Research

Evaluation of Thousand Cankers Disease-Symptomatic *Juglans nigra* in Southwestern Ohio Following Population Collapse of *Pityophthorus juglandis*

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Abstract

Mass attacks of *Juglans nigra* by *Pityophthorus juglandis* and its introduction of the canker pathogen *Geosmithia morbida* lead to branch dieback and, commonly, tree death in locations with outbreaks of thousand cankers disease (TCD). Following initial detection of TCD in Butler County, Ohio, in 2012, and trapping of thousands of *P. juglandis*, a dramatic decrease in insect population occurred. Dissections of "lingering" TCD-symptomatic *J. nigra* in 2014 and 2015 were conducted to document the presence of "typical" *Geosmithia* cankers and other types of damage on branches and main stems. Isolation and subsequent molecular identification of *G. morbida* and other selected fungi were attempted from representatives of the various canker-like and insect-associated damage observed. Of the fungi obtained, *G. morbida* was most commonly isolated, primarily from "typical" *Geosmithia* cankers, but it was also obtained at lower frequencies from other damage types. In addition, *G. morbida* commonly co-occurred with *Fusarium solani* (*F. solani* species complex) but infrequently with *Diplodia seriata*. These other fungal species could contribute to the severity of canker development. These results suggest that the TCD pathogen persists in the landscape following population collapse of the primary insect vector and would be of interest to state regulatory agencies in the eastern United States.

Keywords: *Diplodia seriata*, *Fusarium solani*, *Geosmithia morbida*, *Juglans nigra*, *Pityophthorus juglandis*, thousand cankers disease

Thousand cankers disease (TCD) is an emerging problem on *Juglans* and *Pterocarya* species in the United States. It has been considered a serious threat to the health of high-value eastern black walnut (*J. nigra*), particularly within its native range in the eastern United States (Newton et al. 2009). However, the disease has caused significant levels of mortality in western states since it was first reported in Colorado in 2009 (Seybold et al. 2019; Tisserat et al. 2009). Thousands of infections by the canker pathogen, *Geosmithia morbida* M. Kolarik, E. Freeland, C. Utley, & N. Tisserat (Ascomycota: Hypocreales, Bionectriaceae) cause death of branches, major limbs, and entire trees. The walnut twig beetle (WTB) (*Pityophthorus juglandis* Blackman) is the primary insect vector of the pathogen. Mass attacks by WTB and subsequent beetle inoculation of the inner phloem with *G. morbida* result in large numbers of cankers within the inner bark of host trees. Coalescing of the numerous small cankers can lead to girdling and dieback of infested trees (Sitz et al. 2017).

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The disease and its associated biotic agents were first detected in the eastern United States in 2010 in Knoxville, Tennessee (Grant et al. 2011). Tree surveys and insect trapping programs in eastern states have led to the discovery of TCD (symptomatic trees plus WTB and *G. morbida*) in five other states, whereas the fungus and/or the beetle only has been detected in Indiana and Michigan. The disease, the pathogen, and WTB have also been documented in Italy (Montecchio and Faccoli 2014; Moricca et al. 2019). In Ohio, eight WTB were captured in 2012 over a 3-month period in insect traps set at a walnut veneer mill in Butler County by the Ohio Department of Natural Resources (Fisher et al. 2013). During the subsequent year, hundreds to thousands of WTB were captured as part of an intensive and systematic survey conducted by the Ohio Department of Agriculture (ODA) in the vicinity of the mill (Dan Kenny, Plant Health Administrator, ODA, *personal communication*). Most of those captures were from two adjoining residential neighborhoods located approximately 5 km northeast of the mill. Several clusters of declining and dead *J. nigra* were found in these areas. Interestingly, either zero or one WTB was trapped annually in these same locations between 2014 and 2018, suggesting that the insect population had collapsed. In Virginia, trapping surveys have been conducted since 2011. TCD was first confirmed in *J. nigra* in the Richmond area in that year and subsequently documented in Fairfax and Prince William Counties near the Washington, DC area. Between 2016 and 2021, high WTB counts have been documented in locations of these two counties; however, the insect was not detected in other parts of Virginia during that same period

(Devin Bily, State Plant Pathologist, Virginia Department of Agriculture and Consumer Services, *personal communication*). Griffin (2015) documented the progression of TCD in individual trees and new occurrences on the landscape in the Richmond, Virginia, and Knoxville, Tennessee, areas between 2010 and 2013. In addition to observing trees with actively declining branches, the researcher noted trees with both slower symptom progression and quiescent dieback or those whose disease-affected crowns had recovered over the study period.

Multiple types of damage besides that associated with WTB colonization and cankers caused by *G. morbida* were found on branches of TCD-symptomatic and asymptomatic *J. nigra* trees in the Knoxville, Tennessee, area in 2012, where TCD was well established (McDermott-Kubeczko et al. 2020). Furthermore, other known pathogens of *Juglans* (*Botryosphaeria dothidea*, *Fusarium solani*, and *Biscogniauxia atropuntata*) were isolated from damage areas not characteristic of *G. morbida* and WTB, such as other cankers and from other insect galleries. *F. solani* species complex (FSSC) 25 was associated with *Geosmithia* cankers on branches of *J. nigra* and *J. regia* in Italy (Montecchio et al. 2015).

The goal of this study was to conduct a quantitative assessment of cankers caused by *G. morbida* on TCD-symptomatic trees within the disease epicenter in Butler County, Ohio, during the 2 years following the WTB population collapse. In addition, we sought to characterize and quantify the assemblage of putative pathogenic fungi associated with *Geosmithia* cankers and other observed damage types on affected trees. The specific objectives of the 2-year study were (i) to document numbers of "typical" *G. morbida* cankers (roughly circular, two-toned, often with galleries of WTB) (Seybold et al. 2013) with and without obvious WTB galleries on branches and stems of moderately to severely affected *J. nigra*, and (ii) to determine the frequencies of selected pathogenic fungi associated with those cankers, as well as a representative subset of other fungus- or insect-associated damage types observed.

Materials and Methods

Study trees and sample collection

All *J. nigra* study trees were located on residential properties in a cul-de-sac of a Hamilton, Ohio, neighborhood. Crowns of the trees exhibited active dieback symptoms characteristic of TCD, with the percentage of crown affected ranging from 40 to 95%. Four trees (20 to 23 cm DBH [diameter at 1.4 m stem height]) were felled on 9 September 2014. Three trees on one lot were growing in a cluster of five *J. nigra* in a backyard lawn setting. The fourth tree was on the wooded edge of an adjacent lot. The remaining study trees (15 to 25 cm DBH) were felled on 3 August 2015. Three trees were located within a small woodlot, and one was open grown in a backyard lawn on property adjacent to the 2014 sites.

Four actively declining branches ranging from 5 to 12 cm in diameter were cut from the crowns of each downed tree. Two 30-cm-long segments, or subsamples, were cut from each branch; however, only one subsample per branch was evaluated in this study. The cut ends of each subsample were coated with melted paraffin, placed in two 5-mm-thick plastic bags and closed with ties. The bagged collections from each branch were stored in a 19-liter plastic bucket with a tightly fitting lid that was labeled with tree and branch numbers. Eight sections for the main stem (30 cm long) were marked in a systematic pattern along the stem of each downed tree, and the distance from the bottom of each section to the tree base was recorded. The sections were then cut from the main stems, and the cut ends were sealed with paraffin before each sample was double bagged in plastic and placed in a bucket with an appropriately labeled lid. The packaging protocols were based on the conditions of annual compliance agreements with the ODA. In 2014, the buckets were transported to the ODA plant quarantine (BSL-2) facility on the Ohio State University campus, Reynoldsburg, and stored at 4°C until processed. The 2015 collection was transported by vehicle to the USDA ARS Cereal Disease Laboratory, St. Paul, Minnesota, for processing in a quarantine laboratory (BSL-2 plus) under the conditions of a compliance agreement with the Minnesota Department of Agriculture and USDA APHIS PPQ Permit P526-14-00978. The buckets were kept in a cold room (4°C) until processing.

Sample processing

In the quarantine laboratory, the outer bark of each branch subsample or stem section was carefully removed using a sterilized drawknife. All observed damage resembling cankers, lesions, and insect galleries or tunnels were marked. Damage was then categorized by size, shape, and/or color of cankers or lesions and by type of insect damage. Numbers of "typical" *Geosmithia*-like cankers (Seybold et al. 2013) on branch and stem samples were counted and recorded. Due to time constraints and coalescing of damage areas, the numbers of the different types of damage on branch and stem samples were counted only for samples from two trees felled in 2015. Fungal isolation was attempted from the margins of damaged tissue, with samples taken from a maximum of five "typical" *Geosmithia* cankers and a maximum of two representing each of the other fungus- and insect-associated damage categories. Four thin chips of sampled host tissue were placed on quarter-strength potato dextrose agar (PDA) amended with 100 mg/liter of streptomycin sulfate and 100 mg/liter of chloramphenicol (Fisher et al. 2013). The plates were incubated in plastic storage containers with lids and stored on the bench top under ambient room conditions (fluorescent room lighting; 24°C temperature). The plates were observed twice weekly for fungal growth, and subculturing was performed, as needed, to half-strength PDA. Hyphal tip or single spore transfers were made from any colonies that resembled *G. morbida*, *F. solani*, or *Botryosphaeria* spp. Preliminary identifications were based on culture morphology and microscopic features (conidiphores and conidia). Fungi were stored on ½-strength PDA slants in screw-cap glass vials stored at 4°C until identifications were verified using molecular methods.

Molecular methods for fungal identification

All putative *F. solani* and *Botryosphaeria* spp. isolates obtained and representative isolates of putative *G. morbida* were grown on PDA for 7 to 14 days under ambient room conditions (24°C; fluorescent lighting). A small amount of fungal material was scraped for each isolate, placed in a 200-µl strip tube with 200 µl of CTAB lysis buffer (1.4 M of NaCl, 0.1 M of Tris-HCl, 20 mM of EDTA, 2% CTAB), and stored frozen at –20°C until further processing. DNA extraction was performed using a modified protocol for a commercial DNA extraction kit (Gene Clean III, Qbiogene, www.qbiogene.com) (per Lindner and Banik 2009). PCR amplification was performed in 15-µl reaction mixtures as described by McDermott-Kubeczko et al. (2020), with the following exceptions: PCR reactions for putative *G. morbida* and *Botryosphaeria* spp. isolates were run with the general fungal primer pair *its1F* and *its4* based on the ITS gene region, whereas the primer pair *ef1* and *ef2* based on the translation elongation factor 1-α gene region were used for putative *F. solani* isolates. Amplification reactions were performed in 96-well plates and temperature-cycled using the equipment and conditions described by McDermott-Kubeczko et al. (2020). PCR products were visualized on 2% agarose gels stained

with ethidium bromide and viewed under UV light. Products were cleaned and sequencing reactions conducted as per a previously published protocol (Lindner and Banik 2009). Sequenced products in direct inject 96-well plates were analyzed using ABI 3730xl equipment from a commercial laboratory (Functional Biosciences, Madison, WI).

Fungal ITS sequences were viewed and edited using commercial software programs (2014 isolates – 4Peaks, available at nucleobytes.com; 2015 isolates – Chromas Pro, Technelysium.com). The resulting sequences for *G. morbida* and *Botryosphaeria* spp. isolates were compared with known sequences in the NCBI database using BLAST and Clustal Omega. Final identification was based on ≥98% homology with a known sequence in GenBank. Sequences for the putative *F. solani* isolates were compared to those in GenBank in 2014 and to those in the FUSARIUM ID database (Geiser et al. 2004) for 2015 isolates. Identities of the isolates with sequences that matched (≥99% homology) with a known sequence of a specific haplotype of the FSSC were recorded.

Data summarization and analyses

The combined numbers of "typical" *Geosmithia* cankers observed on the four branch subsamples were the values used for the branch canker density calculations, and the number of observed cankers on each stem section was used for the main stem canker density determinations. The bark area for branch and main stem samples were estimated using the outer surface area of a cylinder with the measured sample length and the top and bottom diameters of each subsample [π × ½ (top diameter + bottom diameter) × (length)]. Surface areas of the branch subsamples were added together. Density on a 100 cm^2 basis was calculated as [(number of observed cankers/surface area) × 100]. Differences in sample surface area and in canker density among branches or main stems for each tree of a collection year were analyzed using the analysis of variance (aov) function in R (version 1.0.143, R Foundation for Statistical Computing, Vienna, Austria). Post-hoc comparisons of means were performed using Tukey honestly significant difference (HSD) test. Canker densities (*Geosmithia*-like only) were combined for similar main stem height samples from each tree within a year. Linear regression analyses in R (version 1.0.143, R Foundation for Statistical Computing, Vienna, Austria) were then used to explore the relationship between main stem height and canker density within each year. Comparisons of slopes resulting from regression analyses were also completed (GraphPad Prism, version 8.1; available at graphpad.com). Differences in numbers of each damage type observed on main stem samples (combined) of two of the trees felled in 2015 were summarized graphically, but no statistical analyses were performed.

Results for selected fungi isolated from the five damage types (three canker-like and two insect-associated) recorded were summarized by only one fungus species isolated (*G. morbida*, *F. solani* only, *Botryosphaeria* sp. only) and by co-occurrence of different combinations of these species. Separate summaries were made for branches and main stems by year of sampling. No statistical analyses were performed on isolation frequencies due to highly variable occurrence of different damage types and number of taxa of interest isolated from trees each year.

Results

Geosmithia canker frequencies

The frequencies of "typical" *Geosmithia* cankers on both branch and main stem samples were at least three times greater from samples collected in 2014 than in 2015; thus, results were not combined. In 2014, the estimated surface areas of 12 branches from 3 trees ranged from 1,114 to 1,186 cm^2, and the mean surface areas were similar ($P > 0.05$) (Table 1). Estimated surface area of 29 main stem sections collected from 4 trees ranged from 1,714 to 2,226 cm^2. The mean surface areas were largest for Tree BB2 samples compared with Trees BB1 and BB4 and similar for Tree BB3 ($P \leq 0.05$). The numbers of cankers per branch ranged from 6 to 404 for this collection. Estimates of mean canker density (i.e., number of cankers per 100 cm^2) on each branch were also highly variable and ranged from 4.2 to 16.6; thus, log transformations of density values were used in the data analysis. Although not significantly different, the highest mean density of branch cankers was found for Tree BB4, whose lower branches were TCD symptomatic and top was dead. The lowest density of branch cankers was recorded for Tree BB1, which had a dead top, but the remainder of the crown was asymptomatic. No living tissue was found on branches collected from Tree BB3. Similar and lowest mean canker densities on main stem samples ($P > 0.05$) were found for Tree BB1 (described above) and for BB2 that had one main fork with TCD-symptomatic branches, and the remainder of the crown was asymptomatic.

For the four study trees evaluated in 2015, the mean branch surface areas ranged from 803 to 1,143 cm^2 for four branches and from 1,557 to 2,139 cm^2 for main stem samples. Mean surface areas were similar for branch samples ($P > 0.05$) (Table 2). When present, the numbers of cankers per branch ranged from 1 to 45 for the 2015 collection. The estimated mean branch densities were not normally distributed; however, log transformation of data resolved the issue and was used for the analyses. The highest mean density of branch cankers ($P < 0.05$) was found along the edge of the woodlot (Tree 145) that had the highest diseased crown rating (75%) compared with the mean canker densities on the other study trees (30 and 40% for within woodlot Trees 138 and 139) and Tree 140 (30% disease rating and open-grown) (Table 2). Mean canker densities on the main stem samples of Tree 145 were only slightly higher than the mean densities for the main stem samples of the other trees ($P = 0.118$).

Although the mean canker densities on the main stems of study trees varied by tree within evaluation year, a general trend in mean density was observed by the height of the stem from which the sample was obtained (Fig. 1A). The greatest increase by height (i.e., slope) occurred for two (Trees BB2 and BB4) of four trees in 2014. However, slopes of regression lines for the four trees were different from each other ($P = 0.008$). For 2015 trees, the greatest increase by height occurred on Tree 145 (Fig. 1B); however, slopes of regression lines for the four trees were different from each other ($P = 0.0194$). The same relationship was observed for two (Trees 140 and 145) of the four trees evaluated in 2015 (Fig. 1B).

Frequencies of all damage types

The numbers of disease-like damage and insect-type damage observed were recorded for the eight stem sections sampled from Trees 140 and 145 in 2015 (Table 3). For this study, "typical" *Geosmithia* cankers (GM; previously described) were distinguished from elliptically shaped, brown (one or two tone), or uniform black (EL) cankers and from long, narrow, dark brown or black (LN) cankers. Tunnels characteristic of buprestid (B) beetle (Coleoptera: Buprestidae) colonization and of bark weevil (W) colonization (Coleoptera, subfamily Cossoninae) were also distinguished. The highest numbers of GM damage type were recorded for both trees (Table 3). Low numbers of buprestid and bark weevil tunnels were found on the main stems of both trees.

Fungi isolated by damage type

Fewer numbers of EL, LN, B, and W damage types were sampled from the branches and main stems of study trees in 2014; thus, results are presented separately by year for each sample type (Tables 4 and 5). *Botryosphaeria obtusa* (anamorph *Diplodia seriata*) was isolated from all damage types, both sample types, and in each collection year. In contrast, *B. dothidea* was only isolated five times, with three isolates obtained from Tree 138 branches and one each from a branch of Trees 140 and 145. Results are presented for both *Botryosphaeria* species combined. *F. solani* (FSSC) and *G. morbida* were isolated from all damage types, both sample types, and in each collection year, except for bark weevil tunnels on main stem samples in 2015.

G. morbida was isolated from GM cankers on the branches of study trees at similar frequencies for 2014 (57.3%; $n = 117$) and 2015 (55.1%; $n = 107$) (Table 4). The pathogen was found to co-occur with *Botryosphaeria* and/or FSSC at a higher rate in the same assayed cankers (25.6%) in 2014 than those in 2015 (5.6%). On a percentage basis, *G. morbida* was frequently isolated from the other canker types (EL and LN pooled data) in both years. The pathogen was isolated from buprestid and bark weevil tunnels on branches collected in both years but was detected more commonly in buprestid tunnels in 2014 than in 2015. In comparison, *G. morbida* was isolated more frequently from weevil tunnels on branches in 2015 than in 2014.

Isolation of *G. morbida* from GM cankers on main stems of study trees was higher (36.8%; $n = 125$) for assayed cankers in 2015 than for those assayed in 2014 (22.8%; $n = 114$) (Table 5). However, similar isolation rates were found in assayed GM cankers that also yielded *Botryosphaeria* and/or FSSC in 2014 (9.8%) and 2015 (8.9%). On a proportional basis, *G. morbida* was isolated from 30 to 40% of EL and NL cankers assayed in 2014 and EL cankers assayed in 2015. Low frequencies of pathogen isolation (0 to 12%) were obtained from buprestid and bark weevil tunnels on the main stems of study trees, regardless of year.

Molecular identification of fungal taxa

DNA sequencing was performed on 19.2% of the *G. morbida* morphologically identified isolates ($n = 120$) that were obtained from damage areas on branches and stems in 2014. BLAST search

TABLE 1
Characteristics and frequencies of cankers on branch and main stem sections of thousand cankers disease-symptomatic *Juglans nigra* trees in 2014, Hamilton, Ohio

	Sample collection[y]		Bark surface area (cm^2)			No. of cankers per 100 cm^2 bark	
Sample type	Number	Tree	Mean[z]	SE	No. of cankers per sample (range)	Mean[z]	SE
Branch	4	BB1	1,183 a	58.6	6–164	4.2 a	2.9
	4	BB2	1,186 a	37.8	68–176	10.3 a	1.7
	4	BB4	1,114 a	132.5	50–404	16.3 ab	6.7
Main stem	8	BB1	1,714 cd	67.4	1–6	0.2 c	0.05
	8	BB2	2,226 e	71.5	3–60	0.9 cd	0.4
	6	BB3	1,966 cde	88.7	20–93	2.5 d	0.75
	7	BB4	1,874 cd	71.9	3–148	4.6 d	1.3

[y] Explanation for unequal sample numbers: No live branches on Tree BB4 at time of sampling. Portions of main stems of BB3 and BB4 were decayed or dead, so numbers of cankers could not be determined reliably; thus, no canker data were collected for two or one sample(s), respectively.

[z] Analysis of variance performed with log-transformed values and means were compared using Tukey honestly significant difference in R (R Foundation for Statistical Computing, Vienna, Austria). Values followed by different letters are significant ($P < 0.05$).

TABLE 2
Characteristics and frequencies of cankers on branch and main stem sections of thousand cankers disease-symptomatic *Juglans nigra* trees in 2015, Hamilton, Ohio

	Sample collection[y]		Bark surface area (cm^2)			No. of cankers per 100 cm^2 bark	
Sample type	Number	Tree	Mean[z]	SE	No. of cankers per sample (range)	Mean[z]	SE
Branch	4	138	803 a	135.4	3–23	1.8 ab	0.51
	2	139	1,143 a	184.2	7–16	1.0 a	0.25
	4	140	1,128 a	196.2	1–16	0.6 a	0.24
	4	145	953 a	96.8	32–45	4.1 b	0.63
Main stem	8	138	1,844 d	49.2	0–13	0.4 c	0.08
	7	139	1,557 c	64.4	0–6	0.2 c	0.06
	8	140	2,139 e	46.6	0–44	0.6 c	0.25
	8	145	2,077 de	103.7	0–58	1.1 c	0.55

[y] Explanation for unequal sample numbers: Two branches on Tree 139 were dead. Portions of one main stem section of 139 were decayed, so numbers of cankers could not be determined reliably; thus, no canker data were collected for this sample.

[z] Analysis of variance performed with log-transformed values and means were compared using Tukey honestly significant difference in R (R Foundation for Statistical Computing, Vienna, Austria). Values followed by different letters are significant ($P < 0.05$).

results revealed that the sequences fell into two groups of *G. morbida* isolates. Group 1 (for 22 isolates) were all ≥99% identical to *G. morbida* GenBank accession KF656717 but different from Group 2 (for 1 isolate), which was ≥99% identical to GenBank accession FN434081. DNA sequences were obtained for 29 of the 196 morphologically identified *G. morbida* isolated from damage areas on the branches and stems of 2015 evaluated trees. Based on BLAST searches, the resulting sequences with >99% identities to GenBank accessions fell into three groups: Group 1 sequences for 20 isolates (accession KF656717), Group 2 sequences for 6 isolates (accession FN434081), and Group 3 sequences for 3 isolates (accession KJ664795).

For *Botryosphaeria*-like isolates ($n = 66$) from 2014 branches and main stems, only five were subjected to DNA sequencing. All were identified as '*B.*' *obtusa* based on ≥99% identities to GenBank accession *D. seriata* MF136589. In 2015, 70% of the tentatively identified *Botryosphaeria* species isolates ($n = 102$) from collected samples were identified using DNA sequencing. Based on GenBank BLASTn results, 65 isolates were identified as '*B.*' *obtusa* with ≥99% identities to *D. seriata* accession KJ93.56, 5 as *B. dothidea* with ≥99% identities to accession KM435318, and 1 non-*Botryosphaeria* sp. (i.e., *Mortiella* sp.).

Eleven percent of tentatively identified *F. solani*-like isolates ($n = 130$) obtained from 2014 collected branch and main stem samples were subjected to DNA sequencing using the translation elongation factor 1-α primers *ef1*/*ef2*. GenBank BLASTn search results revealed that the sequences fell into three groups of FSSC (Table 6) and were identified to phylogenetic species with ≥99% identity matches: FSSC 13 for 10 isolates (GenBank accession HQ647286); FSSC 6 for 1 isolate (GenBank accessions LC1453335, DQ246873, HE647960); and FSSC 25 for 1 isolate

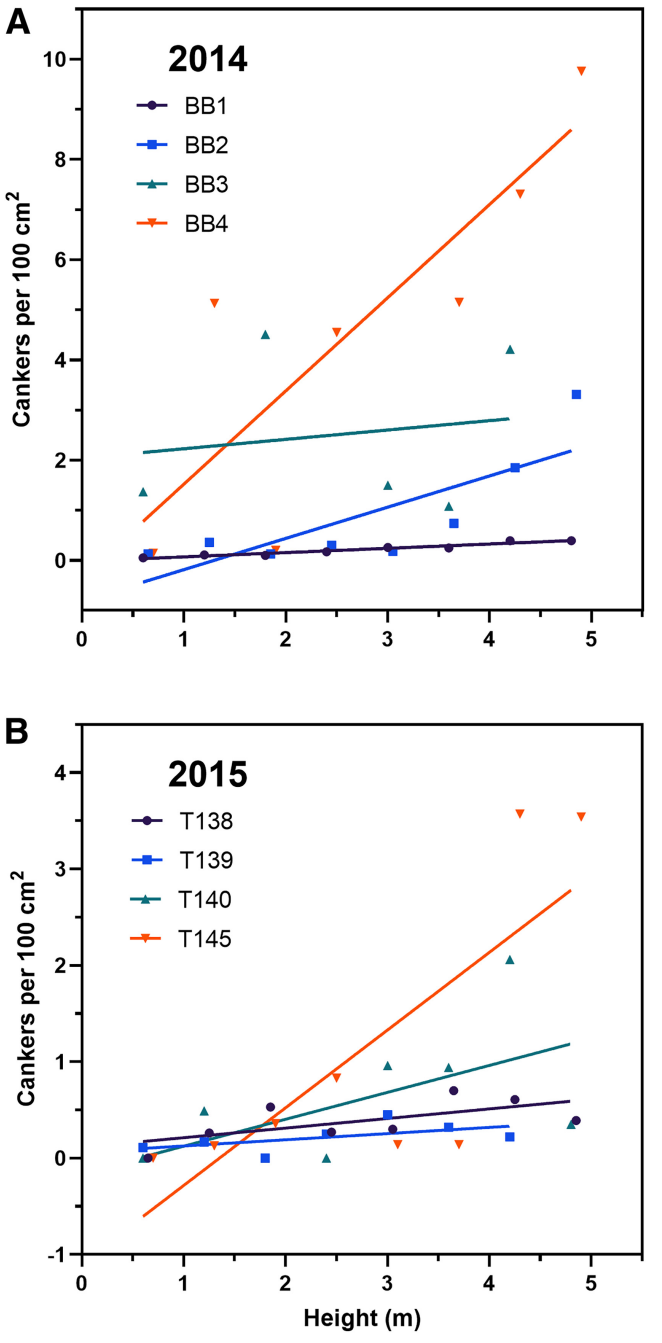

FIGURE 1

Relationship between canker density (*Geosmithia*-like only) and main stem height on thousand cankers disease-symptomatic *Juglans nigra* trees in **A,** 2014, and **B,** 2015, in Hamilton, Ohio. Lines are based on linear regression analyses with best linear fit as follows. BB1: $y = 0.086x - 0.016$, $P < 0.0001$, $r^2 = 0.941$; BB2: $y = 0.623x - 0.808$, $P = 0.016$, $r^2 = 0.647$; BB3: $y = 0.187x + 2.041$, $P = 0.796$, $r^2 = 0.026$; BB4: $y = 1.861x - 0.339$, $P = 0.018$, $r^2 = 0.705$; T138: $y = 0.996x + 0.114$, $P = 0.081$, $r^2 = 0.422$; T139: $y = 0.064x + 0.063$, $P = 0.178$, $r^2 = 0.329$; T140: $y = 0.279x - 0.154$, $P = 0.133$, $r^2 = 0.334$; and T145: $y = 0.806x - 1.088$, $P = 0.026$, $r^2 = 0.590$.

TABLE 3
Numbers of three morphological types of cankers and of two insect damage types observed on main stems of two thousand cankers disease-symptomatic *Juglans nigra* trees in 2015, Hamilton, Ohio

		Canker type[y]			Insect damage type[z]	
Tree number	Section ID[x]	GM-like	EL	LN	Buprestid	Weevil
140	MS2	0	3	0	0	1
	MS4	10	10	2	0	0
	MS5	0	4	0	1	1
	MS7	5	3	0	1	0
	MS9	20	40	8	0	0
	MS12	19	4	2	0	0
	MS14	44	16	4	0	0
	MS16	7	16	3	1	0
145	MS2	1	2	2	1	2
	MS3	3	2	0	4	3
	MS5	8	5	3	0	1
	MS8	17	2	7	3	1
	MS9	5	1	3	1	1
	MS11	5	6	4	2	2
	MS14	58	0	4	2	0
	MS15	58	0	2	2	2

[x] Main stem sections (0.30 m long) were obtained between the base of felled trees to approximately 5 m height. Outer bark on each section was carefully removed with a drawknife to expose any visible damage.

[y] Three types of cankers or necrotic lesions were observed on the sections: GM-like = *Geosmithia morbida*-like cankers; EL = elliptical lesions; LN = long and narrow lesions.

[z] Insect-type damage observed on the sections: tunnels characteristic of bark weevils such as *Stenomimus pallidus* and *Himatium erans*; larval tunnels (and the occasional larva) characteristic of buprestids.

TABLE 4
Isolation frequencies for *Geosmithia morbida* (GM), *Fusarium solani* sensu lato (FS), and *Diplodia seriata* (DS) from observed cankers and insect tunnels on branches of thousand cankers disease-symptomatic *Juglans nigra* trees in Hamilton, Ohio, late summer 2014 and 2015[w]

		Number of assayed damage areas yielding fungi by species[z]						
Damage type[x]	Number of damage areas assayed[y]	GM only	FS only	DS only	GM + FS	GM + DS	FS + DS	GM + FS + DS
2014 evaluations								
Canker								
GM-like	117	37	18	16	13	13	5	4
EL	5	2	1	1	0	0	0	0
LN	7	3	0	1	1	0	0	0
Insect								
Buprestid	9	5	2	0	0	1	0	0
Weevil	4	0	1	2	0	0	0	0
2015 evaluations								
Canker								
GM-like	107	53	6	19	3	3	0	0
EL	29	17	5	3	2	1	0	0
LN	19	10	8	2	3	0	0	0
Insect								
Buprestid	17	1	1	6	1	0	0	0
Weevil	13	5	1	2	1	1	0	0

[w] Four trees sampled in each year. One 30-cm-long subsample was collected from each of four branches per tree for this evaluation.
[x] Three types of cankers or necrotic lesions were observed on the sections: GM-like = *Geosmithia morbida*-like cankers; EL = elliptical lesions; LN = long and narrow lesions. Insect-type damage observed on the sections: tunnels characteristic of bark weevils such as *Stenomimus pallidus* and *Himatium erans*; larval tunnels (and the occasional larva) characteristic of buprestids.
[y] Outer bark was carefully removed with a drawknife to expose any damage.
[z] Fungal taxa by abbreviation: GM = *Geosmithia morbida*; FS = *Fusarium solani* sensu lato; DS = *Diplodia seriata*.

TABLE 5
Isolation frequencies for *Geosmithia morbida* (GM), *Fusarium solani* sensu lato (FS), and *Diplodia seriata* (DS) from observed cankers and insect tunnels on main stems of thousand cankers disease-symptomatic *Juglans nigra* trees in Hamilton, Ohio, late summer 2014 and 2015[w]

		Number of assayed damage areas yielding fungi by species[z]						
Damage type[x]	Number of damage areas assayed[y]	GM only	FS only	DS only	GM + FS	GM + DS	FS + DS	GM + FS + DS
2014 evaluations								
Canker								
GM-like	114	16	35	1	9	0	6	1
EL	15	3	5	2	3	0	0	0
LN	22	5	6	2	2	0	0	0
Insect								
Buprestid	29	0	7	4	0	0	1	0
Weevil	26	1	5	3	2	0	3	0
2015 evaluations								
Canker								
GM-like	125	35	20	22	8	3	0	0
EL	53	14	3	5	2	0	1	0
LN	46	25	4	8	1	3	0	0
Insect								
Buprestid	33	2	4	12	2	0	0	0
Weevil	30	0	0	11	0	0	0	0

[w] Four trees sampled in each year. Eight 30-cm-long main stem sections were collected from each tree.
[x] Three types of cankers or necrotic lesions were observed on the sections: GM-like = *Geosmithia morbida*-like cankers; EL = elliptical lesions; LN = long and narrow lesions. Insect-type damage observed on the sections: tunnels characteristic of bark weevils such as *Stenomimus pallidus* and *Himatium erans*; larval tunnels (and the occasional larva) characteristic of buprestids.
[y] Outer bark was carefully removed with a drawknife to expose any damage.
[z] Fungal taxa by abbreviation: GM = *Geosmithia morbida*; FS = *Fusarium solani* sensu lato; DS = *Diplodia seriata*.

(GenBank accession HM347154). In 2015, 67% of the tentatively identified *F. solani*-like isolates ($n = 76$) from collected TCD tree samples were identified using DNA sequencing with the same primer pairs as above. All but one isolate (a *Fusarium* sp.) were identified to FSSC phylogenetic species with >99% identities using the search function in the Fusarium ID database (Geiser et al. 2004). The isolate frequencies for each identified species and their closest match in GenBank were as follows: 40 isolates, FSSC 13b (accession HQ647286); 6 isolates, FSSC 6 (accessions LC145335, DQ246873, HE647960); and 3 isolates, FSSC 14b (accession DQ246841).

Discussion

Disease progression in one of the 2014 and three of the 2015 TCD-symptomatic trees examined appeared to have "slowed and quiescent dieback" per the description of Type 1 TCD symptoms in Griffin (2015). In general, numerous *Geosmithia* cankers were found on branches and many on main stems, as expected for TCD-symptomatic *J. nigra*. Assuming most new infections by *G. morbida* are associated with *P. juglandis* attacks, most of the observed *Geosmithia* cankers were likely up to 1 (for 2014 trees) or 2 years old (for 2015 trees) because no WTB were found in annual survey traps in those years. However, *G. morbida* was still viable in these old cankers. In companion studies, additional branch and main stem sections were collected from the same eight trees of this study at the time of tree harvest and placed in insect rearing buckets. No *P. juglandis* were obtained from the 2014 trees (Juzwik et al. 2016), but two emerged from 2015 sections and yielded *G. morbida* in molecular and/or standard isolation assays (Moore et al. 2019).

Two other known or putative canker pathogens of *J. nigra* were isolated solely or in conjunction with *G. morbida* from typical *Geosmithia* cankers. FSSC isolates were commonly obtained from branch and main stem samples, but relative isolation rates of "*F. solani* only" to "*G. morbida* only" were not consistent (i.e., varied by year and type of sample). Haplotypes of the FSSC isolates (particularly 6 and 25) are consistent with those reported for *Geosmithia* cankers or TCD trees in Colorado and Italy (Montecchio et al. 2015; Sitz et al. 2017). Thus, at least two FSSC haplotypes could contribute to annual size expansion of single cankers and to subsequent coalescing of cankers in the eastern United States. In addition, natural infection by *F. solani* alone could result in canker production and subsequently contribute to branch dieback in TCD-symptomatic trees (McDermott-Kubeczko et al. 2020). *D. seriata* (anamorph of *Botryosphaeria obtusa*) was also commonly isolated from typical *Geosmithia* cankers on branches and main stems. This fungus causes cankers and dieback in cherry laurel (*Prunus laurocerasus*) and olive (*Olea europaea*) (Kaliterna et al. 2012; Quaglia et al. 2014); however, its pathogenicity *J. nigra* has not been tested. Further field inoculation studies would be helpful in determining how these other fungi might contribute to branch dieback in eastern black walnut.

TABLE 6
Number of *Fusarium* isolates obtained from thousand cankers disease-symptomatic *Juglans nigra* trees and listed according to *Fusarium solani* species complex (FSSC) haplotype identities

	FSSC haplotype designation[z]			
Source tree number[y]	6	13	14	25
2014 isolations				
BB1		1		
BB2	1	4		
BB3		1		1
BB4		4		
2015 isolations				
138		2		
139		17	3	
140	5	8		
145	1	13		

[y] Branch and main stem sections were sampled from each of four trees in each year.

[z] Representative *Fusarium solani* sensu isolates that were obtained from various damage types (canker/lesion or insect damage) were subjected to DNA extraction and PCR analysis. Resulting sequences were compared to accessions in GenBank and in Fusarium ID databases to obtain isolate haplotype identities.

G. morbida was infrequently isolated from damage associated with branch and stem colonization characteristic of buprestid woodborers and Cossoninae bark weevils. The feeding galleries of these insects might intersect with those of *P. juglandis* where *G. morbida* would be acquired. In addition, several weevil species, as well as other insect taxa that colonize *J. nigra*, have been found to carry viable propagules of *G. morbida* (Juzwik et al. 2015, 2016). Thus, other insects might also introduce the pathogen to *J. nigra* and sustain TCD presence in the landscape (Chahal et al. 2019) even in the absence of *P. juglandis* or in known TCD locations (Juzwik et al. 2015; Moore et al. 2019). Further investigation into the role other insect species play in the spread of the pathogen and development of TCD is needed.

The observations of diagnosed TCD-affected *J. nigra* with "slow and quiescent dieback" or "recovering" crown conditions in Virginia and Ohio locations where WTB populations have collapsed and the presence of viable *G. morbida* in "older" cankers on such trees are notable. The authors hypothesize that continuation of a TCD outbreak in a location in the eastern United States requires sustained populations of WTB that lead to mass attacks of *J. nigra*. Although *G. morbida* might remain viable on *J. nigra* in the years following TCD outbreak in a locale, we further hypothesize that it is insufficient on its own to continue disease expansion in the landscape. The "weak pathogen" status of *G. morbida* assigned by Ploetz et al. (2013) supports this hypothesis. Furthermore, viable propagules and/or DNA of *G. morbida* has been detected on multiple insect species (other than *P. juglandis*) in four eastern states, with three (Illinois, Indiana, and Minnesota) of these having no known occurrence of TCD in the landscape (Moore et al. 2019). Thus, it appears that only when *G. morbida* is introduced to *J. nigra* during massive WTB attacks will the pathogen contribute to significant *J. nigra* mortality in the eastern United States. Based on research results of a disease outbreak in a plantation of largely *J. nigra* and 10% *J. regia* in central Italy, the authors concluded that the disease could potentially have disastrous effects on local economies relying on *J. regia* nut production. Specifically, high *P. juglandis* population densities could lead to massive attacks and associated high *G. morbida* population pressure resulting in TCD in *J. regia* plantations (Moricca et al. 2020).

Further investigations of factors promoting levels of *P. juglandis* populations are needed. Precipitation patterns and their relationship to physiological stress in *J. nigra* could be significant. Specifically, drought-stressed *J. nigra* could contribute to high WTB populations in local areas of the eastern United States, followed by population collapse when normal precipitation patterns resume and subsequently support *J. nigra* vigor and health.

Additional field studies have been conducted to determine the pathogenicity of *D. seriata* on *J. nigra* and to compare the relative virulence of *G. morbida* to that of selected FSSC haplotypes, *D. seriata*, and *Botryosphaeria dothidea* on branches of *J. nigra* growing in forest plantation or park landscapes where WTB is absent or below detectable levels. Preliminary results of multiple-point branch inoculations (50 per branch) from these studies have been published (Juzwik et al. 2020). The largest and similar sized (1.2 and 1.1 cm^2) cankers resulting 15 months after mid-June inoculations were found for *G. morbida* and *F. solani*, respectively, in Ohio park reserve trees. However, cankers developing over 24 months following September inoculations with *G. morbida*, *F. solani*, and *D. seriata* were largest (average 2.6 cm^2) for *G. morbida* treatments compared with for the other fungi (average 0.8 cm^2). However, branch condition ratings did not differ among treatments over the 24 months, and no *G. morbida*-inoculated branches died. These results support the previous assessment (Ploetz et al. 2013) of *G. morbida* as a weak pathogen. Smallwood et al. (2022) concluded that reduction of WTB populations should be the focus of TCD control because of the lower virulence of the canker pathogen. The insect vector species' population was reduced to, and maintained at, consistently low levels in two *J. nigra* plantations in Washington state by the authors. Furthermore, emamectin benzoate-only treatment of crop trees was associated with similar or improved healthy crown conditions compared with those of trees treated with a combination of the insecticide and propiconazole (fungicide) or the untreated control trees.

Conclusions and Significance

Careful dissection of branch and main stem sections of TCD-symptomatic eastern black walnut trees in Butler County, Ohio, revealed numerous *Geosmithia*-like cankers and other necrotic lesions, as well as insect-associated damage comparable to observations reported for similar diseased trees in the greater Knoxville, Tennessee, area (McDermott-Kubeczko et al. 2020). Furthermore, *G. morbida* was commonly isolated alone or in co-occurrence with *F. solani* sensu lato and *D. seriata* from typical *Geosmithia*-like cankers. Two of the FSSC haplotypes (6 and 25) associated with our study trees had been reported as TCD-symptomatic tree associates in Colorado, United States (Sitz et al. 2017), and in Italy (Montecchio et al. 2015). The common isolation of *G. morbida* from diseased but "lingering" TCD-symptomatic *J. nigra* 1 and 2 years after a precipitous drop in *P. juglandis* populations attests to the persistence of the TCD pathogen in the landscape without new, massive attacks by its primary insect vector. The results of our study will be of particular interest to state plant health regulatory agencies in the eastern United States.

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