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Research Article

Preventing Trunk Diseases with Fungicide Applications to Pruning Wounds in Washington Wine Grapes

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Abstract

Background and Goals: Grapevine trunk diseases in the Columbia River Basin of eastern Washington include Cytospora dieback, Eutypa dieback, and Esca. Although some of the causal fungi are known (as *Cytospora viticola*, *Eutypa lata*, and *Phaeomoniella chlamydospora*, respectively), basic epidemiology is not. This makes it difficult to time management practices. The common assumption is that these pathogens infect through pruning wounds during the

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dormant season, as has been shown for causal fungi of some grapevine trunk diseases in California. As such, we evaluated fungicides for protecting wounds after pruning under eastern Washington conditions.

Methods and Key Findings: In March of 2019, 2020, and 2021, we evaluated the protection efficacy of pyraclostrobin + fluxapyroxad and thiophanate-methyl sprayed within 3 days of pruning, at an established *Vitis vinifera* 'Chardonnay' vineyard in Prosser, WA. Within 2 days of fungicide treatment, *C. viticola*, *E. lata*, or *P. chlamydospora* (2,000 spores per wound) were inoculated separately onto spurs, and molecular-detection attempts were made 5 to 8 weeks later (after budbreak). Compared to water-treated spurs, detection rates of *C. viticola* and *P. chlamydospora* from thiophanate-methyl-treated spurs were lower in all three study years. Detection rates of *E. lata* from thiophanate-methyl-treated spurs were lower in one year.

Conclusions and Significance: This suggests that dormant-season spray applications of thiophanate-methyl as a pruning-wound protectant can reduce grapevine spur infection by these pathogens. Little-to-no rain during the dormant season in eastern Washington may limit opportunities for disease spread, but winter injury to the permanent, woody structure of the vine may create additional infection courts.

Introduction

Grapevine trunk diseases impact vineyards worldwide. The fungal pathogens cause chronic infections in the permanent, woody structure of the vine. They can kill fruiting shoots, as is the case with the dieback-type trunk diseases (Gubler et al. 2013), or prevent fruit ripening, a symptom of Esca (Gramaje et al. 2018). Eradicating the causal pathogens from a vineyard is possible if done early in the infection process. A practice known as 'trunk renewal' or 'vine

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surgery' (Calzarano et al. 2004, Sosnowski et al. 2011) involves removing the entire vine canopy (typically the cordons and often including the trunk, and the fungal infections that go along with it) and retraining the vine from a presumably healthy base of the trunk. This is a labor-intensive and thus expensive approach, which takes a vine out of production for 2 years, while a new trunk and canopy are being retrained (Baumgartner et al. 2019). A more cost-effective approach to managing trunk diseases in the long term is to prevent the infections from happening in the first place (Kaplan et al. 2016). This can be done through annual practices that minimize the risk of pruning-wound infection, namely delayed pruning (Úrbez-Torres and Gubler 2011), double pruning (Weber et al. 2007), or applications of fungicides and other protectants to pruning wounds (Rolshausen and Gubler 2005, Rolshausen et al. 2010, Brown et al. 2021).

While nursery stock can potentially be infected at the time of planting (Gramaje and Armengol 2011), the causal pathogens can also originate outside of a newly planted vineyard in the form of airborne spores, released after rain events or prolonged periods of high relative humidity [e.g., spores of the Eutypa-dieback pathogen *Eutypa lata* (Carter 1991), and the Esca pathogens *Phaeoacremonium minimum* (Rooney-Latham et al. 2005) and *Phaeomoniella chlamydospora* (González-Domínguez et al. 2020)]. The time it takes for one of these pathogens to complete its life cycle (from spore interception on a pruning wound, to spore germination, to host infection, to establishment of an internal wood infection, and finally to subsequent spore production on the infected host) can range from one year [e.g., Phomopsis-dieback pathogen *Diaporthe ampelina* (Anco et al. 2012)] to several years [e.g., *E. lata* (Ramos et al. 1975)].

In growing regions with a Mediterranean climate, these spores are thought to spread primarily during the dormant season, given that most rain falls during this period. Spore-

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trapping experiments show a trend of numerous spore-dispersal events by a broad range of pathogenic species with rain throughout the entire dormant season [e.g., northern California (Úrbez-Torres et al. 2010, Fujiyoshi et al. 2021b), eastern and northern Spain (González-Domínguez et al. 2020), western Cape province of South Africa (van Niekerk et al. 2010)]. Also in the dormant season of Mediterranean climates, the risk of vine infection is thought to be high, as the dormant-season practice of pruning creates wounds, which are susceptible infection courts for many of the causal fungi [e.g., *E. lata* (Petzoldt et al. 1981, Weber et al. 2007) and Botryosphaeria-dieback pathogen *Neofusicoccum parvum* (Úrbez-Torres and Gubler 2011)]. In western North America, most field trials of preventative pruning [e.g., delayed pruning (Úrbez-Torres and Gubler 2011)] or applications of fungicides after pruning [e.g., thiophanate methyl and pyroclostrobin (Brown et al. 2021)] have been done in California. However, studies on spore dispersal and pruning-wound susceptibility from regions where the rainy season is not synchronous with the timing of dormant-season pruning suggest that spores are produced and dispersed over a longer period of time. For example, spores of Botryosphaeria-dieback pathogens are trapped year-round in the maritime climate of New Zealand (Amponsah et al. 2009) and spores of *Eutypella* species (fungi in the same fungal family, Diatrypaceae, as *E. lata*) are trapped year-round in the desert climate of southern California (Úrbez-Torres et al. 2020). Without knowing which vine tissues are susceptible, when such tissues are at highest risk for infection in these climates, or if the trapped spores are actually infectious to such tissues, it is difficult to adapt the timing of practices originally developed for Mediterranean climates.

In a previous survey of Washington vineyards with trunk diseases, we identified Esca and Eutypa dieback (Travadon et al. 2022 In Press). A unique finding of that survey was the

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prevalence of the trunk disease Cytospora dieback and its causal pathogen *Cytospora viticola*, which was originally described as a new species from vineyards in the northeastern US and southeastern Canada (Lawrence et al. 2017) and has since been reported from vineyards in the northern midwestern US (Dekrey et al. 2022). Eastern Washington is the second largest US producer of wine grapes and the largest producer of juice grapes (USDA National Agricultural Statistics Service 2018). The climate characteristics of this semi-arid steppe region are much colder and drier than that of the major grape-growing areas in California, especially during the dormant season. While the dormant season is when the predominate of this region's 200 to 500 mm of annual precipitation falls, it often falls as snow (non-liquid form) and is accompanied by prolonged periods of freezing temperatures. Further, cold damage to vines during the dormant season can result in entire vineyards needing to be retrained from the base of the trunk, every 10 to 20 years.

Our goal is to identify effective protectants as fungicide applications against pathogens we identified in eastern Washington: *Cytospora viticola* (Cytospora dieback), *Eutypa lata* (Eutypa dieback), and *Phaeomoniella chlamydospora* (Esca) (Travadon et al. 2022 In Press). No studies to date (to our knowledge) have tested fungicides against trunk diseases in Washington. As such, we evaluated fungicides previously shown to be effective against at least one of the pathogens. Without knowing the exact timing of pruning-wound susceptibility or spore dispersal in the cold, dry winter of the lower Columbia Basin of eastern Washington, we carried out the experimental steps based on the timing of dormant-season pruning.

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Materials and Methods

Study vineyard in eastern Washington. A replicated field trial was conducted for three years, from 2019 to 2021, in an experimental vineyard in Prosser, Washington USA (Washington State University, Irrigated Agriculture Research and Extension Center). The vineyard was planted in 2011 with *V. vinifera* 'Chardonnay' on its own roots. Planting materials were certified at the time of planting by the Washington State Department of Agriculture (https://agr.wa.gov/departments/plant-health). Spacing is 3 m between rows and 1.5 m between vines, at a density of approximately 1,749 vines/hectare (726 vines/acre). Vines are trained to a dual-trunk bilateral cordon, spur pruned, and the canopy trained to a modified vertical-shoot positioned system. The vineyard is drip-irrigated, and the irrigation season runs from approximately April to October (per Sunnyside Valley Irrigation District allocations).

Prosser is located within the Yakima Valley American Viticultural Area of eastern Washington. The climate is a semi-arid steppe, characterized by hot, dry summers, and cold winters (USDA Cold Hardiness Zone 7a; https://planthardiness.ars.usda.gov/). The majority of the average 203 mm of annual precipitation falls between November and March. Weather data for this study (Figure 1) were recorded at an AgWeatherNet station (weather.wsu.edu; "Prosser.NE), located approximately 500 m from the vineyard location.

Our experimental approach (Table 1) included the following steps: 1) prune all vines in data rows and buffer rows to spurs of 40 cm length; 2) apply fungicide treatments to data vines within 3 days after pruning; 3) carry out inoculation treatments of spurs on data vines within 2 days of fungicide treatments; and 4) collect spurs that received the inoculation treatments for pathogen detection, when shoots growing from those spurs were approximately 20-cm long.

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Pruning cuts were made horizontally, to prevent the droplet of inoculum from running off. The relatively long length of the retained spurs (40 cm) after pruning was intentional to create enough space to minimize the risk of infection progressing to the cordon from the pruning wounds, which are inoculated; it allowed us to remove inoculated tissue while still being able to leave sufficient segments of canes to allow normal canopy growth and development during the growing season. Also, the fungicide applications are more efficient and thorough when all the pruning wounds are at the same height in the canopy. The short incubation period between steps 3 and 4 (ranging from 39 to 56 days; Table 1) was also necessary to minimize the risk of disease spread from the inoculated spurs to the rest of the vineyard, which does not have a history of trunk diseases.

Fungicides applied after pruning. Fungicide treatments were as follows: a water-treated control, pyraclostrobin + fluxapyroxad [Merivon Xemium, BASF, EPA 7969-310; Fungicide (FRAC) groups Quinone outside inhibitor (QoI) + succinate-dehydrogenase inhibitor (SDHI)], and thiophanate-methyl [Topsin M, United Phosphorus, Inc., EPA CA-030001; FRAC group methyl benzimidazole carbamate (MBC)]. Treatments were applied to pruning wounds at a spray volume equivalent of 935 L/ha, using a backpack sprayer (Solo Model 425, Newport News, VA, USA) with an air induction spray nozzle (TeeJet AITXA 8002). The application rate of pyraclostrobin + fluxopyroxad (0.42 mL formulated product/L or 1.5 lbs per 100 gal) was the maximum allowable rate on the manufacturer's label. The application rate of thiophanate-methyl (1.8 g formulated product/L or 5.5 fl oz per 100 gal) was recommended by the manufacturer for management of trunk diseases. During application, the spray nozzle was directed at each and every pruning wound and applied to run-off. Thorough coverage of spray

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applications was confirmed as 100% each year, using two, 2 cm^2 pieces of water- and oil-sensitive paper (TeeJet, Wheaton, IL) attached to the pruned spurs on one data vine per treatment per block.

Fungicide treatments (including the water-treated control) were applied in a randomized complete block design (RCBD) with three blocks, each of which consisted of one treated row separated by five buffer rows. Within each treated row (i.e., block), fungicide treatments (water-treated control, pyraclostrobin + fluxapyroxad, thiophanate-methyl) were distributed among eight-vine sets, and these fungicide treatments were randomized within the three blocks. For each eight-vine set, all eight vines were treated on both sides of the row, but the spurs did not receive inoculation treatments on the exterior cordons of vine 1 or vine 8 (i.e., 0.5 vine on either end of the eight-vine set was treated, but not subject to pathogen inoculation). On the remaining 14 cordon lengths (the total of 14 comes from the fact that there were two cordons per each of the eight vines, less the exterior cordons on both ends of the treated area), spurs of the central three vines (data vines) were randomly flagged for inoculation treatment, with five spurs per inoculation treatment per data vine (including the water-treated control). This gave a total of 540 spurs per year (3 blocks x 3 fungicide treatments x 4 inoculation treatments x 3 data vines per fungicide treatment x 5 spurs per inoculation treatment per data vine). The same eight-vine sets within the three blocks received the same fungicide treatments each year. Within data vines, however, inoculation treatments were randomized among spurs each year, as spurs are renewed annually in grape production systems.

Pathogens inoculated to pruning wounds after fungicide applications. Inoculation treatments were as follows: a water-inoculated control, *C. viticola* (Bent901), *E. lata* (ascospores

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collected from environmental stromata on the wood of *Nerium oleander* L., in Yolo County, California), and *P. chlamydospora* (Bent708). *Cytospora viticola* (Bent901) and *P. chlamydospora* (Bent708) were originally isolated from vineyards in eastern Washington with symptoms of Esca, and were shown to be virulent in greenhouse assays (Travadon et al. 2022 In Press). Water-inoculated controls (i.e., the spurs that we pipetted water onto instead of pathogen inoculum, in the eight-vine sets that were first sprayed with either water - the water-treated controls - or one of the fungicides) were included in the experimental design as a type of negative control, to determine background levels of local pathogens in the vineyard.

For *C. viticola*, inoculum consisted of spores (conidial) from pycnidia produced in culture on autoclaved grape wood (Lawrence et al. 2017). To induce development of pycnidia, one-year-old grape canes (approx. 1 cm in diameter) were collected in the vineyard and cut into 5 cm long segments. Wood segments were autoclaved in glass Petri plates twice, 24 h apart, at 122 °C for 25 min. Autoclaved wood segments were placed in Petri plates (9 cm diam.), with two segments per plate, and autoclaved potato dextrose agar (PDA; Difco Laboratories) was poured to the level at which the segments were almost completely submerged. An agar plug from an actively growing culture on PDA was placed between the two wood segments, and plates were incubated at room temperature under natural lighting for 4 weeks. During the 4 weeks of incubation, when pycnidia appeared, mature pycnidia were crushed with a flame-sterilized probe in 1 ml of sterile, distilled water, the concentration was estimated with a hemocytometer, and then adjusted with sterile water to 1×10^5 spores/mL.

Because *E. lata* does not produce its infectious sexual spores (ascospores) in culture, ascospores were harvested from fruiting bodies (perithecia) we collected in the field, from the

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infected wood (visibly covered in stromata, within which perithecia were imbedded) of a *Nerium oleander* L., in Solano County, California. To collect ascospores from perithecia, stromata were sliced with a sterile razor blade, to reveal and cut open the perithecial cavities, and a drop of sterile water was placed on the perithecia. Masses of ascospores were then collected with a sterile probe and transferred to 1 ml sterile water. The spore concentration was estimated with a hemocytometer, and then adjusted with sterile water to 1×10^5 spores/mL.

To produce spore (conidial) suspensions of *P. chlamydospora*, a liquid culture was first established by inoculating ten, 2 mm agar plugs from a 7-day-old culture on PDA to a 250-mL Erlenmeyer flask containing 100 mL of potato dextrose broth (PDB; Difco Laboratories). After incubation at 25°C and 150 rpm for 5 days, a hand-held disperser (IKA-ULTRA-TURRAX T8) was used to homogenize the culture (1 min, speed 5), and 100 µl of homogenate was spread onto each of three PDA plates (9 cm diam.). After 14 days, spores were harvested by pipetting 2 mL of sterile water onto the agar surface, filtering the suspension through two layers of sterile cheesecloth to remove fragments of aerial mycelium, the concentration was estimated with a hemocytometer, and then adjusted with sterile water to 1×10^5 spores/mL.

Each year, the day before inoculations, inoculum (i.e., the spore suspensions described above) was prepared in Davis, CA and shipped overnight to Prosser, WA. On the day of inoculation in the vineyard, inoculum viability for each pathogen was tested in the lab by plating it on PDA. For each inoculation treatment with a pathogen, inoculum was pipetted onto the cut surface of the pruning wounds of data vines (20 µL or 2,000 spores per spur). For water-inoculated spurs, 20 µL of sterile water was pipetted onto the cut surface of the pruning wound.

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Incubation of the inoculation treatments in the field, between the time of inoculation and when spurs were collected for pathogen detection, ranged from 39 to 56 days (Table 1).

Detection of pathogens to measure fungicide efficacy. From spurs of each inoculation treatment, we collected the distal 15 cm of the spur wood, removing any shoots that had emerged. Spurs were shipped overnight to our lab in Davis, CA, for pathogen detection. DNA-based detection was used for spurs inoculated with *C. viticola*, using published primers for genus-level detection of *Cytospora* species (Luo et al. 2017), as detailed below. Because of our past experience with low recovery of *C. viticola* in culture from inoculated grape in the greenhouse (Lawrence et al. 2017), we were hopeful for higher and especially more consistent data with DNA-based detection. DNA-based detection was also used for *E. lata* and *P. chlamydospora*, as both species have proven difficult to isolate in culture. Further, the species specificity against a range of other wood-colonizing fungi of grape has been tested for qPCR primers for *E. lata* (Pouzoulet et al. 2017, Fujiyoshi et al. 2021a) and *P. chlamydospora* (Pouzoulet et al. 2013). We have used these qPCR primers to detect *E. lata* and *P. chlamydospora* in the field (Brown et al. 2021, Fujiyoshi et al. 2021b). From the distal 15 cm of wood from each inoculated spur, the bark was first scraped off the surface with a flame-sterilized knife and the approximately 0.5 cm of dried wood at the cut surface of the pruning wound was cut away. A 2.5 cm section of wood from below this discarded 0.5 cm end was sealed in a pre-labeled glass vial and stored at -80°C. Wood samples were ground to a powder in chilled containers (Grinder MM400, Retsch, Haan, Germany) and stored at -80°C in 2 mL microcentrifuge tubes. For DNA extraction, 1 mL of extraction buffer [Tris-HCl 100 mM, EDTA 20 mM, NaCl 1.4 M, CTAB 2%, PVPP 2%, β-mercaptoethanol 0.5%, RNAse A 0.4% v/v

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(Qiagen, Germantown, MD)] was added to 100 mg of wood powder in the 2 mL tube. Tubes were briefly vortexed, 500 µL of chloroform-isoamyl-alcohol (24:1) was added, tubes were incubated on ice for 5 min, and then centrifuged (2,300 g, 10 min, 4°C). The supernatant was transferred to a new tube and mixed with AP2 buffer and the rest of the manufacturer's protocol for the DNeasy plant mini kit (Qiagen) was followed.

For qPCR, 1 µl of 1X DNA extraction was used as template in a 25 µL reaction volume consisting of: 1X Brilliant SYBR Green q-PCR Master Mix (Stratagene, La Jolla, CA), 150 nM per primer (Operon Biotechnologies), 30 nM ROX Reference Dye (Invitrogen), and sterile molecular biology-grade water (GIBCO). All reactions were performed in 200 µL tubes in 96-well plates, in an Mx3000p Real-time PCR Thermal Cycler (Stratagene). The PCR program was as follows: initial denaturation step at 95°C for 3 min, 50 cycles of 20 s at 94°C, followed by 20 s at 65°C for both annealing and extension (62°C for *C. viticola*), and additional melting analysis. After the amplifications were completed, dissociation curves were obtained based on a standard protocol from manufacturer's instructions, and the temperature of the peak of the curve was checked to confirm the correct PCR product. The threshold level for fluorescence was set arbitrarily within the log–linear phase of increase. Genomic DNA from pure cultures was used as positive controls. Amplification of target DNA was based on the dissociation temperature (81.5°C for *C. viticola*, 79.0-79.5°C for *E. lata*, 75.9°C for *P. chlamydospora*). Positive detections were samples crossing the threshold level by 40 cycles for *C. viticola*, and 45 cycles for *E. lata* and *P. chlamydospora*. Detection (%) for each data vine was the percentage of spurs positive for the inoculated pathogen, out of five inoculated spurs.

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For DNA-based detection of *C. viticola*, we used *Cytospora* primers CtBTFF1 and CtBTFR1, which amplify a portion of the β-tubulin gene, and were originally developed for genus-level detection of *Cytospora* species from the tree crops walnut and plum (Luo et al. 2017). Luo et al. (2017) screened the primers against other genera of wood-colonizing fungi of walnut and plum, to demonstrate their specificity to the genus *Cytospora*. We further evaluated the specificity of the *Cytospora* primers within our study system by first screening *Cytospora* species known to be pathogenic to grape, using DNA of virulent isolates from our previous surveys of vineyards with trunk diseases (US states from which isolates were collected are in parentheses; Supplemental Table 1): *Cytospora species* 1 [Washington (Travadon et al. 2022 In Press)], *Cytospora vinacea* [New Hampshire (Lawrence et al. 2017)], and *C. viticola* [California (Lawrence et al. 2017) and Washington (Travadon et al. 2022 In Press)]. We also evaluated the *Cytospora* primers from all *Cytospora* species cultured from the water-inoculated (control) spurs, from which we made culture attempts each of the three study years, as detailed below. Species-level identity of the *Cytospora* isolates was confirmed by sequencing the rDNA internal transcribed spacer region (ITS) (White et al. 1990) and translational elongation factor 1-α (TEF) (Carbone and Kohn 1999), both of which have been shown to be informative for species delineation in the genus *Cytospora,* especially for some of the *Cytospora* species reported from grape (namely, *C. vinacea* and *C. viticola*) (Lawrence et al. 2017).

Although there were no vines with external symptoms of trunk diseases in the vineyard, our experimental design included water-inoculated (control) spurs on the data vines within each fungicide treatment, to determine the presence of local pathogens, originating possibly from asymptomatic vines in the vineyard or from symptomatic vines/alternate hosts outside the

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vineyard. Spores from such hosts may have infected spurs between the time of pruning and the collection of spurs (i.e., coinciding with the incubation period of the pathogens that were inoculated to the spurs). Groups of pathogens we were particularly interested in isolating from water-inoculated (control) spurs were as follows: 1) The three species inoculated as part of the experimental design (*C. viticola*, *E. lata*, and *P. chlamydospora*), the presence of which would be expected to contribute to the detection rates we measured; 2) Causal fungi of Cytospora dieback, Eutypa dieback, and Esca, other than the three pathogens we inoculated to spurs; and 3) Causal fungi of other trunk diseases, namely Botryosphaeria dieback and Phomopsis dieback. To check this 'background level' of local pathogens, we attempted to culture fungi from all water-inoculated spurs on both fungicide-treated and water-treated data vines. From the distal 15 cm of wood from each water-inoculated spur, the bark was first scraped off the surface with a flame-sterilized knife and the approximately 0.5 cm of dried wood at the cut surface of the pruning wound was cut away. From the 2.5 cm section of wood from below this discarded 0.5 cm end, we cut 16 small (each approx. 5 mm^3) pieces of wood from apparently healthy wood and discolored wood (if present). Wood chips were surface sterilized in 0.6% sodium hypochlorite (pH 7.2) for 15 s, rinsed twice in sterile distilled water for 1 min, plated on two PDA dishes amended with tetracycline (1 mg/L, Sigma-Aldrich) per spur, and incubated at 23°C. At 3, 6, 10, 15, and 30 days (to accommodate fast and slow-growing pathogens) subcultures were hyphal-tip purified to PDA for identification. Isolates were identified to the species level based on sequencing of ITS and TEF.

Statistical analyses. For each pathogen, separate analyses of variance (ANOVAs) were used to determine the main effects of *year* and *fungicide treatment*, and their interaction on

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detection (%). Normality and homogeneity of variances were evaluated, and confirmed, using normal probability plots and Levene's test, respectively. ANOVAs were performed using the MIXED procedure in SAS v. 9.4 (SAS Institute, Cary, NC, U.S.A.), with year and treatment as fixed effects, and block and block interactions as random effects. Year was considered a repeated measure. Appropriate covariance models were selected for ANOVAs of each pathogen based on comparisons of information criteria (AIC, AICC, BIC), as specified in the REPEATED statement (unstructured for *C. viticola* and *P. chlamydospora*, autoregressive for *E. lata*). For significant main or interactive effects ($P < 0.05$), means were compared by Tukey's tests. When interactive effects were not significant, means for significant main effects were compared by Tukey's tests. To aid in the presentation and interpretation of the detection rates, we also calculated efficacy (%) as $100 \times [1 - (\text{Detection}_{\text{Fungicide}}/\text{Detection}_{\text{Control}})]$, where $\text{Detection}_{\text{Fungicide}}$ is detection (%) of the pathogen from fungicide-treated spurs and $\text{Detection}_{\text{Control}}$ is detection (%) of the pathogen from water-treated spurs. In this way, a high detection rate corresponds to a low efficacy. Efficacies of the fungicides against the pathogens were calculated from detection rates, but efficacies were not analyzed statistically.

Results

Differences in detection and environmental variables among study years. In order to ensure that our experimental approach evaluated fungicide efficacy consistently among fungicide treatments and over time, we used the same procedures to prepare inoculum each year, and then we evaluated inoculum viability in culture on the day of inoculation. However, spores of *E. lata* in 2019 (year 1) did not germinate on the day of inoculation, which was consistent with no detection of *E. lata* from the inoculated spurs (Table 2), which were collected 56 days later.

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Fortunately, *E. lata* inoculum was viable and inoculations were successful in 2020 and 2021 (P = 0.006 for *year* x *fungicide treatment*), with detection rates from the water-treated spurs of 40% and 83%, respectively (Table 2). Detection rates of *C. viticola* were consistently high in all study years, in spite of statistically significant variation over time (P= 0.03 for *year*). Detection rates of *C. viticola* from water-treated spurs ranged from a low of 91% in 2021 to a high of 98% in 2019. In contrast, detection rates of *P. chlamydospora* were much more variable (and statistically significant at $P < 0.0001$ for *year*) over time. Detection rates of *P. chlamydospora* from water-treated spurs ranged from a low of 29% in 2019 to a high of 98% in 2021.

The timing of pruning at the start of the experiment corresponded to the time of pruning by growers in commercial vineyards. Because early March 2019 was cold and the vineyard under snow (Figure 1), pruning and subsequent activities were later than in other years (pruning on 28 March 2019 and spur collection on 28 May 2019). That said, 2019 was characterized by the highest temperatures on the day of inoculation, during the week following inoculation, and during the entire incubation period (Table 3). In contrast, 2020 was characterized by the lowest temperatures during these same periods. Total precipitation during the incubation period was highest in 2019 (37.6 mm), but was extremely low in 2020 in 4.1 mm. There was no rain during the 2021 study period. Each year, on the day of inoculation, there was no precipitation (Figure 1), but relative humidity ranged from a low of 44.5% in 2019 to a high of 59.4% in 2020 (Table 3). Regardless, climate differences among study years did not seem to correspond to differences in detection rates of *E. lata* or *P. chlamydospora* from water-treated spurs.

Fungicide efficacy. Thiophanate-methyl was the most effective fungicide against *C. viticola* infection. Detection of *C. viticola* was lower for pruning wounds treated with

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366 thiophanate-methyl ($P < 0.0001$ for *fungicide treatment*) versus the water-treated control in all
367 three study years (Table 2). Detection of *C. viticola* from pruning wounds treated with
368 thiophanate-methyl ranged from 22 to 62%, which corresponded to efficacies ranging from 33 to
369 77% (Table 2). Detection of *C. viticola* was also lower for pruning wounds treated with
370 pyraclostrobin + fluxapyroxad ($P < 0.0001$ for *fungicide treatment*); detection rates ranged from
371 71 to 87%, which corresponded to efficacies ranging from 7 to 27% (Table 2). The mean
372 detection rates of *C. viticola* from water-treated spurs ranged from 91 to 98%, averaged across
373 three blocks per year.

374 Thiophanate-methyl was the most effective fungicide against *P. chlamydospora* in two of
375 three years. Detection of *P. chlamydospora* was significantly lower for pruning wounds treated
376 with thiophanate-methyl ($P = 0.04$ for *fungicide treatment*) versus the water-treated control
377 (Table 2). Nonetheless, in 2021, detection of *P. chlamydospora* from thiophanate-methyl-treated
378 spurs was high at 93%; as such, the efficacy of thiophanate-methyl against *P. chlamydospora*
379 was only 5% in 2021, as compared to efficacies of 62% and 38% in 2019 and 2020, respectively.
380 Pyraclostrobin + fluxapyroxad was not effective against *P. chlamydospora*; detection of *P.*
381 *chlamydospora* from pruning wounds sprayed with pyraclostrobin + fluxapyroxad was in each
382 year either close to or higher than that of the water-treated controls.

383 Thiophanate-methyl was the most effective fungicide against *E. lata*, but only in 2021
384 ($P = 0.006$ for *year* x *fungicide treatment*). Of the two study years when *E. lata* inoculations
385 were successful (2020 and 2021), detection of *E. lata* was lower in 2021 for pruning wounds
386 sprayed with thiophanate-methyl versus the water-treated control (mean detection rates of 24%
387 versus 83%, averaged across three blocks per year), with a corresponding efficacy of 71% (Table

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2). Detection of *E. lata* was intermediate in 2021 for pruning wounds sprayed with pyraclostrobin + fluxapyroxad (mean detection rate of 60%, averaged across three blocks per year, with a corresponding efficacy of 28% (Table 2).

Pathogens and other fungi isolated from water-inoculated (control) spurs. Across the three years, we cultured 22 isolates, based on culture attempts from 405 total water-inoculated (control) spurs (45 water-inoculated spurs per fungicide treatment x three fungicide treatments x three years = 405 total water-inoculated spurs). These 22 isolates were identified as the following species (number of spurs per fungicide treatment are shown in parentheses): *Cryptovalsa ampelina* (nine water-treated spurs), *Cytospora chrysosperma* (one water-treated spur), *Cytospora parakantschavelii* (one water-treated spur), *Cytospora parasitica* (three water-treated spurs, one pyraclostrobin + fluxopyroxad-treated spur), *Cytospora ulmicola* (one pyraclostrobin + fluxopyroxad-treated spur), *Cytospora viticola* (one water-treated spur, one thiophanate-methyl-treated spur), undescribed species *Cytospora species* 2 (one water-treated spur), and *Diplodia seriata* (one water-treated spur). Prior to the experiment, we found that in addition to *C. viticola*, the *Cytospora* primers used for detection of *C. viticola* also detected other *Cytospora* species known to be pathogenic to grape, based on positive amplification of isolates from our culture collection of *Cytospora species* 1 and *C. vinacea* (Supplemental Table 1). During the experiment, we found that the *Cytospora* primers detected isolates from the non-inoculated (control) spurs, namely *C. chryosperma*, *C. parakantschavelii*, *C. parasitica*, *Cytospora species* 2, and *C. ulmicola*; the pathogenicity of these species on grape is not known. Altogether, the *Cytospora* primers detected nine of the 10 total *Cytospora* isolates we tested (Supplemental Table 1), with amplification products ranging in size from 85 to 93 bp. We

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assume therefore that some of our qPCR detections might represent a species of *Cytospora* other than *C. viticola*. However, given that *Cytospora* species other than *C. viticola* were isolated from only eight of the 405 water-inoculated spurs we made culture attempts from (2.2%), we assume their detection rate is negligible.

Discussion

The high efficacy of thiophanate-methyl against *C. viticola* and *P. chlamydospora* suggests this fungicide could minimize dormant-season pruning-wound infection by these pathogens. Thiophanate-methyl is a grower standard fungicide treatment in California, based on current recommendations (Gubler et al. 2013), but was not registered for dormant-season use in Washington at the start of our study. Thiophanate-methyl was also the most effective fungicide against *E. lata* in one of two study years, which was the year in which the *E. lata* inoculations were most successful. Pyraclostrobin + fluxapyroxad was associated with lower detection rates of *C. viticola* than those of the water-treated controls in all three years, but its efficacy was below 27%. Our findings are novel, with respect to *C. viticola*, a trunk-disease pathogen which has not been included in past studies on pruning-wound protectants. No studies have evaluated pruning-wound protectants for a *Cytospora* species virulent on grape, although studies on other *Cytospora* species that attack tree crops (e.g., peach and almond) report that thiophanate-methyl is effective against preventing their infection (Miller et al. 2019, Holland et al. 2021).

Thiophanate-methyl has been previously shown to have moderate to high efficacy against a broad range of trunk-disease pathogens: *E. lata* (Rolshausen et al. 2010), Botryosphaeria-dieback pathogens [(*B. dothidea*, *D. viticola*, *D. seriata*, and *L. theobromae* (Rolshausen et al. 2010), *D. seriata* (Diaz and Latorre 2013, Martinez-Diz et al. 2021), *N. luteum* (Amponsah et al.

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2012), and *N. parvum* (Brown et al. 2021)], Phomopsis-dieback pathogen *Diaporthe ampelina* (Brown et al. 2021), and Esca pathogen *P. chlamydospora* (Diaz and Latorre 2013, Martinez-Diz et al. 2021). Most of these studies cited above relied on the application of thiophanate-methyl at an experimental scale and with spray bottles, whereas we used a backpack sprayer and timed the applications when and how a grower would (i.e., soon after pruning). Our findings confirmed thiophanate-methyl could protect pruning wounds, when applied with a back-pack sprayer to each and every pruning wound to the point of run-off, from taxonomically diverse pathogens.

Pyraclostrobin has been tested in various formulations and was moderately effective against *E. lata* [e.g., in California (Rolshausen et al. 2010, Brown et al. 2021) and in Australia (Sosnowski et al. 2008, Sosnowski et al. 2013, Ayres et al. 2017, Ayres et al. 2022)], against Botryosphaeria-dieback pathogens [e.g., *Botryosphaeria dothidea*, *Diplodia seriata*, *Dothiorella viticola*, and *Lasiodiplodia theobromae* in California (Rolshausen et al. 2010), *Neofusicoccum parvum* in California (Brown et al. 2021), and *Neofusicoccum luteum* in Australia (Ayres et al. 2022)] and against *P. chlamydospora* (Rolshausen et al. 2010, Diaz and Latorre 2013). However, we did not see similar promising results; pyraclostrobin + fluxapyroxad had low efficacy against *C. viticola*, *E. lata*, and *P. chlamydospora.* When we consider variable efficacy of pyraclostrobin against the same pathogen species in different studies, differences could result from variable isolate virulence, differing formulations, concentrations, application methods, and/or inoculation methods. For example, we found low efficacy of pyroclostrobin, with *P. chlamydospora* spores inoculated to pruning wounds, after applying pyraclostrobin + fluxapyroxad as a liquid formulation via a back-back sprayer. In contrast, Diaz and Latorre (2013) found high efficacy of pyroclostrobin, with agar plugs from an actively growing culture

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454 of *P. chlamydospora* inoculated to pruning wounds, after applying pyroclostrobin as a paste
455 formulation. To further compare and contrast studies on pyroclostrobin versus *P.*
456 *chlamydospora*, Rolshausen et al. (2010) found high efficacy of pyroclostrobin, with *P.*
457 *chlamydospora* spores inoculated to pruning wounds, after applying pyraclostrobin at 10 times
458 the concentration we used. Our findings of low efficacy of pyroclostrobin against *E. lata* are
459 consistent with those of other studies on grape (Sosnowski et al. 2013, Ayres et al. 2017) and
460 almond (Holland et al. 2021).

461 The ideal environmental conditions for spore germination and subsequent infection of
462 pruning wounds are not known for the pathogens we tested. In addition to freezing temperatures,
463 other factors that may negatively impact the viability of the spore suspensions after they are
464 pipetted onto pruning wounds include exposure to UV light and dry conditions caused by wind.
465 As such, we try not to inoculate on sunny, windy days that are either very hot or very cold. Each
466 year, we inoculated with higher spore concentrations (2,000 spores per spur) than would likely
467 occur naturally, in order to achieve consistently high pathogen pressure across fungicide
468 treatments and years. Nonetheless, this approach may not sufficiently compensate for the effects
469 of poor environmental conditions at the time of pathogen inoculation and colonization in some
470 years more than others; hence the variable detection rates we found from year to year for *P.*
471 *chlamydospora* and *E. lata*. Higher detection rates of *P. chlamydospora* and *E. lata* in 2021 may
472 possibly be due to a combination of conditions that favored infection on the day of inoculation or
473 during incubation, for e.g., no freezing temperatures and moderate relative humidity, and/or
474 higher pruning-wound susceptibility. Variable detection rates from year to year for *P.*
475 *chlamydospora* (2021 > 2020 and 2019) and *E. lata* (2021 > 2020) suggest there is annual

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476 variation in either inoculum viability in the laboratory or spore survival/pruning-wound
477 susceptibility in the field. Conidia of *P. chlamydospora* consisted of conidia from a single
478 isolate produced under the same laboratory conditions each year, whereas ascospores of *E. lata*
479 had to be harvested from an infected oleander tree each year. The reliance on such
480 environmental samples may explain the lack of viability (and the lack of infections in the field)
481 of *E. lata* ascospores in 2019; this is one of the risks of having to use field-collected spores of *E.*
482 *lata* for our research, as spore maturity and viability cannot be under our control.
483 Because we made culture attempts from all water-inoculated (control) spurs within each
484 fungicide treatment, we were able to characterize the 'background level' of local pathogens each
485 year, albeit from isolates from a low proportion of samples (22 of 405 spurs). We identified *C.*
486 *viticola*, which was one of our inoculation treatments (albeit on inoculated spurs), and other
487 *Cytospora* species known to be pathogenic to grape, *Cytospora species* 1 (and undescribed
488 species, which we have in our culture collection) and *C. vinacea* (Lawrence et al. 2017). Other
489 species pathogenic to grape we identified included *Cryptovalsa ampelina*, which is in the same
490 fungal family as *Eutypa lata* (Diatrypaceae) and also causes dieback symptoms, and
491 Botryosphaeria-dieback pathogen *Diplodia seriata*, which was identified in a previous survey of
492 Washington vineyards (Holland et al. 2015). Although characterization of natural fungal
493 infections of pruning wounds is rarely published [e.g., (Luque et al. 2014)], and could potentially
494 be very site and year-specific, this step helped us evaluate our DNA-based detection method of
495 *C. viticola*. Certainly, we knew prior to the field trial that the qPCR primers were not species-
496 specific. Indeed, the primers amplified five additional *Cytospora* species, which we identified
497 along with *C. viticola* from a combined 2.2% of the 405 water-inoculated (control) spurs

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examined throughout the 3 years. The diversity of *Cytospora* species, many of which are of unknown pathogenicity to grape, suggests that Cytospora dieback may be caused by more species than just *C. viticola*.

The main benefit of DNA-based detection via qPCR over culture-based detection of the three pathogens was being able to store the samples (540 spurs each year) at -20°C for processing over a period of time. Our study coincided with the global COVID pandemic, which limited our ability to process samples in the time-sensitive manner necessitated by culture-based detection; DNA-based detection allowed us to continue the study in consecutive years through lab-occupancy restrictions. We acknowledge, though, that this approach does have limits. For example, in this study, the consistently high detection rates of *C. viticola*, even from fungicide-treated spurs, may reflect detection of DNA from dead spores. It is also possible that in some cases we detected DNA of dead *E. lata* spores that traveled within xylem vessels below the surface of the pruning wound, as has been shown at depths of 5 to 8 mm after artificial inoculations under dry conditions, similar to inoculation conditions in this study (Carter 1960, Larignon 2010). However, we discarded the distal approximately 0.5-cm end of the spur, and extracted DNA from the wood below. Further, in 2019, *E. lata* inoculum was not viable and we had 0% detection via qPCR. As such, it seems that our DNA-based detection method did not have a high rate of false positives (i.e., did not detect the DNA of dead spores of *E. lata*). Consequently, we assume that positive detection through qPCR reflects detection of the corresponding, metabolically active pathogens.

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Conclusions

Because we timed the fungicide applications in our study to closely follow the typical time of final pruning in commercial vineyards, we assume our findings on fungicide efficacy would be relevant if spores of *C. viticola*, *E. lata*, or *P. chlamydospora* are dispersed after pruning. We demonstrated that fresh pruning wounds are susceptible to infection by the three pathogens, albeit at higher spore concentrations than they may encounter naturally. Additionally, environmental conditions during the dormant season in eastern Washington are cold and dry, which may also limit disease pressure. Pruning wounds, however, may not be the only point of infection for these pathogens. Wounds to the permanent, woody structure of the vine that are created by winter injury may be an important infection court, in addition to or more so than pruning wounds. More work is needed on the susceptibility of such wounds and their natural healing process in eastern Washington, to reveal the best time for preventative practices, like fungicide applications and the timing of pruning.

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675 **Table 1** Timing of pruning, application of fungicide treatments and inoculation treatments, and
676 spur-collection steps in the Prosser, Washington vineyard. Step 4 was carried out after budbreak,
677 when shoots growing from inoculated spurs were approximately 20 cm long.

Practice	2019	2020	2021
Step 1: Pruning	28 March	14 March	22 March
Step 2: Fungicide treatments	1 April	16 March	23 March
Step 3: Inoculation treatments	2 April	17 March	25 March
Step 4: Collection of spurs for pathogen detection	28 May	28 April	3 May
Incubation period of pathogens in inoculated spurs	56 days	42 days	39 days
Total study period	62 days	46 days	43 days

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679 **Table 2** DNA-based detection of pathogens inoculated to pruning wounds, after application of fungicide treatments, compared to that
680 of a water-treated, inoculated control. Detection (%) is the percentage of spurs from which the pathogen was detected, out of the total
681 number of inoculated spurs [95% confidence limits (CL) are shown in parentheses]. Efficacy (%) is 100 x [1 –
682 ($Detection_{Fungicide}/Detection_{Control}$)]. Fungicide treatments with detection rates higher than that of the water-treated control were
683 completely ineffective; they were given an efficacy of 0, rather than the calculated negative value. Each value is the mean of three
684 blocks (with three data vines per block and five spurs per pathogen per data vine). Detection rates within a year with different letters
685 are significantly different (Tukey's test, $P < 0.05$).

		Cytospora viticola		*Phaeomoniella chlamydospora*		*Eutypa lata*	
Year	Fungicide treatment	Detection (%) (95% CL)	Efficacy (%)	Detection (%) (95% CL)	Efficacy (%)	Detection (%) (95% CL)	Efficacy (%)
2019	Water-treated	98 a (87 - 109)		29 a (21 - 37)		0	
	Pyraclostrobin + fluxapyroxad	71 b (60 - 82)	27	24 ab (16 - 32)	15	0	-
	Thiophanate-methyl	22 c (11 - 33)	77	11 b (3 - 19)	62	0	-
2020	Water-treated	93 a (74 - 112)		29 a (24 - 34)		40 a (23 - 57)	
	Pyraclostrobin + fluxapyroxad	87 b (68 - 106)	7	27 ab (22 - 32)	8	51 a (35 - 68)	0
	Thiophanate-methyl	62 c (43 - 81)	33	18 b (13 - 23)	38	47 a (30 - 63)	0
2021	Water-treated	91 a (83 - 99)		98 ab (95 - 101)		83 a (63 - 104)	
	Pyraclostrobin + fluxapyroxad	73 b (65 - 81)	20	100 a (97 - 103)	0	60 a (43 - 77)	28
	Thiophanate-methyl	33 c (25 - 41)	64	93 b (90 - 96)	5	24 b (8 - 41)	71

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687 **Table 3** Weather (total precipitation, maximum temperature, minimum temperature, and relative
688 humidity) on the day of pathogen inoculation, during the week following inoculation, and during
689 the entire incubation period (i.e., between inoculation treatment and collection of spurs for
690 pathogen detection), in the Prosser, Washington vineyard.

Climate conditions	2019	2020	2021
Day of inoculation			
Total precipitation	0 mm	0 mm	0 mm
Daily maximum temperature	19.5°C	12.9°C	13.6°C
Daily minimum temperature	9.6°C	-4.5°C	1.9°C
Daily relative humidity	44.5%	59.4%	56.2%
Week following inoculation			
Total precipitation	17.5 mm	0 mm	0 mm
Average maximum temperature	16.2°C	16.2°C	16.0°C
Average minimum temperature	7.1°C	-0.7°C	0.4°C
Average relative humidity	69.2%	50.1%	48.1%
During incubation period			
Total precipitation	37.6 mm	4.1 mm	0 mm
Average maximum temperature	21.1°C	17.9°C	19.5°C
Average minimum temperature	7.4°C	3°C	3.4°C
Average relative humidity	53.7%	47.2%	39.1%

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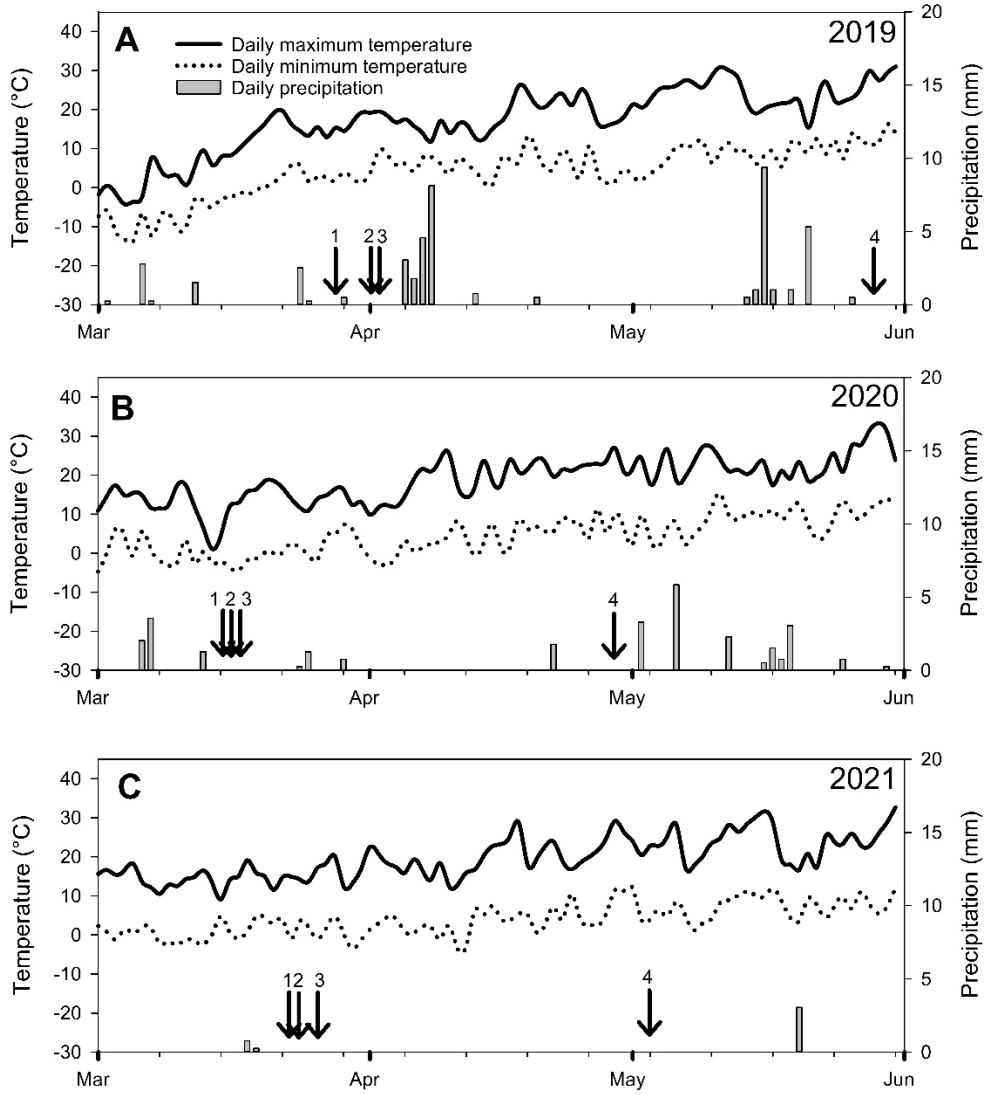

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695 **Figure 1** Daily maximum temperature (solid line), minimum temperature (dotted line), and
696 precipitation (columns) from March 1 to June 1 of 2019 to 2021, in an experimental vineyard in
697 Prosser, Washington USA. Arrows in each panel denote the dates of the following steps: 1)
698 Pruning, 2) Application of fungicide treatments, 3) Application of inoculation treatments; and 4)
699 Collection of spurs for pathogen detection.

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Supplemental Table 1 Identity of *Cytospora* isolates from water-inoculated (control) spurs in the Prosser, Washington vineyard, in comparison to related *Cytospora* previously reported from grape.

					Internal Transcribed Spacer Region (ITS)				Elongation Factor (TEF)					
						BLASTn results of curated specimen				BLASTn results of curated specimen				
Species	Isolate	Origin	Amplification results for qPCR primers CtBTFF1 and CtBTFR1	Curated specimen identified from DNA sequence database BLASTn searches of ITS and TEF sequences	GenBank#	% Coverage	Number of gaps	% Identity	GenBank#	% Coverage	Number of gaps	% Identity	Species reported as pathogenic on grape	Reference citing species' pathogenicity
Cytospora chrysosperma	AD	Isolated from water-inoculated (control), water-treated spur in 2019	-	*Cytospora chrysosperma* CFCC 89619	OM976605	100	1	99.65	ON012572	100	5	94.96	Yes	Arsenious et al. (2015)
Cytospora parakantschavelii	AM	Isolated from water-inoculated (control), water-treated spur in 2019	+	*Cytospora parakantschavelii* MFLUCC 15-0857	OM976607	97	2	99.64	ON012574	100	1	98.55	Unknown	NA
Cytospora parasitica	WA215	Isolated from water-inoculated (control), pyraclostrobin + fluxopyroxad-treated spur in 2020	+	*Cytospora parasitica* MFLUCC141055a	OM976611	100	0	100	ON012578	100	1	98.17	Unknown	NA
Cytospora parasitica	AG	Isolated from water-inoculated (control), water-treated spur in 2019	+	*Cytospora parasitica* MFLUCC141055a	OM976606	98	0	100	ON012573	100	1	98.53	Unknown	NA
Cytospora parasitica	CF	Isolated from water-inoculated (control), water-treated spur in 2019	+	*Cytospora parasitica* MFLUCC141055a	OM976608	98	0	100	ON012575	100	1	98.53	Unknown	NA
Cytospora parasitica	WA207	Isolated from water-inoculated (control), water-treated spur in 2020	+	*Cytospora parasitica* MFLUCC141055a	OM976609	100	0	100	ON012576	100	1	98.53	Unknown	NA
Cytospora species 1	Bent902	Isolated from previous survey of Washington vineyards	-	*Cytospora chrysosperma* CFCC 89619	OM976602	96	1	99.66	ON012569	100	2	94.60	Yes	Travadon et al.
Cytospora species 1	Bent903	Isolated from previous survey of Washington vineyards	+	*Cytospora chrysosperma* CFCC 89619	OM976603	96	1	99.66	ON012570	100	2	94.60	Yes	Travadon et al.
Cytospora species 2	WA209	Isolated from water-inoculated (control), water-treated spur in 2020	+	*Cytospora ulmicola* MFLUCC 18-1227	OM976610	100	1	99.33	ON012577	NA	NA	NA	Unknown	NA

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Cytospora ulmicola	WA310	Isolated from water-inoculated (control), pyraclostrobin + fluxopyroxad-treated spur in 2021	+	*Cytospora ulmicola* MFLUCC 18-1227	OM976614	100	0	99.82	ON012581	NA	NA	NA	Unknown	NA
Cytospora vinacea	CBS141585	Isolated from previous survey of New Hampshire vineyards	+	*Cytospora vinacea* Cyt5	KX256256	100	0	100	KX256277	100	0	100	Yes	Lawrence et al. (2017)
Cytospora viticola	Kern504	Isolated from previous survey of California vineyards	+	*Cytospora viticola* Cyt6	OM976604	72	0	99.76	ON012571	90	0	98.02	Yes	Lawrence et al. (2017)
Cytospora viticola	Bent401	Isolated from previous survey of Washington vineyards	+	*Cytospora viticola* Cyt6	OM976600	70	0	100	ON012567	79	0	99.60	Yes	Lawrence et al. (2017)
Cytospora viticola	Bent901	Isolated from previous survey of Washington vineyards	+	*Cytospora viticola* Cyt6	OM976601	73	0	99.76	ON012568	79	1	98.03	Yes	Lawrence et al. (2017)
Cytospora viticola	WA302	Isolated from water-inoculated (control), thiophanate-methyl-treated spur in 2021	+	*Cytospora viticola* Cyt6	OM976612	73	1	99.76	ON012579	98	0	98.70	Yes	Lawrence et al. (2017)
Cytospora viticola	WA305	Isolated from water-inoculated (control), water-treated spur in 2021	+	*Cytospora viticola* Cyt6	OM976613	73	0	100	ON012580	98	1	96.97	Yes	Lawrence et al. (2017)

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