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ASSESSING THE TRANSMISSION, INFECTION, AND PATHOGENICITY OF WOOD DECAY FUNGI IN CALIFORNIA ORCHARDS

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ASSESSING THE TRANSMISSION, INFECTION, AND PATHOGENICITY OF WOOD DECAY FUNGI IN CALIFORNIA ORCHARDS

By

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ABSTRACT

Ganoderma species are well-documented pathogens of a wide range of woody hosts. Recently, *Ganoderma adspersum* has been observed to be associated with wood decay and tree failure in California almond orchards. The purpose of this study was to investigate the pathogenicity, infectivity, and transmission of *G. adspersum* associated with increasing tree failure in California's Central Valley.

To date, no studies have investigated the pathogenicity of *Ganoderma* species in agricultural and native trees in California. Pathogenicity tests were conducted to determine the pathogenicity of *G. adspersum* and *G. brownii* (a native *Ganoderma* species in California*)* on almond, walnut, pistachio, and oak trees. Inoculations were made by coring into the sapwood of the saplings' basal stem and pipetting *Ganoderma* spore solutions into the wounds. In two greenhouse trials, 10 months post-inoculation, trees had no visual, external symptoms of disease, although there were differences in the extent of internal wood decay and xylem discoloration. *Ganoderma adspersum* caused extensive discoloration and was reisolated from Nemaguard and Viking rootstock cultivars. The data suggest that these *Ganoderma* species were pathogens capable of infecting healthy sapwood of almond.

Disease surveys in almond orchards revealed substantial tree losses associated with *G. adspersum.* In one case, tree losses resulted in early removal of a 19-20-year-old orchard. A two-year long spore survey in almond orchards revealed that the majority of spores were trapped during increased temperatures and almond harvest from August to September. These findings suggest the need to assess management practices to reduce dust production and *G. adspersum* spore transmission in California orchards.

In field conditions, *G. adspersum* was often associated with bacterial colonization of *Agrobacterium tumefaciens,* causal agent of crown gall. The high co-incidence of the fungal and bacterial pathogens suggests potential synergystic effects in pathogenesis. However, co-inoculation assays have shown that the pathogens do not significantly interact with each other nor promote increased initial disease development. The rise of multi-pathogen diseases in almonds likely results from the rapid adoption of high-density planting, mechanized harvesting, and increased inputs of irrigation and fertilization, which create an environment that facilitates the infection and establishment of multiple pathogens.

CHAPTER 1: PATHOGENICITY OF GANODERMA SPECIES IN CALIFORNIA AGRICULTURAL AND NATIVE HOSTS

Abstract

Ganoderma species have been associated with wood decay of dead or dying trees and failure of living trees. Past studies have not investigated the pathogenicity of *Ganoderma* species in agricultural and native trees in California. Pathogenicity tests were used to determine the pathogenicity of *Ganoderma adspersum* and *G. brownii* on young, healthy trees (*Prunus dulcis* rootstock cultivar Nemaguard, Viking, and Lovell; *Juglans microcarpa* x *Juglans regia* rootstock Rx1, and VX211; *Pistacia vera* rootstock cultivar UCB1; *Quercus douglasii*; and *Quercus lobata*. Inoculations were made by coring into the sapwood of the crown and pipetting *Ganoderma* spore solutions into the wounds. In two greenhouse trials, 10 months post inoculation, trees had no visual, external symptoms of disease, though there were differences in the extent of internal wood decay and xylem discoloration. *Ganoderma adspersum* caused extensive wood decay and discoloration and was reisolated from Nemaguard and Viking cultivars. The data suggests that this species was capable of infecting healthy sapwood and may be a pathogen of young trees.

Keywords: Ganoderma, almond, walnut, pistachio, oak, spores

Introduction

Ganoderma is one of the more diverse genera of wood decay fungi in the world with over 300 described species (Bhosle et al. 2010) and causes economically important diseases in a wide range of woody crops (Seo and Kirk, 2000). Infections of *Ganoderma* spp. are typically associated with the presence of applanate reddish-brown basidiomata which produce high volumes of inoculum (Kadowaki et al., 2010). Infected trees often fail due to windthrow or breaks caused by a loss of strength and rigidity due to decay of the cellulose and lignin of the lower bole or root flare (Sinclair and Lyon 2005).

Recent studies have shown that *Ganoderma adspersum* is established within the Sacramento and Central Valley of California as the causal agent of tree failure and quick decline of almond orchards (Johnson 2020). *Ganoderma adspersum* is mainly limited to almond rootstock cultivar Nemaguard (*P. persica* × *P. davidiana*), though there have been observations of the fungus in walnut rootstock cultivar Rx1 (*Juglans microcarpa* x *Juglans regia)* and a rose bush (*Rosa* sp.). Maximum-likelihood phylogenetic analysis revealed clustering of California isolates with isolates from Europe and Asia, suggesting that *G. adspersum* has been introduced to California (Johnson 2020).

In its native range in Eurasia, *Ganoderma adspersum* is an aggressive root and butt rot pathogen capable of penetrating and decaying through reaction zones of infected wood, causing extensive decay over a relatively short period of time (Schwarze et al. 2003). Pathogenicity tests with *G. adspersum* on hosts from its native range have been conducted on *Tilia* sp. (linden) and *Aesculus* sp. (horse-chestnut) in Italy, and *Pseudotsuga menziesii* (Douglas fir), *Fagus sylvatica* (European beech), *Quercus robur* (English oak), and *Acerpseudo platanus* (Sycamore maple) in Germany (Nicolotti et al., 2004; Deflorio et al. 2008). A native species of *Ganoderma, G.*

brownii, has also been observed on almond trees although at a much lower incidence than *G. adspersum* (Johnson 2020). Pathogenicity tests have not been previously conducted for *G. brownii*.

While *G. brownii* may have jumped from native hosts to opportunistically infect nearby almond trees, it is also possible for spillover from infected hosts in environments with low biodiversity to novel, native hosts (Beckstead et al. 2009). There are over 500,000 ha of *Prunus* crop species in California alone (USDA National Agricultural Statistics Service, 2022), making up a homogenous landscape capable of carrying high loads of fungal inoculum. High loads of inoculum and susceptible hosts combined with the statewide shaking and sweeping of *Prunus* (almond) orchards during harvest-time could allow pathogenic fungi to spread into other tree crops or natural forests.

Ganoderma propagules must be in contact with or in close proximity to an infection court for colonization of a host to occur (Adaskaveg and Gilbertson 1987; Bakshi et al. 1976; Rees et al. 2007). In the surrounding natural forests, disturbances, such as fire may result in wounds that increase the susceptibility of vulnerable novel hosts (Gara et al., 1985; Littke and Gara 1986; Gara 1988, Parker et al. 2007). Additionally, *G. adspersum* predominately infects and establishes in hardwoods (Nicolotti et al., 2004; Deflorio et al. 2008), a dominant tree type in lower elevation forest regions of California (Fryer 2007).

Disease of native hosts may lead to the fundamental change of the forest ecosystems, given the infection of an abundant tree species (Loo 2009). Implementing proper management practices to prevent spillover will require bridging the gap of knowledge on systems involving pathogenic fungi. Therefore, it is crucial to examine the potential of *G. adspersum* to infect California native species.

The goal of this project is to investigate the pathogenicity of *G. adspersum* (putatively introduced) and *G. brownii* (native) on agricultural tree crops with known [almond and walnut] (Johnson 2020) or possible [pistachio] infections. In addition, *Quercus* species are known as hosts of *G. adspersum* in its native range (Tortic 1970, Gašparcová et al., 2017a, Náplavová 2021). California has large areas of oak woodlands found in proximity to almond orchards affected by *Ganoderma* (Howard 1992, Fryer 2007). Although *G. adspersum* has not been found on oak in California, our goal is to conduct preliminary experiments to determine their potential as hosts.

Most previous inoculation studies with *Ganoderma* species have used inoculum grown on wooden dowels that were inserted into wounds (Nicolotti et al. 2004, Deflorio et al. 2008, Loyd et al. 2018). However, it is likely that *G. adspersum* transmission in almond orchards is mainly due to aerial spores (Johnson, 2020) because *Ganoderma* species are poor competitors in the soil environment (Bakshi et al. 1976; Rees et al. 2007). Therefore, in our studies pathogenicity tests were conducted using *Ganoderma* spore inoculum.

Materials and Methods

Ganoderma identification and spores

Ganoderma adspersum and *G. brownii* were observed during 2016 - 2020 surveys of almond orchards in the Central Valley of California and non-agricultural areas in California, respectively (Johnson 2020). Attempts to grow *G. adspersum* and *G. brownii* basidiocarps for spores were made following modified protocols of previous *Ganoderma* cultivation methods (Stamets 2000). However, spore production was insufficient using these *in vitro* methods. Therefore, spores for inoculations were collected in Petri dishes from *G. adspersum* basidiomata growing on two almond orchards in Fresno, California, and *G. brownii* basidiomata removed from bay laurel (*Umbellularia californica*) in Big Sur, California.

Basidiomata were initially identified based on the macromorphological features of basidiomata and were validated through sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA). A portion of the basidiomata context tissue was removed and cut into small pieces (2 mm x 2 mm). The pieces were plated (9 pieces per plate) onto 90-mm-diameter Petri dishes of 1.4% Malt Extract Agar (MEA) amended with benomyl (4 µg a.i./ml) and streptomycin sulfate (100 µg/ml) and sub-cultured onto MEA after incubation at 25 ± 1°C in dark conditions for 10 days (Rizzo et al. 1998). To confirm spore viability and taxa, spores were also plated onto amended MEA and subcultured, as previously described.

Genomic DNA was extracted from 10- to 14-day old fungal colonies from basidiomata and spores growing on MEA using Prepman Ultra Sample Preparation Reagent (ThermoFisher Scientific), according to the manufacturer's instructions. The internal transcribed spacer (ITS) regions, including the 5.8s gene was amplified by PCR using primers ITS1-F (Gardes and Bruns 1993) and ITS4TT (Bourret et al. 2018). For each PCR reaction, the following reagents were

used: 12.5 µl of Immomix Red Master Mix (Bioline, London, UK), 8.5µl of PCR-grade H2O, 1µl BSA (3% w/v), 1µl of each primer, and 1ng/µl of DNA template (Loyd et al. 2018). Reactions were performed on an Eppendorf AG 22331 mastercycler (Eppendorf, Hamburg, DE) with the following thermocycler conditions: cycle of 95° C for 10 min, 35 cycles of 94° C for 30 sec, annealing temperature of 55° C for 1 min, a final extension step of 72° C for 5 min, and then 4° C (Loyd et al. 2018).

Following confirmation of amplification with gel electrophoresis, PCR products were sequenced. Amplicons were purified with exonuclease I and shrimp alkaline phosphatase (New England BioLabs Inc., Ipswich, MA), according to the manufacturer's recommendations. All ITS sequences generated were deposited and accessioned in the GenBank sequence database.

Host plants

For each pathogenicity trial, bare root trees of almond rootstock cultivars Nemaguard (*P. persica* × *P. davidiana*), Viking [*P. persica x (P. dulcis x (P. cerasifera x P. armeniaca*)], and Lovell (*Prunus persica*); *Juglans microcarpa* x *Juglans regia* rootstock cultivars Rx1 and VX211; *Pistacia vera* (pistachio) rootstock cultivar UCB1, *Quercus douglasii* (Blue oak), and *Quercus lobata* (Valley oak) were sourced from commercial growers in northern California and potted into 19 liter containers with sterilized UC mix soil made up of sand, redwood compost, and sphagnum peat moss (expanded) at a ratio of 1:1:1. The rootstock cultivars were selected based on their prominent use in California orchards. *Quercus* species were selected due to their range in the Sierra foothills of California in relative proximity to almond orchards in the southernmost area of the Central Valley. Individual trees within a species were of similar size; almond and pistachio trees were ~2 cm in diameter and walnut and oak trees were ~1 cm in diameter. Almond rootstock cultivar Lovell and walnut rootstock Rx1 trees failed to establish

and died within one month of potting, likely due to prolonged exposure to heat and wind prior to potting. The rootstocks were not available for disease assessment.

Pathogenicity tests

Pathogenicity tests were conducted twice in a 2400 SF, Level of Control and Protection (LCP) 2 greenhouse at the University of California, Davis. For each trial, the pathogenicity tests were set up in a completely randomized design with spore inoculations of *G. adspersum* and *G. brownii*, as well as mock inoculations of controls across the eight host types with five replicates per *Ganoderma*-host combination (n=120). Trees were irrigated and fertilized every 2-3 days with drip irrigation, fertilized, and sprayed with insecticides monthly for spider mites.
Prior to the trials, 1 ml of $1x10^9$ CFU/ml spore solutions were pipetted onto MEA plates and 5 cm x 5 cm sterilized almond wood blocks. Growth on plates and wood confirmed viability of spores for use in inoculations.

Inoculation of MEA plates and wood blocks were repeated with fresh spore solutions the day of the trials. Trees were inoculated on November 22, 2021 (trial 1) and April 14, 2022 (trial 2) by pushing a 5 mm diameter increment borer at least 3 mm into the bark and cambium of the trees, 5 cm above the soil line; removing the bark of the 5 mm wound with a scalpel; and pipetting 1 ml of $1x10^9$ CFU/ml spore solutions or sterilized deionized water into the wound. All wounds were sealed with Parafilm and covered with aluminum foil to avoid contamination and desiccation.

Destructive harvest, disease assessment, and reisolation

Inoculated trees were monitored monthly and assessed for disease symptoms 10 months post-inoculation: September 19, 2022 (trial 1) and February 15, 2022 (trial 2). Trees were cut below the inoculation point and sectioned into approximately 10-cm long sections. Longitudinal

cuts were made through the inoculation point and lesion lengths were measured (Fig. 1.1), by taking five 2mm diameter pieces plating them into yeast malt extract (YME) amended with benomyl (4µg a.i./ml) and streptomycin sulfate (100 µg/ml) to confirm the presence of the inoculated *Ganoderma* species. Subcultures of suspected *Ganoderma* isolations were made onto MEA and confirmed using morphology and ITS sequencing.

Statistical analyses.

To test for differences in length of discoloration or decay with the different *Ganoderma* inoculations relative to the mock inoculated control, analysis of variance (ANOVA) and Dunnetts Means comparison test were calculated independently for each experiment and tree species in R, with *Ganoderma* species or mock inoculated control as the independent variable and length of discoloration or decay as the dependent variables (Loyd et al. 2018).

Results

Disease assessment, wound responses, and reisolation

In trial 1, significant internal discoloration was observed in almond rootstock cultivars Nemaguard ($P=0.03$), Viking ($P=2.5*10^{-5}$), and UCB1 ($P=5.8*10^{-8}$) inoculated by *G. adspersum* (Fig. 1.1-1.2). In trial 2, significant internal discoloration was observed in almond rootstock cultivars Viking ($P=0.05$), and VX211 ($P=0.03$) inoculated with *G. adspersum*¸ and VX211 ($P=0.01$) inoculated with *G. brownii* (Fig. 1.3). In both trials, no inoculated tree types had significant decay ($P>0.05$, Table 1.1, Fig. 1.4-1.5) with any inoculation. No symptoms were observed on any of the mock inoculated controls. No external disease symptoms were observed in any trees.

In both trials, all inoculation sites had or were in the process of healing over with response wood and bark for all *Ganoderma* and mock inoculated Nemaguard, UCB1, and oak trees. Generally, *Ganoderma* and mock inoculated Viking and VX211 had also healed over with response wood and bark, though few *Ganoderma* and mock inoculated Viking and VX211 trees displayed a 5 mm wide x 3-7 mm long outgrowth of sapwood at the inoculation site.

In walnut rootstock VX211, pistachio rootstock UCB1, Valley oak, and blue oak; the inoculated species were not reisolated (Table 1.2). In almond rootstocks Nemaguard and Viking, inoculated *G. adspersum* was successfully reisolated from the decayed or discolored wood in 60% and 20-40% of inoculated trees, respective of rootstock. In Nemaguard, *G. brownii* was successfully reisolated from the decayed or discolored wood of 20% and 40% of inoculated trees, respective of trial. In both trials and all hosts, reisolation failure was frequently associated with ‘contaminant’ microorganisms, such as *Trichoderma* sp., *Penicillium* sp., *Rhizopus* sp., and *Mucor* sp.

Discussion

While past studies have attempted inoculations with *Ganoderma*-inoculated dowels (Nicolotti et al. 2004, Deflorio et al. 2008, Loyd et al. 2018), this study is the first to attempt the use of *Ganoderma* spores as part of the inoculations of wounded trees. Using spore-based inoculum, *G. adspersum* was seen to be a major pathogen of sapwood in young, actively growing almond trees, while *G. brownii* was seen to be a minor pathogen of almond. As a native fungus, *G. brownii* has been present in California for longer than *G. adspersum*, though the strongly invasive behavior of *G. adspersum* observed in the inoculation trials explicate the substantially higher incidence of *G. adspersum* and associated tree failures in almond orchards (Johnson 2020).

Spore inoculations were used to mimic the likely sources of infection in the field. Wound inoculation was conducted on lower trunks, due to its success in past studies and observations of *Ganoderma* basidiomata on trunk wounds from shaker damage in orchards and fire scars in forests (Pataky 1999). Most *Ganoderma* and mock inoculated trees had wounds that were partially or completely closed over, suggesting active compartmentalization (Boddy and Rayner 1983, Gilbertson 1980). Other *Ganoderma* and mock inoculated trees had outgrowths of sapwood, likely due to the inoculation method. In both trials, no external symptoms were observed across all tree species inoculated with either *Ganoderma* species, or in the mock inoculated controls.

Successful reisolation of *Ganoderma* species from the discolored wood in Nemaguard and Viking confirmed the cause of symptoms. The internal xylem discoloration in all agricultural tree species is likely due to *Ganoderma* infection, as mock inoculation trees exhibited minimal discoloration in the tested agricultural tree hosts. Trees respond to wounding of sapwood by

producing wood-staining antimicrobial compounds, such as phenolics that discolor wood and create well-defined colored reaction zones which prevent the establishment and spread of decay microbes (Shigo 1985, Scheffer and Morrell 1998, Deflorio et al. 2008). Discoloration and decay may extend further through the sapwood and subsequently, heartwood, when trees are unable to effectively restrict fungal colonization (Green et al. 1981, Shigo et al. 1977). Bacteria and wood-staining fungi, often prevalent in wounded sapwood tissues, may outcompete slower-growing decay fungi (Scheffer and Cowling 1966).

Internal discoloration was commonly observed in oak controls, which indicate a greater response to wounding (Deflorio et al. 2008). The frequent isolation of Mucor/Rhizopus (Zygomycota) species from discolored oaks may have outcompeted the establishment of the decay species in oaks. These factors may explain the unsuccessful infections of *G. adspersum* and *G. brownii* and the lack of significant discoloration and decay in the inoculated oak trees. The results of this study coincide with past studies, in which *G. adspersum* behaved as a weakly invasive saprobe in oaks (Deflorio et al. 2008). There is a low risk that *G. adspersum* will cause substantial disease in California's oak-woodlands.

The results of this study coincide with past observations and studies showing *G. adspersum* is capable of degrading both lignin and cellulose, and invading living host sapwood (Wilkins 1943, Pearce and Woodward 1986, Pearce 1991, Schwarze and Fink 1997). Orchard trees often experience mechanical wounding on trunks and roots from tree shakers (Abdel-Fattah et al. 2003), and presumably these wounded tissues can become infection courts for decay fungi, such as species of *Ganoderma*. This study's data suggests that *G. adspersum* is likely to cause infection in young, wounded trees that may only possess sapwood. Wood composition and host

defense may play a role in virulence, as the extent of discoloration varied greatly between rootstocks.

In conclusion, *G. adspersum* was a sapwood pathogen of the almond rootstocks tested following the wounding of the lower trunk tissue. *Ganoderma adspersum* typically affects the basal stem and major lateral roots of infected almond trees, which are predominantly sapwood for the first several decades before structural, nonfunctional heartwood is formed within the sapwood (Adaskaveg and Ogawa 1990). The fungal pathogen causes structure failure in trees of 12 years, on average (Johnson 2020), well before the development of heartwood, though *G. adspersum* can overcome reaction wood (Schwarze and Baum 2000, Schwarze et al. 2003) in sapwood to advance and cause decay within maturing trees.

Future studies may explore the role of wood composition among different rootstocks and tree types in the pathogenicity of *Ganoderma* species. Inoculations of wounded roots may provide knowledge on the role of different infection courts in *G. adspersum* pathogenicity. Long term pathogenicity studies may also investigate the development of wood decay disease and basidiomata on mature trees.

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Tables

Table 1.1. *Ganoderma* spp. isolates from basidiomata, in which spores were collected and used in this study, with associated GenBank accession, location of collection, and host information.

Isolate	**Taxon**	**Host**	**County (CA)**	**ITS GenBank accession**
FD0404	*G. adspersum*	*Prunus dulcis*	Fresno	
FD0428	*G. adspersum*	*Prunus dulcis*	Fresno	
KBS1105	*G. brownii*	*Umbellularia californica*	Monterey	
KBS0119	*G. brownii*	*Umbellularia californica*	Monterey	

Table 1.2. Average total lengths of discolored wood and wood decay, and inoculum recovery for almond rootstock cultivars Nemaguard and Viking; walnut rootstock cultivar VX211; pistachio rootstock cultivar UCB1; *Quercus douglasii*; and *Quercus lobata* inoculated with *Ganoderma adspersum* and *G. brownii.*

	Trial 1			**Trial 2**		
	Discolored wood (mm) [a]	**Decayed wood (mm)**	**Inoculum recovery**	**Discolored wood (mm)**	**Decayed wood (mm)**	**Inoculum recovery**
Nemaguard						
Control	0	0	- [c]	10.65 ± 2.18	0	-
G. adspersum	50.62 ± 37.2* [b]	39.94 ± 36.24	+++ [d]	20.29 ± 7.90	4.97 ± 2.98	+++
G. brownii	4.60 ± 6.51	2.50 ± 3.53	+	11.7 ± 7.43	5.10 ± 7.43	++
Viking						
Control	0	0	-	0	0	-
G. adspersum	18.68 ± 6.22*	3.57 ± 4.89	+	18.97 ± 15.76*	5.04 ± 4.61	++
G. brownii	0	0	-	3.83 ± 4.01	0	-
VX211						
Control	0	0	-	0.93 ± 1.87	0	-
G. adspersum	8.13 ± 8.81	0.42 ± 0.84	-	6.35 ± 3.69*	0	-
G. brownii	1.61 ± 2.78	0	-	7.32 ± 1.50*	0	-
UCB1						
Control	0	0	-	0	0	-
G. adspersum	7.47 ± 0.80*	0	-	7.81 ± 2.54	0	-
G. brownii	0	0	-	4.83 ± 3.00	0	-
Valley Oak						
Control	0	0	-	2.18 ± 2.86	0	-
G. adspersum	6.37 ± 5.24	0	-	8.36 ± 4.97	0	-
G. brownii	6.67 ± 5.55	0	-	3.66 ± 4.48	0	-
Blue Oak						
Control	1.08 ± 1.52	0	-	7.00 ± 4.09	0	-
G. adspersum	12.24 ± 17.31	0	-	2.68 ± 2.98	0	-
G. brownii	3.80 ± 5.37	0	-	3.78 ± 3.83	0.40 ± 0.57	-

[a] Each cell represents averages of replicates where n = 3, 4, or 5. Values represent the average discolored area ± the standard deviation.

[b] Values with * are statistically significant (P≤0.05) with Dunnets test relative to the control.

[c] – indicates an unsuccessful reisolation

[d] + indicates successful reisolation, where each + indicates success in one replicate.

Figures

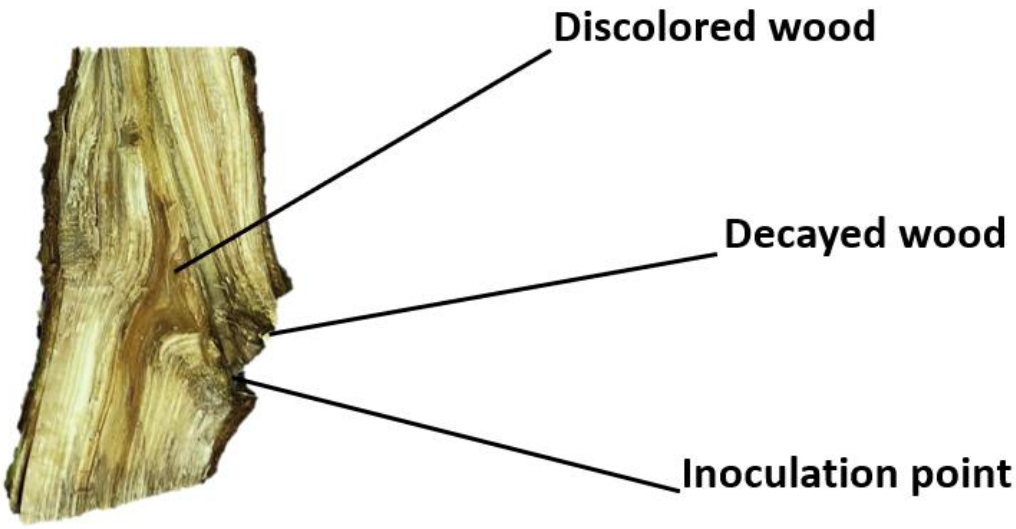

Figure 1.1. Longitudinal cross section through inoculation site, showing discolored wood and decay on sample from almond rootstock cultivar Nemaguard inoculated with *Ganoderma adspersum*.

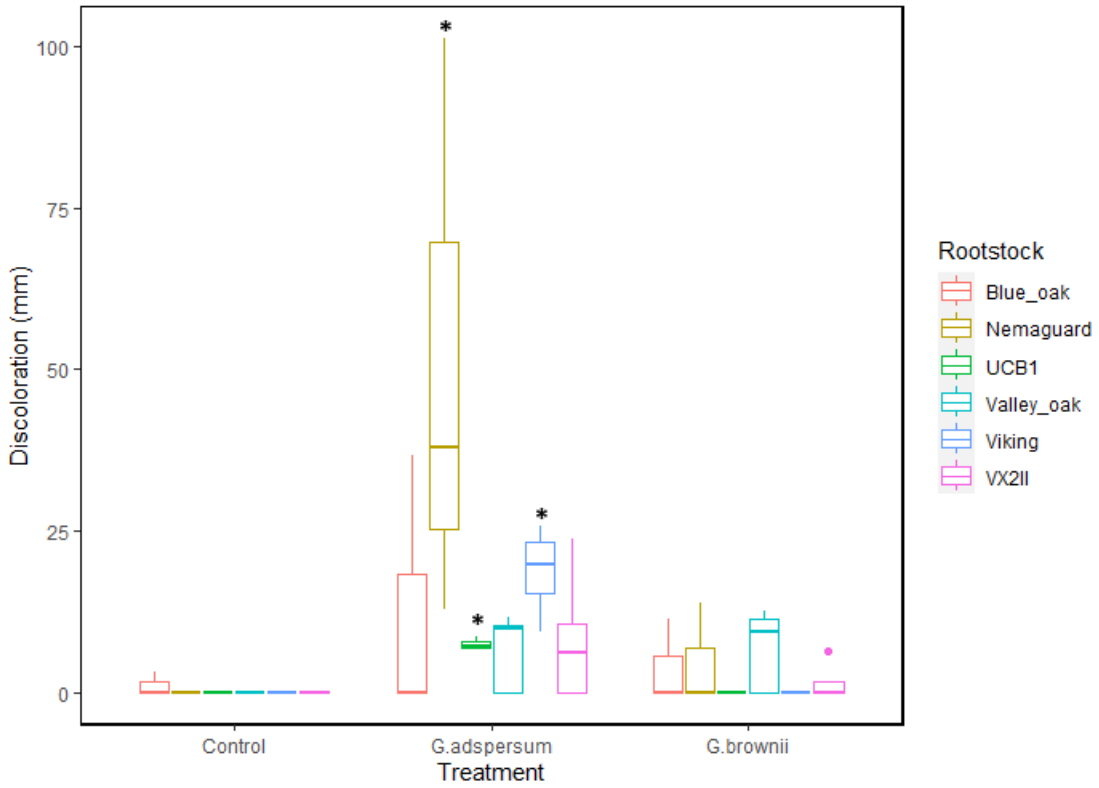

Figure 1.2. Average total length of discolored wood for almond rootstock cultivars Nemaguard (yellow) and Viking (dark blue), walnut rootstock cultivar VX211 (pink), pistachio rootstock cultivar UCB1 (green), *Quercus douglasii* (orange), and *Quercus lobata* (teal) inoculated with *Ganoderma adspersum* and *G. brownii* in trial 1. Treatments with asterisk were statistically significantly ($P < 0.05$) compared to the control.

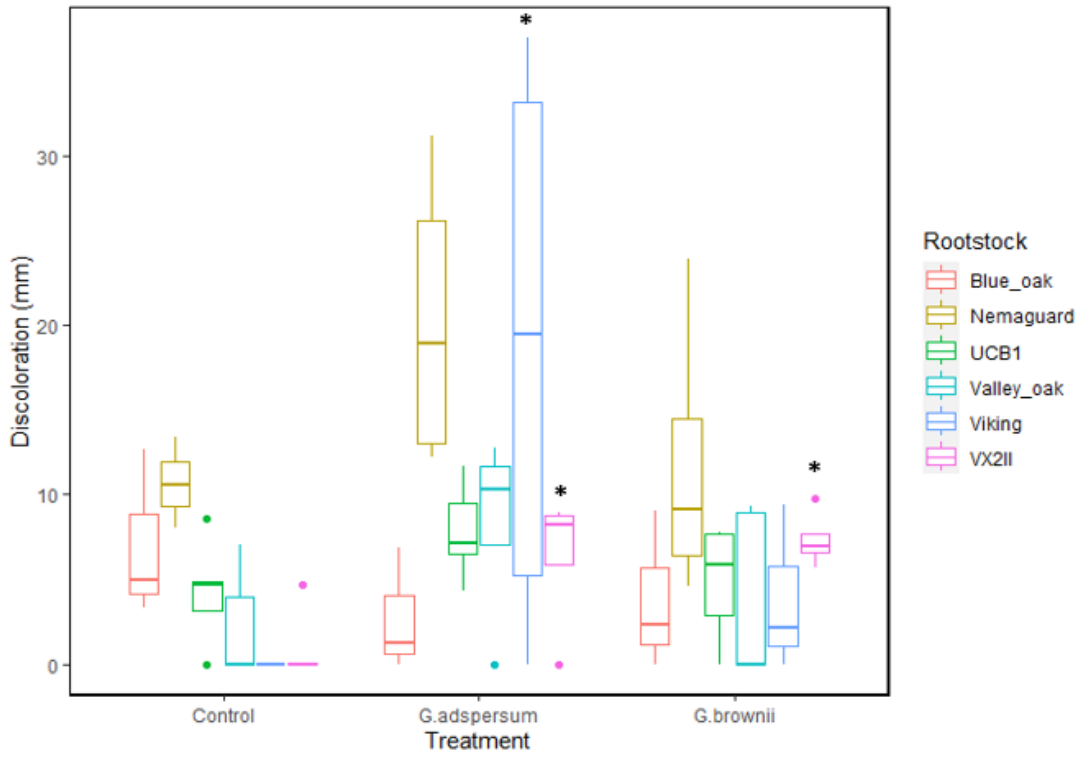

Figure 1.3. Average total length of discolored wood for almond rootstock cultivars Nemaguard (yellow) and Viking (dark blue), walnut rootstock cultivar VX211 (pink), pistachio rootstock cultivar UCB1 (green), *Quercus douglasii* (orange), and *Quercus lobata* (teal) inoculated with *Ganoderma adspersum* and *G. brownii* in trial 2.

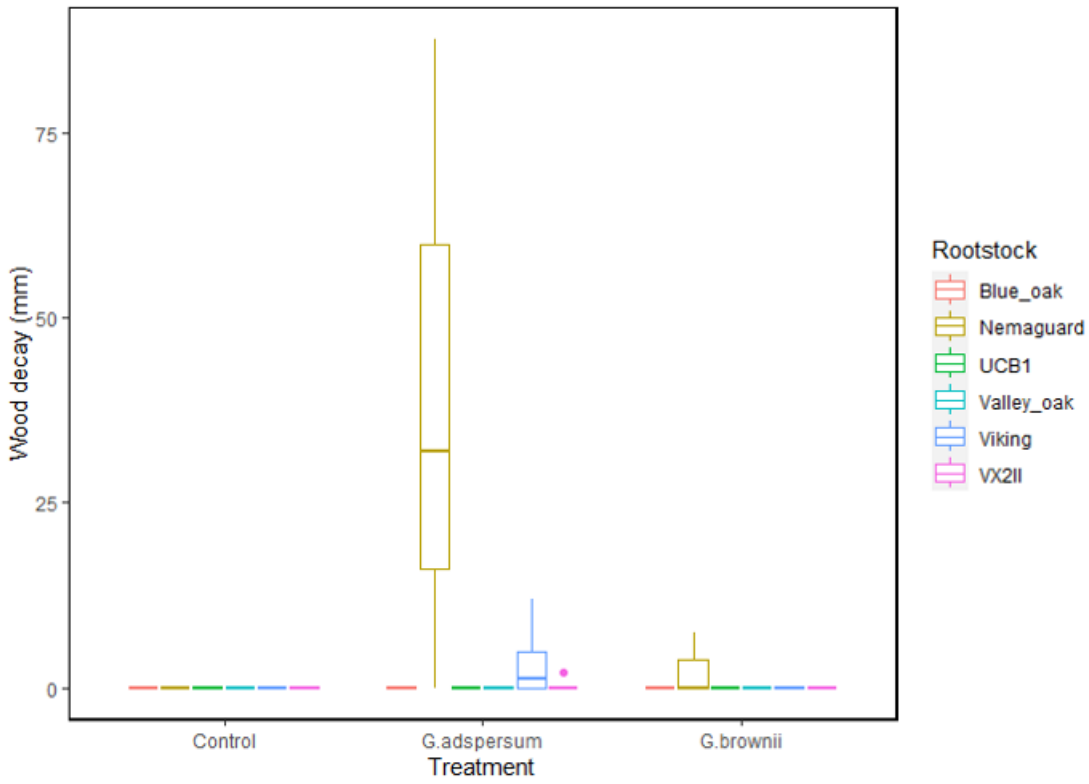

Figure 1.4. Average total length of wood decay for almond rootstock cultivars Nemaguard (yellow) and Viking (dark blue), walnut rootstock cultivar VX211 (pink), pistachio rootstock cultivar UCB1 (green), *Quercus douglasii* (orange), and *Quercus lobata* (teal) inoculated with *Ganoderma adspersum* and *G. brownii* in trial 1. Treatments with asterisk were statistically significantly ($P < 0.05$) compared to the control.

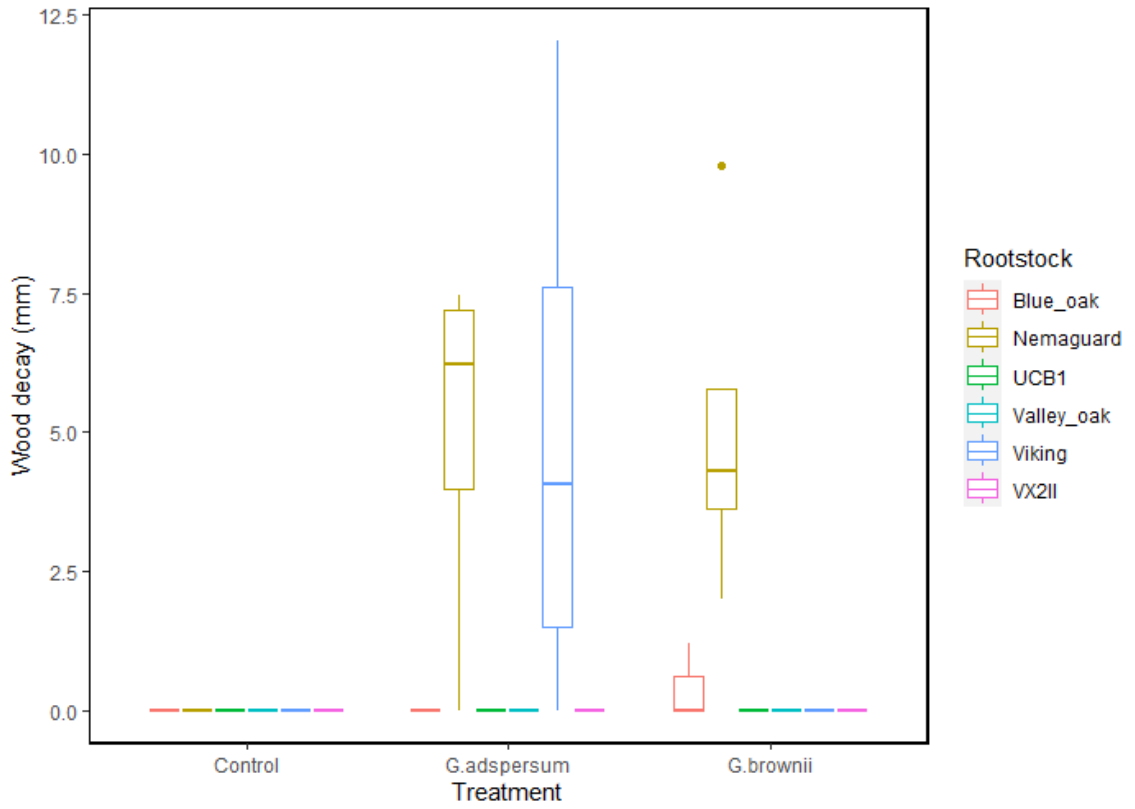

Figure 1.5. Average total length of wood decay for almond rootstock cultivars Nemaguard (yellow) and Viking (dark blue), walnut rootstock cultivar VX211 (pink), pistachio rootstock cultivar UCB1 (green), *Quercus douglasii* (orange), and *Quercus lobata* (teal) inoculated with *Ganoderma adspersum* and *G. brownii* in trial 2.

CHAPTER 2: AIRBORNE GANODERMA ADSPERSUM SPORE ASSESSMENT IN CALIFORNIA ALMOND ORCHARDS

Abstract

Ganoderma adspersum has been observed to be more prominent in California, in association with wood decay and tree failure in almond orchards. Disease progression and tree losses were studied in California almond orchards every eight months for two years. Substantial tree losses associated with wood decay were observed throughout the study. The seasonal abundance of *G. adspersum* spores was studied using mesh-covered buckets. Regression analysis resulted in a strong positive relationship between average temperature and *G. adspersum* spore abundance. The highest number of spores trapped in almond orchards during this study were during harvest from August to September. These findings warrant the need to assess management practices to reduce dust production and fungal spore transmission in California orchards.

Keywords: Ganoderma adspersum, almond, spore trapping, harvest, epidemiology, air-borne inoculum

Introduction

Fungal species in Ganodermataceae are saprobes and pathogens that cause wood rot diseases in a wide range of deciduous and coniferous trees (Seo and Kirk, 2000). While *Ganoderma* species have been described as economically important pathogens in Eurasia and Southeastern United States (Loyd et al. 2018, McKay 2011, Idris et al. 2004), only recently has *Ganoderma adspersum* been recognized as a detrimental pathogen of almond (*Prunus dulcis*) in the San Joaquin and Central Valley of California (Johnson 2020). First noticed in Kings County, the pathogen's geographic range currently extends over a range of 350 km in 9 California counties (Johnson 2020). High incidences of *G. adspersum* have led to substantial tree losses and early orchard removals throughout the 500,000 ha of California almond orchards (U.S. Department of Agriculture 2021).

Trees infected by *G. adspersum* may exhibit reddish-brown basidiomata growing from the trunk and crown. *Ganoderma* basidiomata can produce up to 30 billion spores per day (Levetin, 1990), which may travel long distances via air mass transport (Edman et al., 2004; Sesartic and Dallafior, 2011). Limited studies have characterized the seasonal patterns of *Ganoderma* spore abundance as a contributor of aeroallergens (Sadyś et al. 2016, Jędryczka et al. 2015, Craig and Levetin 2000). To date, no studies have investigated *Ganoderma* spore abundance in agricultural landscapes in the United States. Mechanical harvest practices (e.g., tree shaking, blowing nuts) of agricultural nut and fruit trees in California may lead to increased inoculum dispersal. For example, in the San Joaquin Valley one-third of inhalable particles, with diameters 10 micrometers and smaller (PM10), originates from farming operations, fungi, and chemical constituents (Englert 2004, Clausnitzer and Singer 1996).

Once established, many root rot pathogens can survive on woody debris for long periods of time and continue to infect surrounding hosts in perennial systems (Mohammad et al. 2014). Under these circumstances, control through eradication of the pathogen becomes nearly impossible. This suggests that prevention of initial infection should be the main disease management approach. A cost-effective approach to prevention will require a focus of management efforts during times of highest transmission risk. While previous studies have determined the seasonality of *Ganoderma* spp. in urban environments (Oliveira et al. 2005, Grinn-Gofron and Strzelczak 2011, Jothish and Nayar 2004), little is known about the epidemiology of *G. adspersum* in almond orchards, warranting an investigation into annual inoculum availability and the environmental factors favoring spore dispersal.

This study focuses on understanding the relationship between environmental conditions, cultural practices (i.e. orchard harvest), and seasonality of spore dissemination. Our objectives are to assess the seasonal variations in *G. adspersum* spore abundance, and to identify the environmental factors that influence the seasonality of spore distribution. Knowledge of the transmission biology of *G. adspersum* in orchards will provide initial data to predict periods of high infection risk, forecast dispersal distances through air, and determine appropriate preventative measures.

Materials and Methods

Survey location

Spore and disease surveys were conducted from March 30, 2021, to April 1, 2023, within and around three orchards in California's Central Valley spanning an 87 km area. This area has a Mediterranean climate with cool, wet winter and hot, dry summers. At elevations ranging from 83 to 93 m, rainfall in the area ranged between 190 to 260 mm per year and wind speeds of up to 14 m/s during 2021 to 2022. The area experienced atypical storms in early 2023 leading to increased rainfall of up to 350 mm and wind speeds of up to 17 m/s in the first three months (NOAA 2023). Temperatures ranged from -0.5°C to 46°C during 2021 to 2023 (NOAA 2023).

Prior to surveying, orchards were confirmed for *G. adpsersum* infection by initial sampling of characteristic basidiomata and BLAST identification using the ITS sequences of isolates obtained from collected basidiomata. The surveyed sites were two 28-year-old, 15 ha almond orchards in Madera (M1) and Fresno (F1) counties and one 6-year-old, 4 ha almond orchard in Madera (M2) county. The cultivars of M1 were Nonpareil, Fritz, Monterey, and Aldrich; cultivars of F2 were Nonpareil and Alrich; and cultivar of M2 was Independence. All varieties were planted on Nemaguard cultivar rootstock.

Information on fumigation, irrigation, fertilization, dormancy sprays, shaking times, and tree disposal methods were collected from growers for each orchard. M1 and M2 did not undergo fumigation prior to planting, though F1 underwent pre-planting soil fumigation with 1,3-dichloropropene (Telone). F1 and M2 were drip irrigated, while M1 was irrigated with microjet sprinklers. All orchards were fertilized with nitrogen at approximately 490 lbs/ha. All orchards were sprayed with dormant oil after harvest, but no orchard underwent a secondary sanitation shaking. Approximately 3 months prior to the survey, *G. adspersum* basidiomata were removed

from trees in F1 and M1, in the growers' attempts to control the incidence of *G. adspersum*. F1, wounds from removed basidiomata were sprayed with tree prune sealer. For all orchards, felled trees were stacked at the perimeter of the orchards and burned.

Climatic data was collected from weather stations, GHCND:USW00093193 for F1 and GHCND:USW00093242 for M1 and M2, available at the Physical Science Division of the National Oceanic and Atmospheric (NOAA) Administration's Earth System Research Laboratory (ESRL) (https://www.ncdc.noaa.gov/cdo-web/). The collected information was used to assess the effects of harvest shaking, precipitation, average temperature, average relative humidity, and average wind speed on *G. adspersum* spore loads.

Disease surveys

Every eight months, every tree of each orchard was assessed for symptoms and signs of *G. adspersum* infections. Symptoms and signs of *G. adspersum* infections are mainly internal wood decay and mycelium (Johnson 2020), which limits diagnostics until a tree has felled or once basidiomata are produced. Trees with *G. adspersum* basidiomata were marked as 'diseased' trees. Decay of main roots by *G. adspersum* may lead to tilting (Johnson 2020), from lack of structural support, so trees tilted at a maximum 65° angle were marked as 'tilted' and were considered likely infected. Trees that were apparently younger than the orchards age and/or recently planted were marked as 'replant' trees. Since *G. adspersum* has been commonly observed with crown gall caused by *A. tumefaciens* (Johnson 2020), trees with crown gall were also monitored and marked as 'gall' trees. Some trees were marked with more than one label, when observed. Trees with no apparent symptoms or signs were marked as 'healthy,' though not to say that the trees were not diseased, but rather could not be confirmed as diseased. Disease

incidence was calculated as the percentage of diseased trees relative to the total number of almond trees inspected.

Spore dissemination surveys

Ganoderma adspersum spores are readily distinguished from other fungi, as they are hardy, ovoid, melanized and double-walled (Niemela and Miettinen 2008, Southworth 1973). The external spore wall is transparent and smooth, while the internal wall may be dark brown to golden brown. The spores also exhibit a flattened basal apiculus, the projection on the spore from where it was attached to the basidia (Southworth 1973). The spores have an average length and width of 10.95 and 7.05 microns (Niemela and Miettinen 2008), allowing the spores to be filtered out of soil and water to be observed and quantified under a light microscope.

Ganoderma adpsersum spores were collected from basidiomata growing from the crown of infected almond trees in F1. Preliminary tests showed that short-term (24 hrs) and long-term (2 months) exposure of *G. adspersum* spores to algaecide, tween80 (Sigma-Aldrich, St. Louis, MO), 4% tween80, and 10% glycerol (Sigma-Aldrich, St. Louis, MO) in temperatures 25°C, 4°C, and -80°C had no apparent effect on the appearance and structural integrity of the spores. At least 1 month after collection, few *G. adspersum* spores (<1%) would have a more flaccid and wrinkled external spore wall.

Spore traps consisted of 7.5-liter square buckets (Uline, Pleasant Prairie, WI) with 0.5 liter of water set up with a metal mesh lid (0.635 cm spacing) to exclude large debris and wildlife from traps (Davidson et al. 2005). Algaecide, “Algae Guard” (HTH, Wellborn, FL), was added to the water, as recommended on the label, to prevent algae blooms in the buckets: n-Alkyl (60% C14, 30% C16, 5% C12, 5% C18) Dimethyl Benzyl Ammonium Chlorides 9.96%, n-Dialkyl (60% C14, 30% C16, 5% C12, 5% C18) Methyl Benzyl Ammonium Chlorides .04%.

Bucket traps were set up in March 2021, and used to collect settled-air samples every 4 weeks until April 2023. Each orchard was split into 3.64-hectare quadrants. Twenty-four buckets were placed within randomly selected quadrants for orchards F1 and M1. Due to the smaller area, 15 buckets were placed in M2 (Figure 2.1-2.3). The bucket traps were staked onto the ground within tree rows, out of the way of machinery, and the two surrounding trees were spray painted with the bucket's assigned number. A numbered flag was also place directly next to the bucket location to ensure that the trapping locations were kept consistent throughout the study.

For each sampling period, the buckets were in the orchard for 7 days before being collected. During times of higher temperatures, the water in the bucket traps would be dried out by the time they were collected. All bucket traps were processed within 2 weeks. Using deionized water, the contents of each spore trap were individually washed through 2 layers of 100% cotton cheesecloth into a wide-mouth 2L Erlenmeyer flask (Pyrex, Rosemont, IL) to filter out large particles, such as twigs, leaves, flowers, and large sand particles. Pieces of cheesecloth were observed under a light microscope to confirm *Ganoderma* spores were not lost at the initial filtering stage. The filtrate was filtered through a Whatman grade 50 hardened low ash filter with 2.7 µm pore size (Sigma-Aldrich, St. Louis, MO) set on a Buchner funnel with reinforced polyurethane tubing [3 cm internal diameter] (United States Plastic Corp, Lima, OH).

The solutes on the filter paper were rinsed into a beaker with at least 100 ml of 4% tween80 (Sigma-Aldrich, St. Louis, MO) solution and DI water. The filter papers were rinsed until no particles were observed on the filters, and the final volume of the rinse was recorded. The melanized, ovate, double-walled *G. adpsersum* spores were counted using a hemocytometer to obtain the total CFUs/ml, which, along with the final rinse volume, was used to calculate the total CFUs/traps and CFUs/orchard. While spores could not be quantified on media, due to

contamination of *Rhizopus*, *Trichoderma,* and unidentified yeasts, spores were plated to confirm viability. Given the prevalence of *G. adspersum* fruiting bodies in the orchards, the spores were identified as *G. adspersum* spores.

Weekly total numbers of *G. adspersum* spores were correlated with the environmental variables recorded on the same week at each location using a stepwise-backward multiple regression analysis in R (Craig and Levetin 2000). In addition, a correlation analysis was performed to determine the correlation between harvest and aerial spores, as well as diseased trees and aerial spores in the California almond orchards.

Results

Disease survey

Throughout the two-year study, orchards exhibited differing incidences of *G. adspersum* basidiomata with F1 exhibiting a significantly higher percentage of basidiomata than the other orchards at each surveying time: April 2021, December 2021, August 2022, and April 2023 (P = 0.004). *Ganoderma adspersum* basidiomata incidence in F1 was 3.6%, 5.2%, 4.8%, and 2.2%; in M1 was 0.6%, 0.4%, 0.8%, and 2.0%; and in M2 was 0.0%, 0.1%, 0.1%, and 0.1%. At each time point, approximately 18-20% of the trees in F1 and M1 with *G. adspersum* basidiomata also had crown galls caused by *A. tumefaciens*. Trees in M2 did not exhibit comorbidity.

Though *G. adspersum* basidiomata are more commonly observed at the base of the tree scion, basidiomata may also be observed on wounds from tree trunks severely damaged by harvesting equipment, as well as decaying pruning wounds and cracks at the crotch of a tree. Most *G. adspersum* basidiomata would be less than 10 cm in width, though basidiomata on trees exposed to more frequent or thorough irrigation, usually due to faulty irrigation equipment, would grow 15-20 cm.

At each surveying time, tilting occurred in 2.3%, 5.1%, 4.8%, and 19.2% of the trees in F1; 3.3%, 5.0%, 12.3%, and 12.0% in M1; and 14.6%, 14.6%, 31.9%, and 38.4% in M2, respectively.All orchards experienced approximately a 1% tree loss in the first 8 months. Orchards F1 and M1 experienced 2.9% and 9.9% tree loss over 16 months and 17.4% and 12.6% in 24 months, respectively. After the initial 1.2% loss in 8 months, M2 did not lose more trees within 16 months, but exhibited a 3.2% loss in 24 months. While orchard F1 was not replanted with new trees after losses during the two-year study, orchards M1 and M2 were replanted soon after tree removal with a 27% and 56% increase in replants within 24 months, respectively.

Spore dissemination surveys

Regression analysis indicated a positive relationship between the presence of *G. adspersum* spores recovered in the buckets and temperature ($R^2 = 0.05$, $P \leq 0.05$, $n = 1218$). *Ganoderma adspersum* spores were trapped throughout the year, with increases from early-summer to late-fall at temperatures from 25°C to 30°C. The total number of spores trapped varied depending on the season. In all orchards, the highest counts of *G. adspersum* spores were observed from August to September, during almond harvest season (Figure 2.7-2.9). Correlation analysis revealed a strong, positive relationship between aerial spores and harvest ($P = 2.2*10^{-16}$). Spore concentrations differed significantly between F1 and M2 ($P = 0.008$).

Increases in *Ganoderma* aerial spores also coincided with increases of dematiaceous hyphomycetes. These other fungi were identified by spore morphological characteristics (citation). The most common hyphomycetes identified were *Alternaria* spp. dictyospores, *Alternaria*-like dictyospores, *Bipolaris* spp., *Cladosporium* spp. and *Pseudopithomyces* spp.

Discussion

Increased tree losses in California's Central Valley have been associated with *G. adspersum* (Johnson et al. 2020). While previous almond orchard life-cycle studies have assumed an annual tree loss of 1%, at 311 trees per ha (Marvinney et al. 2015), the survey revealed higher tree losses in infected orchards with a 1% tree loss within 8 months, and up to 17% losses within 24 months. Failed trees were often tilted, likely due to decaying roots, or had *G. adspersum* basidiomata at least 8 months prior to the failure.

Orchard F1 had experienced the highest tree losses with the highest incidence of *G. adspersum* basidiocarps. Severe losses in orchard F1 led to the grower's decision to remove the orchard after the study, an early removal at 19-20 years old. A cost study analysis for the southern region, conducted in 2016, decreased the expected lifespan for an almond orchard from 25 in 2008 to 23 years (Yaghmour et al. 2016, Freeman et al. 2008), acknowledging that lifespan in this region has decreased. Though the cause of the decrease was not noted, it is possible that early removals of infected orchards have contributed to this analysis.

A few months prior to the study, *G. adspersum* basidiomata were removed from orchards F1 and M1, although there was still a high incidence of basidiomata in the orchards at the start of the study. In orchard F1, wounds where basidiomata were removed were sprayed with tree pruning sealer. Most removed *G. adspersum* basidiomata had regrown within 3-4 months after removal, regardless of treatment, indicating that basidiomata removal is not an effective management strategy.

Saplings were often replanted into the orchards alongside of wood-decayed stumps and *G. adpsersum* basidiomata. Many stumps initially without basidiomata would grow basidiomata throughout the study. Preliminary studies have shown that *G. adspersum* is a poor competitor in

soil (Appendix B) and is unlikely to move through soil to infect new wood. However, close contact of sapling roots to infected roots and sporulating basidiomata of stumps may still contribute to transmission, warranting the need to completely remove stumps prior to replanting, in order to reduce possible inoculum.

This study showed higher *G. adspersum* spore loads in early-summer to late-fall with peaks in mid-August to early-September, during almond harvest. The results indicate the significance of temperature and harvesting practices in the prevalence of aerial *Ganoderma* basidiospores. The temperature results are similar to those from previous studies of *Ganoderma* spp. in other locations around the world (Grinn-Gofron and Strzelczak 2011, Oliveira et al. 2005, Jothish and Nayar 2004, Mitakakis and Guest 2001, Craig and Levetin 2000). Other studies have found a significance of precipitation in the prevalence of aerial *Ganoderma* spores (Grinn-Gofron and Strzelczak 2011, Craig and Levetin 2000) though this correlation could not be assessed properly, as little to no precipitation occurred in the droughted Central Valley until the latter end of the study in which record amounts of precipitation occurred.

Incidence of *G. adspersum* basidiomata in the orchards did not correlate with the prevalence of *G. adspersum* spores. Given the perennial nature of the fungus and its host, *G. adspersum* spores may not only originate from an actively sporulating basidiomata but may also accumulate in the soil over time before being wind-blown or picked up by equipment. Since growers tend to harvest from orchards within overlapping time frames, *G. adspersum* spores may also be transmitted from nearby sources, such as other infected orchards. The age of the *G. adspersum* spores could not be confirmed, though spore plating confirmed viability of trapped spores of at least 1% of spores at any point.

The management of wood-decay diseases is under-studied. This study provides valuable

information in understanding the epidemiology of *G. adspersum* in California almond orchards. The study shows the need to implement integrated pest management strategies that will not only reduce the incidence of spore-producing *G. adspersum* basidiomata in almond orchards, but also reduce fugitive dust during high wind and harvesting events.

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Figures

Figure 2.1. Fresno orchard F1 map showing location of spore traps.

Figure 2.2. Fresno orchard M1 map showing location of spore traps.

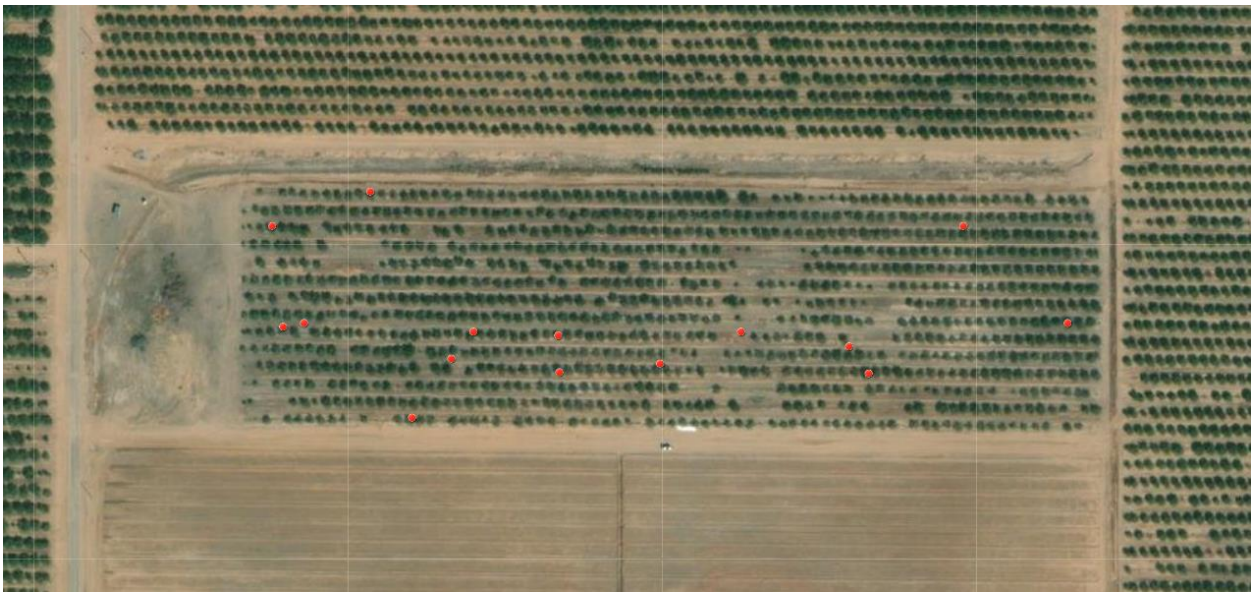

Figure 2.3. Fresno orchard ‘M2’ map showing location of spore traps.

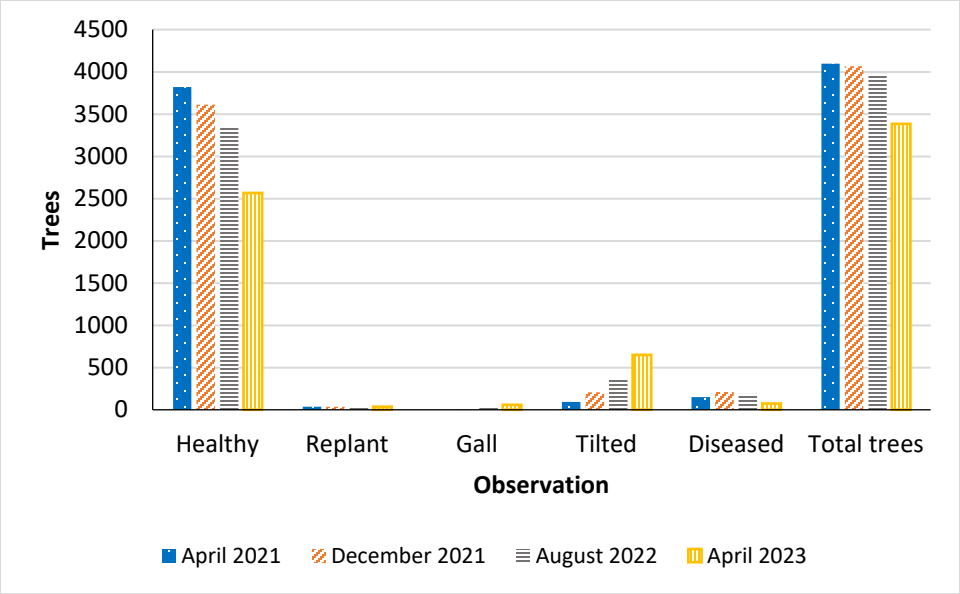

Figure 2.4. Disease survey data for orchard F1 showing number of seemingly healthy, replanted, galled, tilted, and diseased trees, and total trees for April 2021 (dotted blue), December 2021 (diagonally striped orange), August 2022 (horizontally striped gray), and April 2023 (vertically striped yellow).

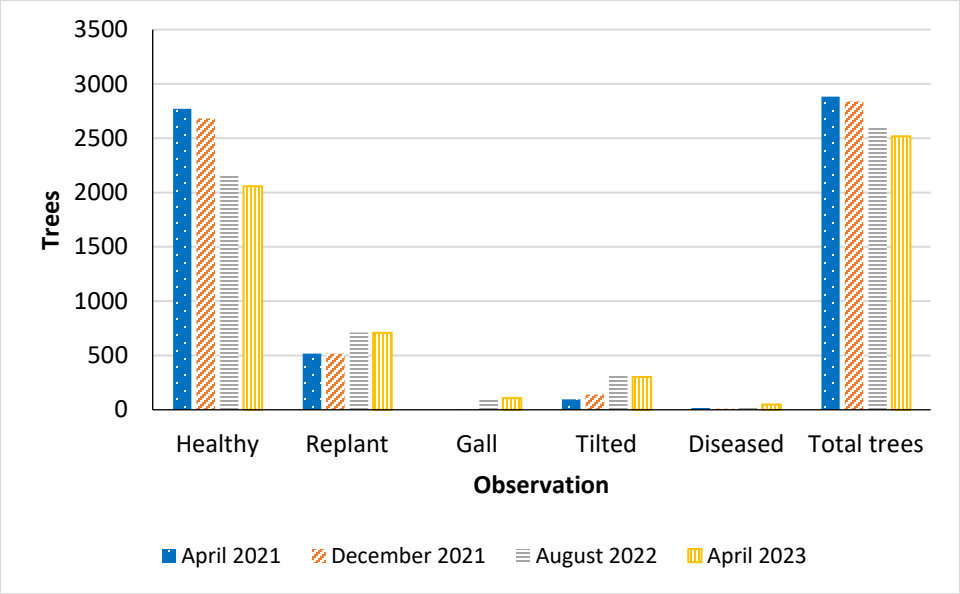

Figure 2.5. Disease survey data for orchard M1 showing number of seemingly healthy, replanted, galled, tilted, and diseased trees, and total trees for April 2021 (dotted blue), December 2021 (diagonally striped orange), August 2022 (horizontally striped gray), and April 2023 (vertically striped yellow).

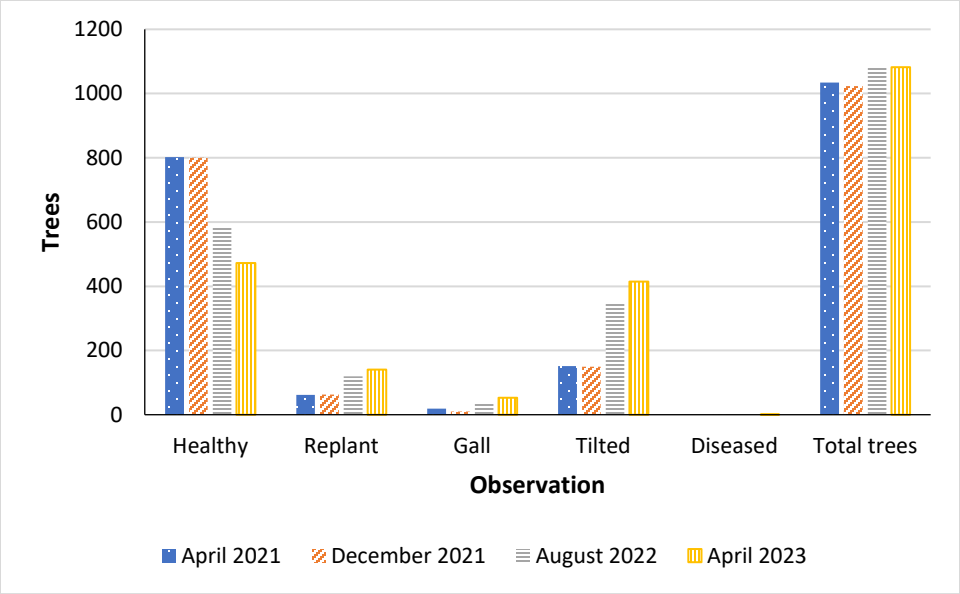

Figure 2.6. Disease survey data for orchard M2 showing number of seemingly healthy, replanted, galled, tilted, and diseased trees, and total trees for April 2021 (dotted blue), December 2021 (diagonally striped orange), August 2022 (horizontally striped gray), and April 2023 (vertically striped yellow).

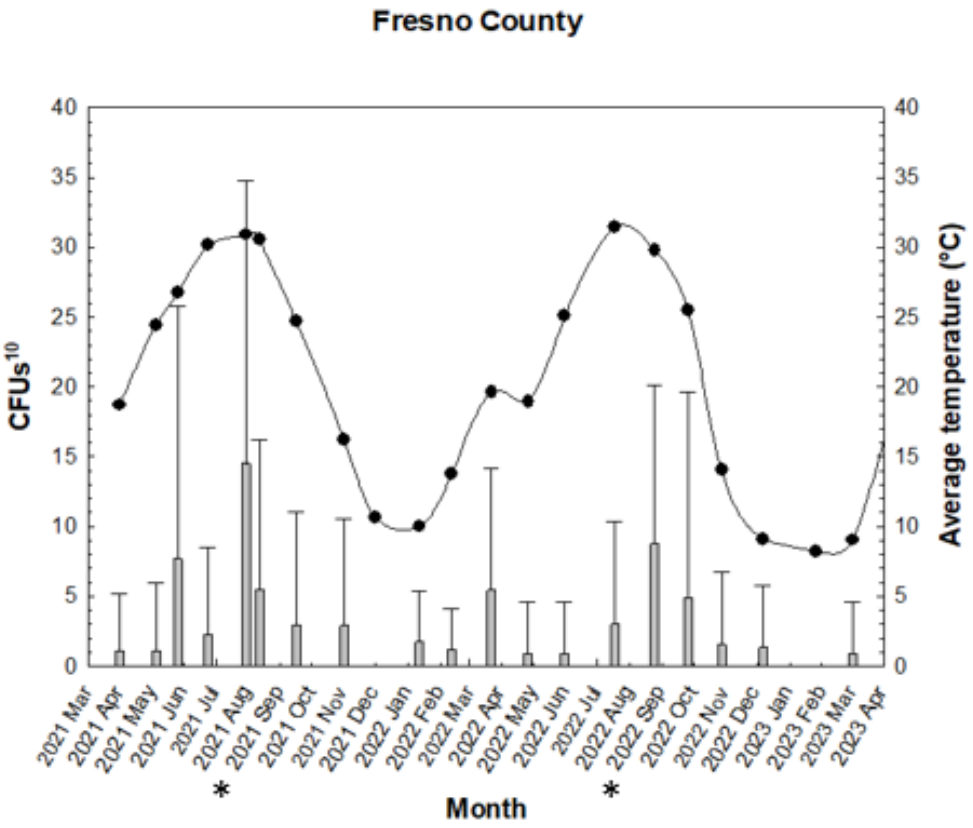

Figure 2.7. Monthly spore trap counts with temperature (°C) for Fresno County orchard F1. Vertical bars represent total colony forming units (CFUs) counted in the orchard each month. Line represents average monthly temperature (°C); * = almond fruit harvest.

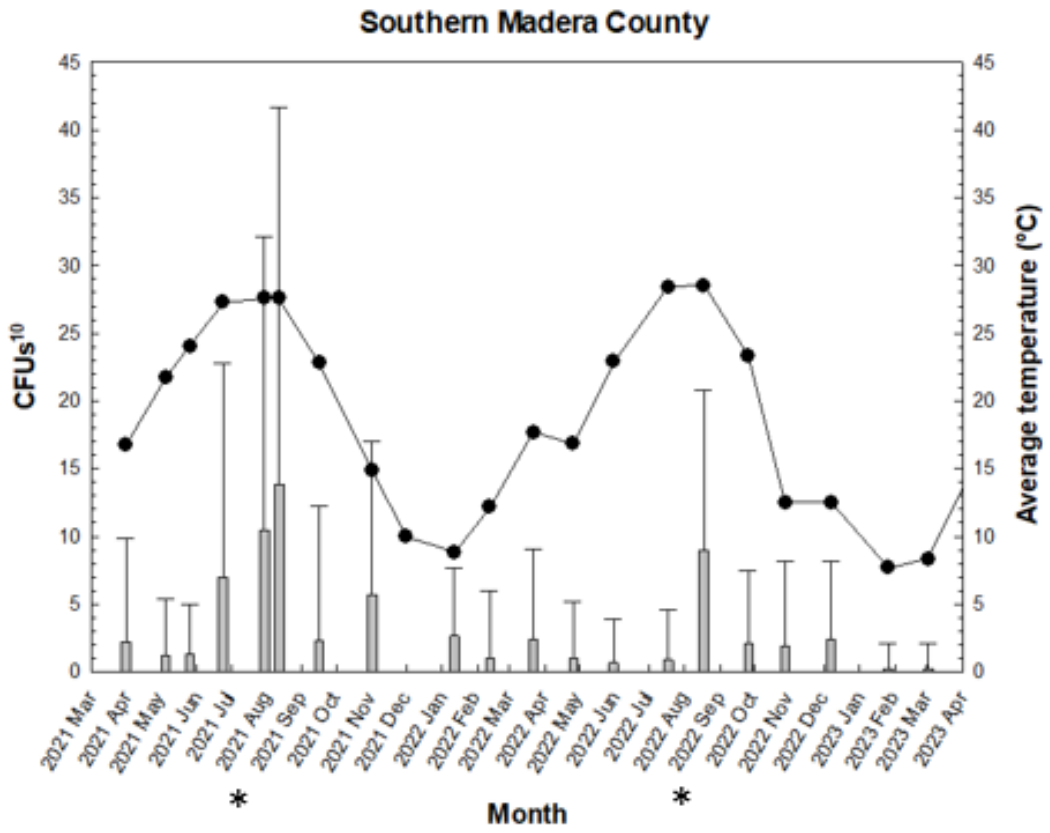

Figure 2.8. Monthly spore trap counts with temperature (°C) for Madera County orchard M1. Vertical bars represent total colony forming units (CFUs) counted in the orchard each month. Line represents average monthly temperature (°C); * = almond fruit harvest.

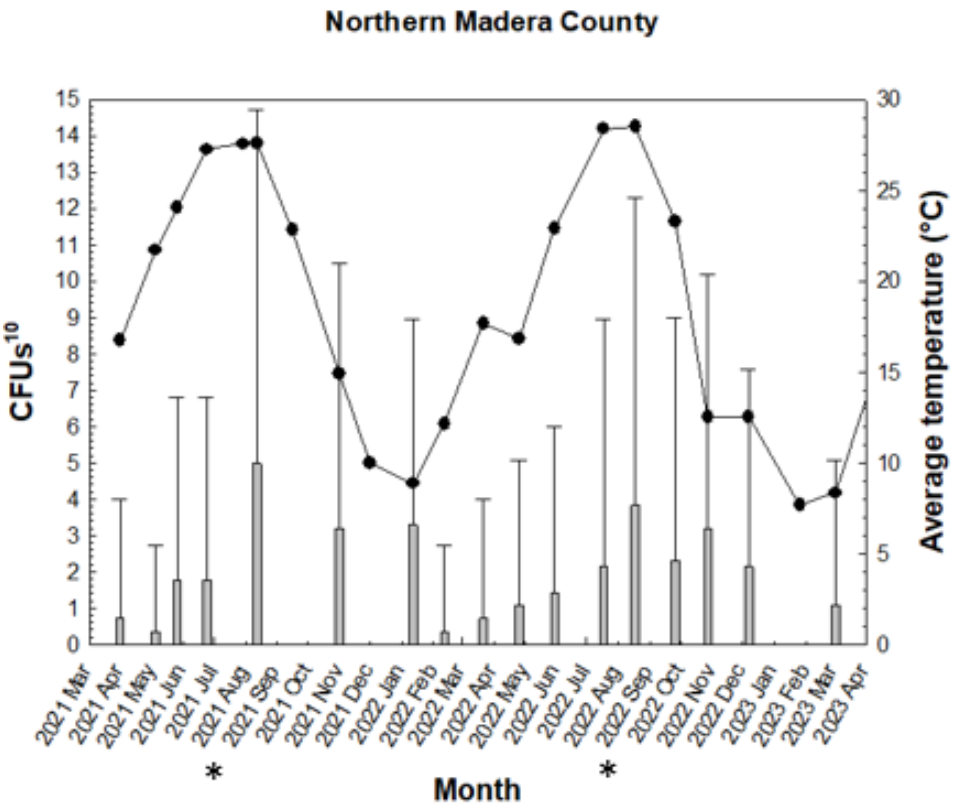

Figure 2.9. Monthly spore trap counts with temperature (°C) for Madera County orchard M2. Vertical bars represent total colony forming units (CFUs) counted in the orchard each month. Line represents average monthly temperature (°C); * = almond fruit harvest.

CHAPTER 3: CO-INFECTIONS OF *AGROBACTERIUM TUMEFACIENS* AND *GANODERMA ADSPERSUM* IN CALIFORNIA ORCHARDS

Abstract

While plant diseases often involve co-infections of multiple pathogens, the interactions. To understand the complex interactions in cross-taxa co-infections, we investigate the interactions between an almond, wood-decay pathogen, *Ganoderma adspersum*, and a bacterial pathogen, *Agrobacterium tumefaciens*. The high co-incidence of the fungal and bacterial pathogens suggests mutualistic effects in pathogenesis. However, co-inoculation assays revealed that the pathogens do not significantly interact directly with each other nor promote increased initial disease development. The rise of multi-pathogen diseases in almonds likely results from the adoption of management practices that have created a system capable of sustaining multiple pathogens.

Keywords: Ganoderma, Rhizobium, Agrobacterium, almond, walnut, co-infections

Introduction

While plant pathology studies have previously focused on individual host-pathogen interaction, recent plant epidemiological studies have reviewed the complex interactions of plants co-infected with multiple pathogen species/genotypes (Abdullah et al. 2017, Kozanitas et al. 2017, Tollenaere et al. 2017, Tollenaere et al. 2016). Pathogen species sharing a common host may interact in a direct or indirect manner or both simultaneously (Pedersen and Fenton 2007). Direct interactions between pathogens may lead to facilitation or interference competition (Tollenaere et al. 2016, Kohlmeier et al. 2005). Indirect interactions may be mediated by the attribution of host resources or host defense response (Tollenaere et al. 2016).

In the past decade, a substantial increase in tree losses in almond orchards within the Sacramento and Central Valley of California has been observed (Johnson 2020). *Ganoderma adspersum,* an introduced pathogen from Eurasia, was identified as the main causal agent of wood decay (Johnson 2020), in whichthe fungus degrades tyloses, lignin, polyphenols, and cellulose of the lower bole or root flare (Schwarze 2000, Sinclair and Lyon 2005). The loss of strength and rigidity in infected trees often led to failure due to windthrow or harvest shaking.

Trees infected by *G. adspersum* would often exhibit reddish-brown basidiomata, clefting, and shallow roots (Johnson 2020). Additionally, the pathogen would be frequently associated with crown gall symptoms (Johnson 2020) caused by bacterial pathogen *A. tumefaciens* (syn. *Rhizobium radiobacter*), which can reduce fruit yields in infected trees (Epstein et al. 2008). The bacterium induces gall formation in plants by transferring a single-stranded segment of T-DNA into plant cells via the Ti plasmid (Gelvin 2017). The galls produce opines which are utilized by *A. tumefaciens* (Moore and Cooksey 2013). On trees, galls are perennial and increase in size with growth of the tree, causing roots to grow poorly and reducing crop yields (Pulawska 2010).

Ganoderma adspersum and *A. tumefaciens* both cause diseases that originate at the base of live trees. Though pathogenesis requirements are less understood for *G. adspersum*, it is known that *A. tumefaciens* requires wounds to cause infection (Escudero et al. 1997). The main source of below-ground wounds in almond trees may be the twice-yearly mechanical tree shaking that generally begins in the 3rd or 4th year of an almond orchard's establishment (Abdel-Fattah et al. 2003). Mechanical shaking is used to drop almonds during harvest and to remove fruit mummies during dormancy (Haviland et al. 2017). Additionally, crown gall infections may increase the incidence of wood decay in orchards by serving as natural sites of infection (Ohlendorf and Strand 2002).

Ganoderma adspersum and *A. tumefaciens* require hosts to balance the tradeoff between tree host defense and wood and fruit growth (Wang and Wang 2014). Tree defense relies on signaling by chemical compounds such as jasmonic acid (JA) or ethylene that allow for the induction of pathogenesis-related proteins, some of which exhibit antimicrobial activities (Costet et al., 1999; Durrant and Dong, 2004, Morris et al. 2020). *Agrobacterium tumefaciens* is known to bypass these defenses to settle in tumors (Lee et al. 2009, Someya et al. 2013, Nonaka and Ezura 2014, Nonaka et al. 2017). Trees may respond to *Ganoderma* infections by producing reaction wood (Morris et al. 2020), which *G. adspersum* can grow through (Schwarze and Ferner 2003).

Past studies have investigated co-infections involving multiple genotypes of viruses, different genotypes or species of fungi, or viral and bacterial pathogens; with unpredictable results in disease development (Tollenaere et al. 2016, Rochow et al. 1955, Schurch and Roy 2004). Few studies have examined the interaction between fungal and bacterial pathogens (Grube et al. 2011, Spoel et al. 2007). The goals of this project serve to investigate the

interactions of pathogens and their hosts in cross-taxa co-infections. In this study, we explore the physical interactions of *G. adspersum* and *A. tumefaciens,* as well as how the cross-taxa co-infections affect disease severity and symptom expression. Understanding these cross-taxa multiple pathogen interaction will provide a basis of knowledge which will contribute to the development of holistic disease management decisions.

Material and Methods

Microbial identification and spores.

Attempts to grow *G. adspserum* basidiomata for spores were made following modified protocols of Stamet's 2000 *Ganoderma* cultivation methods (Stamets 2000), though in any case, spore production was insufficient. Spores were collected in Petri dishes from *G. adspersum* basidiomata growing on almonds in orchards in Fresno, California. Basidiomata were initially identified based on the macromorphological features of basidiomata and were validated through sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA).*Ganoderma adspersum* cultures were maintained at room temperature (25℃), in dark conditions, on yeast malt extract (YME) agar containing 4 g/L yeast extract, 10 g/L malt extract, 4 g/L glucose and 20 g/L agar, pH 7.3 (Shirling and Gottlieb, 1966; Travadon et al. 2013).

A rifampicin-resistant mutant of *A. tumefaciens* biovar 1, strain 186r was obtained from Dr. Daniel Kluepfel, USDA ARS (Yakabe et al. 2014). The bacterial mutant was selected for co-inoculation studies, as it could be more readily isolated from complex environments using trypticase soy broth agar (TSBA) amended with rifampicin sulfate at 100 ppm (TSBA + rif) (Yakabe et al. 2012). Bacterial solutions of *A. tumefaciens* were prepared in trypticase soy broth (TSB) (Sigma-Aldrich) or yeast extract peptone broth (YEPB) (Iqbal et al. 2021) from independent colonies at 1 colony per 2 mL of TSB. Bacterial suspensions were centrifuged for 5 min. at 7.500 × g and resuspended in sterile deionized water. The suspensions were quantified using a spectrophotometer and the final concentration was adjusted (optical density of 1) to 1×10^9 CFU/ml (Yakabe et al. 2014).

In vitro plate co-inoculation assays

A modified protocol from Passera et al. 2021 was used to conduct *in vitro* co-inoculation assays for *G. adspersum* strain W0125 and *A. tumefaciens* strain 186r. A 4 mm plug from a 2-week-old *G. adspersum* colony was centrally inoculated on YME medium in a 90 cm Petri dishes. On each plate, except the fungal-only controls, four 0.5 cm discs of sterilized filter paper were placed approximately 4 cm from the fungal plug in the center. Treatments included: (1) fungal-only controls, (2) controls with uninoculated filter paper, (3) controls with sterilized deionized water on each filter paper, (4) inoculated filter paper on the same day the fungal plug was placed, (5) inoculated filter paper one day after fungal plug, (6) inoculated filter paper two days after fungal plug, and (7) inoculated filter paper three days after fungal plug (Passera et al. 2017). All water and bacterial inoculations were made with 10 µl of solution. All plates were incubated at 28℃ for 10 days, and the diameter of the pathogen colonies was measured daily. Three replicates were performed for each treatment. The trials were replicated three times for *A. tumefaciens* prepared in TSB and *A. tumefaciens* prepared in YEPB to determine if the bacteria's nutrient source affected the study. The growth inhibition percentage (GIP) was calculated as ((GC-GT)/GC) x 100, where GC (growth control) is the mean colony diameter on the control and GT is the mean colony diameter on the treated plates (Zygadlo et al. 1994).

***In vitro* tube co-inoculation assays**

A modified protocol of Mille-Lindblom et al. (2006) was used to conduct *in vitro* tube co-inoculation assays for *G. adspersum* and *A. tumefaciens*. Tubes of 15 ml of TSB and YEPB were inoculated with 10 ul of $1 * 10^9$ *A. tumefaciens* 186r and/or a 5 mm plug of *G. adspersum* mycelium, as follows: uninoculated control, bacterial control, fungal control, bacteria then fungus, fungus then bacteria, and bacteria and fungus. In sequential inoculations, the secondary inoculation was conducted 10 hr after the initial inoculation. Fifteen replicates were made for

each treatment for each medium and were incubated for at 28°C in dark conditions. After 24 hr, *A. tumefaciens* was quantified by determining the OD600 in a spectrophotometer. The experiment was conducted twice.

Pathogen mobilization

A modified protocol from Kohlmeier et al. (2005) was used to access mobilization of bacteria via fungal hyphae. Bacteria were inoculated together (simultaneous inoculation) or after *G. adspersum* had been established (sequential inoculation). A 5 mm plug from a 7-day old *G. adspersum* colony and 1 μL of 1×10^9 CFU/ml *A. tumefaciens* solution, prepared as previously described, was placed on a 13 mm plug of YME for simultaneous inoculations. The 13 mm YME section was removed from the Petri dish and placed in a 90 cm Petri dish 1 mm away from a 1 cm by 3 cm rectangular strip of potato dextrose agar (PDA). In sequential inoculations, the bacterium was inoculated on the fungal mycelium 2 days after the fungus, in which the fungus had grown throughout the YME plug. The bacterium was inoculated on fungus-free Petri dishes, as controls. The fungus was allowed to completely overgrow the PDA strip in dark conditions at 28°C. The gap of 0.5−1 mm width served to prevent motile bacteria from swarming over the agar surface. After 12 days of incubation, the fungal-colonized PDA strips were cut into three sections and placed in 1.7 ml tubes. One ml of 0.15 M NaCl was added and vortexed several times to separate bacteria attached to fungal hyphae. The suspensions were plated on TSB and incubated at 28°C for 72 hrs. The inoculated YME plug was also cut into two sections and processed as previously described to confirm bacterial growth at the inoculation point.

Additional experiments were conducted on 90 cm YME agar plates, in which a 0.5 mm strip of agar was removed from the center of the plate. Simultaneous and sequential inoculations were inoculated as described above, except with a 30 mm fungal plug rather than a 5 mm plug.

The fungus was allowed to grow through one-third of the medium on the uninoculated side of the gap. The agar was cut into 1 cm^2 sections and placed in 1.7 ml tubes to be processed as previously described.

Host plants.

Bare root trees of *Prunus dulcis* (almond) rootstock cultivars Nemaguard (*Prunus persica*), Viking [*P. persica x (P. dulcis x (P. cerasifera x P. armeniaca*)], and Lovell (*Prunus persica*); and *Juglans 58acrocarpa* x *Juglans regia* (walnut) rootstock cultivars Rx1 and VX211 were sourced from commercial growers in northern California. Trees were potted in 19-liter containers with sterilized UC mix soil made up of sand, redwood compost, and sphagnum peat moss (expanded) at a ratio of 1:1:1 (Evans 2014). Rootstock cultivars were selected based on their prominent use in California orchards and their known (Nemaguard and Rx1) or possible (Viking, Lovell, and VX211) susceptibility to *G. adspersum* and/or *A. tumefaciens* (Hernandez, 2023). Almond rootstock cultivar Lovell and walnut rootstock Rx1 trees failed to establish and died within one month of potting, likely due to prolonged exposure to heat and wind prior to potting. The rootstocks were not viable for disease assessment. Individual trees within a species were of similar size; almonds were ~2 cm in diameter and walnuts were ~1 cm in diameter.

***In vivo* co-inoculation**

In vivo co-inoculations were conducted twice in a 2400 SF, Level of Control and Protection (LCP) 2 greenhouse at the University of California, Davis Vegetable Crops Greenhouses. For each repetition, the pathogenicity tests were set up in a completely randomized block design (Loyd et al. 2018) with spore inoculations of *G. adspersum* and suspensions of *A. tumefaciens*, as well as mock inoculations of controls across the five tree types with five

replicates per pathogen-host combination. Trees were irrigated and fertilized every 2-3 days with drip irrigation, fertilized, and sprayed with insecticides monthly for spider mites.

Prior to the trials, 1 ml of $1x10^9$ CFU/ml spore solutions were pipetted onto MEA plates and 5 cm x 5 cm sterilized almond wood blocks. Growth on plates and wood confirmed viability of spores for use in pathogenicity trials. Inoculation of MEA plates and wood blocks were repeated with fresh spore solutions the day of the trials. Trees were inoculated on November 22, 2021 (trial 1) and April 14, 2022 (trial 2) by pushing a 5 mm increment borer at least 3 mm into the bark and cambium of the trees, 5 cm above the soil line; removing the bark of the 5 mm wound with a scalpel; and pipetting 1 ml of $1x10^9$ CFU/ml spore solutions or sterilized deionized water into the wound. All wounds were sealed with Parafilm and covered with aluminum foil to avoid contamination and desiccation.

Co-inoculations were conducted at the same time and in different sequences. Simultaneous co-inoculations with *G. adspersum.* And an *A. tumefaciens* strain were conducted with spore inoculations and bacterial inoculations at the same time, in which two wounds were made, as previously described, one directly above the other. The microbe inoculated in the upper or lower wound was randomized. Sequential co-inoculations were conducted 5 months apart, in which a second wound was randomly made directly over or under the previous wound. Uninoculated wounded controls were used for comparison.

Ten months after the initial inoculation, trees were evaluated for signs and symptoms: gall appearance, decayed wood, and wood discoloration. Diameter and weight of the galls, and length of decayed wood and discoloration were measured using calipers and a calibrated scale.

Inoculated trees were monitored monthly and assessed for disease symptoms 10 months post-inoculation: September 19, 2022 (trial 1) and February 15, 2022 (trial 2). Trees were cut

below the inoculation sites and sectioned into approximately 10 cm long sections. Two longitudinal cuts were made perpendicular and through the inoculation point to make four wood sections. Photographs were taken prior to cutting through inoculation points and for all longitudinal cross sections of the inoculation sites. The length of the discoloration and wood decay, and diameter of galls were measured using calipers.

Isolations from discolored wood and decay were attempted, when present (Fig 1), by taking five 2 mm pieces plating them into yeast malt extract (YME) amended with benomyl (4µg a.i./ml) and streptomycin sulfate (100 µg/ml) to confirm the presence of the inoculated *G. adspersum*. Subcultures of suspected *G. adspersum* isolations were made onto MEA and confirmed using morphology.

Isolations from galls or inoculations points were attempted by rinsing the areas with deionized (DI) water, then 70% ethanol, and allowed to dry. The outside layer of the galls or inoculation point were removed, and small pieces (2 mm x 2 mm) of the inner layer were plated (5 pieces per plate) onto the surface of 90-mm-diameter Petri dishes of *A. tumefaciens* semi-selective 1A medium for Rhizobiaceae (Mougel et al. 2001) amended with tellurite at 80 ppm Yakabe et al. 2012) and trypticase soy broth agar (TSBA; trypticase soy broth at 30 g liter–1 and bacto agar at 15 g liter–1; Difco Bacto BBL) amended with rifampicin sulfate at 100 ppm (TSBA+rif) for 186r (Yakabe et al. 2012). 1A was incubated for 72 h at 28°C and 25°C for up to 5 days. TSBA+rif was incubated for 48 h at 28°C and then at 25°C for up to 7 days. Bacterial colonies were picked at first appearance and subcultured on TSBA+rif (Yakabe et al. 2012). Morphology and growth on TSBA+rif confirmed isolation of 186r.

Statistical analyses

To test for differences in length of discoloration or decay and gall diameter with the different inoculations relative to the mock inoculated control, analysis of variance (ANOVA) and Dunnett's Means comparison test were calculated independently for each experiment and tree species in R, with pathogen species or mock inoculated control as the independent variable and length of discoloration or decay or gall diameter as the dependent variables (Loyd et al. 2018; Yakabe et al. 2014). Tukey's honest significance test were also calculated independently for each experiment and tree species in R to compare symptoms between treatments (Loyd et al. 2018).

Results

In vitro co-inoculation assays

Radial growth of *G. adspersum* W0125 was generally similar in the presence of *A. tumefaciens* 186r prepared in TSB and YEPB, with a high initial inhibition percentage which decreases over time and may exhibit growth promotion. However, there were no significant differences between co-inoculation treatments or days of each treatment ($P>0.5$, Fig. 3.2, Table 3.1). The presence of the bacterium caused morphological differences when compared to the non-treated control, in particular mycelium becoming more compact over the bacterial colonies (Fig 3.1). Though the fungal mycelium had overgrown the bacteria by the end of the experiments, bacterial subcultures on TSB agar from each co-inoculation treatment showed that *A. tumefaciens* was viable, in all cases. In all co-inoculation assays, there was no inhibition zone between the pathogen species. In tube co-inoculations, the growth of *A. tumefaciens* 186r was not significantly affected by the presence of *G. adspersum* mycelium (Table 3.2).

Pathogen mobilization

In all cases, *A. tumefaciens* was only detected on the inoculated sections of YME agar. *Agrobacterium tumefaciens* was not detected on the surface of the PDA gel slice nor on the uninoculated side of the YME agar, suggesting that it is unable to mobilize on *G. adspersum* mycelium.

In vivo co-inoculation

In trial 1, significant internal discoloration was observed in Nemaguard inoculated by *Ganoderma adspersum* ($P=0.02$) and simultaneous inoculations of *G. adspersum* and *A. tumefaciens* ($P=8.0*10^{-3}$, Table 3.3, Fig. 3.3). Significant internal discoloration was observed in Viking inoculated by *Ganoderma adspersum* ($P=5.7*10^{-5}$) and both pathogens simultaneously

($P=5.5*10^{-4}$). Significant decay in Nemaguard (P=0.04) inoculated with *G. adspersum* (Fig. 3.5). Significant galling occurred on Nemaguard inoculated by *A. tumefaciens* ($P=4.3*10^{-5}$), *A. tumefaciens* followed by *G. adspersum* ($P=2.3*10^{-3}$) and *A. tumefaciens* and *G. adspersum* inoculated simultaneously ($P=8.2*10^{-6}$). Significant galling occurred in Viking inoculated by *A. tumefaciens* ($P=7.4*10^{-5}$); and VX211 inoculated by *A. tumefaciens* (P=2.8), *A. tumefaciens* followed by *G. adspersum* ($P=8.9*10^{-3}$), and both pathogens simultaneously ($P=5.4*10^{-4}$, Fig. 3.7). No significant differences were observed between inoculation types in any tested tree host.

In trial 2, significant internal discoloration was observed in VX211 (P=0.02) inoculated by *A. tumefaciens* and Viking (P=0.01) simultaneously inoculated by *G. adspersum* and *A. tumefaciens* (Table 3.3, Fig. 3.4). Significant decay was observed on Nemaguard ($P=8.5*10^{-4}$) inoculated by *G. adspersum* (Fig. 3.6). Significant galling occurred in Nemaguard, Viking, and VX211 inoculated with *A. tumefaciens* ($P=4.5*10^{-4}$, $P=9.3*10^{-9}$, $1.3*10^{-9}$, respectively), *G. adspersum* followed by *A. tumefaciens* inoculations (P=2.5*10-3, 1.9*10-9, 1.7*10-2, respectively), *A. tumefaciens* then *G. adspersum* (P=6.1*10-4, P=5.9*10-8, P=0.04, respectively), and both pathogens simultaneously ($P=4.6*10^{-3}$, $P=2.7*10^{-6}$, P=0.08, respectively). Significant galling occurred in VX211 inoculated with *A. tumefaciens* compared to the three co-inoculation treatments ($P=1.4*10^{-3}$, $P=1.1*10^{-4}$, $P=6.0*10^{-5}$, respectively, Fig. 3.8).

All inoculation sites completed or were in the process of forming response wood and bark for all *G. adspersum* and mock inoculated trees. No significant symptoms were observed on any of the mock inoculated controls. *Ganoderma adspersum* was successfully reisolated from the decayed or discolored wood of Nemaguard treated with singular and simultaneous inoculations of *G. adspersum* and *A. tumefaciens* and Viking treated with singular inoculations (Table 3.4).

Agrobacterium tumefaciens was reisolated from galls growing on Nemaguard treated with singular and simultaneous inoculations of *G. adspersum* and *A. tumefaciens* (Table 3.4).

Discussion

This study on the interactions between *G. adspersum* and *A. tumefaciens*, and their hosts reveals changing relationship dynamics of the fungal and bacterial pathogens. In *vitro* assays investigating pathogen growth and mobility suggested that the high co-incidence of *G. adspersum* and *A. tumefaciens* in the field are not due to direct interactions between the pathogens. While co-inoculations often led to less severe symptoms, the common occurrence of *G. adspersum* and *A. tumefaciens* co-infections in almond orchards leading to substantial tree failures, indicates likely dynamic shifts in the pathogens' interactions from competition in the initial stages of pathogenesis to coexisting commensalism (Xu 2021) in the later stages of disease development.

In vivo co-inoculations with *G. adspersum* and *A. tumefaciens* indicate that plant response to co-inoculations with *G. adspersum* and *A. tumefaciens* differs among cultivars. In both trials, only Nemaguard exhibited significant wood decay when inoculated only with *G. adspersum*, a known susceptible rootstock to the fungal pathogen. Wood decay and discoloration were not observed, or observed to a lesser extent, in galled trees. *Agrobacterium tumefaciens* induces rapidly growing galls which may initially reduce the development of wood decay, due to the extra wood being produced. At the end of the inoculation experiments, all galls were soft and green, while field observations of co-infections revealed wood decay and *G. adspersum* mycelium within older, dry galls. *Ganoderma adspersum* may likely utilize matured galls as an infection court and source of nutrients.

In trial 1, Nemaguard and VX211 trees exhibited significant galling, when initially inoculated with *A. tumefaciens*, though co-inoculations where *A. tumefaciens* was inoculated after *G. adspersum* did not lead to significant galling, likely due to the bacterium's inability to

induce galls during dormancy in winter months (Garrett et al. 1978), when the secondary inoculations were conducted. Confirming this phenomenon, significant galling was observed in all trees inoculated with *A. tumefaciens* in trial 2. *Agrobacterium tumefaciens* produces significantly larger galls in the absence of *G. adspersum* in VX211, a walnut rootstock with moderate resistance to *A. tumefaciens* (Hackett et al. 2010), indicating there is little to no competitive advantage from the pathogen experiencing lower resistance from the host (Estrada et al. 2012) due to *G. adspersum* infection. Overall, the pathogenesis of *A. tumefaciens* is generally unaffected by *G. adspersum* co-infections, demonstrating commensalism between the pathogens.

The common occurrence of the studied fungal and bacterial pathogens in almonds is likely due to the rapid increase in high-density planting undergoing mechanized harvesting and high irrigation and fertilization inputs (Holland et al. 2021), resulting in an environment can sustain the survival and infection of multiple coexisting pathogens. While more studies are required, substantial failures of co-infected almond trees may be a result from the long-term tradeoff in carbon allocation between growth and defense, as well as the amplified weakening of the trees' structure from the development of wood decay and galls at the basal stem.

Long-term studies may investigate the effect of host age, host immune response, time between inoculations, and environmental conditions on disease development from co-infections of these pathogens. Further studies may further explore cross-taxa interactions in plant disease systems, as the understanding of transmission, host immune response, and disease development in co-infection relationships will inform the development of integrated plant disease management practices.

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Tables

Table 3.1. Results of dual-culture growth assays with *A. tumefaciens* 186r prepared in TSB or YEPB. For each of the pathogenic fungi are reported the results of mycelial growth inhibition percentage (GIP) measured daily post inoculation (d.p.i.). The results are organized in columns according to the treatment they refer to: T0 (strain 186r inoculated the same day as the fungi), T1 (strain 186r inoculated 1 day ahead of the fungi), or T2 (strain 186r inoculated 2 days ahead of the fungi). P values obtained with Dunnett's Means comparison test are reported for the statistical comparison between the results obtained for each treatment and the fungal control. In the column headed inhibition halo are reported the average values measured of minimum distance between strain 186r colonies and fungal mycelium in dual-culture assays.

Fungal strain	**Measurement**	**T0 TSB**	**T1 TSB**	**T2 TSB**	**T0 YEPB**	**T1 YEPB**	**T2 YEPB**	**Inhibition Halo (cm)**
G. adspersum W0125	GIP 1 d.p.i	0.00	0.00	0.00	0.00	0.00	0.00	0
	GIP 2 d.p.i	30.87	0.80	-5.98	18.29	31.09	5.78	
	GIP 3 d.p.i	0.74	2.54	-0.58	8.63	16.04	8.89	
	GIP 4 d.p.i	18.51	2.30	0.38	6.37	11.88	12.98	
	GIP 5 d.p.i	6.30	-3.09	-9.97	-2.53	10.07	8.13	
	GIP 6 d.p.i	5.60	1.86	-7.96	2.60	10.67	6.81	
	GIP 7 d.p.i	-0.05	-5.69	-17.68	-8.42	3.53	-1.02	
	GIP 8 d.p.i	-1.83	-6.55	-16.23	-2.70	2.03	-7.08	
	GIP 9 d.p.i	-2.25	-6.83	-13.61	-4.20	0.93	-3.71	
	GIP 10 d.p.i	-1.87	-7.26	-13.10	-3.91	4.18	-9.39	
	P value	0.35	0.97	0.06	1.00	0.05	0.97	

Table 3.2. Results of dual-culture growth assays with 10 ul of *A. tumefaciens* 186r and a 5 mm plug of *G. adspersum* W0125 in 15 ml of TSB. P values obtained with Dunnett's Means comparison test are reported for the statistical comparison between the results obtained for each treatment and the bacterial control.

Measurement	***A. tumefaciens* control**	***A. tumefaciens* → *G. adspersum***	***G. adspersum* → *A. tumefaciens***	***A. tumefaciens* + *G. adspersum***
Concentration (CFU/ml)	$1.55 * 10^8$	$1.25 * 10^8$	$1.30 * 10^8$	$1.16 * 10^8$
Growth inhibition (%)	-	19.9	16.5	25.6
P value	-	0.97	0.98	0.91

Table 3.3. Average total lengths of discolored wood and wood decay for almond rootstock cultivars Nemaguard and Viking, and walnut rootstock cultivar VX211 inoculated with *Ganoderma adspersum* and *Agrobacterium tumefaciens.*

	Trial 1			**Trial 2**		
	Discolored wood (mm)[a]	**Decayed wood (mm)**	**Gall (mm)**	**Discolored wood (mm)**	**Decayed wood (mm)**	**Gall (mm)**
Nemaguard						
Control	0	0	0	10.65 ± 2.18	0	0
G. adspersum	50.62 ± 37.2* [b]	39.94 ± 36.24*	0	20.29 ± 7.90	4.97 ± 2.98*	0
A. tumefaciens	0	0	11.57 ± 2.49*	3.58 ± 4.39	0	24.45 ± 6.21*
Ganoderma before *Agrobacterium*	14.90 ± 15.27	8.60 ± 5.10	1.42 ± 1.44	13.69 ± 13.96	0	21.15 ± 2.98*
Agrobacterium before *Ganoderma*	14.53 ± 4.29	0	8.16 ± 0.64 *	9.11 ± 9.98	0	23.89 ± 9.20*
Ganoderma and *Agrobacterium*	58.92 ± 28.16 *	29.17 ± 21.81	12.88 ± 4.92 *	7.16 ± 5.50	0	21.35 ± 7.46*
Viking						

Control	0	0	0	0	0	0
G. adspersum	18.68 ± 6.22*	3.57 ± 4.89	0	18.97 ± 15.76	5.04 ± 4.61	0
A. tumefaciens	0	0	7.77 ± 2.07 *	16.72 ± 11.50	0	29.83 ± 4.44*
Ganoderma before *Agrobacterium*	17.01 ± 5.47*	7.02 ± 3.67	0	9.52 ± 6.48	0	31.07 ± 5.39*
Agrobacterium before *Ganoderma*	2.50 ± 3.54	0	3.79 ± 2.68	4.94 ± 5.64	2.11 ± 2.98	27.29 ± 5.67*
Ganoderma and *Agrobacterium* VX211	2.80 ± 3.96	2.77 ± 3.91	3.42 ± 2.49	30.87 ± 11.73	1.63 ± 2.31	24.99 ± 1.46*
Control	0	0	0	0.93 ± 1.87	0	0
G. adspersum	8.13 ± 8.81	0.42 ± 0.84	0	6.35 ± 3.69	0	0
A. tumefaciens	3.42 ± 4.84	0	13.88 ± 9.82 *	0	0	49.98 ± 12.46*[c]
Ganoderma before *Agrobacterium*	3.22 ± 4.56	0	7.94 ± 5.63	10.59 ± 5.06*	1.15 ± 1.63	22.2 ± 7.19*
Agrobacterium	2.08 ±	0	16.32 ±	5.58 ± 4.17	0	14.45 ± 2.88*

Agrobacterium before *Ganoderma*	1.50		4.67 *			
Ganoderma and *Agrobacterium*	2.79 ± 3.80	0.53 ± 0.92	20.38 ± 6.94 *	1.75 ± 2.47	0	12.56 ± 2.09*

[a] Each cell represents averages of replicates where n = 4 or 5. Values represent the average discolored area ± the standard deviation.

[b] Values with * are statistically significant (P≤0.05) with Dunnet's test relative to the control.

[c] Values are statistically different (P≤0.05) with Tukey test with other treatments on the same tree type.

Table 3.4. Inoculum recovery for almond rootstock cultivars Nemaguard and Viking, and walnut rootstock cultivar VX211 inoculated with *Ganoderma adspersum* and *Agrobacterium tumefaciens.*

	Trial 1		Trial 2	
	***Ganoderma* recovery**	***Agrobacterium* recovery**	***Ganoderma* recovery**	***Agrobacterium* recovery**
Nemaguard				
Control	- [a]	-	-	-
G. adspersum	+++ [b]	-	+++	-
A. tumefaciens	-	+	-	+++
Ganoderma before *Agrobacterium*	-	-	++	++
Agrobacterium before *Ganoderma*	-	-	+	++
Ganoderma and *Agrobacterium*	++	+	+	-
Viking				
Control	-	-	-	-
G. adspersum	+	-	++	-
A. tumefaciens	-	-	-	+++
Ganoderma before *Agrobacterium*	-	-	+	++
Agrobacterium before *Ganoderma*	-	-	++	+++
Ganoderma and *Agrobacterium*	-	-	+	-
VX211				
Control	-	-	-	-
G. adspersum	-	-	-	-
A. tumefaciens	-	-	-	++++
Ganoderma before *Agrobacterium*	-	-	-	++
Agrobacterium before *Ganoderma*	-	-	-	++
Ganoderma and *Agrobacterium*	-	-	-	+++

[a] – indicates an unsuccessful reisolation

[b] + indicates successful reisolation, where each + indicates success in one replicate.

Figures

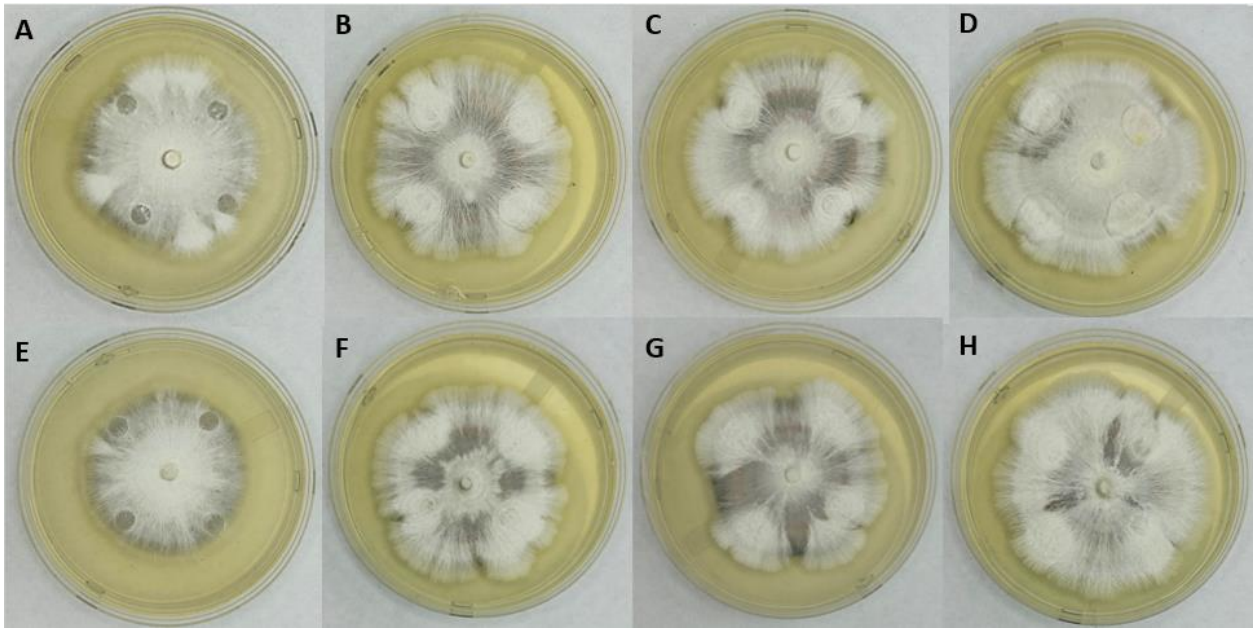

Figure 3.1. Comparison between dual-culture treatments with *G. adspersum* strain W0125 and *A. tumefaciens* strain 186r. Photographs of one representative plate per treatment, taken at 10 days post inoculation, showing white mycelium (discoloration due to markings on the bottom of plate). From left to right are presented: non-treated control (**A, E**), T0 treatment (strain 186r inoculated the same day as the fungus) (**B, F**), T1 treatment (strain 186r inoculated 1 day ahead of the fungus) (**C, G**), and T2 treatment (strain 186r inoculated 2 days ahead of the fungi) (**D, H**). Top row presents treatments with strain 186r grown in TSB, and bottom row presents treatments with strain 186r grown in YEPB.

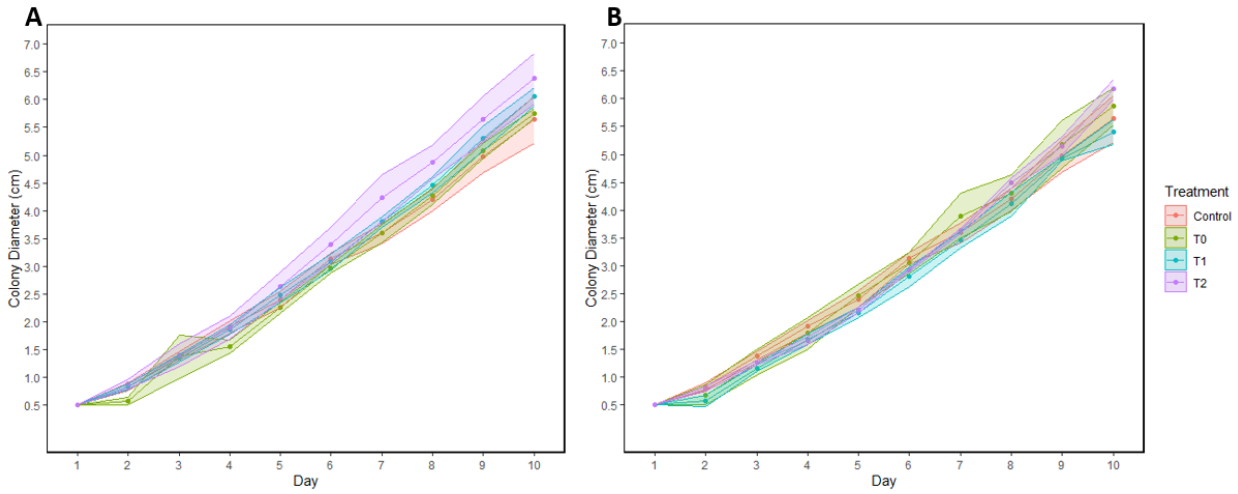

Figure 3.32. Radial growth of *G. adspersum* for 10 days with non-treated control (**red**), T0 treatment (strain 186r inoculated the same day as the fungus) (**green**), T1 treatment (strain 186r inoculated 1 day ahead of the fungus) (**blue**), and T2 treatment (strain 186r inoculated 2 days ahead of the fungi) (**purple**). Co-inoculations conducted with *A. tumefaciens* 186r grown in TSB (**A**) and YEPB (**B**).

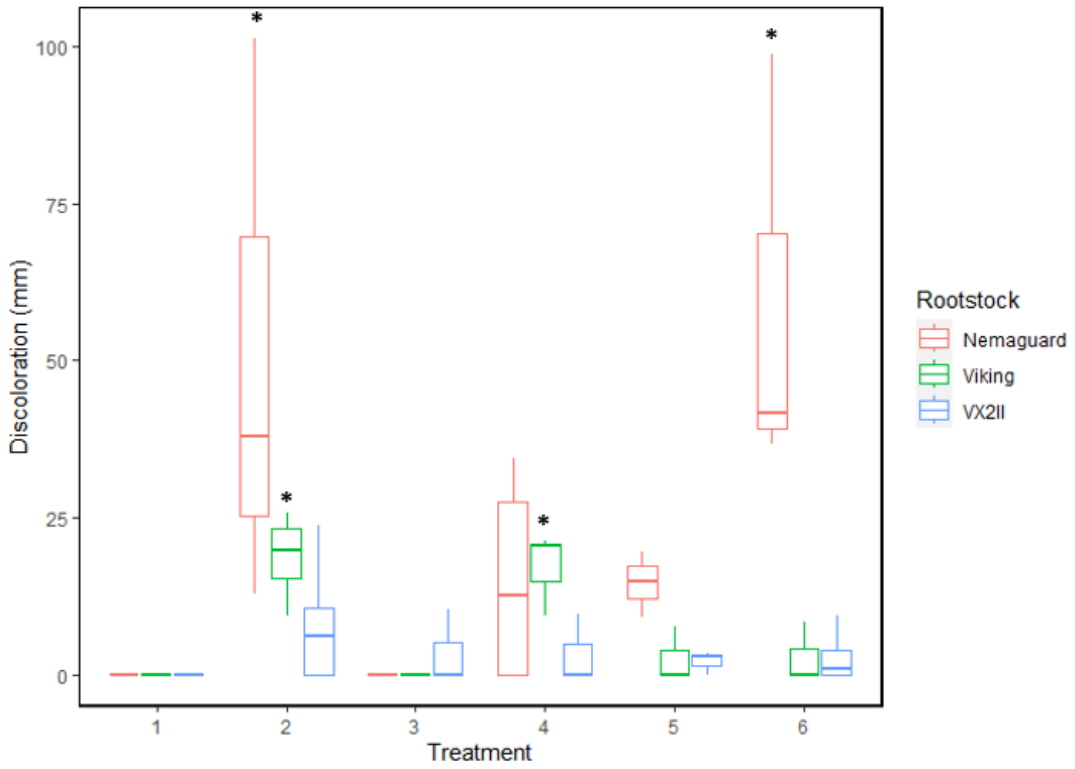

Figure 3.3. Average total length of discolored wood for almond rootstock cultivars Nemaguard (red) and Viking (green) and walnut rootstock cultivar VX211 (blue) inoculated with water (1); *Ganoderma adspersum* (2); *Agrobacterium tumefaciens* (3); *G. adspersum* then *A. tumefaciens* (4); *A. tumefaciens* then *G. adspersum* (5); and *G. adspersum* and *A. tumefaciens* (6) in trial 1. Treatments with asterisk were statistically significantly ($P < 0.05$) compared to the control.

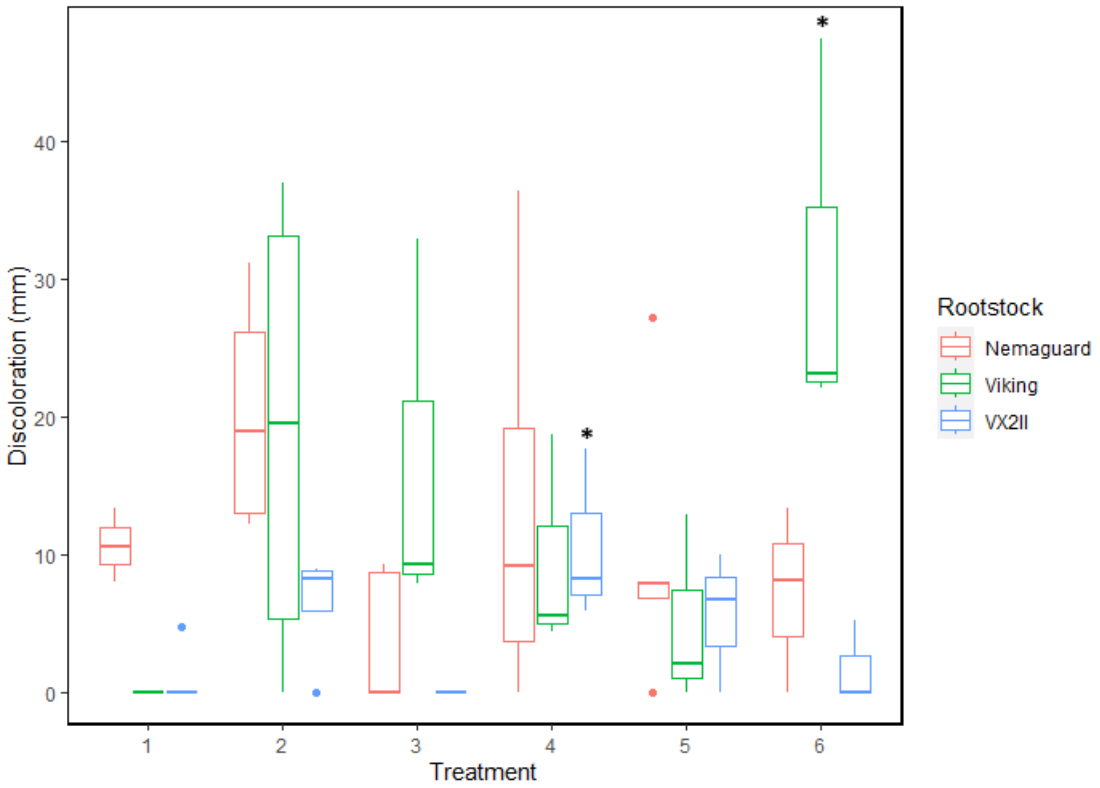

Figure 3.4. Average total length of discolored wood for almond rootstock cultivars Nemaguard (red) and Viking (green) and walnut rootstock cultivar VX211 (blue) inoculated with water (1); *Ganoderma adspersum* (2); *Agrobacterium tumefaciens* (3); *G. adspersum* then *A. tumefaciens* (4); *A. tumefaciens* then *G. adspersum* (5); and *G. adspersum* and *A. tumefaciens* (6) in trial 2.

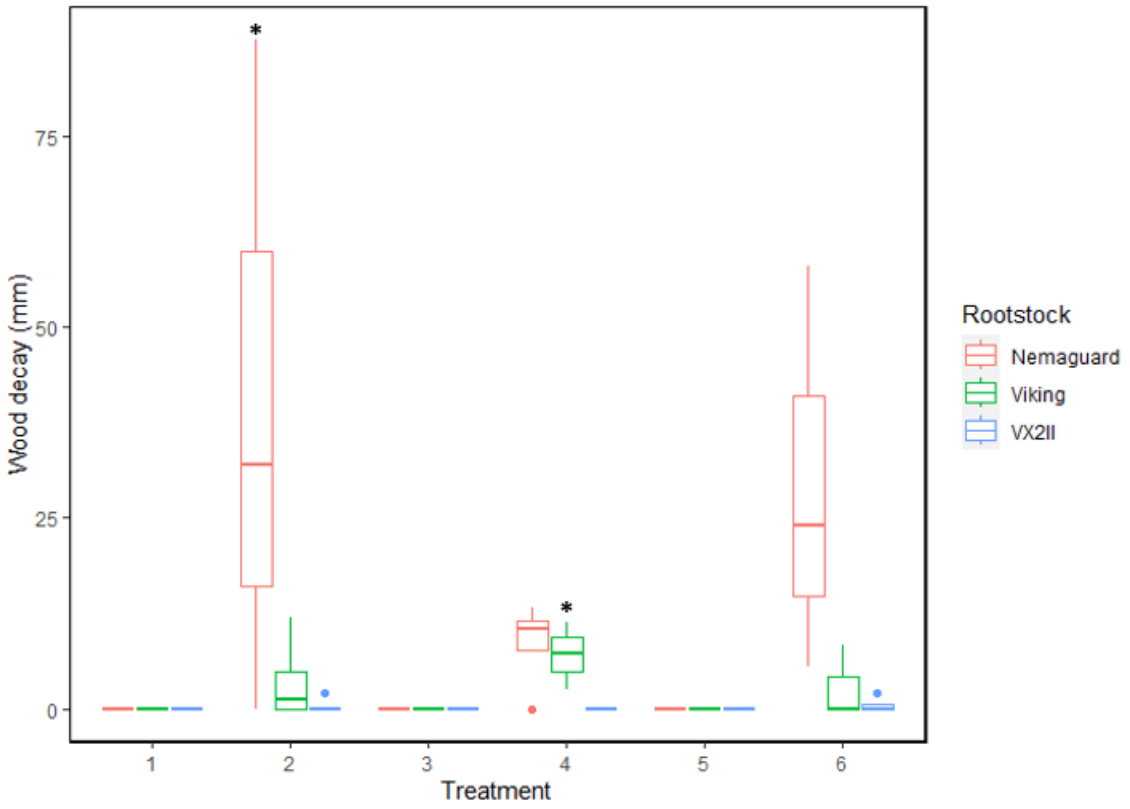

Figure 3.5. Average total length of wood decay for almond rootstock cultivars Nemaguard (red) and Viking (green) and walnut rootstock cultivar VX211 (blue) inoculated with water (1); *Ganoderma adspersum* (2); *Agrobacterium tumefaciens* (3); *G. adspersum* then *A. tumefaciens* (4); *A. tumefaciens* then *G. adspersum* (5); and *G. adspersum* and *A. tumefaciens* (6) in trial 1. Treatments with asterisk were statistically significantly ($P < 0.05$) compared to the control.

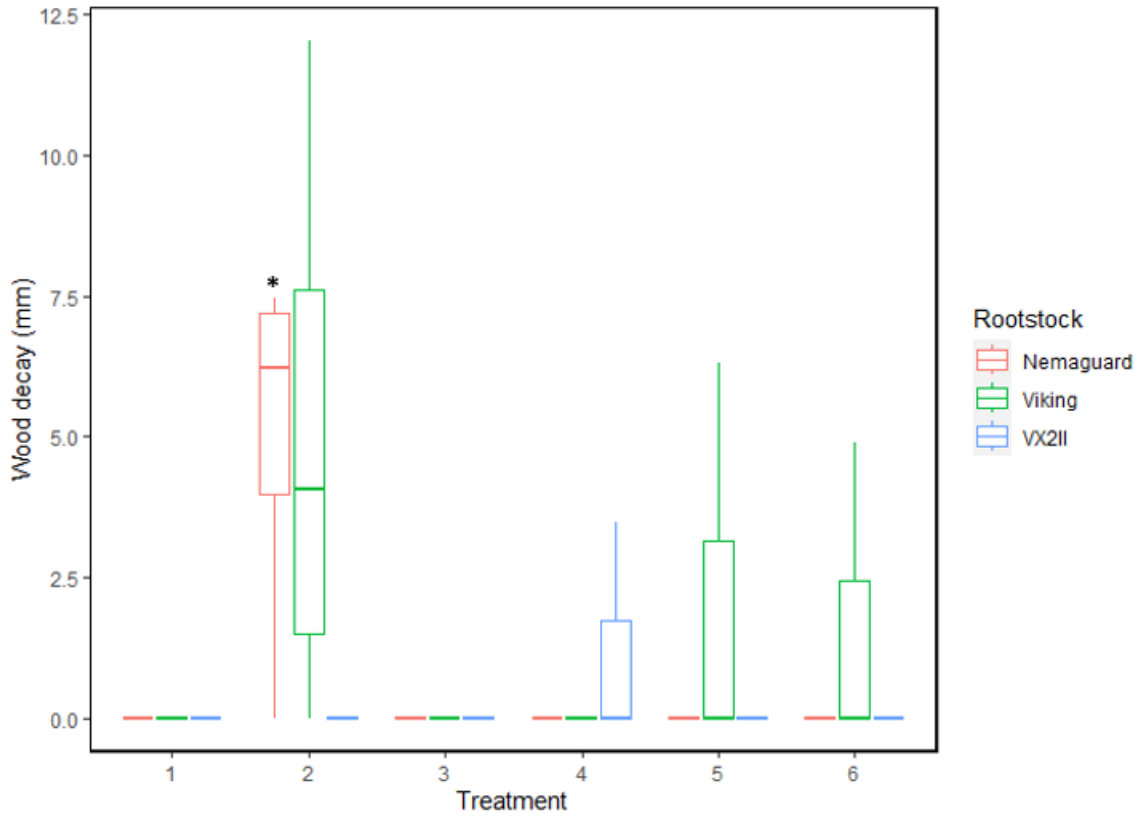

Figure 3.6. Average total length of wood decay for almond rootstock cultivars Nemaguard (red) and Viking (green) and walnut rootstock cultivar VX211 (blue) inoculated with water (1); *Ganoderma adspersum* (2); *Agrobacterium tumefaciens* (3); *G. adspersum* then *A. tumefaciens* (4); *A. tumefaciens* then *G. adspersum* (5); and *G. adspersum* and *A. tumefaciens* (6) in trial 2.

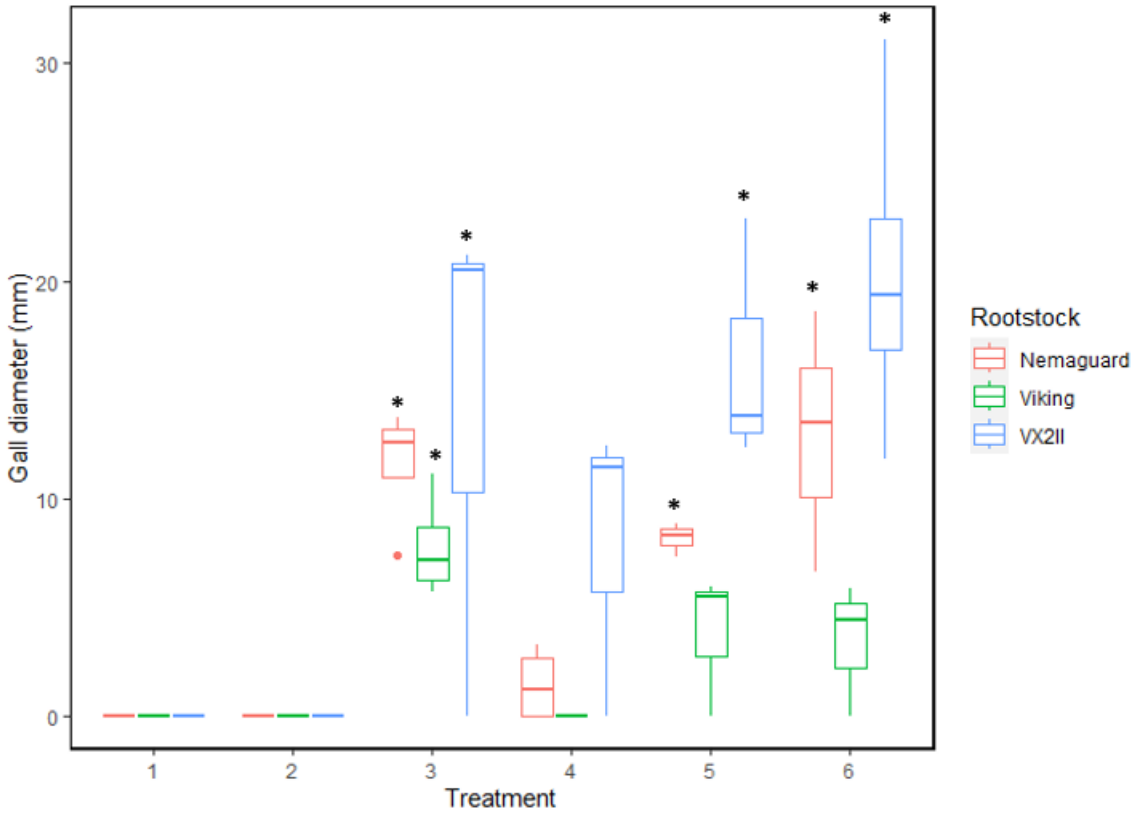

Figure 3.7. Average total width of galls for almond rootstock cultivars Nemaguard (red) and Viking (green) and walnut rootstock cultivar VX211 (blue) inoculated with water (1); *Ganoderma adspersum* (2); *Agrobacterium tumefaciens* (3); *G. adspersum* then *A. tumefaciens* (4); *A. tumefaciens* then *G. adspersum* (5); and *G. adspersum* and *A. tumefaciens* (6) in trial 1. Treatments with asterisk were statistically significantly ($P < 0.05$) compared to the control.

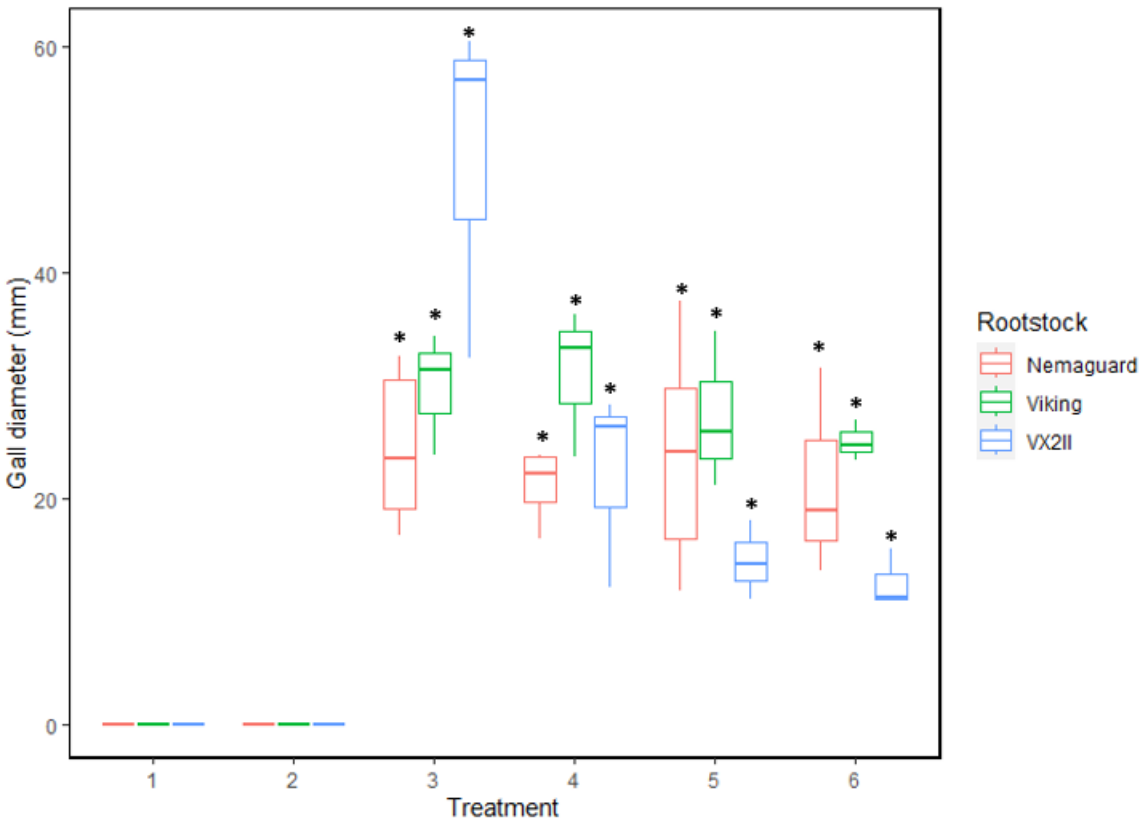

Figure 3.8. Average total width of galls for almond rootstock cultivars Nemaguard (red) and Viking (green) and walnut rootstock cultivar VX211 (blue) inoculated with water (1); *Ganoderma adspersum* (2); *Agrobacterium tumefaciens* (3); *G. adspersum* then *A. tumefaciens* (4); *A. tumefaciens* then *G. adspersum* (5); and *G. adspersum* and *A. tumefaciens* (6) in trial 2.

APPENDIX A

Ganoderma diagnostics

Wood-decay species vary in their ability to oxidize gallic acid, resulting in variations in colony growth and color. To develop selective media to improve non-molecular diagnostic of *Ganoderma* species, *Ganoderma adspersum* AD161 and AD293, *G. brownii* B358 and B144, *G. polychromum* RCR273 and PC355, *G. applanatum* Ap2, *Phellinus pomaceous* Ph134D2, and *Trametes versicolor* T1 were tested on 1.5% malt extract (ME) broth and agar amended with 0.5% gallic acid (Van Der Westhuizen 1958). All cultures were incubated in dark conditions at 25 ± 1°C for up to 10 days.

All isolates displayed various levels of discoloration on ME amended with gallic acid with no notable differences between species. *Ganoderma adspersum* growth was not inhibited by gallic acid in ME agar while all other wood-decay species were inhibited (Fig A1). In ME broth amended with gallic acid, *Ganoderma adspersum* displayed the lowest level of oxidation (Fig A2).

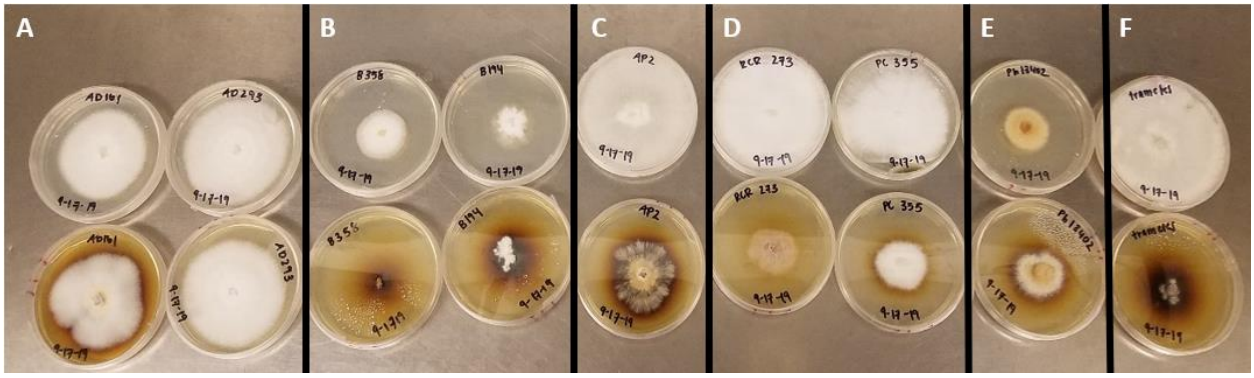

Fig A1. A: *Ganoderma adspersum*, B: *Ganoderma brownii*, C: *Ganoderma applanatum*, D: *Ganoderma polychromum*, E: *Phellinus pomaceous*, and F: *Trametes versicolor* on 1.5% malt extract (ME) agar (top row) and ME amended with 0.5% gallic (bottom row).

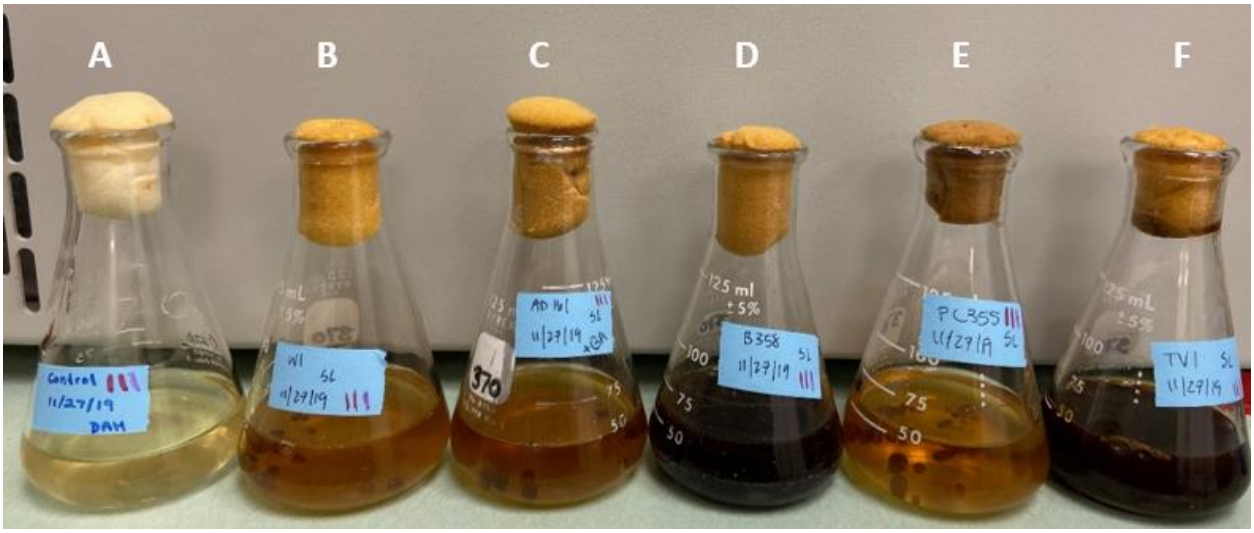

Fig A2. A: Uninoculated control, B-C: *Ganoderma adspersum*, D: *Ganoderma brownii*, E: *Ganoderma polychromum*, and F: *Trametes versicolor* in ME broth amended with 0.5% gallic.

Literature Cited

Van Der Westhuizen, G.C.A., 1958. Studies of Wood-rotting fungi: 1. Cultural characteristics of some common species. Bothalia 7(1):83-99.

APPENDIX B

Ganoderma transmission: spore percolation

Soil cores from five orchard sites positive for *Ganoderma adspersum* were collected from up to 30 cm into the soil. Spores were extracted from the soil at every depth up to 30 cm using a sucrose spore extraction method (Allen et al. 1979, Ianson and Allen 1986). Spores were quantified using a hemocytometer. Soil dilution quantification was attempted on ME agar amended with streptomycin and benomyl, though the slow growth of *Ganoderma* compared to other soil inhabitants, such as *Rhizobium* spp. and *Penicillium* spp. prevented proper quantification.

To determine how much water is required for *Ganoderma* spores to percolate into the soil, soil columns were made using 10 cm wide x 60 cm long PVC pipe (ACE Hardware) which was split in half longways. The sides of the split PVC pipe were sealed with black electrical tape, while the bottom of the pipe was sealed with two layers of cheesecloth to keep the soil in the pipe. The pipe was filled with autoclaved, sieved (4 mm) sand. 1 ml of 1 x 10^9 spore solution was pipetted onto the top of the soil and various quantities of autoclaved, deionized water were poured onto the top of the column: 50 ml, 100 ml, 150 ml, and 200 ml. All treatments were made in triplicate. Spores were found up to 33 cm into the soil column when treated with 50 ml, though in all other treatments, spores were found at depths up to 60 cm.

Ganoderma transmission: mycelial growth

Wood-consuming basidiomycete fungi, such as *Ganoderma* spp., may not simply passively explore their surroundings, but actively forage by sensing the presence of woody resources in the environment and responding adaptively to them. To determine mycelial foraging behavior, *Ganoderma adspersum* AD161 and AD293, *G. brownii* B358, *G. polychromum*

PC355, and *Armillaria mellea* Arm1 were inoculated onto wood blocks. Isolates were grown on 1.5% malt extract (ME) agar in dark conditions at 25 ± 1°C. Liquid cultures were made in 125 ml flasks with 50 ml of YME by scraping the mycelium off 7-day old cultures and placing them in the broth. The flasks were plugged with a sponge plug, and the flasks were incubated on a stirring incubator (150 rpm) in dark conditions at 25 ± 1°C for up to 10 days.

Prunus dulcis (almond) branches (~2 cm diameter) were autoclaved in 100 ml of water before being debarked and cut in ~2 cm blocks using a bandsaw. Up to 15 wood blocks were autoclaved in batches in autoclavable airflow spawn bags (Fungi Perfecti). Over half of the broth from the liquid cultures was removed from the flask before using the liquid cultures to inoculate the wood blocks. The wood blocks were incubated in dark conditions at 25 ± 1°C for 15 weeks.

In sterilized 15 cm x 24 cm plastic boxes (Ziploc), a 1 cm layer of the following media was placed: autoclaved sieved (4 mm) sand, unautoclaved sieved (4 mm) sand, or unautoclaved, sieved sand mixed with 20% sandy loam topsoil and sawdust. An inoculated wood block was placed on the center of the box and incubated for 7 days to allow for moisture equilibrium. 3 autoclaved, uncolonized 2 cm x 2 cm wood blocks (baits); 3 autoclaved, uncolonized 1 cm x 1 cm wood blocks (small baits); and 3 plastic 2 cm x 2 cm tube caps (controls) were randomly placed around the center inoculum (Dowson et al. 1989). Each treatment was conducted in triplicate. All boxes were closed with a lid and were incubated in dark conditions at 20 ± 1°C for 5 weeks.

In treatments with autoclaved soil, all *Ganoderma* spp. grew mycelium on the surface of the sand towards the 2 cm and 1 cm wood blocks, but not the plastic caps. Growth through the sand was not visible though was present, as it caused sections the sand to clump together. The *Armillaria* isolate grew rhizomorphs on the surface and through the sand towards the wood

blocks. Minimal growth occurred at the bottom of the boxes in all cases. In treatments using unsterilized sand, there was no mycelial growth from the *Ganoderma* spp., though there was growth of *Rhizobium* spp. and *Trichoderma* spp., demonstrating that the wood-decay fungi are weak competitors in the soil. Pictures were not included of growth on unautoclaved soil, due to the sheer growth of the contaminants.

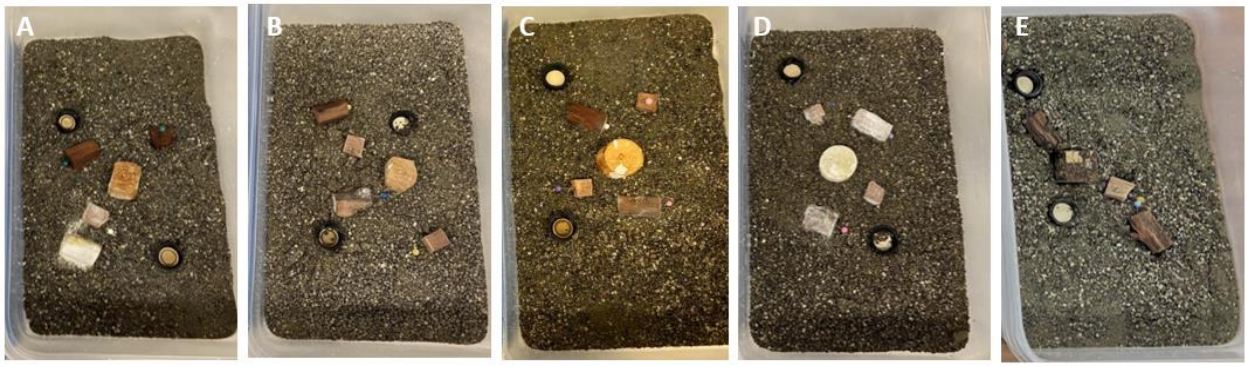

Figure B1. Growth of *Ganoderma adpsersum* (**A**), *G. brownii* (**B**), *G. applanatum* (**C**), *G. polychromum* (**D**), and *Armillaria melea* (**E**) on autoclaved, sieved (4 mm) sand.

Literature Cited

Allen, E. B., Moore Jr., T. S., and Christensen, M. 1979. Growth of vesicular-arbuscular mycorrhizal and non-mycorrhizal *Bouteloua gracilis* in a defined medium. *Mycologia* 71: 666-669.

Dowson, C.G., Springham, P., Rayner, A. D. M., and Boddy, L., 1989. Resource relationships of foraging mycelial systems of *Phanerochaete velutina* and *Hypholoma fasciculare* in soil. *New Phytologist* 111(3): 501-509.

Ianson, D. C., and Allen, M. F. 1986. The Effects of Soil Texture on Extraction of Vesicular-Arbuscular Mycorrhizal Fungal Spores from Arid Sites. *Mycologia* 78(2): 164-168.

APPENDIX C

Ganoderma basidiocarp and spore production

Attempts to grow Ganoderma basidiocarps for spores were made following modified protocols of Stamet's 2000 *Ganoderma* cultivation methods (Stamets 2000). *Ganoderma adspersum* AD161, AD293 and W1, *G. brownii*, and *G. polychromum* RCR273 and PC355 were inoculated into sawdust, almond wood, and rye substrates. Isolates were grown on 1.5% malt extract (ME) agar or yeast malt extract (YME) agar in dark conditions at 25 ± 1°C. Liquid cultures were made in 125 ml flasks with 50 ml of YME by scraping the mycelium off 7 day old cultures and placing them in the broth. The flasks were plugged with a sponge plug, and the flasks were incubated on a stirring incubator (150 rpm) in dark conditions at 25 ± 1°C for up to 10 days.

Rye was rinsed and soaked in deionize water for 24 hours. After draining, 200 g of rye was placed into autoclavable airflow spawn bags (Fungi Perfecti) and sterilized twice with a 24-hour cooling period after each sterilization. Rye bags were inoculated with 10-day-old liquid cultures. Over half of the broth from the liquid cultures was removed from the flask before using the liquid cultures to inoculate the rye bags. Inoculated rye bags were sealed and incubated in dark conditions at 25 ± 1°C for up to 14 days.

The following substrates were tested: ~2300 g of sawdust (substrate 1); four almond wood branches (15 cm length x 3 cm diameter), and ~1100 g of sawdust (substrate 2); four almond wood branches (15 cm length x 3 cm diameter), 200 g rye, and ~1100 g of sawdust (substrate 3); four almond wood branches (15 cm length x 3 cm diameter), 200 g rye, ~1100 g of sawdust, and 22 g calcium carbonate (substrate 4); and four almond wood branches (15 cm length x 3 cm diameter), 200 g rye, ~1100 g of sawdust, 22 g calcium carbonate, and 100 g

soybean meal (substrate 5). Almond branches were autoclaved in 100 ml of water before being debarked and autoclaved for a second time. All substrates were autoclaved twice as previously described. Substrate bags were inoculated with rye spawn, and incubated in dark conditions at various temperatures from 5°C to 28°C.

After 8-10 weeks, some substrate bags were also moved to different temperatures after 5-8 weeks: 5°C or 7°C to 25°C or 28°C. All bags were transferred to ambient daylight cycles (14 h light, 10 h dark) and two X-shaped cuts (5 cm long) were made to allow more air flow into the bags. All bags were kept moist by placing a water bin in the chamber and daily spraying and were monitored for basidiocarp growth and sporulation for up to 16 weeks.

Ganoderma polychromum RCR273 and PC355 produced basidiocarps and sporulated readily in all substrates within 3 weeks of cutting the substrate bags (Fig. C1). *Ganoderma adspersum* and *G. brownii* basidiocarps were produced in substrate 4 and 5 when maintained at 25-28°C, though growth was stunted at 5-7 cm and no sporulation occurred (Fig C1). Additionally, *Ganoderma brownii* maintained on YME agar covered with 10-15 wooden dowels (balsam) produced no fruiting bodies though did produce few spores when maintained in ambient daylight cycles (14 h light, 10 h dark) for up to ten weeks.

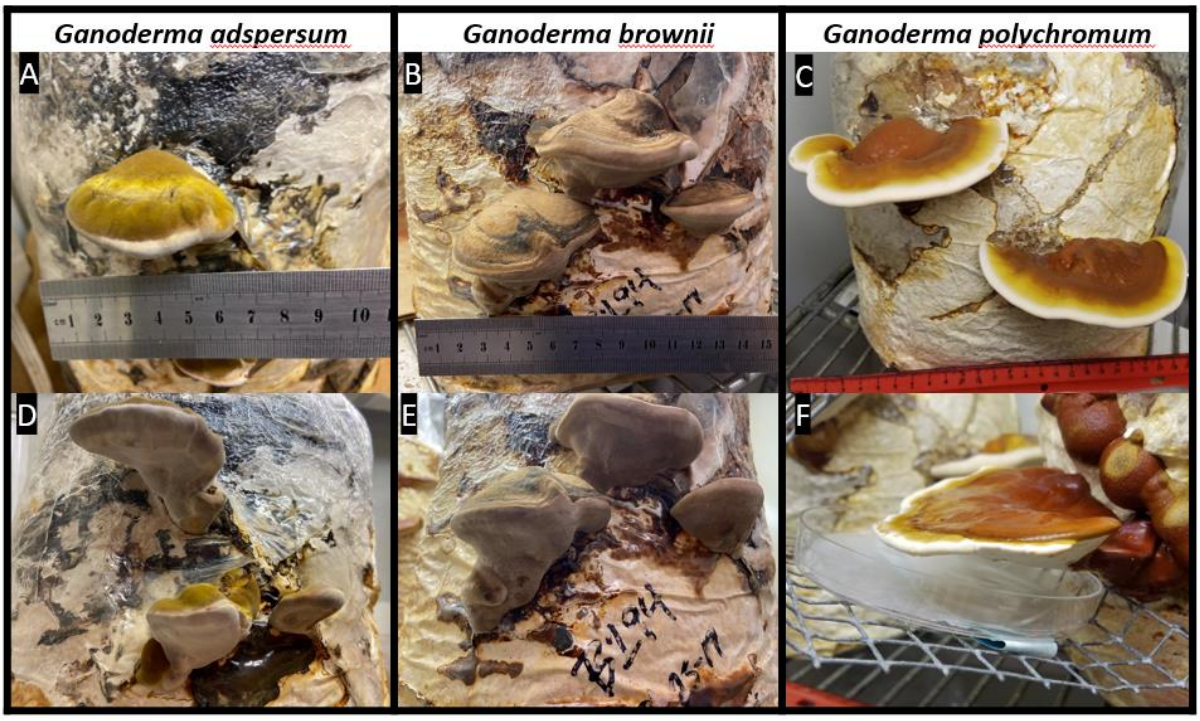

Fig C1. Fruiting bodies of *Ganoderma adspersum* (A & D), *Ganoderma brownii* (B & E), and *Ganoderma polychromum* (C & F).

Literature Cited

Stamets, P. 2000. Growing gourmet and medicinal mushrooms. Ten Speed Press, Berkeley, CA.