Preventive Fungicidal Control of *Cytospora leucostoma* in Peach Orchards in Colorado

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Abstract

In western Colorado, *Cytospora leucostoma* is ubiquitous in peach orchards and has developed into a major limiting factor of peach production. The pathogen is unable to invade healthy intact phloem tissue of the tree, but instead, it requires a wound as a mode of entry. Bark injuries caused by cold and pruning in commercial orchard systems provide infection courts that, in suitable environment conditions, can lead to many successful fungal infections. Preventive fungicide control is an integral component of management in tree fruit production. Eighteen fungicides were tested at selected label dose rates for *C. leucostoma* control. All treatments were initially tested in vitro in fungicide-amended media dishes. Successful treatments were then tested under controlled conditions on detached peach branch segments. Effective

fungicides identified in the laboratory assays (thiophanate-methyl, captan, lime sulfur, and copper hydroxide) were further tested as spray applications in the field and as wound sealant applications in combination with latex paint and kaolin clay. Of the treatments evaluated, thiophanate-methyl, captan, 50% latex paint, thiophanate-methyl amended in 50% latex paint, captan amended in 50% latex paint, and lime sulfur were most effective in reducing *C. leucostoma* necrotic area. Copper hydroxide was ineffective in all field trials and in some instances, yielded larger necrotic areas than the nontreated positive control shoots.

Keywords: Cytospora canker, *Leucostoma persoonii*, stone fruit

The peach (*Prunus persica* [L.] Batsch) industry in western Colorado over the last 10 years has had an annual estimated value of \$17 to \$30 million (U.S. Department of Agriculture 2016). A survey conducted in western Colorado peach-producing regions, more specifically the Grand Valley, North Fork of the Gunnison River, and the Olathe region, found that 75% of trees in orchards were infected with Cytospora canker, with some orchards having incidence rates of 100%. Cytospora canker, caused by *Cytospora leucostoma* ([Pers.] Sacc.) (teleomorph *Leucostoma persoonii* Höhnelt, syn. *Valsa leucostoma* Höhnelt) (Adams et al. 2002; McNeill et al. 2012; Rossmann et al. 2015), is estimated to account for 15 to 20% of annual losses in peach production in Colorado (Pokharel and Larsen 2009), with an estimated \$3,375,000 loss in crop value per year (U.S. Department of Agriculture 2016). Along with continual pathogen pressure, environmental factors unique to the high elevation of western Colorado, such as low winter temperatures, late spring freezes, diminishing water supplies, and alkaline soils, have only exacerbated the problem, weakening trees and allowing for increased pathogen aggression.

In stone fruit production, manipulating tree architecture by pruning and training is essential for improved light interception, canopy aeration, crop load management, and optimized fruit quality (Minas et al. 2018). The biology of *C. leucostoma* fits very well with the required pruning management practices, because the fungus is considered a “weak parasite” unable to invade healthy, intact bark, therefore

requiring a wound as a mode of entry (Biggs 1989). Tree susceptibility is highest during pruning periods, because wounded tissue is exposed to pathogens. In western Colorado, growers typically prune during dormancy after leaf drop through prior bloom (mid-November through mid-March), with the vast majority of growers pruning late to adjust for any midwinter floral bud cold damage. Summer pruning is also a standard practice for commercial peach production, especially in high-density plantings, to control tree vigor, increase sunlight penetration within canopies, increase floral bud initiation, and improve fruit coloration and quality (Minas et al. 2018). Several studies have shown that *C. leucostoma* is able to release conidia in all seasons, yielding trees susceptible to infections all year round (Bertrand and English 1976; Grove and Biggs 2006; Regner et al. 1990). Cytospora canker is difficult to control after an infection occurs, because the fungus disrupts the conductivity of the xylem (Hampson and Sinclair 1973). Cankers forming low, close to, or on the tree trunk are very difficult to remove without permanently damaging the tree. Preventive measures for disease-free trees are critical for pathogen control, and cultural management practices should include fungicidal treatments of pruning sites and other injuries. Furthermore, diseases, such as brown rot, should be managed, because twig infections by *Monilinia fructicola* may also be invaded by *C. leucostoma* (Biggs 1989).

Fungicides have yet to be registered for Cytospora canker, and previous studies examining fungicide efficacy have yielded conflicting results regarding pathogen control (Biggs and Grove 2005). The fungicide captan was found ineffective as a canopy spray application during spring (Northover 1976), but it was effective in inhibiting fungal growth on amended media and excised branches (Biggs et al. 1994; Dhanvantari 1968). Lime sulfur was also found to be ineffective in canopy sprays in previous studies (Northover 1976). Benomyl and captafol were found to be effective treatments, but they are no longer available for application in the United States because of their teratogenic and carcinogenic effects (Kavlock et al. 1982; Patnaik 2004). In vitro inhibition of *Cytospora* spp. has been studied for a wide array of fungicides in the past (Dhanvantari 1968; French and Helton 1962; Rohrbach and Helton 1965). The main objective of this research was to evaluate commercially available fungicides, including novel treatments, for Colorado peach growers for Cytospora

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canker control and test effective fungicides as wound-sealing treatments to develop a preventive strategy for disease management.

Materials and Methods

Isolation of *C. leucostoma*. Cankers were collected from symptomatic peach trees with *C. leucostoma* infections on the western slope of Colorado (Grand Junction, CO) and transported to Colorado State University (Fort Collins, CO) for isolations. Canker margins were cut into pieces <1 cm³ and placed on half-strength potato dextrose agar (PDA; 4.0 g potato starch [BD Difco; Franklin Lakes, NJ], 19.5 g dextrose [BD Difco], and 7.5 g agar per liter [BD Difco]). Plates were incubated at 25°C for 5 days, and fungal colonies were then transferred onto Leonian's agar (LA; 6.25 g peptone [BD Difco], 6.25 g maltose [Oxchem; Monmouth Junction, NJ], 6.25 g malt extract [BD Bacto; Franklin Lakes, NJ], 1.25 g KH₂PO₄ [Fisher Scientific; Hampton, NH], 0.625 g MgSO₄ · 7H₂O [Millipore Sigma; St. Louis, MO], and 20.0 g agar per liter [BD Difco]) (Booth 1971). Isolates were then hyphal tipped onto LA plates. Spore suspensions were prepared from eight isolates (CP5.1, CP11.3, CP56.2, CP64.1, CP84.1, CP92, CP108.1, and CP124), and these isolates were used in the fungicide studies. All isolates were used for testing fungicide fungal growth inhibition. Isolates CP5.1 and CP92 produced the largest lesions in inoculation trials and were used for testing fungicidal efficacy on detached branch segments. Lastly, isolate CP5.1 was used for inoculations in the field efficacy trials, because it produced a high amount of pycnidia and spores.

In vitro toxicity of fungicides. Nineteen conventional fungicides or fungicides certified by the Organic Materials Review Institute (OMRI) were added to autoclaved, cooled LA medium at label mid-rates (Table 1). Four-millimeter-diameter agar plugs taken from the edge of hyphal-tipped 3-day-old cultures were inoculated in the center of fungicide-amended plates and incubated at 25°C for 7 days. Colony diameters of each sample were measured, and fungal growth area (πr²) was determined. Results are presented as relative growth inhibition of fungal colonies: relative growth inhibition = control – treatment × 100/control. Each fungicide was evaluated with three replications per isolate, and the assay was conducted twice.

Fungicidal efficacy studies using detached branch segments. The most active fungicides from the in vitro assays were selected for additional investigation. Peach branches of similar diameter (~1.5 cm) were collected in April and cut into 20-cm-long segments. Cut ends were sealed with hot polymer melt adhesive (Hot Glue

Stick; Gorilla, Cincinnati, OH) to prevent desiccation, and segments were sterilized with 10% sodium hypochlorite (Clorox, Oakland, CA) for 5 min and rinsed with sterile distilled water. Segments were then wounded at a depth only to remove the outer layer of bark with a 4-mm core borer (two wounds per segment). Segments were then

Table 2. Field studies conducted at the Colorado State University's Western Colorado Research Center–Orchard Mesa (Grand Junction, CO)^x

Season	Experiment group (wound type, spray type, and inoculation strategy) ^y	Sample size (n) ^z
Summer 2016	Pruning, standard fungicide spray, 1 day postfungicide	180
	Razor, standard fungicide spray, 1 day postfungicide	180
	Pruning, standard fungicide spray, 7 days postfungicide	180
	Razor, standard fungicide spray, 7 days postfungicide	180
	Pruning, wound-sealing spray, 1 day postfungicide	210
	Razor, wound-sealing spray, 1 day postfungicide	210
	Pruning, wound-sealing spray, 7 days postfungicide	210
	Razor, wound-sealing spray, 7 days postfungicide	210
Fall 2016	Pruning, standard fungicide spray, 1 day postfungicide	120
	Razor, standard fungicide spray, 1 day postfungicide	120
	Pruning, wound-sealing spray, 1 day postfungicide	140
	Razor, wound-sealing spray, 1 day postfungicide	140
Spring 2017	Pruning, standard fungicide spray, 1 day postfungicide	120
	Razor, standard fungicide spray, 1 day postfungicide	120
	Pruning, wound-sealing spray, 1 day postfungicide	140
	Razor, wound-sealing spray, 1 day postfungicide	140

^x Various trials were conducted in different seasons.

^y Experimental group refers to the experimental trial conducted. Two wound types were investigated: pruning and razor wounds. Two spray types were investigated: standard fungicide sprays without 50% latex or kaolin clay and wound-sealing fungicide sprays amended in 50% latex or kaolin clay. Inoculation strategy refers to the time point of inoculation: 1 or 7 days post-fungicide treatment.

^z Sample size varies because of the number of treatments used. Sprays embedded with paints and kaolin clay coating (wound-sealing sprays) contained more treatments, because a latex control and a kaolin clay control were also used.

Table 1. Fungicide treatments used at label midrates to test in vitro fungal growth inhibition of *Cytospora leucostoma*

Common name	Product name	Recommended rate (per 757 liters [200 gallons])	Rate used	Registration use
Sulfur	Microthiol Disperss	4.5–9 kg	6.8 kg	Conventional
Penthiopyrad	Fontelis	400–560 g	480 g	Conventional
Cyflufenamid	Torino	90 g	90 g	Conventional
Pyraclostrobin and boscalid	Pristine	300–410 g	350 g	Conventional
Fosetyl-Al	Aliette	4.5 kg	4.5 kg	Conventional
Thiophanate-methyl	Topsin-M	450–680 g	570 g	Conventional
Benomyl	Benlate-WP	680–910 g	790 g	Conventional
Captan	Captan	2.8–3.8 liters	3.3 liters	Conventional
Difenoconazole and cyprodinil	Inspire Super	450–560 g	510 g	Conventional
Zinc dimethyldithiocarbamate	Ziram	1.4–2.4 kg	1.8 kg	Conventional
Latex	Latex Paint	300–410 g	340 g	Conventional
CaCl	CaCl	1.4 kg	1.4 kg	OMRI ^z
Neem oil	Neem Oil	2.8 liters	2.8 liters	OMRI
Potassium salts	M-pede	7.5–15 liters	11.3 liters	OMRI
Potassium bicarbonate	Kaligreen	1.1–1.3 kg	1.2 kg	OMRI
<i>Bacillus subtilis</i>	Serenade	400–560 g	480 g	OMRI
Copper hydroxide	NuCop-WP	3.6–9.1 kg	4.5 kg	OMRI
Copper hydroxide and copper oxychloride	Badge X2	1.6–2.4 kg	1.9 kg	OMRI
ZnSO ₄	ZnSO ₄	1.8–2.7 kg	2.2 kg	OMRI
29% Calcium polysulfide	BSP Lime Sulfur	75–91 liters	83 liters	OMRI
Kaolin clay	Surround WP	4.5 kg	4.5 kg	OMRI

^z Treatments labeled Organic Materials Review Institute (OMRI) are certified for use in organic production systems by the U.S. Department of Agriculture National Organic Program.

dipped into fungicide solutions for 5 min. Six conventional fungicides were included: fosetyl-Al (Aliette; Bayer Crop Science, Triangle Park, NC), thiophanate-methyl (Topsin-M; United Phosphorus Inc., King of Prussia, PA), benomyl (Benlate-WP; DuPont, Wilmington, DE), captan (captan; Loveland Products, Greeley, CO), zinc dimethyldithiocarbamate (Ziram; United Phosphorus Inc., King of Prussia, PA), and difenoconazole (Inspire Super; Syngenta, Greensboro, NC). Eight OMRI-certified fungicides were included: clarified hydrophobic extract of neem oil (Neem Oil; Bonide, Oriskany, NY), potassium salts (M-Pede; Gowan, Yuma, AZ), potassium bicarbonate (Kaligreen; Toagosei; Nishi Shimbashi Minato-KU, Tokyo, Japan), *Bacillus subtilis* (Serenade; AgraQuest, Davis, CA), copper hydroxide (Nucop-WP; Agri Star, Ankeny, IA), copper oxychloride and copper hydroxide (Badge X2; Isagro, Morrisville, NC), zinc sulfate (ZnSO₄; Maximo 360, Monterrey, Mexico), and lime sulfur (BSP lime sulfur; Ag Formulators, Fresno, CA). All fungicidal solutions were prepared at midrate label specifications (Table 1). Two controls were included: a wounded negative control without inoculation or treatment and a positive control that was inoculated with *C. leucostoma* but not treated.

Cytospora isolates CP5.1 and CP92 were used for inoculations. Two inoculation strategies were followed; branch segments were either inoculated the same day after fungicide treatments were applied

or inoculated 7 days postfungicide treatment (DPFT). For the delayed inoculations, branch segments were placed inside clear 1-gallon plastic bags, stored in the greenhouse, and rotated daily to ensure even exposure to solar radiation. The inoculation technique was identical for both strategies. Hyphal agar disks (4 mm in diameter) taken from 3-day-old cultures were placed on each of the two branch segment wounds ~10 cm apart from each other and wrapped with Parafilm (American National Can, Chicago, IL). Branch segments were then placed inside 1-gallon bags with two 6 × 6 × 13-cm moistened sponges (not in contact with branches) for moisture retention and stored in the dark at 28°C. This temperature was chosen from previous trials highlighting that it was optimal for colony growth. Lesion lengths were measured 8 days postinoculation, and reisolations were conducted to confirm Koch's postulates. Host tissue wound reaction lesions were subtracted from all measurements. Eighty-millimeter length was the maximum length permitted based on branch size.

Five branch segments were used for each fungicide treatment for each inoculation strategy and isolate. Thus, with two isolates used, 16 treatments, two inoculation strategies, and five replications, a total of 320 branch segments were used. The experiment was repeated twice.

Fungicidal efficacy trials in the field. Studies were completed in an experimental orchard at the Western Colorado Research

Fig. 1. In vitro toxicity of selected conventional and Organic Materials Review Institute-approved fungicides to the growth of *Cytospora leucostoma* after 7 days of incubation at 25°C. Results are presented as growth inhibition percentages. The positive control consists of growth on nutrient agar (Leonian's agar). Columns labeled with different letters are statistically different ($P < 0.05$).

Center–Orchard Mesa in Grand Junction, Colorado in the summer (June) and fall (November) of 2016 and spring (March) of 2017 to simulate grower standard dormant and summer pruning timing. The orchard was planted in the spring of 2013 with cultivar Cresthaven peach scions grafted on cultivar Viking rootstocks and irrigated by microsprinklers. Before fungicide applications and inoculations, 1-year old tree shoots were wounded. Two wound types were used: one to simulate pruning wounds and one to simulate winter freeze damage cracking wounds. Pruning cuts were made with a hand-pruning shear at 45° to facilitate precipitation runoff of the wound. To simulate winter freeze cracking of bark tissues, longitudinal cuts were made with a generic box cutter $\sim 35 \pm 1$ mm long, 1 mm wide, and 2 mm deep. The four most effective treatments from the laboratory trials were selected for application on wounds. These treatments included thiophanate-methyl, captan, lime sulfur, and copper hydroxide (Table 1). Furthermore, for the development of a preventive strategy against *C. leucostoma*, conventional fungicides were suspended in a 50% white latex paint/water mixture (latex paint; Rust-oleum, Vernon Hills, IL). Similarly, OMRI-approved fungicides were suspended in a midrate application of a kaolin clay coat mixture that is registered for organic production systems (Surround WP; Engelhard, Iselin, NJ). Thus, for specific field trials, fungicidal sprays were either applied alone at their label midrates (defined as “standard fungicide sprays” throughout the text) or applied in combination with a 50% white latex paint/water mixture or kaolin clay (defined as “wound-sealing sprays” throughout the text). Treatments were randomized to minimize directional effects of solar

radiation and temperature. Applications were done the day of wounding at the label midrate (Table 2) using hand-spray bottles and sprayed until runoff or ~ 3 ml per branch.

Inoculum was prepared from 2-month-old fungal cultures with well-formed pycnidia. Each plate was immersed in 20 to 40 ml distilled water for 3 to 5 min with slight agitation. Pycnidia were cracked open with sterilized tweezers, and the spore suspension was adjusted to 10^5 spores per 1 ml using a hemocytometer. Mycelial and empty pycnidia debris was filtered out from the spore suspension with a cheesecloth filter. One hundred microliters of spore suspension was then applied to each wound individually using a pipette. Following this application, the wounds were wrapped in Parafilm.

Two inoculation strategies were followed for the 2016 summer trials; tree shoots were either inoculated 1 or 7 DPFT. However, in the 2016 fall trials and the 2017 spring trials, inoculations were only done 1 DPFT (Table 2). Shoot infections were allowed to develop for 90 days in the spring trials, 60 days in the summer trials, and 150 days in the fall trials because of different pathogen growth rates through the different seasons of the year. Pruning cut necrotic areas were measured using a digital caliper, and volume of the decayed tissue was calculated based on branch diameter and necrotic area length. For the razor wounds, the decay area was calculated based on the length and width of the necrotic area. Wound reaction lesions of the host were subtracted from all measurements. Koch’s postulates were satisfied by the plating of symptomatic plant tissue on one-half PDA media and confirming *C. leucostoma* presence.

A randomized complete block design was created for each season, wound type, and inoculation time point. For the 2016 summer trials,

Fig. 2. Fungicide toxicity and reduced growth of *Cytospora leucostoma* in response to selected conventional and Organic Materials Review Institute-approved fungicides. Inoculations were made on detached peach branch segments and incubated at 28°C for 8 days after wounding and fungicide applications. The positive control included *C. leucostoma* inoculations with no fungicide application, and the negative control consisted of wounding with no fungicide application and no inoculation. Columns labeled with different letters are statistically different ($P < 0.05$).

six blocks (trees) were used for each experimental group. For the 2016 fall and 2017 spring trials, four blocks (trees) were used for each experimental group. Each block contained all treatments. Within each tree, each fungicide treatment was applied to five 1-year-old shoot replications.

Statistical analysis. RStudio was used for statistical analyses, and packages used included lme4, lmerTest, pbrTest, and lsmeans (Bates et al. 2015; Halekoh and Højsgaard 2014; Kuznetsova et al. 2016; Lenth 2016; R Core Team 2017). Summary statistics and graphical depictions were created using the plyr package and the ggplot2 package (Wickham 2009, 2011). A mixed model was used for the analysis of each experiment. For the in vitro trials, a continuous response variable of fungal growth area (millimeters squared) with a fixed fungicide treatment predictor variable and two random effect predictor variables of isolate and run were assigned in the mixed model. Triplicates within run and treatment were averaged by unique isolate and not across isolates. A likelihood ratio test was conducted on the random effect variable (isolate) of the mixed model to evaluate inhibitory differences among isolates used. For the detached branch segment trials, the continuous response variable for both models included lesion length (millimeters), with a fixed predictor variable composed of 16 treatment levels, and two random effect variables, including isolate and run. To correct for assumptions of normality and equal variances, the data from the same-day inoculation time point were log transformed. A likelihood ratio test was conducted on the random effect variable (isolate) of the mixed model to evaluate inhibitory differences among isolates used. For the field trials, a

mixed model was built for each experimental group (wound type, spray type, and inoculation strategy) (Table 2). The model was based on a randomized complete block design. The continuous response variable for pruning wounds equaled lesion volume (millimeters cubed), whereas the continuous response variable for razor wounds equaled lesion area (millimeters squared). The mixed model predictor variables consisted of fixed fungicide treatment predictor variables and a random effect blocking variable. A linear model analysis of variance was run on all mixed models followed by Tukey adjusted pairwise comparisons ($\alpha = 0.05$; Tukey's honest significant difference-adjusted P values are $P = 0.05$). Additionally, multicomparison mixed models were used to evaluate chemical efficacy by each experimental group (wound type, spray type, and inoculation strategy) (Table 2) among seasons to evaluate seasonal effects (Supplementary Table S1). If appropriate, additional analyses were conducted to examine differences among chemical treatments across all seasons to evaluate fungicide efficacy (Supplementary Table S2).

Results

In vitro toxicity of fungicides. Effective in vitro fungicide treatments for the dose rates evaluated (Table 1) were identified through *C. leucostoma* colony growth inhibition. After 7 days of growth, fosetyl-Al, thiophanate-methyl, benomyl, captan, and difenoconazole were all found to significantly inhibit colony area expansion compared with the control ($P < 0.0001$) (Fig. 1). The OMRI-certified fungicides, including potassium salts, potassium bicarbonate, *B. subtilis*, copper hydroxide, copper oxychloride/copper

Fig. 3. Fungicide toxicity and reduced growth of *Cytospora leucostoma* in response to selected conventional and Organic Materials Review Institute-approved fungicides 7 days postfungicide treatment (DPFT). Inoculations were made on detached peach branch segments 7 DPFT and incubated at 28°C for 8 days. The positive control included *C. leucostoma* inoculation with no fungicide treatment, and the negative control consisted of wounding with no fungicide treatment and no inoculation. Columns labeled with different letters are statistically different ($P < 0.05$).

hydroxide, ZnSO₄, and lime sulfur, were also effective ($P < 0.0001$) (Fig. 1). The conventional fungicides pyraclostrobin and boscalid, cyflufenamid, penthiopyrad, and sulfur also showed inhibition activity ($P < 0.0001$); however, it was significantly lower than that in the rest of the conventional fungicides tested (Fig. 1). Calcium chloride and neem oil were ineffective in colony growth inhibition ($P = 0.9823$ and $P = 0.9910$, respectively) (Fig. 1). A likelihood ratio test analysis of the inhibitory differences between the random effect factor (isolate) yielded no differences among the six isolates tested ($P = 0.4$).

Fungicidal efficacy studies using detached branch segments.

For the same-day inoculations (Fig. 2), thiophanate-methyl, benomyl, and captan were significantly different than the positive control, yielding smaller lesion lengths ($P < 0.0001$), and they were similar in size to the negative control ($P > 0.9$) (Fig. 2). Copper hydroxide and lime sulfur were also significantly different than the positive control ($P < 0.0001$) (Fig. 2), and they were similar in size to the negative control ($P = 0.5153$ and $P = 0.5524$, respectively) (Fig. 2). Zinc dimethyldithiocarbamate was significantly different than the positive control ($P < 0.0001$), indicating pathogen inhibition, but it was also significantly different than the negative control ($P = 0.0003$), suggesting that control activity was not equal to a wounded but noninoculated branch. All other treatments (fosetyl-Al, difenoconazole, neem oil, potassium salts, potassium bicarbonate, *B. subtilis*, copper oxychloride/copper hydroxide, and ZnSO₄) were ineffective compared with the positive control ($P > 0.90$, $P = 0.3343$, $P = 0.7466$, $P = 0.3819$, $P = 0.4888$, $P = 0.4652$, $P = 0.9271$, and $P > 0.90$, respectively) (Fig. 2). An analysis of the random effect variable (isolate) yielded inhibitory differences between the two isolates used for inoculations ($P = 0.00003$).

For inoculations made 7 DPFT (Fig. 3), thiophanate-methyl, benomyl, captan, and zinc dimethyldithiocarbamate were significantly different than the positive control ($P = 0.0059$, $P < 0.0001$, $P < 0.0001$, and $P = 0.0012$, respectively) (Fig. 3), and they were not significantly different than the negative control, suggesting that control

activity of these fungicides was similar to a wounded but noninoculated branch ($P = 0.4515$, $P > 0.90$, $P > 0.90$, and $P = 0.7221$, respectively) (Fig. 3). Copper hydroxide was also significantly different than the positive control ($P = 0.0001$) and was not significantly different compared with the negative control ($P = 0.9741$) (Fig. 3). All other treatments (fosetyl-Al, difenoconazole, neem oil, potassium salts, potassium bicarbonate, *B. subtilis*, copper oxychloride/copper hydroxide, and ZnSO₄) were ineffective in delayed inoculations, because no differences were detected between the fungicides and the positive control ($P > 0.95$) (Fig. 3). Lime sulfur was also found ineffective after 7 DPFT inoculations compared with the positive control (Fig. 3) ($P = 0.3253$). An analysis of the random effect variable (isolate) yielded inhibitory differences between the two isolates used for inoculations ($P = 0.0002$).

Fungicidal efficacy trials in the field. Four fungicide treatments (two effective conventional and two effective OMRI-certified treatments from the in vitro and detached branch segments assays) were chosen for field trials. When applied as standard isolated sprays on pruning wounds during the summer trial, captan was the only fungicide to have efficacy for inoculations 1 DPFT (Fig. 4A) ($P = 0.0085$). Thiophanate-methyl was not effective for inoculations made 1 DPFT during the summer on pruning wounds; however, average lesion volume was lower than the positive control and higher than the negative control (Fig. 4A) ($P = 0.2296$ and $P = 0.0359$, respectively). Lime sulfur and copper hydroxide were also found ineffective during the pruning wound summer trials (Fig. 4A) ($P = 0.4991$ and $P = 0.7724$, respectively). When 7 DPFT inoculations were made during the summer on pruning wounds, only thiophanate-methyl showed efficacy in comparison with the positive control (Fig. 5A) ($P = 0.0084$). Treatments with captan, lime sulfur, and copper hydroxide were not effective (Fig. 5A) ($P = 0.7329$, $P = 0.8845$, and $P > 0.95$, respectively).

For the summer trial, treatments were also investigated on razor wounds. Captan, thiophanate-methyl, and lime sulfur were not found to be effective in the inoculations 1 DPFT. The efficacy of captan

Fig. 4. Field trials (standard fungicide sprays): growth of *Cytospora leucostoma* inoculated (100 μ l of 1×10^5 spores per 1 ml) 1 day postfungicide treatment (DPFT). Inoculations were made on branches. **A, B, and C**, Pruning wounds in summer, fall, and spring, respectively. **D and E**, Razor wounds in summer and spring, respectively. The positive control consisted of growth on wounded branches with no fungicide treatment, and the negative control consisted of wounding with no *C. leucostoma* inoculation and no fungicide treatment. Columns labeled with different letters are statistically different ($P < 0.05$).

could not be confirmed or rejected, because no significant differences were found between captan and the positive or negative controls (Fig. 4D) ($P = 0.5228$ and $P = 0.5930$, respectively). Thiophanate-methyl was also ineffective (Fig. 4D) ($P = 0.7501$ and $P = 0.3825$, respectively). Similarly, lime sulfur was ineffective compared with the controls (Fig. 4D) ($P = 0.9101$ and $P = 0.2136$, respectively). Copper hydroxide yielded lesions significantly larger than the positive control (Fig. 4D) ($P = 0.0012$). When inoculations were made 7 DPFT on razor wounds during the summer, thiophanate-methyl, captan, and lime sulfur all showed efficacy compared with the positive control (Fig. 5C) ($P = 0.0167$, $P = 0.0006$, and $P = 0.016$, respectively). Copper hydroxide, however, was not effective, because lesion areas were similar to the positive control (Fig. 5C) ($P = 0.9686$).

Fungicidal efficacy was also investigated in the fall on pruning and razor wounds. Captan, lime sulfur, and copper hydroxide showed no efficacy on inoculations made 1 DPFT on pruning wounds compared with the positive control (Fig. 4B) ($P = 0.2100$, $P = 0.9545$, and $P > 0.95$, respectively). In contrast, thiophanate-methyl was effective on pruning wounds during the fall, and they had significantly smaller

lesion volumes compared with the positive control (Fig. 4B) ($P = 0.0193$).

Similar to the fall trial, captan, lime sulfur, and copper hydroxide were not effective in the spring trial after inoculations 1 DPFT on pruning wounds compared with the positive control (Fig. 4C) ($P = 0.2170$, $P = 0.0694$, and $P = 0.9738$, respectively). However, thiophanate-methyl did inhibit pathogen growth, because lesion volumes were significantly smaller than the positive control and did not differ significantly from the negative control (Fig. 4C) ($P = 0.0068$ and $P = 0.9235$, respectively). When fungicide treatments were tested on razor wounds in the spring, none of the fungicides were effective. However, as in the summer trials, copper hydroxide yielded lesions significantly larger than those of the positive control (Fig. 4E) ($P = 0.0023$). Because of limited successful infections, no fungicides were found effective during the fall on razor wounds.

Fungicidal efficacy trials in the field (amendments in latex paint and kaolin clay). Efficacy of paints amended with fungicides was also tested as an alternative to standard fungicide sprays. During the summer field trial inoculations 1 DPFT on pruning wounds,

Fig. 5. Field trials: growth of *Cytospora leucostoma* inoculated ($100 \mu\text{l}$ of 1×10^5 spores per 1 ml) 7 days postfungicide treatment (DPFT). Inoculations were made on branches. **A.** Pruning wounds in summer: chemical sprays. **B.** Pruning wounds in summer: wound-sealing sprays. **C.** Razor wounds in summer: chemical sprays. **D.** Razor wounds in summer: wound-sealing sprays. The positive control consisted of growth on wounded branches with no fungicide treatment, and the negative control consisted of wounding with no *C. leucostoma* inoculation and no fungicide treatment. Columns labeled with different letters are statistically different ($P < 0.05$).

conventional fungicides combined with 50% latex paint were all effective in lesion inhibition compared with the positive control (Fig. 6A) ($P < 0.0001$). Latex paint alone was equally effective compared with latex paint combined with thiophanate-methyl or captan ($P < 0.0001$). Lime sulfur combined with kaolin clay was also effective ($P = 0.0005$), although it was not as effective as the latex paint combination treatments (Fig. 6A). When inoculations were made 7 DPFT during the summer trials, all fungicide treatments combined with latex paint were effective (Fig. 5, B and D) ($P < 0.0001$). Inoculations made 7 DPFT on lime sulfur combined with kaolin clay did not show activity on pruning wounds (Fig. 5B) ($P = 0.4079$). When latex paint and kaolin clay-amended fungicide treatments were tested on razor wounds during the summer, no significant differences were observed (Fig. 6D). Copper hydroxide in combination with kaolin clay had significantly larger lesions than the positive control (Fig. 6D) ($P = 0.0005$). Thiophanate-methyl and 50% latex, captan and 50% latex, and 50% latex alone were all effective and yielded significantly reduced lesion sizes compared with the positive control (Fig. 5D) ($P = 0.0095$, $P = 0.033$, and $P = 0.001$, respectively) during the summer on inoculations occurring 7 DPFT on razor wounds. Inoculations made 7 DPFT on lime sulfur combined with kaolin clay exhibited efficacy on razor wounds during the same season and inoculation time (7 DPFT) (Fig. 5D) ($P = 0.0058$). Kaolin clay applied alone or with copper hydroxide was ineffective in all trials (Fig. 6).

During the fall 1 DPFT trials, none of the treatments were significantly different than the positive control, although a trend for reduction in lesion volume was found for thiophanate-methyl in combination with 50% latex (Fig. 6B). In the spring trials, thiophanate-methyl and captan in combination with 50% latex paint were effective in reducing lesion sizes in comparison with the positive control (Fig. 6C) ($P = 0.0003$ and $P = 0.0182$, respectively). Because of limited successful infections, no fungicides were found effective during the fall on razor wounds. Similarly, during the summer razor wound trials, no fungicidal efficacy was shown (Fig. 6D).

In the spring trials, however, all fungicide treatments were effective on razor wounds compared with the positive control, with the exception of copper hydroxide combined with kaolin clay (Fig. 6E).

Results examining the effect of season on chemical group yielded few significant differences, whereas results examining the most effective chemicals across seasons yielded various significant differences. The only treatment that was significantly different across seasons was in the application of latex on pruned wounds ($P = 0.0020$). The most effective chemicals across all seasons included thiophanate-methyl and captan alone and in combination with latex. Similarly, lime sulfur was effective across all seasons when applied as a standard chemical spray on pruning and razor wounds and when combined with kaolin clay on razor wounds.

Discussion

The efficacy of several fungicides for the control of *C. leucostoma* was investigated in controlled laboratory studies and field trials. All fungicide doses were selected from label midrates (Table 1); thus, results are representative of doses evaluated. Although no fungicides were consistently effective in all evaluated seasons during field trials, several showed promise. Of the treatments tested over the three seasons, thiophanate-methyl, captan, and lime sulfur were the most effective fungicides when applied alone at their label midrates. Thiophanate-methyl and captan amended in 50% white latex paint and lime sulfur amended with kaolin clay were the most effective when used as preventive wound-sealing treatments.

Fosetyl-Al, thiophanate-methyl, benomyl, captan, difenoconazole, potassium salts, potassium bicarbonate, *B. subtilis*, copper hydroxide, copper oxychloride/copper hydroxide, ZnSO_4 , and lime sulfur all inhibited hyphal growth in amended agar plate assays. Captan and thiophanate-methyl were previously shown to inhibit growth of *C. leucostoma* on agar media (Biggs et al. 1994). This fungicide list was narrowed further, because only thiophanate-methyl, benomyl, captan, zinc dimethyldithiocarbamate, copper hydroxide, and lime sulfur were

Fig. 6. Field trials (wound-sealing sprays): growth of *Cytospora leucostoma* inoculated ($100 \mu\text{l}$ of 1×10^5 spores per 1 ml) 1 day postfungicide treatment (DPFT). Inoculations were made on branches. **A, B, and C**, Pruning wounds in summer, fall, and spring, respectively. **D and E**, Razor wounds in summer and spring, respectively. The positive control consisted of growth on wounded branches with no fungicide treatment. Columns labeled with different letters are statistically different ($P < 0.05$).

effective in the detached branch segment assays. Despite effectively reducing pathogen growth, efficacy from each fungicide was reduced compared with amended agar assays. This was likely caused by the woody tissue composition of a detached branch and fungicide spread on the branch compared with continuous contact within the fungicide-amended agar. Nonetheless, results were consistent with previous studies that reported that captan and thiophanate-methyl were both effective in preventing *C. leucostoma* growth on excised branches immersed in fungicide solutions (Biggs et al. 1994).

Field trials elucidated the efficacy of captan, thiophanate-methyl, and lime sulfur alone; captan and thiophanate-methyl mixed with white latex paint; and lime sulfur mixed with kaolin clay. Contrary to previous studies, which found that captan and lime sulfur were ineffective as canopy field applications (Northover 1976), our study showed evidence of efficacy for these two fungicides when applied in the summer on 1 DPFT pruning wounds and 7 DPFT on razor wounds, respectively. Methods for fungicide application varied significantly in previous studies, where 14 liters of a fungicide were applied to individual trees, and 1-year old shoots were evaluated for disease incidence without pathogen inoculations. Lack of fungicide efficacy may have been because of infections already being present. Results of our wound inoculation treatments confirm earlier reports showing latex efficacy as a temporary sealant on wounded tree shoots (Biggs and Peterson 1990).

Efficacy of treatments varied depending on the season. This was also observed in earlier studies in Grand Junction, Colorado, where the greatest increase in canker surface area occurred between March and June compared with summer and fall (Jones and Luepschen 1971). In our study, inoculations made during March and June also yielded the quickest increase of lesion size; however, inoculations made during November yielded much slower lesion size growth, and thus, shoots were harvested 150 days postinoculation. Pathogen growth rates were consistent in this study with previous investigations conducted in western Colorado (Jones and Luepschen 1971).

Paint treatments to wounds were only effective during the summer and spring. This concurs with previous research suggesting that latex paint may only prevent infections for a short period of time (Biggs and Peterson 1990). The latex paint failed to provide long-term (5-month) protection in our fall/winter studies, likely because the latex cover cracked, revealing underlying susceptible tissues that could be invaded, similar to the observations by Biggs and Peterson (1990). Furthermore, thiophanate-methyl combined with 50% latex paint showed a lower average lesion volume compared with the positive control in the fall trial. Thus, latex paint may provide protection up to 3 months, and this protection may be extended with the use of amended fungicides; this can be inferred from seasonal differences when comparing latex paint group across seasons.

In our study, copper hydroxide was found to be consistently ineffective, and in some cases, it yielded larger lesion sizes than the positive control. Previous studies also indicated that copper lime Bordeaux mixtures are ineffective and phytotoxic to peach trees, increasing the severity of *Cytospora* canker (Northover 1976), with phytotoxicity of copper hydroxide varying depending on tree variety (Lalancette and McFarland 2007). Our results confirm copper phytotoxicity and suggest that lesion sizes may be larger because of phytotoxic effects.

This study presents fungicidal efficacy on pruning and razor wounds. Razor wounds were selected to mimic cold damage that causes phloem tissue cracking and splitting, a common wound that results in *Cytospora* cankers. Fungicide performance in razor wound studies was inconsistent, perhaps because of inconsistency in wounding depths that may have impacted the ability of fungicides to uniformly penetrate the wound, the healing of the wounds, or the ability of the pathogen to infect. When fungicide treatments were tested on razor wounds during the fall, no significant differences were found owing to reduced branch infections in the positive control; thus, the data are not presented.

The larger pruning wounds increased exposed tissue areas; they healed more slowly and were more consistently infected by *C. leucostoma*. Other limitations of our studies included the difficulty in

differentiating between discoloration from the plant tissue oxidation, host responses, and fungal infection. We also isolated for *C. leucostoma* in the negative controls to confirm infections. Of the 12 large lesions isolated, 92% were confirmed as infected with *C. leucostoma*. Negative controls were excluded from the wound-sealing paint trials because of the high incidence of infections by natural field inoculum.

Conclusion

In conclusion, this research provides practical information for growers of western Colorado. We confirmed that field inoculum levels in western Colorado were high enough to cause infections on non-treated negative controls. Thus, treating tree tissue close to injury time is highly recommended. Given the results of the seasonal field trials that simulated standard commercial pruning and canopy management practices, shoots can be treated with thiophanate-methyl alone, captan alone, lime sulfur alone, or thiophanate-methyl or captan mixed with 50% latex paint. For organic production systems, lime sulfur and lime sulfur in combination with kaolin clay can be applied to wounded shoots. Given the lack of efficacy and the high risk of phytotoxicity, use of copper hydroxide for *C. leucostoma* control is not recommended. Considering the high-risk category for resistance development to thiophanate-methyl (FRAC Code List 2018), fungicides should be used in rotation to optimize preventive treatments. Different sealant products or sealant concentrations should be evaluated in future studies.

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