Wood Decay Fungi Associated with Windfall

Project No.: Path11.Rizzo

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A. Summary

Ganoderma adspersum is a likely recently introduced wood decay fungus in California. We have confirmed infestations and accompanying tree failure in more than 50 orchard blocks spread across 5 counties of the southern growing region ranging in age from 4 to 35 years. More than half of the sampled orchards are 13 years old or younger. While these orchards are in various states of decline, we are aware of hundreds of acres in which a quick decline of many trees resulted in complete orchard removal at 12 years old or less. Our research questions center around learning basic biology of Ganoderma. For example, how does *Ganoderma* spread, howdoes it infect trees, and what is the long-term survival of the pathogen, and how do root stocks vary in their susceptibility to infection. We are also developing protocols to diagnose disease and evaluate management strategies. Preliminary data suggests *Ganoderma* infects trees via spores at the root crown area. We believe spores infect wounds that are caused by shaking and are working on experiments to confirm this. Current experiments and surveys suggest that Nemaguard is the most susceptible rootstock. There is still much to learn about this new pathogen.

B. Objectives

1. Confirm infection pathway and process of *Ganoderma adspersum*
    - Ongoing- have developed a number of experiments to look at infection pathways in order to link to cultural practices (e.g., shaking)
    - Expanded experiments to include interactions with crown gall
2. Access rootstocks for susceptibility to infection and decay
    - Ongoing- have completed first round of inoculations and second round initiated

3. Increase capacity of private diagnostic labs to identify *G. adspersum*

- Ongoing- have completed a number of experiments that can now be translated to practical lab approaches

4. Provide outreach and extension

- Ongoing – continue to make new contacts with growers, POCAs, and farm advisors

C. Results and Discussion

Objective 1. Confirm infection pathway and process

Our approach to studying *G. adspersum* has been to understand the biology of the pathogen in order to design representative experiments around management. Based on our initial research, our main hypotheisis is that infection of almonds by *Ganoderma* is via spores. To perform realistic pathogenicity tests and confirm infection pathways of *G. adspersum* via spores (rather than mycelium), the use of spore-based inoculum is required. Once we have a method to produce spores, we can then begin simulated wounding experiments and inoculation of trees. In 2019, we began the first step in confirming the infection pathway and process, which is to reliably produce spores in a laboratory setting. We are adapting protocols from commercial production of Rieshi mushrooms, a medicinal *Ganoderma* species. To date, *Ganoderma adspersum, G. brownii,* and *G. polychromum* fruiting bodies have been grown in bags, with only the latter shown to actively sporulate (Fig.1). *Ganoderma adspersum* and *G. brownii* fruiting bodies develop a pore layer without signs of sporulation. Continued modification of the sawdust substrate to include nutrients from soybean meal and calcium carbonate is in process.

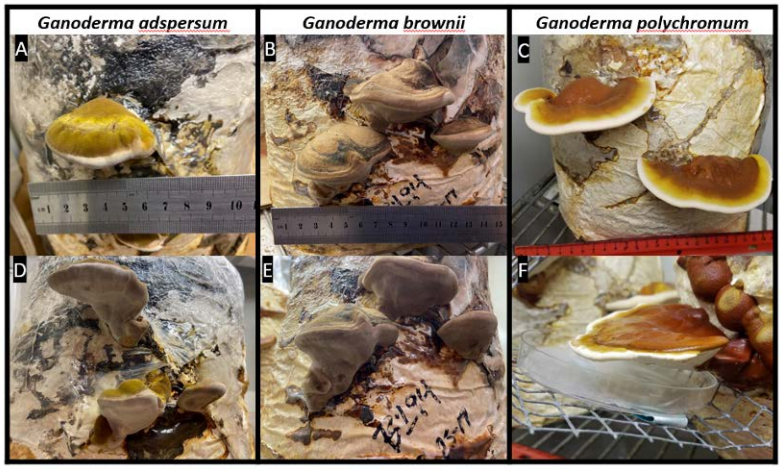


Figure 1. Fruiting bodies with pore layers of (A,D) *Ganoderma adspersum,* (B,E) *G. brownii,* and (C) *Ganoderma polychromum*. (F) Spore collection of actively sporulating G. *polychromum* fruiting body.

In 2020, 15 bags of almond wood have been inoculated with *G. adspersum* and *G. browni* individually, and mycelia is currently growing in the bags. The bags are monitored twice weekly for the presence of pinning and fruiting bodies. We also initiated an experiment to cultivate *G. adspersum* and *G. brownii* fruiting on live trees (Fig. 2). Nine almond trees were inoculated with *Ganoderma* inoculated dowels by drilling 2-3 holes into the base of the trees ~2-3 cm above the soil line at a downward angle. Three trees each were assigned for three *Ganoderma adspersum* isolates. The wounds and dowels were covered with Parafilm

and foil. No fruiting directly on Almond trees has been observed as of December 2020. In addition to developing a method to collect spores, this inoculation experiment will help us determine how long it will take for an almond tree to produce a fruiting body on an infected tree. This experiments will also add to our understanding of infection processes at the orchard level and help with diagnosis (e.g., how long after infection will we see external fruiting bodies on an almond tree).

Figure 2. Almond tree inoculated with *Ganoderma adspersum* colonized dowels

A new area of research that we have initiated is the interaction between *Ganoderma* and crown gall. Our orchard surveys have shown that nearly 50% of the *Ganoderma*-infected and failed trees also exhibited crown gall symptoms. Crown gall is caused by *Rhizobium radiobacter*, which enters plants through fresh wounds at the base and roots of plants. Galls are perennial and increase in size with growth of the tree, causing roots to grow poorly and reducing crop yields. Trees appear to be more prone to breaking at the crown and site of galls, increasing tree susceptibility for secondary infections. Understanding the interaction between *Ganoderma* and crown gall in almond trees will assist in management decisions involving wound and crown gall prevention.

Our preliminary experiments focused on direct *in vitro* antagonism assays. Dual-culture antagonism assays were conducted with the modified use of 10 µl of bacterial solution. Measurement were taken 2, 5, 9, and 10 days post inoculation (dpi) as mycelial growth diameter. Each test was carried out with plates in triplicate and three independent measures were made for each plate at each measuring time. Growth inhibition percentage (GIP) was calculated as [1-(D1/D2)]x100, where D1 is the radial colony growth on bacteria-treated plate, D2 is the radial colony growth in the control plate. The results, in Table 1, Fig. 3, and Fig. 4, show that *Rhizobium* strain 186r does not significantly stunt the growth of *G. adspersum* mycelium regardless of timing.

Table 1. Results of dual-culture antagonism assays with *Rhizobium* strain 186r and a *G. adspersum* strain shown as mycelial growth inhibition percentage (GIP) measured at 2, 5, 7, and 10 days post inoculation (d.p.i.). Columns refer to T0 (*Rhizobium* strain 186r inoculated the same day as *G. adspersum*), T1 (*Rhizobium* strain 186r inoculated 1 day

ahead of *G. adspersum*), or T2 (*Rhizobium* strain 186r inoculated 2 days ahead of *G. adspersum*). Inhibition halo is the average minimum distance between strain 186r colonies and fungal mycelium in dual-culture assays.

Measurement	T0	T1	T2	P value	Inhibition halo (cm)
GIP 2 d.p.i	22.24362	40.13	27.13048	0.1342	0
GIP 5 d.p.i	40.28021	31.74081	29.74431	0.1742	
GIP 9 d.p.i	-1.79309	2.846919	2.264952	0.3608	
GIP 10 d.p.i	0	0	0	-	

Figure 3. Dual-culture treatments from left to right: *G. adspersum*, blank control, water mock inoculation, *G. adspersum* and *R. radiobacter* strain inoculated on same day, *G. adspersum* inoculated one day after *R. radiobacter*, and *G. adspersum* inoculated two days after *R. radiobacter*. Top row is day 2. Bottom row is day 10.

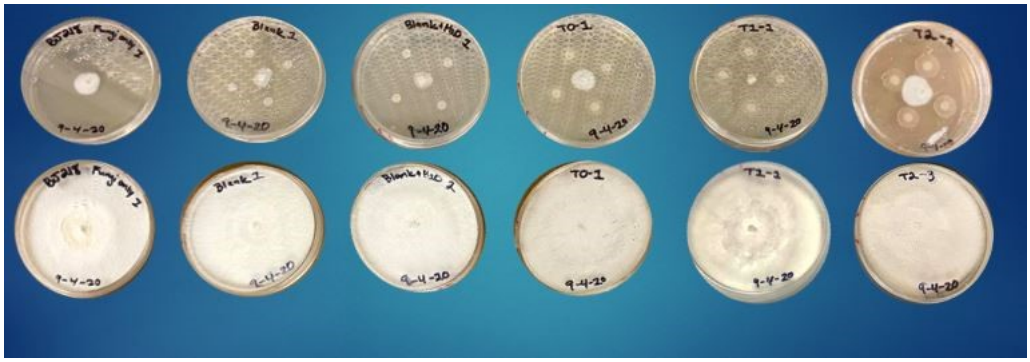


Figure 4. Dual-culture assays from left to right: *G. adspersum*, *G. adspersum* and *R. radiobacter* strain inoculated on same day, *G. adspersum* inoculated one day after *R. radiobacter*, and *G. adspersum* inoculated two days after *R. radiobacter*.

Our next step is to test effect of dual inoculations of *G. adspersum* and *R. radiobacter* on almond rootstocks: Nemaguard, Viking, Lovell, Rx1, and VX211. Dual inoculations will be done at the same time and in different sequences. Twelve months after the initial inoculation, plants will be evaluated for symptoms, such as gall appearance, decayed wood, and wood discoloration. The diameter and weight of the galls, and length of decayed wood and discoloration will be measured using calipers. The symptoms of individual and dual infections will show the effect of dual inoculations on almond health.

Objective 2. Assess rootstocks for susceptibility to infection and decay

In our initial surveys, we investigated decay related root and butt failures in 75 almond orchard blocks in 11 counties in the Central Valley's three almond growing regions from 2016 through 2019. Investigations were based on reports from growers. A majority of the sites were in the southern almond growing region comprising Fresno, Kern, Kings, Madera, and Tulare Counties. *Ganoderma* was the most prevalent genus associated with root and butt failure in almond orchards and *G. adspersum* was by far the most prevalent species. *G. adspersum* was only found infecting almond orchards using Nemaguard peach seedling cultivar as a rootstock. *Ganoderma adspersum* caused tree mortality in orchard blocks ranging in age from 4 to 34-years. In order to examine the role of rootstock in in fections we set up several inoculation experiments with six different rootstocks.

In September 2019, 10 months after initial inoculation two trials evaluating the decayability of almond rootstocks by *Ganoderma* were terminated. When 6 different rootstocks were inoculated with *G. adspersum*, 10 months after inoculation white rot was evident, but there were no differences between rootstocks. Discoloration was greated in Empyrean, Viking and Krymsk 86 (Figure 3).

Figure 3. *G. adspersum* decay in almond rootstocks

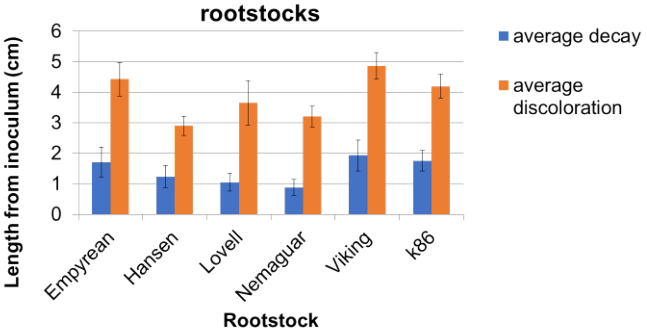
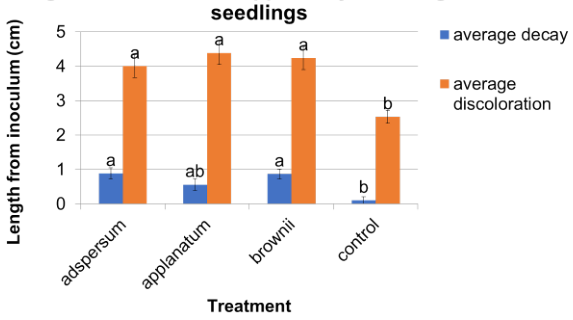


Figure 4. *Ganoderma* spp. decay in nemaguard seedlings

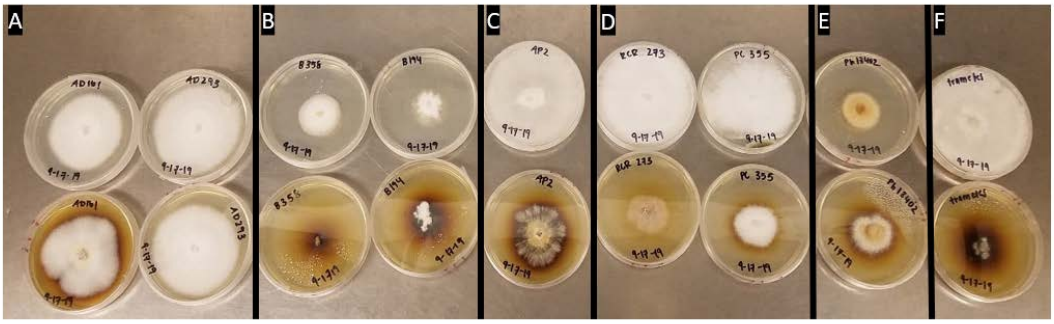


The second trial, where Nemaguard seedlings were inoculated with three different *Ganoderma* species, *G. adspersum* and *G. brownii* resulted in more decay than the wounded control (Fig. 4). In spring 2020, these inoculuation experiments were repeated with the same rootstocks to confirm the original data. We are letting these incouations go for 10 months to a year and will collect data in spring 2021.

Objective 3. Increase capacity of private diagnostic labs to identify *G. adspersum*

In our lab, diagnosis of *G. adspersum* has been conducted through morphological observation and confirmed with genetic analysis. The lengthy nature and cost of genetic analysis makes culture diagnostics a favored choice for diagnostic labs. Our inital studies have shown that cultures of *Ganoderma* species vary in their tolerance to the toxicity of gallic added to nutrient media, resulting in varied growth patterns. Differences in their ability to oxidize gallic lead to varied color changes. We would like to complete these studies to provide information to diagnostic lab around the state. Results of a growth trial would provide a characterization of the growth and color of *Ganoderma* species on different types of media, which allows or an official reference for culture diagnostics.

In order to provide diagnostic labs with a protocol for *G. adspersum* diagnosis, we will be performing a growth trial using various types of selective media. Cultures of wood-decay species vary in their tolerance to the toxicity of gallic added to nutrient media, resulting in varied growth patterns. Differences in their ability to oxidize gallic lead to varied color changes. Isolates of common wood decay fungi and other common almond fungal pathogens will be plated on malt extract agar (MEA), MEA with streptomycin and benomyl, and MEA with 0.5% gallic acid, and incubated in the dark for 10 days at room temperature. We will organize a diagnostic flowchart based on the results of the growth trials.



Example of 10-day old cultures of wood decay species grown on MEA (top) and MEA + gallic acid (bottom). A) *G. adspersum*. (B) *G. brownii.* (C) *G. applanatum.* (D) *G. polychromum.* (E) *Phellinus tuberculosus.* (F) *Trametes* sp.

Objective 4. Provide outreach and extension

See section D below.

D. Outreach Activities

Due to COVID, we had fewer in-person activities in 2020 than expected. We have been in communication with ~15 growers via phone and email due to a flyer we circulated, with help from Katherine Jarvis, about our upcoming *Ganoderma* spore survey (flyer attached). This flyer and outreach has set the stage for a large experiment to be initiated in spring 2021. Several growers were able to realize that they may have *Ganoderma* in their orchards after seeing the flyer. Daisy hernandez in the lab has answered questions for all the grower's orchards and plans to confirm possible infections. Several growers and a farm advisor (Phoebe Gordon) have sent us fruiting bodies and/or pictures of their fallen trees in lieu of us visiting them. A virtual presentation was given at the annual California Forest Pest Council meeting in November 2020 by Daisy Hernandez.The goal of this presentation was to introduce issues around *Ganoderma* to a wider audience consisting of professionals working in forest and urban tree health. Having aadditional professionals observing Ganoderma, will allow us to gain a better distribution of these pathogens in California and how this can assist us in determining risk to almond orchards.

In 2019, we visited approximately 15 orchards in the San Joaquin Valley to meet with growers and/or PCAs. During each visit, practitioners were trained in *Ganoderma* identification and proper sample collection techniques. In November 2018, a presentation on *Ganoderma* in almond was given at a public meeting in Visalia organized by Soil Basics. Approximately 35 individuals were in attendance. In June 2019, another *Ganoderma* presentation was made at a public meeting in Tulare County organized by Soil Basics.

Approximately 55 people were in attendance. A poster and presentation were made at the 2019 Almond Conference and 3 podcast interviews were conducted.

Materials and Methods *(500 word max.)***:**

Objective 1. Confirm infection pathway and process

Cultures of *Ganoderma spp.* were isolated from fruiting bodies collected from *Prunus* orchards. Inoculum was prepared by placing mycelial pieces into malt extract broth (MEB) and incubating in a shaking incubator for 1 week at room temperature before blending of the mycelia and transferring inoculum into new MEB flasks to incubate for another week at room temperature. Autoclaved sawdust bags were inoculated with the rye, and incubated in the dark at room temperature until fully colonized. Sporulating fruiting bodies developed within 1-2 months depending on the *Ganoderma spp.* Spores were collected by placing a Petri dish with wax paper within 2 cm of the bottom of the fruiting bodies or by gently brushing the spores from the top of the fruiting bodies. Spores were stored in dry, dark conditions at room temperature and monitored monthly for longevity.

To grow fruiting bodies on live trees, nine trees were inoculated with *Ganoderma* dowels by drilling holes into the base of the trees ~2-3 cm above the soil line. Three trees were assigned for three *Ganoderma adspersum* isolates. One tree for each isolate was inoculated with three dowels, and the other two trees were inoculated with two dowels. The wounds and dowels were covered with Parafilm.

Objective 2. Assess rootstocks for susceptibility to infection and decay

Saplings of six almond rootstocks: Nemaguard, Lovell, Krymsk 86, Empyrean 1, Hansen 536 and Viking were grown in 6-gallon pots. *G. adspersum* colonized hardwood dowels were inserted into a hole drilled approximately 2 cm above the soil surface. Uncolonized dowels and unwounded trees served as controls. Trees were monitored for disease symptoms. The trial was terminated 10 months after inoculation. A 10 cm section of trunk, centered on the site of inoculation was collected and cut length wise for evaluation. The length of discoloration and column of decay was recorded and then re-isolation was attempted. A similar trial using only Nemaguard rootstock was conducted to evaluate the decay potential of three different *Ganoderma* species; two isolates each of *G. adspersum, G. brownii*, and *G*. *applanatum*. Protocol was the same as described above.

Objective 3.Increase capacity of private diagnostic labs to identify *G. adspersum*

Cultures of wood-decay species vary in their tolerance to the toxicity of gallic or tannic added to nutrient media, resulting in varied growth patterns. Differences in their ability to oxidize gallic or tannic acid also leads to varied color changes. Isolates of common wood decay fungi were plated on MEA with 0.5% gallic acid and MEA with 0.5% tannic acid, and incubated in the dark for 7-10 days at room temperature. Isolates were also grown in flasks of MEB with 0.5% gallic acid incubated in a shaking incubator at room temperature.

E. Publications that emerged from this work

1. Publications in preparation (attached)

Johnson B, Hernandez DA, Rizzo DM. *Ganoderma adspersum*, the cause of quick decline of almond orchards in California. (to be submitted to Plant Disease in spring 2021)

Johnson B, Marvinney E, and Rizzo DM. Challenging prevailing assumptions of almond orchard productive lifespan in California. (to be submitted to PLOS One in spring 2021)

2. Outreach material:

Flyer sent to growers announcing a spore collection experiment (attached)