DIAGNOSIS, ETIOLOGY, EPIDEMIOLOGY, AND MANAGEMENT OF CANKER DISEASES IN DRIED PLUMS

Themis J. Michailides, Yong Luo, Franz Niederholzer, David P. Morgan, Dan Felts, Ryan Puckett, Michael Luna

and

Richard Buchner, Elizabeth Fichtner, and Daniella Lightle

OBJECTIVES

1) To develop a molecular approach for the efficient and accurate diagnosis of canker disease, to quantify inoculum densities, and infection level in shoots.

2) To determine susceptibility of pruning wounds, the year-round disease development, and the critical time period of pathogen infection.

3) To compare efficacy of potential fungicides against canker diseases.

4) To determine whether protecting sunburned tissues or tissues damaged mechanically (hail, storms, shaker, etc.) reduces infection and canker development.

INTRODUCTION

In the last several years, wood-cankers, wood decay, and branch killing of dried plum and other *Prunus* spp. have become a major concern to growers in California. Thus far, there are two types of problems in dried plums: a) the canker diseases caused by ascomycete fungi and b) wood decay caused by basidiomycete fungi. Frequently, one can find both types of diseases occurring in the same dried plum tree. Such infections can be very severe, leading to tree weakening, reduction in yields, killing of major scaffolds and in extreme cases the entire tree. We continued helping farm advisors, pest control advisers, and also growers by diagnosing canker disease problems and at the same time we advanced our knowledge on monitoring techniques of various canker pathogens. Over the years we received samples from Tulare, Madera, Fresno, Sutter, Yuba, Butte, Colusa, Glenn, and Tehama Counties. We have isolated from these samples *Cytospora leucostoma,* other *Cytospora* species, *Lasiodiplodia citricola, Neoscytalidium dimitiatum* (synonyms: *Nattrassia mangiferae* and *Hendersonula toruloidea*), *Botryosphaeria dothidea,* other *Botryosphaeria* and *Phomopsis* species, *Diplodia seriata, Paecilomyces variotii,* and *Fusarium* species. Furthermore, from trees with heart rot we isolated *Schizophyllum commune,* a *Phellinus* species, and a third basidiomycete fungus that may also play a role in heart rot attacking dried plum trees. However, our studies will

emphasize canker fungi and not wood decay fungi, unless we detect an interaction between the two groups of pathogens.

PROCEDURES

Objective 1. **To develop a molecular approach for the efficient and accurate diagnosis of canker disease, to quantify inoculum densities, and infection level in shoots.** In order to effectively identify the canker-causing pathogens for disease diagnosis and quantification of latent infection level, we developed molecular approaches by using real-time PCR (qPCR) to identify and distinguish 6 canker-causing fungal species, i.e. *Phomopsis* spp., *Botryosphaeria dothidea*, *Lasiodiplodia* spp., *Cytospora* spp., *Neofusicoccum* spp., and *Diplodia* spp. The genus-specific DNA primers for each of the 6 pathogens were designed, and specificity tests were performed to confirm the applicability of each primer pair to specifically target the corresponding pathogen. In this study, the primer pairs, LcBT-F2/LcBT-R2 and CtBTFF1/CtBTFR1, were used to target *Lasiodiplodia* spp. and *Cytospora* spp., respectively. A Real-time PCR system by using a SYBR method was developed to identify each pathogen. The standard curve for each of the two pathogens was developed by using a serial dilution of the template DNA and the corresponding primers. The standard curves were used to quantify the latent infection level in shoot samples.

To quantify the infection level of shoots using molecular procedures, we introduced the concept of molecular severity (MS): $MS= Log_{10}(P/H)$, where P is the pathogen DNA weight in femtogram (fg) which is calculated by using the equation of the standard curve for the corresponding pathogen, and H is the shoot weight in gram (g). Thus, by using this method, we were able to build up a system to quantitatively study the latent infection level of the pathogens causing canker diseases.

In order to confirm the applicability of the above method to quantify infection level, shoot inoculation was conducted in May 2015. Two isolates, 7F93 (*Lasiodiplodia citricola*) and 9D71 (a *Cytospora* species) were used. For each of the two pathogens, two-year old shoots were wounded by using a sterile cork borer, inoculated by spraying 2 ml of the pathogen's spore suspension (10^4 spore/ml), and covered with a plastic bag for 48 hours to create high humidity. About 8 to 10 inoculated shoots were randomly collected for each pathogen in July, 2015. Each shoot was cut into about 4-cm-long section in the margin between living and symptomatic tissues. Each piece was vertically split into two parts and processed separately as described below.

One part of a shoot sample were continuously cut into smaller pieces as about 0.5 cm long, surface sterilized in 1% chlorine (10% solution of a 5.25% NaOHCl commercial bleach) for 10 min, washed three times with sterile distilled water, and plated in a plate containing LA-PDA (acidified potato dextrose agar). The plates were incubated at room temperature for 7 to 10 days. The cultures were examined with a dissecting microscope for the typical pycnidia and the characteristic spores of each pathogen used in the inoculation experiments. A plate showing at least one piece with the typical cultural characteristics of the corresponding pathogen was counted as an infected shoot sample.

The second half of the shoot sample was cut continuously to smaller pieces as 0.2 cm long, and grinded with a blender for 1 min into fine small pieces for DNA extraction by using the protocols developed in our lab. The qPCR was applied to quantify infection level for each pathogen by using the corresponding DNA primers described above.

Objective 2. To determine susceptibility of pruning wounds, the year-round disease development, and the critical time period of pathogen infection. The study for this objective was performed in a dried plum orchard located at the University of California, Kearney Agricultural Research and Extension Center (UC-KARE). To determine susceptibility of pruning wounds, shoots were pruned during 18 to 21 February 2015 and inoculated 0, 3, 7, 14, and 30 days later. Infection of pruning wounds and infection, in general, was recorded in mid-April 2015. An isolate 7F93 each of *Lasiodiplodia citricola*, 9D71 of *Cytospora leucostoma*, and 3D75 of *Paecilomyces variotii* were used in the experiments. Two different methods, pruning and side wounding, were applied on different inoculation dates before inoculation (Table 1). For each inoculation, a 10^5 to 10^6 spores/ml (depending on spore availability) suspensions were used. In each inoculation date, 30 shoots with different ages were marked on each tree for each pathogen. For the pruning method, each marked shoot was pruned, inoculated with 2 ml of spore suspension, and covered with a plastic bag for 48 hours to create high humidity. For the wounding method, each marked shoot was wounded by using a sterile cork borer, inoculated by spraying 2 ml of spore suspension directly on the wound, and covered with a plastic bag for 48 hours to create high humidity. Since canker disease developed very slowly, the disease recording and sample process were conducted in early December of 2015.

For disease recording, the number of shoots showing typical canker symptoms or dead shoots from the 30 inoculated shoots for each inoculation date was recorded to calculate disease incidence. Various numbers (around 10) of dead shoots were cut off and processed with two methods. The sampled shoots were cut vertically into two pieces. The same methods described in the Objective 1 were used. Briefly, small fine pieces were cut from the one half shoot samples, cultured on LA-PDA medium under conditions as same as described in Objective 1 for at least 10 days. For the samples which did not show the typical cultural characteristics of the corresponding pathogen, another half piece of the samples were used to extract DNA and processed with qPCR system described above to determine the existence of the pathogen in the sampled tissues.

In order to determine the effects of the contaminated pruning shears on pathogen infection, we also conducted field experiments. On March 4 (for *Lasiodiplodia citricola* and *Paecilomyces variotii*), March 13 (for *Schizophyllum commune*) and April 29, 2015 (for *Cytospora leucostoma*), two trees for each pathogen were selected, and 30 shoots of each tree showing no canker symptoms were marked. Thus, a total of 60 shoots of different ages (from 1 to 4 years old) for each pathogen were used. For each *L. citricola*, *P. variotii,* and *C. leucostoma,* a spore suspension of 10^5 spores/ml was prepared. On the corresponding inoculation date, a pruning shear was dipped in the spore suspension for 5 seconds, and was used to prune each marked shoot sequentially. All the inoculated shoots were covered with plastic bags for 48 hours to create high humidity. Because *Schizophyllum* does not produce spores in culture, only mycelial

plugs were used in the inoculation experiments using this putative pathogen. The marked shoots were wounded with a sterile cork borer, placed with a piece of mycelial plug on the wound, and covered with parafilm for the whole season. The inoculated shoots were maintained on trees and disease recording was conducted at the end October 2015.

Table 1. Inoculation dates in 2015 and methods for the corresponding pathogens used in the Objective 2 of this study. The inoculation experiments were conducted in the dried plum orchards at University of California, Kearney Agricultural Research and Extension Center (KARE).

		Pathogen species			
Order	**Inoculation date**	***Lasiodiplodia citricola***	***Cytospora leucostoma***	***Paecilomyces variotii***	***Schizophyllum commune***
1	3/4/15	no wounding & prunning		no wounding & prunning	
2	3/13/15				wounding
3	4/8/15	prunning		prunning	
4	4/29/15		prunning		
5	5/11/15	prunning			
6	5/13/15			prunning	
7	6/5/15	prunning	prunning	prunning	
8	7/10/15	prunning		prunning	
9	7/20/15		prunning		
10	8/7/15	wounding	wounding	wounding	
11	9/9/15	wounding	wounding	wounding	
12	10/14/15	wounding	wounding	wounding	
13	11/18/15	wounding	wounding	wounding	

Objective 3. To compare efficacy of potential fungicides against canker diseases. We conducted a fungicide trial in a dried plum orchard located in Yuba County showing severe Cytospora canker disease. Six fungicides were used: Topsin, Quilt Xcell, VitiSeal, Pristine + Pentra Bark, tebuconazole, Pristine + VitiSeal, plus an untreated control. Regular pruning was conducted in this orchard on November 5, 2014 and fungicide treatments were conducted on November 6, 2014. A rate of 5g/L for each fungicide was used to paint on the pruning wounds. For each fungicide treatment, 10 pruned branches were used. The experiment was conducted again on November 12, just few hours before a rain. The fungicide treated wounds were maintained on trees for the whole season of 2015 and disease recording was conducted in early December of 2015 for canker incidence for each fungicide treatment.

Objective 4. To determine whether protecting sunburned tissues or tissues damaged mechanically (hail, storms, shaker, etc.) reduces infection and canker development. This year, we also conducted an experiment to determine whether the sunburn could affect the disease development. On August 8, 2015, some trees of the very south row of the orchard described above were selected. Two methods to promote sunburn of shoots were tried. One was to bend the shoots and tie them to a metal stick so that they are exposed to direct sunlight (Treatment A).

The second method was to wrap a portion of a shoot as 3 cm long with a piece of black plastic sheet and making sure that it is exposed to direct sunlight (Treatment B) (Figure 1). Five shoots were used for each of the two methods for each pathogen, and another 5 shoots without sunburn treatment were used as control. The inoculations were conducted on September 12, 2015 and the same inoculation methods used in the Objective #2 were applied for the corresponding pathogens as described above. The whole experiment was replicated twice and disease recorded in mid-November 2015.

Figure 1. Experimental design to study the effects of sunburn of shoot on canker disease development. Two methods to promote sunburn were used: placing shoots at special direction for two weeks to face on sunlight (left, Treatment A) and wrapping portion of shoots with a black plastic sheet for 2 weeks (right, Treatment B).

RESULTS, DISCUSSION, AND CONCLUSIONS

Objective 1. To develop a molecular approach for the efficient and accurate diagnosis of canker disease, to quantify inoculum densities, and infection level in shoots.
The primer pairs LcBT-F2/ LcBT-R2 (Figure 2C) and CtBTFF1/ CtBtFR1 (Figure 2D) were used to target *Lasiodiplodia* spp. and *Cytospora* spp., respectively. Figure 2 shows the gel electrophoresis demonstrating the specificities of the designed primers to the corresponding canker-causing pathogens. These two primer pairs were also used to test the protocols for quantification of latent infection level.

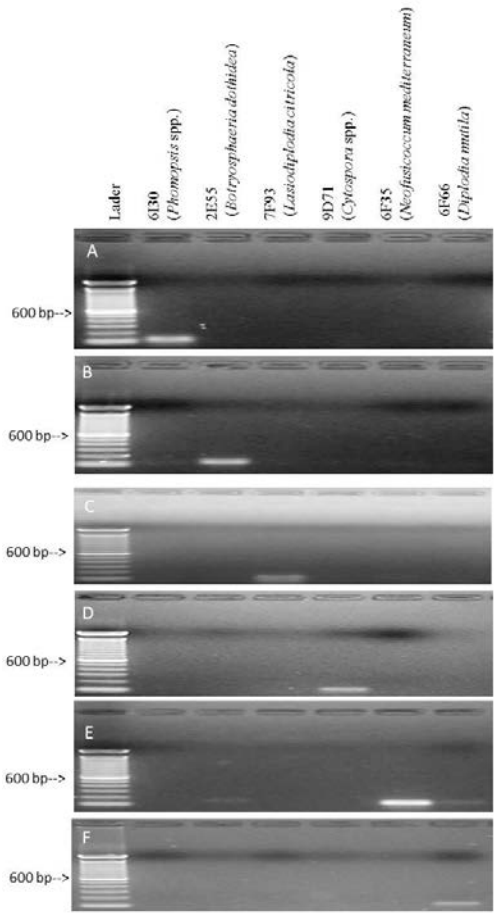

Figure 2. Gel electrophoresis showing the specificity of the designed primer pairs to target the corresponding canker-causing pathogen species. Six primer pairs were designed and the corresponding six representative isolates of these species were used in primer specificity tests. The primer pairs used in these electrophoreses are: PhBT-F1/ PhBT-R1(**A**), BdF/ BdR (**B**), LcBT-F2/ LcBT-R2 (**C**), CtBTFF1/ CtBtFR1 (**D**), NpBT-F2/ NpBT-R2 (**E**), and DpF/ DpR (**F**), respectively.

Application of qPCR to quantify shoot infection level. For the shoot samples collected from the dry plum orchard which were inoculated with *L. citricola* and *Cytospora* spp. the two species were isolated from the margins of the cankers in some of the inoculated shoots, using the traditional culturing method (Table 2). The incidences of *Lasiodiplodia citricola* and *Cytospora* spp. were 0.88 and 0.78, respectively. However, this method cannot provide information about the infection level by each species. The qPCR method revealed a 100% incidence for each of the two species from the same samples as processed with the culturing method, and average molecular severities were 6.72 and 8.84 for *L. citricola* and *Cytospora* spp., respectively (Table 2).

Table 2. Comparison in infection level of canker disease in dry plum shoots inoculated with *Lasiodiplodia citricola* and *Cytospora* spp. between traditional culturing method and the qPCR method developed in this study. The samples were collected from the inoculated shoots in a dried plum orchard at KARE for other research objectives.

Lasiodiplodia citricola Shoot code	Culturing method[z]	Molecular severity
L2-1	+	6.95
L2-2	+	7.03
L2-15	+	5.76
L3-1	+	7.37
L3-3	-	6.24
L3-7	+	6.50
L3-9	+	7.23
L3-10	+	6.66
Incidence	0.88	1.00
Mean Mol. Severity		6.72
S.D.		0.54

***Cytospora* spp.** Shoot code	Culturing method[z]	Molecular severity
C2-1	+	11.25
C2-13	-	9.33
C2-24	+	10.83
C2-26	-	10.01
C3-10	+	9.99
C3-12	+	10.67
C3-13	+	9.40
C3-18	+	7.73
C3-24	+	8.18
Incidence	0.78	1.00
Mean Mol. Severity		8.84
S.D.		2.97

z: +:the pathogen was isolated, -: the pathogen was not isolated.

The above results demonstrated the applicability and the potential usefulness of the designed primer pairs for disease diagnosis and the corresponding qPCR method for quantification of latent infection levels for these two pathogens. The applications of these protocols could be also extended to quantification of inoculum densities in dried plum orchards from air, irrigation water, rain and so forth.

Objective 2. To determine susceptibility of pruning wounds, the year-round disease development, and the critical time period of pathogen infection.

Susceptibility of pruning wounds. The results for *Cytospora leucostoma* inoculations have been summarized in Table 3.

Table 3. Susceptibility of pruning wounds to *Cytospora leucostoma* spore inoculum after inoculations at 0 to 30 days after pruning.

Treatment	Date of pruning	Date of inoculation	Length of canker (cm)*
0 days	2/21/14	2/21/14	15.4 abc
3 days	2/18/14	2/21/14	20.0 bc
7 days	2/20/14	2/27/14	13.8 abc
15 days	2/19/14	3/06/14	20.3 c
30 days	2/18/14	3/20/14	5.5 a
Non-inoculated	2/21/14		9.8 ab

* Shoots were recorded in April 2015.

Apparently, wounds are susceptible to infection for 15 days after pruning when conditions are favorable for disease development. Tissues killed in shoots pruned and inoculated 30 days after pruning did not differ significantly from those developed on pruned and non-inoculated shoots (Table 3). Because the majority of fungicide sprays can protect tissues for about 2 weeks, spraying an effective fungicide after

pruning should protect the pruning wounds during the time they are susceptible to infection by *Cytospora leucostoma.*

The results of sequential inoculations on pruning wounds from March to July of 2015 showed high incidence of death of shoots inoculated from April to June for all the three pathogens (Figure 3). The comparatively lower incidences of symptomatic shoots were observed on those inoculated in March and July, respectively (Figure 3). However, the percentages of dead shoots from which the corresponding pathogens were isolated varied during the season. Basically, the isolation rates of *L. citricola* and *Cytospora* spp. were higher than those of *P. variotii* from the samples inoculated in May of 2015. However, the isolation rates of *Cytospora* decreased on shoots inoculated from June to August of 2015 (Figure 3). *L. citricola* showed higher pathogenicity over the season than the other two species. The results also implied that perhaps other factors might have contributed to killing of the shoots in addition to the inoculated canker fungi. Improper timing of pruning may be also important to induce shoot death. These experiments need to be repeated in order to make sure how much the canker pathogens used in this study contribute to killing of these shoots.

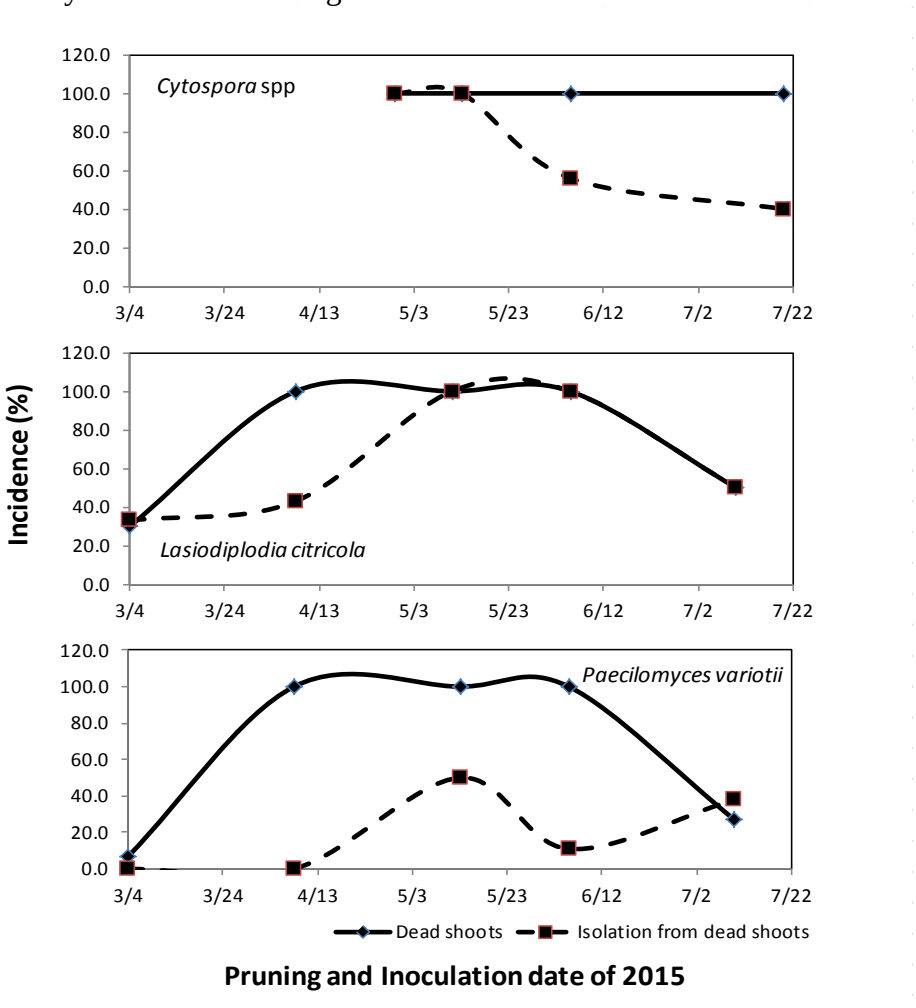

Figure 3. Dynamics of incidence of dead shoots inoculated with three canker- causing pathogens, *Cytospora* spp., *Lasiodiplodia citricola*, and *Paecilomyces variotii* on dried plum, and the percentage of

the dead shoots from which the corresponding pathogen were isolated for each sampling date. The shoots were pruned on 5 different dates of 2015, and inoculations were conducted after pruning. The disease recording was conducted in early December of 2015. Both traditional culturing and molecular methods were used to determine the percentage of the isolations of the corresponding pathogens from the dead shoot samples.

The experiments to study the effects of wound on infection started in August of 2015. Shoots were wounded and inoculated immediately, and the inoculations were conducted from August to November of 2015. The disease was recorded in early December. However, only shoots inoculated in August showed some symptoms, and those inoculated in other dates have not yet shown symptoms. Figure 4 demonstrated that the incidence of cankered shoots were 30, 40 and 30% for *Cytospora* spp., *L. citricola*, and *P. variotii*, respectively, while, the dead shoots from which the corresponding pathogens were isolated were 10, 30, and 40% for the respective pathogens (Figure 4). The results implied that at least three months were needed to show canker symptom after inoculation with comparatively higher inoculum density than natural ones. The results also demonstrated that killing of the shoots might have been the result of other factors in the orchard in addition to pathogen infection. Thus, confirmation of infection by molecular approaches is needed in addition to the conventional culturing method.

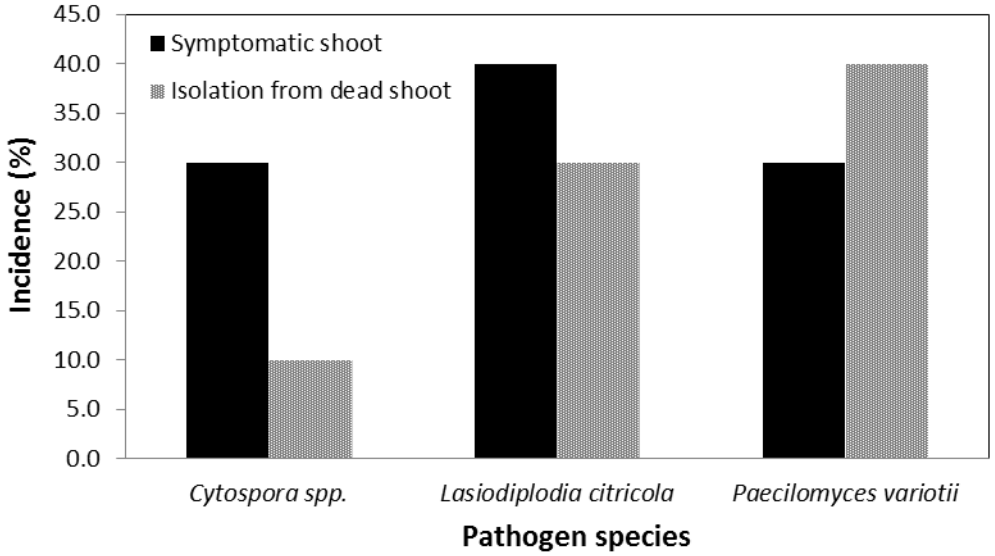

Figure 4. Incidence of inoculated shoots of dried plum showing canker symptoms or shoot death and percentage of dead shoots from which the corresponding pathogen was isolated. Three canker causing pathogens were used. The shoots were wounded and immediately inoculated on **August 7, 2015,** and the disease was recorded in early December of 2015. (The shoots wounded and inoculated from September to November of 2015 have not yet shown any canker symptoms yet.)

Spread of disease with pruning shears. In the experiments of using contaminated shears in pruning, four pathogens were used in inoculation in different dates of spring of 2015 immediately after pruning, and 60 pruned and inoculated shoots were used for each pathogen. The disease recording was conducted in early December of 2015, and almost all inoculated shoots were died at that time for *Cytospora* spp. *L. citricola* and *P. variotii*. However, only 10% shoots inoculated with *S. commune* were died (Figure 5). The percentages of the dead shoots

from which the corresponding pathogens were isolated were 80, 80, 50 and 30% for *Cytospora* spp. *L. citricola, P. variotii* and *S. commune*, respectively (Figure 5). The result implied that the spring pruning could greatly promote pathogen infection and canker development. Among the four pathogens, *S. commune* showed much less pathogenicity than other three pathogens (Figure 5).

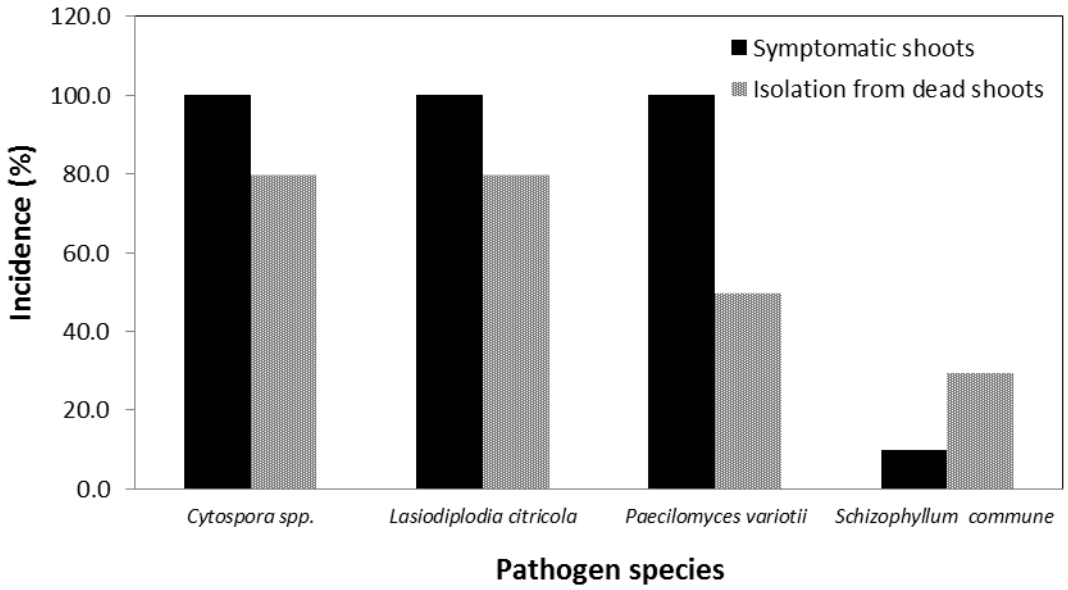

Figure 5. The incidence of inoculated shoots of dried plum showing canker symptoms or shoot death, and the percentage of dead shoots from which the corresponding pathogens was isolated. Four canker-causing pathogens were used. The shoots were pruned with shears contaminated with the corresponding pathogens on March 4, 2015 (for *Lasiodiplodia citricola* and *Paecilomyces variotii*), March 13, 2015 (for *Schizophyllum commune*) and April 29, 2015 (for *Cytospora* spp.), respectively. For each pathogen, 60 shoots were used. Disease was recorded in early December of 2015.

Objective 3. To compare efficacy of potential fungicides against canker diseases.

The 2015 experimental results of the fungicide trial in the prune orchard in Yuba County demonstrated that the fungicides tebuconazole and Topsin M (70 WP) were effective in reducing the incidence of infection significantly under natural infection conditions in (Figure 6). The fungicide treatments Quilt Xcell and Pristine + VitiSeal had no effect on disease control compared with the control. The canker incidences were also significantly reduced by using the fungicide treatments Pristine + Pentra Bark and VitiSeal, in comparison with the control, while, the effect was not as good as that of tebuconazole and Topsin M (Figure 6).

The experiments showed the usefulness of some fungicides selected in this study for canker disease management, especially in the dried plum orchards showing severe canker disease occurrence. Efforts to register an effective fungicide to be sprayed immediately after pruning should be of high priority for the dried plum industry.

The same experiments using the same fungicide and fungicide combinations had been repeated in the December 2015 and results will be collected in November 2016.

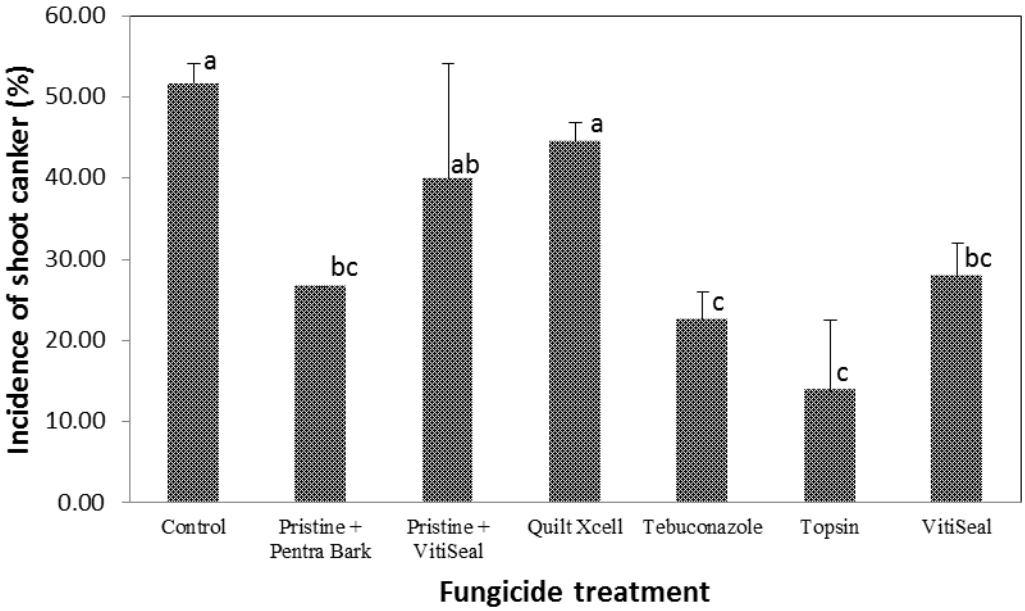

Figure 6. Efficacy of fungicide treatments applied after pruning dried plum shoots against infection by *Cytospora* spp. in a mature dried plum orchard under infection by natural spore inoculum. The trees were pruned on November 6 of 2014, and the pruning wounds were sprayed with different fungicides before a rain event. The disease was recorded on December 8, 2015, and the average value of disease incidence for each fungicide treatment was calculated from two replicates each with 15 pruned shoots.

Objective 4. To determine whether protecting sunburned tissues or tissues damaged mechanically (hail, storms, shaker, etc.) reduces infection and canker development.

The disease was recorded in late November of 2015 for all the four pathogens. From two replicates of each of the three sunburn treatments for *Cytospora* spp. *L. citricola* and *P. variotii*, no inoculated shoots showed typical symptoms, including the no-treated control. For *S. commune*, only one inoculated shoot from the second replicate of treatment A showed a typical canker symptom. The disease will be recorded in the 2016 growing season.

SUMMARY

Four objectives were involved in this 2015 project. In order to develop the methodology for efficiently processing large number of samples for epidemiological studies, we designed DNA primers to specifically target the six canker-causing pathogens, including *Phomopsis* spp., *Botryosphaeria dothidea*, *Lasiodiplodia* spp., *Cytospora* spp., *Neofusicoccum* spp., and *Diplodia* spp. Real-time PCR approaches have been developed to quantify the inoculum densities from air and rain samples in orchards and to quantify the infection level in plant tissues. The potential usefulness of the molecular approaches in epidemiological studies was demonstrated by testing various kinds of real samples collected in this study. Field experiments were conducted in a dried

plum orchard at the University of California, Kearney Agricultural Research and Extension Center (KARE). Pruning wounds remained susceptible to infection by *Cytospora leucostoma* for 2 weeks after pruning. Effects of pruning wound on pathogen susceptibility and infection and canker disease development were studied in field inoculation experiments in the spring using four pathogens: *Cytospora* spp., *L. citricola, Paecilomyces variotii,* and a wood decay fungus, *Schizophyllum commune* commonly found on dead wood in prune orchards. Spring pruning greatly promoted pathogen infection and canker development. *S. commune* found to be less pathogenic than the other three pathogens. Monthly inoculations were also conducted on pruned or wounded shoots using *Cytospora* spp., *L. citricola,* and *P. variotii*. High incidences of killing the shoots inoculated from April to June were observed for all the three pathogens. The isolation rates of *L. citricola* and *Cytospora* spp. from the dead shoots were higher than those of *P. variotii* from the samples inoculated in May of 2015. However, the isolation rates of *Cytospora* decreased on shoots inoculated from June to August of 2015. The pathogen *L. citricola* showed higher pathogenicity over the season than the other two species. The results also implied that the dead shoots may be caused by other factors, in addition to infections. Fungicide trials were conducted in a dried plum orchard in Yuba County where the canker disease is severe. Compared with the no-fungicide control, the fungicides Tebuconazole and Topsin significantly reduced the incidence of canker disease, while Quilt Xcell and Pristine + VitiSeal had no effect on disease control. The fungicide treatments Pristine + Pentra Bark and VitiSeal also significantly reduced canker incidence, but the efficacies were not as strong as those of tebuconazole and Topsin. The possible effects of sunburn of shoots on pathogen infection and disease development are being also studied at the KARE experimental dried plum orchard. The disease development is now in progress, and the eventual development of cankers will be recorded in the 2016 growing season.