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 (LLDB)

1. Select four orchards with a history of 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 (LLDB) 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 regards 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, "waiting" to initiate disease with the right conditions. 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.

Materials and Methods:

<u>Lower Limb Dieback</u>

1. <u>Select orchards with a history of LLDB and make isolations periodically to determine the succession of colonization by fungi (*Botryosphaeria* and *Phomopsis*).</u>
   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 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. **Figure 1** shows the isolation results from symptomatic and symptomless limbs for these orchards.

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 1)**. We inoculated various ages of wood; thus the inoculations were done using mycelial plugs in one year-old wood and wood formed in past years as well. Inoculated wounds were wrapped with Parafilm to prevent desiccation of the inoculum and disease symptoms 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.

3. Determine if herbicide drift contributes to LLDB symptoms.
   This experiment was performed at the Kearney Ag Center (KAC) 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. Glyphosate 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.

   In order to determine if drift of herbicide reaches the lower limbs, two almond and two pistachio orchards were selected. Trials were conducted in two pistachio orchards at the Kearney Ag Center and in one almond orchard at the Kearney Ag Center and second one in a Stanislaus County that Bruce Lampinen is working in (Stanislaus County #1).

   Young, 2-week-old squash plants were placed on the berm or on poles in the tree rows 3, 5, and 7 feet high at five replicate sites per orchard. Squash plants placed on the orchard floors distant from the spray served as unsprayed controls. After the herbicide spray had dried, the squash plants were collected and brought to the Kearney Agricultural Center, placed in a greenhouse, and observed for herbicide symptom development over a 3-week period. Killing of the plants, stunting, or other typical symptoms of glyphosate damage as described in the literature would indicate that they received drift of this herbicide. The effects of the herbicide on the squash plants kept at different heights would indicate if drift has reached the squash plants and at what height. By running the spray equipment at the Kearney Ag Center trials using parameters somewhat outside of what most applicators use, we attempted to intentionally cause drift.

   We grew Jackpot F1 hybrid summer squash. Plants used in the field trials had two fully expanded true leaves and two additional small new leaves. Glyphosate (Roundup Weather Max) was sprayed at 4 pints/Acre at 25 GPA for the KAC trials. For the KAC trials, the pressure was increased from 35 to

55 psi and the nozzle size was decreased from 8004 to 8002. Two tips per boom were used for the berm sprays: OC-02 and T-Jet. The wind speed for January 27, 2009 at 10 am was 2.4 mph from the south and the temperature was 42°F. Two pistachio blocks with rows running east to west were used. Because the wind was from the south, the berm on the row with the squash and the adjacent berm to the south were sprayed.

On January 29, 2009, the same experiment was repeated in a KAC almond orchard with rows running east to west. The temperature was 45°F at 10 am and the wind speed was 3.9 mph from the south. In this test, the two berms to the south of the berm with the squash plants were also sprayed.

On February 25, 2009, we conducted the same experiment at the Stanislaus County #1 almond orchard that Bruce Lampinen is working in Ceres, CA. The temperature was 58°F at the 10 am spray time and the wind was reported to be calm at Modesto Airport, but there appeared to be about a 2 mph wind from the north in the orchard in Ceres. The herbicide sprayer parameters were 30 GPA, with 28 lbs pressure and air injected nozzles were used to reduce drift. Three herbicides were tank mixed. Prowl H_2O (EPA Reg 241-418 at 1 Gal/Ac, Orchard Star at 3 pints/Ac (2, 4, D) (42750-19), and Glyphosate X-tra (4787-23) at 3 pints/Ac. First Choice herbicide activator (CA 11656-50024-2C) was sprayed at 12 oz/Ac. In addition, to this experiment that would measure possible drift of herbicide onto the lower portions of limbs, we examined lower limbs by scraping the bark and observed the tissues around the lenticels. This is done because in LLDB limbs there are brown necrotic tissues around the lenticels, an indication that perhaps something toxic enters through the lenticels killing the tissues.

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*.

4. <u>Band Canker - Determine when infections by *Botryosphaeria dothidea* occur.</u>

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 for symptom development, in case the infections are latent due to unfavorable severe drought conditions in the last two years.

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, Botryosphaeria parva*, as well as *Botryosphaeria cf parva* and *Botryosphaeria 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.

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 (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.

Results and Discussion:

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

   The frequency of isolation of *B. dothidea* and *Phomopsis* sp. from limbs with symptoms was higher than that from isolations from the symptomless limbs (**Figure 1**). 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.*

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

   Results from inoculations of almond with *Botryosphaeria* and *Phomopsis* isolates on 2 July 2008 are shown in Table 1. With the exception of isolate of *Botryosphaeria* 661, the two isolates of *Botryosphaeria* resulted in larger cankers than the cankers produced by *Phomopsis.* Only *Phomopsis* 3774 caused a small canker while the other two isolates were not pathogenic (**Table 1**). In general, the two *Botryosphaeria* isolates produced larger cankers in the thrifty trees than those in the unthrifty trees.

3. <u>Determine if herbicide drift contributes to LLDB symptoms.</u>

   In the first four tests, we did not observe any herbicide damage symptoms on squash plants placed at 3, 5, and 7 feet above the berm, but those placed on the berm which received direct contact with the herbicide spray were killed. The lack of symptoms on the squash plants may be attributed to the fact that the wind speed was very low on the dates when these experiments took place and thus there was not much drift.

   Later in season, we will go back in these orchards to examine the lower limbs by scraping the bark and observe the tissues around the lenticels. This will be done because in LLDB there are brown tissues around the lenticels, an indication that perhaps something toxic enters through the lenticels killing the tissues. Thus we will look for this characteristic symptom, which is very typical of LLDB.

   Very preliminary observations showed that there may be some damage in the scars where the leaves were removed. No other symptoms were observed, but we will continue monitoring these limbs, just in case, that the damage takes longer time to reveal symptoms.

   In addition, all the sites in the commercial orchards where we exposed the squash will again visited and any symptoms of LLDB will be recorded.

<u>Band Canker:</u>

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

   We have determined, based on two years of monthly inoculations that early spring seems to be the period when canker development is most rapid. From serial inoculations of potted trees, we found that the infections that occur during March to May developed the most and apparently those will be the ones causing the most severe damage to the trees. When groups of trees were exposed periodically under trunks bearing pycnidia of *B. dothidea* (inoculum of the pathogen) no symptoms of infection develop. After growing these trees for two more seasons, we did not observe any gumming when evaluated nor did we see signs of infection or cankers development. The lack of infection of these trees may be attributed to the fact, that the young trees are resistant to infection, and perhaps they need to develop rough bark until the fungus is established in them. We will check this hypothesis in a future experiment with older trees. In previous research we showed that *Botryosphaeria* species colonize the bark of trees first and then we see symptoms of band canker, after the development of growth cracks, pruning wounds, or other type of wounds (tissue splitting due to wind shaking, branch breaking, etc.). We will continue monitoring these trees and have installed 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, LLDB, cankers in the canopy, and fruit blights of almond.

   The results from determining species of *Botryosphaeria* fungi from almond are shown in **Figure 2**. 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.

   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 3**). 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 2009.

7. Other studies.

   a) Chemical control of band canker.
      We have just initiated this study to manage band canker by painting the tree trunks with fungicides mixed with latex paint. This orchard is located in Glenn County and showed uniform distribution of the disease in the section where the experiment was set (**Figure 4**). In addition, the disease was at the early stages of development and showed various degrees of band canker severity. The reason for initiating this experiment was based on the premise that it may be possible to manage band canker at an earlier stage of development. Details of this experiment are given in **Table 2**. It is too early to provide results since thi experiment was initiated on May 14, 2009.

   b) Band canker distribution.

      On July 1, 2009 we mapped six rows for band canker symptoms on four year old Nonpareil cultivar almonds running north south. The cankers were rated for severity on a scale of 0 to 3: with 0 meaning symptomless trees; 1 with cankers < 25% around the trunk; 2 with cankers < 50% around the trunk; and 3 with cankers > 50% around the trunk. Canopy symptoms were categorized as either light or severe thinning of the canopy. For the purposes of the graph, all six rows and groups of 10 trees per row (incidence per 60 trees) were averaged to determine the percentage of trees with band canker symptoms per section of the orchard. In **Figure 4**, trees with any symptom (Categories 1, 2, and 3) of band canker were included while in **Figure 5**, only trees with severe band canker symptoms were included (Category 3). **Figure 5** shows that disease was more severe in the north side of the orchard which is located close to a row of old eucalyptus trees. Eucalyptus trees have been reported to harbor *Botryosphaeria* species. Examination of samples from those Eucalyptus trees revealed both the pycnidia and pseudothecia of *Botryosphaeria* species, which suggests that infections in this orchard started first in the north side and it is spreading to the south (**Figures 4 & 5**).

Conclusions:

1. Isolations from limbs of trees with symptoms of LLDB and without symptoms revealed both *Botryosphaeria* and *Phomopsis* species. In general, the frequency of these fungi increased with time during the growing season.
2. Isolation of *Botryosphaeria* species from symptomless limbs suggests that these fungi can live in the almond bark “waiting” for the right conditions to initiate disease.
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. Virulence studies showed that *B. parva* was the most virulent species followed by *N. mediterraneum* and *B. cf parva* in causing cankers after inoculation of potted Carmel almond trees.
6. In inoculations studies, the *Botryosphaeria* isolates were more aggressive and caused larger cankers than the *Phomopsis* isolates, after artificial inoculation of Nonpareil almonds in an experimental fields.
7. In general, the involvement of five different species of *Botryosphaeria* as causes of band canker makes the management of this disease challenging and very difficult.

Table 1. Inoculation with *Phomopsis* and *Botryosphaeria* isolates and 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, corresponding to 0.5, 1, 2, and 3-5 cm diameter sites on the limb. Rating scale: a rating of 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).

Table 2. Effect of fungicides applied in white latex paint on the control of Almond band canker in David Lohse Nonpareil almonds in Glenn County (May 14, 2009).

Treatment	Color	Rate per acre	Rate per gallon
Abound	BW	15.4 fl oz	91 ml
Pristine	Blue	14.5 oz	82 g
Captan 4L	B-dot	1.125 gallon	851 ml
Plant Shield	Y	2.5 lb	227 g
Control with paint only	Or-dot		
Unpainted control	Pink		
Extra Pristine Treatment – Painted on severe band cankers(see below)	RBL	14.5 oz	82 g
Unpainted control of severe band cankers	RBL		

Methods: We applied about 10 gallons per acre. We used Glidden Interior flat latex paint (part number HM 1211 Base 1) and the paint was diluted 1 part paint interior latex paint and 1 part water. (White = no canker; Red = middle size canker (canker was < 25% around the trunk); RBL = large canker (canker extended around the trunk – the same color was used for trees either painted with Pristine).

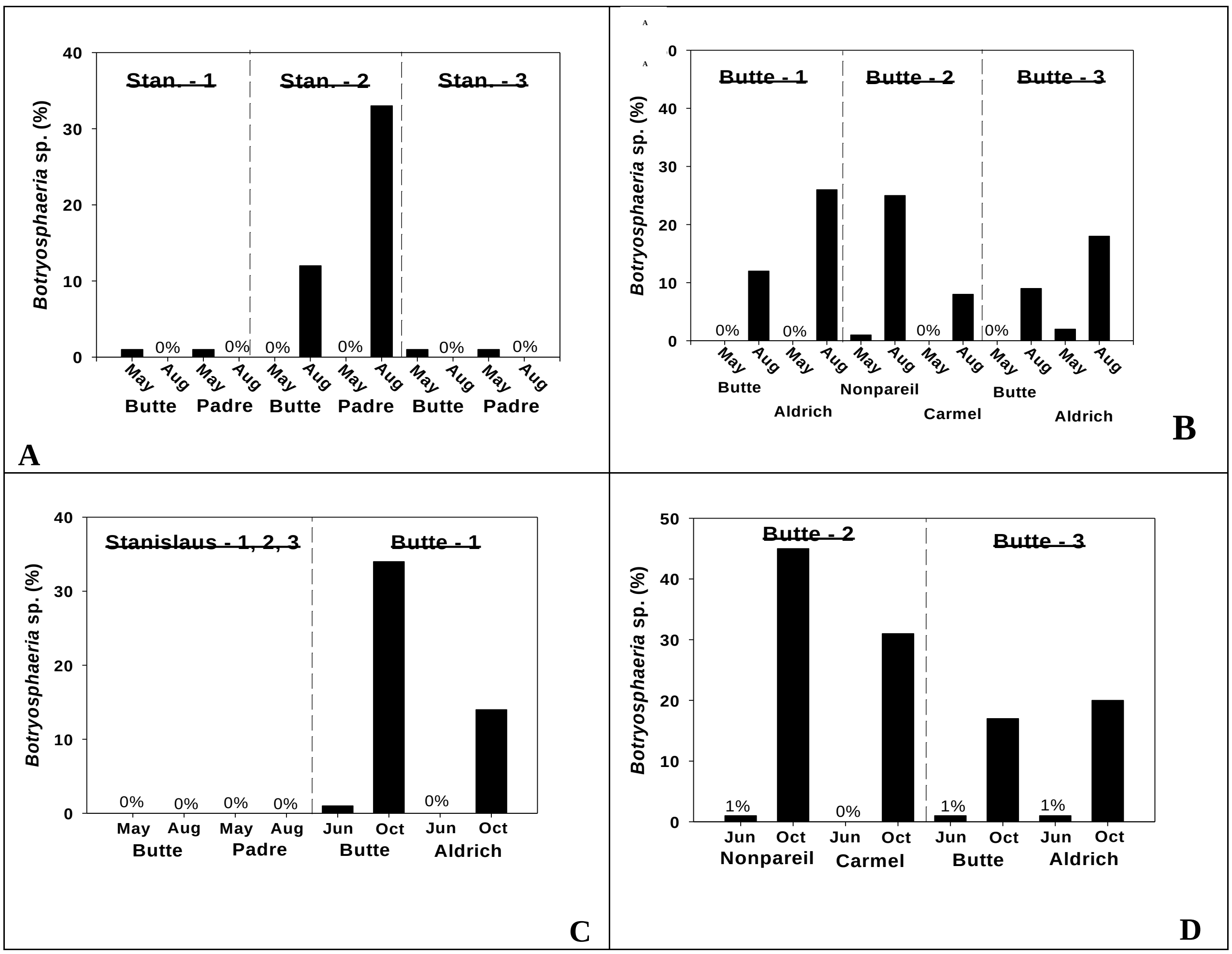

Figure 1. Incidence of isolations of *Botryosphaeria* species isolated from **A** and **B** cankers in limbs killed by lower limb dieback (LLDB) in three orchards each in Stanislaus and Butte Counties and from those **C** and **D** without any symptoms of LLDB collected from the same orchards (2008).

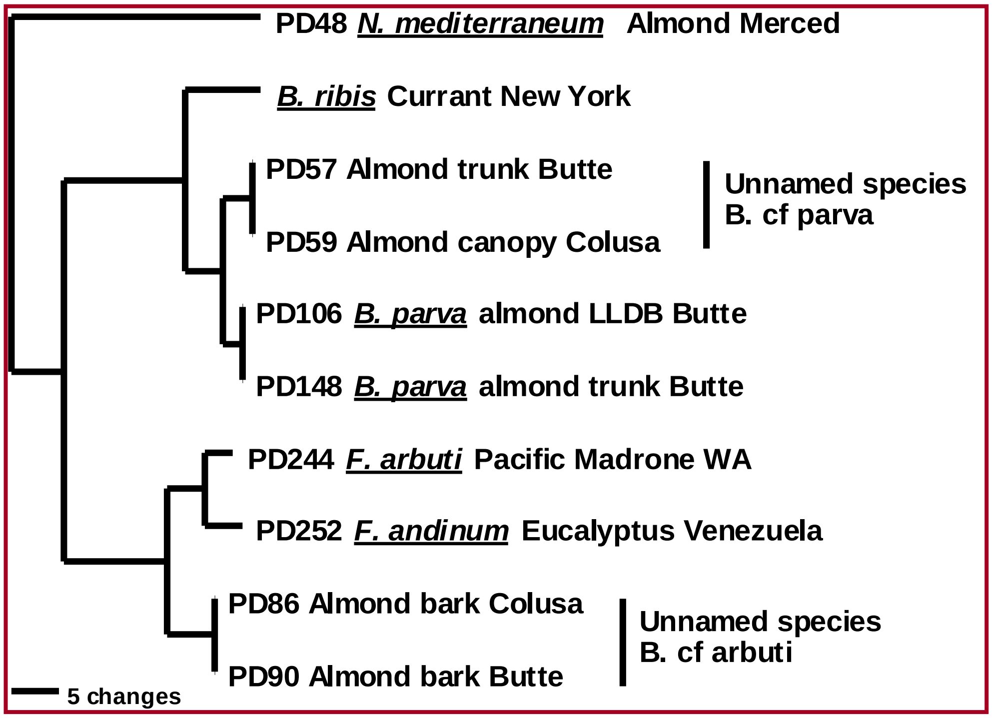

Figure 2. Phylogenetic tree showing the relationship of the *Botryosphaeria* spp. isolated from almond and the two new species of *Botryosphaeria* and a few of other related species from other tree hosts.

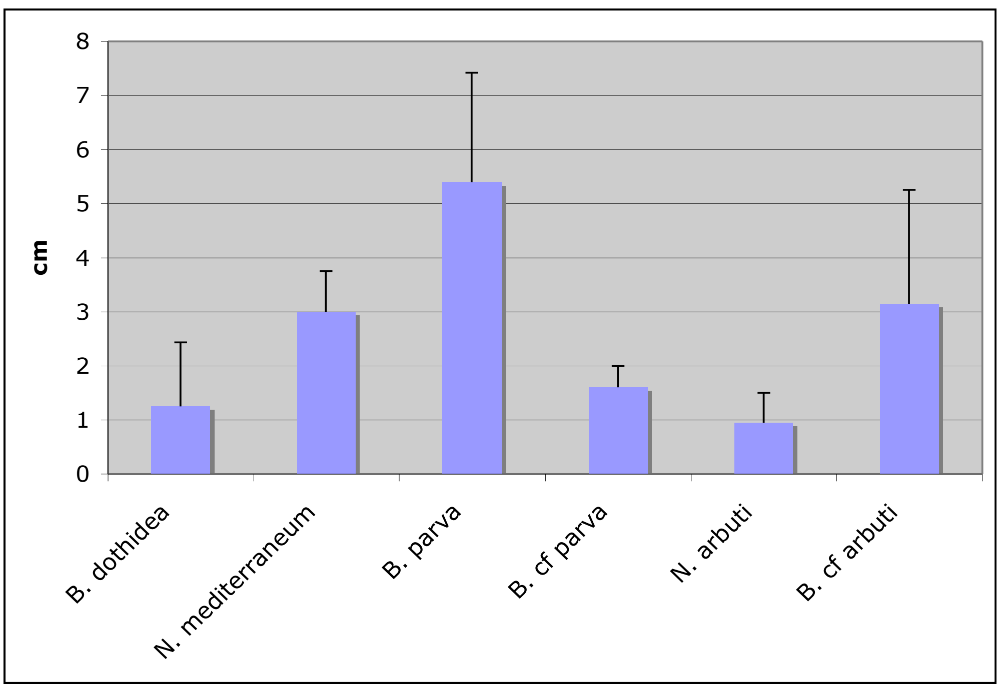

Figure 3. Mean stem canker lengths and standard errors for six different species in the *Botryosphaeria* group of fungi on almond potted trees. Note that cankers in *N. mediterraneum* and *B. parva* were significantly longer than in *B. cf parva* and *N. arbuti.* (*N. arbuti* is a fungus from Pacific madrone not known to occur on almond.).

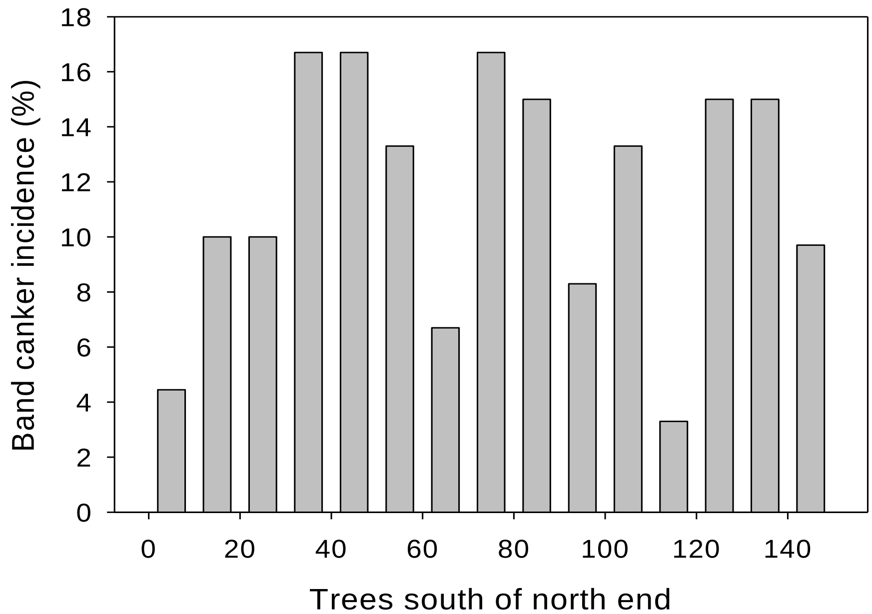

Figure 4. Distribution of almond band canker in a Glenn County orchard (average of six rows). All three categories of cankers included a scale of 1, 2, and 3 severity categories, corresponding to cankers with <25, <50, and >50% of trunk circumference.

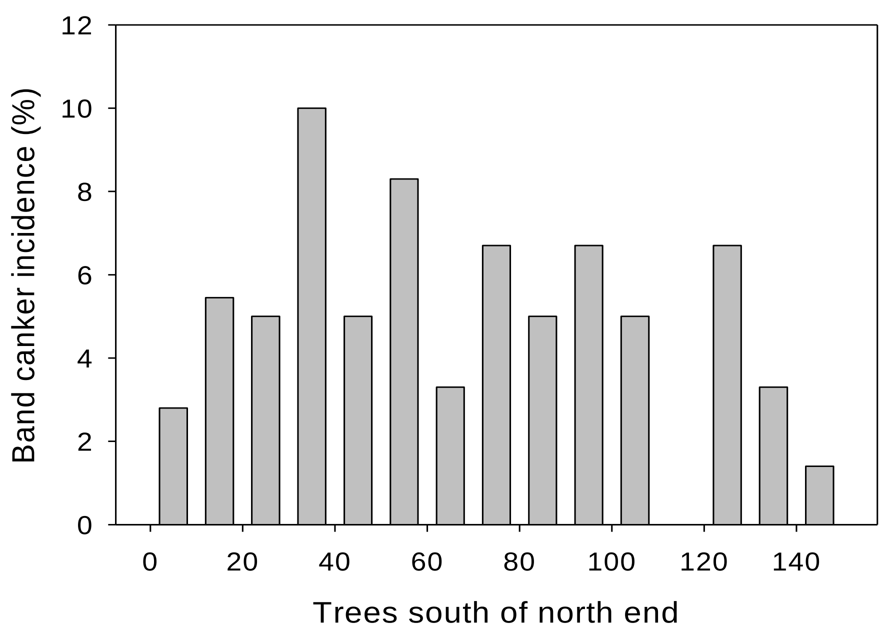

Figure 5. Distribution of almond band canker in a Glenn County orchard (average of six rows). Only cankers in category 3 (severe cankers) included which corresponds to cankers with >50% of trunk circumference.