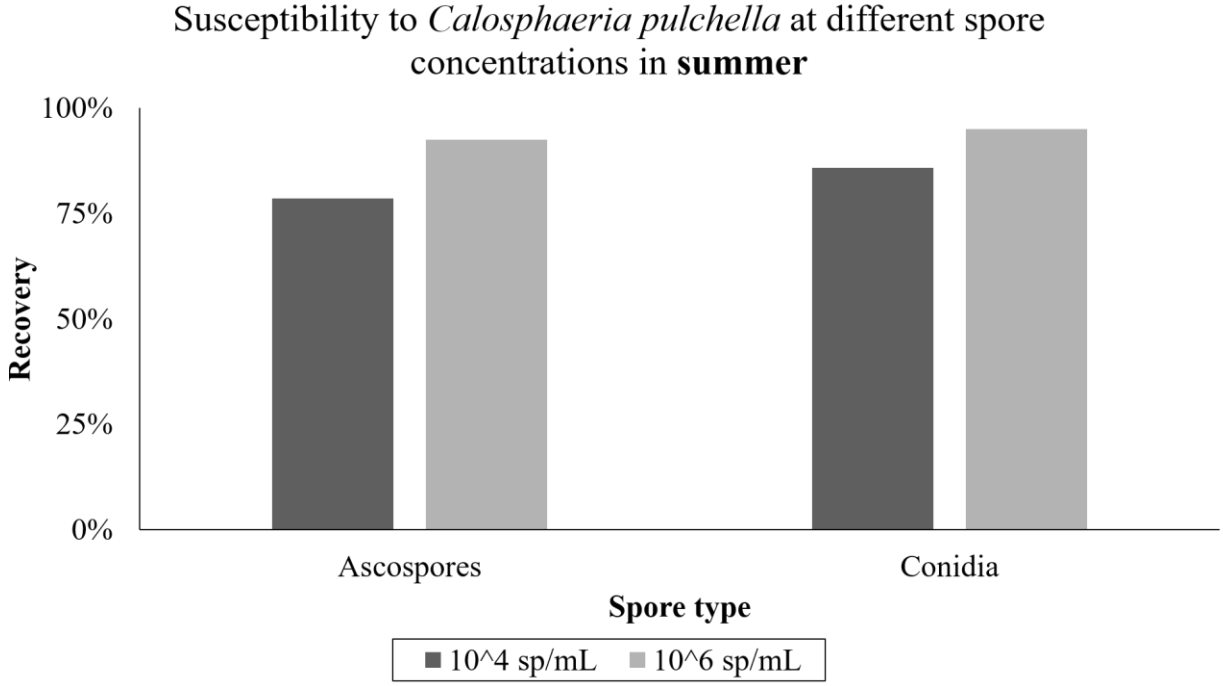
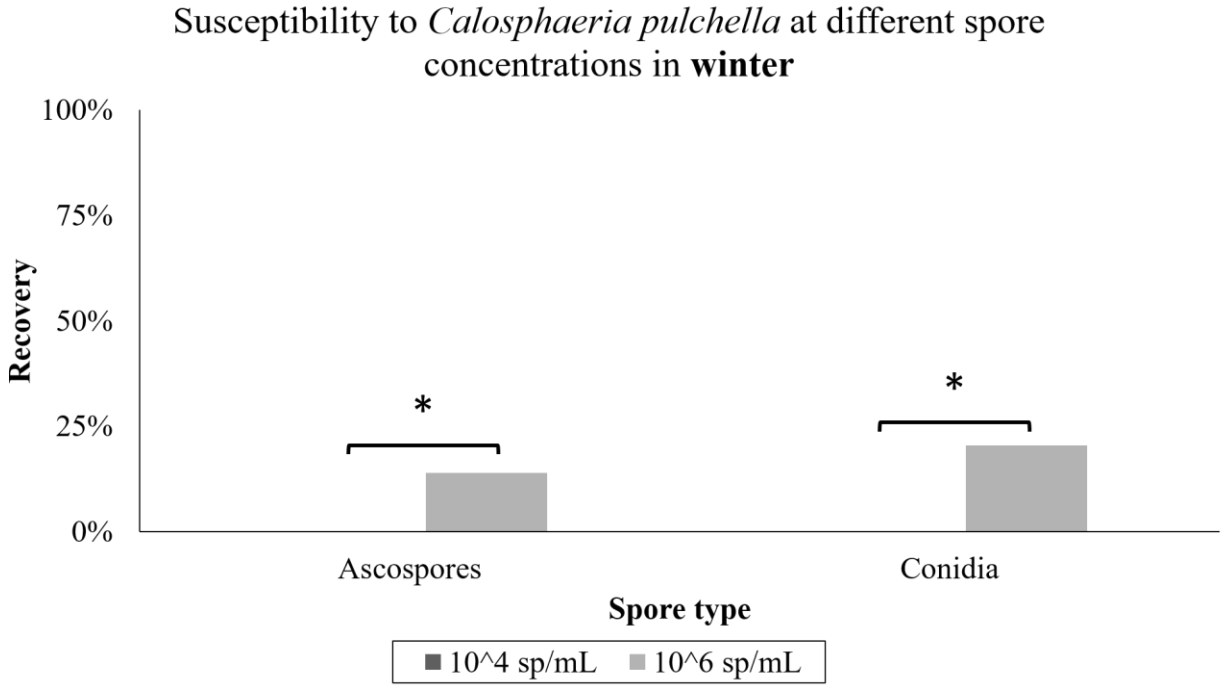


Figure 4b.


Susceptibility to *Calosphaeria pulchella* at different spore concentrations in **winter**
100%
75%
50%
25%
0%
Recovery
*
*
Ascospores
Conidia
Spore type
10^4 sp/mL
10^6 sp/mL