Characterization of a Phytoplasma Associated with Almond Brown Line Disease and Development of a Molecular Assay for Its Detection

Project No.: **10-PATH9-Sudarshana**

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Objectives:

Objective 1: Survey the orchard for symptomatic trees in spring, summer and fall season.

Objective 2: Graft bud chips from symptomatic trees on to peaches and almonds on Marianna 2624 rootstock.

Objective 3: Conduct PCR-based assays on leaf and petioles and developing green almonds from 'Winters' and 'Sonora' trees for the presence of PYLR phytoplasma.

Objective 4: Develop specific primers for quantitative real time PCR and monitor the titer of PYLR phytoplasma in peach and almond trees maintained at UCD orchard.

Interpretive Summary:

Investigations were conducted on an outbreak of almond brown line (ABL) disease in a 2nd leaf stage almond orchard in Sutter County to determine the etiology of the pathogen and to develop a molecular assay to detect the agent. The outbreak lasted from 2nd leaf (year 2008) to 4th leaf (2010) with most of the trees showing disease in the 2nd and 3rd leaf stage. Annual surveys for symptomatic trees indicated there was no horizontal spread of the disease in the orchard and the diseased trees died within a year or two thus self-eliminating the source of the pathogen. Previous study conducted by Uyemoto et al. (1992) provided anecdotal evidence

for the association of peach yellow leafroll phytoplasma as the causal agent of ABL and this investigation was focused on determining the association of a phytoplasma.

Polymerase chain reaction (PCR) assays using phytoplasma specific general primers P1 and Tint, and primers specific to 16S-23S rDNA regions of peach yellow leafroll phytoplasma (PYLR-P) were able to amplify products of 1.6 kb and 530 bp, respectively, from nucleci acid extracts from diseased trees. However, primers specific to non-rDNA regions of PYLR-P and PD-P failed to amplify a product of expected size from almond trees with ABL symptoms and indicated the phytoplasma in question is related to PYLR-P only in the rDNA region, but not elsewhere in the genome. A quantitative PCR (qPCR) for real-time detection of the phytoplasmas, PYLR-P, PD-P and ABL-P, has been optimized to detect these phytoplasmas in peach, almond and pear samples, respectively. A two-year incubation period following graft inoculations using sidegrafts from almond tree showing ABL symptoms has not been able to reproduce ABL symptoms on test plants of almonds on Marianna 2624. Future studies have to address establishment of the genetic nature of ABL-P, its role in ABL development, and natural reservoirs.

Background:

Almond brown line (ABL) disease was first reported in California in 1987 on Carmel, Peerless and Price almond trees on plum rootstock Marianna 2624 (M2624; *Prunus cerasifera* X *P. munsoniana* (Uyemoto et al., 1992). The affected trees barely had new shoot growth and had small chlorotic leaves rolled upward. Diseased trees, showed bark split and union disorder, and a brown line consisting of necrotic phloem tissue and pits and grooves on the woody cylinder. Most of the affected trees died within a year or two. Attempts to characterize the etiological agent were not successful because the bud chips from brown line affected trees failed to reproduce disease symptoms. However, bud chips from peach trees infected with Peach yellow leafroll phytoplasma (PYLR-P) produced brown line on Marianna 2624 trees indicating the likely involvement of PYLR-P in causing the brown line disease in orchards on Marianna 2624.

Almonds in California are predominantly grown on peach rootstocks. However, plum rootstock Marianna 2624 is a preferred in marginal lands and heavy soils. This plum rootstock is also moderately resistant to *Armillaria*, phytopthora crown and root rots, and root-knot nematodes. Brown line disease has been found only on almond trees on Marianna 2624 but never on peach rootstock. A few years after the brown line disease was recognized, ‘Nonpareil’ trees on peach rootstock ‘Nemaguard’ in an orchard near Modesto, CA, were reported to have a late bud break and stunted growth with nuts containing shriveled kernels (Uyemoto et al., 1999). A year later, similar symptoms were noticed in an adjacent orchard of ‘Butte’ trees also propagated on peach. Nucleic acid extracts from these trees tested negative for viruses, but reacted positively with a DNA probe specific for PYLR-P and X disease phytoplasma (WX-P). Injection of oxytetracycline into affected trees caused new shoot growth indicating the association of phytoplasmas with the shriveled kernel disease. When peach bud chips from diseased trees were grafted on healthy ‘Peerless’ almond on Marianna 2624, typical brown line disease developed, while control bud chips from trees infected with WX-P did not show the brown line disease. Together, these evidences point to PYLR-P as the etiological agent of

almond brown line disease. However, the identity of the agent that caused brown line disease in nature could not be established.

Genetic comparison of PYLR phytoplasma with the European Pear decline phytoplsama (= *Candidatus* phytoplasma pyri) by restriction fragment length polymorphism analysis of PCR amplified rDNA has shown that these two phytoplasmas are very closely related (Kison et al., 1997). *Cacospylla pyricola*, a vector of pear decline phytoplasma, was found in traps set in peach orchards with peach yellow leafroll disease and it was suggested to be a possible vector of PYLR-P (Purcell and Suslow, 1984). The role of PYLR-P in causing the disease on peaches has been well established. However, the association of PYLR-P with ABL has not been seen.

In spring 2008, inspection of a second-leaf almond orchard in Sutter County revealed several trees of cv. Winters exhibiting stunted growth. The affected trees had poor canopy with chlorotic leaves that had rolled up. Some of the affected trees had snapped at the graft union, and upon careful examination the graft unions were found necrotic, thus making the trees vulnerable to breakage by high winds. Examinination of symptomatic trees, by lifting the bark at the union, revealed that these trees were affected by brown line disease. The symptoms were very similar to those described by Uyemoto et al. in 1992. Several symptomatic trees died during the following months. This orchard had been planted with three almond cultivars 'Aldrich', Sonora' and 'Winters', on plum rootstock Marianna 2624. In a survey in 2008, we found 13 trees of cv. Winters and one tree of cv. Sonora affected by the disease. In the following year, 11 and 8 more trees of cvs. Sonora and Winters, respectively, showed ABL symptoms.

This project was initiated with a long term objective of (1) establishing the causal agent of ABL, and (2) to develop molecular assays for its detection so that ABL could be avoided in young almond orchards.

Materials and Methods:

Objective 1.

Surveying the orchard for symptomatic trees: We conducted surveys during spring, summer, and fall, in 2010 and 2011 in an almond orchard in Sutter County where almond brown line (ABL) was first noticed in spring 2008. A total of 1737 trees were surveyed in a block containing 27 rows, each containing upto 68 trees (rows 1 to 15), and examined for ABL disease symptoms such as pale green canopy, rolled up leaves and brown line at the graft union. Symptomatic trees were flagged for future observations.

Objective 2.

Bioassays using sidegrafts: In 2010 and 2011, trees showing symptoms for the first time were not seen and trees that showed symptoms in year 2009 failed to survive to allow more budding. Almond trees sidegrafted in year 2009, using 20 to 25 cm long side grafts from a sympotamtic tree that tested positive in PCR assays, were monitored for symptom development.

Objective 3
Conduct PCR-based assays on leaf and petioles and developing green almonds from 'Winters' and 'Sonora' trees for the presence of ABL phytoplasma.

This objective was not pursued because of the death and subsequent removal of diseased almond trees in Sutter county orchard targeted in this research project.

Objective 4:
Develop specific primers for quantitative real time PCR and monitor the titer of phytoplasmas in peach and almond trees maintained at UCD orchard.

Molecular assays for the detection of the phytoplasmas: Leaf samples were collected from symptomatic and asymptomatic almond trees in the Sutter County orchard in fall 2008 and 2009. Leaf samples from almond and peach trees inoculated with PYLR-P, and a Bing cherry tree inoculated with WX-P in our research plots were included as controls. In addition, columellae (endocarp) from pear fruits from trees infected with pear decline phytoplasma (PD-P) were also collected. Nucleic acid extract from plant samples were obtained using Plant DNeasy mini kit (www.qiagen.com). Phytoplasma 16S-23S rRNA specific general primers OP1 and Tint (Smart et al., 1996) were used to amplify ~ 1.6 kb product from nucleic acid (NA) extracts on an Eppendorf *Mastercycler* ***ep****gradient* (www.eppendorf.com). PCR conditions were 1 cycle of 94 °C for 2 min; 40 cycles of 94 °C for 30 sec, 62 °C for 30 sec, 72 °C for 2 min; and 1 cycle of 72 °C for 5 min. The amplified products were analyzed by electrophoresis using 1% agarose in Tris acetate EDTA buffer (TAE).

Design of a new set of primers for the detection of almond brown line phytoplasma: The amplified product from NA extracted from ABL-P samples was cloned into plasmid vector using a TOPO-TA cloning kit (www.invitrogen.com) and sequenced at UCD sequencing facility. The sequences were aligned with those of PYLR-P and PD-P. A new set of forward and reverse primers, PYLRrRNAf606 and PYLRrRNAr1135, respectively, were designed to amplify a 530 bp product specifically from ABL-P, PD-P and PYLR-P. PCR conditions were 1 cycle of 94 °C for 2 min; 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec; 72 °C for 1 min; and 1 cycle of 72 °C for 5 min. The amplified products were analyzed as above.

Determination of genetic relationship between phytoplasmas causing almond brown line, peach yellow leafroll, and pear decline: Two primers, fCPD and rCPD, specific to non rDNA region of PD-P (Kison et al. 1997), were used to amplify ~1.5 kb product from NA extracts from symptomatic and healthy trees. PCR conditions were 1 cycle of 94 °C for 2 min; 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec; 72 °C for 2 min 30 sec; and 1 cycle of 72 °C for 5 min. The amplified products were analyzed using 1% agarose as described above.

Development of a Quantitaive PCR (qPCR) assay for the detection of almond brown line phytoplasma: Assays were performed on NA extracts using Brilliant SYBR®green qPCR master mix on a Mx3000P qPCR system (www.stratagene.com). A 530 bp amplified product from ABL-P obtained using CPPrRNAf606 and CPPrRNAr1135 primers was cloned using the TOPO-TA cloning kit. Plasmid DNA containing the cloned product was spiked into NA extract from healthy almond leaves and assay conditions were standardized to detect ABL-P specific

DNA and obtain dissociation constants. The PCR conditions were one cycle of denaturation at 95 °C for 10 min; 40 cycles of 95 °C for 30 sec, 60 °C for 1 min, and 72 °C for 1min 40 sec; and one cycle of 72 °C for 3 min. Dissociation constants were obtained by denaturation at 95 °C for 1 min followed by annealing at 55 °C for 30 sec and continuously monitored till melted completely at 95 °C for 30 sec.

Results and Discussion:

Survey of an almond orchard with almond brown line in 2010 and 2011.

Yearly surveys were conducted in the years 2010 and 2011 in a commercial orchard in Sutter County affected by ABL in 2008. We did not see development of almond brown line (ABL) in fourth (Year 2010) and fifth leaf trees (Year 2011). Our results indicate that brown line disease did not spread horizontally in almond orchards. These results were similar to those observed by Uyemoto et al. (1992) wherein only young trees succumbed to the disease. It appears that removal of symptomatic trees and replanting with new healthy trees can be an effective strategy to quickly overcome the impact of ABL in young almond orchards. However, this argument will be strengthened by future surveys, because in the event the putative agent is delivered by insect vectors into almond scions, the agent may take several years to reach the graft union to initiate development of brown line in the tree.

The absence of new infections, coupled with the death of trees symptomatic in 2009, did not allow us to continue grafting experiments to determine the graft-transmissibility of the putative agent responsible for ABL in symptomatic trees. Sidegrafts from a symptomatic tree, positive for phytoplasma in PCR assays in 2009, remained alive on grafted almond trees of Peerless on M2624. However, none of these trees have developed ABL symptoms. It has to be noted that bud chip inoculations done by Uyemoto et al. (1992) also failed to reproduce ABL symptoms during the study period of four years post inoculation. The absence of disease symptoms, perhaps, indicates a slow movement of the etiological agent from side grafts to the scion and a delay in brown line development at the graft union.

Development of specific primers for quantitative real time PCR (qPCR).

It is well established that a 1.6 kb product specific to phytoplasmas could be amplified using P1 and Tint primers (smart et al., 1996). We were able to amplify ~1 6 kb product product from NA obtained from leaves of almond trees showing ABL symptoms (**Figure 1**). However, not always, could a product be amplified from trees with ABL symptoms (**see lanes 2 to 4 in Figure 1**). Even when a product was seen, the PCR conditions required 40 cycle amplification, while NA from PYLR-P infected almond and peach trees and WX-P infected cherry trees consistently gave a positive reaction in 30 cycles.

When the amplified products were cloned and sequenced, the sequence of P1 primer was found having a change at the very 3'-end base with respect to the sequences of 16S-23S rDNA regions of several stone fruit phytoplasmas (**Figure 2**). We believed this base change, as well as the longer length (1.6 kb) of the amplified product, may not permit detection of the phytoplasmal DNA at extremely low titer. To overcome some of these problems, new primers were designed to amplify a 530 bp product from the 16S-23S rDNA regions of ABL-P, PD-P and PYLR-P. These primers, PYLRrRNAf606 and PYLRrRNAr1135, consistently amplified a product of expected size in 30 cycles (**Figure 3**) from ABL-P, PD-P and PYLR-P NA, but not

from a cherry tree infected with WX-P or a healthy almond tree. However, NA from majority of almond trees with ABL required 40 cycles of amplification even with these primers (**see lane 2 in Figure 3, 30 cycles; and Figure 4, 40 cycles**). Also, along with the expected size product, several smaller size products were also amplified from NA from ABL trees, but not from PYLR-P infected almond tree and healthy almond tree (**Figure 4**).

<u>Genetic relationship of Almond brown line phytoplasma with Peach yellow leafroll phytoplasma.</u>

The rDNA regions of several phytoplasmas are known to be identical, but elsewhere the genomes can differ significantly. To determine if the phytoplasma associated with ABL is the same as PYLR-P and PD-P, primers fCPD and rCPD (Lorenz et al., 1995), specific to non-rDNA region, were used to amplify products from nucleic acid extracts from trees infected with phytoplasmas. These primers amplified a product of similar in size from nucleic acid extracts made from PYLR-P and PD-P infected trees (**Figure 5, lanes 3, 5 & 7**), but not from ABL trees and cherry trees with WX-P (**Figure 5, lanes 2 & 9**). These results indicate that ABL-P is genetically distinct to PD-P and PYLR-P, even though in the 16S-23S rDNA region they are nearly identical to each other.

<u>A quantitative PCR assay for the detection of almond brown line phytoplasma.</u>

To estimate the titer of phytoplasma in infected trees and to develop high throughput screening, CPPrRNAf606 and CPPrRNAr1135 primers were used along with a SYBR®green based master mix for quantitative PCR (qPCR). We were able to detect the putative phytoplasma causing ABL, PYLR-P, and PD-P, consistently in qPCR reactions (**Figure 6; Table 1**). Cloned plasmid DNA containing a 530 bp product amplified from NA obtained from an almond tree showing ABL was used to spike NA from healthy almond trees at various concentrations. Positive Ct values were obtained for samples containing as low as 0.1 ng of DNA (data not shown). Also, when cloned DNA was spiked at 10^2 to 10^6 copies of DNA in the qPCR reaction mix, positive results were obtained by as low as 10^2 copies of cloned DNA (Table 1). Nucleic acid extracts from WX-P infected cherry trees, healthy almond, peach and pear were consistently negative in qPCR assays. We were also able to detect PYLR-P in dormant buds from infected trees, but the Ct value was very high.

Our results provide evidence for the presence of a phytoplasma in nucleic acid extracts from almond trees with ABL and this phytoplasma does not appear to be genetically related to PYLR-P. However, unlike in case of PYLR, bud chip inoculations from ABL trees have not reproduced the ABL symptoms, a key feature required to establish the nature of the causal agent. Future studies should address detailed observations on inoculations made with bud chips from ABL trees. New technology such as whole genome amplification and next generation sequencing might be the way to go to determine genetic nature of the phytoplasma present in nucleic acid extracts from ABL trees.

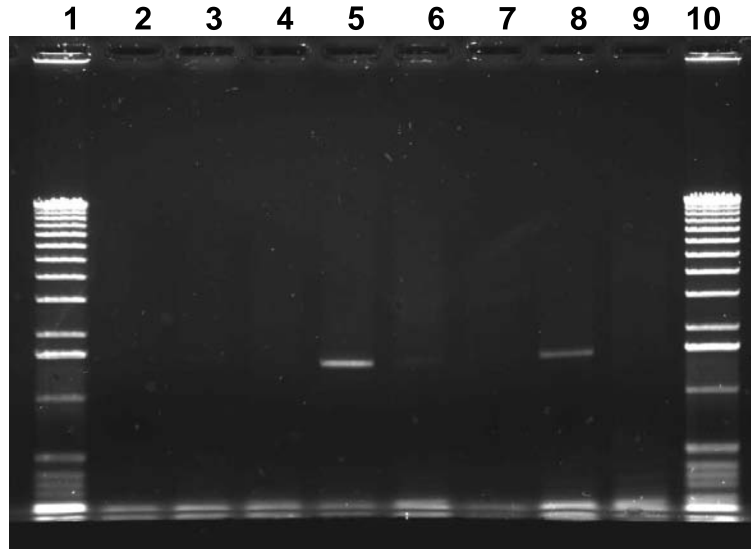

Figure 1. Agarose gel showing 1.6 kb PCR product amplified from an almond tree affected by brown line disease using P1 and Tint primers. Lanes 1 and 10, 1 kb DNA ladder (Invitrogen); lanes 2 to 7, samples from almond trees with brownline; lane 8, almond tree inoculated with PYLR phytoplasma; lane 9, healthy almond tree.

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O-P1                  5'-AAGAGTTTGATCCTGGCTCAGGATT-3'
PD-P    AJ542543.1    5'- AGAGTTTGATCCTGGCTCAGGATG-3'
CPM     AJ542542.1    5'- AGAGTTTGATCCTGGCTCAGGATG-3'
PYLR-P  Y16394.1      5'-      TTGATCCTGGNTCAGGATG-3'
WX-P    AF533231.1    5'-AAGAGTTTGATCCTGGCTCAGGATG-3'
PD-P-T  DQ011588.1    5'-AAGAGTTTGATCCTGGCTCAGGATT-3'
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Figure. 2. Alignment of nucleotide sequence of phytoplasma 16S-23S rDNA spacer region specific forward primer P1 with corresponding sequences from selected phytoplasmas of apple proliferation group. O-P1= Sequence of Primer O-P1 (Smart et al., 1996); PD-P = *Candidatus* Phytoplasma pyri (Germany); PD-P-T= *Candidatus* Phytoplasma pyri (Taiwan); CPM= *Candidatus* Phytoplasma mali (France); PYLR-P= Peach yellow leafroll phytoplasma (California); WX-P= Western X phytoplasma (California).

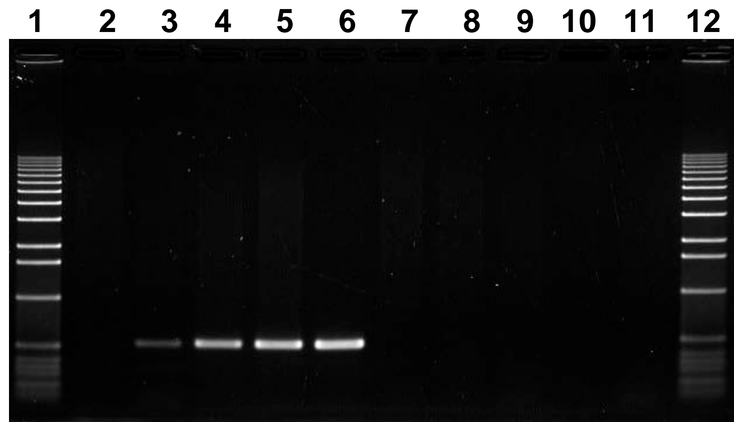

Figure 3. Agarose gel showing 530 bp product amplified by PCR from nucleic acid extracts of trees infected with phytoplasmas using primers PYLRrRNAf606 and PYLRrRNAr1135 after 30 cycles of amplification. Lanes 1 and 12, 1 kb DNA ladder (Invitrogen); lanes 2 and 3, almond trees with ABL symptoms; lanes 4 and 5, PYLR-P infected almond and peach, respectively; lane 6, Pear with PD-P; lane 7, WX-P in cherry; 8, 9, and 10, healthy almond, peach and cherry, respectively; lane 11, water.

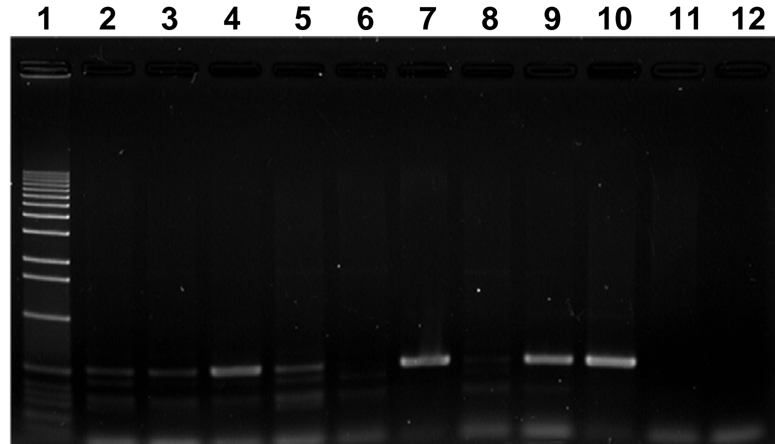

Figure 4. Agarose gel showing PCR products amplified from nucleic acid extracts of almond trees with ABL using primers PYLRrRNAf606 and PYLRrRNAr1135 after 40 cycles of amplification. Lanes 1 and 12, 1 kb ladder; lanes 2 to 9, almond trees with with ABL symptoms; lane 10, almond tree infected with PYLR-P; lane 11, healthy almond tree; lane 12, water.

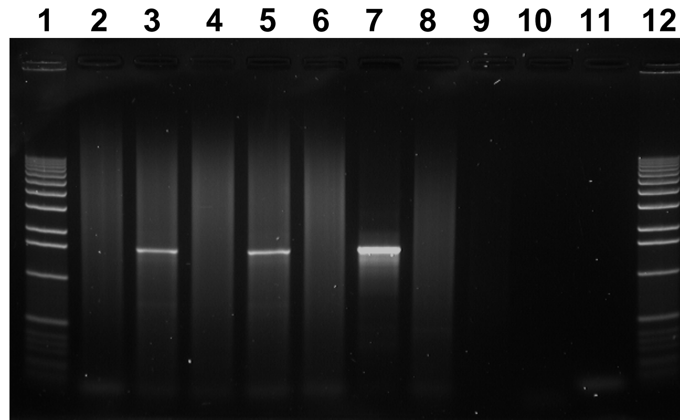

Figure 5. Agarose gel showing PCR products amplified by fCPD and rCPD using nucleic acid extracts obtained from trees infected with phytoplasmas. Lanes 1 & 12, 1 Kb ladder; Lane 2, nucleic acid extract from almond tree with brown line disease; lane 3, almond tree infected with peach yellow leafroll phytoplasma; lane 4, healthy almond tree; lane 5, peach tree infected with peach yellow leafroll phytoplasma; lane 6, healthy peach tree lane 7, pear tree infected with pear decline phytoplasma; lane 8, healthy pear tree; lane 9, cherry tree infected with Western X phytoplasma; lane 10, healthy cherry tree; lane 11: water.

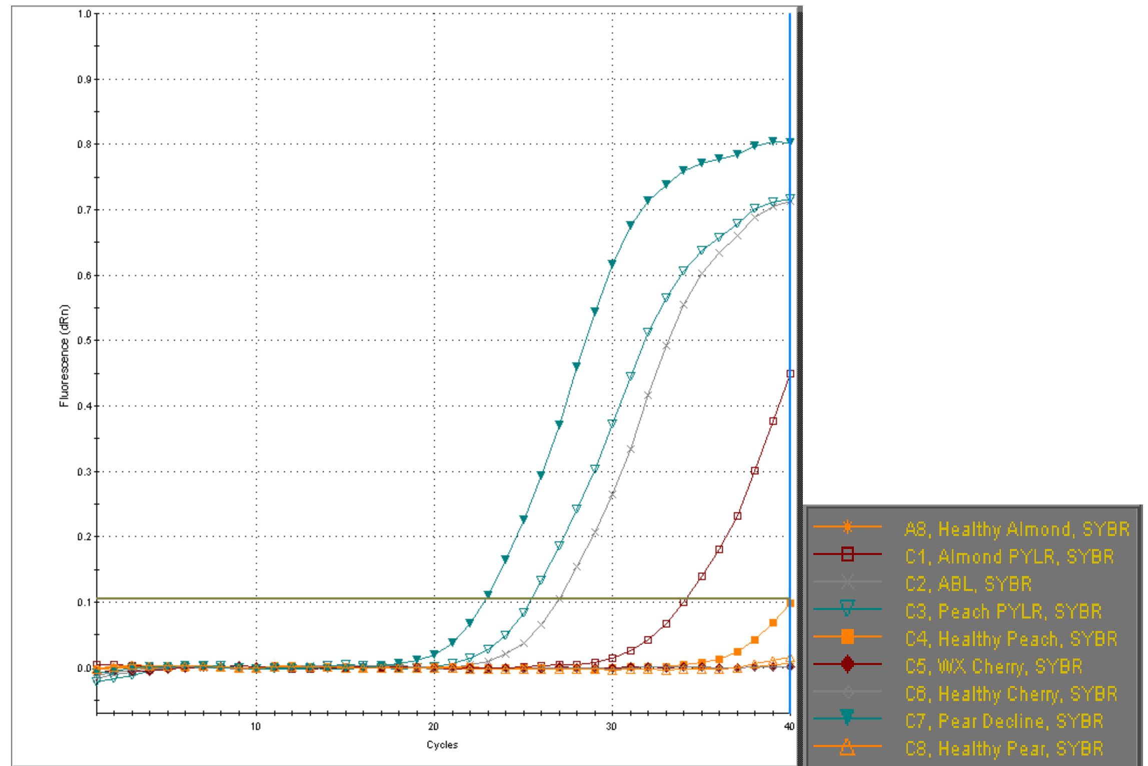

Figure 6. Graph showing real-time monitoring of accumulation of PCR products from nucleic acid extracts using SYBR green. Note almond brown phytoplasma (C2), peach yellow leafroll phytoplsma in almond (C1) and peach (C3), and pear decline phytoplasma in pear (C7) are positive.

Table 1. Sensitivity and specificity of detection of phytoplasma DNA by quantitative real-time PCR for stone-fruit phytoplasmas

DNA	Mean Ct	Tm °C
a) **Cloned DNA**		
10^6	19.9	81.9
10^5	21.9	81.9
10^4	25.5	81.9
10^3	23.4	81.9
10^2	22.8	81.9
b) **Phytoplasmas**		
1) In Leaf extracts		
ABL - almond	27.0	81.9
PYLR - almond	34.1	82.3
PYLR - peach	25.5	82.3
PDP - pear	22.9	82.3
2) In dormant buds		
PYLR- Almond buds	34.6	82.3
PYLR- Peach buds	39.8	82.3
c) **Negative controls**		
WX Cherry	No Ct	
Healthy almond	No Ct	
Healthy peach	No Ct	
Healthy pear	No Ct	
Water	No Ct	

Ct = cycle threshold (Ct); Tm = dissociation constant.

Research Effort/Recent Publications:

Sudarshana, M.R., Gonzalez, A., Dave, A.,Uyemoto, J.K. 2011. A quantitative PCR assay for the detection of phytoplasmas causing almond brownline, peach yellow leafroll, and pear decline diseases in California. Phytopathology 101:S172. Poster presented at the annual meeting of the American Phytopathological Society.

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References Cited:

Lorenz, K. H., Schneider, U, and Seemüller, E. 1995. Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and nonribosomal DNA. The American Phytopathological Society. 85: 771-776.

Kison, H., Kirkpatrick, B. C., and Seemüller, E. 1997. Genetic comparisons of the peach yellow leaf roll agent with European fruit tree phytoplasmas of the apple proliferation group. Plant Pathol. 46:538-544.

Purcell, A.H. and Suslow, K.G. (1984) Surveys of leafhoppers (Homoptera: Cicadellidae) and pear psylla (Homoptera: Psyllidae) in pear and peach orchards and the spread of peach yellow leaf roll disease. J. Econ. Entomol. 77:1489–94.

Smart, C.D., Schneider, B., Blomquist, C.L., Guerra, L.J., Harrison, N.A., Ahrens, U., Lorenz, K.H., Seemüller, E., and Kirkpatrick, B.C. 1996. Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. Appl Environ Microbiol. 62:2988-93.

Sudarshana, M.R., F. J. Niederholzer, N. Sharma, and J. K. Uyemoto. 2009. Etiology of almond brown line disease in Northern California, Phytopathology 99:S126.

Uyemoto, J.K., Connell, J.H., Hasey, J.K., Luhn, F.C. 1992. Almond brown line and decline: a new disease probably caused by a mycoplasma-like organism. Annals of Applied Biology 120:144-424.

Uyemoto, J.K., Asai, and Kirkpatrick, B.C. 1999. Etiology of almond shriveled kernel disease. New Zeland Journal of Crop and Horticultural Sciences 27:225-228.