THE PATHOGENICITY OF *DIPLODIA CORTICOLA* AND *DIPLODIA QUERCIVORA* ON OAK SPECIES OF THE SOUTHEASTERN COASTAL PLAIN: A HOST-RANGE STUDY

By

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To Wendy and Dave
Best Buds

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Abstract of Thesis Presented to the Graduate School
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A host-range study was conducted in 2011 and 2012 on 29 species of oaks, along with four cultivars and two seed sources of live oak (*Quercus virginiana* Mill.) commonly grown on the Southeastern Coastal Plain of the United States, to assess the pathogenicity of two presumed strains of the ascomycete *Diplodia corticola*. Two isolates with 99% and 97% homology with the ITS region of *D. corticola*, dubbed "J" and "P," have been discovered in Florida, which vary by 10 nucleotides in the ITS region. Inoculations were performed on the stem and Koch's postulates completed. All controls developed callus and healed. Only one of the 382 trees inoculated with either pathogen formed callus, in contrast, and none healed. All inoculated trees developed long vascular necroses which also girdled the stem to varying degrees, most exhibited stem bleeding and pycnidia, and some died. Stem diameter was negatively correlated with lesion length, and red oaks (section Lobatae) had both significantly longer lesions and more girdling than white oaks (section Quercus). During preparation of this thesis, a new species, *Diplodia quercivora*, was identified, with which isolate P has 99% homology in the ITS and EF-α regions. Results support a conclusion that both fungal species inhibit wound repair in oaks.

CHAPTER 1
INTRODUCTION

In September 2010, the University of Florida Forest Pathology Laboratory received reports of dieback and cankers[1] on small branches of landscape live oaks (*Quercus virginiana* Mill.) on a horse farm in Marion County, Florida. Isolations from the cankers led to identification of the pathogen *Diplodia corticola* A.J.L. Phillips, Alves & Luque, sp. nov., by comparison of the internal transcribed spacer (ITS) and beta tubulin (βt) nucleotide sequences with sequences deposited in Genbank (16). A review of the literature revealed that *D. corticola* had been identified as the primary cause of a canker disease causing serious decline of the cork oak forests (*Quercus suber*) in Mediterranean countries (2).

In December 2010, a first report appeared in *Plant Disease* documenting *D. corticola* in California, and establishing the pathogen as a factor in the widespread mortality seen since 2002 in coast live oaks, *Quercus agrifolia* Nee, in San Diego County (30). This report prompted a statewide survey in Florida for declining live oaks, leading to identification of *D. corticola* from several regions of the state (Figures 1-1 and 1-2). Unlike the situation in California, disease incidence in Florida has been almost entirely limited to landscape plantings. It has not been documented in forests.

The symptoms reported in response to the survey have fallen into two general categories. The first is characterized by dieback of outer twigs throughout the crown, giving the canopy a sparse appearance. The dead twigs frequently bear cankers as well as black pycnidia, which are flask-shaped fruiting bodies of the fungus. The second is distinguished by localized flagging of

[1] Cankers are "localized wounds or dead areas in the bark of the stem or twigs of woody or other plants that are often sunken beneath the surface of the bark." Dieback is the "[p]rogressive death of shoots, branches, and roots, generally starting at the tip" (1).

large branches randomly distributed in the crown, also frequently bearing cankers and pycnidia, with or without twig dieback.

Molecular studies of fungi cultured from these affected trees in Florida revealed the existence of two presumed strains of *Diplodia corticola*, referred to here as "P" and "J," which differ at 10 nucleotide sites in the ITS region. "P" was isolated from a declining live oak in a grocery store parking lot in Gainesville, and "J" from a condominium complex in Jacksonville.[2] Isolate P corresponded with the first syndrome described above, and isolate J with the second. Since the first submission of this thesis, the ITS sequence of a species newly identified on three oak species in Tunisia has been deposited in GenBank, with which the ITS and EF-α regions of isolate "P" have 99% homology (Dreaden, T., personal communication). The new species has been named *Diplodia quercivora* (24). It will continue to be referred to as *Diplodia corticola* isolate P in this thesis.

The research objectives of this study were to assess the susceptibility of various members of the family Fagaceae to these two emergent and closely related pathogens, P and J, by completing Koch's postulates. The host tree species selected are native to or commonly cultivated on the coastal plain of the Southeastern United States. A first study was conducted in 2011 on eleven oak species native to Florida, including a Florida seed source of live oak (*Q. virginiana* Mill.). An expanded study was conducted in 2012-13 on an additional 17 species of oaks commonly grown in the Southeastern U.S., including an Asian species (*Quercus acutissima*); four cultivars of live oak; a live oak grown from a Louisiana seed source; and *Castanea pumila*, a non-oak member of family Fagaceae.

[2] "P" is culture no. PL 1345, and "J" is culture no. PL 1325, in the UF Forest Pathology Lab.

Figure 1-1. Live oak (*Q. virginiana*) showing dieback. Photo: Jason Smith, Univ. of Florida

Figure 1-2. Cankers on infected live oak branch. Photo: Tyler Dreaden, University of Florida

CHAPTER 2
LITERATURE REVIEW

An Emergent Pathogen on Oaks and Grapevines

Since the 1960's, the ancient cork oak forests in Spain and Portugal have been declining, a condition which accelerated dramatically in the 1980's (27, 2). In 1989, the fungal pathogen believed to be the primary cause was identified as *Botryosphaeria stevensii* Shoemaker (27, 36). In 2004, the causative organism was re-evaluated and recognized as a new species, *Botryosphaeria corticola* A.J.L. Phillips, Alves & Luque, sp. nov. (2). The name has since been changed to *Diplodia corticola*, referring to its anamorph (asexual) stage (17). Renaming is due to reorganization of the genus *Botryosphaeria* to exclude species having pigmented conidia at maturity. The pigmented species were thus placed in the genera *Diplodia* and *Neofusicoccum* (12, 13).

Since its identification as the cause of cork oak mortality (2), *D. corticola* has also been implicated in the die-off of other species of European oaks, a malady which had been referred to as "oak decline" (36, 8, 25). In 2010, *Diplodia corticola* was then identified as the cause of rapidly spreading dieback and occasional death of live oaks in California and Florida (*Q. agrifolia* Nee; *Q. chrysolepis* Liebm.; *Q. virginiana* Mill.), as well as grapevine mortality in Texas, California, and Mexico (16, 42, 43, 30, 29, 9). A more in-depth examination of the literature has revealed that the pathogen might, in fact, have originated in North America and been identified as the cause of decline of chestnut oak (*Q. prinus*) and other oak species in Pennsylvania in 1912 (45, 22, 40, 6).

Although referred to as "Bot canker," that term was already commonly used for diseases caused by other members of the family Botryosphaeriaceae, which are generalist pathogens on

hundreds of plant genera (7, 14, 21). *Diplodia corticola*, in contrast, has been found only on oak and grapevine.[1]

The Botryosphaeriaceae are known to enter plants through wounds, including leaf scars, but can also enter through stomata and lenticels open for gas exchange (46, 7). These fungi are spread by air, water splash, or contaminated pruning tools (7). The traditional--and environmentally sustainable--enterprise of removing cork from oak trees[2] causes mild injury, thus providing entry for *Diplodia corticola* into *Quercus suber* trees (18). In addition, the Botryosphaeriaceae often live harmlessly as endophytes within plants, but are believed to become pathogenic when the plant is stressed by environmental factors such as drought, heat, freezing, herbicide use, or soil compaction (7, 25).[3]

In California, *Diplodia corticola* has been implicated in the death of "tens of thousands of acres of coast and canyon live oaks since 2002," commonly occurring with other, less aggressive pathogens (29). There is equivocal evidence that the fungus may be vectored in California by insects. *D. corticola* was isolated from coast live oak trees, both living and dead, that had been colonized by bark and ambrosia beetles in Marin County following artificial inoculations with *Phytophthora ramorum* (32). The exotic gold spotted oak borer, *Agrilus auroguttatus* Schaeffer

[1]From GenBank sequences, Alves *et al*. (2) identified *D. corticola* on two other hosts: Eastern Redbud, *Cercis canadensis* L., in the District of Columbia, and *Tsuga* sp. in North Carolina. Both sequences were deposited by Jacobs in 1998. *Cercis canadensis* is listed as a host of *D. corticola* also in Table 2 of Slippers *et al*. (41) (GenBank accession number AF027752), but *Tsuga* (GenBank accession number AF02755) is not. The USDA Fungus Host database includes *C. canadensis* as a host (17).

Botryosphaeria stevensii (anamorph: *Diplodia mutila*) has now been determined not to infect *Quercus*.. Thus, prior reports of *B. stevensii* infecting oaks must be interpreted as referring to *Diplodia corticola*, instead (Luque, J., personal communication). Affected European oak species from which *B. stevensii* was identified, which are not listed as hosts in GenBank because the identifications predated molecular characterization, are: *Q. cerris* L., *Q. ilex*, *Q. petraea* (Matt.) Liebl., *Q. pubescens* Willd., *Q. robur* L., and *Q. trojana* Webb. *See* citations in (28).

[2] "Cork harvesting," http://www.youtube.com/watch?v=ztr-RP0XYd8 (accessed 6-27-13).

[3] Luque *et al.* (26) note that it may be the other way round: that infection by a pathogen usually negatively affects water transport in the tree, in the absence of drought, as well as that the symptoms of pathogenic infection resemble those of drought stress in uninfected trees.

(Coleoptera: Buprestidae) ("GSOB"), has also been associated with the colonization of coast live oak by *D. corticola*; however, trees *not* infested by the GSOB are also infected by *D. corticola*, and declining. Attack by the GSOB increases drought stress in the trees, possibly awakening the latent fungal pathogen already in residence (29, 30, 10). Lastly, a report of lesions and branch dieback in Virginia in 1959 in *Q. prinus* and *Q. alba* found infections by the fungus *Dothiorella quercina* (Cke. & Ell) Sacc. often occurring in association with scale insects (6). Vajna, in 1986, concluded *D. quercina* was *Diplodia corticola* (45).

Surveys in Florida have most often found symptomatic live oaks (*Q. virginiana*) in landscape plantings, and most reports have concerned the cultivar 'Cathedral'. Oaks in landscape settings, unlike those in natural forests, are exposed to various stresses such as transplanting, pruning, and herbicides. Most of Florida also experienced severe drought in 2010, the same year the decline was first reported.

Reported Symptoms of *D. corticola* Infection in Oak

In the cork oak in Europe (*Q. suber*), discolored bark is the first disease symptom, appearing 2-3 months after cork removal (8). In the early period following infection there may be foliar yellowing and discoloration, as well as epicormic shoot development (shoots which develop from buds under the bark) (8). After approximately six months, the tree develops necroses in the cambium of varying lengths (cankers) on branches and the trunk, and bark comes off easily. Wilting follows due to loss of vascular function and pycnidia are observed in and around necrotic areas. Death of the tree, if it occurs, usually comes between one and three years after symptoms are first noted (27).

Infection by *D. corticola* in mature live oak trees in California and Florida is not visible in the early stages, since it is not preceded by cork removal. In Florida, clumps of dead branches appear randomly distributed in the crown (16). Cankers are evident on branches, and cutting into

the branch at these points reveals sapwood streaking and phloem necroses (dark brown or black discoloration) (Figure 1-2). Although trunks are frequently cankered in California (29), only once have cankers been found on a trunk in Florida. Bleeding sap and bark cracking are observed with the branch cankers, along with pycnidia, which erupt through the bark or cankers. In artificial inoculations of coast live oak seedlings with *D. corticola* in California, Lynch *et al*. (29) also reported epicormic shoots, and leaf desiccation. They report that *D. corticola* "killed the xylem in advance of the living phloem and moved into the taproot on 70% of inoculated seedlings."

Luque *et al*. (28) conducted a study of the effects of 38 different fungal species isolated from cork oaks in Catalonia (Northeastern Spain) on *Q. suber* by inoculating stems, leaves, and roots under two watering regimes, one adequate and the other water-stressed. Among the inocula was *Botryosphaeria stevensii* Shoemaker (*Diplodia corticola* on oak). In contrast to the later findings of Lynch *et al.* (29), Luque *et al*. (26) found that *D. corticola* was not pathogenic on roots or leaves. On stems, however, *D. corticola* was the most virulent of the pathogens tested, not only inducing long cankers and vascular lesions, but causing death of almost all plants (one-year-old cork oak seedlings) within two weeks of inoculation. Notwithstanding reports such as those cited above--that plants become more susceptible to infections by Botryosphaeriaceae during drought--the Luque group determined that the length of lesions caused by the *D. corticola* was independent of water stress in the plant.

Mechanism of Host Death.

Cork oaks and holm oaks (*Q. ilex*) artificially infected with *D. corticola* by Linaldeddu *et al*. (25) exhibited significant reduction in net photosynthetic rate and stomatal conductance. Because the impact of the infection on gas exchange rates was independent of stem lesion length in both species, the authors suggested the cause was "diffusible toxins."

Diplodia corticola produces several phytotoxins as secondary metabolites. The most important appears to be the compound diplopyrone, which is toxic to cork oak at concentrations from 0.01 to 0.1 mg/mL, and causes collapse of internal stem tissue on tomato cuttings at 0.1 to 0.2 mg/mL (31). Other pathogenic *Botryosphaeria* species also produce plant toxins.[4] It is unclear from these reports which of the other metabolites are phytotoxins or whether they become phytotoxic acting synergistically.

Fungal Morphology.

Identification of *D. corticola* based on morphology is problematic, as is the *Diplodia* genus generally. There is a substantial history of misidentification. Different lighting conditions or temperatures have different effects on development and pathogenicity (38), and members of the Botryosphaeriaceae are notoriously similar one to another, with closely related species often inhabiting the same plant (42). In some reports, for example, in culture on potato dextrose agar, *Diplodia corticola*, when viewed from above, initially appears fluffy white, turning to dark gray after five to seven days, while the underside turns olive-green, then black (16, 2). The description of "abundant gray mycelium with a diurnal zonation that gradually became dark olivaceous" was assigned to *D. corticola*'s cousin *Neofusicoccum mediterraneum*, however, where both pathogenic species were isolated from the same sample of grapevines in Spain, while *Diplodia corticola* was described as "whitish, dense, aerial mycelium [which] remained white up to 10 days on PDA and darkened to *gray* thereafter" (35).

[4] *Botryosphaeria obtusa*, for example—which causes black rot on apple fruit and frogeye leaf spot on apple and other plants—produces a veritable smorgasbord of phytotoxins: mullein, tyrosol, 4-hydroxymellein, 5-hydroxymellein, 7-hydroxymellein, 4,7-dihydroxymellein, and 4-hydroxybenzaldehyde. This production is puzzling on the fruit, which is undefended tissue. *B. obtusa* also does not require a wound for entry. It enters through lenticels and stomata. (46,15).

Identification of particular structures, generally by microscopy, although also a challenge provides an additional tool to differentiate these species. *Diplodia corticola* is an ascomycete with both sexual and asexual spore stages. The sexual stage, or teleomorph, is rarely found in nature, and in many cases the teleomorphic characters are too similar to distinguish among species in the Botryosphaeriaceae (23). Thus, the anamorphic (asexual stage) features, conidia and conidiophores, are used, since size, shape, color, wall thickness, and septation in these structures are often distinct. They are described for *D. corticola* as follows:

> *Conidiomata* pycnidial, separate or aggregated, globose, dark brown to black, immersed, multilocular, thick-walled; outer wall layers thick-walled *textura angularis*, inner layers thin-walled, hyaline; ostiole central, papillate. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* holoblastic, integrated or discrete, determinate, cylindrical, hyaline, smooth, forming a single, apical conidium, proliferating percurrently to form one or two indistinct annelations, or proliferating at the same level to form periclinal thickenings. *Conidia* smooth, unicellular, cylindric with broadly rounded ends, some with a large central guttule, smooth, with a thick glassy wall that remains hyaline even after the conidia have been discharged from the pycnidium, rarely becoming light brown and one- or two-septate after discharge (23.5–)26–35(–41) · (10–)11–17(–18.5) µm, average of 250 conidia = 29.9 ± 2.6 × 13.6 ± 1.4 µm. Length/width ratios in the range of (1.6–)2.2–2.3(–3.1) with a mean and standard deviation of 2.2 ± 0.3.
>
> (11).

The length-width ratio of approximately 2.2 of the conidia of *Diplodia corticola* is what primarily distinguished it from *Neofusicoccum mediterraneum*, the conidia of which have a length-width ratio of about 4.0, in the example above (35).

CHAPTER 3
METHODS AND MATERIALS

The impetus for this host range study was the discovery of two strains of *Diplodia corticola*, "P" and "J," which were isolated from cankers in live oaks, characterized molecularly, and found to be somewhat different. The study, which took place in 2011 and 2012, was to assess the effects of the two isolates on different oak species native to, or grown commonly in, the Southeastern U.S. The experiment involved inoculation of (usually) 12 trees per species with one of the two fungal isolates, P and J, in the greenhouse, and isolation of the fungal strains from the plants after disease developed, pursuant to the design described below.

Isolation and Characterization of Fungal Strains P and J

Samples approximately 4 mm square in size were taken with sterile razor blades from the margins of cankers on small branches from declining live oaks (*Quercus virginiana* Mill.) in Jacksonville, Florida, between August 8 and 12, 2011. Samples were similarly taken from declining live oaks growing in a grocery store parking lot in Gainesville, Florida, first in 2010 and again for this host range study on Sept. 20, 2011. These samples were surface-sterilized in a 50% solution of 8.25% sodium hypochlorite (regular bleach) for 30 sec, the drops shaken off, and three to four placed per plate on acidified potato dextrose agar (APDA). Cultures were kept in the dark at approximately 25 ºC for 7-10 days, after which time each morphologically different fungus on the plates was separately subcultured on APDA.

A tentative identification as *Diplodia corticola* was made of those subcultures matching the published description of this organism in culture. Salient features included initially white mycelia which turned dark grey-olivaceous on top, with the underside black, after 5-10 days (2). Molecular confirmation that both were *D. corticola* was obtained by extracting DNA from mycelia with use of the DNeasy Plant Mini Kit (Qiagen). PCR (Polymerase Chain Reaction)

was run using primers ITS 1 and ITS 4. After confirming the presence of DNA amplicons at approximately 650 bp, 5 µL of PCR product was purified with 2 µL of Exosap (Affymetrix) and sequenced at the University of Florida's Interdisciplinary Center for Biotechnology Research.

The ITS sequences were aligned and edited using Geneious Pro 5.6.6 software. A BLASTn search revealed that the nucleotide sequences from two isolates matched sequences of *Diplodia corticola* deposited in GenBank to different degrees. The ITS sequences of these isolates had 99% and 100% homology with the ITS region of *Diplodia corticola* isolate CBS 112074 (GenBank Accession No. AY268421), which is the type specimen (2). The βt (beta tubulin) regions of the two isolates had 99 and 97% homology with the βt sequences of *D. corticola* strain UCD2397TX (Gen Bank Accession No. GU294724), and *D. corticola* strain CBS 112550 (Gen Bank Accession No. AY259097.1), respectively. The isolates were then dubbed "J" and "P." Aligned with each other, their ITS regions differ at 10 nucleotide positions.

These subcultures, of J and P, were stored on slants of APDA at 5 °C. New subcultures were prepared from them and used as the inoculum source in the 2011 study.

Despite the similarity of their DNA sequences, there are differences between the two isolates in pure culture when they are grown in the dark at 25 °C. J fills the plate rapidly and begins piling up aerial mycelia around the edges. While P also has an initial rapid growth rate, it drastically slows before filling the plate entirely. The colony margin is wavy and clearly defined, usually keeping a space between it and the plate edge, and the colony is appressed, of denser texture, and darkening much sooner than J (Figures 3-1 A – F).

Plant Materials

2011 Study

In October 2011, saplings of indeterminate ages in each of 11 species of oaks from commercial seed sources were acquired from two nurseries near Gainesville and Orlando,

Florida. These species, followed by abbreviations used to designate them, were: *Quercus virginiana* from a Florida source (QVA); *Quercus shumardii* (QSH); *Quercus alba* (QAL); *Quercus michauxii* (QMI); *Quercus laurifolia* (QLA); *Quercus austrina* (QAU); *Quercus falcata* (QFA); *Quercus margaretta* (QMA); *Quercus geminata* (QGE); *Quercus chapmanii* (QCH); and *Quercus myrtifolia* (QMY).

The trees were acclimated over several weeks, then arranged in two randomized north/south blocks on the floor of a campus greenhouse at the University of Florida in Gainesville, FL (lat. 29.6514°, long. -82.3250°, 35.052 a.s.l.) Each block contained eight trees per species, for a total of 16, with the exception of *Quercus geminata* (QGE), *Quercus margaretta* (QMA), and *Quercus falcata* (QFA), due to limited availability. There were eight QGE, QMA, and QFA trees total, and those were distributed randomly within the first block only.

All trees were in 3-gal pots, except for QFA, which was in 1-gal pots. Trunk diameter ranged from 7 mm in the 1-gal pots to 3 cm in 3-gal pots at the inoculation site, which was approximately 5 cm above soil level, except for QFA, which had the smallest stems, so was inoculated approximately 2 cm above soil level.

Temperatures ranged from 19 °C to 29 °C throughout the experiment, and the trees were irrigated daily to field capacity.

2012 Study

The second study began in August 2012 with 19 different species from the family Fagaceae from commercial seed sources, including a live oak, *Q. virginiana,* from Louisiana, along with four *Q. virginiana* cultivars. These were: *Castanea pumila* (CPU); Q*uercus acutissima* (QAC); *Quercus bicolor* (QBI); *Quercus coccinea* (QCO); *Quercus hemisphaerica* (QHE); *Quercus incana* (QIN); *Quercus inopina* (QIO); *Quercus palustris* (QPA); *Quercus laevis* (QLV); *Quercus lyrata* (QLY); *Quercus macrocarpa* (QMC); *Quercus marilandica*

(QML); *Quercus muehlenbergii* (QMU); *Quercus nigra* (QNI); *Quercus nuttallii* (QNU); *Quercus phellos* (QPH); *Quercus pumila* (QPU); *Quercus rubra* (QRU); *Quercus virginiana* (Louisiana seed source) (QVL); *Q. virginiana* 'Highrise' (QVH); *Q. virginiana* 'Cathedral' (QVC); *Q. virginiana* 'Augustina' (QVU); and *Q. virginiana* 'Sky Climber' (QVS).

Again the trees were arranged in two blocks of eight trees per species, except that CPU was short two trees in the second block; QIO was short two trees in each block; QNI was short one tree in the first block; and QNU was short one tree in the second block. The trees were completely randomized within each block. All experimental parameters in 2012 were as in 2011, except that *Q. inopina* and *Q. incana*, the smallest trees, were inoculated in the same stem location as *Q. falcata*. Some of the *Q. virginiana* cultivars and *Castanea pumila*, and all the *Q. laevis*, were in "Accelerator" pots (Nursery Supplies, Inc.), which have holes on the sides, making their rootballs more susceptible to drying than the other pots. These are shown in Table 4-1. *Q. pumila* spreads via rhizomes (stems branching from shallow, laterally growing roots), so had multiple small-diameter stems rather than a single trunk. The *Q. inopina* and *Q. incana* trees also were small and shrubby, several having multiple stems.

Pathogenicity Tests

In each block, three trees per species were inoculated with isolate J and three with isolate P,[1] with two trees serving as controls inoculated with sterile APDA. In the 2011 study, inoculations of the first block, 79 trees, took place Oct. 11, 2011, and inoculations of the second block, also 79 trees, took place Oct. 27, 2011. In the 2012 study, inoculations of the first block, 181 trees, took place Sept. 21, 2012, and of the second block, 179 trees, on Oct. 16, 2012.

[1] Exceptions were CPU, which had only two of each isolate in the 2nd block; QIO, which had only two of each isolate in each block; QNU, which was short a "J" in the second block; and QNI, short a "P" in the first block.

In both the 2011 and 2012 studies the inoculum consisted of an approximately 4-mm^2 plug of APDA, colonized by mycelia, removed with a razor blade from a two-week-old subculture of P or J. The plug was placed, with the mycelium side facing inward, inside a flap approximately 2.5 cm-long cut in the stem cambial tissue with a sterile single-edged blade approximately 5 cm up from soil level. The flap was then pressed back against the wood with the inoculum inside, and wrapped with Parafilm to bind it tightly against the stem.

Intermediate external disease assessments were done in the 2012 study, on Nov. 12, 2012, for block 1 and Dec. 6, 2012, for block 2, noting the presence of bark cankers, fungal fruiting bodies, stem bleeding, whether necrosis extended beyond the flap, and other signs of distress such as branch flagging, leaf desiccation, epicormic shoots, or death. Cankers were measured on the bark vertically, as well as horizontally (by estimating the percentage of stem girdling). Intermediate assessments were not done in the 2011study.

Final destructive sampling for the 2011 study took place approximately three months after inoculations, and, for the 2012 study, four months after inoculations. Prior to the final assessments, external signs and symptoms, as described above, were recorded and the internal extent of phloem necrosis was measured. In the destructive sampling itself, several measures of disease were employed. These included length of necrosis in phloem; percent girdling; presence of pycnidia; stem bleeding; whether or not the flap was dead; and whether necrosis extended beyond the flap. In 2012 xylem discoloration was measured separately from phloem necrosis. (In 2011, xylem discoloration and phloem necrosis were not distinguished.) Dead trees were retained in the dataset, with an artificial value assigned for lesion length equal to 110% of the longest lesion measured for that species-isolate pair on a living tree in that block (19). Girdling was naturally at 100% in the dead trees.

To complete Koch's postulates one symptomatic tree per isolate/species/block was chosen for sampling, to reisolate the pathogen. Sampling was done at canker margins as far away from the inoculation site as possible to avoid any inoculum that might have remained within the flap without colonizing the host tissue. No isolations were made from controls, nor from any trees prior to inoculation. The tissue samples were cultured and the morphology in cultures was examined visually to distinguish between isolates P and J. DNA extraction and PCR were also performed via the methods described in the first section of this chapter, "Isolation and Characterization of Fungal Strains," with an important difference. By the time these isolations were made, Ph.D. student Tyler Dreaden had developed forward primers "Dcort 1" and "Dcort1A," and reverse primer "Dcort3," which selectively amplified the J and P strains of *Diplodia corticola* (and did not amplify other fungi). The amplicons were then trimmed with a restriction enzyme, Msel, which produced different-sized restriction products, so they could be differentiated (Dreaden, T., personal communication).

Experimental Design

A complete randomized block design was used. There were two blocks in 2011 and two blocks in 2012, a "block" usually consisting of eight trees of each species tested in that year. The exceptions were QGE, QMA, and QFA in 2011, which had only eight trees total, so four per block; and the 2012 exceptions noted in n.1, above. Because equal numbers were not used for all species, the design was not balanced.

Statistical Analysis

The purpose of the experiment was to evaluate the effects of the two *Diplodia corticola* isolates P and J on different oak species. The null hypotheses were that there would be no significant difference in the mean responses of (1) any one tree species to the two fungal isolates; (2) any two tree species to the same fungal isolate; (3) any one combination of species*isolate

with any other combination of species*isolate; and (4) any inoculated tree and a control of the same species.

Statistical processing was performed via JMP 8 (SAS Institute, Cary, NC), and data from the two study years were analyzed separately. Blocks were ignored, since the conditions between blocks were very similar.

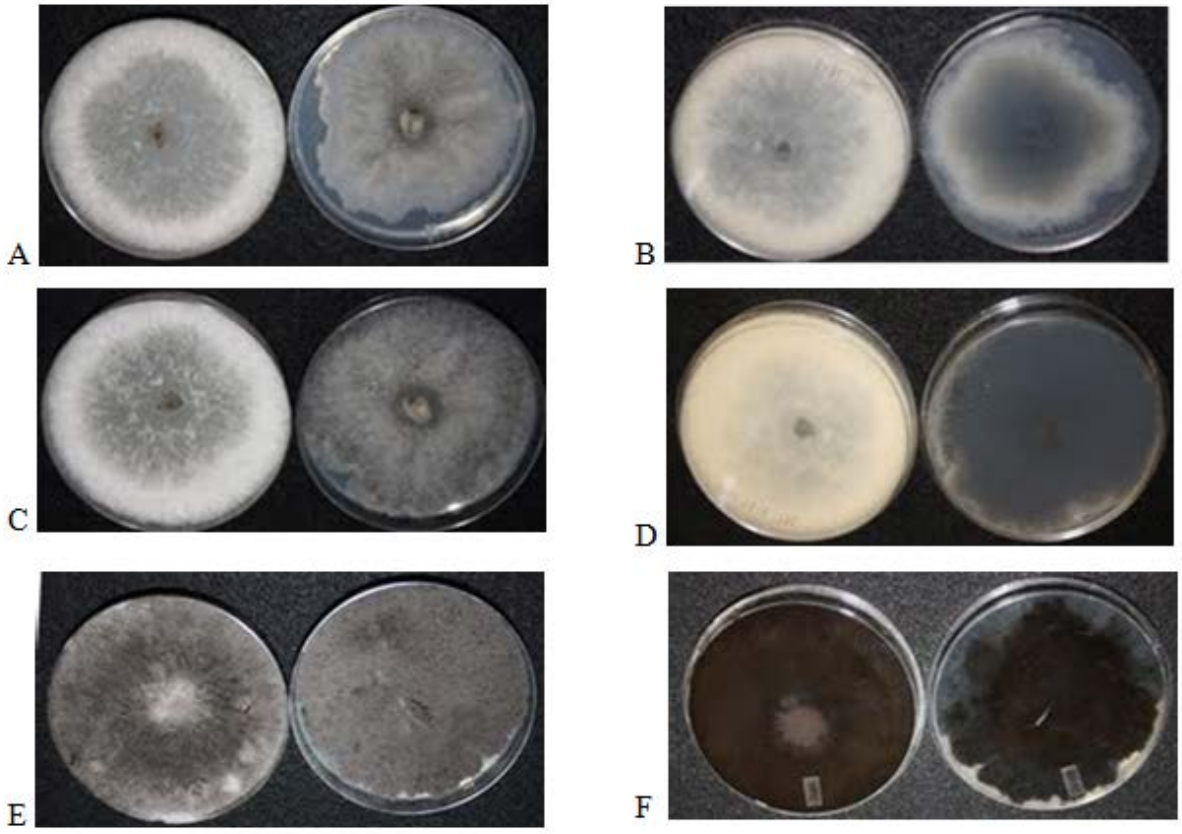

Figure 3-1. Isolates J (left) and P (right) in culture. A) 5 days old, top; B) 5 days old, bottom; C) 9 days old, top; D) 9 days old, bottom; E) 30 days old, top; F) 30 days old, bottom (appearing browner in photo than it was). Photos and cultures: Adam Black, UF

CHAPTER 4
RESULTS

Differences in Wound Response between Infected Trees and Controls.

There were significant differences in wound response between infected trees and controls. All controls formed callus, which is tissue formed by a tree during the healing process (1), ringing the wound. In contrast, callus did not form in 381 of 382 trees inoculated with either fungal isolate. The one inoculated tree that did form callus was a *Q. michauxii* inoculated with isolate P in block 1, in 2011; and the fungus was reisolated from the callus. Fisher's Exact Test yielded P <.0001 for each study regardless of isolate, meaning that there was a statistically significant difference in the ability of trees of all species inoculated with either *D. corticola* or *D. quercivora* to heal wounds, compared to controls.

There were also no lesions on controls, while lesions were produced both vertically and horizontally (around the stem between 15 and 100%) on every tree inoculated with the pathogen.

Tissue created by the wound in the bark (the "flap") remained alive and green in all controls except in the live oak cultivar 'Highrise' in 2012. In both controls of that cultivar in block 1, the flap died, but the tissue underneath healed. In contrast, in every tree inoculated with either strain of *D. corticola*, save five individuals—thus 377 trees--the flap died in its entirety, except for two cases where part of it was still alive at the end. This difference compared to controls was again significant for both studies, with P < .0001. The exceptions were three *Q. shumardii* inoculated with J in block 1; a *Q. virginiana* inoculated with J in block 2 (both 2011); and a *Q. virginiana* 'Augustina" inoculated with J in 2012 (Table 4-1). The dead flap on inoculated trees was black, the necrosis almost always extending beyond the wound in both directions. The dead flap was also the site where pycnidia were most often found.

Stem bleeding (sap exudation) occurred on none of the controls, with the exception of the two *Quercus lyrata* controls in block 1 in 2012, which bled briefly, then healed. Stem bleeding--of a translucent amber-colored exudate--did occur, profusely, on all but one tree inoculated with isolate P and one with J, in 2012, and all but 18 trees inoculated in 2011, as shown in Table 4-1. These results were again statistically significant, with no difference in the ability of J or P to induce stem bleeding.

Controls were not sampled for the presence of *D. corticola,* so it is unknown whether the organism could be found naturally in any of these species, as an endophyte. If present, it did not cause disease, even though these trees were wounded.

Finally, fruiting bodies or mycelia of fungi which were *not Diplodia corticola* appeared at the wound sites of several inoculated trees, but never on controls. Only in the case of *Q. phellos* was the fungus cultured and sequenced. It was bright orange with a rubbery texture initially, fading to salmon, then buff, which appeared on two of the *Q. phellos* trees inoculated with J, and four inoculated with P, finally disappearing altogether. It was cultured, subcultured, and sequenced. There were two products, having 100% homology with *Pestalotiopsis photinia* and an unidentified *Fusarium* species.

Relative Susceptibility of Oak Species to the Two Isolates

Categorical Variables of Disease Assessment (Visual Observations)

All host species and cultivars challenged with either isolate of *D. corticola* developed stem lesions extending both upwards and downwards from the inoculation site. Representative photographs are presented in Figures 4-1 – 4-3. The specific strain of *Diplodia corticola* with which a tree was inoculated (P or J) was reisolated from one symptomatic tree per isolate/ species/block at the end of the experiment in both 2011 and 2012, with two exceptions: QLA-J, which did not show up, and QMI-J, for which a culture was not made.

In 2012, symptoms appeared within two weeks of inoculation on most species. Certain northern species in the red-oak group, such as *Quercus palustris* (pin oak) and *Quercus rubra* (red oak), exhibited rapidly progressing external symptoms. By the end of the study one red oak tree, a *Quercus phellos* (willow oak), had produced a 46-cm-long phloem necrosis with a 46-cm-long xylem discoloration, and another had a 49-cm-long xylem discoloration (in connection with a 2.4-cm phloem necrosis), both in reaction to isolate J. The mean canker length of red oaks was significantly longer than it was in white oaks (more detail under "Statistical Analysis," below).

The frequency of disease indications at the final destructive sampling, along with mean lesion lengths and mean percent girdling (the metrics used for statistical analysis), for all species are set forth in Table 4-1. Stem bleeding co-occurred with flap death in 266 of 268 inoculated trees (99%), in 2012; the co-occurrence of these symptoms was 96 of 114 (82.5%) in 2011. Phloem necrosis extended beyond the flap in most cases. No foliar symptoms (such as leaf yellowing or flagging), nor twig dieback or branch cankers, were observed in any of the trees.

Xylem streaking, measured only in 2012, was extensive, in every tree much longer than phloem necrosis. In all cases, the extent of xylem discoloration above the inoculation site was at least equal to—and often much longer than--that below the site, since it stopped at the soil line (5 cm below the inoculation point). Staining was thickest at the inoculation site, tapering down to hair-thin streaks (Figure 4-3).

Results of Statistical Analysis

Relationship between lesion length and stem diameter in 2012

In 2012, canker vertical measurement (CVM), combining results from both isolates, was negatively correlated with stem diameter, with $P < .0001$. The statistical test employed was a one-way ANOVA, confirmed via a multivariate analysis followed by Spearman's correlation.

The correlation is shown graphically in Figure 4-4. No significant relationship was found between these parameters in the 2011 results.

Relationship between oak section and lesion length

"Section" is a subgeneric taxonomic division. All but one of the oak species tested fell into Section Lobatae ("red oaks") or Section Quercus ("white oaks"), the exception being *Q. acutissima*, from Asia (which is in Section Cerris). (*Castanea pumila* is in a different genus.) In the 2012 study, 12 red oak and nine white oak species were tested. The differences in mean canker vertical measurement (CVM) and mean girdling scaled by π [(%HC ×πd)/π] between the red oaks (6.83 cm; 0.67 cm) and white oaks (4.10 cm; 0.52 cm) were both statistically significant, with P <.0001. In 2011, four red oak and seven white oak species were tested. In contrast with 2012, where the species were more northerly, the means in 2011, where the species were native to Florida, were almost the same (3.58 cm and 0.48 cm for red vs. 3.28 cm and 0.46 cm for white). Table 4-2 shows the section to which each species belongs.

Statistical significance of the pathogenicity tests

Different response variables were developed and tested to try to remove the "small stem effect" from the data; however, at least in 2012, as discussed, this effect was real: species with small stem diameter were more susceptible to both pathogens, not only becoming girdled more quickly, but having disproportionately longer cankers. Most informative were simply the raw data—mean girdling (the percentage of circumference covered by the lesion, also referred to here as "percent horizontal coverage" or %HC), and mean canker vertical measurement (CVM)--which are graphed and reported first, below. Because the cankered area is a portion of a cylinder, an approximate lesion volume (CVM × %HC × r^2) (a "volume" again scaled by π), where r is ½ stem diameter and CVM is the length of the "cylinder," was also used as a response variable, and the outcome of the statistics program for it is reported for both 2012 and 2011.

This lesion volume is an imperfect approximation, since it assumes that the lesion extends in all cases to the center of the stem.

In 2012, using mean CVM as the response variable, there was a statistically significant (species*isolate) interaction, $P < .0399$. The statistics program output is presented in Figure A-1, showing where significant differences occurred, via the "different letters" test (Least Square Means Differences, Tukey's HSD). A graph showing the mean CVM for each isolate, across species, appears as Figure 4-5.

Using mean girdling as the response variable did not produce a statistically significant (species*isolate) interaction, although the species effect was significant at $P < .0001$. The "species" effect means that the difference between mean girdling of at least two species caused by the same isolate is not likely due to chance. A graph showing the mean girdling for each isolate (scaled by π), across species, is at Figure 4-7, for comparison purposes with Figure 4-5, which shows the same species' mean canker lengths.

Using lesion volume as the response variable produced no significant result for the (species*isolate) interaction in 2012. Species had a significant effect, however ($P < .0012$). The statistics program output for the species test using this variable is at Figure A-2. A graph of the lesion volume approximation for each isolate, by species, appears as Figure 4-6, so that it, too, can be compared directly with the graphs of CVM at Figure 4-5 and girdling at Figure 4-7.

In 2011, neither girdling, CVM, nor volume yielded a significant species*isolate interaction. Both girdling and CVM did yield a significant species effect, $P<.0001$ (Figure A-5) and $P < .0112$ (Figure A-4), respectively, in 2011. *Q. laurifolia* (QLA) had the longest mean lesions in 2011. Other significant differences are as shown by the different-letters test in Figures A-4 and A-5.

Using the approximation of lesion volume (%HC × CVM × r^2) resulted in a significant interaction for both species ($P < .0002$) and isolate ($P < .0064$) in 2011. The statistics program outputs are in Figures A-6 and A-7 and the graph is at Figure 4-10.

Figure 4-1. QMU-J1-1: Pycnidia around flap. Photo: Adam Black.

Figure 4-2. QMU-J1-1 Phloem necrosis (canker length = 3.3 cm) Photo: Adam Black.

Figure 4-3. QMU-J1-1. Xylem streaking = 16.2 cm. Photo: Adam Black.

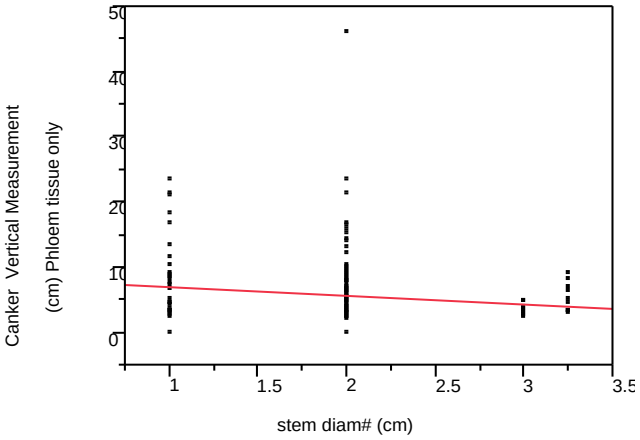

Figure 4-4. Negative correlation between canker length and stem diameter in 2012. (Stem diameters were estimated based on photographs, not measured.)

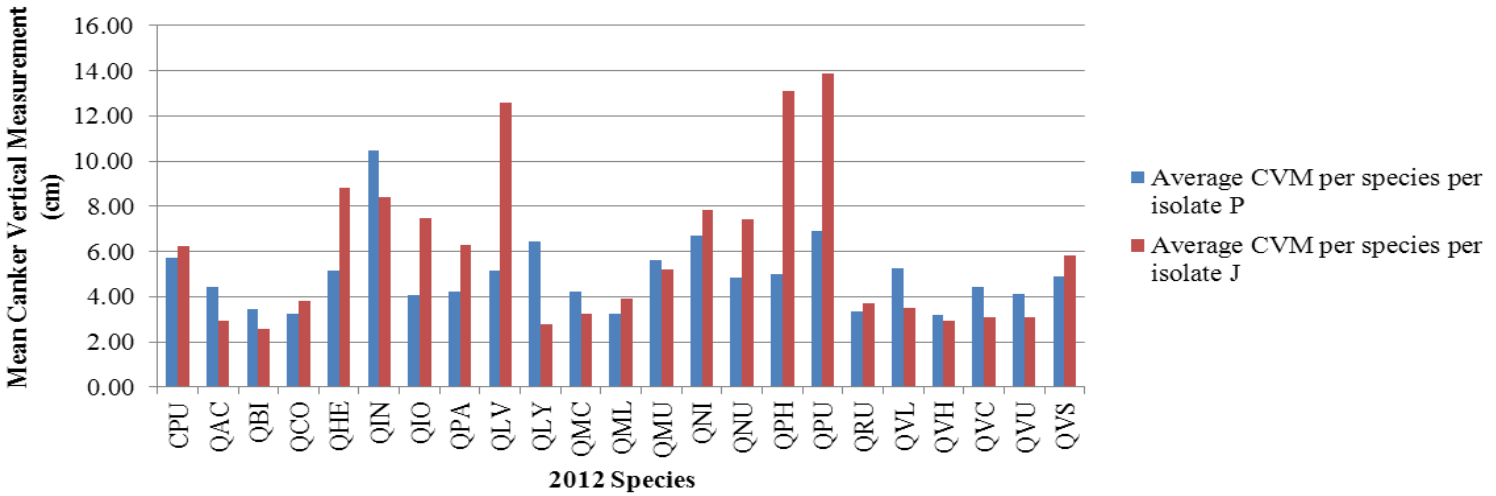

Figure 4-5. Mean canker length for isolates P and J, by species, 2012.

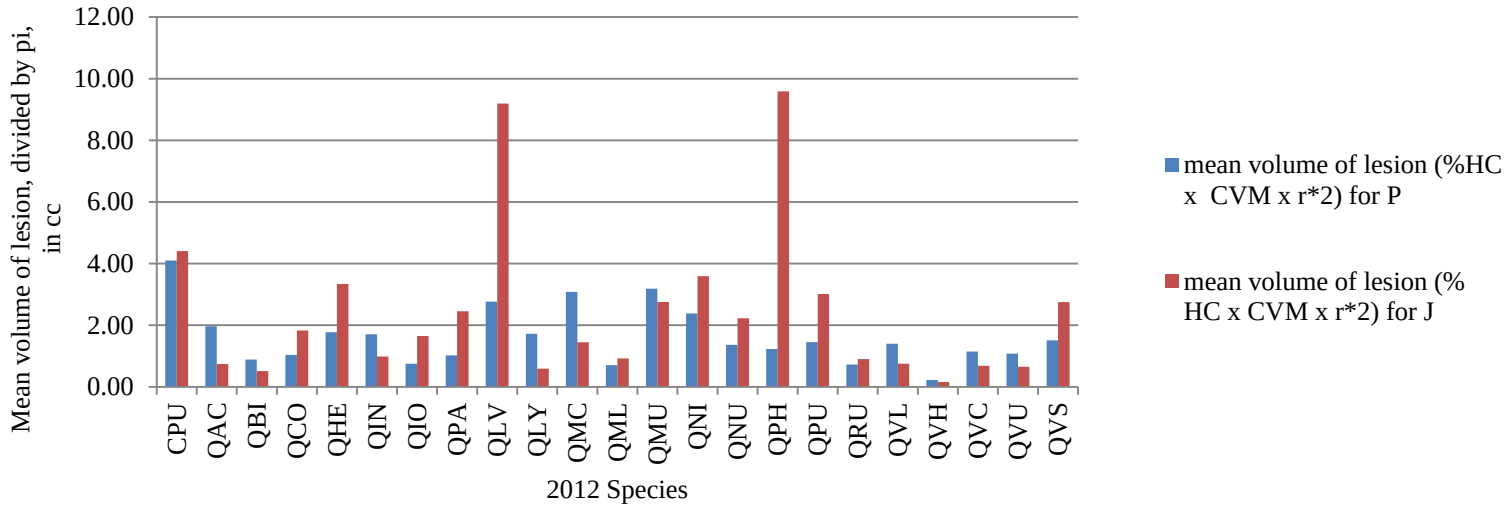

Figure 4-6. Mean lesion volume (r^2×CVM×%HC) for isolates P and J, by species, 2012 (r = 1/2 estimated stem diameter)

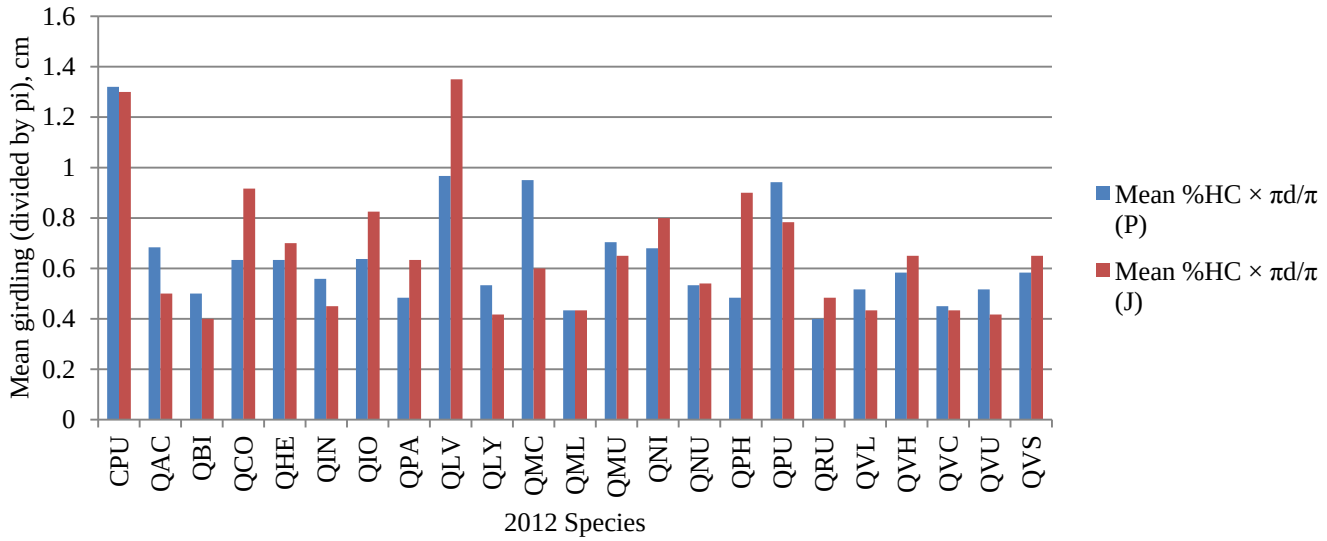

Figure 4-7. Mean girdling (%HC x πd)/π, in cm, for isolates P and J, by species, 2012.

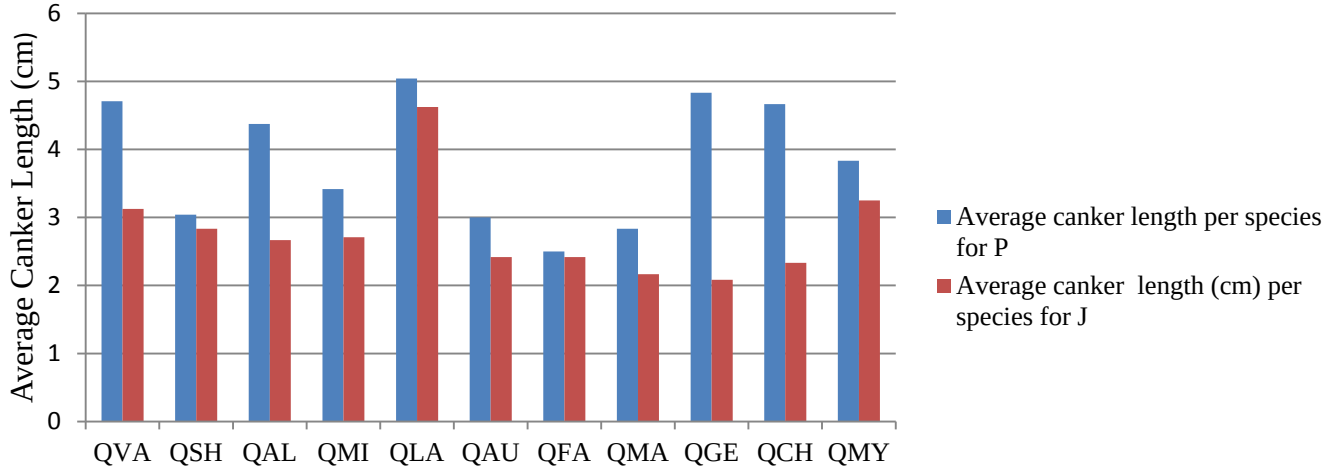

Figure 4-8. Mean canker length for isolates P and J, by species, 2011

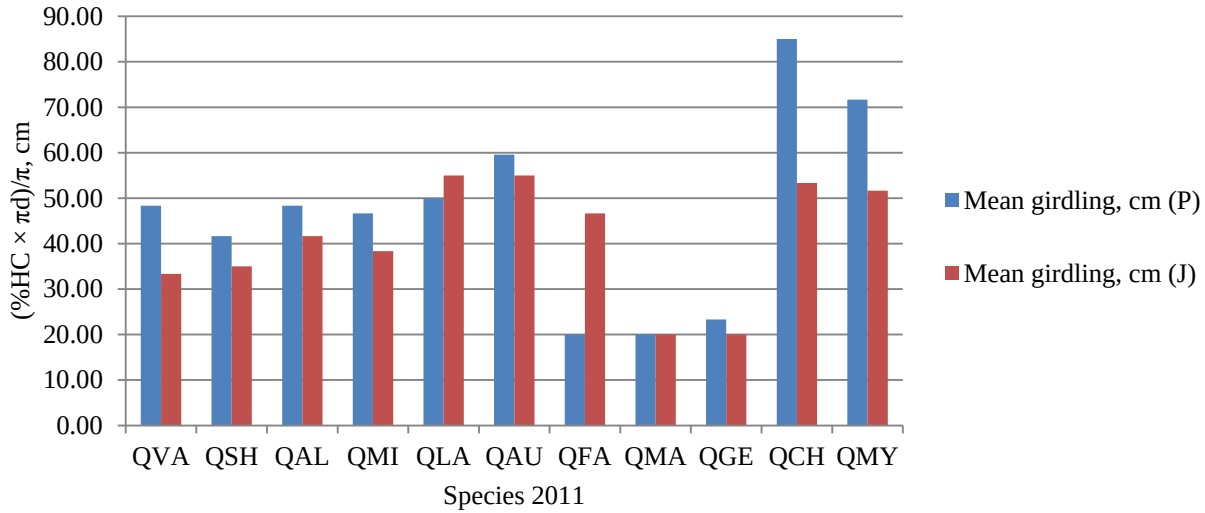

Figure 4-9. Mean girdling (cm) for isolates P and J, by species, 2011

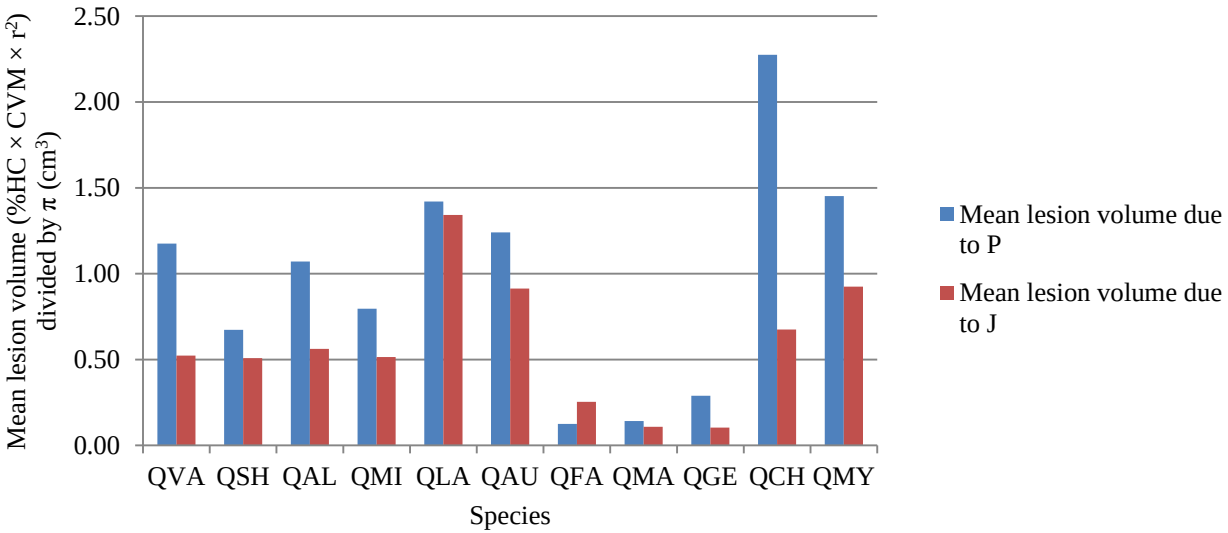

Figure 4-10. Mean lesion volume ($r^2 \times CVM \times \%HC)/\pi$ for isolates P and J, by species, 2011 (r = 1/2 estimated stem diameter)

Table 4-1. Frequencies of signs and symptoms; mean canker length[a,b]; and mean per cent girdling in trees inoculated with *Diplodia corticola* strains P and J, by isolate. Dead tree frequencies denoted by asterisk.

Host	No. of Plants Inoculated		Dead Plants		in Accelerator Pot?[c]		Pycnidia		Bleeding		Flap Death		Necrosis Extent Beyond Flap		Mean Canker Length[a,b] (cm)		Mean Percent Girdling	
	P	J	P	J	P	J	P	J	P	J	P	J	P	J	P	J	P	J
2012																		
C. pumila	5	5	2*	2*	2	3	3	2	5	4	5	5	5	5	5.71	6.23	59.17	56.25
Q. acutissima	6	6	-	-	-	-	1	2	6	6	6	6	4	5	4.43	2.93	34.17	25.00
Q. bicolor	6	6	-	-	-	-	-	1	6	5	6	6	5	-	3.45	2.57	25.00	20.00
Q. coccinea	6	6	-	-	-	-	1	1	6	6	6	6	6	6	3.23	3.82	31.67	45.83
Q. hemisphaerica	6	6	-	-	-	-	1	4	6	6	6	6	5	6	5.17	8.82	31.67	35.00
Q. incana	6	6	-	-	-	-	1	-	6	6	6	6	6	6	10.45	8.38	55.83	45.00
Q. inopina	4	4	-	2*	-	-	-	-	4	4	4	4	4	4	4.05	7.50	63.75	82.50
Q. palustris	6	6	-	-	-	-	4	3	6	6	6	6	5	6	4.20	6.28	24.17	31.67
Q. laevis	6	6	1*	2*	-	-	2	5	6	6	6	6	6	6	5.178	12.60	48.33	67.50
Q. lyrata	6	6	-	-	-	-	2	1	6	6	6	6	6	1	6.45	2.80	26.67	20.83
Q. macrocarpa	6	6	-	-	-	-	2	1	6	6	6	6	6	3	4.20	3.22	31.67	20.00
Q. marilandica	6	6	-	-	-	-	-	-	6	6	6	6	6	6	3.25	3.92	21.67	21.67
Q. muehlenbergii	6	6	-	-	-	-	4	1	6	6	6	6	6	6	5.60	5.22	21.67	20.00
Q. nigra	5	6	-	-	-	-	3	4	5	6	5	6	5	6	5.57	7.85	28.33	40.00
Q. nuttallii	6	5	-	-	-	-	1	3	6	5	6	5	5	5	4.85	7.40	26.67	27.00
Q. phellos	6	6	-	-	-	-	1	5	6	6	6	6	6	6	5.02	13.08	24.17	45.00
Q. pumila	6	6	2*	3*	-	-	1	3	5	6	6	6	6	6	6.93	13.90	77.50	78.33
Q. rubra	6	6	-	-	-	-	-	1	6	6	6	6	4	5	3.37	3.68	21.67	24.17
Q. virginiana (LA seed source)	6	6	-	-	--	-	5	2	6	6	6	6	5	4	5.23	3.52	25.83	21.67
Q. virginiana 'Highrise'	6	6	-	-	-	-	1	1	6	6	6	6	6	4	3.18	2.93	28.33	21.67
Q. virginiana 'Cathedral'	6	6	-	-	6	6	1	-	6	6	6	6	6	4	4.43	3.10	25.00	21.67
Q. virginiana 'Augustina'	6	6	-	-	6	6	--	-	6	6	6	5	6	5	4.10	3.08	25.83	20.83
Q. virginiana 'Sky Climber'	6	6	-	-	6	6	1	-	6	6	6	6	6	5	4.92	5.82	29.17	32.50
2011																		
Q. virginiana (FL seed source)	6	6	-	-			1	-	5	1	6	5	4	-	4.71	3.13	24.17	16.67
Q. shumardii	6	6	-	-			-	-	6	5	6	3	2	3	3.04	2.83	20.83	17.50
Q. alba	6	6	-	-			-	-	6	6	6	6	4	2	4.38	2.67	24.17	20.83
Q. michauxii	6	6	-	-			-	-	6	6	6	6	5	1	3.42	2.71	23.33	19.17
Q. laurifolia	6	6	-	-			1	1	6	6	6	6	5	5	5.04	4.63	25.00	27.50
Q. austrina	6	6	-	-			1	-	5	1	6	6	5	-	3.00	2.42	21.67	20.00
Q. falcata	3	3	-	1*			-	-	3	3	3	3	2	1	2.50	2.42	20.00	46.67
Q. margaretta	3	3	-	-			-	-	3	3	3	3	3	-	2.83	2.17	20.00	20.00
Q. geminata	3	3	-	-			1	-	2	1	3	3	3	1	4.83	2.08	23.33	20.00
Q. chapmanii	6	6	-	-			3	--	5	3	6	6	5	3	4.67	2.33	42.50	26.67
Q. myrtifolia	6	6	-	-			-	2	6	6	6	6	5	5	3.83	3.25	35.83	25.83

a. Dead plants were included in this mean by assigning an artificial lesion length equal to 110% of the longest lesion measured in that species/isolate/block.
b. Necroses measured in phloem (cm).
c. Accelerator pots were used only in 2012, for plants referring to this note (c). Of the dead *C. pumila*, one P and one J were in Accelerator pots.

Table 4-2. Species[a], showing section ("W" = Quercus; "R" = Lobatae)

Species	Species codes	Section (white or red)
2012		
Q. bicolor	QBI	W
Q. coccinea	QCO	R
Q. hemisphaerica	QHE	R
Q. incana	QIN	R
Q. inopina	QIO	R
Q. palustris	QPA	R
Q. laevis	QLV	R
Q. lyrata	QLY	W
Q. macrocarpa	QMC	W
Q. marilandica	QML	R
Q. muehlenbergii	QMU	W
Q. nigra	QNI	R
Q. nuttallii	QNU	R
Q. phellos	QPH	R
Q. pumila	QPU	R
Q. rubra	QRU	R
Q. virginiana (LA seed source)	QVL	W
Q. virginiana 'Highrise'	QVH	W
Q. virginiana 'Cathedral'	QVC	W
Q. virginiana 'Augustina'	QVU	W
Q. virginiana 'Sky Climber'	QVS	W
2011		
Q. virginiana	QVA	W
Q. shumardii	QSH	R
Q. alba	QAL	W
Q. michauxii	QMI	W
Q. laurifolia	QLA	R
Q. austrina	QAU	W
Q. falcata	QFA	R
Q. margaretta	QMA	W
Q. geminata	QGE	W
Q. chapmanii	QCH	W
Q. myrtifolia	QMY	R

a. QAC (*Quercus acutissima*) and CPU (*Castanea pumila*) were omitted, QAC being a member of the group Cerris, in China, and CPU belonging to a different genus.

CHAPTER 5
DISCUSSION

A singular finding in this experiment is that, on the wounded stems inoculated with *Diplodia corticola* or *Diplodia quercivora,* callus tissue never formed, while it did form on all controls. Equally important is that all trees tested were highly susceptible to both pathogens.

Absence of callus was observed by Sanchez *et al*. (38) in wintertime field inoculations of oak species with *D. corticola* in Mediterranean regions, while callus *was* formed in plants inoculated with the other pathogenic fungi they tested, *Diplodia sarmentorum* and *Botryosphaeria dothidea*. The lesions produced by *D. corticola* were also significantly longer. In the stem inoculations done by Luque *et al*. in 2000 (28), again callus formed only on controls and the lesions produced by *D. corticola* were much longer (21 cm in adequately irrigated plants, and 18 cm in water-stressed plants) than those produced by any of the other 31 pathogens tested, ¾ of which measured ≤ 2.5 cm and the longest of which was 9 cm. Moreover, almost all the plants inoculated with *D. corticola* died within two weeks.

Callus would limit the spread of the pathogen by sealing off the opening to the outside so that air could not dry out plant tissues, allowing oxygen to reach the fungus (34), as well as by virtue of antimicrobial properties. Its absence, at least under the conditions of this experiment, indicates that both *Diplodia corticola* and *Diplodia quercivora* interfere with callus formation. The alterations in cell walls which normally occur to compartmentalize the decay or disease agent by "walling it off" inside the stem, per the Compartmentalization of Decay in Trees ("CODIT") model (34), are apparently also not occurring, since nothing is limiting the vertical growth of the lesion in the bark, its expansion around the cambium, its radial entry into the xylem, or the extensive discoloration in the xylem, particularly above the inoculation point.

While there may be many reasons for the failure of wound healing in the plant, which would involve both lignification and suberization, the interference with suberin production, or the enzymatic degradation of suberin, seems likely, since suberin has been shown to have a more important role than lignification in host/pathogen interactions in peach bark (5). Biggs and co-workers concluded that "[i]n trees, the rapid production of high levels of suberin may be a determining factor in resistance against fungal pathogens" (5). Suberin is a component of all four walls in the CODIT model (34, 5), and is formed *de novo* upon wounding, extensively in *Quercus* (34, 33). A small amount also pre-exists in some cells. Wall 1 (which provides a conical "cap" across the xylem above and below the lesion) consists of suberized vessel occlusions (tyloses), while wall 4 is formed in the cambium and would limit girdling. Suberized fibers are found beneath newly forming callus (3).

Tyloses are outgrowths of the parenchyma cells which protrude through pits into xylem vessels in response to infection (3, 33, 34). White oaks overproduce tyloses, the reason white oak is exclusively used for wine casks. Suberin, a primary component of tyloses, is hydrophobic and makes a waterproof seal; it also has antimicrobial properties (34, 19, 3). The difference in disease response between white oaks and red oaks may be due to this difference in tylosis production. Lesion lengths and girdling in response to *D. corticola* infection were significantly less in white oaks than in red oaks in this study in 2012. Ironically, the disease so far has been observed in Florida almost exclusively in a white oak, *Quercus virginiana*, but this could be a result of the greater market share this species commands.

A great deal of suberin is produced in cork cells, as reflected in the name "*Quercus suber*," explaining why the removal of cork may leave *Q. suber* particularly vulnerable to *Diplodia corticola*. Accumulation rates of suberin at wound sites in bark tissues were positively

correlated with disease resistance in peach cultivars (4). In the present study, no resistance was seen in any of the inoculated trees, save the single *Q. michauxii* tree that did form callus, despite the presence of fungus in that tissue.

Small stem diameter is a risk factor for disease. There was a significant negative correlation between small stem size and not simply girdling, which would seem obvious, but canker length, in 2012. Figure 4-4. The species most girdled in 2012 were CPU and QLV, both of which are considered "shrubby." Cankers were longest in QPU, QPH, and QLV, all in response to isolate J, and QPU and QLV are also considered shrubby. Figs. 4-6 and 4-10. The two QPH (*Q. phellos*) individuals with 46- and 49-cm long cankers were *not* particularly small-stemmed. It is a red oak, however, so more susceptible as a rule.

The conclusion that small stem size is a risk factor is consistent with the twig dieback seen in natural infections caused by both pathogens, but more consistently in Florida by isolate "P," which has now been identified as *Diplodia quercivora*. Interference with either lignification or suberization might explain why *D. corticola* and *D. quercivora* in nature, without any obvious wounding, kill the smallest twigs. Although no express authority was found for this proposition, these twigs are the youngest, and presumably still in the process of forming bark.

In fact, because *no* twig dieback was observed on the artificially infected trees in this study, where the trees were inoculated in the trunk--nor is twig dieback reported as a symptom in the cork oak infections, where the wound is on the trunk—this symptom must occur because the pathogen has entered through (or become active in) the outermost twigs initially. It is unknown how the fungus gains entry where there are no obvious wounds. There could be physiological factors governing entry, such as gas exchange rates or lenticel size; environmental influences; and (probable) differences in infective capability between the two fungal isolates. Some of the

hosts which reacted to the pathogen in trunk inoculations in this study may never be infected naturally, therefore. Possible pathogen entry through lenticels in twigs might be investigated by spraying a spore suspension on them directly, without wounding.

The death of young twigs due to girdling by cankers may be due, as Agrios said, to their inability to produce defensive tissues faster than the fungus grows (1). However, in the present study, trunks much larger (1-3.25 cm in diameter) and older (1-3 years) than small twigs were girdled. This fact, along with the stem bleeding and long xylem streaking, are diagnostic not simply of a fast-growing fungus, but of a phytotoxin readily translocated through the xylem, particularly since the fungus itself was not isolated away from the phloem necrosis boundary. Investigations of canker formation by *Hypoxylon mammatum* in quaking aspen (*Populus tremuloides*) similarly found that, in addition to bark necrosis and collapse, the failure to form callus was due to a pathotoxin which interfered with wound healing, and was produced as a normal metabolite by *H. mammatum* (39).

As mentioned in the Introduction, diplopyrone, the phytotoxin produced by *Diplodia corticola*, caused the collapse of internal stem tissue in tomato plants (31) and was toxic to cork oak in tiny concentrations. In another fungus which causes stem lesions, *Botrytis cinerea*, phytopathogenicity is caused by a suite of factors which are believed to act synergistically, including "cell wall-degrading enzymes,… phytotoxins, … reactive oxygen species, … or membrane transporters for secretion of plant defence compounds" (37). Diplopyrone, along with other *D. corticola*/*D. quercivora* metabolites, may be involved in degrading suberin or interfering with cell signaling necessary for its production, and synergistic activity among them is likely, as with *Botrytis cinerea*. It should further be remarked that *Quercus* has an impressive arsenal of phytoalexins, defensive chemicals such as tannins and terpenes, that many other

pathogens cannot tolerate (34). *Diplodia corticola* and *Diplodia quercivora* are obviously not deterred. The pathogens may be interfering with these inducible oak defenses, as well, therefore.

Respecting the use of approximated lesion volume as a response variable: ideally, rather than %HC × $\pi r^2 h$ (percent girdling times cylinder volume, with "h" = lesion length, and r = ½ stem diameter), as was used here (scaled by π), the actual thickness of the lesion (difference between stem radius with and without lesion) should be used, for a better approximation. That measurement was not available in this study. If it had been, the formula would be $\pi (R^2 - r^2)h \times$ %HC, where r is the radius of the cankered (depressed) area on the stem and R is the radius of the uncankered stem. This would describe a wedge, the shape described by Urbez-Torres for cankers formed by *D. corticola* on grapevine (42).

The appearance of non-*Diplodia* fungal fruiting bodies on many inoculated trees remains a mystery worthy of more study. They could possibly be endophytes forced out by the invader, or mycoparasites. They were not observed on controls, and the same fungi appeared on the same oak species.

In sum, under the conditions of this experiment all species were susceptible to both pathogens, and the difference in their responses to the two strains was in the main not statistically significant, as noted. These members of the Fagaceae have some common genetic susceptibility to these closely related species of *Diplodia.* This paper concludes that the high susceptibility of oaks is due to the impairment of wound repair processes by the phytotoxin (or suite of phytotoxins) produced by *Diplodia corticola* and *Diplodia quercivora*.

APPENDIX A
RESULTS FROM STATISTICS PROGRAM

Level					Least Sq Mean
QPU,J	A				13.896667
QPH,J	A	B			13.083333
QLV,J	A	B	C		12.598333
QIN,P	A	B	C	D	10.445000
QHE,J	A	B	C	D	8.816667
QIN,J	A	B	C	D	8.383333
QNI,J	A	B	C	D	7.850000
QIO,J	A	B	C	D	7.500000
QNU,J	A	B	C	D	7.400000
QPU,P	A	B	C	D	6.933333
QNI,P	A	B	C	D	6.680000
QLY,P	A	B	C	D	6.450000
QPA,J	A	B	C	D	6.283333
CPU,J	A	B	C	D	6.232000
QVS,J	A	B	C	D	5.816667
CPU,P	A	B	C	D	5.710000
QMU,P	A	B	C	D	5.600000
QVL,P	A	B	C	D	5.233333
QMU,J	A	B	C	D	5.216667
QLV,P	A	B	C	D	5.171667
QHE,P	A	B	C	D	5.166667
QPH,P	A	B	C	D	5.016667
QVS,P	A	B	C	D	4.916667
QNU,P	A	B	C	D	4.850000
QVC,P	A	B	C	D	4.433333
QAC,P	A	B	C	D	4.433333
QPA,P		B	C	D	4.200000
QMC,P		B	C	D	4.200000
QVU,P		B	C	D	4.100000
QIO,P	A	B	C	D	4.050000
QML,J		B	C	D	3.916667
QCO,J		B	C	D	3.816667
QRU,J		B	C	D	3.683333
QVL,J		B	C	D	3.516667
QBI,P			C	D	3.450000
QRU,P			C	D	3.366667
QML,P			C	D	3.250000
QCO,P			C	D	3.233333
QMC,J			C	D	3.216667
QVH,P			C	D	3.183333
QVC,J			C	D	3.100000
QVU,J			C	D	3.083333
QVH,J				D	2.933333
QAC,J				D	2.933333
QLY,J				D	2.800000
QBI,J				D	2.566667

Levels not connected by same letter are significantly different.

Figure A-1. 2012, species*isolate interaction using mean canker length as response variable, LS Means Differences Tukey's HSD

Level				Least Sq Mean
QLV	A			5.9787500
QPH	A	B		5.4079167
CPU	A	B	C	4.2540000
QNI	A	B	C	2.9857500
QMU	A	B	C	2.9707031
QHE	A	B	C	2.5545833
QMC	A	B	C	2.2640625
QPU	A	B	C	2.2329167
QVS	A	B	C	2.1279167
QNU	A	B	C	1.7933333
QPA	A	B	C	1.7383333
QCO	A	B	C	1.4362500
QAC	A	B	C	1.3520833
QIN	A	B	C	1.3478125
QIO	A	B	C	1.1989063
QLY	A	B	C	1.1558333
QVL	A	B	C	1.0754167
QVC		B	C	0.9125000
QVU		B	C	0.8645833
QML		B	C	0.8133333
QRU		B	C	0.8129167
QBI		B	C	0.6987500
QVH			C	0.1913542

Levels not connected by same letter are significantly different.

Figure A-2. 2012: Lesion volume (%HC × CVM × r^2), LS Means Differences, Tukey's HSD, for species

Level						Least Sq Mean
QLV,J	A					1.3500000
CPU,P	A	B				1.3200000
CPU,J	A	B	C			1.3000000
QLV,P	A	B	C	D		0.9666667
QMC,P	A	B	C	D		0.9500000
QPU,P	A	B	C	D		0.9416667
QCO,J	A	B	C	D		0.9166667
QPH,J	A	B	C	D		0.9000000
QIO,J	A	B	C	D	E	0.8250000
QNI,J	A	B	C	D	E	0.8000000
QPU,J	A	B	C	D	E	0.7833333
QMU,P	A	B	C	D	E	0.7041667
QHE,J		B	C	D	E	0.7000000
QAC,P		B	C	D	E	0.6833333
QNI,P	A	B	C	D	E	0.6800000
QMU,J		B	C	D	E	0.6500000
QVS,J		B	C	D	E	0.6500000
QIO,P	A	B	C	D	E	0.6375000
QPA,J			C	D	E	0.6333333
QCO,P			C	D	E	0.6333333
QHE,P			C	D	E	0.6333333
QMC,J				D	E	0.6000000
QVS,P				D	E	0.5833333
QIN,P				D	E	0.5583333
QNU,J				D	E	0.5400000
QLY,P				D	E	0.5333333
QNU,P				D	E	0.5333333
QVL,P				D	E	0.5166667
QVU,P				D	E	0.5166667
QBI,P				D	E	0.5000000
QAC,J				D	E	0.5000000
QRU,J				D	E	0.4833333
QPA,P				D	E	0.4833333
QPH,P				D	E	0.4833333
QVC,P				D	E	0.4500000
QIN,J				D	E	0.4500000
QVC,J				D	E	0.4333333
QVL,J				D	E	0.4333333
QML,P				D	E	0.4333333
QML,J				D	E	0.4333333
QLY,J				D	E	0.4166667
QVU,J				D	E	0.4166667
QRU,P				D	E	0.4000000
QBI,J				D	E	0.4000000
QVH,P				D	E	0.3250000
QVH,J					E	0.2166667

Levels not connected by same letter are significantly different.

Figure A-3. 2012: Mean girdling, LS Means Difference Tukey's HSD, for species.

Level			**Least Sq Mean**
QLA	A		4.8333333
QVA	A	B	3.9166667
QMY	A	B	3.5416667
QAL	A	B	3.5208333
QCH	A	B	3.5000000
QGE	A	B	3.4583333
QMI	A	B	3.0625000
QSH		B	2.9375000
QAU		B	2.7083333
QMA		B	2.5000000
QFA		B	2.4583333

Levels not connected by same letter are significantly different.

Figure A-4. 2011: Canker vertical measurement, LS Means Difference Tukey's HSD, for species.

Level					Least Sq Mean
QCH	A				0.69166667
QMY	A	B			0.61666667
QAU	A	B	C		0.57291667
QLA	A	B	C		0.52500000
QAL		B	C	D	0.45000000
QMI		B	C	D	0.42500000
QVA		B	C	D	0.40833333
QSH			C	D	0.38333333
QFA			C	D	0.33333333
QGE				D	0.21666667
QMA				D	0.20000000

Levels not connected by same letter are significantly different.

Figure A-5. 2011: Mean girdling (cm), LS Means Difference, Tukey's HSD, for species.

Level			**Least Sq Mean**
QCH	A		1.4750000
QLA	A		1.3812500
QMY	A	B	1.1885417
QAU	A	B	1.0772624
QVA	A	B	0.8489583
QAL	A	B	0.8166667
QMI	A	B	0.6552083
QSH	A	B	0.5906250
QGE		B	0.1968750
QFA		B	0.1895833
QMA		B	0.1250000

Levels not connected by same letter are significantly different.

Figure A-6. 2011: Approximation of lesion volume (%HC × CVM × r2) , LS Means Difference, Tukey's HSD, for species

Level		Least Sq Mean
P	A	0.96904297
J	B	0.58458807

Levels not connected by same letter are significantly different.

Figure A-7. 2011: Approximation of lesion volume (%HC × CVM × r2), LS Means Differences, Tukey's HSD, for isolate

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BIOGRAPHICAL SKETCH

"Sonja Mullerin," who is called "Sunny" by her family and friends, is the name legally adopted by Alison Maynard in 2011, after 24 years as a lawyer in Colorado. In her law practice, Sunny specialized in water, land-use, and civil rights law, litigating on behalf of citizens and environmental groups often *pro bono* or on a contingent basis against powerful and well-funded developers and governmental officials. She published several law review articles and was the 2002 Green Party candidate for Colorado Attorney General, campaigning against both the Democrat and the Republican on an anti-corruption platform rooted in facts uncovered in her litigation.

In 2005, Sunny began to take biology and chemistry courses one or two at a time at the University of Colorado Denver, and to realize she had been diverted from her childhood passion, which was biology. She began to work towards a career switch, the first step of which was taken when she moved to Gainesville in June 2011 to begin graduate school in Forest Resources and Conservation at the University of Florida, working as a research assistant for Ass't Professor Jason A. Smith. Her original project was to explore whether a gene-for-gene interaction was at play between fusiform rust and its oak host. That led to work on the *Botryosphaeriaceae*, including the *Diplodia corticola* host range study which is subject of this thesis.

Aside from her degrees from UF and UCD, Sunny obtained her Bachelor of Arts in physics in 1976 from Cornell University, College of Arts and Sciences, where she went on a National Merit Scholarship, and her Juris Doctor from the University of Denver in 1986. Between those two educational experiences she traveled and worked in various countries in Europe and Africa, as well as in the geophysical industry in California, Colorado, Wyoming, and North Dakota. Sunny's father, Robert G. Maynard, was an oil company geologist who had been an Army major in World War II awarded the Silver Star for bravery. He died in 2007. Sunny's

mother, Emy, who was schooled in Germany and enrolled in UCLA at the tender age of 16 as a geology major, instilled in Sunny a love of books, as well as science. She died in 1972.

The name "Sonja Mullerin" is an invention. "Sonja" was Sunny's name in Russian class at Cornell, as well as the name of her best friend in 4th grade (with whom she lost contact long ago). It means "wisdom" in Greek. "Mullerin" seemed to follow "Sonja" naturally on the tongue, as the name of a Schubert song cycle ("die Schoene Mullerin").