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 investigation of the susceptibility of pruning wounds to infection by three canker-causing species during the growing season
2) To continue to study the inoculum dynamics during rainfall in dried plum orchards showing severe canker disease
3) To continue the experiments to quantify latent infections of canker-causing pathogens and their corresponding "endophytic" phase in shoot tissues.
4) To study the effect of water stress and fertilizer on predisposing shoots to infection by *Cytospora* spp.
5) To manage cankers by using fungicides and/or biological agents applied onto pruning wounds.

PROCEDURES

Objective 1. To continue the investigation of the susceptibility of pruning wounds to infection by three canker-causing species during the growing season

In 2017, experiments were conducted in a prune orchard at KARE. Wounds of 1- to 3-year old shoots were created by pruning shoots with a pair of sterile pruners. About 30 shoots per tree were randomly selected and pruned on March 8-9, 2017 and marked for later inoculations. The dates of monthly inoculations in 2017 on the wounded shoots were March 9, April 5, May 10, June 13, July 19, and August 16. 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) of each of the three pathogen species: *Lasiodiplodia citricola, Botryosphaeria dothidea* and *Cytospora* spp. The inoculated shoots were covered with a plastic bag for 48 hours to maintain humidity. The recording of disease symptoms was performed on November 3, 2017 for all the inoculated shoots. The previously established canker scoring system was used to assess 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 dead because of canker. The canker severity was assessed for each of the inoculated shoots for all inoculation dates for each pathogen. The incidence and average canker severity were calculated and used to compare the different inoculation dates.

Objective 2: To continue to study the inoculum dynamics during rainfall in dried plum orchards showing severe canker disease

In 2017, we continuously quantified the pathogen concentrations in rain samples in the three prune orchards identified in Yuba County designated as Orchard 1, Orchard 2, and Orchard 3. In each orchard, a rain collector consisting of a 500-ml plastic bottle with a funnel cap was set at canopy height. The rain samples were collected in October, November and December 2016, January, 2017, February to April, 2017, and May to June, 2017. For each sample, 30 – 50 ml of rainwater was centrifuged at 10,000 rpm for 10 min and the supernatants were carefully discarded, leaving about 10 μl of precipitates in the tubes for DNA extraction. The specific primers for each of the six pathogen groups: *Phomopsis* spp., *Botryosphaeria dothidea*, *Lasiodiplodia* spp., *Cytospora* spp., *Neofusicoccum* spp. and *Diplodia* spp. (Luo et al., 2017), were used to target the specific pathogen in rain samples by using real-time PCR (*q*PCR). Our previously-developed equations of standard curves (data not shown) were used to quantify inoculum density for each pathogen in terms of number of spores per ml of rainwater.

Objective 3: To continue the experiments to quantify latent infections of canker-causing pathogens and their corresponding "endophytic" phase in shoot tissues.

In each of the three orchards mentioned above, periodic shoot samplings were conducted in December 2017, March, June, and September 2017, and will be continued every three months during the dormant period as well and during 2018. For each sampling, about 10-20 cm-long shoots, including the new-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 kinds 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). Our published pathogen group-specific primers and the real-time PCR (*q*PCR) assay were applied to obtain the corresponding Ct values. The published equation of the standard curve for each pathogen group (Luo et al., 2017) was used to quantify the DNA of each pathogen in each sample.

In our data analysis, we continuously used 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). The range of MS value is 0 – 15, since 1 femtogram is equal to 10^{-15} g). However, when no infection is detected we assigned MS = 0, since the theoretically detectable amount of pathogen DNA in one gram of shoot should be >1fg. The incidence of latent infection (I), in terms of percentage of shoots showing positive results out of all detected shoots, was obtained for each pathogen on each sampling date. The average MS from 32 shoot samples was obtained for each pathogen and each sampling date.

We also introduced the concept of Index of Latent Infection (ILI) of shoots as: ILI = I × MS / 100. The ILI could be used to estimate the overall risk for the latent infection status of an orchard that has been sampled. In our data analysis, we combined two years of data (2016 and 2017), in total 7 points in time, to draw the dynamic curves of incidence, MS, and ILI of latent infection for both new-growth and 1-year-old shoots for each of the three orchards.

Objective 4. To study the effect of water stress and fertilizer on predisposing shoots to infection by *Cytospora* spp.

We obtained 100 potted young prune trees in August 2017. The experiment was set at KARE. Four water stress treatments: 25%, 50%, 75%, and 100% of regular water supply were used, each with 25 trees. The irrigation treatments were started in August 2017. Inoculation was started in October, 2017. A wound on each tree was made on the position between two lower branches by using a cork borer. A piece of culture of *Cytospora* spp. from PDA medium was placed in the wound and wrapped with a piece of parafilm. The inoculated trees were irrigated according to the water treatments that was stopped in December of 2017 for winter dormancy. The treatments will be used again starting in February 2018. The fertilizer treatments will be conducted in an appropriate month of 2018 when growth is active.

Objective 5. To manage cankers by using fungicides and/or biological agents applied onto pruning wounds.

In 2017, we continued a fungicide trial in a prune orchard located in Yuba County showing severe Cytospora canker disease. Similar to a previous experiment, 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 2016 and fungicide treatments were conducted on December 9, 2016. We used the rate of 5g/L for each fungicide to paint on the pruning wounds. For each fungicide treatment, three replicates each with 10 pruned branches were used.

Additionally, we conducted another trial with treatments on wounds by using some biological agents. The pruning wounds were generated in February 2017 by using the same method described above. Four treatments: Biologicals (Bacillus + Trichoderma), Topsin, Biologicals + Topsin, and water were used. Similarly, three replicates, each with 10 wounds, were used for each treatment.

The canker disease symptoms for all above treatments were recorded on December 7, 2017 for incidence of cankers within each replicate.

RESULTS AND CONCLUSIONS

Objective 1. To continue the investigation of the susceptibility of pruning wounds to infection by three canker-causing species during the growing season.

High incidences (> 80%) of canker disease were observed among the different inoculation dates for each of the three pathogens and no significant differences among inoculation dates was found (data not shown). Variations in canker severity among different inoculation dates for different pathogens were observed. Figure 1 shows the dynamics of average canker severity for *Botryosphaeria dothidea* (3F33), *Cytospora leucostoma* (9D71), and *Lasiodiplodia citricola* (7F93) during the 2017 season. For *B. dothidea*, inoculations on all dates did not cause significant differences in canker severity, except for the inoculation in July that promoted the least canker severity. For *C. leucostoma*, the canker severities were lowest for the inoculations in May and August, while those on other inoculation dates were significantly higher (Figure 1).

For *L. citricola*, the canker severity was the lowest for the inoculation in May, and moderate for the inoculations in April and June. The severities were highest for the inoculations early or late in season over the other periods of time (Figure 1).

In general, for all the three pathogens, the inoculations early in the season promoted higher canker severity compared with the other periods time of the season, even with high variation observed among inoculation dates. Thus, we conclude that the riskiest period of time for severe canker opportunity could occur early in the growing season.

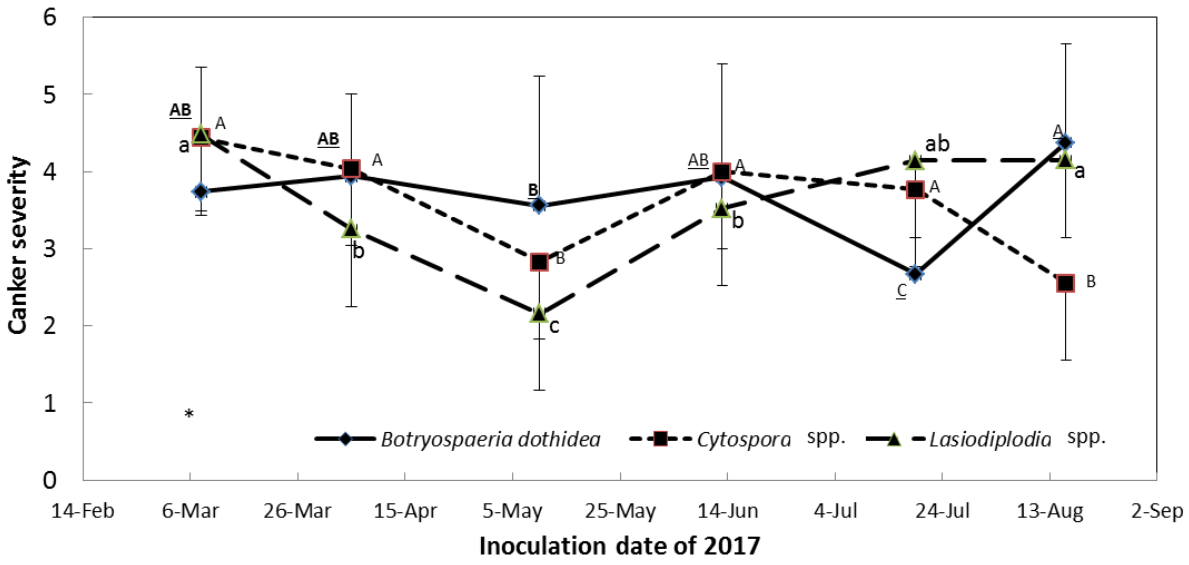

Figure 1. Dynamics of canker disease severity cause by *Botryosphaeria dothidea*, *Cytospora* spp. and *Lasiodiplodia* spp. on wounded shoots in 2017. Inoculations were conducted on wounded shoots monthly. Each dot represents a mean value of severity from 30 wounded shoots. The * indicates the mean value from non-inoculated shoots as control.

Objective 2: To continue to study inoculum dynamics during rainfall in dried plum orchards showing severe canker disease

The rain samples were periodically collected from three prune orchards from October 2017 to June 2017. The pathogen groups *Phomopsis* spp. and *Diplodia* spp. were not found in any of the samples. The other four pathogen groups were detected in different months with variation. *Cytospora* spp. was detected in the period from Februarys to April in all three orchards (Figure 2) with comparatively higher concentrations than the other three pathogen groups. None of the pathogens were detected from April to June, except in Orchard 2, where a low concentration of *Cytospora* spp. was detected. Different pathogen groups may be detected in different seasons in different orchards, while Orchard 2 showed relatively lower concentrations of pathogen groups in the rain samples than the other two orchards (Figure 2). *Lasiodiplodia* spp. were detected from

fall to winter in the three orchards, while, *B. dothidea* was detected from winter to spring in most samples. *Neofusicoccum* spp. also appeared in winter and spring. Generally, the rain samples collected from winter through spring carried different concentrations of spores from canker-causing pathogen groups, but those in summer did not. Thus, we conclude that the riskiest period of time when rains may carry pathogens could be from winter to spring, or the rainy season. The findings also indicate that the spores from pathogens are an inoculum source mostly produced during the rainy season that could be released from pycnidia on shoots.

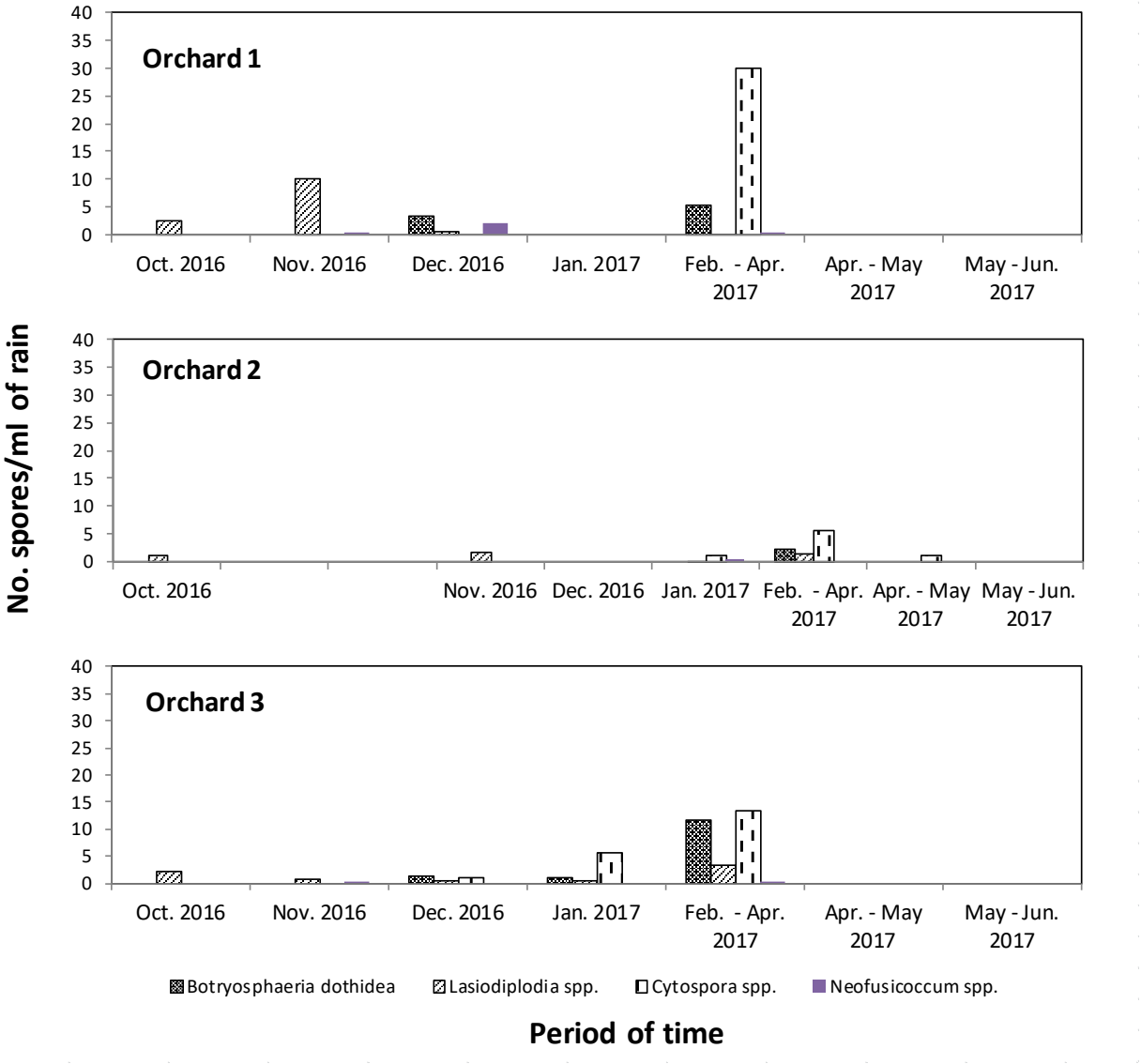

Figure 2. Spore densities of four canker-causing species obtained from fall 2016 to summer 2017. The rain samples were collected from three prune orchards in Yuba County. The real-time PCR assay was applied to quantify these spore densities in rainwater samples.

Objective 3: To continue the experiments on quantification of latent infection of canker-causing pathogens and their corresponding "endophytic" phase in shoot tissues.

To study the dynamics of latent infection by different pathogen groups in the three orchards, we combined 2016 with 2017 data in our analysis to draw the dynamic curves of incidence, molecular severity, and index of latent infection for the three orchards. Figure 1 shows the dynamics of incidence of latent infection on new-emerged shoots from March 2016 to September 2017. The fungal pathogen *Diplodia* spp. was not detected at any sampling date in any of the three orchards while *Phomopsis* spp. and *Neofusicoccum* spp. showed very low levels of incidence of latent infection in new-emerged shoots. Thus, these two pathogen groups demonstrated less important roles among populations involved in latent infection. The dynamics of incidence of latent infection caused by *B. dothidea* showed variation among the three orchards and the incidence levels were higher than those of other pathogen groups (Figure 3). Variation of incidence caused by *Lasiodiplodia* spp. among different orchards was also observed. It seemed that the incidence of latent infection caused by *Cytospora* spp. showed a trend to decrease over time, but this should be confirmed by later samplings (Figure 3).

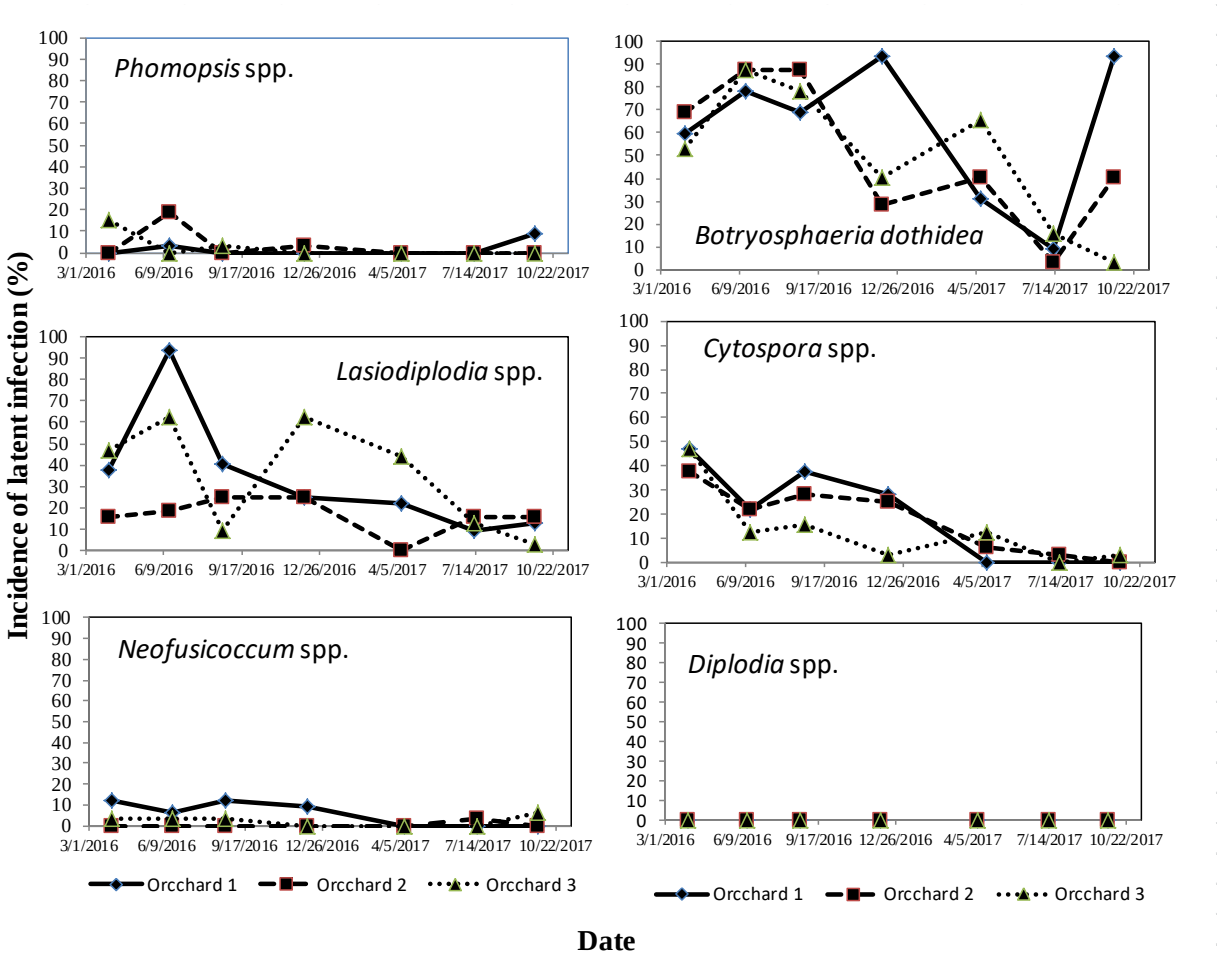

Figure 3. Dynamics of incidences of latent infections in new-emerged shoots caused by six canker-pathogen groups for three prune orchards in Yuba County during the period of time from March 2016 to September 2017. The real-time PCR assay was applied to process these shoot samples.

The samples of old shoots showed similar patterns of latent infections as for the new-emerged shoots (Figure 4). *B. dothidea*, *Lasiodiplodia* spp. and *Cytospora* spp. were three predominant pathogen populations in old shoots, while *Phomopsis* spp. and *Neofusicoccum* spp. showed lower levels of incidence over the season. Less variation in incidence among the three orchards was observed compared with those in new-emerged shoots (Figure 4). However, it also seemed that the incidence decreased over time for most pathogen groups (Figure 4) and this trend was quite consistent among the three orchards.

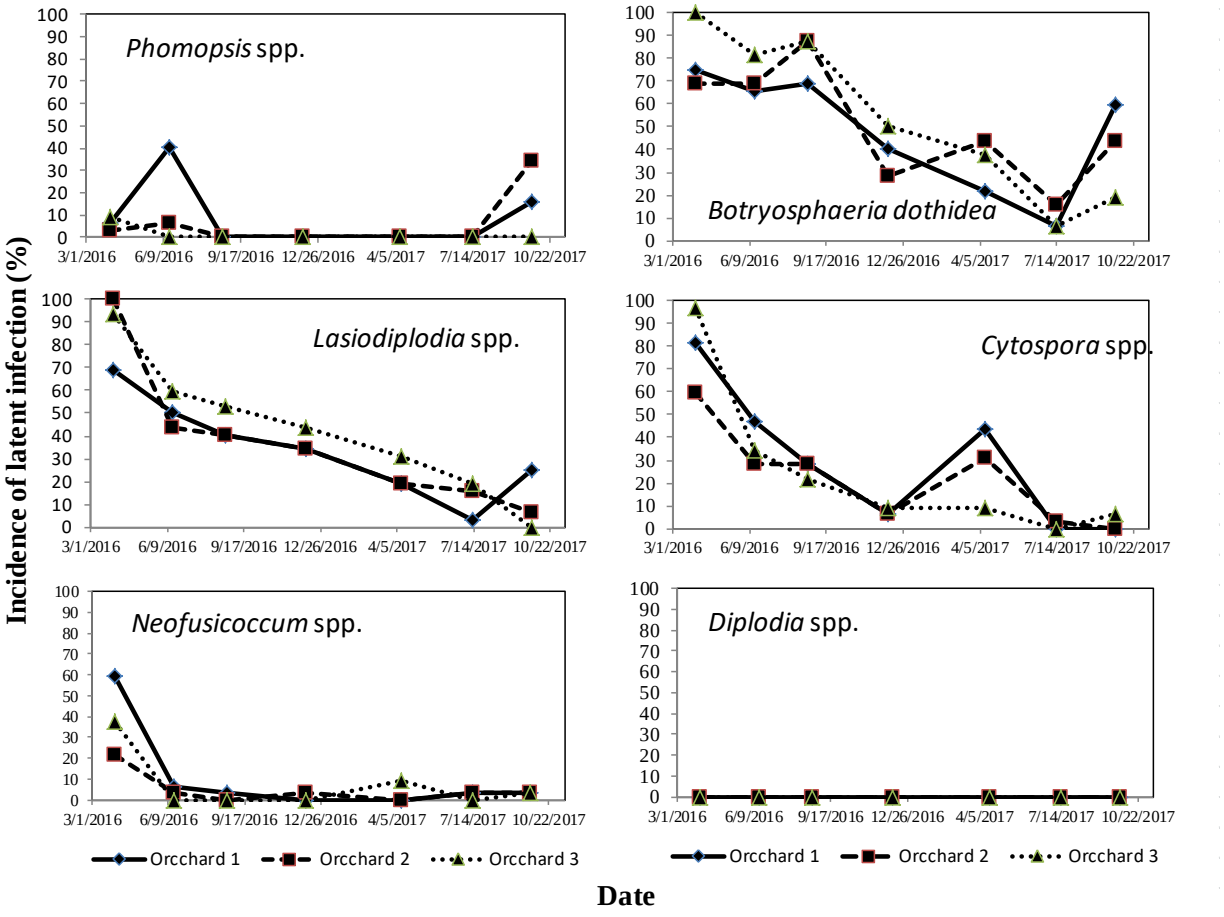

Figure 4. Dynamics of incidences of latent infections in old shoots caused by six canker-pathogen groups for three prune orchards in Yuba County during the period of time from March 2016 to September 2017.. The real-time PCR assay was applied to process these shoot samples.

The molecular severity (MS) reflects the level of infection in individual shoots that were positive. We used average MS and the corresponding standard deviation from 32 samples for each sampling time for data analysis. The dynamics of MS from March 2016 through September 2017 were studied for both new-emerged and old shoots for the three orchards. For the new-

emerged shoots, large variations in MS among orchards were observed for most pathogen groups, mainly because a few samples with low incidence showed high MS levels that increased variation. However, *B. dothidea* showed much less variation in MS among the orchards and among sampling dates (Figure 5). Basically, the average values of MS were in the range of 4 and 6. However, some orchards also showed very low levels of the MS (Figure 5). For all the pathogen groups, there is no clear trend of MS development over the time, namely, even as variations occurred, the changes in average MS were within a certain range, except for *Phomopsis* spp. and *Neofusicoccum* spp. (Figure 5).

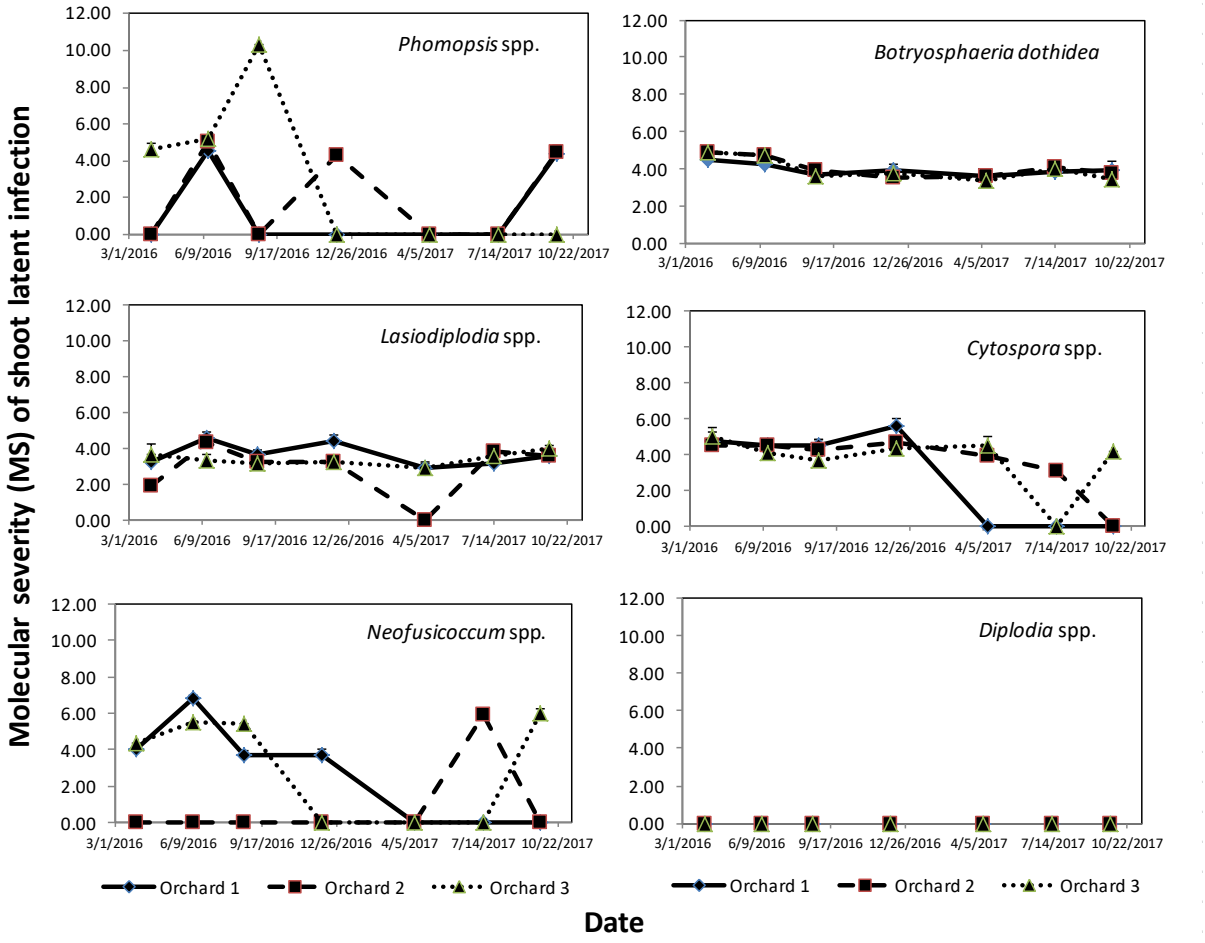

Figure 5. Dynamics of Molecular Severity (MS) of latent infections in new-emerged shoots caused by six canker-pathogen groups for three prune orchards in Yuba County during the period from March 2016 to September 2017. Thirty-two samples were collected for each orchard at each point in time, and the real-time PCR assay was applied to quantify the MS values for these shoot samples.

For the old shoot samples, similar patterns were observed for all the pathogen groups (Figure 6). Large variation was observed for *Neofusicoccum* spp. due to the reason explained above. The range of MS over the season for *B. dothidea* remained consistent, while for *Lasiodiplodia* spp. this increased compared with the new-emerged shoots. Obviously, the MS caused by *Cytospora*

spp. in old shoots was higher than in new-emerged shoots, especially in the early season of 2016 (Figures 5 and 6). This may imply an accumulation of the pathogen in shoots over the season. More samplings will be needed to determine the possible trends after the winter of 2017 and spring of 2018.

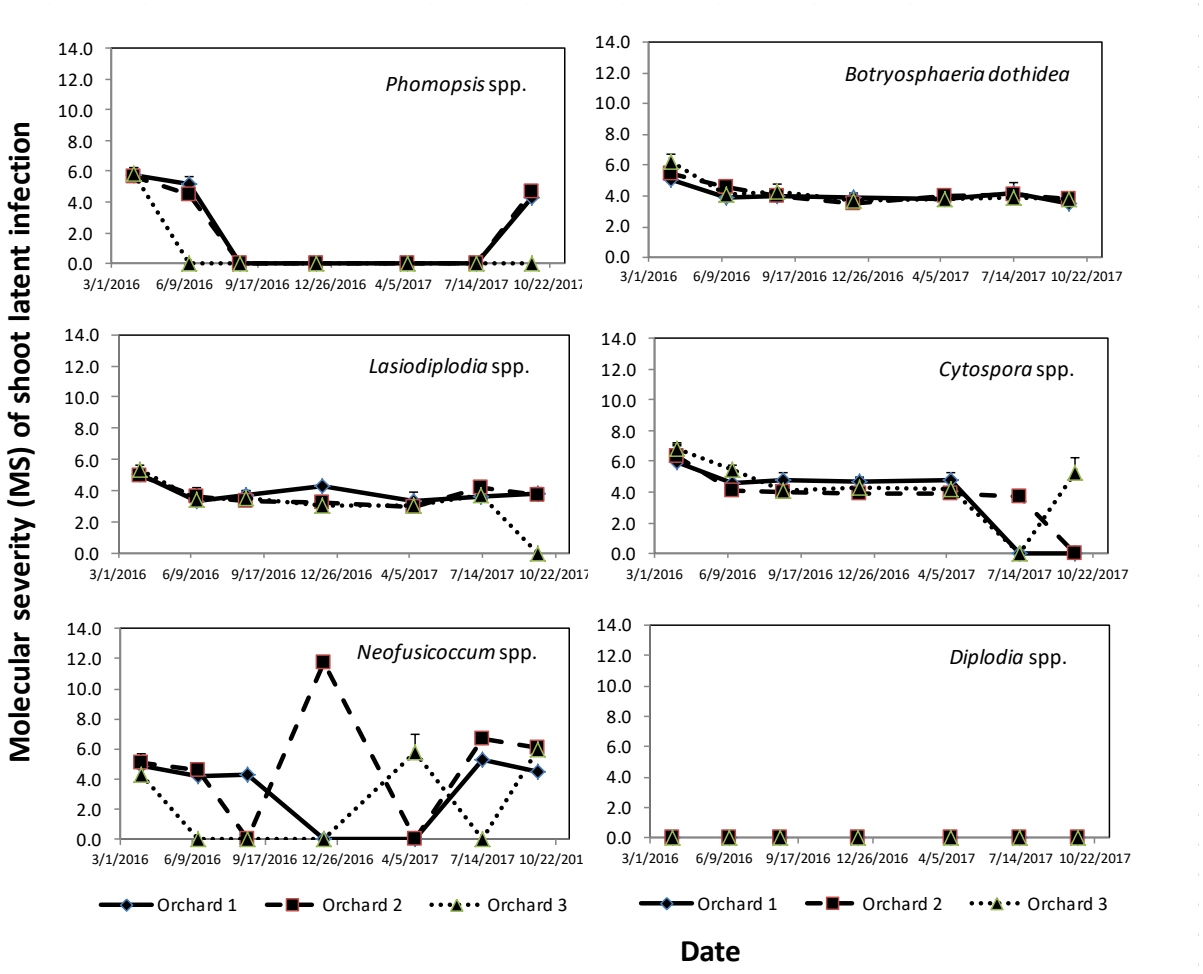

Figure 6. Dynamics of Molecular Severity (MS) of latent infections in old shoots caused by six canker-pathogen groups for three prune orchards in Yuba County during the period from March 2016 to September 2017. Thirty-two samples were collected for each orchard at each point in time, and the real-time PCR assay was applied to quantify the MS values for these shoot samples.

To evaluate the overall situation of latent infection level for an individual orchard, the Index of Latent Infection (ILI) is an applicable parameter for epidemiological research to compare the infection intensity among orchards and for different sampling times. The ILI could also be used to estimate the establishment of the pathogen population and the possible future risk of pathogen development, as well as the canker development potential. Figure 7 shows the ILIs on new-emerged shoots for the 6 pathogen groups for the three orchards. We can conclude that, in addition to *Diplodia* spp. which was not detected for all samples, *Phomopsis* spp. and *Neofusicoccum* spp. were not well developed in latency in new-emerged shoots and that the three

orchards showed quite consistent trends (Figure 7). The ILI caused by *B. dothidea* changed extensively (from 1 to 4 for most cases) over time and large variation existed among orchards. The ILI caused by *Lasiodiplodia* spp. also showed a large change in early season 2016, but the variation was significantly reduced later in the sampling time, and the range of ILI change was within 0 to 1 for most cases (Figure 7). For *Cytospora* spp. relatively larger variations of ILI among the orchards were observed from mid of 2016 to mid of 2017, and the range of change in ILI for most cases was from 0 to 2 (Figure 7).

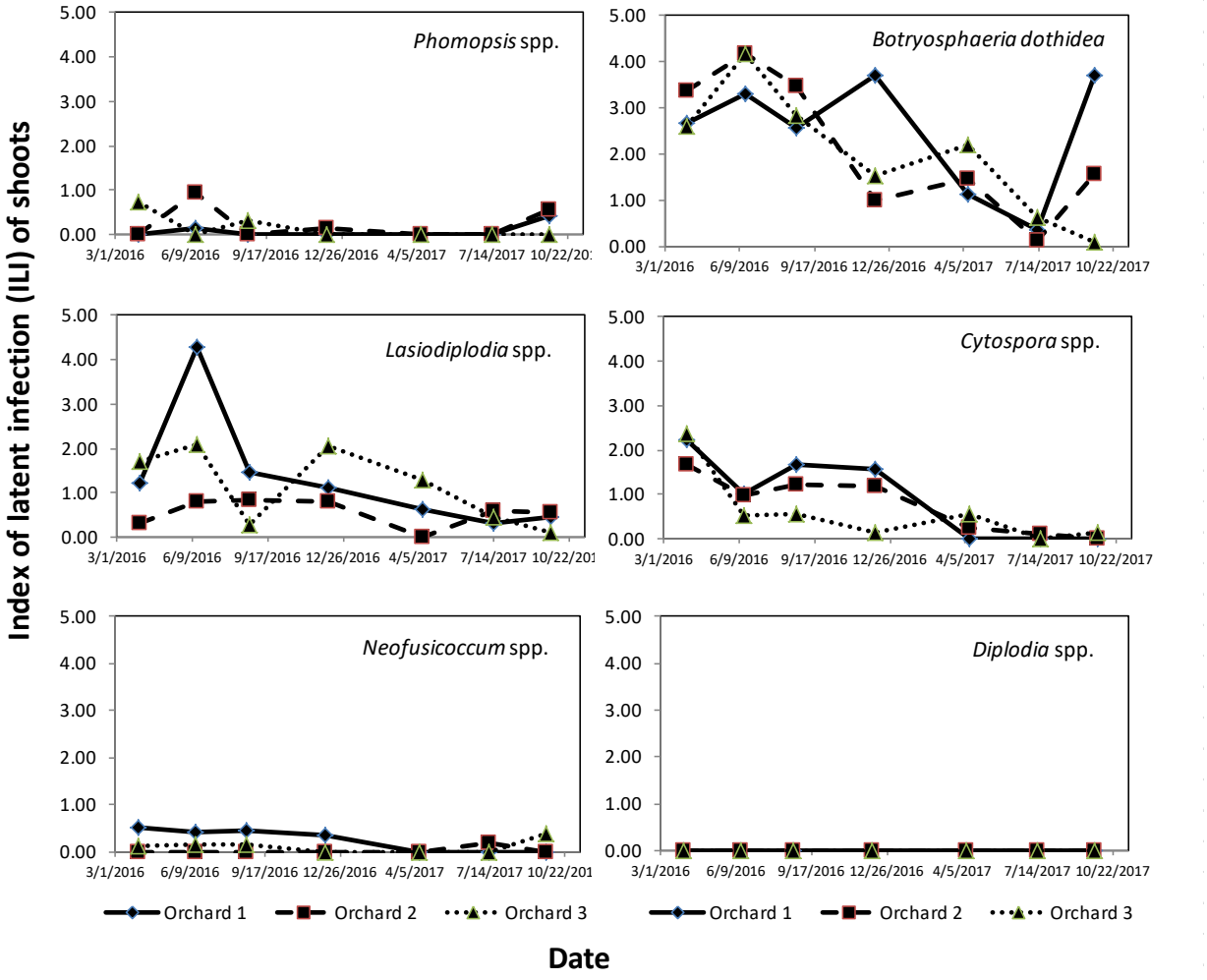

Figure 7. Dynamics of Index of Latent Infection (ILI) in new-emerged shoots caused by six canker-pathogen groups for three prune orchards in Yuba County during the period from March 2016 to September 2017. The ILI values were calculated based on Incidence and Molecular Severity (MS) from 32 samples for each sampling time for each orchard.

For the samples collected from old shoots, smaller variations in ILI were observed compared with those from new-emerged shoots. For *B. dothidea*, lower variations were observed for old shoots (Figure 8) than for new-emerged shoots (Figure 7) over the sampling dates, but the range

of ILI did not change much. However, for *Lasiodiplodia* spp., it is very clear that the variations in old shoots were significantly reduced compared to those from new-emerged shoots and the range of most ILI values was between 1 and 2 (Figure 8). The ILI values for *Cytospora* spp. in spring of 2016 (Figure 8) were much higher than those in new emerged-shoots (Figure 7). This clearly indicates a certain level of accumulation of *Cytospora* spp. as the shoot ages. The results also showed little change in ILI range for both *Phomopsis* spp. and *Neofusicoccum* spp. over the sampling dates (Figure 8).

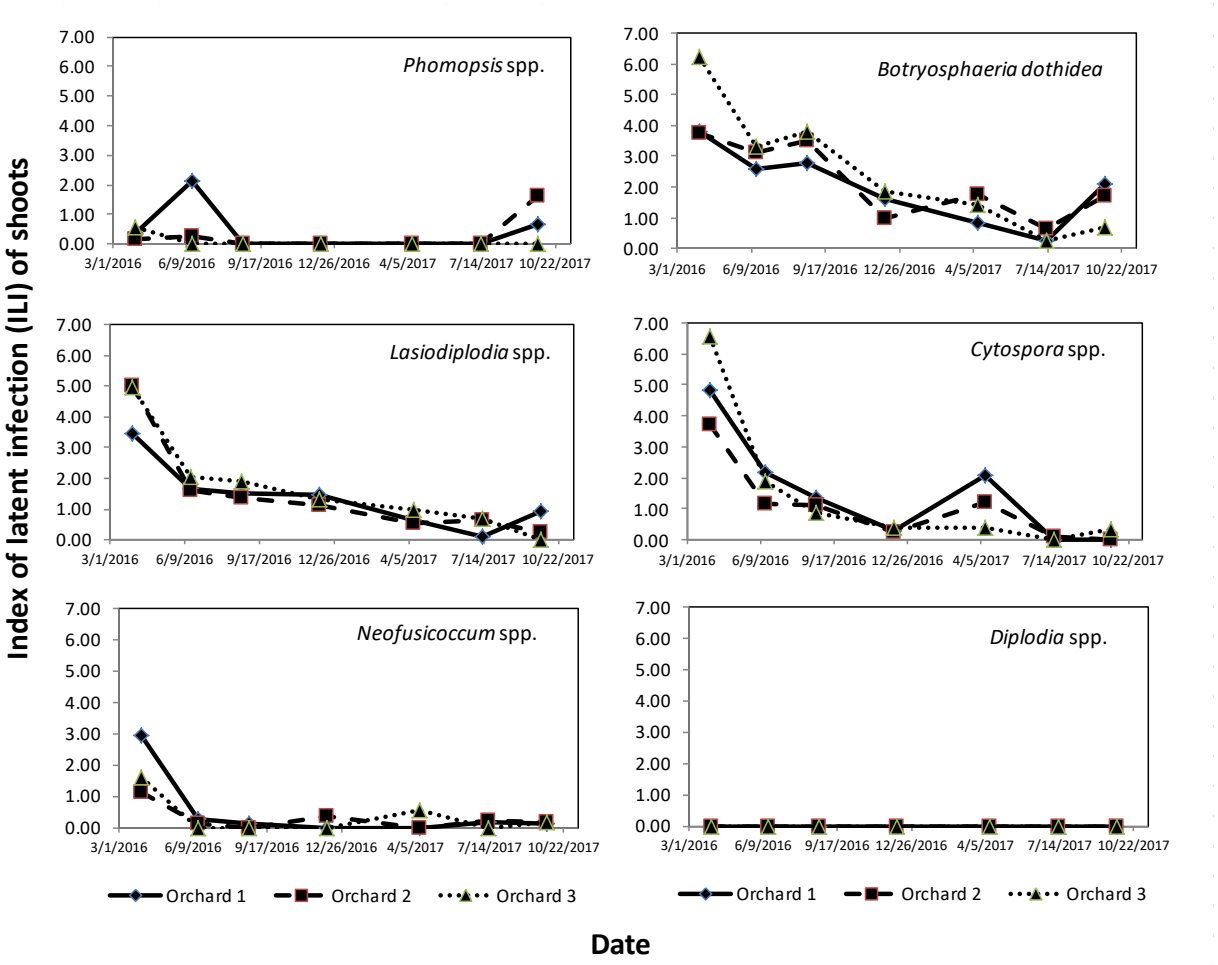

Figure 8. Dynamics of Index of Latent Infection (ILI) in old shoots caused by six canker-pathogen groups for three prune orchards in Yuba County during the period from March 2016 to September 2017. The ILI values were calculated based on Incidence and Molecular Severity (MS) from 32 samples for each sampling time for each orchard.

It seems that there is a trend that the ILIs were reduced over time for the sampling dates for the predominant three pathogen groups. Further samplings are needed to determine whether this ILI reduction was related to the season or not.

Objective 4. To study the effect of water stress and fertilizer on predisposing shoots to infection by *Cytospora* and other canker causing fungi.

Figure 9 shows the setting of potted prune trees at KARE. The four levels of water treatments, 25%, 50%, 75%, and 100% of regular irrigation were started on August 23, 2017. The inoculations with *Cytospora* app. were conducted on each branch of each tree on October 3, 2017. The shoot sampling was conducted on August 8, 2017 to determine the latent infection level by using *q*PCR assay described above. We will report the results on canker disease after water stress and nutrient deficiency treatments are conducted in 2018. The nutrient deficiency experiment will be conducted in spring to summer 2018.

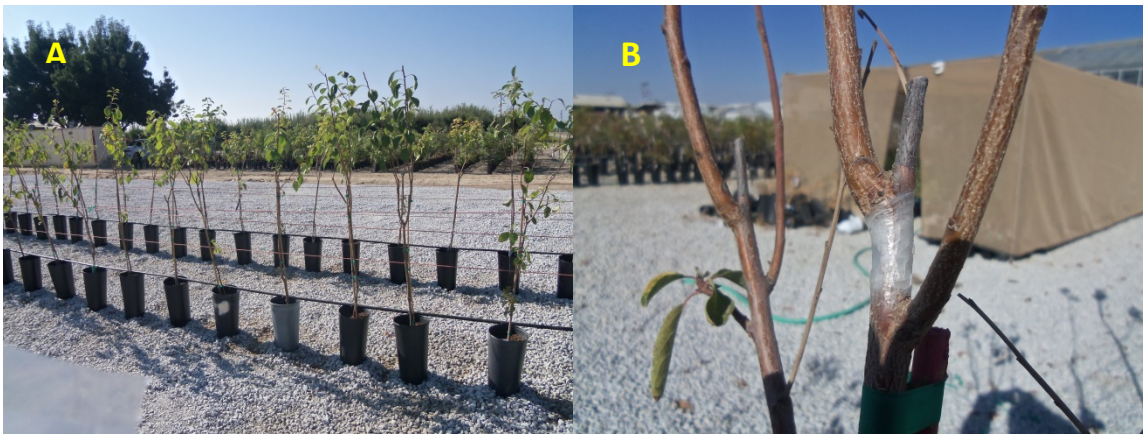

Figure 9. Photos of the potted prune trees for the experiment to study the effects of water stress and nutrient deficiency on canker development (A). Inoculations with *Cytospora* spp. were conducted on shoots of each potted tree on October 4, 2017(B)

Objective 5. To manage cankers by using fungicides and/or biological agents applied onto pruning wounds.

The 2017 experimental results of the fungicide trial in the prune orchard in Yuba County demonstrated that the fungicides Pristine+VitiSeal and Topsin M (70 WP) were the most effective in reducing the incidence of infection under natural infection conditions (Figure 10). The results showed that the incidences of cankers on wounds treated with Pristine + Pentra Bark, Tebuconazole and VitiSeal were not significantly lower than that of control, indicating that there was no effect on reducing canker disease. Although Quilt Xcell showed no significant differences in canker incidence compared with Pristine + Pentra Bark and Tebuconazole, the average incidence was significantly lower than those of VitiSeal and control (Figure 10).

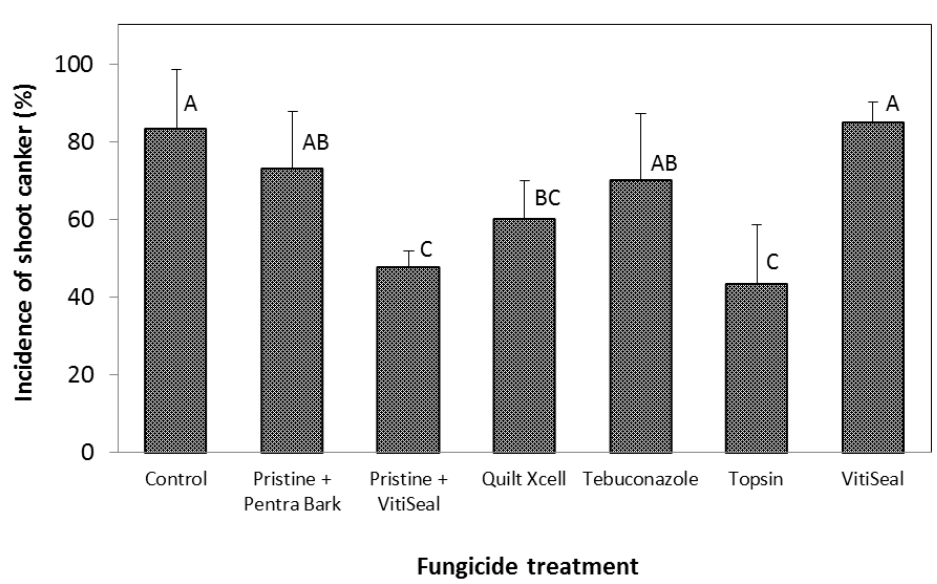

Figure 10. Incidence of shoot cankers with the fungicide treatments applied on shoot wounds after pruning in Orchard 1 under infection by natural spore inoculum. The trees were pruned on December 9, 2016, and the pruning wounds were painted with different fungicides. The disease was recorded on December 7, 2017, and the average value of disease incidence for each fungicide treatment was calculated from three replicates each with 10 pruned shoots.

We did not find an effect of Biologicals (*Bacilius* + *Tricoderma*) on reduction of canker incidence (Figure 11) on wounds treated in February of 2017. The results show that the canker incidence with this treatement was even higher than that of the water control (Figure 11). There was no significant difference in canker incidence between Topsin and water control in this experiment (Figure 11). This might be because of late pruning, large variation, or other environmental effects on canker development. No differences in incidence between Biologicals + Topsin and water control were found (Figure 11).

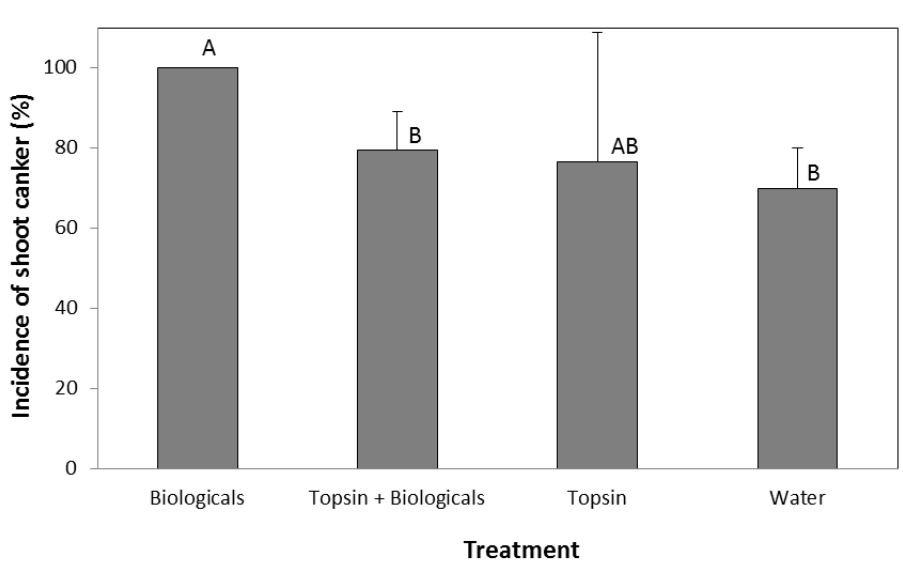

Figure 11. Incidence of shoot cankers with biocontrol agent and combination of biocontrol with fungicide Topsin treatments on shoot wounds after pruning in Orchard 1 with infection by natural spore inoculum. The trees were pruned on February of 2017, and the pruning wounds were painted with different biocontrol agent or fungicides. The

disease was recorded on December 7, 2017, and the average value of disease incidence for each fungicide treatment was calculated from three replicates each with 10 pruned shoots.

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

In 2017, five objectives were planned for this project. The monthly inoculations on wounded shoots starting in March 2017 were continuously conducted at Kearney Agric. Research and Extension Center. Three canker-causing pathogens, *B. dothidea, L. citricola and Cytospora* spp. were used in inoculations. Based on disease recording in November 2017, high canker incidences were observed for inoculated samples but no differences among inoculation dates for the three pathogens were observed. However, large variations in canker severity among different inoculation dates were observed for all three pathogens. In general, for the three pathogens, the inoculations early in the season could promote higher canker severity compared with other time periods throughout the season. Thus, the riskiest period of time for promoting higher chance of severe cankers could occur early in the growing season. Our previously-developed real-time PCR quantification assay could be used in several aspects of the research. For quantification in rainwater, although different canker-causing pathogens were observed in rain samples, the period of time when the most pathogens could be detected in rainwater was from winter to spring. Thus, rainy season could be the highest risk period for occurrence of pathogen infection on shoots. The pathogen groups *B. dothidea, Lasiodiplodia* spp., *Cytospora* spp., *Neofusicoccum* spp. were detected in rain samples in different time periods and in different orchards. Using the *q*PCR assay, we continuously studied the dynamics of latent infection in three orchards showing severe canker disease and we used three parameters: Incidence, Molecular Severity (MS) and Index of Latent infection (ILI), to describe the latent infection level. The pathogen groups *B. dothidea, Lasiodiplodia* spp., and *Cytospora* spp. were detected as the predominant pathogens for latent infections in both new-emerged and old shoots, while *Phomopsis* spp. and *Neofusicoccum* spp. caused very low levels of latent infections. *Diplodia* spp. were not detected in any of the samples. *Cytospora* spp. may be accumulated as shoots age, while more samplings may be needed to confirm for other pathogens. Variations in dynamics of incidence of latent infection among orchards existed, which implied that the canker disease could develop in individual orchards separately without exchange of pathogen populations among orchards. Thus, the initial conditions in an orchard such as initial infection, pathogen component, inoculum source, and other environmental factors may play an important role in later disease development. The

possible existence of pathogen accumulation as shoots age needs to be further examined. Based on this year's trial, treatment on wounds with the fungicides Pristine+VitiSeal and Topsin M (70 WP) could significantly reduce the incidence of canker development. Quilt Xcell also showed an effect on reduction of canker development compared with other fungicides. The biocontrol agent *Bacilius* + *Trichoderma* was not found to be effective for surpressing canker development on wounds. We set up an experiment to study the effects of water stress and nutrient deficiency on canker development and finished the inoculation this year. We will continue this experiment and gather results in 2018.

References

Chen, S. F., Morgan, D. P., Hasey, J. K., Anderson, K., and Michailides, T. J. 2014. Phylogeny, morphology, distribution, and pathogenicity of *Botryosphaeriaceae* and *Diaporthaceae* from English walnut in California. Plant Disease 98: 636-652.

Adams, G. C., and Jacobi, W. R. 2016. Cytospora canker of Hardwoods. USDA Forest Service RMRS-GTR-335: 91-93.

Luo, Y., Gu, S., Felts, D., Puckett, R. D., Morgan, D. P. and Michailides, T. J. 2017. Development of qPCR systems to quantify shoot infections by canker-causing pathogens in stone fruits and nut crops. Journal of Applied Microbiology 122: 416-428.