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

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and

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OBJECTIVES

1) To continue the experiment of monthly inoculations to determine the critical period of pathogen infection and disease development.
2) To investigate the inoculum dynamics during rain events in three commercial orchards with severe canker disease.
3) To investigate the development of pathogens' latent infection and their corresponding endophytic stage in shoot tissues during and over the growing season.
4) Continue to study the efficacy of certain fungicides to control canker disease.
5) Continue to study the putative effects of sunburn of shoots on pathogen infection and canker disease development.

PROCEDURES

Objective 1: To continue the experiment of monthly inoculations to determine the critical period of pathogen infection and disease development.

The experiments were conducted in a prune orchard at KARE. Wounds of 1- or 2-year old shoots were generated by pruning shoots with a pair of sterile pruners. About 30 shoots per tree were randomly selected and pruned on March 7-9, 2016 and marked for later inoculations. The inoculation dates of 2016 on the wounded shoots were March 9, April 23, May 11, June 8, July 7, August 9, September 15 and October 10. On each inoculation date, all wounded shoots of each tree were individually inoculated by spraying about 3 ml of spore suspension (about 10^5 spores/ml per shoot) of each of the two pathogen species, *Lasiodiplodia citricola* and *Cytospora* spp. The inoculated shoots were covered with a plastic bag for 48 hours to maintain the humidity. The disease recording was performed in mid-November of 2016 for all the inoculated shoots except for those inoculated in October 10. The following scoring system was used to access canker disease severity: **0**: no canker symptom; **1**: Canker length <=1cm; **2**: Canker length was1-3 cm; **3**: Canker length was 3-5 cm; **4**: Canker length was >5 cm; **5**: the shoot was died because of canker. Thus, the canker severity was assessed for each of the inoculated shoot for all inoculation dates for each pathogen. The average canker severity was calculated and used to compare among different inoculation dates.

Objective 2: To investigate the inoculum dynamics during rain events in three commercial orchards showing severe canker disease.

Three prune orchards were identified in Yuba County, designated as Orchard 1, Orchard 2, and Orchard 3, and rain samples were collected from these orchards. In each orchard, a rain collector as a 500-ml plastic bottle with a funnel cap was set at canopy height. The rain samples were collected four times in 2016, on February 9, March 2, March 17 and May 5. For each sample, a total of 120 ml of rain water was processed using 4 centrifuge tubes. The centrifuge was set at 10,000 rpm for 10 min, and the supernatants were carefully discarded, leaving about 10 µl precipitates in the tube. The precipitates of the 4 tubes were combined into a 16-ml centrifuge tube and centrifuged again under the same conditions described above. After carefully removing the supernatant, 40 µl precipitates were left to extract DNA. The specific primers for each of the six pathogen groups, *Phomopsis* spp., *Botryosphaeria dothidea, Lasiodiplodia* spp., *Cytospora* spp., *Neofusicoccum* spp. and *Diploid* spp., were used to target the specific pathogen in rain samples by using real-time PCR. Our previously-developed equations of standard curves (data not shown) were used to quantify inoculum density for each pathogen in terms of Log_{10} (number of spores/ml).

Objective 3: To investigate the development of pathogens' latent infection and their corresponding endophytic features in shoot tissues during and over the growing season.

In each of the three orchards mentioned above, periodical shoot samplings were conducted in March, June and September of 2016, and will be continued every three months during the dormant period as well. For each sampling, about 10-20 cm-long shoots including the newly-emerged shoots and part of the old shoot in the proximity of the new shoot (usually 1-year old) were collected. Thus, each sample contained two parts: new shoots and old shoots. The two age kind of shoots were numbered and processed separately. For each sampling, 32 such shoots were randomly collected in each orchard. These shoots were washed twice with regular water, soaked in 10% commercial bleach for 10 min for surface sterilization, washed three times again, and air dried for two days. A pencil sharpener was used to grind shoot samples into fine wood pieces which were used to extract DNA by sing the FastDNA kit (MP Biomedical, CA). Briefly, the pathogen group-specific primers were used in real-time PCR to obtain the corresponding Ct values. The published equation of standard curve for each pathogen group (Luo et al., 2017) was used to calculate the DNA quantity for each pathogen in each sample.

To quantify the infection level of shoots, we introduced the concept of molecular severity (MS): $MS= Log_{10}(P/H)$, where P is the weight of the pathogen's DNA in femtograms (fg), which is calculated by using the equation of the standard curve for the corresponding pathogen (Luo et al., 2017) based on the Ct value from its reaction with the corresponding primers, and where H is the shoot weight in grams (g). Thus, if the minimum detectable amount of pathogen DNA in one gram of shoot is theoretically assigned as 1 fg, the MS would be 0. The maximum of the amount of pathogen DNA in one gram of shoot tissue could be theoretically one gram ($=10^{15}$ fg), and the maximum value of MS should be 15. Thus, the range of MS value is 0 - 15. However, since when no infection is detected we assign MS = 0, the theoretically detectable amount of pathogen DNA in one gram of shoot should be >1fg. The concept and calculation of MS were used to determine the infection level for all the shoot samples used in this study.

Incidence of latent infection in terms of proportion of shoots showing positive results over all detected shoots was obtained for each pathogen on each sampling date. Average MS from 32 shoot samples was obtained for each pathogen and each sampling date. Comparisons in MS between new and old shoots were conducted for each pathogen and on each sampling date.

Objective 4: Continue to study the efficacy of certain fungicides to control canker disease.

We continued a fungicide trial in a dried plum orchard located in Yuba County showing severe Cytospora canker disease. Same as used in 2014-2015 trials, 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 in December 2015 and fungicide treatments were conducted on December 9, 2015. 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 fungicide treated wounds were maintained on trees for the whole season of 2016 and disease recording was conducted on 7 December of 2016 for canker incidence for each fungicide treatment.

Objective 5: Continue to study the putative effects of sunburn of shoots on pathogen infection and canker disease development.

We continuously conducted an experiment to determine whether the sunburn could affect the disease development in this year. On August 5, 2016, some trees of the very south row of the orchard at KARE described above were selected. The shoots were bended at certain degree to face the sun and tied to a metal stick so that they are exposed to direct sunlight. About 10 shoots were used for each of the three pathogens. The inoculations were conducted on August 26, 2016. Each marked shoot was treated by using a sterile cork borer to make the wound, inoculated by spraying 2 ml of spore suspension (10^5 spores/ ml) of each pathogen directly on the wound, and covered with a piece of parafilm for 48 hours to create high humidity. The whole experiment was replicated twice and disease was recorded in mid-November 2016.

RESULTS AND CONCLUSIONS

Objective 1: To continue the experiment of monthly inoculations to determine the critical period of pathogen infection and disease development patterns.

Since two trees with inoculated shoots died because of wood decay fungi and pulled out during this experiment, the results relevant to inoculations with *Paecilomyces variotii* were excluded in data analysis. Figure 1 shows the dynamics of mean canker severity for *Lasiodiplodia citricola* (7F93) and *Cytospora leucostoma* (9D71) during 2016. Comparisons in average canker severity among different inoculation dates demonstrated that for both pathogen species, early inoculations (from March and May) on wounds generally promoted significantly higher canker severity than did later inoculations (Figure 1), especially for *Cytospora* spp. Compared with the non-inoculated control, all inoculated shoots showed significantly higher canker severities. Thus, we concluded that the risky period time promoting higher chance of severe canker occurs in early in the growing season.

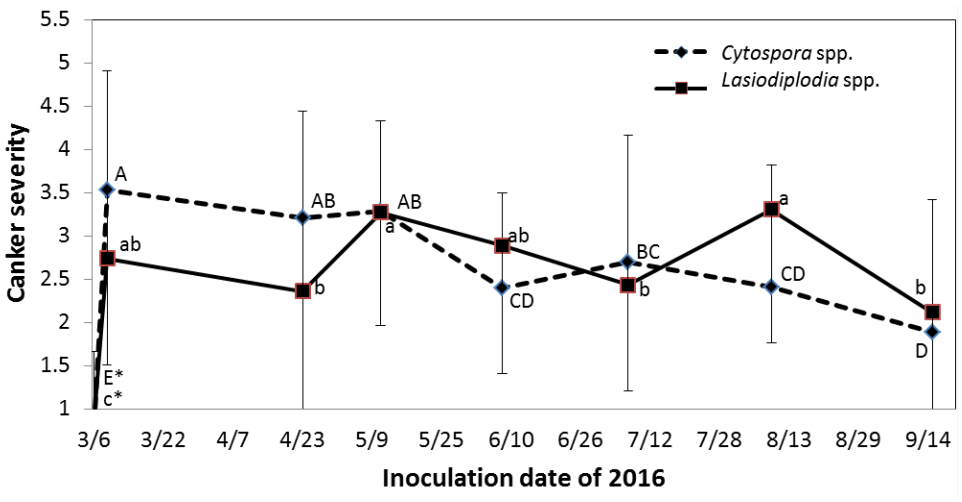

Figure 1. Dynamics of canker disease severity cause by *Cytospora* and *Lasiodiplodia* spp. on wounded shoots in 2016. Inoculations were conducted on wounded shoots periodically. Each dot represents a mean value from 30 wounded shoots. * indicates the mean value from non-inoculated shoots as control.

Objective 2: To investigate the inoculum dynamics during rain events in three commercial orchards showing severe canker disease.

Four times of rain samples from each of the three prune orchards in Yuba County mentioned above were analyzed. We did not find any spores of *Phomopsis* spp. and *Diplodia* spp. in any of the rain samples. *Cytospora* spp. is major pathogen detected in most rain samples in these orchards. Thus, this species was predominant in the pathogen populations throughout the season (Figure 2). *Lasiodiplodia* spp. was found in all the three orchards especially in early season. It was also major species detected in rain water, although the amounts were not as high as *Cytospora* spp. (Figure 2). However, both *Botryosphaeria dothidea* and *Neofusicoccum* spp. were detected from only some rain samples (Figure 2), indicating that these can be considered as minor species of the pathogen populations in the rain water.

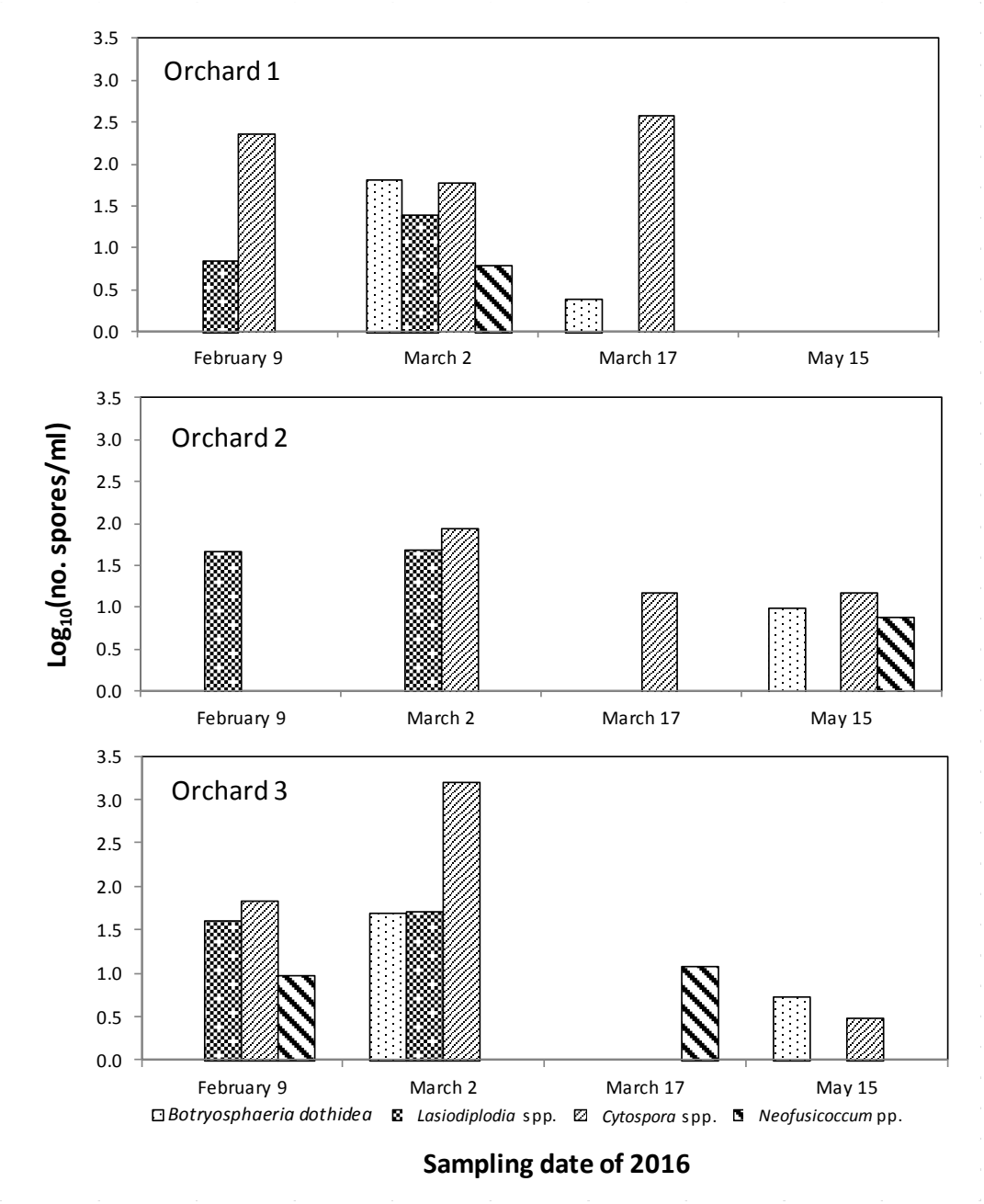

Figure 2. Spore densities of four canker-causing species obtained from four times of rain samples collected from three prune orchards in Yuba County. The real-time PCR assay was applied to quantify these spore densities in samples of rain water.

Objective 3: To investigate the development of pathogens' latent infection and their corresponding endophytic stage in shoot tissues during and over the growing season.

Similar patterns of latent infections in shoots were observed among the three orchards in Yuba County. Basically, three major species in shoot tissues detected as latent infections were *B. dothidea*, *Lasiodiplodia* spp. and *Cytospora* spp. The *Phomopsis* spp. and *Neofusicoccum* spp.

were detected in some samples with very low level of incidence and MS and high flexibility. The *Diplodia* spp. was not detected in all shoot samples in all the three orchards, indicating that this species seemed not important in prune orchards so far in this area.

In Orchard 1, the high incidences of latent infections (over 60%) caused by *B. dothidea* in both new and old shoots were observed in all three samplings (Figure 3). Relatively lower incidences of infections caused by *Lasiodiplodia* spp. and *Cytospora* spp. were observed, which also varied between new and old shoots (Figure 3).

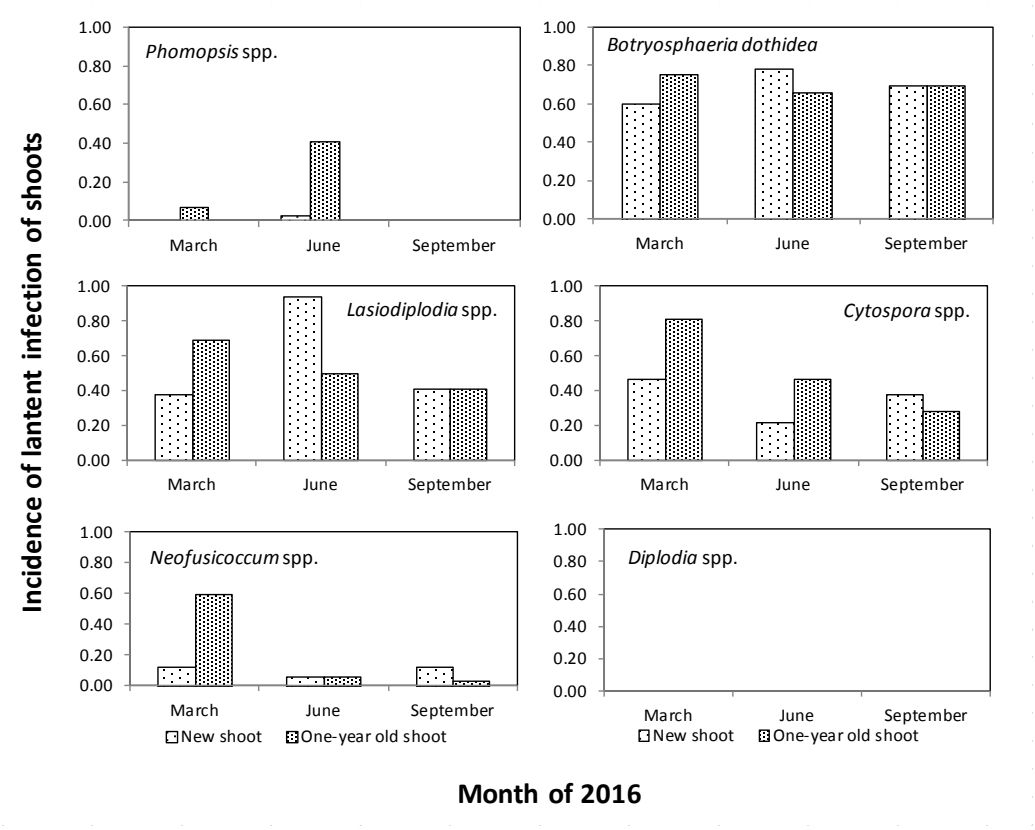

Figure 3. Incidences of latent infections of new-emerged shoots and old 1-year shoots caused by six canker-pathogen groups from prune Orchard 1 in Yuba County. The real-time PCR assay was applied to process these shoot samples collected from 3 time points in 2016.

Comparison in MS between new and old shoots demonstrated that the MSs were significant higher in old shoots than in new shoots in the first samples for most pathogens when the new shoots just emerged (Figure 4). While there was no significant difference in MS between the new and old shoots for most samples in later two samplings (Figure 4). The results showed that even at the very shoot emergence at least three predominant species could be isolated from new shoots, implying some endophytic features of pathogen species in shoots.

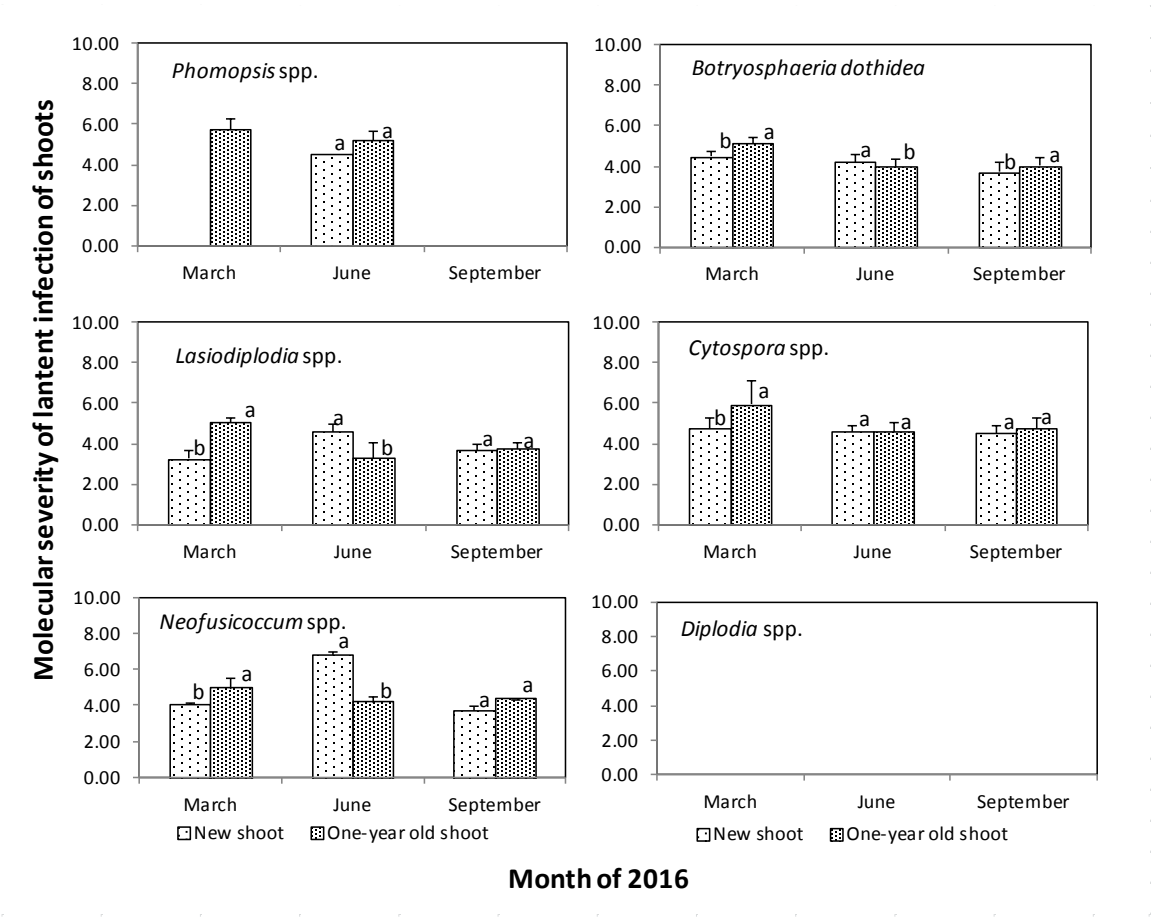

Figure 4. Comparison of mean molecular severities (MS) between new-emerged shoots and the old prune shoots quantified for each of the six canker-causing pathogens. Three samplings were conducted in prune Orchard 1 in Yuba County. The real-time PCR was applied to obtain MS data and 32 shoots were processed for each sampling.

In Orchard 2, similar situations were observed that *B. dothidea* existed in all new and old shoot samples with high incidences (Figure 5). Following that, *Lasiodiplodia* spp. and *Cytospora* spp. were the second predominant pathogens that were recovered from shoot samples, while the incidences were lowers than those of *B. dothidea*. *Phomopsis* spp. and *Neofusicoccum* spp. were infrequently isolated in some samples, indicating less importance of these two species in both new and old shoots (Figure 5).

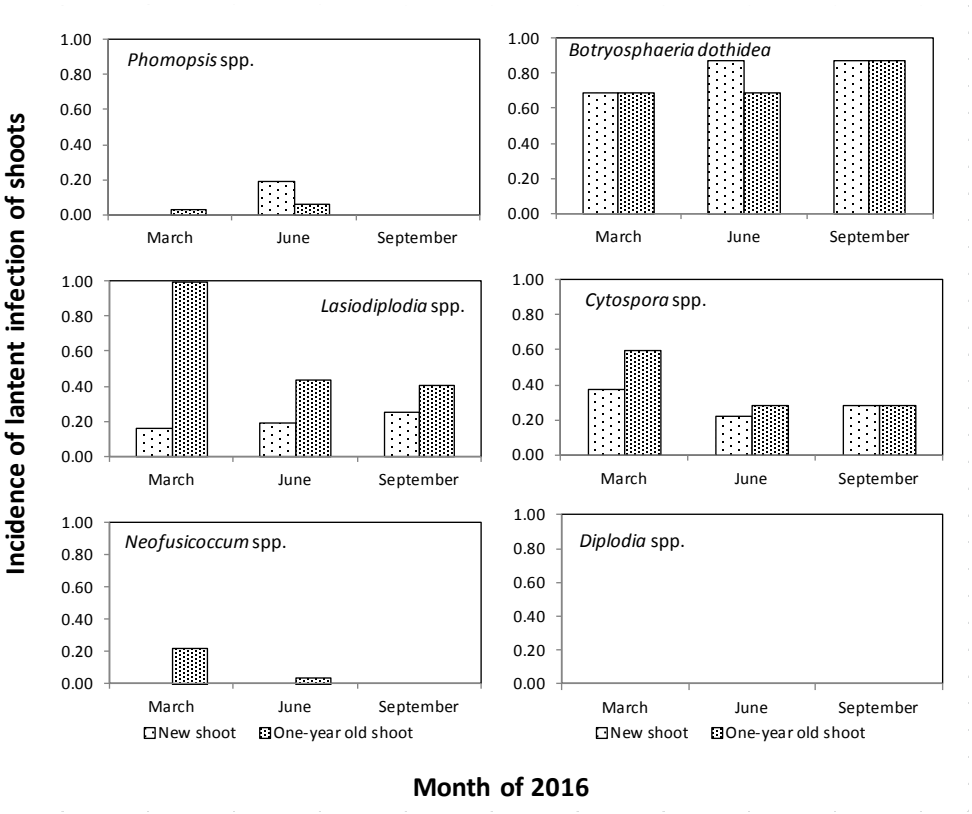

Figure 5. Incidences of latent infections of new-emerged shoots and old 1-year shoots caused by six canker-pathogen groups from Orchard 2 in Yuba County. The real-time PCR assay was applied to process these shoot samples collected from 3 time points in 2016.

Similarly, the average MSs were significantly higher in old shoots than in new shoots at very early stage for the three predominant pathogens mentioned above (Figure 6). This significance appeared also in the second samplings, while, there was no clear patterns in difference in MS between new and old shoots in the last sampling (Figure 6). The result implied that the predominant pathogens existed in new-emerged shoots and could develop during the growing season.

Results from prune Orchard 3 showed higher incidence of shoot latent infection in old than in new shoots for the three predominant pathogens for the first sampling (Figure 7). The situations in incidence of latent infection caused by *Lasiodiplodia* spp. and *Cytospora* spp. was quite the same as that in prune Orchard 1.

Comparisons showed that means of MSs in old shoots were significantly higher than in new shoots at very early stage for *Phomopsis* spp. *B. dothidea*, *Lasiodiplodia* spp. and *Cytospora* spp. (Figure 8). However, no such clear difference patterns were observed in latter samplings.

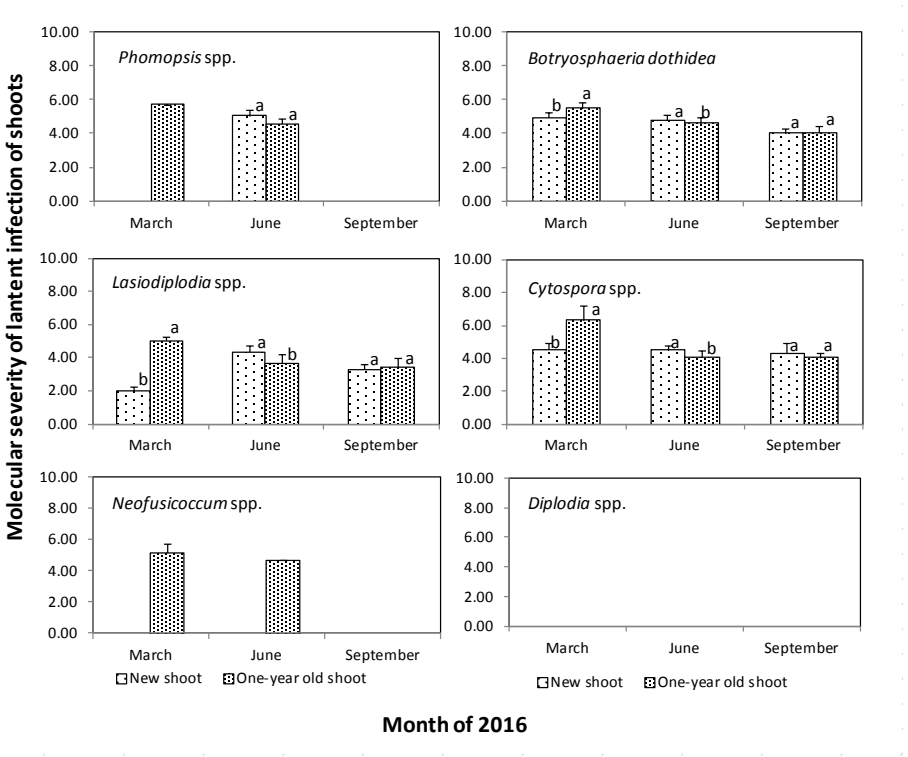

Figure 6. Comparison in mean molecular severities (MS) between new-emerged shoots and the old shoots quantified for each of the six canker-causing pathogens. Three samplings were conducted in prune Orchard 2 in Yuba County. The real-time PCR was applied to obtain MS data, and 32 shoots were processed for each sampling.

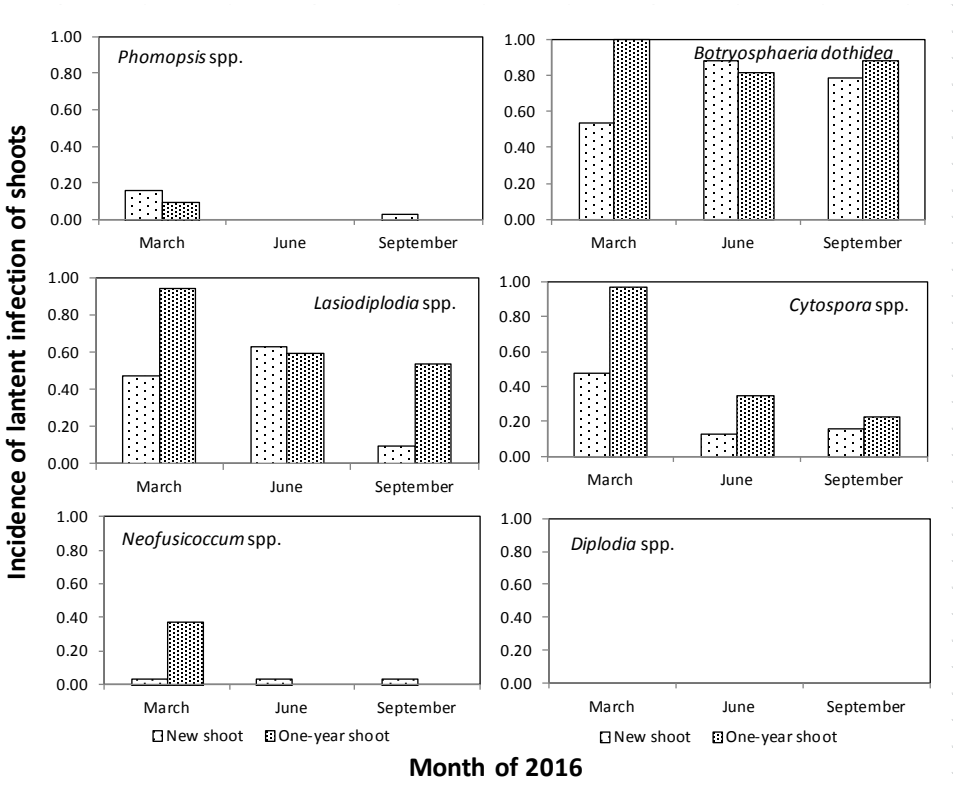

Figure 7. Incidences of latent infections of new-emerged shoots and old 1-year shoots caused by six canker-pathogen groups from prune Orchard 3 in Yuba County. The real-time PCR assay was applied to process these shoot samples collected from three time points in 2016.

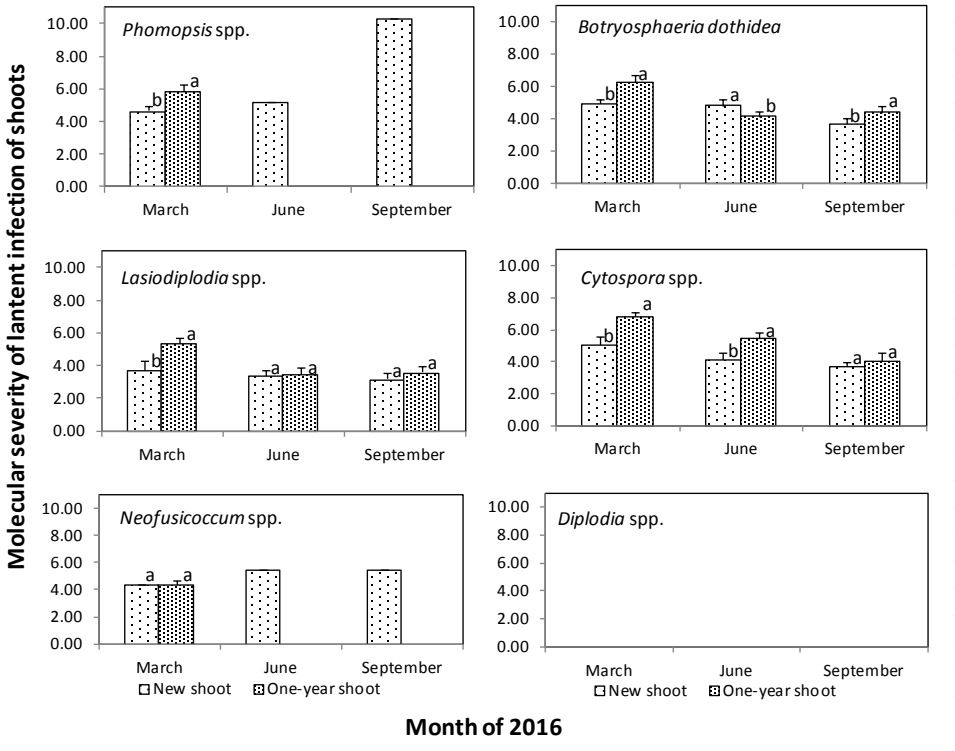

Figure 8. Comparison in mean molecular severities (MS) between new-emerged shoots and 1-year-old shoots quantified for each of the six canker-causing pathogens. Three samplings were collected and processed in prune Orchard 3 in Yuba County. The real-time PCR was applied to obtain MS data, and 32 shoots were processed for each sampling.

Objective 4: Continue to study the efficacy of certain fungicides to control canker disease.

The 2016 experimental results of the fungicide trial in the prune orchard in Yuba County demonstrated that the fungicides Quilt Xcell and Topsin M (70 WP) were the most significantly effective in reducing the incidence of infection under natural infection conditions in (Figure 9). Results showed that compared with control, the incidences of canker on wounds treated with other fungicides, including Pristine + Pentra Bark, VitiSeal, Tebuconazole and Pristine + VitiSeal, were not significantly reduced. Thus, this year's experiment showed that these fungicides were not effective in reducing canker development. In the 2015 experiment, again the Topsin M was the most effective fungicide treatment while the efficacy of Quilt Xcell was not consistent between the two years.

The same experiments using the same fungicide and fungicide combinations had been repeated in the December 2016 and results will be collected in December 2017. In addition, a new fungicide trial will be initiated using Topsin as the standard and other different combinations of fungicides in an orchard in Yuba County.

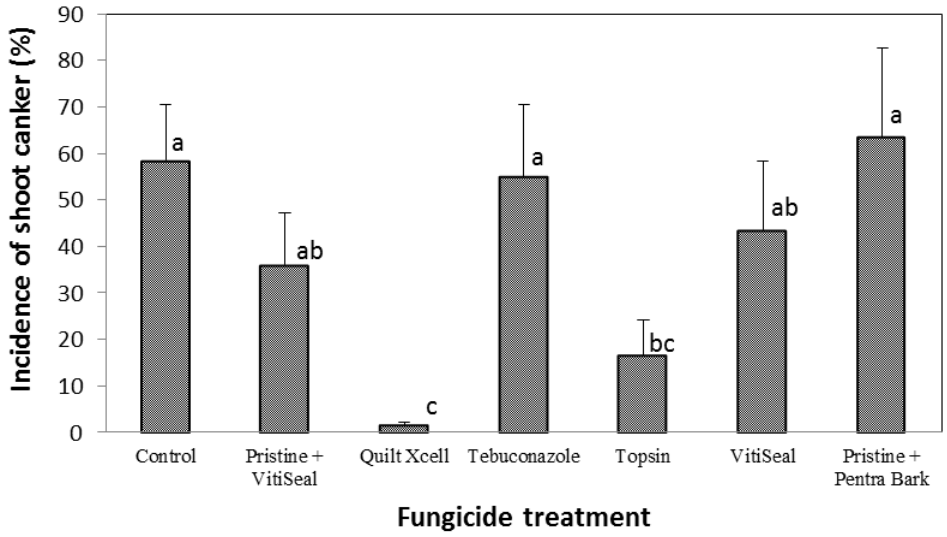

Figure 9. Efficacy of fungicide treatments applied after pruning dried plum shoots to reduce canker development in Orchard 1 under infection by natural spore inoculum. The trees were pruned on December 9, 2015, and the pruning wounds were painted with different fungicides before a rain event. The disease was recorded on December 7, 2016, and the average value of disease incidence for each fungicide treatment was calculated from the two replicates each with 10 pruned shoots.

Objective 5: Continue to study on the putative effects of sunburn of shoots on pathogen infection and canker disease development.

The disease was recorded in late November of 2016. From two replicates, there was no significant difference in canker incidence between sunburn treatment and non-sunburn treatment for each of the pathogens *Cytospora* spp. and *L. citricola*. However, the canker incidence caused by *Cytospora* spp. was significantly lower than the incidence of *L. citricola* (Figure 10), demonstrating the higher virulence of *Lasiodiplodia* than that of *Cytospora* in causing canker development.

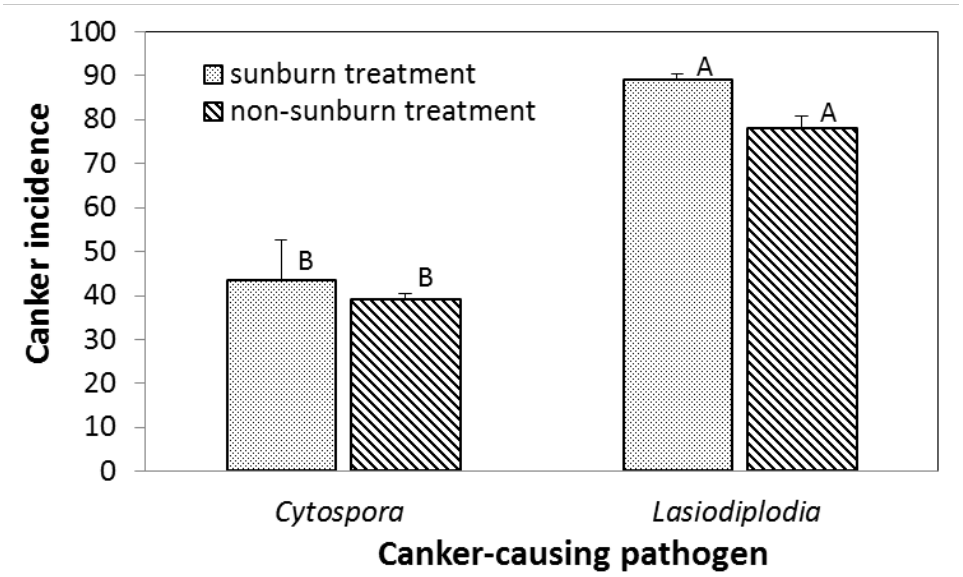

Figure 10. Incidence of canker disease on shoots for two canker-causing pathogens, Cytospora *and* Lasiodiplodia spp. Two treatments: sunburn treatment (see text for details) and non-treatment were conducted in a prune orchard at Kearney Agricultural Research and Extension Center to study the possible effect of sunburn on canker incidence for each of the two pathogens.

ECONOMIC BENEFITS

Managing canker diseases of dried plum will lead to longer lifespan of the trees. Although it is difficult to estimate the benefits, the results of treating pruning wounds with Topsin-M show a significant reduction of infections by Cytospora and one would think that this fungicide could be sprayed after pruning to protect wounds from infection.

SUMMARY

This study in 2016 focused on 5 objectives. We continuously involved in monthly inoculations on wounded shoots by pruning in March in a prune orchard at Kearney Agric. Research and Extension Center. Two canker-causing pathogens, *Lasiodiplodia citricola and Cytospora* spp. were used in inoculations. Disease assessments in November showed similar patterns of canker development for both pathogens. The early inoculations from March to May resulted in significantly higher incidence and canker severity than did the later inoculations with these fungi. This implies that after pruning in early spring, infections that occur in spring and early summer could bring about high risk of canker development. Thus, treating pruning wounds with fungicides in early season is very important to reduce canker diseases. In 2016, three prune orchards in Yuba County of California were identified. Rain samples were collected separately from each of these orchards four times in spring and early summer. Our previously-developed

real-time PCR quantification assay was applied to quantify the spore densities of each of six canker-causing pathogens in each rain sample: *Phomopsis* spp., *Botryosphaeria dothidea, Lasiodiplodia* spp., *Cytospora* spp., *Neofusicoccum* spp. and *Diplodia* spp. We did not find any spores of *Phomopsis* or *Diplodia* spp. in any of the rain samples. *Cytospora* spp. was major pathogen detected in most rain samples in these orchards. *Lasiodiplodia* spp. was found in all the three orchards especially in early season. Both *B. dothidea* and *Neofusicoccum* spp. were detected from only some rain samples, indicating that they serve as minor species of the pathogen populations in rain water. Newly emerged shoots and old shoots where the new shoots emerged from were also sampled in these three orchards in March, June and September 2016. Our published real-time PCR quantification assay was also applied to quantify the latent infection level for each of the six canker-causing species mentioned above. We introduced Molecular Severity (MS) by quantifying the fungal DNA from the latent infections of these shoots. Similar patterns of latent infections were observed among the three orchards. The overall results showed that *B. dothidea, Lasiodiplodia* spp. and *Cytospora* spp. were the three predominant species causing latent infection of shoots. *Phomopsis* spp. and *Neofusicoccum* spp. infrequently existed in shoots and they were not consistent. *Diplodia* spp. did not exist in any of the shoot samples. In few days after the new shoots developed (early stage samples), the MSs were significant lower than the MSs in old shoots. However, this difference was reduced later in season, indicating an increase (accumulation with time) of latent infections in the new shoots. The results also implied that these pathogens indicate endophytic features in young healthy shoots that need further investigation. For instance, if an inoculum source of these pathogens were close to a nursery, it would be possible these infections to establish in the young looking plants as early as before leaving the nursery and/or even in the field during the first year of planting. Thus, it is essential to protect the wounds created during the selection of primary scaffolds of trees. We continuously involved in fungicide trials in 2015 and 2016. The results demonstrated that the fungicide Topsin M (70 WP) significantly reduced canker incidence as compared with other fungicides and the results were consistent in both 2015 and 2016 trials. In contrast, the fungicide Quilt Xcell (propiconazole+azoxystrobin) reduced canker incidence in 2015, had no effect in 2016. In 2015 we showed effect of sunburn increasing the incidence of canker, but in 2016 did not affect the incidence of cankers. When the two canker pathogens were compared, it was determined that *Lasiodiplodia* spp. were more aggressive (virulent) than *Cytospora* spp. in causing canker disease.

References

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