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

Project No.: **11-PATH9-Sudarshana**

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Goals and Objectives:

This project was initiated with a goal of (1) establishing the causal agent of almond brown line (ABL) disease, which has been elusive so far, and (2) develop molecular assays for the detection of the causal agent so that ABL disease could be avoided in young almond orchards and the detection method can be used as a tool for certification of plant material and reduce disease incidence. Research to date indicates a phytoplasma closely related to the peach yellow leaf roll phytoplasma (PYLR) is associated with almond brown line disease. Work done this last year toward these goals includes:

Objective 1: Survey orchards 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, petioles, and developing green almonds from 'Winters' and 'Sonora' trees for the presence of peach yellow leaf roll (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 second leaf 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 no horizontal spread of the disease in the orchard and the diseased trees died within a year or two, thus eliminating the source of the pathogen for subsequent spread. It appears that the disease is self-eliminating in affected orchards and removal of symptomatic trees and replanting healthy trees can overcome the impact of ABL in young almond orchards. However one caution: in the event the putative agent is delivered by insect vectors into mature almond trees, the agent may take several years to reach the graft union and trigger development of brown line in the tree.

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 nucleic acid extracts obtained from diseased trees. However, primers specific to non-rDNA regions of PYLR-P failed to amplify a product of expected size from nucleic acid obtained from almond trees showing 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. Bottle grafts from almond trees showing ABL symptoms which also tested positive for a phytoplasma in PCR analysis in fall 2008 failed to reproduce disease symptoms on test plants of almonds on Marianna 2624. Also, the test plants were found to be negative in PCR tests for phytoplasmas.

In spring of 2011, from a batch of sixty trees of cv. Winters on Marianna 2624 suspected to be exhibiting ABL symptoms from a nursery, four trees showed ABL symptoms. However, PCR analysis failed to reveal the presence of a phytoplasma in these trees. Approach grafting of two declining Winters almond trees with young healthy almond trees of cv. Mission on a peach rootstock allowed the canopy of Winters trees to recover from disease symptoms. It is believed that the biological agent responsible for ABL is able to survive in the rescued trees and these trees are expected to provide: (1) inoculum for performing critical graft experiments and (2) nucleic acid extracts for analysis by shotgun sequencing to determine the nature of the biological agent responsible for ABL. We have optimized a qPCR using SYBR®Green real-time PCR and found leaf petioles taken in fall season are ideal for the detection of PYLR-P in almonds and peaches.

Introduction:

Almond brown line (ABL) disease was first reported in California in 1987 on almond trees of cultivars Carmel, Peerless and Price on plum rootstock Marianna 2624 (M2624; *Prunus cerasifera* X *P. munsoniana)* (Uyemoto et al., 1992). The affected trees showed very little new shoot growth and developed small chlorotic leaves that rolled upward. The diseased trees, also exhibited bark split, and pits and grooves on the woody cylinder, as well as union disorder, which presented as a brown line consisting of necrotic phloem tissue. Most of the affected trees died within a year or two. Attempts to characterize the etiological causal agent were unsuccessful due to failure of the bud chips from trees showing ABL to reproduce disease symptoms. However, bud chips from peach trees infected with peach yellow leafroll phytoplasma (PYLR-P) used as control also caused brown line on Marianna 2624, trees suggesting 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, the plum rootstock Marianna 2624 is preferred in marginal lands and heavy soils. This plum rootstock is also moderately resistant to *Armillaria*, phytopthora crown, root rots, and root-knot nematodes. Until recently brown line disease had only been found on almond trees on Marianna 2624, but never on peach rootstock. A few years after almond brown line disease was recognized, 'Nonpareil' trees on peach rootstock 'Nemaguard' in an orchard near Modesto, CA, were reported having late bud break, stunted growth, and 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 antibiotic oxytetracycline into affected trees caused new shoot growth indicating the association of a phytoplasma 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 findings point to PYLR-P as the etiological causal 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 (RFLP) analysis of PCR amplified rDNA region 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 documented.

In spring 2008, inspection of a second-leaf almond orchard in Sutter County in California revealed several trees of cv. Winters exhibiting stunted growth (**Figure 1**).

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 to be necrotic, thus making the trees vulnerable to breakage by high winds. Examination 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 in 1992 by Uyemoto et al. 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.

During the early spring of 2011, two nurseries expressed concerns regarding some of their young trees of cv. Winters on M2624, propagated for commercial distribution, having symptoms similar to ABL and one nursery provided sixty trees for our examinations. This project was initiated with a long term objective of (1) establishing the causal agent of ABL which has been elusive so far, and (2) to develop molecular assays for the detection of the causal agent so that ABL disease could be avoided in young almond orchards.

Materials and Methods:

Survey of an almond orchard in Sutter County for almond brown line disease symptoms. We conducted surveys during spring, summer, and fall, in the years 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 up to 68 trees (rows 1 to 15), and examined for ABL symptoms such as pale green canopy, rolled up leaves and brown line at the graft union.

Examination of almond trees from a nursery for ABL disease symptoms. Seventy bare rooted almond trees of cv. Winters on M2624 were received from a Nursery in February 2011. Trunks of 10 trees were immersed in water in a stainless steel container and autoclaved for 5 minutes. After the bark was stripped the trunks were examined for markings and pits. The remaining sixty trees were planted in research plots and observed for symptoms during the year.

Detection of the phytoplasmas by Polymerase chain reaction using rDNA specific primers. 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, columella (endocarp) from fruits from Bartlet pear trees in an orchard near Fairfield in Solano County infected with pear decline phytoplasma (PD-P) was also collected. Nucleic acid extract from plant samples were obtained using the 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 extracts using 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) and the expected size (1.6 kb) products were cloned and sequenced using a TOPO-TA cloning kit (www.invitrogen.com).

Bioassays using graft inoculations. Wood was collected from two Winters trees that tested positive for the association presence of a phytoplasma in PCR analysis in 2008 and 2009. Bottle grafts using 20 to 25 cm long mature shoot were established on almond trees of cv. Peerless on M2624 and monitored for symptom development. Bottle grafts using shoots from almond trees infected with Peach yellow leafroll phytoplasma were used as positive control.

From sixty almond trees received from a nursery, two trees that showed ABL symptoms during fall of 2011 were approach-grafted with a healthy tree of cv. Mission on peach rootstock Lovell, planted very close to the symptomatic trees. Approach-grafting was done by inserting three to four branches of Mission tree into the incisions made on the trunk of symptomatic Winters tree. One symptomatic trees of cv. Winters was left ungrafted.

Design of new primers for the detection of almond brown line phytoplasma. The amplified product from nucleic acid extracted from almond trees showing ABL symptoms was cloned into a plasmid vector using a TOPO-TA cloning kit (www.invitrogen.com) and sequenced at the 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. The 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 by electrophoresis using 1% agarose gel.

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 nucleic acid 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 by electrophoresis using 1% agarose gel.

Development of a quantitative PCR (qPCR) assay for the detection of almond brown line phytoplasma. Assays were performed on acid extracts using Brilliant SYBR®green qPCR master mix on an Mx3000P qPCR system (www.stratagene.com). A 530 bp amplified product from ABL-P obtained using PYLRrRNAf606 and PYLRrRNAr1135 primers was cloned using the TOPO-TA cloning kit. Plasmid DNA containing the cloned product was spiked into nucleic acid extract from healthy almond leaves and assay conditions were standardized to detect ABL phytoplasma specific

DNA and to 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 with ramp temperature of 0.2 °C per second.

Determination of titer of peach yellow leafroll phytoplasma in almond and peach trees.
Beginning May 2011, leaf samples were collected once every month from almond and peach trees inoculated with peach yellow leafroll phytoplasma in our experimental orchard till the leaves dropped in fall. This was followed by collection of dormant buds and collection of leaves from April through June 2012. DNA was extracted from leaf blades and petioles and 10 ng of total DNA was subjected to qPCR as described above using primers PYLRrRNAf606 and PYLRrRNAr1135.

Results and Discussion:

Survey of an almond orchard with almond brown line in Sutter County 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. Development of almond brown line (ABL) in fourth (Year 2010) and fifth leaf trees (Year 2011) was not seen indicating brown line disease did not spread horizontally in almond orchards. These results are consistent with those observed by Uyemoto et al. (1992) wherein only young trees succumbed to the disease. It appears that the disease is self-eliminating in affected orchards and removal of symptomatic trees and replanting healthy trees can overcome the impact of ABL in young almond orchards. However, in the event the putative agent is delivered by insect vectors into mature almond trees, the agent may take several years to reach the graft union and trigger development of brown line in the tree.

The absence of new infections in years 2010-2011 and the death of trees that were symptomatic in 2008 and 2009 did not allow us to continue grafting experiments to determine the graft-transmissibility of the putative agent responsible for ABL in symptomatic trees.

Bioassays using graft inoculations. Bottle grafts 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 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. Absence of disease symptoms perhaps indicates a slow movement of the etiological agent from grafts to the scion and a delay in brown line development at the graft union. It has to be noted that nursery supplied stock material had almond chip budded on plum rootstock thus allowing a rapid development of ABL. In our graft assays, the phytoplasma has to translocate from grafts into the almond scion and establish itself there before reaching the graft union to cause ABL.

Status of phytoplasma in Nursery supplied almond trees in Year 2011.
Examination of trunks of ten trees after autoclaving and stripping of bark revealed early signs of development of ABL in two trees as evidenced by pits near the graft union. The remaining sixty trees of cv. Winters on Marianna 2624 were planted in our orchard in March 2011 and monitored periodically for symptoms associated with ABL. Of these, three trees exhibited ABL symptoms in fall. Two symptomatic trees of cv. Winters on M2624 were approach-grafted onto healthy trees of cv. Mission on Lovell in fall 2011 and these were found recovering from disease symptoms in Spring 2012 (**Figure 2**). The shoot growth is normal and the canopy is green. The ungrafted symptomatic tree continues to show ABL symptoms. We hope to recover the etiological agent in approach grafted trees and continue our studies in the coming years by conducting graft assays and shotgun sequencing.

Development of specific primers for quantitative real time PCR (qPCR). It has been 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 a 1.6 kb product from acid obtained from leaves of almond trees showing ABL symptoms (**Figure 3**). However, PCR assays did not always amplify a product from nucleic acid obtained from almond trees exhibiting ABL symptoms (see lanes 2 to 4 in **Figure 3**). Even when a product was seen, the PCR conditions required 40 cycles of amplification while nucleic acid from PYLR-P infected almond and peach trees and WX-P infected cherry trees consistently gave a positive reaction in 30 cycles. These results indicate a low titer of the putative phytoplasma in trees affected by ABL.

When the amplified products were cloned and sequenced, the sequence of the P1 primer was found to have a change at the very 3'-end base with respect to the sequences of 16S-23S rDNA regions of several stone fruit phytoplasmas (**Figure 4**). 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 when present at extremely low titer. To overcome these problems, new primers, PYLRrRNAf606 and PYLRrRNAr1135, were designed to amplify a 530 bp product from the 16S-23S rDNA regions of ABL-P, PD-P and PYLR-P. The new primers consistently amplified a product of expected size in 30 cycles (**Figure 5**) 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, nucleic acid from most of the almond trees with ABL required 40 cycles of amplification even with these primers (see lane 2 in **Figure 5**, 30 cycles; and **Figure 6**, 40 cycles). Also, along with the expected size product, several smaller size products were also amplified from nucleic acid from ABL trees but not from PYLR-P infected almond tree and healthy almond tree (**Figure 6**).

Genetic relationship of Almond brown line phytoplasma with Peach yellow leafroll phytoplasma. 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, the primers fCPD and rCPD, which are specific to non-rDNA regions of PYLR-P and PD-P (Lorenz et al., 1995) were

used to amplify products from nucleic acid obtained from trees infected with phytoplasmas. These primers amplified a product similar in size to that from nucleic acid extract from PYLR-P and PD-P infected trees (**Figure 7**, lanes 3, 5 & 7), but not from ABL tree and cherry tree with WX-P (**Figure 6**, lanes 2 & 9). These results indicate that ABL-P is genetically distinct from PD-P and PYLR-P despite relatedness in the 16S-23S rDNA region. A shotgun sequencing of the entire genome using next generation sequencing technology is likely to provide genetic information on the phytoplasma associated with ABL.

A quantitative PCR (qPCR) assay for the detection of almond brown line phytoplasma. To estimate the titer of phytopalsma in infected trees and to develop high-throughput screening, PYLRrRNAf606 and PYLRrRNAr1135 primers were used along with a master mix containing SYBR®green for qPCR analysis. We were able to detect the putative phytoplasma causing ABL, PYLR-P, and PD-P, consistently in qPCR reactions (**Figure 8**; **Table 1**). Cloned plasmid DNA containing a 530 bp product amplified from nucleic acid obtained from an almond tree showing ABL was used to spike nucleic acid 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**). The nucleic acid extracts from WX-P infected cherry trees, as well as healthy almond, peach, and pear trees, were negative in qPCR assays. We were also able to detect PYLR-P in dormant buds obtained from symptomatic trees using this qPCR assay.

During 2011 and 2012, we periodically sampled leaves from almond and peach trees inoculated with PYLR-P. These trees had been symptomatic for at least two years before sampled. The leaf blade and petiole samples were separated and nucleic acid extracted and analyzed by qPCR using SYBR®Green. Comparison of Ct values indicated usually a higher titer in petioles and hence leaf blades were not sampled after the first four samples (data not shown). The titer of PYLR-P, which is inversely proportional to Ct values, was lower during spring and early summer and increased as the season progressed (**Table 2**). This trend was observed in both almond and peach trees. One sample of dormant buds taken in February 2012 also showed PYLR. However the titer was lower when compared to leaves and petioles. Our results indicate it is best to use petioles in fall for molecular detection of PYLR-P in almonds and peaches.

Our study has provided evidence for the presence of a phytoplasma in nucleic acid from almond trees showing ABL symptoms and this phytoplasma does not appear to be genetically related to PYLR-P. Also, the agents involved in the ABL outbreak in Sutter County and in the outbreak on almond trees in a nursery appear to be different. However, unlike in the case of PYLR-P, bud chip inoculations and bottle grafts from ABL trees have not reproduced the ABL symptoms, a key feature to establish the nature of the causal agent. The remission of ABL symptoms by approach grafts indicates that ABL disease is due to a biological agent in nature and not due to abiotic factors. Our ongoing and future studies will address shotgun sequencing approaches using nucleic

acid from the scion and rootstocks portion of approach-grafted trees showing remission of ABL symptoms to understand the nature of the etiological agent. Most importantly, this study has demonstrated that horizontal spread is not seen in case of ABL. In the event of an outbreak, the disease can be managed in affected orchards by removal of symptomatic trees and replanting.

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Research Effort/Recent Publications from this Study:

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.

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Figure 1. Almond trees showing ABL symptoms in Sutter County. The tree in the top is showing stunted growth and the tree in the bottom is snapped at the graft union and is showing brown line disease.

Figure 2. A Winters almond tree on Marianna 2624 showing brown line disease (left) and a previously symptomatic Winters almond tree showing remission of symptoms after approach grafting on to a Mission almond tree grown on peach rootstock Lovell (Middle). Close up view (Right) of the grafted limb of a Mission almond tree on Lovell (unpainted) callused with Winters almond tree (white painted). Black arrow points to grafted region.

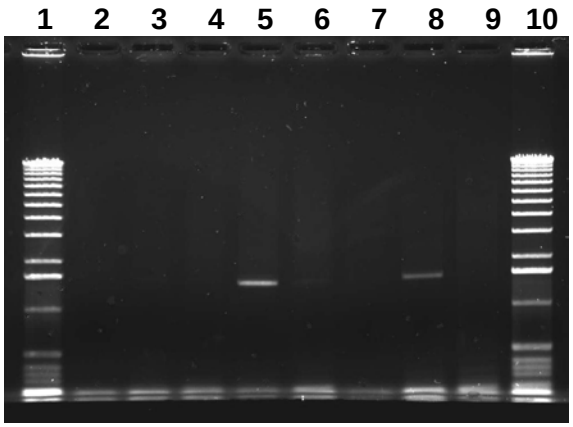

Figure 3. Agarose gel showing 1.6 kb PCR product amplified from an almond tree affected by brown line disease using P1 and Tint primers. Lanes 1 and 10, 1 kb DNA ladder (Invitrogen); lanes 2 to 7, samples from almond trees with brownline; lane 8, almond tree inoculated with PYLR phytoplasma; lane 9, healthy almond tree.

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'**

Figure 4. 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).

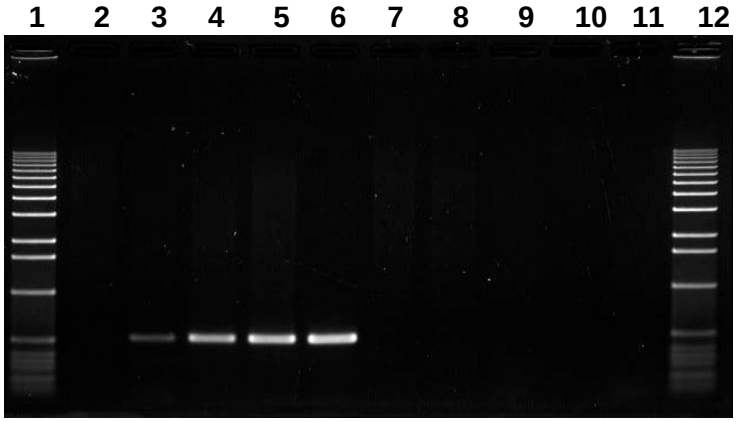

Figure 5. Agarose gel showing 530 bp product amplified by PCR from nucleic acid extracts of trees infected with phytoplasmas using primers PYLRrRNAf606 and PYLRrRNAr1135 after 30 cycles of amplification. Lanes 1 and 12, 1 kb DNA ladder (Invitrogen); lanes 2 and 3, almond trees with ABL symptoms; lanes 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.

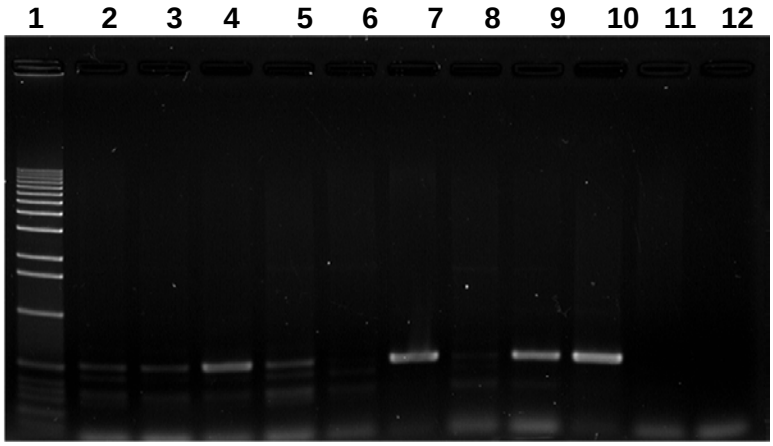

Figure 6. Agarose gel showing PCR products amplified from nucleic acid extracts of almond trees with ABL using primers PYLRrRNAf606 and PYLRrRNAr1135 after 40 cycles of amplification. Lanes 1 and 12, 1 kb ladder; lanes 2 to 9, almond trees with ABL symptoms; lane 10, almond tree infected with PYLR-P; lane 11, healthy almond tree; lane 12, water.

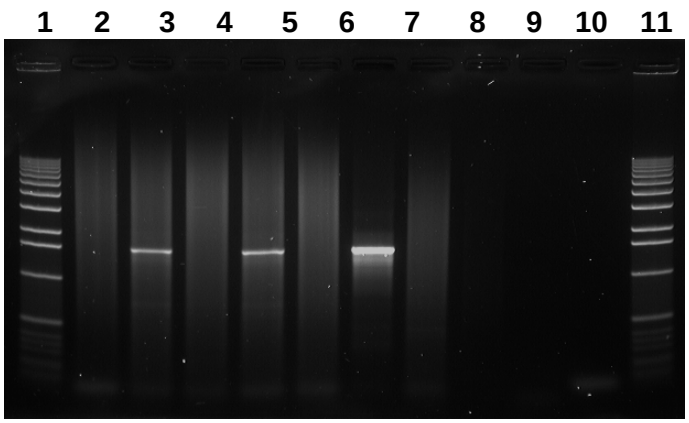

Figure 7. Agarose gel showing PCR products amplified from nucleic acid extracts obtained from trees infected with phytoplasmas using primers fCPD and rCPD specific to Peach yellow leafroll phytoplasma. Lanes 1 & 12, 1 Kb ladder; Lane 2, nucleic acid extract from almond tree with brown line disease; lane 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.

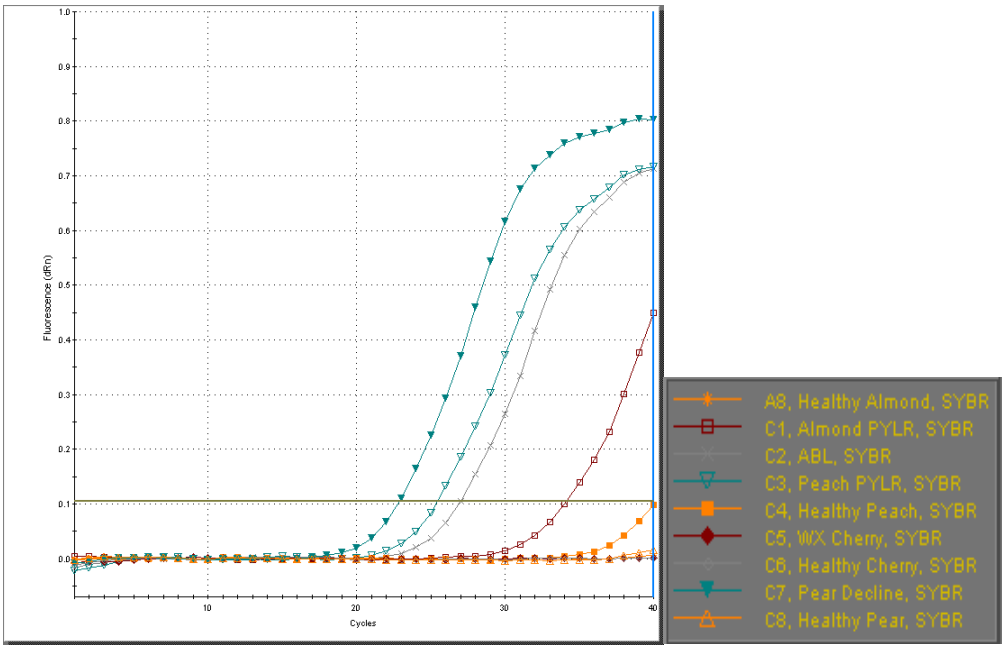

Figure 8. Graph showing real-time monitoring of accumulation of PCR products from nucleic acid extracts using SYBR green. Note almond brown phytoplasma (C2), peach yellow leafroll phytoplasma in almond (C1) and peach (C3), and pear decline phytoplasma in pear (C7) are positive.

Table 1. Sensitivity and specificity of detection of peach yellow leafroll phytoplasma by quantitative SYBR®Green real-time PCR in almond and peaches.

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.

Table 2. Titer of peach yellow leafroll phytoplasma in leaf petioles of almond and peach trees as indicated by cycle threshold values (Ct) in quantitative SYBR®Green real-time PCR.

Sampling	Month	Almond	Peach
Yr. 2011	May	27.3	29.3
	June	22.8	25.4
	July	24.7	22.3
	August	24.1	21.9
	October	21.5	20.6
	November	21.5	21.0
Yr. 2012	February*	31.5	
	April	19.2	30.0
	May	21.0	23.1
	June	23.9	30.0

* In February 2012, dormant buds were used for DNA extraction.