DISSERTATION

INFORMATION ON EMERGENT INSECT ASSOCIATED TREE DISEASES INCLUDING EPIDEMIOLOGICAL STUDIES OF DRIPPY BLIGHT DISEASE OF OAK AND THOUSAND CANKERS DISEASE OF WALNUT

Submitted by

Rachael A. Sitz

Department of Bioagricultural Sciences and Pest Management

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2017

Doctoral Committee:

Advisor: Whitney Cranshaw
Co-Advisor: Jane Stewart

Cris Argueso
Tony Koski

ABSTRACT

INFORMATION ON EMERGENT INSECT ASSOCIATED TREE DISEASES INCLUDING EPIDEMIOLOGICAL STUDIES OF DRIPPY BLIGHT DISEASE OF OAK AND THOUSAND CANKERS DISEASE OF WALNUT

Two emergent disease complexes called drippy blight and thousand cankers have become injurious to hardwood trees in communities across the urban corridor of Colorado. In order to better understand these disease complexes, studies were initiated to identify the causal agents, symptomology, and epidemiology of drippy blight disease as well as determine if multiple fungi were involved in thousand cankers disease.

Chapter I, published in the *Journal of Arboriculture and Urban Forestry*, provides information on the basic biology of drippy blight disease. This article explains the signs and symptoms of drippy blight, and shows that the bacterium *Lonsdalea quercina* subsp. *quercina* is able to cause the disease. Finally, it was noted that a kermes scale insect was abundant on all diseased oaks, but its role as a vector is unknown. Overall, this research documents the microbe and insect causal agents in this disease complex. These findings are biologically significant as the isolated bacterium was able to produce disease on several species of sapling red oaks. This, taken into consideration with other diseases caused by *Lonsdalea quercina*, implies that this bacterium has the potential to become widespread.

Chapter II provides a detailed life history of *Allokermes galliformis*, the insect causal agent in drippy blight disease. Management decisions to mitigate the effects of insect damage are largely based on an understanding of the life cycle. This research documents periods when *A. galliformis* may be most exposed and vulnerable to controls and notes areas of the tree where it

inflicts damage when feeding. Therefore, the information provided in this chapter can serve as a guide for more informed decisions when considering management of drippy blight-affected trees.

Chapter III considers what insects may be involved in the dissemination of *Lonsdalea quercina* subsp. *quercina* from oaks with symptoms of drippy blight disease. Although kermes scales are the most consistent and abundant insects found in association with drippy blight disease, other more mobile insects are frequently observed on diseased trees and it was hypothesized that these may play a role in spreading the bacterial pathogen within trees and to new trees. After testing insects collected on diseased oak trees for bacterial contamination, various insects were documented to carry the bacterium, providing insight into ways the disease may move in a population of susceptible hosts.

Chapter IV, published in *Plant Disease*, involves a tree disease characterized by an insect and fungal pathogen interaction, thousand cankers disease of walnut. This study investigates whether genetically distinct isolates of the fungal pathogen *Geosmithia morbida* vary in pathogenicity. Also examined was whether *Fusarium solani*, commonly found on diseased trees, may contribute to production of thousand cankers disease. A range of *Geosmithia morbida* isolates were tested alone and in combination with a *Fusarium solani* isolate to determine the relative contribution of each fungal pathogen to thousand cankers disease. Importantly, in Colorado *Geosmithia morbida* does not interact synergistically with *F. solani.*

Overall, this research is the first to describe the emergent disease complex drippy blight and provides information to better understand the bacterial and insect causal agents. Furthermore, the investigations of drippy blight of red oak and thousand cankers disease of walnut, improve our understanding of the epidemiology of woody plant diseases that involve both arthropod and pathogen partners.

ACKNOWLEDGEMENTS

My goal of attaining my PhD would have been impossible without the tremendous support provided by my mentors, colleagues, friends, and family.

I am very grateful to my advisor Whitney Cranshaw who has encouraged me to explore countless opportunities and always provided excellent mentorship.

I am also grateful to my influential current and past co-advisors, Jane Stewart and Ned Tisserat. In addition, I thank my current and past committee members, Cris Argueso, Toni Koski, James Klett, and Bill Jacobi, who consistently provided invaluable advice and advocated for my best interests.

I appreciated the funding for my work, provided by the Colorado Agricultural Experiment Station and the TREE Fund. In addition, I thank my collaborators on these projects including Boulder Arborists and City Foresters as well as California Department of Food and Agriculture taxonomists for their help, as well as the current and past members of the Cranshaw, Tisserat, and Stewart labs.

The support and companionship from my close friends and colleagues, Emily, Jorge, Stacy, and Esby has been invaluable.

I am infinitely privileged to have the love and support from my family; especially Ransom, Wayne, Johanna, and Dana.

DEDICATION

To my Mother, who always encouraged me to achieve my goals and instilled the importance of education in my family. She was the strongest, kindest, and most fun loving woman I have known. Above everything else, she prioritized the people she loved and cherished the natural world.

TABLE OF CONTENTS

CHAPTER 1: DRIPPY BLIGHT, A DISEASE OF RED OAKS IN COLORADO PRODUCED FROM THE COMBINED EFFECT OF THE SCALE INSECT *ALLOKERMES GALLIFORMIS* AND THE BACTERIUM *LONSDALEA QUERCINA* SUBSP. *QUERCINA*[1]

SUMMARY

Drippy blight is an emergent disease of red oaks caused by the interaction between a kermes scale insect (*Allokermes galliformis*) and a bacterium (*Lonsdalea quercina* subsp. *quercina*). Multi-locus sequence analysis was used to confirm the bacterial pathogen's identity and its relationship to other phylogenetically related Enterobacteriaceae species. Further, Koch's postulates were performed on sapling red oaks. Prior to the discovery of drippy blight disease in Colorado, within the United States the bacterium was reported on oak trees in California but was limited to acorn infections. The scale insect, *A. galliformis,* was previously known to occur on pin oak in the eastern United States but was not previously associated with this bacterium nor the production of significant branch dieback associated with drippy blight. In addition to a description of this new disease, this research documents a host range expansion of *L. quercina*

[1] Rachael A. Sitz, Graduate Student Department of Bioagricultural Sciences and Pest Management Colorado State University, Fort Collins, CO 80523-1177 and Rocky Mountain Research Station, United States Department of Agriculture Forest Service, 1221 South Main Street, Moscow, ID 83843; Marcelo M. Zerillo, Postdoctoral Researcher, Present Address: Netherlands Institute of Ecology (NIOO/KNAW), Department of Microbial Ecology, 6708 PB Wageningen, The Netherlands; Jacob Snelling; Research Associate, Present Address: Department of Horticulture, Oregon State University, Corvallis, OR 97331-7304; Jorge Ibarra Caballero, Postdoctoral Researcher, Department of Bioagricultural Sciences and Pest Management Colorado State University, Fort Collins, CO 80523-1177; Kathleen Alexander, City Forester, City of Boulder Forestry, 5200 Pearl Street, Boulder, CO 80301-2437; Kendra Nash, City Forester, City of Fort Collins Forestry, 1001 Amy Van Dyken Way, Fort Collins, CO 80521; Ned A. Tisserat, Whitney S. Cranshaw, and Jane E. Stewart, Professors, Department of Bioagricultural Sciences and Pest Management Colorado State University, Fort Collins, CO 80523-1177.

Accepted for publication 24 July 2017.

Corresponding author: Rachael Sitz, rsitz@fs.fed.us

subsp. *quercina* to northern red oak (*Quercus rubra*), Shumard oak (*Q. shumardii*), and pin oak (*Q. palustris*) and extends the reported host range of *A. galliformis* to include northern red and Shumard oaks.

INTRODUCTION

Northern red (*Quercus rubra*), pin oak (*Q. palustris*) and Shumard oak (*Q. shumardii*) comprise a small, but important component of the urban tree landscape along the eastern urban corridor adjacent to the Rocky Mountains in Colorado. Since the early 2000s, some municipalities within this region have noted sites where all ages of these oak species and their hybrids have experienced significant dieback of unknown origin. The disease incidence has accelerated in some locations. For example, in Boulder which has the largest concentration of northern red oaks on public property in Colorado, trees showing extensive twig dieback throughout the crown more than doubled over three years, from 11% (50/450) in 2012 to 24% (110/450) in 2015. As trees have shown progressive declines, this has also resulted in an increase in tree removals (K. Alexander, personal communication, 2016).

Affected trees initially exhibit leaf scorching and leaf drop followed by dieback of small diameter twigs throughout the canopy (Figure 1.1A, B). As branch cankers form or as twigs die they become brittle and snap off the tree, especially during windstorms. At the point of breakage, new shoot growth often results in small witches' brooms, or twig dieback from successive years may result in major limb dieback. Another feature associated with the disease, particularly in northern red oak, is copious gummosis that drips from cankered, damaged twigs onto sidewalks and parked cars creating a nuisance (Figure 1.1C, D). This combination of symptoms has led to this condition being described as "drippy blight" of red oak.

Initially, it was considered that these symptoms resulted from a species of kermes scale insect, *Allokermes galliformis*, consistently found on damaged branches (Figure 1.1C). Kermes scales are insect gall mimics that develop almost exclusively on oak (Gill 1993). *Allokermes galliformis* has been reported from several *Quercus* species but is most often found in association with pin oak, leading to it sometimes being called "pin oak kermes" (Bullington and Kosztarab 1985).

Kermes scales are generally considered minor pests of oak, occasionally causing some twig dieback and can be associated with excretion of waxy substances (Bullington and Kosztarab 1985). However, the extensive production of gummosis and the presence of small cankers noted regularly at scale feeding sites (Figure 1.1C, D) led to suspicions that there may be pathogen involvement. The objectives of this report are to identify the microbe(s) associated with the twig cankers, establish their potential pathogenicity, and to better understand interactions of pathogens and *A. galliformis* in producing the symptoms of drippy blight disease on red oaks.

MATERIALS AND METHODS

Pathogen identification

Cream-colored bacterial colonies were isolated from canker margins in northern red and pin oaks at several locations in Boulder and Denver, Colorado. For molecular identification of the bacteria, single-spore cultures were grown in nutrient broth for 24 hours on a rotary shaker and genomic DNA was extracted using an Invitrogen Easy-DNA kit (Invitrogen, Carlsbad, CA). The 16S ribosomal DNA was amplified using the methods of Jiang et al. (2006). Amplified products were Sanger-sequenced at Colorado State University's Proteomics and Metabolomics

Facility, and compared to the NCBI nucleotide database using BlastN (Altschul et al. 1990) search in order to identify the pathogen.

To ensure each sequence belonged to *Lonsdalea quercina* subsp. *quercina*, multiple genomic regions of other closely-related species were also used for comparison (Table 1.1). Sequences were retrieved from the NCBI Nucleotide Database, either from individual gene submission (Genbank accession numbers in Table 1.1) or from complete genome sequences (Zhao et al. 2014; Caballero et al. 2014). These were trimmed and aligned using ClustalW (Larkin et al. 2007) and Mega5 (Tamura et al. 2011), respectively. Bayesian phylogenetic analyses were performed in MrBayes using the general time-reversible model with inverse-gamma rates of evolution for 500,000 generations and a burn-in equal to 0.25 (Huelsenbeck and Ronquist 2001). *Erwinia piriflorinigrans* was used as the outgroup.

Pathogenicity studies

One-year-old potted northern red, pin, and Shumard oaks were grown in a shade house at Colorado State University, and used to confirm microbe pathogenicity. Trees were inoculated with a northern red oak isolate of *L. quercina* subsp. *quercina* (NCCB100490) collected in Boulder, Colorado. Two experiments were conducted with different inoculation sites: emerging leaf whorls and one-year old stems. A single colony was streaked onto full strength nutrient agar, incubated for 2 days at 26°C, then diluted in 1mL of sterile water for each inoculation experiment. In the first experiment, five trees of each species were used, and each tree was inoculated three times (n=45). Emerging leaf whorls were mechanically wounded with a 1 cc syringe needle as trees broke dormancy, then 50µl of the $5x10^8$ CFU bacterial suspension was injected between the layered whorls. In a second experiment, three trees of each species were

inoculated three weeks post bud break with 5µl of a bacterial suspension injected into one-year-old stems. Each tree was inoculated twice (n=18). In each experiment, control inoculations using sterile water were also made twice, slightly (3-4 cm) above the inoculation sight, or on a different twig. Each site was wrapped in Parafilm®. The canker lengths were recorded and the pathogen was re-isolated from cankers on nutrient agar five to fifteen days after inoculation.

Data were analyzed using JMP software (11.1.0v; SAS Institute). For each inoculation experiment, a mixed model was fit using canker lesion length as the response variable with tree species as a fixed effect. Tukey's HSD was used to obtain pairwise comparisons between lesion lengths on the different tree species.

Disease description

Three drippy blight diseased trees measuring 50-75 cm diameter at breast height located in Boulder, Colorado parks were felled in December 2010. Approximately 100 twigs <1 cm diameter were collected from each tree, and evaluated for the presence of kermes scales and cankers. In 2015 and 2016, diseased trees throughout Boulder, Colorado were monitored weekly from May to October to document the disease progression.

RESULTS AND DISCUSSION

The red oak (NCCB100490) and pin oak (NCCB100489) isolates of *L. quercina* subsp. *quercina*, shared 100% of nucleotide identity when the four gene sequences were compared (Caballero et al. 2014), and formed a monophyletic clade with *L. quercina* subsp. *quercina* ATCC 29281, based on the multi-locus sequence analysis (Figure 1.2). The Colorado strains are closely related to, but genetically distinct from *L. quercina* subsp. *quercina* ATCC 29281 found in California. Strains are less closely related to *L. quercina* subsp. *iberica* (LMG 26264T, LMG 26265, LMG 26266, R-43277) or *L. quercina* subsp. *britannica* (LMG 26267T, LMG 26268,

LMG 26269, R-43661, LMG 6054). *Lonsdalea quercina* subsp. *iberica* causes bark cankers and drippy bud on holm oak and Pyrenean oak in Spain (*Q. ilex* and *Q. pyrenaica,* respectively) (Biosca et al. 2003; Poza-Carrión 2008), whereas in the United Kingdom *L. quercina* subsp. *britannica* is thought to contribute to acute oak decline of *Q. ruber* and *Q. petrea* (Denman et al. 2012; Brady et al. 2012). Oozing bark cankers on hybrid poplar (*Populus* x *euramericana*) have been attributed to *L. quercina* subsp. *populi* (Li et al. 2014; Tóth et al. 2013).

Following inoculation with *L. quercina* subsp. *quercina*, canker development associated with copious production of bacterial exudates was noted in tested trees involving all three red oak species (northern red, pin, and Shumard), except for one of eight Shumard oak saplings. *Lonsdalea quercina* subsp. *quercina* was consistently re-isolated from canker margins on each oak species. Trees inoculated on leaf whorls exhibited bacterial ooze within five to seven days of inoculation (Figure 1.3A) and small lesions formed as the shoots elongated. Shoot and leaf dieback were also observed. Cankers on red oak were significantly longer than cankers on pin oak (P=0.04) with lengths of 1.02 cm and 0.55 cm, respectively. Shumard oak cankers measured 0.77 cm and did not differ from cankers on northern red or pin oaks (P=0.53 and P=0.35 respectively). Oaks inoculated on stems three weeks post bud break developed bacterial exudates within ten to fifteen days of inoculation, and small cankers formed on stems within 21 days of inoculation (Figure 1.3B). Although the cankers on northern red oak trees averaged 1.25 cm and were over twice as long as cankers on Shumard and pin oak trees, there were no statistical differences in the canker lengths (P=0.12 and P=0.47 respectively). Cankers found on Shumard and pin oak were also similar in size (0.53 cm and 0.57 cm respectively, P=0.76).

In the course of field collections of three northern red oak trees in 2010, *A. galliformis* was found on 70-81 percent of the twigs. Cankers typical of those produced by *L. quercina*

subsp. *quercina* were present at one or more feeding sites of scales on 51-57 percent of the kermes scale infested twigs. Further, cankers were only present at scale insect feeding sites, which strongly supports an interaction between *A. galliformis* and *L. quercina* subsp. *quercina*. This combination of kermes scale feeding and subsequent bacterial canker formation resulted in 30-42 percent twig dieback.

The occurrence of twig cankers associated with the production of large amounts of bacterial ooze from infection with *L. quercina* has not previously been reported. In California, *L. quercina* subsp. *quercina* infections are limited to acorns of coast live oak (*Q. agrifolia*) and interior live oak (*Q. wislizenii*), producing a condition sometimes described as "drippy nut of acorns" because of the copious bacterial ooze manifesting at acorn wound sites (Hildebrand and Schroth 1967).

Although *L. quercina* causes infections of acorns in California (Hildebrand and Schroth 1967), a canker disease of *Populus* in China and Hungary (Li et al. 2014; Tóth et al. 2013), and contributes to oak decline in Britain and Spain (Denman et al. 2012; Biosca et al. 2003; Brady et al. 2012), drippy blight of red oak differs from these previous documentations. Branch dieback, canker formation, twig abscission at the junction to the current season's growth, leaf drop, epicormic branching, and witches' brooms are symptoms associated with this newly described condition. Furthermore, during the spring and summer, bacterial oozing occurs throughout the canopy. Exudates may be so copious that dripping ooze results in large sticky areas on sidewalks and other surfaces under the canopy throughout the middle of the summer. Twig cankers, indicated by maroon discoloration and clear margins (Figure 1.1D), appear in the late summer near kermes scale feeding sites and wounds. If the bacterium is present in the fall, it dries and hardens.

Historically, tree damage accompanying kermes scale infestations was attributed exclusively to the insect feeding (Kosztarab 1996; Turner and Buss 2005; Turner et al. 2005; Pellizzari et al. 2012). In contrast, our results document a situation where the combined activity of a kermes scale insect and a bacterium produce the tree decline symptoms described as drippy blight of red oak (Snelling et al. 2011; Caballero et al. 2014). The exact manner of how these two organisms interact to produce drippy blight disease remains unclear. In drippy nut of acorns the bacterium establishes at wound sites caused by seed feeding weevils, filbertworms, and cynipid gall wasps (Swiecki and Bernhardt 2006). Similarly, in drippy blight of red oak, the wounding associated with kermes scale feeding injuries may provide entry or exit courts for the pathogen. Alternatively, the interaction may be more indirect, where kermes scales act as a stressor to facilitate growth and spread of the pathogen within the host.

Additional outstanding questions on drippy blight disease remain. For example, efforts to manage this disease complex have been disappointing and are complicated by the presence of two causal agents. Although scale effective management programs such as removing scales by hand, pruning and destroying infested materials, treating with horticultural oils, and applying contact or systemic insecticides are likely the best way to control drippy blight disease (Turner et al. 2005; Turner and Buss 2005), studies to quantify the efficacy of control treatments are pertinent to maintaining red oaks in drippy blight diseased regions. If horticultural oils or insecticides are used to control drippy blight disease, monitoring for the most susceptible life stage of *A. galliformis* is necessary to determine the proper timing of applications. The life history of *A. galliformis* is similar to *A. kingii* (Kosztarab 1996; Hamon et al. 1976), but checking for *A. galliformis* egg hatch is pivotal to ensuring that the timing of application targets susceptible life stages (Turner et al. 2005).

Drippy blight disease of red oaks is an emergent disease caused by the interaction between *A. galliformis* and *Lonsdalea quercina* subsp. *quercina*. In addition to a description of this new disease, this report documents a host range expansion of *L. quercina* subsp. *quercina* to northern red oak, Shumard oak, and pin oak and extends the reported host range of *A. galliformis* to include northern red and Shumard oaks.

Table 1.1. Genbank Accession Numbers. The species, isolate number, and genbank accession numbers are provided for the species used to compare the pathogen *Lonsdalea quercina* subsp. *quercina* to closely related Enterobacteriaceae species. The species comparison was based off of several loci including 16S ribosomal RNA, DNA gyrase subunit B genes (*gyr*B), beta subunit of ATP synthase (*atp*D), and translational initiation factor genes (*inf*B) sequences.

Species	Isolate number	16S ribosomal DNA	DNA gyrase subunit B genes (*gyr*B)	Beta subunit of ATP synthase (*atp*D)	Translational initiation factor genes (*inf*B)
Cronobacter sakazakii	CMCC 45402	retrieved from genome: NC_023032	retrieved from genome: NC_023032	retrieved from genome: NC_023032	retrieved from genome: NC_023032
Erwinia piriflorinigrans	CFBP 5882	GQ405203	JF311583	JF311470	JF311696
E. toletana	LMG 24162	FN547375	EU145274	EU145258	EU145290
Lonsdalea quercina subsp. *britannica*	LMG 26267T	JF311442	JF311666	JF311553	JF311779
	LMG 26268	JF311443	JF311667	JF311554	JF311780
	LMG 26269	JF311444	JF311669	JF311556	JF311782
	R-43661	JF311445	JF311671	JF311558	JF311784
	LMG 6054	JF311446	JF311672	JF311559	JF311785
Lonsdalea quercina subsp. *iberica*	LMG 26264T	JF311441	JF311665	JF311552	JF311778
	LMG 26265	JF311439	JF311662	JF311549	JF311775
	LMG 26266	JF311440	JF311663	JF311550	JF311776
	R-43277	JF311438	JF311661	JF311548	JF311774
Lonsdalea quercina subsp. *quercina*	NCCB100490	KX537747	retrieved from genome: JIBQ01000000	retrieved from genome: JIBQ01000000	retrieved from genome: JIBQ01000000
	NCCB100489	KX537748	retrieved from genome: JIBP01000000	retrieved from genome: JIBP01000000	retrieved from genome: JIBP01000000
	ATCC 29281	KX537749	retrieved from genome: JIBO01000000	retrieved from genome: JIBO01000000	retrieved from genome: JIBO01000000

Figure 1.1. Signs and symptoms of drippy blight disease. A) A symptomatic pin oak showing witch's brooms and dieback as a result of drippy blight disease. B) Northern red oak branches exhibiting flagging and dieback from drippy blight disease. C) An adult kermes scale insect (*Allokermes galliformis*, indicated by an arrow) next to bacterial exudates (*Lonsdalea quercina* subsp. *quercina*, indicated by a star). D) Dead (shriveled) and live (round) kermes scales surrounded by bacterial cankers.

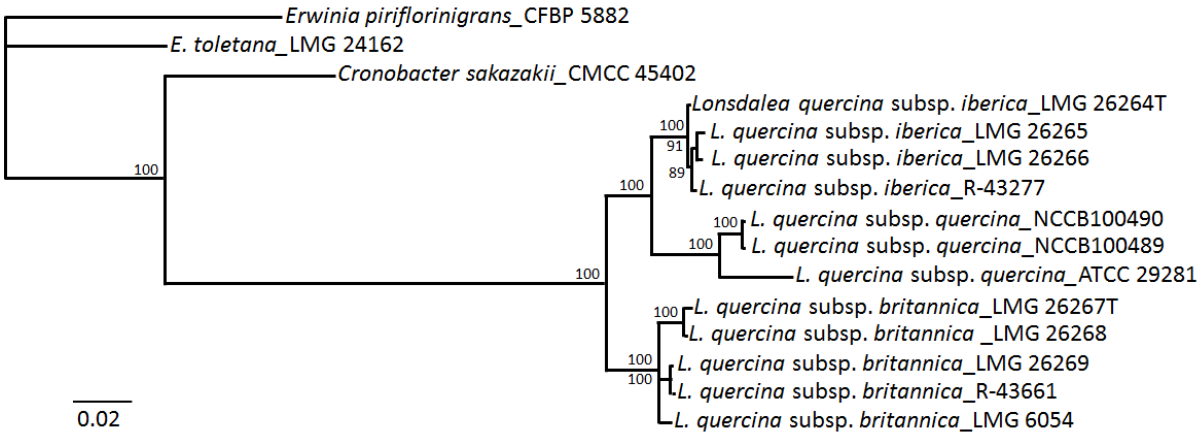

Figure 1.2. Phylogenetic tree of Enterobacteriaceae species based on 16S ribosomal RNA, DNA gyrase subunit B genes (*gyr*B), beta subunit of ATP synthase (*atp*D), and translational initiation factor genes (*inf*B) sequences. A Bayesian analysis was performed for 500,000 generations using a GTR / gamma distributed with invariant sites model of evolution. Bayesian probabilities are shown next to each branch. Species: *Cronobacter sakazakii*; *Erwinia piriflorinigrans* (CFBP 5882); *Lonsdalea quercina* subsp. *iberica* (LMG 26264T, LMG 26265, LMG 26266, R-43277); *Lonsdalea quercina* subsp. *quercina* (red oak isolate NCCB100490, pin oak isolate NCCB100489, ATCC 2981); *Lonsdalea quercina* subsp. *britannica* (LMG 26267T, LMG 26268, LMG 26269, R-43661, LMG 6054); *Pantoea calida*; and *Erwinia toletana*.

Figure 1.3. Inoculation experiment. A) Shumard oak 14 days post leaf whorl inoculation with *Lonsdalea quercina*. Note the bacterial ooze at inoculation sites, indicated by arrows. B) Canker formation in northern red oak on and one year-old stems.

LITERATURE CITED

Alexander, K. Personal -May 4, 2016. E-mail notes. Colorado State University, Fort Collins, CO.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology 215: 403-410.

Biosca, E. G., González, R., López-López, M. J., Soria, S., Montón, C., Pérez-Laorga, E., and M. M. López. 2003. Isolation and characterization of *Brenneria quercina*, causal agent for bark canker and drippy nut of *Quercus* spp. in Spain. Phytopathology 9:485-492.

Brady, C. L., Cleenwerck, I., Denman, S., Venter, S. N., Rodríguez-Palenzuela, P., Coutinho, T, and P. De Vos. 2012. Proposal to reclassify *Brenneria quercina* (Hildebrand and Schroth 1967) Hauben et al. 1999 into a new genus, *Lonsdalea* gen. nov., as *Lonsdalea quercina* comb. nov., descriptions of *Lonsdalea quercina* subsp. *quercina* comb. nov., *Lonsdalea quercina* subsp. *iberica* subsp. nov. and *Lonsdalea quercina* subsp. *britannica* subsp. nov., emendation of the description of the genus *Brenneria*, reclassification of *Dickeya dieffenbachiae* as *Dickeya dadantii* subsp. *dieffenbachiae* comb. nov., and emendation of the description of *Dickeya dadantii*. International Journal of Systematic and Evolutionary Microbiology 62:1592-1602.

Bullington, S., and M. Kosztarab. 1985. Revision of the family Kermesidae (Homoptera) in the Nearctic Region based on adult and third instar females. Virginia Polytechnic Institute and State University Bulletin 85-11 pp. 118.

Caballero, J. I., Zerillo, M. M., Snelling, J., Cranshaw, W., Boucher, C., and N. Tisserat. 2014. Genome sequences of strain ATCC 29281 and pin and northern red oak isolates of *Lonsdalea quercina* subsp. *quercina*. Genome Announcements 2(3):e00584-14.

Denman, S., Brady, C., Kirk, S., Cleenwerck, I., Venter, S., Coutinho, T., and P. De Vos. 2012. *Brenneria goodwinii* sp. nov., associated with acute oak decline in the UK. International Journal of Systematic and Evolutionary Microbiology 62(10):2451-2456.

Gill, R. J. 1993. The scale insects of California. Analysis and Identification Branch, Division of Plant Industry, California Department of Food and Agriculture.

Hamon, A. B., Lambdin, P. L., and M. Kosztarab. 1976. Life history and morphology of *Kermes kingii* in Virginia. Virginia Polytechnic Institute and State University Bulletin 111:1-31.

Hildebrand, D. C., and M. N. Schroth. 1967. A new species of *Erwinia* causing the drippy nut disease of live oaks. Phytopathology 57:250-253.

Huelsenbeck, J. P., and F. Ronquist. 2001. MrBayes: Bayesian inference of phylogenetic trees. Bioinformatics 17:754-755.

Jiang, H., Dong, H., Zhang, G., Yu, B., Chapman, L. R., and M. W. Fields. 2006. Microbial diversity in water and sediment of Lake Chaka, an athalassohaline lake in northwestern China. Applied and Environmental Microbiology 72(6):3832-3845.

Kosztarab, M. 1996. Scale insects of northeastern North America: identification, biology, and distribution. Virginia Museum of Natural History, Martinsville, VA. pp. 650.

Larkin, M. A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., and J. D. Thompson. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23:2947-2948.

Li, Y., He, W., Ren, F., Guo, L., Chang, J., Cleenwerck, I., Ma, Y., and H. Wang. 2014. A canker disease of *Populus* × *euramericana* in China caused by *Lonsdalea quercina* subsp. *populi*. Plant Disease 98:368-378.

Pellizzari, G., Porcelli, F., Convertini, S., and S. Marotta. 2012. Description of nymphal instars and adult female of *Kermes vermilio* Planchon (Hemiptera, Coccoidea, Kermesidae), with a synopsis of the European and Mediterranean species. Zootaxa 3336:36-50.

Poza-Carrión, C., Aguilar, I., Gallego, F. J., Nuñez-Moreno, Y., Biosca, E. G., González, R., López, M. M., and P. Rodríguez-Palenzuela. 2008. *Brenneria quercina* and *Serratia* spp. isolated from Spanish oak trees: molecular characterization and development of PCR primers. Plant Pathology 57(2):308-319.

SAS Institute. 2012. JMP statistics and graphics guide, version 10. SAS Institute, Cary, NC.

Snelling, J., Tisserat, N. A., and W. Cranshaw. 2011. Kermes scale (*Allokermes galliformis*) and the drippy nut pathogen (*Brenneria quercina*) associated with a decline of red oak species in Colorado. Phytopathology 101:S168.

Swiecki, T. J., and E. A. Bernhardt. 2006. A field guide to insects and diseases of California oaks. Gen. Tech Rep. PSW-GTR-197. Albany, CA: Pacific Southwest Research Station, Forest Service, U.S. Department of Agriculture, pp. 151.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28:2731-2739.

Tóth, T., Lakatos, T., and A. Koltay. 2013. *Lonsdalea quercina* subsp. *populi* subsp. nov., isolated from bark canker or poplar trees. International Journal of Systematic and Evolutionary Microbiology 63:2309-2313.

Turner, J. C. L., and E. A. Buss. 2005. Biology and management of *Allokermes kingii* (Hemiptera: Kermesidae) on oak trees (*Quercus* spp.). Journal of Arboriculture 31(4):198-202.

Turner, J. C., Buss, E.A., and A.E. Mayfield. 2005. Kermes scales (Hemiptera: Kermesidae) on oaks. Florida Department Agriculture and Consumer Services, Division of Plant Industry.

Zhao, Z., Wang, L., Wang, B., Liang, H., Ye, Q., and M. Zeng. 2014. Complete genome sequence of *Cronobacter sakazakii* strain CMCC 45402. Genome Announcements 2(1):e01139-13.

CHAPTER 2: LIFE HISTORY OF *ALLOKERMES GALLIFORMIS* (RILEY) (HEMIPTERA: KERMESIDAE) IN COLORADO

SUMMARY

Allokermes galliformis (Riley), sometimes referred to as the "pin oak kermes", has emerged as a significant pest of red oaks grown in the Front Range area of eastern Colorado. Although kermesid scales are reported to infrequently cause significant tree damage, extensive abscission of twigs and branch dieback has been associated with *A. galliformis* on red oaks in Colorado. Apparently, this damage results from feeding injuries created by the insect along with plant infection by an associated bacterium *Lonsdalea quercina* subsp. *quercina*; collectively these two organisms produce a condition known as drippy blight disease of red oaks. To better understand the life history of this insect and identify points where it may be best managed, field studies were conducted in Boulder, Colorado during 2015-2016. *Allokermes galliformis* was confirmed to a have one-year life cycle, with egg eclosion in September and October. Upon eclosion, crawlers migrated to textured places on limbs to overwinter. The preferred overwintering locations were growth rings, bark fissures, callus tissue around wounds, bud scars, branch crotches, on buds, and areas around the female scale venters respectively. In May, a second migration occurred with the great majority of first instar females moving to new growth and the area near growth rings at the base of the current season growth where they permanently settled, becoming sessile plant gall mimics. Immature male scales remained active and ultimately migrated to the trunk or solid surfaces in the near vicinity of the trunk where they produce a cocoon and subsequently emerge as winged forms in early summer. After mating, females began to produce eggs and egg production continued into September. Peak egg production occurred in

mid-August through mid-September and adult females produced an average of 2,488 and 4,726 eggs in 2015 and 2016, respectively.

INTRODUCTION

Kermes scales (Hemiptera: Kermesidae) are a family of gall-like insects that feed primarily on oak twigs and branches throughout the northern continents of the world. In natural ecosystems, predators and parasitoids typically suppress kermes scale populations to levels that rarely cause noticeable damage to their host (Kosztarab 1996; Spodek and Ben Dov 2012; Spodek and Ben Dov 2014). However, in some landscape plantings kermes scale feeding has been shown to cause significant plant injuries in the form of substantial branch dieback that can lead tree decline and in some cases contributes to mortality (Pellizzari et al. 2012; Podsiadlo 2012; Turner and Buss 2005; Turner et al. 2005; Solomon et al. 1980; Hamon et al. 1977).

Allokermes galliformis (Riley) is the most abundant and widespread species of kermes scale in North America; it is present in 34 of the contiguous United States, in addition to being documented in Canada, Japan, and Mexico (Kosztarab 1996; Bullington and Kosztarab 1985; Gill 1993; García Morales et al. 2016). *Allokermes galliformis* reportedly feeds on more than 40 species of oak trees as well as several species of chinquapin, but tentative identifications are common because *A. galliformis* morphology often changes based on its host plant (Kosztarab 1996; Baer and Kosztarab 1985). Despite this, previous identifications of *A. galliformis* are extensive, but information on this species is not detailed (Cockerell 1894; Bullington and Kosztarab 1985; Kosztarab 1996; García Morales et al. 2016). For example, although there are taxonomic descriptions for the second instar male and all stages of the female (Baer and Kosztarab 1985; Bullington and Kosztarab 1985), the adult male is not described and critical information on the life history of *A. galliformis* is lacking (Kosztarab 1996).

The outbreak of *A. galliformis* in Colorado deserves special attention. Not only is *A. galliformis* often present in high abundance in landscape plantings along the urban corridor in Colorado, but it is also associated with development of drippy blight disease of red oaks (Snelling et al. 2011; Ibarra Caballero et al. 2014; Sitz et al. 2017). Feeding injuries by the scale provide wounds that are entry and exit courts for a pathogenic bacterium, *Lonsdalea quercina* subsp. *quercina*. The combination of scale feeding and the bacterial infection exacerbates the impact of typical kermes scale infestations, producing intensified symptomology involving extensive branch dieback and tree decline. For example, in one community experiencing drippy blight disease, approximately 25% of the public red oak tree plantings have been removed in the last decade (Sitz et al. 2017). Due to the increased risk to red oak trees, several municipalities are looking for detailed life history information to identify the most vulnerable life stage of the insect in order to guide best treatment practices for *A. galliformis*. In a disease similar to drippy blight, the incidence of the bacterium *Brenneria quercina* decreased when the efforts were put forth to control the phytophagous insect causing plant damage (Myhre 1988). Similarly, identifying means to best manage the insect partner involved in drippy blight of red oak may be the best way to manage the bacterium. This research aims to (1) describe the life history of *A. galliformis* in Colorado, and (2) provide a more detailed understanding of the overwintering placement and settled feeding locations of kermes scale insects.

MATERIALS AND METHODS

Life history observations

Branch sampling was used to describe overwintering stages of *A. galliformis*. Branch samples were obtained during city tree removals of drippy blight diseased northern red oaks in Boulder, Colorado on 18 December 2014, 12 February 2015 and in the following season on 22

March 2016 and 21 April 2016. Mature trees were used in this survey and ranged in size from 50-75 cm DBH. Sampling focused on the terminal five years of branch growth, which involved branch lengths averaging approximately 50 cm. The number and distribution of Instar I scales were recorded from these branches with specific sites noted including bark fissures, branch crotches, bud scars, buds, growth rings, around venters of dead mother scales, and wounds with callus tissue. A total of 55 branches were examined in 2014-2015 and 42 in 2015-2016.

Additional life history observations continued throughout the year in 2015 and 2016 at two sites in Boulder, Colorado (40°01'02.4''N, -105°27'04.9''W and 40°01'72.9''N, -105°25'99.0''W). Further recording of scale distribution occurred following the second migration of the scales in spring when Instar I and Instar II stages were present (7 and 27 May 2015 n=23 and 18 May 2016 n=30). Samples included the terminal three years of branch growth, averaging approximately 20 cm in length, and the number and distribution of Instar I and II scales were recorded.

Subsequent collections were made through mid-October involving weekly collections of approximately 50 female specimens to determine female development. From late July through mid-October an additional 50 female scales were collected and cut open weekly to document the timing of egg development (2015 n=332, 2016 n=257). In September, estimates of total egg production, based on numbers of eggs or residual exuvium of hatched eggs, were conducted in September 2015 and 2016 (2015 n=19, 2016 n=19). During the winter, venters remaining on the trees were opened to determine the percent of females that successfully produced offspring (2015 n=229, 2016 n=167).

Histological sectioning was used to visualize the feeding damage incurred by kermes scales. In August of 2015, two samples of adult females feeding on two-year-old branch tissue

were fixed, embedded, and sectioned according to Womack et al. (2016). Briefly, the woody samples were dehydrated through weeklong soaks (Ruzin 1999) in each grade of ethanol (30%, 50%, 70%, 90%, 95%, 100%, and 100%), then embedded in hydroxypropyl methacrylate (HPMA) plastic (Electron Microscopy Sciences, Hatfield, PA, USA), and sectioned at a 5µm thickness using a microtome (RM1265, Leica, Wetzlar, Germany). Every other section was mounted onto Fisher Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA, USA) and stained using Eosin and Toluidine Blue (Fisher Scientific).

In June and July of both 2015 and 2016 targeted collections were made of approximately 500 cocoons containing maturing males on the main trunk. Collections were made using an aspirator and the insects were then held in the lab to record emergence of adult male scales and parasitoids.

Statistical analysis

To determine differences in overwintering and migration locations of first instar scales, mixed effects models were fit using JMP software (11.1.0v; SAS Institute). Log of scale insect number was the response variable in two models to determine first instar abundance at (1) overwintering locations, and (2) settled feeding locations. To account for blocking in the experimental design, tree and branch were random effects. In the first model (overwintering locations), branch age and branch location were considered fixed effects. In the second model (settled feeding location), branch location was a fixed effect. Pairwise comparisons were obtained for both models using a Tukey HSD method, but the data were separated by year.

RESULTS AND DISCUSSION

Allokermes galliformis exhibited a univoltine life cycle (Figure 2.1) that for females involves three nymphal instars (Figure 2.2A-C) and an adult (Figure 2.2D). Males were observed

to produce two nymphal male instars, a male "pupal stage" within a cocoon, and an adult male (Figure 2.3A-B). Typical of Kermesidae, *A. galliformis* displayed extreme sexual dimorphism in the adult stage; females develop into large wingless insects that mimic twig galls while males are minute and winged (Gullan and Kosztarab 1997).

Eclosion started in September and lasted through October, when the rust-colored Instar I crawlers moved to protective crevices on the bark of branches to overwinter (Figure 2.1). *Allokermes galliformis* spent approximately eight months as crawlers (Figure 2.1) migrating twice during this time. Male and female crawlers are not sexually dimorphic like the other life stages, and therefore cannot be discerned from one another. Crawlers of *A. galliformis* present in the upper canopy on smaller branches may be female as was documented with the related species *Allokermes kingii* (Hamon et al. 1976).

The distribution of overwintering crawlers was affected by the age of the branch material the crawlers settled on. Overwintering occurred on wood of various ages with an average of 10% noted on five-year-old wood, 33% on four-year-old wood, 32% on three-year-old wood, 19% on second year wood, and 5% on wood of the previous season (Table 2.1). Furthermore, in 2014-2015 first instars overwintered significantly more on older, two- to three-year-old branch growth (2011-2013) than one-year-old branch growth (2014) (Table 2.1; P=0.0105, P<0.001, and P=0.0021 for 2014 compared to 2011, 2012, and 2013 respectively). In 2015-2016 the same trend occurred, but preferences were not significant (Table 2.1; P=0.1006). There was large variation in the abundance of *A. galliformis* between the two study seasons, with more than ten times as many crawlers detected per branch in 2015 (129 ± 67) than in 2016 (10 ± 6) (P<0.001). Despite this variation, the trend for overwintering crawlers to avoid the most recent growth was

consistent across sampling years (Table 2.1). Very few crawlers were observed to settle on branch material older than five years, and large branches were not evaluated.

There were also clear patterns as to the sites within the branch regions where scales overwintered (Table 2.2). Across both years, an average of 35% were observed on the growth rings and 26% were found at bark fissures of the samples from the two years, together accounting for more than half of the scales' settling sites. Less common sites for overwintered scales were wound areas with callus tissue (17%), bud scars (13%), branch crotches (6%), around old female scale venters (2%), and on buds (1%).

After overwintering, a second migration occurs following bud break in May. Across both years, a large majority of female first instars migrated to new growth (86% and 97% in 2015 and 2016 respectively) and either resettled around the new growth ring (29% and 45% in 2015 and 2016 respectively) or current season growth (62% and 49% in 2015 and 2016 respectively) (Table 2.3). Only a small percentage (9% and 6% in 2015 and 2016 respectively) remained on older growth. Molting to Instar II subsequently occurred at these sites, and females appeared the orange color of bud scales (Figure 2.2A-B). Instar II females and all later stages were sessile and increasingly came to resemble a plant gall mimics. Differences in populations of scales on plants between the two seasons continued through Instar II, with almost five-fold more scales noted per branch in 2015 (52 ± 5) than 2016 (11 ± 5) (P<0.001). In both years, the great majority of female scales ultimately settled around the new growth ring or on the current season growth. Later, branch breakage and dieback was often observed to at these sites.

Instar II and each successive female life stage, lasted persisted for one to two months (Figure 2.1). The majority of Instar II females were present in mid to late June, and the majority of Instar III females occurred in early and mid-July. Instar III and early adult females turned

maroon to brown in color (Figure 2.2C). Adult females collected in August and histologically sectioned were found to produce feeding injuries that extended into the cortex of the branch (Figure 2.4).

Some Instar I crawlers were observed overwintering on the trunk of the tree. This was a habit noted to occur with males of *A. kingii* (Hamon et al. 1976), and it is likely that some males of *A. galliformis* similarly move to trunks at this time. Males of *A. galliformis* were short-lived compared to females, and the pre-pupae and adults were only present in July (Figure 2.1). In early and mid-July, pre-pupae created white waxy pupal cocoons (Figure 2.3A) which were most abundant on the trunk, but also frequently found on rocks, mulch, fencing, and plants or debris around the base of the tree. Pupal cases were documented up to six feet away from the base of the tree. From mid-July to August, males developed into the adult stage and were tan in color with clear to iridescent wings (Figure 2.3B). The adults are weak fliers (Hanks and Denno 1998) with one pair of wings and hamulohalteres, and lack functional mouthparts.

The peak time for reproductive female development is from mid-August through mid-September, and post-reproductive females were easily identifiable because their protective venter turned tan with three rows of dark spots (Figure 2.2D). During the middle of August female scales began to develop eggs which largely packed the interior of the venter. Egg-laying was complete by late October and the eggs began to hatch mid-September.

Examination of the content of female venters remaining on the tree in the winter indicated either egg exuvium produced by viable eggs that had hatched, or dead eggs, barren venters, and/or a white powder indicative of non-viable offspring. In total, an average of 65% of adult females survived to produce viable offspring. Of these, females produced an average of 2,488 ± 592 eggs in 2015 (range 43-7,210) and 4,726 ± 701 eggs in 2016 (range 1,400-9,850).

These levels of egg production are consistent with that reported by other *Allokermes* species. For example, Hamon et al. (1975) reported an average of 2,820 eggs being produced by *A. kingii*. The highest number of eggs recorded from *A. galliformis* (9,850) exceeded the record of 6,676 eggs reported by Himebraugh (1904) from a female of *Allokermes gillettei* Cockerell collected in Manitou, Colorado.

Although scale populations fluctuated during this study, and were lower in 2015-2016, *A. galliformis* was still present in high densities in both years. In Colorado, it was common to see groups of ten to fifteen individuals feeding in one location. Even though *A. galliformis* occurred in sustained high population, there was little evidence observed of effects from natural enemies. Some lady beetles and scavenging wasps were seen on infested trees, but rather than showing predatory behavior, they were observed feeding on scale excrement and bacterial exudates. No parasitism of females was observed, but approximately 5% of male scales were parasitized in the pre-pupae stage by a minute wasp. On three separate occasions during the experiment, a predatory thrips species was collected inside newly exited venters which may have fed on developing eggs. Also, in about 5% of female scales the body contents were reduced to a white, powdery substance suggestive of pathogen infection. The absence of natural enemies of *A. galliformis* in Colorado, and the relatively high populations of the insect that occur in contrast to areas where the insect and host are native, suggests that an exploration of natural enemies in areas of origin and their introduction into Colorado may be valuable for long-term management of *A. galliformis* in the state.

Although the life history of *A. galliformis* is similar in some respects to *A. kingii*, the only other kermesid scale in North America that has been similarly studied, there are important differences. Geographic location and climate impact the life history of kermes scale insects. For

example, *Allokermes kingii* exhibits a one-year life cycle in most of its range (Hamon et al. 1976), but in warmer regions *A. kingii* can exhibit a bivoltine life cycle (Turner and Buss 2005). Where a univoltine life cycle occurs, the period in which the successive life stages are present in Colorado are delayed by approximately one month compared to that univoltine life cycle of *A. kingii* in Virginia (Kosztarab 1996). Furthermore, *A. galliformis* female life stages were commonly observed throughout one or two months during the summer and showed more overlap in life stages than *A. kingii*.

These studies have identified specific sites where *A. galliformis* can be found during its development and plant tissues it feeds on, which can be useful in targeting treatments for its management. *Allokermes galliformis* was most commonly found overwintering around growth rings and other textured areas on the most recent five years of growth. Upon bud break in the spring, Instar I demonstrated a second migration where these individuals began feeding on the new growth and became sessile gall-mimics. Their feeding damage was observed in the cortex. Importantly, the information learned about *A. galliformis* gives insight unto the management of this and other kermes scales species.

Table 2.1. The number of first instar *Allokermes galliformis* (mean ± standard error) that settled on the most recent five years of branch growth. Four trees were evaluated each winter (2014-2015 and 2015-2016). All branch material was obtained from trees located on public grounds in Boulder, Colorado. Means followed by the same letter in a row are not statistically different ($P<0.05$, Tukey HSD).

		Year of Branch Growth					
Year Sampled	***no.*[1]**	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**
2014-2015	55	1.83±0.28ab	2.67±0.51a	2.65±0.39a	2.36±0.37a	0.82±0.12b	.
2015-2016	42	.	0.30±0.08a	0.22±0.04a	0.27±0.06a	0.36±0.06a	0.19±0.04a

[1]Number of branches counted

Table 2.2. The number of first instar *Allokermes galliformis* (mean ± standard error) that overwintered on red oak branches (in either bark fissures, bud scars, branch crotches, wounds with callus tissue, around venters, buds, and growth rings). Five years of branch growth was evaluated each winter (2014-2015 and 2015-2016). All branch material was obtained from trees located on public grounds in Boulder, Colorado. Means followed by the same letter in a row are not statistically different ($P<0.05$, Tukey HSD).

Year	***no.*[1]**	**Bark Fissures**	**Bud Scars**	**Branch Crotches**	**Wounds / Callus**	**Around Venters**	**Buds**	**Growth Rings**
2014-2015	55	3.97±0.83b	1.67±0.20b	0.88±0.22bc	2.34±0.37b	0.49±0.13c	0.10±0.05c	4.99±0.48a
2015-2016	42	0.30±0.08b	0.14±0.03b	0.14±0.05b	0.15±0.4b	0±0b	0.01±0.01b	1.14±0.14a

[1]Number of branches counted

Table 2.3. The number of immature *Allokermes galliformis* (mean ± standard error) that migrated onto either new growth, current season growth ring, or previous growth on the most terminal 20 cm of red oak branch material. Immatures were counted in the spring on branches with (2015 n=45 and 2016 n=30). All branch material in 2015 was obtained from two trees located on public grounds in Boulder, Colorado, and all branch material in 2016 was collected from five red oak trees on University of Colorado, Boulder grounds. Means followed by the same letter in a row are not statistically different (P<0.05, Tukey HSD).

Year	*no.*[1]	Previous Growth	Current Season Growth Rings	New Growth
2015	23	4.55±0.86b	16.13±2.51a	16.21±2.32a
2016	30	0.24±0.13c	6.83±1.16a	4.00±1.28b

[1]Number of branches counted

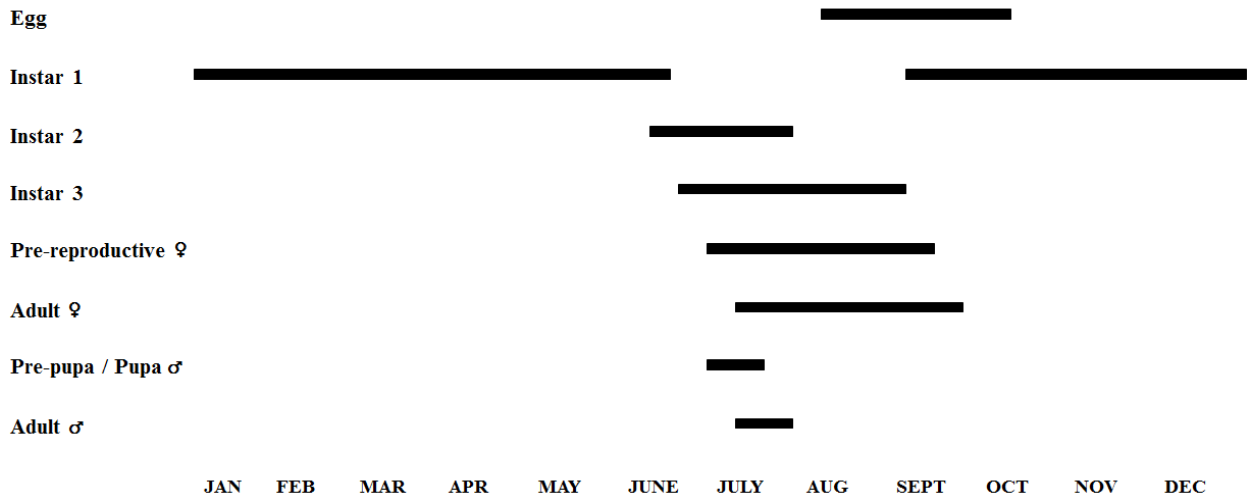

Figure 2.1. Seasonal life history of *Allokermes galliformis* obtained from drippy blight diseased Northern red oak (*Quercus rubra*) from parks in Boulder, Colorado throughout 2015 and 2016.

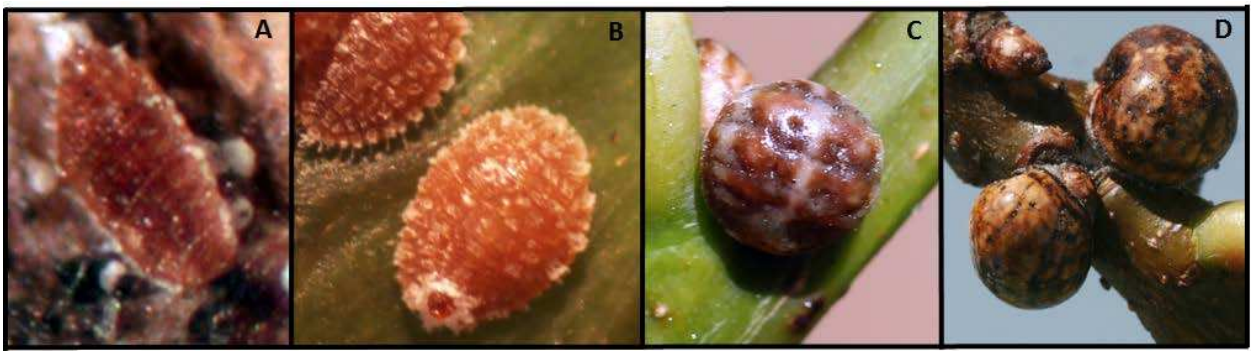

Figure 2.2. Life stages of female *Allokermes galliformis* including (A) first instar, (B) second instar, (C) third instar, and (D) post-reproductive adult female.

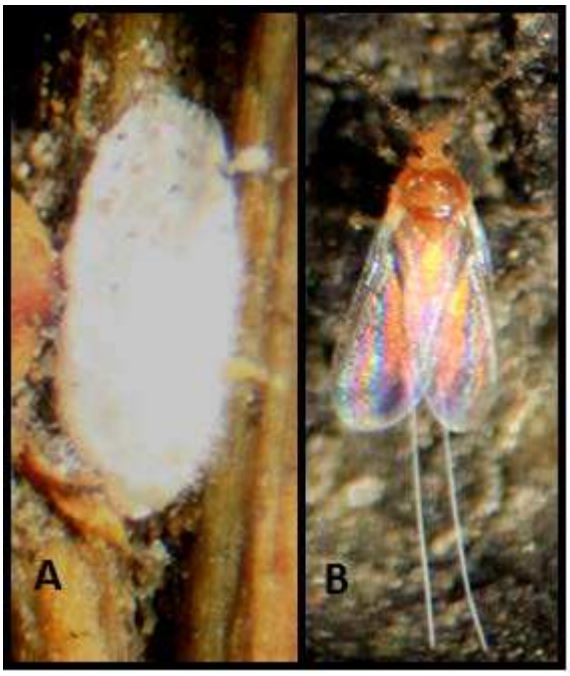

Figure 2.3. *Allokermes galliformis* male (A) pupal case, and (B) adult life stages.

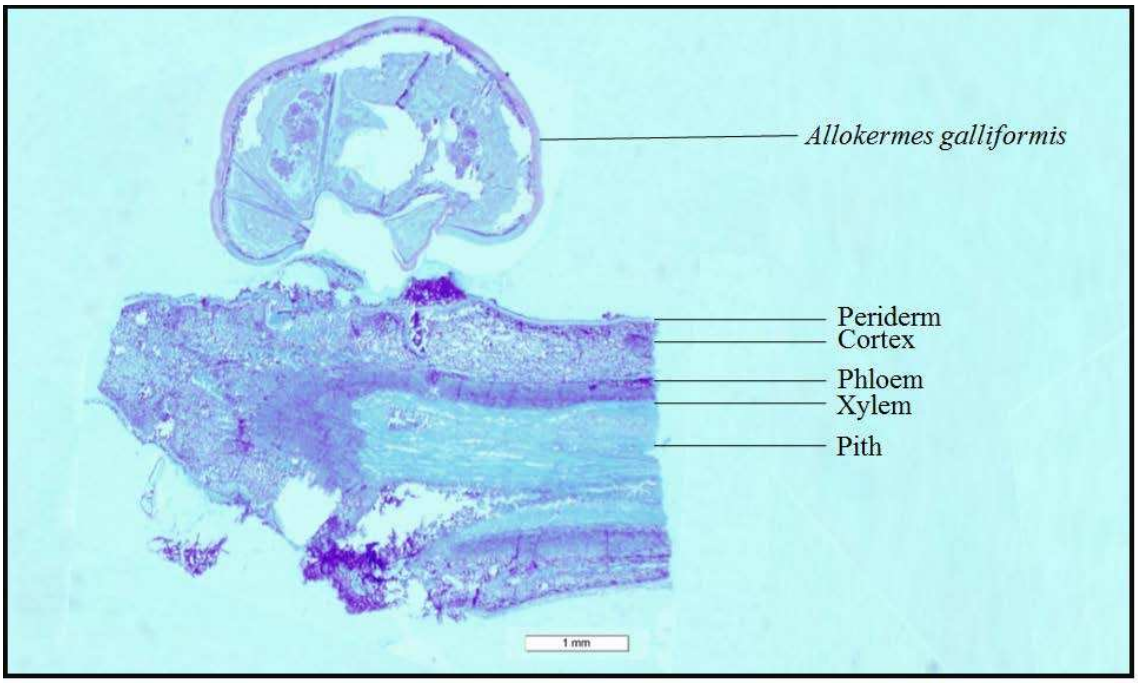

Figure 2.4. Histological section of a female *Allokermes galliformis* feeding on red oak.

LITERATURE CITED

Bae, C., Han, S. W., Song, Y. R., Kim, B. Y., Lee, H. J., Lee, J. M., Yeam, I., Heu, S., and C. S. Oh. 2015. Infection processes of xylem-colonizing pathogenic bacteria: possible explanations for the scarcity of qualitative disease resistance genes against them in crops. Theoretical and Applied Genetics 128(7):1219-1229.

Baer, R. G., and M. Kosztarab. 1985. A morphological and systematic study of the first and second instars of the family Kermesidae in the Nearctic region (Homoptera: Coccidea). Virginia Polytechnic Institute and State University Bulletin 85-11:119-261.

Bullington, S., and M. Kosztarab. 1985. Revision of the family Kermesidae (Homoptera) in the Nearctic Region based on adult and third instar females. Virginia Polytechnic Institute and State University Bulletin 85-11:1-118.

Caballero, J. I., Zerillo, M. M., Snelling, J., Cranshaw, W., Boucher, C., and N. Tisserat. 2014. Genome sequences of strain ATCC 29281 and pin and northern red oak isolates of *Lonsdalea quercina* subsp. *quercina*. Genome Announcements 2(3):e00584-14.

Cockerell, T. D. A. 1894. A check list of Nearctic Coccidae. The Canadian Entomologist 26:31-36.

García Morales M., Denno, B. D., Miller, D. R., Miller, G. L., Ben-Dov, Y., and N. B. Hardy. 2016. *ScaleNet:* A literature-based model of scale insect biology and systematics. Database. doi: 10.1093/database/bav118. http://scalenet.info.

Gill, R. J. 1993. The scale insects of California. Analysis and Identification Branch, Division of Plant Industry, California Department of Food and Agriculture.

Gullan, P. J., and M. Kosztarab. 1997. Adaptations in scale insects. Annual Review of Entomology 42(1):23-50.

Hamon A. B. 1977. Gall-like scale insects (*Kermes* spp.) (Homoptera, Coccoidea, Kermesidae). Entomology Circular 178:1-2.

Hamon, A. B., Lambdin, P. L., and M. Kosztarab. 1975. Eggs and wax secretion of *Kermes kingii*. Annals of the Entomological Society of America 68:1077-1078.

Hamon, A. B., Lambdin, P. L., and M. Kosztarab. 1976. Life history and morphology of *Kermes kingii* in Virginia. Virginia Polytechnic Institute and State University Bulletin 111: 1-31.

Hanks, L. M., and R. F. Denno. 1998. Dispersal and adaptive deme formation in sedentary coccoid insects. Pp. 239-262. *In* Mopper, S.Y., and S. Strauss (eds.), Genetic structure and local adaptation in natural insect populations: effects of ecology, life history, and behavior. Chapman & Hall, New York, 1998.

Himebraugh, L. C. 1904. The eggs of *Kermes gillettei* Cockerell (family Coccidae). Entomological News 15:188.

Kosztarab, M. 1996. Scale insects of northeastern North America: identification, biology, and distribution. Virginia Museum of Natural History, Martinsville, VA, 650 pp.

Myhre, E. A. 1988. Effects of *Lygus* spp. (Hemiptera: Miridae) on Mediterranean white lupin (*Lupinus albus*). M.S. Thesis. Entomology, Washington State University, Pullman, WA.

Pellizzari, G., Porcelli, F., Convertini, S., and S. Marotta. 2012. Description of nymphal instars and adult female of *Kermes vermilio* Planchon (Hemiptera, Coccoidea, Kermesidae), with a synopsis of the European and Mediterranean species. Zootaxa 3336:36-50.

Podsiadlo, E. 2012. Morphology of second instar nymphs of *Kermes quercus* (Linnaeus) (Hemiptera: Kermesidae). Polskie Pismo Entomologiczne 81:35-42.

Ruzin, S. E. 1999. Plant microtechnique and microscopy. Vol. 198. Oxford University Press, Cambridge.

SAS Institute. 2012. JMP statistics and graphics guide, version 10. SAS Institute, Cary, NC.

Sitz, R. A., Zerillo, M. M., Snelling, J., Caballero, J. I, Alexander, K., Nash, K., Tisserat, N. A., Cranshaw, W.S., and J. E. Stewart. 2017. Drippy blight, a disease of red oaks in Colorado produced from the combined effect of the scale insect *Allokermes galliformis* and the bacterium *Lonsdalea quercina* subsp. *quercina.* Accepted manuscript, Journal of Arboriculture and Urban Forestry.

Snelling, J., Tisserat, N. A., and W. Cranshaw. 2011. Kermes scale (*Allokermes* sp.) and the drippy nut pathogen (*Brenneria quercina*) associated with a decline of red oak species in Colorado. Phytopathology 101:S168.

Solomon J. D., McCracken, R. L., Anderson, R., Lewis Jr, Oliveria, T. H., and P. J. Barry. 1980. Oak pests: A guide to major insects, diseases, air pollution and chemical injury. General Report SA-GR11. U.S. Department of Agriculture, Washington, DC.

Spodek, M., Ben-Dov, Y., and A. Protasov. 2012. Taxonomy of *Kermes greeni* Bodenheimer (Hemiptera: Coccoidea: Kermesidae) with a new synonymy. Zootaxa 3545(1):67-75.

Spodek, M., and Y. Ben-Dov. 2014. A taxonomic revision of the Kermesidae (Hemiptera: Coccoidea) in Israel, with a description of a new species. Zootaxa 3781(1):1-99.

Turner J. C. L., and E. A. Buss. 2005. Biology and management of *Allokermes kingii* (Hemiptera: Kermesidae) on oak trees (*Quercus* spp.). Journal of Arboriculture 31(4):198-202.

Turner, J. C., Buss, E. A., and A. E. Mayfield. 2005. Kermes scales (Hemiptera: Kermesidae) on oaks. Florida Department Agriculture and Consumer Services, Division of Plant Industry.

Womack, M. C., Christensen-Dalsgaard, J., and K. L. Hoke. 2016. Better late than never: effective air-borne hearing of toads delayed by late maturation of the tympanic middle ear structures. Journal of Experimental Biology 219(20):3246-3252.

CHAPTER 3: INSECTS VISITING DRIPPY BLIGHT DISEASED RED OAK TREES ARE CONTAMINATED WITH THE PATHOGENIC BACTERIUM *LONSDALEA QUERCINA* SUBSP. *QUERCINA*

SUMMARY

In this study, the potential breadth of insect dissemination of the bacterial causal agent of drippy blight disease, *Lonsdalea quercina* subsp. *quercina,* was investigated. Arthropod specimens were collected and tested for the presence of the bacteria with molecular markers. The presence of *L. quercina* subsp. *quercina* was confirmed on 12 different samples from three insect orders (Coleoptera, Hemiptera, and Hymenoptera), and from eight insect families (Buprestidae, Coccinellidae, Dermestidae, Coreidae, Pentatomidae and/or Miridae, Apidae, Formicidae, and Vespidae), and approximately half of the insects found to carry the bacterium were in the order Hymenoptera. Estimates of the insects that are contaminated with the bacterium and likely carry it between trees is conservative, because the documented insects represent only a subset of the insect orders that were observed feeding on the bacterium or on diseased trees. The insects contaminated with *L. quercina* subsp. *quercina* exhibit diverse life histories, and have commensal relationship with the bacterium. These findings demonstrate that a diverse set of insects that naturally occur on diseased trees likely play a strong role in disseminating the bacterial causal agent of drippy blight disease of red oaks.

INTRODUCTION

Commensal bacterial-insect associations are involved with at least 14 tree diseases, and some are among the most economically important, yet these associations remain largely understudied (e.g. Aleppo pine knot, crown gall, apple rot, walnut blight, fire blight, citrus canker) (Calamassi et al. 2008; Chagas et al. 2001; Harris and Maramorosch 1980; Mansfield et

al. 2012; Nadarasah and Stavrinides 2011). In commensal bacterial-insect associations, bacteria can benefit from the presence of insects in two main ways: insects can become externally contaminated and transfer phytopathogenic bacteria to a new host, or they can create conditions such as feeding wounds that act as entry courts for bacterial pathogens. These two mechanisms are not mutually exclusive (Calamassi et al. 2008), but are often studied independently. In the well-studied system of fire blight, insects present in orchards are commonly contaminated with the bacterium (Hildebrand et al. 2000). Commonly, insects feed at overwintering cankers then later visit and infect flower blossoms (Thomas and Ark 1934) where the the bacterium may multiply in the nectar and be further spread spread by pollinators (Vanneste 2000; Thomson 1992). Second, insect feeding wounds intensify the occurrence of disease by providing a mode of entry and in some cases optimal microclimates for the commensal bacterium in diseases including citrus canker, crown gall, and other bacterial galls of trees (Chagas et al. 2001; Granovsky 1940; Hansen and Smith 1937). In one situation with citrus leafminer (*Phyllocnistis citrella* Stainton), wounds produced by the insect enhance citrus canker infections by *Xanthamonas axonopodis* pv. *citri* but the insect is not deemed important in the dissemination of the bacterium (Chagas et al. 2001; Belasque et al. 2005).

Several subspecies of *Lonsdalea quercina* subsp. *quercina* have been documented near wounds caused by mobile insects that are suspect of movement of the pathogen (Hildebrand and Schroth 1967; Swiecki and Bernhardt 2006; Biosca et al. 2003; Denman et al. 2012; Li et al 2014; Izquierdo and Pulido 2013; Denman et al. 2014; Sitz et al. 2017). In addition, insect presence has long been noted in diseases caused by tree pathogens related to *L. quercina* including genera such as *Brenneria* (Myhre 1988; Lu et al. 2010; Denman et al. 2014; Denman et al. 2016) and *Erwinia* (Thind and Singh 1976; van der Zwet and Keil 1979; Dalmacio et al.

2007). Notably, over 77 insect genera have been documented in the dissemination of the bacterial causal agent of fire blight, *Erwinia amylovora* (van der Zwet and Keil 1979).

Although early studies of commensal phytopathogenic bacterium-insect associations attributed these relationships to chance or accident (Harris and Maramorosch 1980), recent studies have shown that phytopathogenic bacteria can actively recruit insect vectors through volatile cues (Lauzon et al. 1998; Orlovskis et al. 2015). For example, acetoin (3-hydroxy-2-butanone) is produced by plant pathogenic enterobacters in an anaerobic energy-acquiring pathway that breaks down glucose and other fermentable carbon sources (White and Starr 1971;Xiao and Xu 2007), and it is speculated that the acetoin produced by plant pathogenic bacteria is attractive to many insects (del Pilar Marquez-Villavicencio et al. 2011). Acetoin is also well known as an insect pheromone, pheromone synergist, and general attractant (del Pilar Marquez-Villavicencio et al. 2011; Rochat et al. 2002; Saïd et al. 2005; Tolasch et al. 2003; Bengtsson et al. 2009; Buttery et al. 1984; Landolt et al. 2014; Nout and Bartlet 1998; Phelan and Lin 1991).

In drippy blight disease of red oaks, a kermes scale insect, *Allokermes galliformis* (Riley), produces feeding wounds that may then be colonized by the bacterium, *Lonsdalea quercina* subsp. *quercina* first reported as *Erwinia quercina* then *Brenneria quercina* (Hildebrand and Schroth 1967; Biosca et al. 2003; Hauben et al. 1998; Brady et al. 2012; Poza-Carrión 2008). Following host infection, the bacteria exude from these feeding sites (Snelling et al. 2011; Caballero et al. 2014; Sitz et al. 2017). Drippy blight represents an ideal system to study both aspects of commensal phytopathogenic bacterium-insect associations, including the role of wounding and external contamination.

Although *A. galliformis* is associated with production of drippy blight disease, it is a sessile insect for much of its life (Kosztarab 1996) with habits that are not well adapted for dispersal of the bacterium. However, the host plant of the scale, certain oaks, are keystone species throughout North American forests and well known to host a large suite of insects that could be involved in the dissemination of *L. quercina* (García Morales et al. 2016; Fralish 2004). The primary objective of this study was to determine whether insects associated with drippy blight infected oaks become contaminated with *Lonsdalea quercina* subsp. *quercina* under field conditions. Also considered is whether this bacterium may produce the volatile insect attractant acetoin, which may play a role in mobile insects visiting *L. quercina* subsp. *quercina*.

MATERIALS AND METHODS

Study site and experimental design

This study was conducted from May through mid-October in 2015 and 2016 on drippy blight-infected *Quercus rubra* and *Q. palustrus* at the University of Colorado main campus in Boulder, Colorado (2015 *Q. rubra*: 40°01’02.4’’N, -105°27’04.9’’W; 2015 *Q. palustrus*: 40°00’37.0’’N, -105°26’72.8’’W; 2016 *Q. rubra*: 40°00’44.9’’N, -105°26’93.6’’W; 2016 *Q. palustrus*: 40°00’38.1’’N, -105°26’32.9’’W). All insects observed feeding on scale and bacterial exudates from drippy blight diseased trees throughout Boulder, Colorado were recorded. Observations occurred between 8 am and 4 pm weekly. On one occasion, observations occurred between 8 and 10 pm.

Insect specimens collected for testing were identified to order, and further if possible, whereas other Arthropods were identified to class. Two methods to collect specimens and detect the bacterium on arthropod samples were used, including testing for the bacteria with DNA extraction techniques and colony PCR. DNA extraction techniques are limited due to the

difficulty of penetrating hardened insect exoskeletons or encountering inhibitors (Rubin et al. 2014; Hildebrand et al. 2000) whereas colony PCR techniques are time sensitive as faster colonies can overtake slow growing bacteria. To collect samples for DNA extractions, multiple funnel traps were placed in diseased oak trees and checked in two-week intervals throughout the field season. Collection cups were stored at 4°C for up to four days after the collection date, and once identified stored at -20°C until used for DNA extractions. To collect samples for colony PCR, any live arthropods seen on diseased oak trees were captured in sterile collection tubes. Collections occurred once each year (3 September 2015 and 18 July 2016). These specimens were plated on nutrient agar, and the resulting bacterial colonies were used for *L. quercina* subsp. *quercina* detection.

Detection of *Lonsdalea quercina* subsp. *quercina*

To establish whether the arthropods collected from funnel traps then tested with DNA Exraction methods were carrying *L. quercina* subsp. *quercina* either externally or internally, adult female kermes scales visibly contaminated with bacterial exudates were used as a control. DNA was extracted using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA) following the manufacturer's protocol. In some cases, the desired sample weight was obtained by pooling insects in the same order (Table 3.1). In addition to insects collected from the canopy of diseased trees, male kermes scales were tested. Male scales collected as pupae from the trunk of diseased trees were reared into adults, and then 2 individuals and 2 samples with approximately 30 pooled individuals were tested for the bacterium.

For PCR, reaction mixtures contained 20 ng of template DNA (or no DNA template for negative control), 2 µl 10x Standard *Taq* Reaction Buffer (New England BioLabs Inc., Ipswich, MA), 0.4 µl 10 mM dNTPs (Roche Applied Science, Indianapolis, IN), and 1 µl each of 10 µM

primer and 0.2 µl *Taq* DNA Polymerase (New England BioLabs Inc., Ipswich, MA) (total 20 µl). Two primers developed by Shang et al. (2014) to target the flagellar transcriptional regulator (flhD) and DNA gyrase subunit B (gryB) were used to amplify *L. quercina* subsp. *quercina*. Amplifications were performed using the following PCR conditions: 94°C for 3 min, 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, then finally 72°C for 7 min. All PCR reactions were conducted using a MJ PTC-200 thermocycler (Bio-RadLaboratories, Waltham, MA).

Amplified DNA was confirmed with a Sub-Cell GT Wide Mini electrophoresis system (BioRad, Hercules, CA, USA) that yielded a single band (382 kb in length for flhD or 286 kb in length for gryB). PCR products were cleaned using a High Pure PCR Product Purification Kit (Roche Life Science, Indianapolis, IN). Ten ng of amplified DNA per 100 base pairs and 25 pmol of primer were sequenced at Genewiz. Chromatograms were visualized, and sequences were joined to make a consensus and manually edited using Geneious (Kearse et al. 2012). To confirm pathogen presence, *L. quercina* subsp. *quercina* primers and quality nucleotide sequences were first compared against the entire NCBI non-redundant database, and then to the NCBI whole genome shotgun database using the genus *Lonsdalea* as the organism (taxid: 1082702) (Altschul et al. 1990).

After plating insects associated with red oaks, colony PCR was conducted on cream-colored bacterial colonies were tested to determine if *L. quercina* subsp. *quercina* was externally contaminating the insects. Bacterial colonies were lysed at 95°C for 5 min and then used in PCR, electrophoresis, sequencing, and identification as described for DNA extraction methods.

Prediction of *Lonsdalea quercina* subsp. *quercina* synthesis of the volatile compound acetoin

The *L. quercina* subsp. *quercina* genome was annotated using PATRIC and three acetoin reductase enzymes were predicted which are known to reduce 2,3-butanediol into acetoin

(Heidlas and Tressl 1990). To determine their similarity to other acetoin reductase enzymes, sequences were compared against the entire NCBI non-redundant protein sequences database (Gish and States 1993).

RESULTS

Detection of *Lonsdalea quercina* subsp. *quercina*

Individual specimens (ca. 257) representing three arthropod classes, six insect orders, and 28 insect families were collected from drippy blight infected oaks (Table 3.1). Of the various arthropods tested across the two collection seasons, 235 individual specimens were grouped into 115 samples and tested for the bacterium using DNA-extraction techniques, and twenty-two individual samples were tested using colony PCR techniques (Table 3.1). Confirmation of bacterial contamination was performed by nucleotide sequence searches in NCBI that resulted in *L. quercina* subsp. *quercina* as the most similar hit.

Overall, 12 samples from three insect orders (Coleoptera, Hemiptera, Hymenoptera) were contaminated with *L. quercina* subsp. *quercina* (Table 3.1). *Lonsdalea quercina* subsp. *quercina* was confirmed in seven out of the 115 samples tested using DNA extraction techniques (Table 3.1), where approximately 5% of these samples carried the bacterium each year (Table 3.1). Six out of the 22 insects tested using colony-PCR tested positive for the bacterium (Table 3.1). In both sampling years, approximately 25% of the insects collected for colony-PCR tested positive for the bacterium (Table 3.1), and approximately half were contaminated in July. In addition, sequences were comparable in length (97% to 100% coverage) and similarity (95% to 99%) to *L. quercina* subsp. *quercina*, although one poorer quality sample shared 90% similarity.

Females of *A. galliformis* were found to contain the bacterium. However, neither the two samples of individual males nor two pooled samples of males tested positive for the bacterium.

Four of the specimens that carried the bacterium were collected in July, two in August, and one in September.

Eight insect families were represented (Buprestidae, Coccinellidae, Dermestidae, Coreidae, Pentatomidae and/or Miridae grouped together, Apidae, Formicidae, and Vespidae) among the 12 insects found positive for contamination with *Lonsdalea quercina* subsp. *quercina*. Of the three Coleoptera specimens, two were recognized to the species level, including a buprestid, the flatheaded appletree borer, *Chrysobothris femorata* Olivier, and a coccinellid, the multicolored Asian lady beetle, *Harmonia axyridis* Pallas (Figure 3.1E). The remaining coleopteran that tested positive was in the family Dermestidae and genus *Trogoderma* (Figure 3.1B). In the order Hemiptera, two samples tested positive for the bacterium. One was of the western conifer-seed bug *Leptoglossus occidentalis* Heidemann (Coreidae). The second positive sample was combined containing a stink bug (Pentatomidae) and a plant bug (Miridae).

Insects in the order Hymenoptera were most frequently documented to carry *L. quercina* subsp. *quercina*; this group included seven of the 12 positive samples (Table 3.1) and represented three of the eight insect families that carried the pathogen. In addition, Hymenoptera was the only order to have multiple specimens in the same family test positive for *L. quercina* subsp. *quercina* (Table 3.1) including two honey bees (*Apis mellifera* L., Figure 3.1D), and two western yellowjackets (*Vespula pensylvanica* Saussure). In addition, one quarter of the pooled samples of ants (family Formicidae) tested positive for *L. quercina* subsp. *quercina*.

Field observations of insects feeding on the bacterium were indicated that a great many insects visit the bacterial ooze produced by *Lonsdalea quercina* subsp. *quercina* (Table 3.3). Scatopsid flies (Diptera: Scatopsidae) (Figure 3.1A) and cutworm moths (Lepidoptera: Noctuidae) (Figure 3.1F) were observed feeding on the bacterium. Several kinds of beetles,

including additional species (i.e., other than multicolored Asian lady beetle) of lady beetles (Figure 3.1C) and bumble flower beetles, *Euphoria inda* L. (Coleoptera: Scarabeaeidae) were noted feeding on the scale and/or bacterial exudates during the time of collection, but the bacterium was not amplified from these specimens. Hymenopteran specimens were the most frequent order observed on diseased trees, but many specimens found on or near trees were not documented to carry *L. quercina* subsp. *quercina*, including one bumble bee (*Bombus* sp.), two baldfaced hornets (*Dolichovespula maculata* L.), four aerial yellowjackets (*Dolichovespula arenaria* Fabr.), one European paper wasp (*Polistes dominulus* Christ), two paper wasps (*Polistes fuscatus*), and one unidentified yellowjacket (*Vespula* sp.).

Prediction of *Lonsdalea quercina* subsp. *quercina* synthesis of the volatile compound acetoin

It was hypothesized that *Lonsdalea quercina* subsp. *quercina* produces the volatile compound acetoin, which plays a role in attracting a variety of mobile insects. When comparing the three acetoin reductase protein sequences identified in the annotated genome of *L. quercina* subsp. *quercina* to the protein database in NCBI, the high query coverage and low E-values showed high similarity with protein sequences of related species within Enterobacteriaceae (Table 3.2).

DISCUSSION

A suite of potential vectors visited drippy blight diseased trees; the insects sampled were from six insect orders and 28 families. Of them, *Lonsdalea quercina* subsp. *quercina* was found on 12 specimens from three orders (Coleoptera, Hemiptera, and Hymenoptera) and eight families (Buprestidae, Coccinellidae, Dermestidae, Coreidae, Pentatomidae and/or Miridae, Apidae, Formicidae, and Vespidae). Importantly, insects that tested positive for the bacterium exhibited differing life histories and varied in their host specificity. Some displayed qualities of more

specialized herbivores that used red oak trees as a primary host (e.g. *Allokermes galliformis* and *Chrysobothris femorata*), and are likely facultative disseminators due to the potential frequent physical contact with the bacterium. In contrast, many more were generalist, cosmopolitan insects some of which appear to actively seek out bacterial exudates (e.g. *Apis mellifera, Vespula pensylvanica, Leptoglossus occidentalis, Harmonia axyridis,* and insects in the families Pentatomidae and/or Miridae, Dermestidae, and Formicidae). As *L. quercina* subsp. *quercina* does not have a close association with one specific insect vector, both facultative and generalist insects are likely important in the spread of this pathogen.

Female kermes scales *Allokermes galliformis* (Riley) are also regularly contaminated with the bacterial causal agent of drippy blight disease. These are highly sessile insects with habits that do not favor a role in long distance dissemination, especially during the summer months (Bullington and Kosztarab 1985; Gill 1993; Kosztarab 1996). The more mobile males of this species were not among the insects that were contaminated with the bacterium. *Allokermes galliformis* plays several indirect roles in the dissemination of *L. quercina* subsp. *quercina* associated with drippy blight disease. The first instar kermes scales migrate to new growth where they begin feeding in the spring, and during this process may crawl over dried exudates and overwintering cankers before feeding on new growth. Additionally, many insects visit the female scales which are covered with bacterial exudates throughout the summer (Figure 3.1A-E). Perhaps more importantly most of the exudation of the drippy blight bacterium to the plant surface is from scale feeding sites, allowing the bacteria to become accessed by other insects. Scale feeding sites occur throughout the tree canopy (Sitz et al. 2017) and peak production of bacterial ooze typically occurs in July, coincident with peak feeding by the maturing scales.

Other insects that use red oak as a primary host may also create feeding wounds that play an indirect role, but are also mobile and could contribute directly to the onset and spread of drippy blight disease. Specifically, adults of the buprestid borer *Chrysobothris femorata*, which develops as a wood borer within red oak, was found contaminated with *L. quercina* subsp. *quercina*. This finding supports a previously documented association between wood borers in the family Buprestidae and bacterial tree diseases. For example, diffuse cankers occur when galleries of the two spotted oak borer (*Agrilus biguttatus* Fab.) touch bacterial lesions of acute oak decline (Denman et al. 2012; Brown et al. 2013; Denman et al. 2014). In addition, wood borers are responsible for creating wounds that allow new infections of fire blight in apple and pear orchards (Hildebrand et al. 2000). In Aleppo pine knot disease, the Europe pine weevil *Pissodes castaneus* (Degeer) is thought to both directly damage the host as well as indirectly damage the host through spreading the pathogen (Calamassi et al. 2008). Overall, several oak-feeding insects were contaminated with *L. quercina* subsp. *quercina*, however, generalist insects were more abundant on diseased trees and therefore may play a more important role in dissemination.

Red oaks showing symptoms of drippy blight disease were noted to attract to a suite of insects that do not have a close association to oak trees. The most abundant group of insects visiting and becoming contaminated with the bacterium were social Hymenopterans. In particular, ants were the most frequently recorded insects carrying the bacterium (Figure 3.1B) and were observed feeding on kermes scale and bacterial exudates. This finding is supported because of the relationship between scale insects and ants. *Allokermes galliformis* rely on ants to physically remove their sugar-rich excrements (Turner and Buss 2005; Gullan and Kosztarab 1997) to avoid honeydew accumulation close to their bodies (Gullan and Kosztarab 1997; Kosztarab 1996).

Winged hymenopterans that move readily within and between trees may be more important in bacterial dissemination. The honey bee (*Apis mellifera*) was the most abundant insect observed visiting drippy blight affected trees, and during mid-summer hundreds of bees might be seen on a single tree. Honey bees are known to ingest *L. quercina* and related pathogens (Mattila et al. 2012; Budge et al. 2016); Brady et al. (2012) even used a genetic sequence of *L. quercina* subsp. *britannica* isolated from the intestine of a honey bee for the taxonomic reclassification of *Lonsdalea* and closely related bacteria in the family Enterobacteriacea. In addition, adult honey bees are frequently transported across geographic regions (Smith 1962), posing a risk for the long-distance spread of phytopathogenic bacteria. This is relevant to drippy blight disease because dried exudates of *L. quercina* subsp. *quercina* can be viable after one year at room temperature (R. Sitz unpublished data). Although these exudates were on petri plates, a relevant study of the fire blight bacterium showed that it could remain on insect hosts for two days (Hildebrand et al. 2000) suggesting that the drippy blight bacterium could be viable on the insect host for an extended period of time.

Another important group of winged hymenopterans attracted to drippy blight diseased trees are social wasps and two western yellowjackets (*Vespula pensylvanica*) were found contaminated with *L. quercina* subsp. *quercina*. This is the first time this species has been documented carrying a phytopathogenic bacterium. Eusocial wasps may have been attracted to bacterial-derived products such as acetoin and alcohols (Landolt et al. 2014; Landolt and Zhang 2016) or other potential attractants such as scale insect volatiles and sugary tree sap (Martinson et al. 2013; Brown et al. 2015). It is likely that wasps were attracted to the bacterium as: (1) associations between eusocial wasp and bacterial infections of plants involving the genera *Brenneria* and *Erwinia* have been reported (Myhre 1988; van der Zwet and Keil 1979); (2)

western yellowjackets are regularly observed visiting drippy pod diseased lupines and ergot honeydew, and neither diseases involve a scale insect (Myhre 1988; Hardy 1988); (3) plant-associated fungi mediate the attraction of some vespid wasps through the volatile chemicals 3-methyl-1-butanol and 2-methyl-1-butanol (Davis et al. 2002), which are chemically similar to acetoin which is likely produced by *L. quercina* subsp. *quercina*; and (4) other studies have documented behavioral preferences in various wasps, true bugs, and beetles toward acetoin (Buttery et al. 1984; Bengtsson et al. 2009; Rochat et al. 2002; Tolasch et al. 2003; Saïd et al. 2005; Nout and Bartlet 1998), although not within a bacterium. Therefore, future studies that connect insect attraction to volatiles produced by phytopathogenic bacteria is a clear step forward to understanding the role insects play in the dissemination of drippy blight disease.

Estimates of the number of potential disseminators is likely conservative, more insect groups feeding on the bacterium or attracted to diseased trees were observed than those that were contaminated with *L. quercina* subsp. *quercina*. There were limitations in sampling methods used in this study that may have limited which insects were collected. For example multiple funnel traps and live collections during the day limited the collection of insects to day active species, and night visiting species were likely undersampled (Fig. 1F).

Oak trees support thriving communities of insects, and the insect groups that are involved in dissemination of the *L. quercina* diseases likely will vary by region. For example, *L. quercina* subsp. *quercina* has been documented in California in association with seed-feeding weevils, cynipid gall wasps, filbertworms (*Cydia latiferreana* Walsingham) (Swiecki and Bernhardt 2006; Izquierdo and Pulido 2013), but those insects are not associated with red oaks in Colorado.

The kermes scale *A. galliformis* is thought of as the insect causal agent of drippy blight disease. Although this kermes scale is sessile during the summer months, it plays in indirect role

in bacterial dissemination by creating primary sites of bacterial exudation that many opportunistic insects were found visiting. In addition, this study is the first to document a diverse suite of generalist insects that were directly linked to the drippy blight pathogen *L. quercina* subsp. *quercina*. These potential disseminators exhibit starkly different lifestyles; and bacterial contamination was documented not only on insects that use red oak as a primary host, but also on generalist pollinators such as honey bees, herbivores such as plant bugs and wood borers, predators such as lady beetles, and scavengers such as dermestid beetles and yellowjackets. This is important, as environments containing trees infected with *L. quercina* pathogens sustain insect communities, and many of the insects present could play a role in dissemination. In drippy blight, the insects identified as potential disseminators comprise a portion of insects that are capable of moving *L. quercina* subsp. *quercina*. This is particularly threatening as red, white, and live oaks are susceptible to *Lonsdalea quercina* diseases and host a suite of insects that could enhance the movement of this emergent disease. It is likely insects are attracted to volatile compounds produced by the bacterium, and therefore actively seek out diseased trees rather than experiencing accidental encounters.

Table 3.1. Insects and other arthropods collected on drippy blight diseased oaks located in Boulder, Colorado were tested for contamination with the bacterial pathogen *Lonsdalea quercina* subsp. *quercina.* Insect specimens were identified to order and family, and the number of individuals tested for the bacterium, and the percent and number that tested positive for the bacterium using DNA extraction or colony PCR methods are presented.

Classification Subdivision	No. DNA Extraction Samples	No. Individuals tested using DNA Extraction	No. Individuals tested using Colony PCR	Percent of Samples Infected from DNA Extractions (No. Infected)	Percent of Samples Infected from Colony PCR (No. Infected)
Hymenoptera	**26**	**52**	**10**	15% (n=4)	30% (n=3)
Apidae		1	5		
Vespidae		11	5		
Formicidae		33			
Unknown		7			
Hemiptera	**32**	**57**	**1**	3% (n=1)	100% (n=1)
Membracidae		27			
Cicadellidae		7			
Aphididae		8			
Psyllidae		1			
Reduviidae		1			
Coreidae		1	1		
Pentatomidae		3			
Miridae		9			
Coleoptera	**36**	**79**	**9**	3% (n=1)	22% (n=2)
Dermestidae		39	2		
Buprestidae		10	1		
Coccinellidae		2	5		
Latridiidae		5			
Curculionidae		8			
Carabidae		2			
Nitidulidae		1			
Histeridae		1			
Lampyridae		2			
Scarabaeidae		2			
Staphylinidae		7			
Unknown			1		
Diptera*	**8**	**30**	**2**	0% (n=0)	0% (n=0)

Brachycera		18	1		
Nematocera		12	1		
Dermaptera	**4**	**5**	**0**	0% (n=0)	0% (n=0)
Forficulidae		5			
Neuroptera	**2**	**2**	**0**	0% (n=0)	0% (n=0)
Rhaphidioptera*		1			
Chrysopidae		1			
Arachnida[1]	**6**	**9**	**0**	0% (n=0)	0% (n=0)
Acari		5	0		
Aranae		3	0		
Opiliones		1	0		
Diplopoda	**1**	**1**	**0**	0% (n=0)	0% (n=0)

* Diptera is classified into suborder rather than family
[1]Arachnida is a class, Acari a subclass, and Araneae and Opiliones are orders.

Table 3.2. Average nucleotide identity between three predicted diacetyl reductase proteins of *Lonsdalea quercina* subsp. *quercina* (Lqq) isolate B83 and available protein sequences in the NCBI non redundant protein sequences database.

Species	Acetoin (diacetyl reductase) product	Identity to Lqq B83 predicted protein	Query coverage	E-value
Lqq	WP_026739747.1	-	-	-
Klebsiella pneumoniae	WP_071067812.1	87	100	$1e^{-162}$
Cronobacter malonaticus	WP_032992536.1	86	100	$9e^{-162}$
Cronobacter muytjensii	WP_075193029.1	86	100	$1e^{-161}$
Cedecea neteri	WP_061277411.1	86	100	$2e^{-161}$
Klebsiella variicola	WP_012967974.1	86	100	$3e^{-161}$
Lqq	WP_026739748.1	-	-	-
Rouxiella chamberiensis	WP_045047152.1	76	98	$1e^{-144}$
Cronobacter malonaticus	WP_032992536.1	76	98	$2e^{-140}$
Cronobacter sakazakii	WP_004388540.1	76	98	$2e^{-140}$
Enterobacter cloacae	WP_063146548.1	75	98	$1e^{-139}$
Cronobacter condimenti	WP_007667240.1	75	98	$1e^{-139}$
Lqq	WP_026740982.1	-	-	-
Yersinia frederiksenii	WP_050110505.1	92	99	$2e^{-172}$
Yersinia kristensenii	WP_057635221.1	90	99	$1e^{-167}$
Erwinia persicina	WP_062744532.1	85	99	$2e^{-158}$
Pseudomonas fragi	WP_016779238.1	79	100	$3e^{-148}$
Halotalea alkalilenta	WP_064123555.1	80	100	$5e^{-147}$

Table 3.3. The number of insects (order and family) observed feeding on the bacterial causal agent of drippy blight disease, *Lonsdalea quercina* subsp. *quercina*, present in diseased oaks located in Boulder, Colorado.

Insect Classification	No. Individuals Observed
Hymenoptera	
Apidae	hundreds
Vespidae	4
Formicidae	1
Hemiptera	
Coreidae	1
Coleoptera	
Scarabaeidae	2
Coccinellidae	7
Dermestidae	1
Diptera*	
Brachycera	1
Nematocera	
Lepidoptera	
Noctuidae	3

* Diptera is classified into suborder rather than family

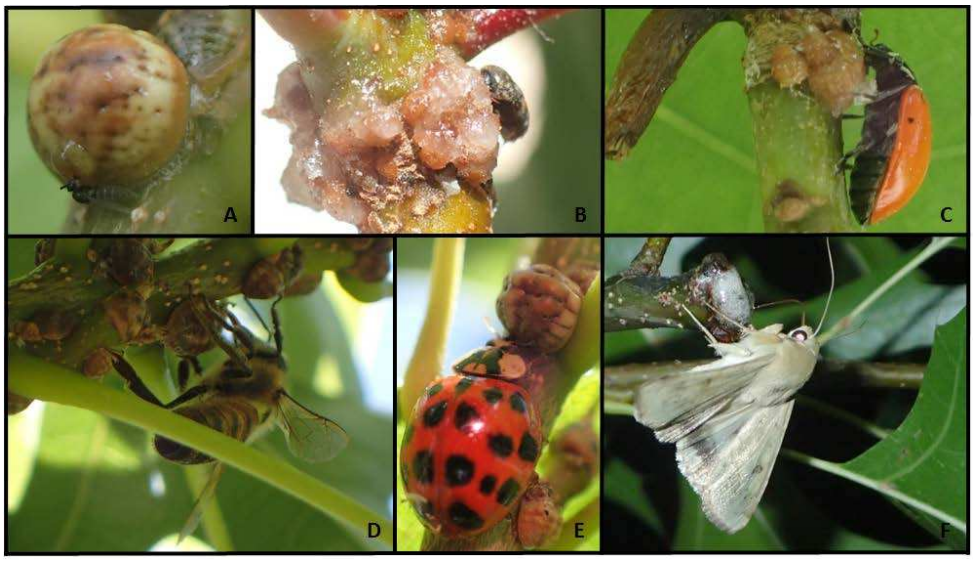

Figure 3.1. Images of insects feeding on *Lonsdalea quercina* subsp. *quercina* on drippy blight diseased red oaks in Boulder, Colorado. The images include insects in the following orders and families: (A) Diptera: Scatopsidae; (B) Coleoptera: Dermestidae; (C and E) Coleoptera: Coccinellidae; (D) Hymenoptera: Apidae; and (F) Lepidoptera: Noctuidae.

LITERATURE CITED

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403-410.

Belasque Jr, J., Parra-Pedrazzoli, A. L., Rodrigues Neto, J., Yamamoto, P.T., Chagas, M. C. M., Parra, J. R. P., Vinyard, B. T., and J. S. Hartung. 2005. Adult citrus leafminers (*Phyllocnistis citrella*) are not efficient vectors for *Xanthomonas axonopodis* pv. *citri*. Plant Disease 89(6):590-594.

Bengtsson, J.M., Wolde-Hawariat, Y., Khbaish, H., Negash, M., Jembere, B., Seyoum, E., Hansson, B.S., Larsson, M.C., and Y. Hillbur. 2009. Field attractants for *Pachnoda interrupta* selected by means of GC-EAD and single sensillum screening. Journal of Chemical Ecology 35:1063-1076.

Biosca, E. G., González, R., López-López, M. J., Soria, S., Montón, C., Pérez-Laorga, E., and M. M. López. 2003. Isolation and characterization of *Brenneria quercina*, causal agent for bark canker and drippy nut of *Quercus* spp. in Spain. Phytopathology 9:485-492.

Brady, C. L., Cleenwerck, I., Denman, S., Venter, S. N., Rodríguez-Palenzuela, P., Coutinho, T, and P. De Vos. 2012. Proposal to reclassify *Brenneria quercina* (Hildebrand and Schroth 1967) Hauben et al. 1999 into a new genus, *Lonsdalea* gen. nov., as *Lonsdalea quercina* comb. nov., descriptions of *Lonsdalea quercina* subsp. *quercina* comb. nov., *Lonsdalea quercina* subsp. *iberica* subsp. nov. and *Lonsdalea quercina* subsp. *britannica* subsp. nov., emendation of the description of the genus *Brenneria*, reclassification of *Dickeya dieffenbachiae* as *Dickeya dadantii* subsp. *dieffenbachiae* comb. nov., and emendation of the description of *Dickeya dadantii*. International Journal of Systematic and Evolutionary Microbiology 62:1592-1602.

Brown, R. L., El-Sayed, A. M., Unelius, C. R., Beggs, J. R., and D. M. Suckling. 2015. Invasive *Vespula* wasps utilize kairomones to exploit honeydew produced by sooty scale insects, Ultracoelostoma. Journal of Chemical Ecology 41(11):1018-1027.

Brown, N., Inward, D. J., Jeger, M., and S. Denman. 2014. A review of *Agrilus biguttatus* in UK forests and its relationship with acute oak decline. Forestry: An International Journal of Forest Research 88(1): 53-63.

Budge, G. E., Adams, I., Thwaites, R., Pietravalle, S., Drew, G. C., Hurst, G. D., Tomkies, V., Boonham, N., and M. Brown. 2016. Identifying bacterial predictors of honey bee health. Journal of Invertebrate Pathology 141:41-44.

Bullington S, and M. Kosztarab. 1985. Revision of the family Kermesidae (Homoptera) in the Nearctic Region based on adult and third instar females. Virginia Polytechnic Institute and State University 85-11. 118 pp.

Buttery R. G., Kamm J. A., and L. C. Ling. 1984. Volatile components of red clover leaves, flowers, and seed pods: possible insect attractants. Journal of Agricultural and Food Chemistry 32:254-256.

Caballero, J. I., Zerillo, M. M., Snelling, J., Cranshaw, W., Boucher, C., and N. Tisserat. 2014. Genome sequences of strain ATCC 29281 and pin and northern red oak isolates of *Lonsdalea quercina* subsp. *quercina*. Genome Announcements 2(3):e00584-14.

Calamassi, R., Mori, B., Moscatelli, F., Alberghini, S., Battisti, A., Mugnai, L., and G. Surico. 2008. Aleppo pine knot disease: histology of the knots, detection of causal agent and mode of transmission. Phytopathologia Mediterranea 47(1):61-72.

Chagas, M., Parra, J. R., Namekata, T., Hartung, J. S., and P. T. Yamamoto. 2001. *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) and its relationship with the citrus canker bacterium *Xanthomonas axonopodis* pv *citri* in Brazil. Neotropical Entomology 30(1):55-59.

Dalmacio, S. C., Lugod, T. R., Serrano, E. M., and G. P. Munkvold. 2007. Reduced incidence of bacterial rot on transgenic insect-resistant maize in the Philippines. Plant Disease 91:346-351.

Davis, T. S., Boundy-Mills, K., and Landolt, P. J. 2012. Volatile emissions from an epiphytic fungus are semiochemicals for eusocial wasps. Microbial Ecology 64(4):1056-1063.

Denman, S., Brady, C., Kirk, S., Cleenwerck, I., Venter, S., Coutinho, T., and P. De Vos. 2012. *Brenneria goodwinii* sp. nov., associated with acute oak decline in the UK. International Journal of Systematic and Evolutionary Microbiology 62(10):2451-2456.

Denman, S., Brown, N., Kirk, S., Jeger, M., and J. Webber. 2014. A description of the symptoms of acute oak decline in Britain and a comparative review on causes of similar disorders on oak in Europe. Forestry 87(4):535-551.

Denman, S., Plummer, S., Kirk, S., Peace, A., and J. E. McDonald. 2016. Isolation studies reveal a shift in the cultivable microbiome of oak affected with acute oak decline. Systematic and Applied Microbiology 39(7):484-490.

Fralish, J. S. 2004. The keystone role of oak and hickory in the central hardwood forest. In: *Spetich, M. A. ed. Upland oak ecology symposium: history, current conditions, and sustainability*. USDA Forest Service General Technical Report SRS-373, Southern Research Station, 78-87.

García Morales M., Denno, B. D., Miller, D. R., Miller, G. L., Ben-Dov, Y., and N. B. Hardy. 2016. *ScaleNet:* A literature-based model of scale insect biology and systematics. Database. doi: 10.1093/database/bav118. http://scalenet.info.

Gill, R. J. 1993. The scale insects of California. Analysis and Identification Branch, Division of Plant Industry, California Department of Food and Agriculture.

Gish, W., and D. J. States. 1993. Identification of protein coding regions by database similarity search. Nature Genetics 3:266-272.

Granovsky, A. A. 1940. The relation of subterranean insects to the Raspberry crown gall. Hoosier Horticulture 22(5).

Gullan, P. J., and M. Kosztarab. 1997. Adaptations in scale insects. Annual Review of Entomology 42(1):23-50.

Hansen, H., R. Smith. 1937. A bacterial gall disease of douglas fir, *Pseudotsuga taxifolia*. Hilgardia, 10(14):567-577.

Hardy, T. N. 1988. Gathering of fungal honeydew by *Polistes* spp. (Hymenoptera: Vespidae) and potential transmission of the causal ergot fungus. Florida Entomologist 71(3):374-376.

Harris, K. F., and K. Maramorosch. (Eds.). 1980. Vectors of plant pathogens. Academic Press. New York, New York.

Hauben, L., Moore, E. R., Vauterin, L., Steenackers, M., Mergaert, J., Verdonck, L., and J. Swings. 1998. Phylogenetic position of phytopathogens within the Enterobacteriaceae. Systematic and Applied Microbiology 21(3):384-397.

Heidlas, J., and R. Tressl. 1990. Purification and characterization of a (R)-2, 3-butanediol dehydrogenase from *Saccharomyces cerevisiae*. Archives of Microbiology 154(3):267-273.

Hildebrand, D. C., and Schroth, M. N. 1967. A new species of *Erwinia* causing the drippy nut disease of live oaks. Journal of Phytopathology 57:250-253.

Hildebrand, M., Dickler, E., and K. Geider. 2000. Occurrence of *Erwinia amylovora* on insects in a fire blight orchard. Journal of Phytopathology 148(4):251-256.

Izquierdo, L. P., and F. Pulido. 2013. Spatiotemporal variation in acorn production and damage in a Spanish holm oak (*Quercus ilex*) dehesa. Forest Systems 22(1):106-113.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., et al. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12):1647-1649.

Kosztarab, M. 1996. Scale insects of northeastern North America: identification, biology, and distribution. Virginia Museum of Natural History, Martinsville, VA. pp. 650.

Lauzon, C. R., Sjogren, R. E., Wright, S. E., and R. J. Prokopy. 1998. Attraction of *Rhagoletis pomonella* (Diptera: Tephritidae) flies to odor of bacteria: apparent confinement to specialized members of Enterobacteriaceae. Environmental Entomology 27(4):853-857.

Landolt, P. J., Cha, D. H., Werle, C. T., Adamczyk, J. J., Meagher, R. L., Gilbride, R. L., Clepper, T. S., Reed, H. C., Teal, P. E. A., and B. J. Sampson. 2014. *Polistes* spp. (Hymenoptera: Vespidae) orientation to wine and vinegar. Florida Entomologist 97(4):1620-1630.

Landolt, P., and Q. H. Zhang. 2016. Discovery and development of chemical attractants used to trap pestiferous social wasps (Hymenoptera: Vespidae). Journal of Chemical Ecology 42(7):655-665.

Li, Y., He, W., Ren, F., Guo, L., Chang, J., Cleenwerck, I., Ma, Y., H. Wang. 2014. A canker disease of *Populus* × *euramericana* in China caused by *Lonsdalea quercina* subsp. *populi*. Plant Disease 98:368-378.

Lu S. E., and D. C. Gross. 2010. Drippy pod of white lupine: A new bacterial disease caused by a pathovar of *Brenneria quercina*. Plant Disease 94:1431-1440.

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V., Beer, S. V., Machado, M. A., Toth, I. A. N., Salmond, G., and G. D. Foster. 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. Molecular plant pathology 13(6):614-629.

Martinson, H. M., Raupp, M. J., and P. M. Shrewsbury. 2013. Invasive stink bug wounds trees, liberates sugars, and facilitates native Hymenoptera. Annals of the Entomological Society of America 106(1):47-52.

Mattila, H. R., Rios, D., Walker-Sperling, V. E., Roeselers, G., and I. L. Newton. 2012. Characterization of the active microbiotas associated with honey bees reveals healthier and broader communities when colonies are genetically diverse. PLoS One 7(3):e32962.

Myhre, E. A. 1988. Effects of *Lygus* spp. (Hemiptera: Miridae) on Mediterranean white lupin (*Lupinus albus*). M.S. thesis. Entomology, Washington State University, Pullman, WA.

Nadarasah, G., and J. Stavrinides. 2011. Insects as alternative hosts for phytopathogenic bacteria. FEMS Microbiology Review 35:555-575.

Nout, M. J. R., and R. J. Bartelt. 1998. Attraction of a flying nitidulid (*Carpophilus humeralis*) to volatiles produced by yeasts grown on sweet corn and a corn-based medium. Journal of Chemical Ecology 24:1217-1239.

Orlovskis, Z., Canale, M. C., Thole, V., Pecher, P., Lopes, J. R., and S. A. Hogenhout. 2015. Insect-borne plant pathogenic bacteria: getting a ride goes beyond physical contact. Current Opinion in Insect Science 9:16-23.

Phelan, P. L., and H. Lin. 1991. Chemical characterization of fruit and fungal volatiles attractive to dried-fruit beetle, *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae). Journal of Chemical Ecology 17(6):1253-1272.

del Pilar Marquez-Villavicencio, M., Weber, B., Witherell, R. A., Willis, D. K., and A. O. Charkowski. 2011. The 3-hydroxy-2-butanone pathway is required for *Pectobacterium carotovorum* pathogenesis. PloS One 6(8):e22974.

Poza-Carrión, C., Aguilar, I., Gallego, F. J., Nuñez-Moreno, Y., Biosca, E. G., González, R., López, M. M., and P. Rodríguez-Palenzuela. 2008. *Brenneria quercina* and *Serratia* spp. isolated from Spanish oak trees: molecular characterization and development of PCR primers. Plant Pathology 57(2):308-319.

Rochat, D., Morin, J. P., Kakul, T., Beaudoin-Ollivier, L., Prior, R., Renou, M., Malosse, I., Stathers, T., Embupa, S., and S. Laup. 2002. Activity of male pheromone of Melanesian rhinoceros beetle *Scapanes australis*. Journal of Chemical Ecology 28:479-500.

Rubin, B. E., Sanders, J. G., Hampton-Marcell, J., Owens, S. M., Gilbert, J. A., and C. S. Moreau. 2014. DNA extraction protocols cause differences in 16S rRNA amplicon sequencing efficiency but not in community profile composition or structure. MicrobiologyOpen 3(6):910-921.

Saïd, I., Renou, M., Morin, J. P., Ferreira, J. M., and D. Rochat. 2005. Interactions between acetoin, a plant volatile, and pheromone in *Rhynchophorus palmarum*: Behavioral and olfactory neuron responses. Journal of Chemical Ecology 31(8):1789-1805.

Shang, J., Liu, B. L., and W. He. 2014. A new method to detect *Lonsdalea quercina* in infected plant tissues by real-time PCR. Forest Pathology 45(1):28-35.

Sitz, R. A., Zerillo, M. M., Snelling, J., Caballero, J. I, Alexander, K., Nash, K., Tisserat, N. A., Cranshaw, W. S., and J. E. Stewart. Accepted Manuscript. Drippy blight, a disease of red oaks produced from the combined effect of the scale insect, *Allokermes galliformis* and the bacterium *Lonsdalea quercina* subsp. *quercina*. Journal of Arboriculture and Urban Forestry.

Snelling, J., Tisserat, N. A., and W. Cranshaw. 2011. Kermes scale (*Allokermes* sp.) and the drippy nut pathogen (*Brenneria quercina*) associated with a decline of red oak species in Colorado. Phytopathology 101:S168.

Smith, M. V. 1962. Establishment of honeybee stocks by transporting immature stages and semen. Journal of Apicultural Research 1(1):19-23.

Swiecki, T. J., and E. A. Bernhardt. 2006. A field guide to insects and diseases of California oaks. Gen. Tech Rep. PSW-GTR-197. Albany, CA: Pacific Southwest Research Station, Forest Service, U.S. Department of Agriculture, 151 p.

Thind, B. S., and N. Singh. 1976. Maize borer [*Chilo partellus* (Swinhoe)] as carrier of *Erwinia carotovora* var. *zeae*, the causal agent of bacterial stalk rot of maize. Current Science 45:117-118.

Thomas, H. E., and P. A. Ark. 1934. Fire blight of pears and related plants. California Agricultural Experiment Station Bulletin 586:1-43.

Thomson, S. V., Hansen, D. R., Flint, K. M., and J. D. Vandenberg. 1992. Dissemination of bacteria antagonistic to *Erwinia amylovora* by honey bees. Plant Disease 76(10):1052-1056.

Tolasch, T., Sölter, S., Tóth, M., Ruther, J., and W. Francke. 2003. (R)-acetoin-female sex pheromone of the summer chafer *Amphimallon solstitiale* (L.). Journal of Chemical Ecology 29(4):1045-1050.

Turner, J. C. L., and E. A. Buss,. 2005. Biology and management of *Allokermes kingii* (Hemiptera: Kermesidae) on oak trees (*Quercus* spp.). Journal of Arboriculture 31(4):198-202.

van der Zwet, T., and H. L. Keil. 1979. Fire blight, a bacterial disease of rosaceous plants. Agriculture handbook no. 510. U.S. Government Printing Office, Washington, D.C.

Vanneste, J. L. (Ed.). 2000. Fire blight: the disease and its causative agent, *Erwinia amylovora*. CABI.

White, J. N., and M. P. Starr. 1971. Glucose fermentation endproducts of *Erwinia* spp. and other enterobacteria. Journal of Applied Bacteriology 34(2):459-475.

Xiao, Z., and P. Xu. 2007. Acetoin metabolism in bacteria. Critical Reviews in Microbiology 33(2):127-140.

CHAPTER IV: VIRULENCE OF GENETICALLY DISTINCT *GEOSMITHIA MORBIDA* ISOLATES TO BLACK WALNUT AND THEIR RESPONSE TO CO-INOCULATION WITH *FUSARIUM SOLANI*[2]

SUMMARY

Geosmithia morbida is well documented as the causal agent of thousand cankers disease of black walnut trees. However, it is not well-understood how *G. morbida* strains differ in virulence and how their interactions with co-occurring pathogens contribute to disease severity. In this study, we systematically investigated virulence of genetically distinct *G. morbida* strains. Overall, we found varying degrees of virulence, although differences were not related to genetic groupings. Furthermore, the pathogen *Fusarium solani* is also commonly isolated from thousand canker-diseased trees. The degree of disease contribution from *F. solani* is unknown, along with interactions it may have with *G. morbida.* This research shows co-inoculation with these pathogens does not yield a synergistic response.

INTRODUCTION

Thousand cankers disease (TCD) is an aggressive emerging disease on *Juglans* spp. and *Pterocarya* spp. that was first identified in Colorado in 2007 and has now spread throughout the western states and seven eastern states (IN, MD, OH, PA, NC, TC, VA) (Utley et al. 2013, Hishinuma et al. 2016, Tisserat et al. 2009, Reed et al. 2013, ODNR/ODA 2012, PDA 2011, NCDACS 2013, Grant et al. 2011, VDACS 2011) and Italy (Montecchio and Faccoli 2015). This

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[2] Rachael A. Sitz, Graduate Student; Emily K. Luna, Research Associate; Jorge Ibarra Caballero, Postdoctoral Researcher; Ned A. Tisserat, Retired Professor; Whitney S. Cranshaw, Professor; and Jane E. Stewart, Assistant Professor, Department of Bioagricultural Sciences and Pest Management, Colorado State University, Fort Collins, CO 80523-1177

Accepted for publication 29 August 2016. Published January 2017.

Corresponding author: Jane Stewart, Jane.Stewart@colostate.edu, accession number KX085029

disease is caused by the symbiotic relationship between the fungal pathogen *Geosmithia morbida* M. Kolařík, E. Freeland, C. Utley, & N. Tisserat (Ascomycota: Hypocreales) and the walnut twig beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae). Adult beetles enter limbs and the main trunk, excavating brood galleries beneath the bark. As beetles create wounds during feeding and brood gallery construction, the phloem is inoculated with *G. morbida*, that is carried by *P. juglandis*. With large masses of beetle feeding, many small diffuse cankers form that eventually can coalesce, leading to girdling and dieback.

Geosmithia fungi are ubiquitous, with a worldwide distribution and occur on broad range of substrates (Kolařík et al. 2004, Pitt and Hocking 2009). However, most are associated with insects including bark beetles (Kolařík et al. 2006, 2007, 2008) and wood-boring ambrosia beetles (Kolařík and Kirkendall 2010, Kolařík et al. 2015). As a symbiotic partner, *Geosmithia* spp. provide nutritional sources for their insect symbionts. While most *Geosmithia* are non-pathogenic, *G. morbida*, and most recently, *G. pallida,* are the first species to be described as plant pathogens (Kolařík and Kirkendall 2010, Kolařík et al. 2011, Lynch et al. 2014), suggesting that plant pathogenicity in *Geosmithia* is a recent evolutionary change (Zerillo et al. 2014). The genetic diversity and pathways of *G. morbida* spread that have occurred in the US were examined (Hadziabdic et al. 2014, Zerillo et al. 2014). Zerillo et al. (2014) found four genetically distinct populations of *G. morbida* that could be clustered into three geographic regions, with no evidence of sexual reproduction or genetic recombination. It is not known whether phenotypic differences (i.e., virulence) may exist among the four genetic clusters identified.

Several species of *Juglans,* including *Juglans nigra* L. (black walnut), are highly susceptible to the pathogen and tree death is typically observed two years after initial symptoms

appear (Tisserat et al. 2009, Seybold et al. 2013). However, according to Tisserat and Cranshaw (personal communication, July 23, 2015), variation in disease progression (stages and timing of tree decline) and canker type have been observed in black walnut stands in Colorado. Typically, an acute phase of disease development occurs during early stages of TCD appearance, with rapid tree decline after first external symptoms are evident. However, in recent years some trees in sites with a long history of the disease exhibit a more chronic phase where disease progression slows and may be largely arrested, allowing trees to survive with persistent, but low levels, of infection.

Whether trees experience acute or chronic phases of infection will determine the severity of the disease in areas where it has been introduced. What drives this variation in TCD progression is unclear, and remains one of the most important unresolved questions outstanding about TCD. Several possible reasons are speculated, including differences in the community of natural enemies affecting *P. juglandis* and/or *G. morbida*, differences in how induced resistance of the host affects *G. morbida* and/or *P. juglandis* survival (Webber 2000, Bonello et al. 2001), and variation in virulence among *G. morbida* strains. Further, in later stages of TCD decline, *Fusarium solani* is commonly isolated from necrotic bark of *J. nigra* (Tisserat et al. 2009). However, it is unclear to what extent *F. solani* can affect the progression of symptoms associated with TCD. Previous studies have shown that *F. solani* can be pathogenic to walnut (*J. nigra* and *J. regia*) (Tisserat et al. 2009, Carlson et al. 1993, Tisserat 1987), and there is likely a spectrum of *F. solani* virulence. Montecchio et al. (2015) inoculated walnut saplings with an isolate of *F. solani* cultured from diseased trees in Italy, and they found that this particular isolate did not increase or decrease the virulence of *G. morbida*, but produced cankers comparable in size. Here,

we investigate how different strains of *G. morbida* influence disease progression and how they interact with a different strain of *F. solani* obtained from diseased trees in the United States.

In this study, our main goals were to determine whether: (1) virulence differences exist among the different genetic clusters of *G. morbida* present in North America, (2) *F. solani* may contribute to TCD, and if so (3) whether this results from a synergistic response that occurs with co-inoculation of *G. morbida* and *F. solani*.

MATERIALS AND METHODS

Plant selection

Black walnut (*Juglans nigra*) trees ranging in size from 4.6 ± 0.2 m tall to 5.6 ± 0.2 cm in diameter breast height, located at the Colorado State Forest Service Nursery in Fort Collins, CO, were selected based on tree health, tree size, and branch length for inoculations with *G. morbida* and *F. solani* and co-inoculations with both species.

Description of the pathogen isolates

In 2014 eight isolates of *G. morbida* (G1217, G1218, G1248, G1274, G1301, G1334, G2071, and G2224) were collected from several locations in Colorado and used in the 2014 inoculation experiment (Table 4.1). The experiment was repeated in 2015 with the exclusion of *G. morbida* isolate G2224 because the strain was no longer viable. Although quarantine restrictions preclude the use of *G. morbida* strains from other states in these Colorado field studies, a recent genetic diversity study of *G. morbida* identified three distinct genetic clusters within Colorado. Six of the eight isolates represented three of the four known unique genetic clusters of *G. morbida* (Table 4.1; Zerillo et al. 2014). The other two isolates include one that was suspected to be a highly virulent strain (G2071), while the second (G2224) was deemed less virulent than other *G. morbida* strains based on disease symptomology. Isolate G2071 was

collected from a black walnut in Larimer County (Fort Collins, CO) that succumbed quickly to TCD in 2014, much like trees observed in the acute phase. Isolate G2224 was selected because it came from a black walnut in Otero County (Rocky Ford, CO) that has shown slow TCD-related decline over the past seven years indicative of the chronic phase.

Fusarium solani is often found in association with *G. morbida* in TCD-affected trees in Colorado, and strain F917 was used in previous pathogenicity studies of TCD (Tisserat et al. 2009). In this study, *F. solani* was again tested both alone and in combination with each isolate of *G. morbida* to determine interactions of the concomitant species in their ability to produce cankers.

To determine what *Fusarium solani* species complex (FSSC) isolate F917 belonged to, a portion of the translation elongation factor 1 alpha (EF1α) was amplified using primers EF1-986R and EF1-728F developed by Carbone and Kohn (1999). The culture was grown on potato dextrose agar for 5-7 days. DNA was extracted using a ZR Fungal/Bacterial DNA MiniPrep (Zymo Research). For PCR, reaction mixtures (total 25 µl) contained 20-40 ng of template DNA (or no DNA template for negative control), 2.5µl 10x Standard *Taq* Reaction Buffer (New England BioLabs Inc.), 0.5µl 10mM dNTPs (Roche Applied Science), 1µl each of 10µM primer and 0.125 µl *Taq* DNA Polymerace (New England BioLabs Inc.). Amplifications were performed using the following PCR conditions: 94°C for 1 min, 35 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s, then finally 72°C for 10 min. All PCR reactions were conducted using an MJ PTC-200 thermocycler (Bio-RadLaboratories, Waltham, MA). PCR products were cleaned using a High Pure PCR Product Purification Kit (Roche Life Science), and sequenced at Eurofins Scientific (www.eurofinsus.com). Forward and reverse sequences for the EF1α were aligned and a consensus sequence was generated. A Bayesian phylogeny was produced to

compare the F917 EF1α sequence to 10 *F. solani* sequences representing *F. solani* isolates that genetically represented *Fusarium solani* species complex (FSSC) lineages 1, 2 and 6 obtained from the Fusarium-ID database (http://isolate.fusariumdb.org/), as implemented in MrBayes using a GTR model of evolution. The Markov chain Monte Carlo search was run with four chains for 1,000,000 generations, generating 11,001 trees, and the first 2,000 trees were discarded as "burnin" of the chains. Sequence identifications (NRRL numbers) are shown in the phylogeny (Figure 4.1). The sequence was deposited in NCBI Nucleotide Database, accession number KX085029.

Inoculations

Single-spore isolates of various test fungi were grown for 10-14 days at 25°C in 100-mm-diameter x 15-mm-deep Petri dishes containing 20 mL of half-strength potato dextrose agar (½ PDA). There were a total of 18 (2014) or 16 (2015) different isolates or isolate combinations, including a ½ PDA control treatment. To inoculate trees, a 5 mm punch was used to wound the branch, tissues were removed to the phloem, and a 5 mm plug containing the designated treatment was placed into the wound with a scalpel. Tree branches had an average diameter measurement of 16 ± 6.57 mm. Each branch represented one replication. The first wound was made 5 cm from the branch crotch on the main stem, and subsequent wounds were made 10 cm apart. At branch junctions the larger diameter section was followed, and in some cases multiple sections of the branch were used.

Trees were inoculated on August 11 or 15 of 2014, and August 14, 2015, and there were 19 replications on four trees over two years. The experiment followed a randomized complete block design where each branch represented a block (ca. 19) containing each of the treatments. There were a total of 18 (2014) or 16 (2015) different isolates or isolate combinations

(treatments), including a ½ PDA control treatment. Each branch was inoculated with the following treatments: G1217, G1217+F917, G1218, G1218+F917, G1248, G1248+F917, G1274, G1274+F917, G1301, G1301+F917, G1334, G1334+F917, G2071, G2071+F917, F917, and a ½ PDA control. In 2014 branches were also inoculated with G2224 and G2224+F917. In cases where *F. solani* was used in combination with *G. morbida*, 5 mm plugs of both fungi were cut in half and united to make one plug. Each inoculated wound was immediately wrapped with Parafilm to covered the wounds until the inoculated branches were harvested after six weeks.

Canker measurements

Branches were harvested on 24 August 2014 and 23 August 2015 and taken back to Colorado State University. Within five days of branch removal, the branches were peeled to expose the cankers and immediately photographed. An image processing software program (Rasband 1997-2016) was used to obtain the area of each canker. Images included a ruler to calibrate the software and standardize the photographs. A digital caliper was used to measure the branch widths at each inoculation site.

Statistical analyses

Mixed effects models were fit using square root canker area as the response. Transformation was used to satisfy model assumptions, and JMP software (11.1.0v; SAS Institute) was used to analyze the data. All data were back-transformed for presentation of figures and descriptive statistics. Isolate (18 levels in 2014 and 16 levels in 2015) or treatment type (*Fusarium solani*, *Geosmithia*, and co-inoculation) and branch diameter were included as fixed effects by year. Branch was included as a random effect to account for blocking. Pairwise comparisons between isolates were considered using a LSD method.

RESULTS

Geosmithia morbida inoculations

In 2014, the canker area produced by inoculations of *G. morbida* isolates ranged from 0.15 cm^2 to 3.77 cm^2 (n=88) whereas in 2015 they ranged from 0.16 cm^2 to 2.26 cm^2 (n=56). A similar trend was observed in the mean *G. morbida* canker areas where in 2014 measurements ranged from 0.96 cm^2 to 1.74 cm^2 and in 2015 were 0.82 cm^2 to 1.03 cm^2. Regardless of treatment, cankers were larger in 2014 than in 2015 (*P*=0.0121) with the exception of isolate combination G1274 + F917 (Figure 4.2A&B). Overall, *G. morbida* virulence did not vary among the three genetic clusters used in years 2014 and 2015 (*P*=0.5104 and *P*=0.7898, respectively). However, comparisons between specific isolates from different genetic clusters did differ significantly. For example, in 2014 isolate G1248 (genetic cluster 3) produced significantly larger cankers than several other *G. morbida* isolates including G1218 (genetic cluster 2, *P=0.0235*), G2071 (*P=0.0153)*, and G2224 (*P=0.0013*). *Geosmithia morbida* isolate G2224 produced the smallest canker areas, and were 45 % smaller than those produced by the G1248 and G1334 isolates (cluster 3, *P*=0.0013 and cluster 2, *P*=0.0148 respectively). Furthermore, all *G. morbida* isolates produced cankers larger than the control wounds, although the canker produced by isolate G2224 was not significantly different than the control wound in 2014 (Figure 4.2A&B). In 2015, all seven *G. morbida* isolates produced similar sized cankers (*P*=0.3705), however, when looking at the mean canker area from isolates in cluster 3, G1301 was 44 % larger than that of G1248 (*P*=0.0889), the isolate that produced the largest canker in 2015.

Fusarium solani identification and inoculations

Based on a phylogeny of the EF1α, isolate F917 falls into the *F. solani* phylogenetic species complex 6 (FSSC 6, Figure 4.1). Using this strain, the average canker area was 47 % smaller than cankers produced by *G. morbida* and 47 % smaller than cankers produced by the combined *F. solani* and *G. morbida* treatment in 2014 (Figure 4.2A). A similar trend was seen in 2015; cankers produced by F917 were 37 % smaller than cankers produced by *G. morbida* and 30 % smaller than cankers produced by all combined treatments (Figure 4.2B). In both years, the necrotic area produced by *Fusarium* isolate F917 was similar (*P=0.8268)*, and no different in size than the control wounds (2014: *P=0.7718 and 2015: P=0.2124)* (Figure 4.2A&B).

Concomitant relationship

Fusarium solani isolate F917 did not increase nor decrease the virulence of *G. morbida* when using a combined inoculum in 2014 (*P=0.3287)* or 2015 (*P=0.2740)*. In fact, in 2014 the canker areas of *G. morbida* only differed from canker areas of *G. morbida* combined with *F. solani* by less than 1 %, and in 2015 canker areas of *G. morbida* were 11 % larger than cankers produced by the combined treatments. Interestingly, in 2015 one canker produced by a combined treatment (1217 + 917) was also a comparable size to the control and *F. solani* alone.

DISCUSSION

Our results indicate that *G. morbida* isolates, in our study, from distinct genetic clusters as identified by Zerillo et al. (2014) do not show differences in virulence. Pathogenicity genes, rather than the genes and genomic regions that were used to determine the genetic clusters, may influence virulence (Zerillo et al. 2014, Idnurm and Howlett 2001). Interestingly, we see broad differences of canker areas within cluster groupings. For example, in 2014 the second largest and the smallest cankers were produced from isolates in genetic cluster 3 (G1301 and G1248,

respectively). This is similar to a review by Kistler (1997) where the degree of genetic diversity of *Fusarium oxysporum* did not correlate to pathogenic phenotype on many different hosts. In addition, Appel and Gordon (1995) compared pathogenic and non-pathogenic isolates of *F. oxysporum*, and showed that genetic similarity did not explain the level of virulence. The lack of, or biologically subtle, virulence differences in *G. morbida* strains imply that other factors such as tree health and/or environmental factors could contribute to the rate of tree decline (Freeland et al. 2012).

We documented smaller canker area measurements with subsequent inoculations. Cankers produced by *G. morbida* in 2015 were 34 % smaller than in those created in 2014. For example, G1248 produced the largest cankers in 2014, whereas in 2015 it produced the smallest cankers. One possible explanation for this response is that prior wounds from the removal of 2014 branches could have initiated a defense response priming the trees for increased resistance in 2015. We saw evidence of this in a greenhouse study where black walnut saplings exposed to a mechanical wound 6 weeks prior to *G. morbida* inoculations experienced smaller cankers than the control (P=0.0198) (Supplementary Table 4.1). Further evidence of this concept is documented by Bonello et al. (2001) and Krokene et al. (2000), where mechanical wounding primed conifer hosts and initiated systemic induced resistance.

In our study, canker areas initiated by *F. solani* were no different than the control, suggesting that this isolate of *F. solani* is not a competitive counterpart. There may be other factors at play when looking at the virulence of *Fusarium* and its interaction with *Geosmithia* and TCD. When examining canker length from *Fusarium solani* inoculations, isolate F917 cankers were 65 % shorter than all other fungal treatments. This is similar to results found by Tisserat et al. (2009), where on average *F. solani* cankers were 61 % shorter than *G. morbida*

cankers. It is clear in these experiments that this isolate of *F. solani* is not as pathogenic as *G. morbida* on black walnut. This phenomenon is also seen with a different species of *Geosmithia* where Čížková et al. (2005) found *Geosmithia langdonii* more pathogenic than *F. solani* on garden cress.

Based on our results, it is unlikely that *G. morbida* and this isolate of *F. solani* exhibit a mutualistic relationship, and it is unknown if *G. morbida* outcompeted the *F. solani* in this experiment. There are documented cases where fungal endophytes or non-pathogenic *Fusarium* sp. decrease the virulence of other fungal pathogens or increased host resistance (Gwinn and Gavin 1992, Schardl et al. 2004, Zabalgogeazcoa 2008, Forsyth et al. 2006), however, it is unknown if *F. solani* is an endophyte. Montecchio et al. (2015) documented that inoculations with *G. morbida* alone produced cankers 35% larger than the cankers produced by co-inoculations with the Italian *F. solani* isolate S1 belonging to phylogenic lineage FSSC 25 (4.8 cm^2 and 3.1 cm^2 respectively). In contrast, our inoculations with *G. morbida* alone produced cankers of comparable size to the co-inoculations with F917 (FSSC 6), a genetically distinct strain from isolate S1, FSSC 25, used in the Montecchio et al. (2015) experiment. In 2014 both cankers measured 1.31 cm^2, and in 2015 they differed by 10% where *G. morbida* cankers averaged 0.87 cm^2 and co-inoculations averaged 0.78 cm^2.

Montecchio et al. (2015) documented canker areas produced by co-inoculations with *Fusarium solani* FSSC 25 S1 isolate and *G. morbida* isolate LM13GMN were the same as the *F. solani* 25 S1 canker areas, both measuring 3.1 cm^2. In contrast, cankers produced by co-inoculations versus *F. solani* F917 (FSSC 6) inoculations were 47 % larger in 2014 and 31% larger in 2015, measuring 1.3 cm^2 and 0.78 cm^2 respectively. This could mean there is a range of

virulence within the FSSC, diverse interactions occurring between the two pathogens, or other factors contributing to the differences.

Through this research, we have determined that *Geosmithia morbida* virulence is most likely not explained by genetic grouping. Furthermore, the *Fusarium solani* isolate used in our study does not increase or decrease the virulence of *G. morbida.* In Colorado, thousand cankers disease causes an initial chronic phase characterized by high tree mortality, and in some cases a later acute phase occurs where disease progression slows. Further research is needed to determine what factors influence disease severity in inflicted communities, and to better understand how *G. morbida* strains differ in their aggressiveness in long-term studies of pathogenicity.

Table 4.1. Fungal isolate number, genetic clusters, and isolate collection location for isolates of *Geosmithia morbida* and *Fusarium solani* used in this study.

Species	**Isolate Number**	**Genetic Cluster***	**Colorado County**
Geosmithia morbida	G1217	1	Boulder
G. morbida	G1218	2	Boulder
G. morbida	G1248	3	Jefferson
G. morbida	G1274	1	Jefferson
G. morbida	G1301	3	Jefferson
G. morbida	G1334	2	Otero
G. morbida	G2071	n/a	Larimer
G. morbida	G2224	n/a	Otero
Fusarium solani	F917	n/a	Boulder

* Based on results of Zerillo et al. 2014

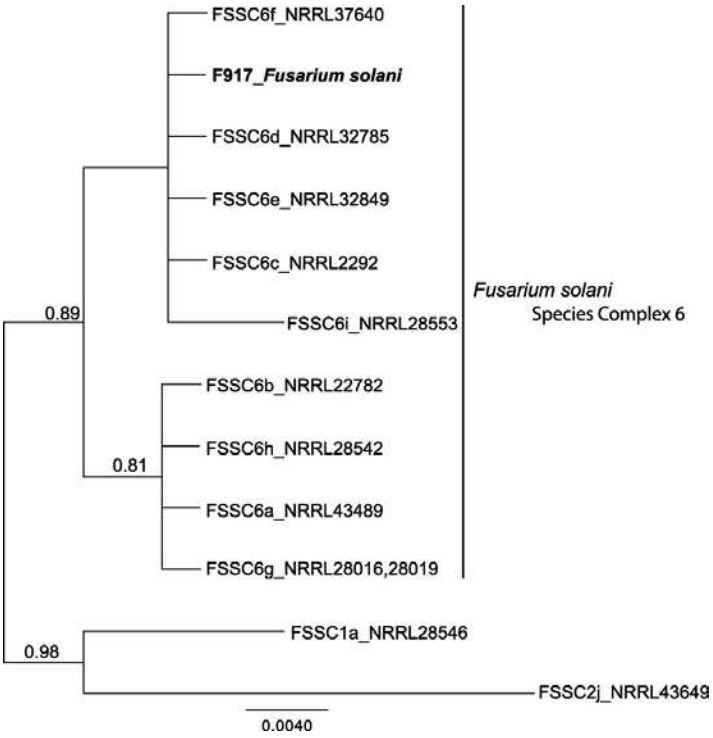

Figure 4.1. Bayesian phylogeny of the elongation factor 1 alpha (EF1α) including *Fusarium solani* isolate F917 (bold) and 10 *F. solani* sequences representing *F. solani* species complex (FSSC) 1, 2 and 6, obtained from the Fusarium-ID database (http://isolate.fusariumdb.org/). Posterior probabilities over 0.50 are labelled above each node. Phylogenetic Sequence identifications (NRRL numbers) are shown in the phylogeny.

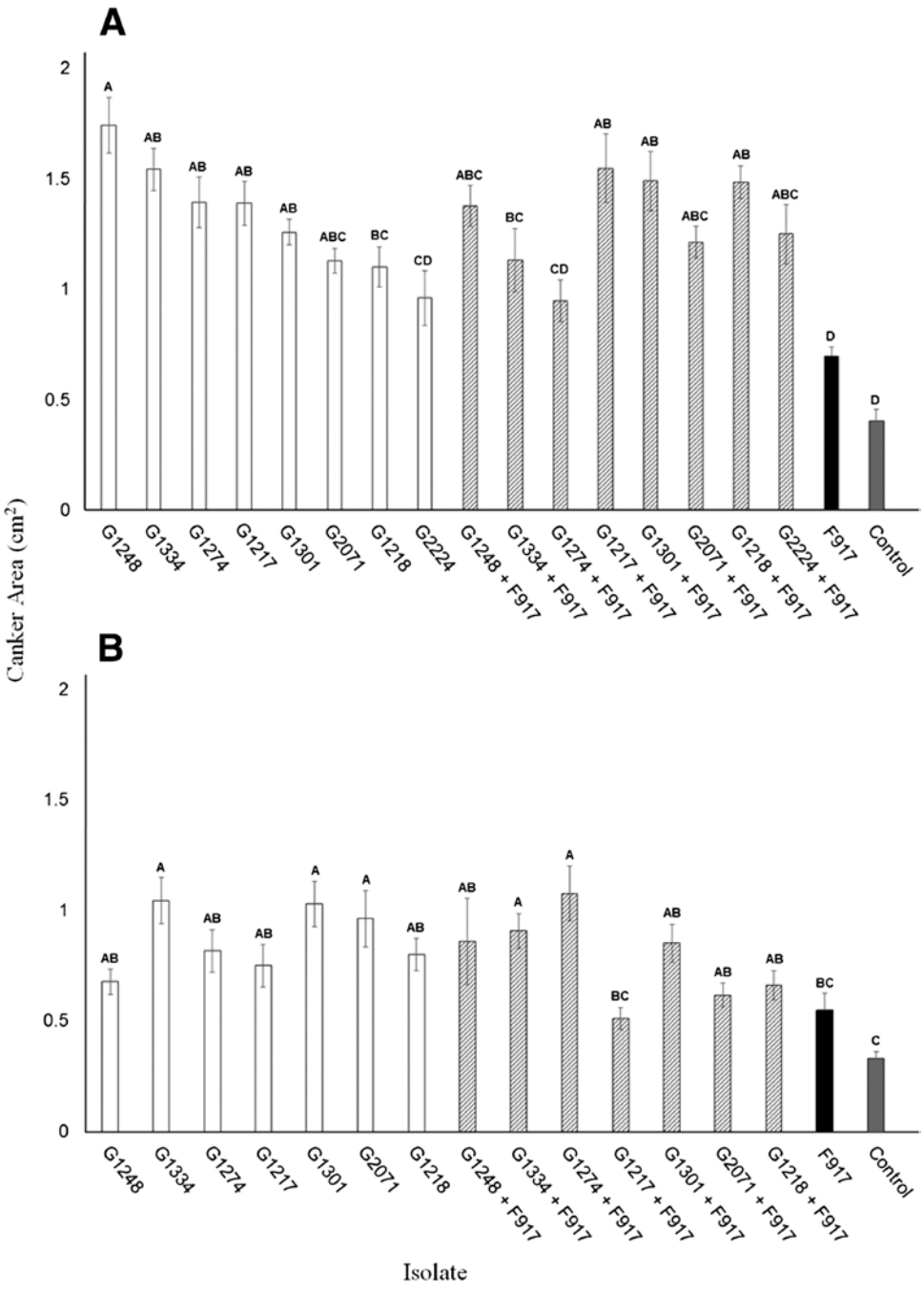

Figure 4.2A&B. A. 2014 Experiment. B. 2015 Experiment. Black walnut branches were inoculated with *Geosmithia morbida* (white), one isolate of *Fusarium solani* (black), *Geosmithia morbida* combined with *Fusarium solani* (hatched), or a ½ PDA control (grey). Canker areas were measured six weeks after inoculation. Differences in letter groupings indicate statistical differences among canker area means at the $P<0.05$ (LSD).

Supplementary Table 4.1. Hypothesized systemic induced resistance of black walnut (*Juglans nigra*) observed in previously wounded saplings. In June 2014, inoculations were performed on black walnut saplings in the greenhouse using inoculation methods similar to those described in this paper. The control treatment was inoculation of either *Geosmithia morbida* isolate G1217 or G2071. The wounding treatment trees were inoculated 6 weeks after wounding. For the analysis, canker isolate areas were combined, square root transformed, and a t-test was performed.

Treatment	***n*[a]**	**Mean Canker Area (cm^2) ± SEM[c]**
Control	8	1.37± 0.24
Wounding	8	0.74*[b] ± 0.11

[a] number of trees per treatment
[b] test statistic= -2.63
***** *P*=0.0216
[c] standard error mean

LITERATURE CITED

Appel, D. J., and T. R. Gordon. 1996. Relationships among pathogenic and nonpathogenic isolates of *Fusarium oxysporum* based on the partial sequence of the intergenic spacer region of the ribosomal DNA. MPMI-Molecular Plant Microbe Interactions 9(2):125-138.

Bonello, P., Gordon, T. R., and A. J. Storer. 2001. Systemic induced resistance in Monterey pine. Forest Pathology 31:99-106.

Carbone, I., and L. M. Kohn. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91(13):553-556.

Carlson, J. C., Mielke, M. E., Appleby, J. E., Hatcher, R., Hayes, E. M., Luley, C. J., O'Brien, J. G., and D. J. Rugg. 1993. Survey of black walnut canker in plantations in five central states. Northern Journal of Applied Forestry 10:10-13.

Čížková, D., Šrůtka, P., Kolařík, M., Kubátová, A., and S. Pažoutová. 2005. Assessing the pathogenic effect of *Fusarium*, *Geosmithia*, and *Ophiostoma* fungi from broad-leaved trees. Folia Microbiologica 50:59-62.

Forsyth, L. M., Smith, L. J. and Aitken, E. A. B. Aitken. 2006. Identification and characterization of non-pathogenic *Fusarium oxysporum* capable of increasing and decreasing Fusarium wilt severity. Mycological Research 110(8): 929-935.

Freeland, E., Cranshaw, W., and N. Tisserat. 2012. Effect of *Geosmithia morbida* isolate and temperature on canker development in black walnut. Online Publication. Plant Health Progress doi: 10.1094/PHP-2012-0618-01-RS.

Grant, J. F., Windham, M. T., Haun, W. G., Wiggins, G. J., and P.L. Lambdin. 2011. Initial assessment of thousand cankers disease on black walnut, *Juglans nigra*, in eastern Tennessee. Forests 2:741-748.

Gwinn, K. D., and A.M. Gavin. 1992. Relationship between endophyte infestation level of tall fescue seed lots and *Rhizoctonia zeae* seedling disease. Plant Disease 76(9):911-914.

Hadziabdic, D., Vito, L. M., Windham, M. T., and Pscheidt, J. W., Trigiano, R. M., and M. Kolařík. 2014. Genetic differentiation and spatial structure of *Geosmithia morbida*, the causal agent of thousand canker disease in black walnut. Current Genetics 60(2):75-87. Online publication. doi:10.1007/s00294-013-0414-x.

Hishinuma, S. M., Dallara, P. L., Yaghmour, M. A., Zerillo, M. M., Parker, C. M., Roubtsova, T. V., Nguyen, T. L., Tisserat, N. A., Bostock, R., Flint, M. L., and S. J. Seybold. 2016. Wingnut (Juglandaceae) as a new generic host for *Pityophthorus juglandis* (Coleoptera: Curculionidae) and the thousand cankers disease pathogen, *Geosmithia morbida* (Ascomycota: Hypocreales). The Canadian Entomologist 148(01):83-91.

Idnurm, A., and B. J. Howlett. 2001. Pathogenicity genes of phytopathogenic fungi. Molecular Plant Pathology 2(4):241-255.

Kistler, H.C. 1997. Genetic diversity in the plant-pathogenic fungus *Fusarium oxysporum.* Phytopathology 87(4):474-479.

Kolařík, M., Sláviková, E., and S. Pažoutová. 2006. The taxonomic and ecological characterization of the clinically important heterobasiodiomycete *Fugomyces cyanescens* and its association with bark beetles. Czech Mycology 58:81-98.

Kolařík, M., Freeland, E., Utley, C., and N. Tisserat. 2011. *Geosmithia morbida* sp. Nov., a new phytopathogenic species living in symbiosis with the walnut twig beetle (*Pityophthorus juglandis)* on *Juglans* in USA. Mycologia 103(2):325-332. doi: 10.3852/10-124

Kolařik M., Hulcr J., and L. R. Kirkendall. 2015. New species of *Geosmithia* and *Graphium* associated with ambrosia beetles in Costa Rica. Czech Mycology 67(1):29-35.

Kolařík, M., and L. R. Kirkendall. 2010. Evidence for a new lineage of primary ambrosia fungi in *Geosmithia* Pitt (Ascomycota: Hypocreales). Fungal Biology 114(8):676-689.

Kolařík, M., Kostovčík, M., and S. Pažoutová. 2007. Host range and diversity of the genus *Geosmithia* (Ascomycota: Hypocreales) living in association with bark beetles in the Mediterranean area. Mycological Research 111(11):1298-1310.

Kolařík, M., Kubátová, A., Hulcr, J., and A. Pažoutová. 2008. *Geosmithia* fungi are highly diverse and consistent bark beetle associates: Evidence from their community structure in temperate Europe. Microbial Ecology 55(1):65-80.

Kolařík, M., Kubátová, A., and P. Srutka. 2004. Morphological and molecular characterization of *Geosmithia putterillii*, *G. pallida* comb. nov. and *G. flava* sp. nov., associated with subcorticolous insects. Mycological Research 108(9):1053-1069.

Krokene, P., Solheim, H., and B. Långström. 2000. Fungal infection and mechanical wounding induce disease resistance in Scots pine. European Journal of Plant Pathology 106(6):537-541.

Lynch, S. C., Wang, D. H., Mayorquin, J. S., Rugman-Jones, P. F., Stouthamer, R., and A. Eskalen. 2014. First report of *Geosmithia pallida* causing foamy bark canker, a new disease on coast live oak (*Quercus agrifolia*), in association with *Pseudopityophthorus pubipennis* in California. Plant Disease 98(9):1276-1276.

Montecchio, L., and M. Faccoli. 2015. First record of thousand cankers disease *Geosmithia morbida* and walnut twig beetle *Pityophthorus juglandis* on *Juglans nigra* in Europe. Environmental Entomology 44:1455-1464.

Montecchio, F., Faccoli, M., Short, D. P. G., Fanchin, G., Geiser, D. M., and M. T. Kasson. 2015. First report of *Fusarium solani* Phylogenetic species 25 associated with early stages of thousand cankers disease of *Juglans nigra* and *Juglans regia* in Italy. Plant Disease 99:1183.

[NCDACS] North Carolina Department of Agriculture and Consumer Services. 2013. Haywood County wood products under NCDA&CS quarantine for thousand cankers disease. Retrieved January 9, 2013 from http://www.ncagr.gov/paffairs/release/2013/1-13-Thousand-Cankers-Quarantine-Haywood-County.htm

[ODNR/ODA] Ohio Department of Natural Resources and the Ohio Department of Agriculture. 2012. Walnut twig beetle now in Ohio County. Retrieved December 9, 2013 from: http://thevwindependent.com/?p=32540

[PDA] Pennsylvania Department of Agriculture. 2012. Thousand cankers disease description. Retrieved December 3, 2012 from http://www.portal.state.pa.us/portal/server.pt/gateway/PTARGS_0_2_24476_10297_0_43/AgWebsite/ProgramDetail.aspx?name=Thousand-Cankers-Disease&navid=12&parentnavid=0&palid=137&

Pitt, J. I., and A. D. Hocking. 2009. *Penicillium* and related genera. In: *Fungi and food spoilage* (pp. 169-273). Springer U.S.

Rasband, W.S. 1997-2016. ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/

Reed, S., J. T. English, J. Juzwik, and M. Ginzel. 2013. Bark and ambrosia beetles and their associated fungi colonizing stressed walnut in Missouri and Indiana. *Walnut Council Bulletin.* 01/2013, 40(2):17-19. Retrieved February 4, 2015 from http://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0CCAQFjAA&url=http%3A%2F%2Fwww.nrs.fs.fed.us%2Fpubs%2Fjrnl%2F2013%2Fnrs_2013_reed_001.pdf&ei=KnvSVLRs1LfIBMihgvAJ&usg=AFQjCNElawmSkScuLVeZJEEGPS6aAsSWbA&bvm=bv.85076809,d.aWw

SAS Institute. 2012. JMP statistics and graphics guide, version 10. SAS Institute, Cary, NC.

Schardl, C. L., Leuchtmann, A., and M. J. Spiering. 2004. Symbioses of grasses with seedborne fungal endophytes. Annual Review of Plant Biology 55:315-340.

Seybold S. J., Haugen D., O'Brien J., and A. D. Graves 2013. Thousand cankers disease. USDA Forest Service, Northeastern Area State and Private Forestry Pest Alert, NA-PR-02-10, originally published May 2010, reprinted Aug. 2010, Oct. 2011, and Feb. 2013.

Tisserat, N. 1987. Stem canker of black walnut caused by *Fusarium solani* in Kansas. Plant Disease 71: 557.

Tisserat, N. and W. Cranshaw. July 23, 2015. Personal communication, meeting notes. Colorado State University, Fort Collins, CO.

Tisserat, N., Cranshaw, W., Leatherman, D., Utley, C., and K. Alexander. 2009. Black walnut mortality in Colorado caused by the walnut twig beetle and thousand cankers disease. Plant Health Progress Online publication. doi:10.1094/PHP-2009-0811-01-RS.

Utley, C., Nguyen, T., Roubtsova, T., Coggeshall, M., Ford, T. M., Grauke, L. J., Graves, A. D., Leslie, C. A., McKenna, J., Woeste, K., Yaghmour, M. A., Cranshaw, W., Seybold, S. J., Bostock, R. M., and N. Tisserat. 2013. Susceptibility of walnut and hickory species to *Geosmithia morbida*. Plant Disease 97(5): 601-607.

[VDACS] Virginia Department of Agriculture and Consumer Services. 2011. VDACS - Plant and pest pervices - Thousand cankers disease. Retrieved November 17, 2011 from http://www.vdacs.virginia.gov/plant&pest/disease-tcd.shtml

Webber, J. F. 2000. 3 Insect vector behavior and the evolution of Dutch elm disease. The Elms: Breeding, Conservation, and Disease Management, 47.

Zabalgogeazcoa, I. 2008. Review. Fungal endophytes and their interaction with plant pathogens. Spanish Journal of Agricultural Research 6(Special Issue):138-146.

Zerillo, M. M., Ibarra Caballero, J., Woeste, K., Graves, A. D Hartel, C., Pscheidt, J. W., Tonos, J., Broders, K., Cranshaw, W., Seybold, S. J., and N. Tisserat. 2014. Population structure of *Geosmithia morbida*, the causal agent of thousand cankers disease of walnut trees in the United States. PLoS One 9(11):e112847. doi:10.1371/journal.pone.0112847.

CONCLUSION

Tree diseases are of major concern as they result in severe ecosystem and economic damage worldwide. The prevalence of emerging tree disease complexes, comprised of insect and microbial causal agents, are rapidly increasingly. Emergent insect-associated plant diseases are particularly threatening, as they are more likely to spread and to incur more damage than diseases caused by pathogens alone. Furthermore, insect-associated diseases are typically comprised of invasive organisms, and they also inflict economic loss, disrupt ecosystem services, and threaten the biodiversity of hardwood trees and the organisms supported by them. Therefore, it is critical to have a better understanding of these disease complexes in order to pave the way for management efforts of current and future emergent diseases.

This dissertation presents materials on two emergent tree diseases: drippy blight and thousand cankers disease. These diseases have microbe and insect causal agents that have shifted to new hosts and altered their life histories in order to become disease agents. Furthermore, both diseases can be considered invasive. For these reasons, drippy blight and thousand cankers diseases are ideal for addressing broader questions about disease interactions while simultaneously providing fundamental information about each disease.

Both of the emergent diseases noted in this dissertation have become injurious to hardwoods in communities across the urban corridor of Colorado. Although the hardwoods affected by this disease are intentionally planted in Colorado, these hardwoods are keystone species that naturally inhabit woodlands across the United States and other countries. Thus, by characterizing these diseases in Colorado, we may be able to help prevent the spread or lessen the environmental and economic impacts if they do spread.

This dissertation provided the basic biology of drippy blight disease. More specifically, it confirmed the causal agents of the disease, noted disease incidence and infected hosts, and described the signs and symptoms. Additionally, it presented the life cycle and common feeding locations of the kermes scale insect associated with the manifestation of drippy blight disease and examined the suite of insects present on drippy blight diseased trees for their role as potential disseminators. This information on drippy blight is essential for foresters making management decisions to combat this disease in Colorado. This research, along with the literature on the bacterium *Lonsdalea quercina* show that this bacterium is a threat to all groups of oaks. Therefore, the implications of this bacterium expanding its geographic range are unyielding which makes it increasingly important to study this emergent disease.

Overall, this work provides a foundation for future studies on drippy blight disease and other bacterial diseases related to drippy blight. Although there are many unresolved questions about this disease complex, several research avenues are precedent. For example, we still do not fully understand the relationship between the kermes scale insect and the bacterium. We know both causal agents are needed to cause drippy blight disease, but the same bacterium causes disease in California without the kermes scale. Therefore, future work should investigate the nature of their commensal relationship. Additionally, in order to quantify the risk of disease spread, it is imperative to identify the host range of the bacterium and kermes scale insect.

In the last chapter of this dissertation, thousand cankers disease of black walnut was introduced. This work investigated the virulence of *Geosmithia morbida,* the fungal causal agent of thousand cankers disease, and looked at its relationship with an additional fungus present on diseased trees (*Fusarium solani*). Overall, this chapter improves upon what is known about

thousand cankers disease by showing that all genetic clusters of this fungus are virulent. This finding givese us insight into the impacts disease spread.

It is becoming increasingly important to take a systems approach when studying disease complexes. In both drippy blight and thousand cankers diseases, the insect and microbe causal agents do not cause disease alone; they are dependent on each other. Historical investigations of disease complexes were approached from either a plant pathology or entomology point of view. Hopefully this work can help shift the current paradigm and begin looking at the causal agents of disease complexes together. Ultimately, this would alleviate the long-term argument of whether the insect or the pathogen is more important, and in turn allow us to recognize the strength of their interactions. Therefore, my goal is to continue asking questions that integrate microbe insect interactions in order to strengthen our knowledge of emergent diseases.