Etiology, Epidemiology, and Management of Lower Limb Dieback and Band Canker of Almonds

Project No.: **08-PATH5-Michailides**

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Objectives:

Lower Limb Dieback

1. Select 4 orchards with a history of Lower Limb Dieback (LLDB) and make isolations periodically starting in March until October/November to determine the succession of colonization by fungi.
2. Repeat an inoculation study using more isolates of *Botryosphaeria* and *Phomopsis* spp. and compare symptoms with those of naturally occurring LLDB symptoms in the field.
3. Initiate herbicide experiment to determine if glyphosate application in almond orchards contributes to LLDB symptom development.

Band Canker

4. Determine when infection by *B. dothidea* of almond occurs (second year experiment using the spore dispersal wooden construction).

Lower Limb Dieback and Band Canker

5. Identify and determine species compositions of *Botryosphaeria* found in association with band canker, LLDB, cankers in the canopy, and fruit blights of almond.
6. Determine any differences of the *Phomopsis* and *Botryosphaeria* species in regard to virulence towards almond trees.

Interpretive Summary:

Isolations from limbs of trees with symptoms of LLDB and without symptoms again revealed both *Botryosphaeria* and *Phomopsis* species. The frequency of these fungi increased later in the season and in general it was higher in limbs with LLDB symptoms than in limbs without symptoms. *B. dothidea* and *Phomopsis* spp. were also recovered to some extend from symptomless limbs, suggesting that these fungi can live in the almond bark. An experiment to study the effect of glyphosate drift as a cause of LLDB is being initiated but no results are available yet. At least five species were identified hidden within the *Botryosphaeria dothidea* "complex" from almond. These are *B. dothidea, Neofusicoccum mediterraneum, B. parva*, as well as *B. cf parva* and *B. cf arbuti*. All species isolated from canopy cankers are also present in band cankers, suggesting that canopy cankers can be caused from band canker inoculum. In addition, the species recovered from band canker of almond are present on other hosts that can serve as additional inoculum sources for the almond band canker disease. The experiment to determine when infections by *Botryosphaeria dothidea* occur is still in progress. No band canker disease has developed yet in potted trees exposed periodically under *B. dothidea* spore inoculum probably because of the very unfavorable long drought conditions. Inoculation studies of Carmel almond trees to compare the virulence of the five species of *Botryosphaeria* encountered on almond showed that *B. parva* was the most virulent species followed by *N. mediterraneum* and *B. cf parva* in causing cankers.

Results and Discussion:

Lower Limb Dieback (causal agent unknown)

1. Select orchards with a history of LLDB and make isolations periodically to determine the succession of colonization by fungi (*Botryosphaeria* and *Phomopsis*).

Six orchards with LLDB were selected; 3 each in Stanislaus and Butte counties and trees with early symptoms of LLDB were selected. Five of these were orchards where Dr. Lampinen (see also Project No. 08-PATH6-Lampinen, Lower Limb Dieback in Almond) was doing measurements of the water/ moisture content in the soil. We collected samples for isolations from these trees and will continue isolating from the same trees from limbs with LLDB until October/November. Collected samples were brought to the laboratory and isolations were made within 1 to 2 days on agar media. The goal of these isolations was to determine if there was a succession and/or an increase of colonization of the LLDB limbs with *Botryosphaeria* and *Phomopsis.* It has been documented in published research that "stressed" plants are predisposed to infection by *Botryosphaeria* spp. Table 1 shows the isolation results from symptomatic and symptomless limbs.

The frequency of isolation of *B. dothidea* and *Phomopsis* sp. from limbs with symptoms was higher than that from isolations from the symptomless limbs. However, both fungi

were found in symptomless branches and limbs of almonds, suggesting that propagules of these fungi can live in the bark of these trees. The higher frequency of isolation from limbs with symptoms indicate that these fungi have grown more, but does not prove that they are really responsible for causing lower limb dieback. Interestingly, the frequency of *Aspergillus niger* was higher in limbs with LLDB symptoms than those without symptoms. In general, isolations made later in the season had higher levels of *B. dothidea, Phomopsis* sp., and *A. niger.*

Table 1. Frequency of fungi isolated from almond limbs[1] with and without symptoms of lower limb dieback collected from orchards in Stanislaus and Butte Counties.

Orchard	Cultivar	Collection time	Limbs with symptoms[1]			Limbs without symptoms[1]		
			***Botryosphaeria dothidea* (%)**	***Phomopsis* sp. (%)**	***Asergillus* spp. (%)**	***Botryosphaeria dothidea* (%)**	***Phomopsis* sp. (%)**	***Asergillus* spp. (%)**
Stanislaus 1	Butte	May	1	3	3	0	0	0
		August	0	1	17	0	0	0
	Padre	May	1	4	2	0	0	0
		August	0	4	39	0	0	0
Stanislaus 2	Butte	May	0[2]	4	0.	0	0	0
		August	12	2	12	0	0	0
	Padre	May	0	0	3	0	0	0
		August	33	4	8	0	0	0
Stanislaus 3	Butte	May	1	1	0	0	0	0
		August	0	17	7	0	0	0
	Padre	May	1	3	1	0	0	0
		August	0	8	17	0	0	0
Butte 1	Butte	June	0	0	4	1	0	0
		October	12	0	20	34	1	16
	Aldrich	June	0	1	2	0	0	6
		October	26	3	1	14	4	9
Butte 2	Nonpareil	June	1	6	3	1	1	3
		October	25	4	21	45	2	2
	Carmel	June	0	4	0	0	0	0
		October	8	1	27	31	3	2
Butte 3	Butte	June	0	16	0	1	0	1
		October	9	2	0	17	3	11
	Aldrich	June	2	11	0	1	4	0
		October	18	11	0	20	1	23

[1] Shoots with LLDB symptoms and asymptomatic shoots from the same tree were collected from 10 trees of each cultivar per orchard.

[2] Numbers refer to the percentage recovery from all plated samples per orchard (10 pieces per limb and 10 limbs per orchard).

2. Determine whether *B. dothidea* and *Phomopsis* spp. can cause LLDB symptoms in the field.

Inoculations were repeated in a Nonpareil almond orchard at the Nickels Soil Lab Estates orchard in 2008. Three limbs in each of 10 trees were selected and inoculated with three *Botryosphaeria* and three *Phomopsis* isolates on 2 July 2008 (Table 2). We inoculated various ages of wood; thus the inoculations were done using mycelial plugs in one year-old wood and wood formed in other years as well. Inoculated wounds were wrapped with Parafilm to prevent desiccation of the inoculum and disease symptoms (yellowing of leaves, development of canker, killing of limb, etc.) were monitored throughout the season. At the end of the season infected shoots were collected, cankers measured, and compared morphologically with symptoms of cankers characteristic of LLDB.

Table 2. Inoculation with *Phomopsis* sp. and *Botryosphaeria dothidea* isolates and LLDB canker formation from the July 2, 2008 inoculation.

Isolate	Inoculation rating score[1]	
	Thrifty trees (good growth)	Unthrifty trees (poor growth)
Phomopsis 07019	0.8 b	0.6 b
Phomopsis 07022	0.6 b	0.0 b
Phomopsis 3774	1.2 b	0.0 b
Botryosphaeria 661	0.6 b	0.4 b
Botryosphaeria 809	3.0 a	1.6 a
Botryosphaeria 3449	2.0 ab	1.5 a

[1] Limbs were inoculated at four sites per limb, measuring from the terminal to the basal sections. Rating scale: 1 means that only the terminal inoculation caused a canker (mildly virulent isolate), while a rating of 4 means that all four inoculation sites down the shoot caused a canker (virulent isolate).

In general, the *Botryosphaeria* isolates were more virulent, especially isolates 809 and 3449. The isolates were more virulent on the thrifty trees, although only *Botryosphaeria* isolate 809 was significantly more virulent on the thrifty vs. the unthrifty trees (statistics not shown in the table). However, when the inoculation rating score of all the *Phomopsis* and *Botryosphaeria* isolates were averaged together, the average score of 1.4 on the thrifty trees was significantly different than the score of 0.7 on the unthrifty trees $P < 0.05$. This indicates that thrifty trees may be more susceptible to LLDB.

3. Spray almond shoots with Roundup to see if this predisposes the limbs to lower limb dieback.

This experiment was performed at the Kearney Ag Center in an almond block planted on January 2006. We used trees of Butte, Nonpareil, and Padre cultivars. Leaves were removed from about 18 inches of the lower scaffolds on July 16, 2008. Roundup was sprayed on the limbs on July 23, 2008. We had three treatments such as Roundup at 1% [commercial rate], 0.1%, and 0.01%, and an unsprayed control. There were three trees of each cultivar in each row and each tree had all four treatments. Thus there were six replicate branches of each treatment per cultivar. Very preliminary observations showed that there may be some damage in the scars where the leaves

were removed. It is too early to observe any effects of these treatment and we are not going to evaluate this experiment any further until next spring when new growth develops.

We would like to follow up on this line of thinking that perhaps the glyphosate is slightly injuring the buds or leaf scars in the late fall or dormant season, and perhaps providing an infection site for *Botryosphaeria* or *Phomopsis*. We will coordinate with growers when they make applications of glyphosate to measure drift in the canopy of the trees by exposing indicator plants.

Band Canker

4. Determine when infections by *Botryosphaeria dothidea* occur.

Answering the question when infections of almond by the band canker pathogen(s) occur is of major importance to be able to develop effective control measures against the disease. Once effective fungicides have been determined, we would know when to apply these fungicides to prevent infection of trunks. We have determined, based on two years of monthly inoculations that early spring seems to be the period when canker development is most rapid. It can be argued that, although this is when cankers appear to develop faster, this may or may not be the time when most of the infections occur in almond trees. Groups of trees were exposed periodically under trunks bearing pycnidia of *B. dothidea* and they are now under continuous observation to determine which group of trees will show most infections. After growing these trees for an additional year, we did not observe any gumming when evaluated nor did we see signs of canker development by mid October 2008, perhaps due to long drought. We will continue monitoring these trees and also install overhead sprinkler irrigation in order to provide enough moisture to trigger any possible infections that might have occurred and remained latent due to unfavorable severe drought conditions in the last two years.

Lower Limb Dieback and Band Canker

5. Identify and determine species compositions of *Botryosphaeria* found in association with band canker, lower limb dieback, cankers in the canopy, and fruit blight of almond.

We found earlier that the fungus so far identified as *Botryosphaeria dothidea* on almond represents at least five different species. These are *Botryosphaeria dothidea, Neofusicoccum mediterraneum, B. parva*, as well as *B. cf parva* and *B. cf arbuti*. Here we report results from our efforts to find names for all *Botryosphaeria* species isolated from California almond, as well as virulence assays to gain insight into the pathogenicity of these species. For two of the above species, temporarily referred to as *B. cf parva* and *B. cf arbuti*, no names could be determined based on DNA analyses, thus requiring morphological investigations done by measuring pycnidia and spore sizes of these isolates and comparing them with characteristics of isolates received from herbaria in the USA and internationally. Based on similarities in conidium size and shape, we then identified candidate names for *F. cf arbuti* and *B. cf parva*. We chose conidium characters as a criterion for selection because these characters were mentioned in most

species descriptions. To find names for *F. cf arbuti* and *B. cf parva*, we expanded our database with morphological information of the 191 known *Fusicoccum* species. We found that *F. cf arbuti* conidia from almond were on average 24 µm long and 7 µm wide (and average length-to-width ratio of 3.3). In our dataset, there were 25 species with similar conidia lengths and shapes. Similarly, *F. cf parva* conidia from almond were 19 × 6.2 µm (average length-to-width ration = 3.1). After completing all the possible comparisons, between *F. cf arbuti* and *B. cf parva*, six candidate species were shared, requiring us to order the type material of 26 species for morphological examination from herbaria in the US, Europe, and South Africa. One herbarium specimen of *Fusicoccum persicae* has so far been received from the US Department of Agriculture Herbarium in Beltsville, MD. Morphological investigations showed that the *F. persicae* type material, comprised clusters of pycnidia breaking through the bark of small peach branches. However, the size and shape of the pycnidiospores and the abundance of the stromatic tissues (black tissues surrounding the pycnidia) differed from those of the *B. cf parva,* and for this reason, *B. cf parva* differs from *F. persicae* morphologically, and can thus not be called *F. persicae*. Our results showed that all species isolated from canopy cankers of almond are also present in band cankers. That makes a recent introduction of the canopy canker fungi into California unlikely but shows that canopy cankers can be caused from band canker inoculum. Also, the almond band canker fungi are present on other hosts that can serve as additional inoculum sources. Further studies should focus on the relative importance of each of these species to band canker, canopy canker, and fruit blight.

6. Virulence Assays.

Virulence assays were performed on approx. 2 ½ year-old potted almond trees (cv. Carmel) at the Kearney Ag Center. Tree stems were wounded and inoculated on 11 July 2008 with a potato dextrose agar plug with fungal mycelium or without as control, and sealed with Parafilm to prevent desiccation. Cankers were measured after 75 days. Treatments were randomized, and consisted of 14 fungal strains representing 6 species, and one agar control, five replicates each, thus requiring a total of 75 trees. This experiment was repeated on August 19, 2008. We found that the cankers on the almond sapling stems did not differ between species, except in *Botryosphaeria parva* and *Neofusicoccum mediterraneum* where the cankers were significantly longer than in *B. dothidea, B. cf parva*, and *N. arbuti*, respectively (Figure 1).

Differences in canker lengths were relatively small, since almost 90% of the cankers were 4 cm in length or shorter. The longest cankers, up to 10 cm in length, were observed in *B. parva*. No cankers were observed in the controls. These data are intriguing, as they suggest that *B. parva* might be more virulent than both *B. dothidea* and *B. cf parva*. However, these results have to be confirmed by the second replicate (second inoculation) to be harvested and evaluated in mid November 2008.

Conclusions:

1. Isolations from limbs of trees with symptoms of LLDB and without symptoms again revealed both *Botryosphaeria* and *Phomopsis* species. The frequency of isolation

seems to be higher for the limbs with symptoms of LLDB than that of limbs without symptoms. In general, the frequency of these fungi increased with time during the growing season.

2. *B. dothidea* and *Phomopsis* spp. were also recovered from symptomless limbs, suggesting that these fungi can live in the almond bark.
3. At least five species were identified hidden within *Botryosphaeria dothidea* from almond. These are *B. dothidea, Neofusicoccum mediterraneum, B. parva*, as well as *B. cf parva* and *B. cf arbuti*.
4. All species isolated from canopy cankers are also present in band cankers, suggesting that canopy canker can be caused from band canker inoculum. In addition, the species recovered from band canker of almond are present on other hosts that can serve as additional inoculum sources for almond infection.
5. The experiment to determine when infections by *Botryosphaeria dothidea* occur is still in progress. No disease developed due to unfavorable long drought conditions.
6. Virulence studies showed that *B. parva* was the most virulent followed by *N. mediterraneum* and *B. cf parva* in causing cankers after inoculation of potted Carmel almond trees.

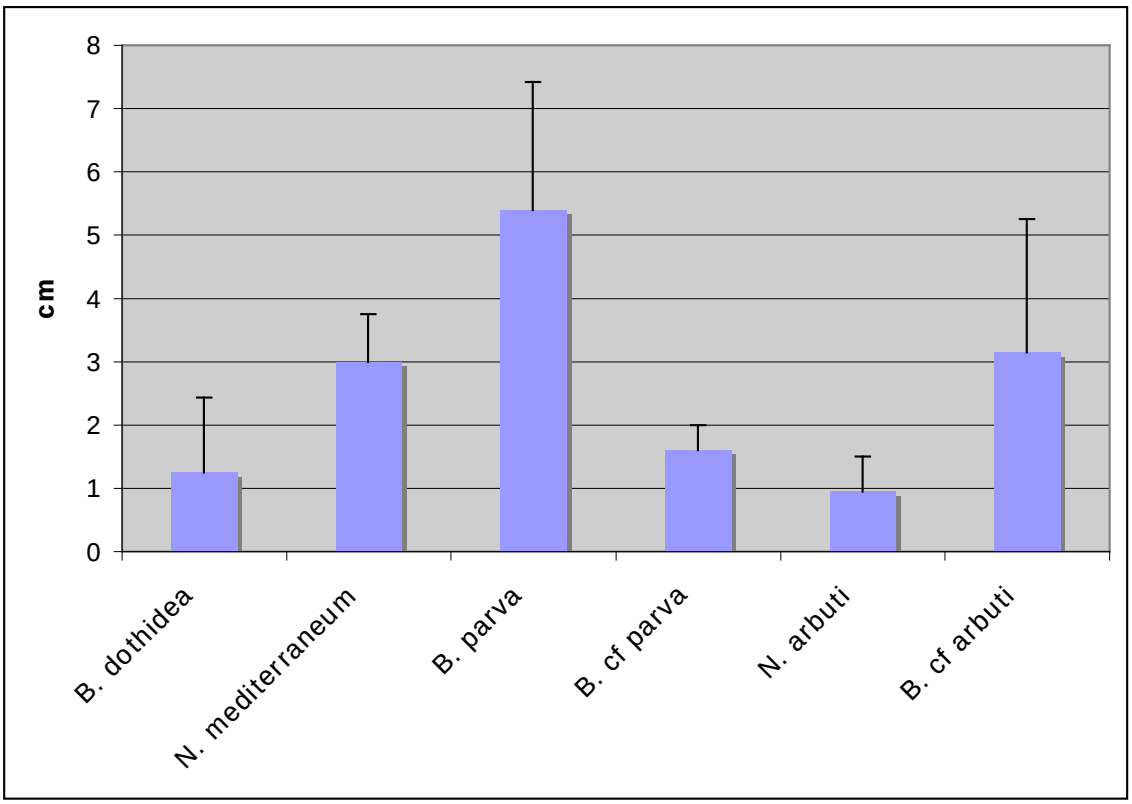

Figure 1. Mean stem canker lengths and standard errors for six different species in the *Botryosphaeria* group of fungi on almond potted trees. Note that cankers in *N. mediterraneum* and *B. parva* were significantly longer than in *B. cf parva* and *N. arbuti.* (*N. arbuti* is a fungus from Pacific madrone not known to occur on almond.)

First Report of *Eremothecium coryli* Kernel Rot (Stigmatomycosis) of Almond in California

Special Report

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Objective:

Describe a new disease (stigmatomycosis) encountered on almond kernels in 2006 through 2008.

Interpretive Summary:

In the spring of 2006, many California almond and pistachio orchards had significant losses due to a severe infestation by leaffooted bugs (*Leptoglossus clypealis*). This damage resulted in a very severe nut drop for some almond cultivars. Although immature almond fruit will drop after injury by this insect, fruit injured later in the season by the same insect will remain on the tree and develop into full size nuts. The kernel of these nuts, however, became jelly like, slimy, and moldy. In the spring and summer 2006, multiple nut samples were collected from the ground and trees and examined. More than 100 nuts from each category were opened and the kernels were examined under a dissecting microscope. Symptoms of the insect damage included clear to amber color gum on the exterior of the nut exocarp, exuding from areas punctured by the insect. Gumming was also observed in the cavity of the shell and on the kernel as well as there were brown spots besides there being gumming. The kernels of affected nuts were partially developed, shriveled, or misshapen. After sectioning the kernels, a cloudy liquid was present on the entire or part of the kernel surface, just beneath the brown testa (skin of kernel). A white-creamy ascomycetous yeast, *Eremothecium coryli* (Peglion) Kurtzman [(syn. *Nematospora coryli* (Peglion)], characterized by spindle-shaped ascospores with long, thin, whip-like terminal appendages, was consistently isolated from diseased tissues on Petri plates containing either acidified potato dextrose agar (APDA) or potato dextrose agar (PDA). The incidence of *E. coryli* was 5% for the fruit collected from the ground and 4% of the nuts collected from the tree.

In late September 2008, samples of Fritz almonds brought to our laboratory with small 2/16-3/16" (2 to 4 mm) black spots on the kernel surface typical of damage done by punctures by large hemiptera insects. Upon removal of the kernel skin, one could see concave cavities underneath with spongy kernel tissues, some of which had white yeast growing in the cavity and sometimes in the inner surface of the skin covering the spot. About 34% of the fruit had multiple (2 to 8) concave spots per nut and about 4% of the spots harbored growth of *E. coryli.* In some cases, there was also growth of *E. coryli* even on the surface of the skin surrounding the feeding spot on the kernel.

Koch's postulates:

To prove whether *E. coryli* is a pathogen on almond, mature almond fruit cv. Carmel prior to hull split and separation from the shell developmental stage were collected from an experimental orchard at the Kearney Agricultural Center where no fungicide sprays were applied. In two experiments, 15 and 30 fruit, respectively were surface sterilized with 10% bleach (0.5% sodium hypochlorite), allowed to dry, and injected using a syringe with a 0.1-ml suspension of a 10,000 vegetative cells and ascospores per milliliter to a depth of 4 to 5 mm to reach the kernel and simulate probing by sucking hemiptera insects. Control fruit were injected with similar amounts of sterile water. Fruit were placed in containers over plastic screens under >95% relative humidity and incubated at 86°F. After incubation for 2 weeks, a few fruit developed external discolored dark brown lesions, and after sectioning, 81% (for experiment 1) and 85% (for experiment 2) (average 83%) of the inoculated fruit developed symptoms typical of stigmatomycosis in the kernels. For instance, kernels had gumming that developed on their surface and were shriveled and covered with a white-creamy mass under the kernel's skin. Microscopic examination revealed masses of spherical vegetative cells, asci, and needle-shaped ascospores with appendages. The density of these structures was 20× higher than what was used to inoculate the almonds and covered most of the kernel surface. In some of the fruit, the yeast spread over a large area of the kernel beyond the injection site, indicating that it had grown and reproduced in the fruit. The fungus was re-isolated on APDA and was identified as *E. coryli.* In two more inoculation experiments conducted in a similar manner, fruit of the almond cultivars Carmel and Butte at the hull split stage (later developmental stage than the fruit used in the two first experiments) were used and 43% Carmel and 29% Butte nuts were similarly infected by the fungus. The lower percentage of infection suggests that fruit may be less susceptible to infection at later developmental stages than fruit before hull split. Controls fruit remained symptomless. Although the same fungus was found and reported to cause stigmatomycosis in pistachio and rot of tomatoes in California, this is the first report of *E. coryli* causing stigmatomycosis on almond worldwide and is associated with leaffooted bug feeding.

Conclusion:

This is a new disease of almond and it is very similar to the stigmatomycosis reported on pistachio. Losses of yields can include not only fruit dropped to the ground due to probing by sucking insects early in the season, but also fruit that can remain on trees

and decay by stigmatomycosis. An additional yield loss could be nuts graded as unmarketable due to the black spotting on the kernel as described above (also see Figures 1 and 2). Isolates of the pathogen are maintained at the Kearney Agricultural Center, University of California at Parlier and transmission experiments using leaffooted bugs and other large hemiptera insects have been initiated.

Figure 1. **Upper left plate:** Almonds damaged (punctured) by the leaffooted bug (*Leptoglossus clypealis*) with sap exuded from the wounds and misshapen and partially developed kernels after they were stung by the insect. **Upper right plate:** Initial symptoms of bug damage and stigmatomycosis decay caused by *Eremopthycium coryli*. **Lower left plate:** Sectioned almond showing kernel infected with stigmatomycosis with white yeast (*E. coryli*) on the surface of the kernel's skin; **Lower right plate:** Infected kernel late in season (notice the change in the texture and color of the kernel cotyledons).

Figure 2. **Upper left plate:** Almonds damaged (punctured) by the leaffooted bug (*Leptoglossus clypealis*) with sap exuded from the wounds in the hulls and small black spots on the kernel due to insect puncturing. **Upper right plate:** Multiple black spots on kernel due to hemiptera damage. **Middle left plate:** An almond punctured by hemiptera insects resulting in decayed and shriveled kernel (notice excess gumming on the hull). **Middle right plate:** Concave cavities colonized by *Eremothycium coryli*, just underneath the black spots (upper right plate) seen after removing the skin of damaged kernels. **Lower left plate:** Cultures of the stigmatomycosis pathogen growing from plated shriveled kernels of affected almonds. **Lower right plate:** Vegetative cells of *E. coryli* along with asci containing needle shape ascospores of the fungus.