Plant Disease • 2017 • 101:116-120 • http://dx.doi.org/10.1094/PDIS-04-16-0535-RE

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Virulence of Genetically Distinct *Geosmithia morbida* Isolates to Black Walnut and Their Response to Coinoculation with *Fusarium solani*

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Abstract

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 that coinoculation with these pathogens does not yield a synergistic response.

Thousand cankers disease (TCD) is an aggressive emerging disease on *Juglans* and *Pterocarya* spp. that was first identified in Colorado in 2007 and has now spread throughout the western states and seven eastern states (Indiana, Maryland, Ohio, Pennsylvania, North Carolina, Tennessee, and Virginia) (Grant et al. 2011; Hishinuma et al. 2016; Reed et al. 2013; Tisserat et al. 2009; Utley et al. 2013) and Italy (Montecchio and Faccoli 2015). This disease is caused by the symbiotic relationship between the fungal pathogen *Geosmithia morbida* M. Kolařík, E. Freeland, C. Utley, & 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 (Kolarík et al. 2004; Pitt and Hocking 2009). However, most are associated with insects, including bark beetles (Kolařík et al. 2006, 2007, 2008) and woodboring ambrosia beetles (Kolařík and Kirkendall 2010; Kolařík et al. 2015). As a symbiotic partner, *Geosmithia* spp. provide nutritional sources for their insect symbionts. Although most *Geosmithia* spp. are nonpathogenic, *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* spp. is a recent evolutionary change (Zerillo et al. 2014). The genetic diversity and pathways of *G. morbida* spread that have occurred in the United States 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.

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Accession number KX085029.

*The ***e*-Xtra** logo stands for "electronic extra" and indicates that one supplementary table is published online.

Accepted for publication 29 August 2016.

Several species of *Juglans,* including *Juglans nigra* L. (black walnut), are highly susceptible to the pathogen and tree death is typically observed 2 years after initial symptoms appear (Seybold et al. 2013; Tisserat et al. 2009). However, according to N. A. Tisserat and W. S. Cranshaw (personal communication), 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 the 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 about TCD. Several possible reasons have been considered, including differences in the community of natural enemies affecting *P. juglandis* or *G. morbida*, differences in how induced resistance of the host affects *G. morbida* or *P. juglandis* survival (Bonello et al. 2001; Webber 2000), 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*) (Carlson et al. 1993; Tisserat 1987; Tisserat et al. 2009), 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 coinoculation of *G. morbida* and *F. solani*.

Materials and Methods

Plant selection. Black walnut (*J. 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, were selected based on tree health, tree size, and branch length for inoculations with *G. morbida* and *F. solani* and coinoculations 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 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 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 7 years, indicative of the chronic phase.

F. 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 1α (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 to 7 days. DNA was extracted using a ZR Fungal/Bacterial DNA MiniPrep (Zymo Research). For polymerase chain reaction (PCR), mixtures (total of 25 μl) contained 20 to 40 ng of template DNA (or no DNA template for negative control), 2.5 μl of 10× Standard *Taq* Reaction Buffer (New England BioLabs, Inc.), 0.5μl of 10 mM dNTP (Roche Applied Science), 1 μl each of 10 μM primer, and 0.125 μl of *Taq* DNA Polymerase (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 assays were conducted using an MJ PTC-200 thermocycler (Bio-Rad Laboratories). 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 FSSC lineages 1, 2, and 6 obtained from the *Fusarium*-ID database, 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 "burn-in" of the chains. Sequence identifications (NRRL numbers) are shown in the phylogeny (Fig. 1). The sequence was deposited in National Center for Biotechnology Information Nucleotide Database, accession number KX085029.

Inoculations. Single-spore isolates of various test fungi were grown for 10 to 14 days at 25°C in petri dishes (100 mm in diameter by 15 mm deep) containing 20 ml of half-strength potato dextrose agar (1/2 PDA). In total, there were 18 (2014) or 16 (2015) different isolates or isolate combinations, including a 1/2 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 11 or 15 August 2014 and 14 August 2015, and there were 19 replications on four trees over 2 years. The experiment followed a randomized complete block design where each branch represented a block (approximately 19) containing each of the treatments. In total, there were 18 (2014) or 16 (2015) different isolates or isolate combinations (treatments), including a 1/2 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 1/2 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

Table 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[a]	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

[a] Based on results of Zerillo et al. (2014); n/a = not available.

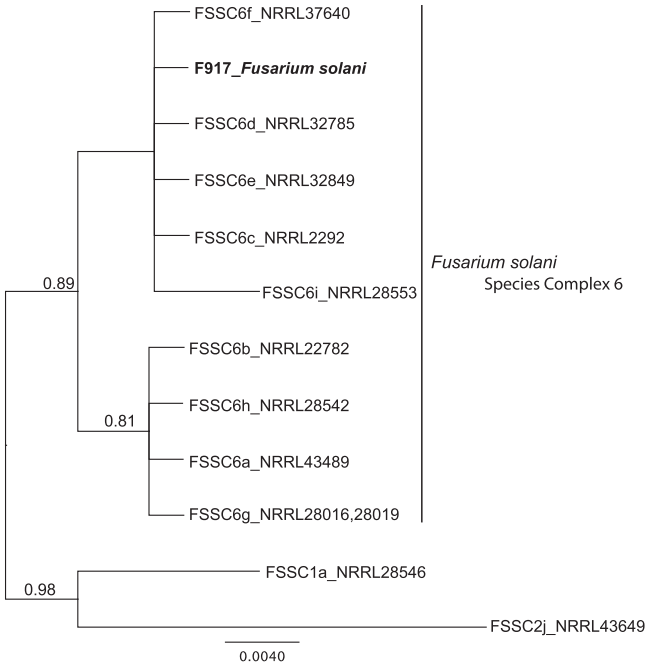

Fig. 1. Bayesian phylogeny of the elongation factor 1α (EF1α), including *Fusarium solani* isolate F917 (bold) and 10 *F. solani* sequences representing *F. solani* species complex (FSSC) 1, 2, and 6, obtained from the *Fusarium*-ID database. Posterior probabilities over 0.50 are labeled above each node. Phylogenetic sequence identifications (NRRL numbers) are shown in the phylogeny.

wrapped with Parafilm to covered the wounds until the inoculated branches were harvested after 6 weeks.

Canker measurements. Branches were harvested on 24 August 2014 and 23 August 2015 and taken back to Colorado State University. Within 5 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. General linear mixed models were fit to the data using SAS software (9.2v; PROC GLIMMIX; SAS Institute). Transformation was used to satisfy model assumptions, including normality of distributions, and all data were back transformed for presentation of figures and descriptive statistics. Models were fit using square root canker area as the response variable. Isolate (18 levels in 2014 and 16 levels in 2015) or treatment type (*F. solani*, *Geosmithia* sp., and coinoculation) 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 Fisher's least significant difference method.

Results

***G. morbida* inoculations.** In 2014, the canker area produced by inoculations of *G. morbida* isolates ranged from 0.15 to 3.77 cm^2 (n = 88) whereas, in 2015, they ranged from 0.16 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 to 1.74 cm^2 and, in 2015, were 0.82 to 1.03 cm^2. Regardless of treatment, cankers were larger in 2014 than in 2015 (P = 0.0024) with the exception of isolate combination G1274 + F917 (Fig. 2A and B). Overall, *G. morbida* virulence did not vary among the three genetic clusters used in 2014 and 2015 (P = 0.4983 and 0.5973, respectively). However,

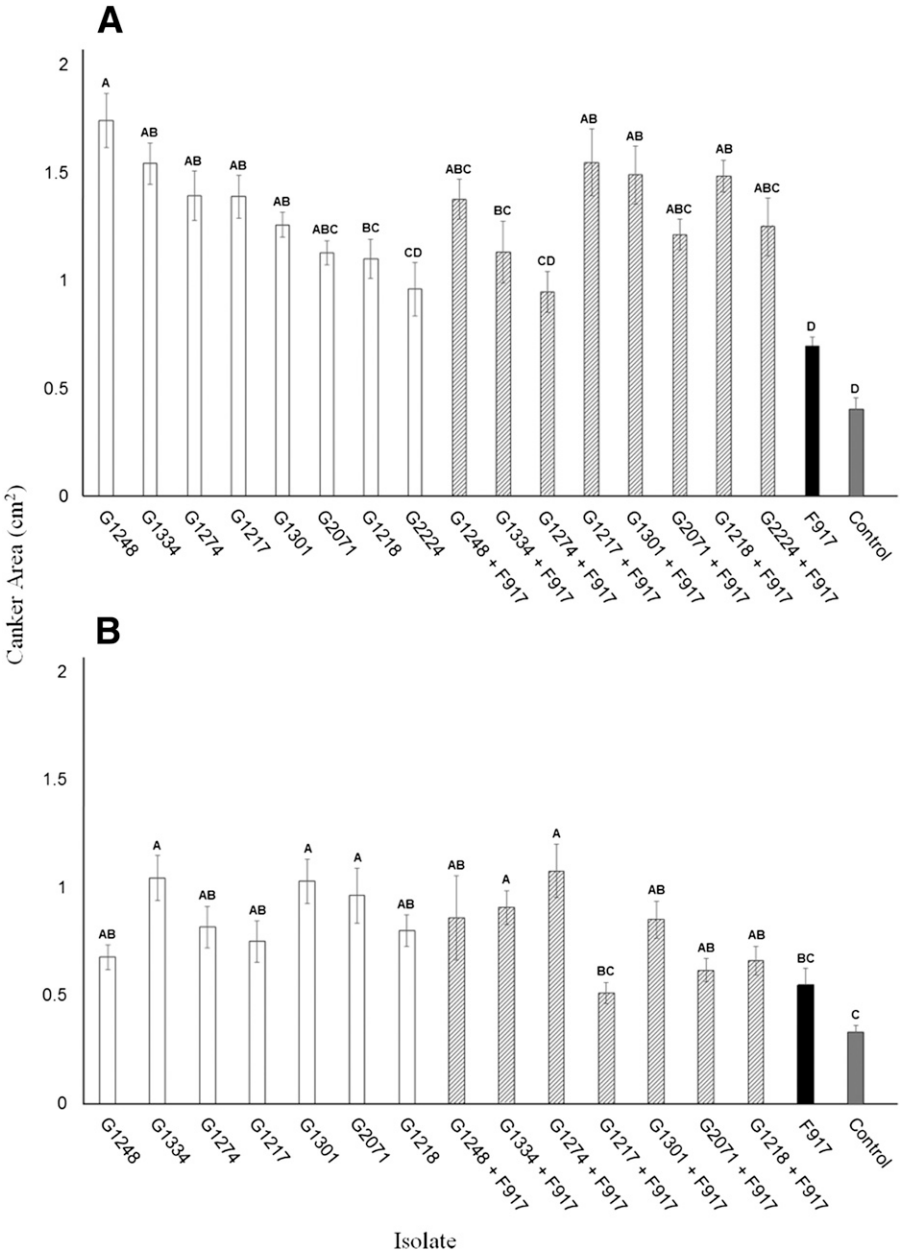

Fig. 2. Experiments in **A,** 2014 and **B,** 2015. Black walnut branches were inoculated with *Geosmithia morbida* (white), one isolate of *Fusarium solani* (black), *G. morbida* combined with *F. solani* (hatched), or half-strength potato dextrose agar as a control (gray). Canker areas were measured 6 weeks after inoculation. Differences in letter groupings indicate statistical differences among canker area means at $P < 0.05$ (Fisher's least significant difference).

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 ($P = 0.0152$), and G2224 ($P = 0.0013$). *G. morbida* isolate G2224 produced the smallest canker areas, which 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 ($P < 0.0001$), although the canker produced by isolate G2224 was not significantly different than the control wound ($P = 0.0918$) in 2014 (Fig. 2A and 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 ($P = 0.1215$), the isolate that produced the largest canker in 2015.

***F. solani* identification and inoculations.** Based on a phylogeny of the EF1α, isolate F917 falls into FSSC 6 (Fig. 1). Using this strain, the average canker area was 47% smaller than cankers produced by *G. morbida* ($P < 0.0001$) and 47% smaller than cankers produced by the combined *F. solani* and *G. morbida* treatment ($P < 0.0001$) in 2014 (Fig. 2A). A similar trend was seen in 2015; cankers produced by F917 were 37% smaller than cankers produced by *G. morbida* ($P = 0.0379$) and 30% smaller than cankers produced by all combined treatments ($P = 0.1418$) (Fig. 2B). In both years, the necrotic area produced by *Fusarium* isolate F917 was similar ($P = 0.7004$) and no different in size than the control wounds (2014: $P = 0.6968$ and 2015: $P = 0.2391$) (Fig. 2A and B).

Concomitant relationship. *F. solani* isolate F917 did not increase or decrease the virulence of *G. morbida* when using a combined inoculum in 2014 ($P = 0.3134$) or 2015 ($P = 0.2226$). 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 of a size comparable with the control and *F. solani* alone ($P = 0.9621$).

Discussion

Our results indicated that the *G. morbida* isolates used in our study did not show differences in virulence among genetic characterized groupings of isolates (Idnurm and Howlett 2001; Zerillo et al. 2014). 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 *F. oxysporum* did not correlate to pathogenic phenotype on many different hosts. In addition, Appel and Gordon (1996) compared pathogenic and nonpathogenic 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 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 S1). 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* spp. and their interaction with *Geosmithia* spp. and TCD. When examining canker length from *F. 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 *G. langdonii* to be 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 whether *G. morbida* outcompeted the *F. solani* in this experiment. There are documented cases where fungal endophytes or nonpathogenic *Fusarium* sp. decrease the virulence of other fungal pathogens or increased host resistance (Forsyth et al. 2006; Gwinn and Gavin 1992; Schardl et al. 2004; Zabalgogeazcoa 2008); however, it is unknown whether *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 coinoculations with the Italian *F. solani* isolate S1 belonging to phylogenic lineage FSSC 25 (4.8 and 3.1 cm^2 respectively). In contrast, our inoculations with *G. morbida* alone produced cankers of a size comparable with the coinoculations 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 coinoculations averaged 0.78 cm^2.

Montecchio et al. (2015) documented that canker areas produced by coinoculations with *F. 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 coinoculations versus *F. solani* F917 (FSSC 6) inoculations were 47% larger in 2014 and 31% larger in 2015, measuring 1.3 and 0.78 cm^2, respectively. This could mean that 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 *G. morbida* virulence is most likely not explained by genetic grouping. Furthermore, the *F. solani* isolate used in our study does not increase or decrease the virulence of *G. morbida.* In Colorado, TCD causes an initial acute phase characterized by high tree mortality and, in some cases, a later, chronic 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.

Acknowledgments

We thank A. Broberg and K. Due for field assistance, K. Otto and E. Peirce for laboratory assistance, S. Endriss for editing the manuscript, J. Stolz and O. Aon at the Colorado State Forest Service Nursery for allowing us to use their trees and facility for the experiments, and A. Hess for statistical consulting through the Franklin Graybill Statistical Laboratory at Colorado State University. Funding for this project was provided by the United States Department of Agriculture (USDA) National Institute of Food and Agriculture Western Region IPM Center and Critical Issues-Emerging and New Plant and Animal Pests and Diseases grant programs, the USDA Forest Service Forest Health Monitoring Program (Detection Monitoring Grant Number INT-DM-09-01 and Evaluation Monitoring Grant Number INT-EM-B-11-03), and USDA Forest Service Special Technology Development Grant R4-2011-01.

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