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Methyl Bromide Fumigation to Eliminate Thousand Cankers Disease Causal Agents from Black Walnut

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Phytosanitary treatments for logs and barked wood products are needed to mitigate the spread of thousand cankers disease through the movement of these commodities. The disease threatens eastern black walnut (*Juglans nigra* L.) populations in the United States. It is caused by repeated attacks by the walnut twig beetle (*Pityophthorus juglandis* Blackman) and subsequent canker development caused by the fungal pathogen *Geosmithia morbida* M. Kolařík et al. Methyl bromide (MB) fumigations were evaluated for efficacy against *P. juglandis* and *G. morbida* in *J. nigra* bolts. Fumigation with 82 mg/L MB for 24 h at 4.5° C eliminated *P. juglandis* in *J. nigra*, but was ineffective against *G. morbida*. Subsequent experiments focused on eliminating *G. morbida*, but results were inconclusive because of low rates of pathogen recovery from naturally infested control bolts. Final experiments used *J. nigra* bolts artificially inoculated with *G. morbida*. Fumigations with 240 and 320 mg/L MB for 72 h at 10° C were effective in eliminating *G. morbida* from *J. nigra* bolts. Results confirm that the USDA fumigation treatment schedule for logs with the oak wilt pathogen will also mitigate the risk of spreading the thousand cankers disease vector and pathogen by movement of walnut bolts and wood products.

Keywords: *Geosmithia morbida*, *Pityophthorus juglandis*, walnut twig beetle, *Juglans nigra*, phytosanitary treatments

Thousand cankers disease (TCD) is a recently described disease that is responsible for decline and mortality of walnut (*Juglans* spp. L.) trees in the United States (Tisserat et al. 2009, Seybold et al. 2013, Utley et al. 2013). The disease is caused by the fungal pathogen *Geosmithia morbida* M. Kolařík, E. Freeland, C. Utley, and N. Tisserat, the spores of which are carried by the walnut twig beetle *Pityophthorus juglandis* Blackman (Tisserat et al. 2009, Kolařík et al. 2011, Seybold et al. 2016). Branches and stems of walnut trees become infected with *G. morbida* following attacks by *P. juglandis*. The beetles construct egg galleries as they feed on the phloem, which is inoculated with spores of the pathogen (Kolařík et al. 2011, Utley et al. 2013). Infections develop in the phloem as small, dark-colored cankers, and repeated attacks by *P. juglandis* on the same tree lead to the formation of numerous cankers, hence the common name "thousand cankers disease" (Tisserat et al. 2009, Utley et al. 2013, Hadziabdic et al. 2014a). Galleries and lesions may overlap and block nutrient translocation within the tree, resulting in disease symptoms that include foliar chlorosis and wilt, crown thinning, and branch dieback, which may not appear until years after the initial infection (Tisserat et al. 2009, Kolařík et al. 2011, Hadziabdic et al. 2014a). Advanced progression of TCD may ultimately lead to tree mortality, but some *J. nigra* trees have survived or even recovered with improved crown

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health after initial disease symptoms had been observed (Utley et al. 2013, Griffin 2015).

The disease was discovered in the 2000s after widespread mortality of eastern black walnut (*Juglans nigra* L.) occurred in the western United States (Graves et al. 2011, Tisserat et al. 2011, Utley et al. 2013). *Juglans nigra* is not native to this region, but has been planted widely across the West for ornamental purposes (Graves et al. 2011, Kolařík et al. 2011). In 2010, TCD was discovered in *J. nigra* populations in Knoxville, Tennessee (Cranshaw 2011, Grant et al. 2011, Hadziabdic et al. 2014a). This was the first observation of the disease within the native range of *J. nigra* in the eastern United States. Since that time, TCD has been documented in other eastern states, including Virginia, Pennsylvania, Maryland, North Carolina, and Ohio (Seybold et al. 2013, Hadziabdic et al. 2014b, Rugman-Jones et al. 2015). In 2013, the disease was discovered for the first time outside North America in northeastern Italy (Montecchio et al. 2014).

Pityophthorus juglandis is native to the southwestern United States and Mexico where it is found on Arizona walnut (*Juglans major* L.), a tree species also native to this area (Kolařík et al. 2011, Zerillo et al. 2014, Rugman-Jones et al. 2015). Walnut twig beetles attack native and introduced *Juglans* spp. in the West, and infection by *G. morbida* and TCD development are thought to follow colonization of host tissues by *P. juglandis* (Graves et al. 2011, Seybold et al. 2016). However, the severity of the disease varies among *Juglans* spp. because of host selection by *P. juglandis* and susceptibility of the host (Utley et al. 2013, Hishinuma 2017, Hefty et al. 2018). *Pityophthorus juglandis* can colonize most *Juglans* spp. but brood production can vary greatly among species, with the highest reproduction rates occurring on *J. nigra*, *J. californica*, and *J. hindsii* (Hefty et al. 2018). Similarly, a field survey in California from 2010 to 2014 indicated that TCD symptoms progressed rapidly in these species but slowly in *J. major* (Hishinuma 2017). It is assumed that *J. major* has co-evolved with *P. juglandis*, resulting in some resistance to disease development (Hishinuma 2017, Hefty et al. 2018). In an evaluation of the susceptibility of several *Juglans* spp. to canker development after artificial inoculation with *G. morbida*, *J. nigra* was found to be the most susceptible species (Utley et al. 2013). However, other pathogenic fungi such as *Fusarium* spp. may also play a role in TCD progression, as they are often associated with canker development in later stages of the disease (Tisserat et al. 2009, Cranshaw and Tisserat 2012). Specifically, *Fusarium solani* has been isolated from cankers on the main stem of *J. nigra* trees with TCD, but it has not been isolated from cankers surrounding *P. juglandis* galleries (Tisserat et al. 2009).

Eastern black walnut is one of the most important commercial tree species in North America, and the occurrence of TCD in its native range threatens commercial trade of its valuable veneer, lumber, and nuts (Haun et al. 2010). The United States averages US$325 million in annual exports of *J. nigra*, and the standing volume of *J. nigra* was recently valued at US$539 billion (Haun et al. 2010). Quarantine regulations restricting the movement of walnut products have been implemented within the United States to prevent the spread of TCD (Newton et al. 2009, Haun et al. 2010, Leslie et al. 2010). However, this has the potential to limit trade and make walnut products more expensive. Additionally, disruption of the market for export of walnut logs could negatively affect the forest industry. The Republic of Korea currently recognizes TCD as a potential threat to their native forests and requires a phytosanitary certificate declaring that walnut logs are free of *P. juglandis* and *G. morbida* prior to importation from states in the United States where TCD is known to occur (EPPO 2015, USDA 2018). Thus, it is urgent to develop effective phytosanitary treatments that mitigate the spread of the TCD vector and pathogen, and allow safe movement of walnut logs domestically and internationally.

Heat treatments are used frequently as a phytosanitary measure for wood products such as pallets and firewood, and previous studies have demonstrated effective thermal treatments for the TCD causal agents in black walnut (IPPC 2002, Mayfield et al. 2014, Mackes et al. 2016). Heat treatments, and other phytosanitary measures such as debarking and reducing sapwood moisture content, are used less frequently for whole logs because these treatments are likely to cause checking and reduce lumber quality. For this reason, fumigation with methyl bromide (MB) has long been the preferred treatment for US log exports. The use of MB for general pest-control purposes has been largely phased out under the Montreal Protocol, because of its ozone-depleting properties. However, there is no planned phase-out for quarantine and preshipment use of MB, and it remains a valuable tool to meet import requirements of trade partners for US logs and other agricultural products where there are no cost-effective alternatives. Current treatment schedules for log exports from the United States include a hardwood log treatment for oak logs to eliminate the the oak wilt pathogen, *Bretziella fagacearum* (Bretz) Z.W. de Beer, Marinc., T.A. Duong, and M.J. Wingf. [formerly *Ceratocystis fagacearum* (Bretz) Hunt] (De Beer et al. 2017, USDA 2017). Other studies have evaluated MB efficacy against a number of wood boring beetles, but none have evaluated efficacy against the vector–pathogen complex that causes TCD (Barak et al. 2005, 2011). The objective of this study was to determine an effective MB fumigation treatment schedule to provide quarantine-level control of *P. juglandis* and *G. morbida* in *J. nigra* logs.

Materials and Methods

A series of five experiments were conducted to evaluate MB fumigation for eradication of *P. juglandis*, *G. morbida*, or both in

Management and Policy Implications

Eastern black walnut (*Juglans nigra* L.) is one of the most important commercial tree species in North America, with a standing volume recently valued at over US$500 billion. The United States averages US$325 million in annual exports of *J. nigra*, but regulations designed to prevent the spread of thousand cankers disease have the potential to make walnut products more expensive and limit both domestic and international trade. Fumigation with methyl bromide has long been a preferred treatment for US log exports, and although its general use has been largely phased out under the Montreal Protocol, there is no planned phase-out for quarantine and preshipment use, and it remains a valuable tool to meet log import requirements of US trade partners. This study demonstrates that a fumigation schedule of at least 240 mg/L methyl bromide for 72 h at 10° C, which is currently approved to eliminate the oak wilt pathogen from oak logs for export, will also eliminate the thousand cankers disease vector and pathogen from black walnut bolts. Use of this schedule will help prevent additional spread of thousand cankers disease and will help minimize negative impacts on the black walnut log export market.

bolts of *J. nigra*. The collection and infestation of *J. nigra* material varied among experiments (Table 1). The first four experiments (2011–14) used *J. nigra* bolts collected from naturally infested trees exhibiting TCD symptoms from several sites in Knox County, TN. Trees were felled and sawn with bark intact into bolts that differed in length and diameter depending on experiment (Table 1).

In the 2012 experiment only, *J. nigra* bolts were baited with a walnut twig beetle pheromone lure (product #300000736, Contech Enterprises Inc., Victoria, British Columbia) to attract *P. juglandis* and maximize the number of adults and brood in the bolts. Five symptomatic *J. nigra* trees with TCD were felled from sites in Knox County, TN and cut into 78 large bolts (90 cm length, approx. 8 cm dia.) used in this experiment. As described in Mayfield et al. (2014), the bolts were hung in groups of three from ropes in the crowns of *J. nigra* trees showing symptoms of TCD at sites in Knox County. Each group of three bolts had one walnut twig beetle pheromone lure stapled underneath a bolt in the group and was field-deployed for 2 months. After field deployment, the logs were transported to the University of Tennessee, Knoxville, and were cut into 234 sample bolts (30 cm length) for use in the experiment.

In the 2017 experiment, efficacy was evaluated against the pathogen only, and *J. nigra* bolts were inoculated with isolates of *G. morbida* to ensure colonization by the pathogen. Four healthy *J. nigra* trees were felled at a site in Oconee National Forest, GA and cut into 16 large bolts (60 cm length, approx. 9 cm dia.). Bolts were transported to the Southern Research Station in Athens, GA and inoculated the following day with two *G. morbida* isolates (GM-8, GM-49) obtained from Dr. Mark Windham, University of TN, Knoxville. A cork borer (7 mm dia.) was used to remove bark plugs, a plug of *G. morbida* (6 mm dia.) cultured on potato dextrose agar (PDA) was placed in the wound, and the bark plug was re-inserted. The inoculation points were located at 7.5, 23.5, 38.0, and 53.5 cm on one side of the bolts. Then, the bolts were flipped 180 degrees, and the second *G. morbida* isolate was inoculated at the same distances along the bolts. Parafilm and duct tape were wrapped around the inoculation points, and the bolts were incubated in a small greenhouse setup in the laboratory at 25° C and 89 percent relative humidity. Bolts used in the May and June 2017 experiments were incubated for seven and nine weeks, respectively. After incubation, the large bolts (60 cm length) were cut into four sample bolts (15 cm length). Each sample bolt had two inoculation points, one for GM-8 and one for GM-49.

In all experiments, the ends of bolts were sealed with paraffin to preserve moisture content and to ensure that measured levels of efficacy from fumigation against the TCD organisms was the result of penetration through the bark rather than the end grain. Paraffin was applied within 24 h of tree harvest and again on the same day that the bolts were sectioned into smaller lengths.

Fumigations

Sample bolts were assigned randomly to a fumigation treatment or control group, which differed in sample size based on experiment. Fumigations were conducted in stainless steel chambers (245 or 672 L) with the exception of the final experiment in 2017, which was performed at a smaller scale in glass chambers (10 L). All chambers were equipped with fans (12 V) to ensure circulation of MB and tube fittings (0.64 cm) with septa (Swagelok Inc., Knoxville, TN) to allow sampling to measure headspace MB concentrations during the treatment. The chambers were housed in temperature-controlled units set at a constant temperature throughout the fumigation. The steel chambers (245 or 672 L) were held inside a standard refrigerated shipping container (6.1 m length; Thermo King Inc., Minneapolis, MN), and fumigations with the glass jars (10 L) were conducted inside a walk-in chamber (Parameter Generation & Control, Black Mountain, NC). Bolts were held for 24 h prior to treatment to allow them to acclimate to the treatment temperature. To further ensure adequate fumigant circulation, the bolts were placed on wire mesh 25 mm above the bottom of the chamber, and load factors did not exceed 45 percent of the chamber volumes (Table 2). Untreated control bolts were stored alongside treated bolts at the same temperature.

Initial concentrations of MB were calculated volumetrically by using the ideal gas law to correct for treatment temperature and atmospheric pressure at the time of fumigation. MB source gas (99.8 percent Meth-o-Gas Q; Great Lakes Chemical Corp., Middlebury, CT) was tempered to the treatment temperature prior to adding it to the fumigation chamber. MB was introduced to the chambers by first pulling a slight vacuum (20–70 mm Hg), allowing for introduction of the fumigant. Predetermined volumes of MB were extracted from tedlar sampling bags (SKC Inc., Eighty-Four, PA) to a gas-tight syringe (Hamilton Co., Reno, NV). The syringe was fitted to either a Luer lock valve or Swagelok fitting on the chamber to facilitate gas delivery. Chambers were returned to ambient pressure for the duration of the experiment.

Gas samples were taken periodically during the fumigations to monitor the concentration of MB in the headspace by using an Agilent 490 micro gas chromatograph (GC) with a thermal conductivity detector (Agilent Technologies Inc., Santa Clara, CA). Samples were drawn through peek tubing (1 m length, 0.12 mm ID) over 60 s sampling time (5 mL sample volume) using a six position stream selector valve (Valco Instruments Inc., Houston, TX). Samples entered the GC via an unheated injection port and were

Table 1. Dimensions of *Juglans nigra* bolts (mean ± SE), infestation methods for source material, and target organisms for fumigation experiments (2011–17).

Experiment	Length (cm)	Diameter (cm)	Sample size (*n*)	Infestation method	Target organism	
					P. juglandis	*G. morbida*
2011	30.0 ± 0.12	8.80 ± 0.15	273	Natural	X	X
2012	30.0 ± 0.15	8.37 ± 0.20	234	Natural and baited[a]	X	
2013	27.4 ± 0.24	10.0 ± 0.32	151	Natural		X
2014	33.7 ± 0.18	10.9 ± 0.18	224	Natural		X
2017	15.9 ± 0.08	8.74 ± 0.28	64	Artificial		X

[a]Bolts were baited with a *Pityophthorus juglandis* pheromone lure to maximize infestation.

Table 2. Fumigation parameters, including exposure times, temperatures, methyl bromide concentrations, and chamber sizes (2011–17).

Experiment	Exposure time (h)	Temp. (°C)	Methyl bromide conc. (mg/L)	Chamber size (L)	Replicates (*n*)	Mean chamber load factor (percent)
2011	24	4.5	32, 64, 96, 128, 160	245	2	7.2 ± 0.7
	24	15.6	32, 64, 96, 128, 160	245	2	9.0 ± 1.1
2012	24	4.5	32, 48, 64, 82	245	3	9.7 ± 1.2
2013	48	4.5	120	245	4	38.2 ± 4.1
2014	48	4.5	240	672	2	44.6 ± 10.1
2017	72	10.0	160, 240, 320	10	8	19.8 ± 0.1

transferred to a PoraPLOT Q column (10 m; Agilent Technologies Inc.) held at 100° C. The carrier gas used was helium (30 psi), and the run time was 85 s. The samples entered the detector at 100° C, and an output chromatogram was computed and analyzed through OpenLAB EZChrom software (Agilent Technologies Inc.). A linear calibration of the instrument's response to varying MB concentrations was used to determine concentrations in the headspace. This was verified by analysis of the peak area at a retention time of 1.03 min, which was indicative of MB. After fumigation, the lids were removed from the chambers, and the bolts were aerated in a well-ventilated area for a minimum of 24 h. In total, five fumigation experiments were conducted that varied in MB concentrations, lengths of exposure, temperatures, and/or chamber sizes (Table 2).

Evaluation of Samples for Emergence of *Pityophthorus juglandis*

In the 2011 and 2012 experiments only, emergence containers were used to evaluate fumigation treatment efficacy against *P. juglandis* and to estimate beetle populations in untreated bolts (Mayfield et al. 2014). Each container was constructed from a standard (19 L) plastic bucket modified to allow a *J. nigra* bolt to be suspended within the structure. Bolts were fitted with a screw hook and attached to an eye bolt secured on the bucket lid. The containers had two holes covered with fine mesh on opposing sides. The bottom of the bucket was removed and fitted with a plastic funnel (1 L, 23 cm dia.; LUBEQ Corp., Elgin, IL) leading to a Nalgene collection jar (250 mL). The jar was filled with polypropylene glycol to preserve insects that were collected over 5 months at room temperature. Jars were monitored for emerging *P. juglandis* adults, which were counted and expressed as the number of beetles per square meter of bark surface.

Evaluation of Samples for *Geosmithia morbida*

After treatment, the bark was removed from each bolt, and samples were obtained from areas of discolored phloem associated with cankers. The samples were surface-sterilized by dipping briefly in 95 percent ethanol and flaming, and then subdivided into 10 chips (1 × 1 cm^2) per bolt. The phloem chips were placed onto PDA media plates, which were amended with tergitol (1 mg/L), ampicillin (0.25 g/L), and rifampicin (0.01 g/L) to suppress the growth of fast-growing fungi and to control bacterial growth. The plates were incubated at 25° C and then evaluated for the presence of *G. morbida* after 4 days. *G. morbida* colonies were confirmed by the presence of characteristic conidia and conidiophores (Kolařík et al. 2011). Plates were reevaluated every 1 to 2 days for up to 9 days. Bolts were classified as positive for *G. morbida* if the pathogen was isolated from at least one of the phloem chips. All tools used to isolate *G. morbida* from samples were sterilized with 95 percent ethanol.

Statistical Analysis

Reductions in beetle survival rates for fumigated and control logs were explored via counts of beetles that emerged from the 2012 experiment, but mortality rates could not be estimated because the number of beetles killed during treatments was unknown. A Poisson regression model was investigated, but was severely overdispersed. A negative binomial distribution was chosen to model the data, which contained a continuous predictor for concentration-time (CT) products and a categorical blocking factor for replicate numbers, with no interaction term. A zero-inflated version of the model was fitted with the pscl package (Zeileis et al. 2008) in R statistical computing software (R Core Team 2018), and the adjustment for zero-inflation was tested with a likelihood ratio test. SAS version 9.4 (SAS Institute Inc., Cary, NC) was used to calculate and fit models by using CT products from each replicate in all experiments with positive and negative data for each chip evaluated for viable *G. morbida*. A total of 6,088 observations across three temperatures were included in the model. A normally distributed model using nontransformed data was chosen to assess the data, as log-transformed data and Gompertz-distributed models did not improve the lack-of-fit statistic.

Results

November 2011 Fumigation

Fumigations at 4.5° C eliminated *P. juglandis* at all MB concentrations tested (32–160 mg/L), but did not eradicate *G. morbida* from the bolts (Table 3). However, survival of *G. morbida* generally decreased with increasing concentrations of MB. Fumigation with 160 mg/L MB provided the best control at this temperature with only 1 of 12 (8.3 percent) bolts yielding *G. morbida* post-treatment.

Fumigations at 15.6° C eliminated *P. juglandis* in most MB treatments, with the exception of fumigations with 96 mg/L where one adult *P. juglandis* was found in an emergence container (Table 3). *G. morbida* was recovered from 2 of the 12 (16.7 percent) bolts fumigated at 15.6° C with 32 mg/L MB, which was the lowest concentration tested. Elimination of *G. morbida* was achieved at this temperature for fumigations using 64 mg/L MB or more.

August 2012 Fumigation

Fumigations at 4.5° C with 82 mg/L MB eradicated *P. juglandis* populations in *J. nigra* bolts (Table 4). In contrast to the 2011 experiment, lower MB concentrations tested (32–64 mg/L) did not eliminate *P. juglandis* in the treated bolts, but survival was reduced with increasing MB concentrations (Table 4). Fumigations with 64 mg/L MB greatly reduced *P. juglandis* emergence from the bolts, with adult beetles emerging from 3 of 39 (7.7 percent) of the treated bolts. The overall number of beetles to emerge from control bolts in 2012 was greater than from bolts used in the 2011

Table 3. *Pityophthorus juglandis* emergence and *Geosmithia morbida* recovery (both mean ± SE) from *Juglans nigra* bolts fumigated with methyl bromide for 24 h (2011).

Temp. (°C)	Initial methyl bromide conc. (mg/L)	Concentration–time product (h*mg/L) (mean ± SE)[a]	*Pityophthorus juglandis*			*Geosmithia morbida*	
			Total no. of bolts	No. of bolts with beetles	Beetle emergence (mean no. per m^2 ± SE)	Total no. of bolts	No. of bolts positive[b]
4.5	0	0	38	30	315.5 ± 21.4	24	13
	32	507.7 ± 79.4	19	0	0 ± 0.0	12	4
	64	1,219.5 ± 6.4	19	0	0 ± 0.0	12	4
	96	1,826.0 ± 57.9	19	0	0 ± 0.0	12	2
	128	2,509.4 ± 117.3	19	0	0 ± 0.0	12	3
	160	3,296.6 ± 19.1	19	0	0 ± 0.0	12	1
15.6	0	0	40	34	114.5 ± 38.0	24	11
	32	601.3 ± 81.5	20	0	0 ± 0.0	12	2
	64	1,316.6 ± 62.5	20	0	0 ± 0.0	12	0
	96	1,855.2 ± 87.6	20	1	0.815 ± 0.8[c]	12	0
	128	2,499.0 ± 144.3	20	0	0 ± 0.0	12	0
	160	3,136.5 ± 174.2	20	0	0 ± 0.0	12	0

[a]Concentration–time product from treatment replicates (n = 2).
[b]Positive recovery based on viable *Geosmithia morbida* from at least one phloem chip plated per treated bolt.
[c]One adult *Pityophthorus juglandis* collected in post-treatment emergence evaluation.

Table 4. *Pityophthorus juglandis* emergence (mean ± SE) from *Juglans nigra* bolts fumigated with methyl bromide for 24 h at 4.5° C (2012), including negative binomial model estimates of beetle emergence reduction rates (percent) at mean concentration–time products.

Initial methyl bromide conc. (mg/L)	Concentration–time product (h*mg/L) (mean ± SE)[a]	*Pityophthorus juglandis*			Reduction rate (percent) of beetle emergence	95 percent confidence limits (min–max)
		Total no. of bolts	No. of bolts with beetles	Beetle emergence (mean no. per m^2 ± SE)		
0	0	78	63	2,226.6 ± 314.4	N/A	N/A
32	701.4 ± 10.4	39	16	17.5 ± 6.8	98.8895	98.6396–99.0935
48	1,022.9 ± 2.3	38	4	3.7 ± 2.5	99.8586	99.8099–99.8948
64	1,332.1 ± 7.8	39	3	0.6 ± 0.4	99.9807	99.9716–99.9869
82	1,683.3 ± 18.8	40	0	0 ± 0.0	99.9980	99.9967–99.9988

[a]Concentration–time product from treatment replicates (n = 3).

experiment, likely because of baiting and hanging the bolts in trees with TCD prior to use.

July 2013 and August 2014 Fumigations

In the 2013 experiment, *G. morbida* was recovered from bolts fumigated for 48 h with 120 mg/L MB (Table 5). The pathogen was isolated from at least one bolt in each trial post-treatment, and overall was found in 9 of 151 (6.0 percent) fumigated bolts. *Geosmithia morbida* was recovered from 29 of 41 (70.7 percent) of the untreated control bolts.

In 2014, fumigations with 240 mg/L MB for 48 h did not eliminate *G. morbida* from the treated bolts (Table 5). Although one trial was successful in eradicating *G. morbida* from all bolts, the pathogen was recovered from 1 of 90 (1.1 percent) treated bolts in the other trial. *G. morbida* was isolated from only 5 of 44 (11.4 percent) untreated control bolts, suggesting that the pathogen was not well established in the source material used in this experiment.

May and June 2017 Fumigations

In the May 2017 trial, *G. morbida* was eradicated in bolts fumigated for 72 h with 240 and 320 mg/L MB (Table 5). One of 8 (12.5 percent) bolts fumigated with 160 mg/L MB was positive for *G. morbida* following treatment. In the June 2017 trial, *G. morbida* was eliminated in bolts when fumigated with 160, 240, and 320 mg/L MB. The pathogen was routinely recovered from all untreated control bolts in both trials.

Statistical Analysis

A negative binomial model estimated that CT had a significant effect on *P. juglandis* emergence rates (χ^2 = 219.54, df = 1, P < .001). The zero-inflated term improved the model (χ^2 = 34.349, df = 3, P < .001), indicating that there were more zeros in the data than predicted by the negative binomial distribution. The fitted CT coefficient was –0.0064 (SE = 0.0003), equating to a multiplicative effect of $e^{-0.0064}$ = 0.9936 on beetle emergence rate, or a 0.64 percent reduction in emergence for every one-unit increase in CT (Table 4). Probit models showed a significant treatment effect of temperature; thus estimates for CT values necessary to provide quarantine level control of *G. morbida* were determined separately for each of the three treatment temperatures (Table 6). Model estimates for CT values from the 15.6° C treatments aligned with experimental data from 2012. However, data from treatments at 10° C did not provide a good fit for the model, and fiducial limits could not be calculated because there was just a single recovery at one treatment other than the control. Similarly, estimates for treatments at 4.5° C were less than the CT values shown in this research to be ineffective for attaining 100 percent control of *G. morbida*.

Discussion

Results from these experiments suggest that the insect vector, *P. juglandis*, is easier to eliminate via MB fumigation than the fungal pathogen, *G. morbida*. Although the lowest MB concentration tested, 32 mg/L, eliminated *P. juglandis* in the 2011 experiment, this dose

Table 5. *Geosmithia morbida* recovery from *Juglans nigra* bolts fumigated with methyl bromide (2013–17).

Experiment	Temp. (°C)	Initial methyl bromide conc. (mg/L)	Concentration–time product (h*mg/L) (mean ± SE)	Replicates (*n*)	*Geosmithia morbida*	
					Total no. of bolts	No. of bolts positive[a]
2013	4.5	0	0	1	41	29
		120	3,891.8 ± 95.3	4	151	9
2014	4.5	0	0	1	44	5
		240	5,800.3 ± 81.0	2	180	1
2017	10	0	0	2	16	16
		160	7,683.1 ± 147.1	8	16	1
		240	11,341.5 ± 138.1	8	16	0
		320	15,581.6 ± 249.4	8	16	0

[a]Positive recovery based on viable *Geosmithia morbida* from at least one phloem chip plated per treated bolt.

Table 6. Probit model estimates for methyl bromide concentration–time products to provide quarantine level control of *Geosmithia morbida* in *Juglans nigra* bolts.

Temp. (°C)	Concentration–time products and fiducial limits[a] (h*mg/L) to attain level (percent) of control	
	99.90 percent	99.99682 percent
4.5	6,093 (5,475–6,896)	8,250 (7,382–9,393)
10	4,398 (N/A)[b]	5,224 (N/A)[b]
15.6	1,214 (820–2,486)	1,650 (1,109–3,430)

[a]Minimum–maximum 95 percent fiducial limits for each model estimate indicated in parentheses.
[b]Fiducial limits not calculated because of a single recovery in one treatment other than the control.

was not efficacious in the 2012 experiment, which used more heavily infested host material. Given the trend of decreasing emergence with increasing dose in 2012 (Table 4), coupled with more heavily infested walnut bolts, it can be concluded that 82 mg/L for 24 h at 4.5° C is an effective MB fumigation schedule for *P. juglandis*. A negative binomial regression model determined that CT had a significant effect on beetle emergence rates, and that a one-unit increase in CT values yielded a 0.64 percent reduction in *P. juglandis* emergence. The model estimated that treatment with 82 mg/L MB would provide a 99.9980 percent reduction in *P. juglandis* survival (Table 4).

In 2011, one adult *P. juglandis* emerged from a bolt fumigated with 96 mg/L MB at 15.6° C, but this beetle likely did not survive the treatment (Table 3). Given the efficacy of a lower dose, 82 mg/L, at a lower temperature, and with more heavily infested material the following year (Table 4), it is suspected that this beetle was killed during treatment but adhered to the bolt surface. It is likely that the beetle fell passively into the collection jar during post-treatment evaluation of *P. juglandis* emergence.

Establishing an effective MB treatment for eradication of *G. morbida* proved to be more difficult. The initial dose–response experiments in 2011 indicated that fumigations at 15.6° C with 64 mg/L MB or higher eliminated *G. morbida* in bolts (Table 3). Treatments at 4.5° C were less successful in controlling *G. morbida*, although survival of the pathogen decreased with increasing concentrations of MB. The relative difficulty in killing fungi versus insects via fumigation is consistent with other work evaluating treatments for oak wilt (Schmidt 1982).

The experiments that followed in 2013 and 2014 focused on determining efficacy of fumigation by extending treatment times from 24 to 48 h and increasing the initial concentration of MB. Despite the longer treatment time, fumigations with 120 mg/L MB were not sufficient to eradicate *G. morbida.* In 2014, the concentration was doubled to 240 mg/L MB, which also proved ineffective for eliminating the pathogen from treated logs in one trial. One trial using 240 mg/L MB was successful in eliminating *G. morbida*, but the results were inconclusive because low rates of pathogen recovery from untreated controls in this experiment suggested that the pathogen was not well established in the source material.

Subsequent experiments reinforced the conclusion that eradication of *G. morbida* from infested *J. nigra* would require a combination of relatively high MB concentrations and/or long exposure times (concentration–time [CT] products), especially at lower fumigation temperatures (Table 5). In the 2017 experiments, focus was shifted toward increasing both the fumigation treatment time and MB concentration in hopes of eliminating the pathogen. This experiment determined that exposure of the bolts to at least 240 mg/L MB for 72 h at 10° C was required for eradication of the pathogen (Table 5).

Probit models were used to estimate CT products necessary to achieve quarantine-level control (99.9 or 99.99682 percent) of *G. morbida* in *J. nigra* bolts (Table 6). The models were calculated from experimental data and grouped based on treatment temperatures. For treatments at 15.6° C, the model predicted CT values similar to experimental CT values that successfully eliminated *G. morbida*. However, the model for treatments at 4.5 and 10° C did not provide a good fit to the data and resulted in estimated CT values that did not align with experimental results. The model for the 4.5° C treatments estimated that CT values used in this research would attain 99.9 percent control of *G. morbida*, yet this mortality level was not achieved experimentally.

The procedure for artificially inoculating walnut bolts used during this study provides a convenient way to yield well-infected sample material for testing purposes. This is particularly useful in light of quarantines that limit the movement of infested walnut materials and the reduced availability over time of naturally infested materials in areas where TCD was once prevalent. In this study, fumigations with artificially inoculated bolts provided a better comparison of treatment efficacy than naturally infested bolts, as evidenced by the high rates of *G. morbida* recovered from the controls. However, there may be differences in the response to treatment of artificially versus naturally infested materials. Additionally, the bolts used in the fumigations were relatively small compared to commercial saw- and veneer-logs that would be subjected to

treatment. While the bolt size enabled statistical replication among the fumigations, investigation into treatment efficacy for large logs is warranted, though efficacy is not expected to vary greatly considering that the beetle and pathogen reside near the bark surface. These and other potential variables, such as geographical source and season of harvest, merit further study. However, the experiments detailed in this research adequately approximate commercial fumigations of *J. nigra* logs infested with TCD.

Results from this study support a treatment schedule of 240 mg/L MB or above for 72 h at 10° C for walnut bolts infested with *P. juglandis* and *G. morbida*. Future research should investigate the efficacy of this treatment on larger logs and other *Juglans*, especially susceptible species such as *J. regia*, *J. hindsii*, and *J. californica*. This schedule is within the bounds of accepted fumigation schedules for logs and lumber with the oak wilt fungus, *B. fagacearum*. Currently, logs infested with *B. fagacearum* are fumigated with 240 mg/L MB for 72 h before export (USDA 2017). This study confirmed the efficacy of this schedule in eliminating both the TCD vector and pathogen from *J. nigra* bolts. Exports of some log species from the United States continue to rely on the MB quarantine and preshipment use exemption to meet the import requirements of trade partners. Therefore, implementing this MB fumigation schedule could provide a useful option until more desirable and cost-effective phytosanitary measures are available.

Conclusions

This study focused on developing an effective fumigation schedule for the TCD vector and pathogen, which are a threat to *J. nigra* populations. Control of *P. juglandis* populations was achieved by fumigating *J. nigra* bolts for 24 h at 4.5° C with at least 82 mg/L MB. *G. morbida* was not eradicated from *J. nigra* bolts as easily. Fumigations with exposure to 240 and 320 mg/L MB for 72 h at 10° C were successful in eliminating *G. morbida*. The results of these experiments indicate that the maximum MB concentration used to treat oak logs for elimination of the oak wilt pathogen prior to export is also effective for treating *J. nigra* bolts infested with the TCD causal agents.

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