Survival of the Oak Wilt Fungus in Logs Fumigated with Sulfuryl Fluoride and Methyl Bromide

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Abstract

Methyl bromide (MB) fumigation of oak (*Quercus* sp.) logs destined for export is required to mitigate risks associated with movement of the oak wilt fungus, *Bretziella fagacearum*. Alternative fumigants with efficacy against *B. fagacearum* are needed because of MB's ozone-depleting properties. Fumigation with sulfuryl fluoride (SF) is considered a promising substitute. Logs (1.8 m long) were obtained from *Quercus* trees (18.3 to 29.2 cm diameter at breast height) that were naturally infested (NI) or artificially inoculated (AI) with *B. fagacearum* to compare pathogen colonization and survival following fumigation with SF and MB. The logs were fumigated with SF for 72 hours with 240, 280, and 320 g/m^3 or 96 hours with 128 and 240 g/m^3. MB fumigations were conducted using the current treatment schedule for oak logs destined for export (240 g/m^3 for 72 h). Frequencies of successful pathogen isolation before treatment were higher for AI logs than for NI logs based on isolation rates from sapwood chips. Treatments greatly reduced frequencies of viable pathogen presence, but no treatment was successful in eradicating the pathogen. Experiments were conducted on blocks (10.2 by 10.2 by 11.4 cm^3) obtained from *Quercus* trees to investigate simulated penetration and diffusion of SF and MB into oak logs. Slow, variable fumigant diffusion never reached concentration–time products lethal to *B. fagacearum*. Based on these results, reliance on SF alone as a quarantine measure may require higher concentration × time products to achieve quarantine level control of the oak wilt fungus in logs.

Oak wilt, a vascular disease caused by the fungus *Bretziella fagacearum* (Bretz) Z.W. deBeer, Marinc., T.A. Duong, and M.J. Wingf. (syn. *Ceratocystis fagacearum* (Bretz) Hunt), currently threatens the health of *Quercus* species in the United States. The disease was first described in the early 1940s following widespread mortality of red oak (*Q. rubra*) populations in Wisconsin and has since been documented in 24 states, primarily around the North Central states and Texas (Juzwik et al. 2011). All oak species can be infected; however, those of the red oak group (section Lobatae) are the most susceptible, dying within 4 to 6 weeks of first crown symptom expression. Oak wilt symptoms are variable among *Quercus* species but commonly include leaf bronzing and necrosis beginning at the margin of wilted leaves.

B. fagacearum is not currently known to exist outside of the United States. Prior to use of biocidal agents to treat logs, the disease had an economic impact on the US forest industry due to the presence of the fungus in commercial oak-producing areas (Jones 1973). Phytosanitary treatments have been established to prevent unintentional spread. Some phytosanitary measures used for lumber, such as bark removal and kiln drying, are not suitable for the treatment of logs (Jones 1973, Liese and Ruetze 1985). Fumigation with methyl bromide (MB) has demonstrated success in killing *B. fagacearum* in logs harvested from infested trees while also maintaining wood quality (MacDonald et al. 1985). The current fumigation schedule (240 g/m^3 MB for 72 h) for oak logs destined for export from the United States was developed from experiments published in the early 1980s (Schmidt et al. 1982, Liese and Ruetze 1985, MacDonald et al. 1985, USDA APHIS 2016). MB is currently recognized

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as a Class I ozone-depleting substance by the United Nations Environmental Programme and an amendment to the 1992 Montreal Protocol outlined a phase-out of the chemical by 2005 (UNEP DTIE 2002). However, exemptions to the phase-out have allowed for the continued use of MB for some quarantine and preshipment (QPS) applications. MB fumigation of oak logs is one of the largest remaining critical-use QPS exemptions; thus, an alternative fumigant with similar efficacy is urgently needed (USDA APHIS 2016).

An alternative fumigant, sulfuryl fluoride (SF), has demonstrated the ability to kill *B. fagacearum* in timber and logs from wilted oak trees (Woodward and Schmidt 1995, Schmidt et al. 1997). However, previous studies utilizing logs from naturally infected trees have reported low levels of fungal colonization in the logs prior to treatment. It has been suggested that the outcome of fumigation trials could be dependent on the level of wood colonization prior to treatment (Tubajika and Barak 2011). Therefore, testing logs with a higher level of pathogen colonization may be needed to accurately evaluate the success of SF fumigation as an alternative phytosanitary treatment.

Chemical fumigation is a practical phytosanitary treatment in situations where log quality is of importance, yet high moisture contents and the presence of bark can make effective fumigation treatments for quarantine-level control of infested logs difficult (Rhatigan et al. 1998). The presence of water and bark are physical barriers that slow fumigant penetration into wood and thus could reduce the effectiveness of fumigations (Liese et al. 1981, Cross 1992, Morrell 1995, Rhatigan et al. 1998). Diffusion of MB and SF into various wood species has been investigated previously and has shown that SF penetrates and diffuses faster in dry wood than MB (Scheffrahn et al. 1992, Ren et al. 2011).

Experiments were conducted in 2016 and 2017 to evaluate the efficacy of SF fumigation as an alternative phytosanitary treatment of logs from oak wilt–affected trees and to compare the rate of *B. fagacearum* survival following fumigation with SF and MB. The objectives of this research were to (1) assess the rates of *B. fagacearum* colonization in logs from naturally infected (NI) and artificially inoculated (AI) trees prior to fumigation, (2) compare the rate of pathogen survival following fumigation with SF and MB, and (3) characterize the diffusion of SF and MB through green (i.e., undried) oak wood with intact bark.

Materials and Methods

Objectives 1 and 2: Oak log fumigations

Selection of study trees.—In July 2015, eight healthy *Q. ellipsoidalis* (18.3- to 26.2-cm diameter at 1.5-m height) were selected in woodlots in Lino Lakes and Blaine, Minnesota, for artificial inoculation with *B. fagacearum*. Inoculations were carried out per the method described in Juzwik et al. (2019), where three primary roots were exposed on each of the selected trees. Small holes (2.0 cm deep, 0.64 diameter) were drilled into each of the three roots approximately 0.3 m lateral to the root collar. An aqueous suspension (1 mL) of *B. fagacearum* conidia (10^6 spores/mL) was dispensed into each of the drilled holes and, following complete uptake of the inoculum by the tree, the holes were sealed with a moldable epoxy putty and roots were covered with the original soil. Trees were subsequently monitored throughout the growing season to document the progression of crown wilt. Trees selected for artificial inoculation were chosen for their proximity to trees with active, naturally occurring oak wilt infections. In addition, six *Q. ellipsoidalis* (18.3- to 29.2-cm diameter at 1.5-m height) that were naturally infected with *B. fagacearum* were located, one in Lino Lakes, Minnesota, in August 2015 and five in Scandia, Minnesota, in February 2016. The 14 study trees were felled between February and March 2016. Logs (1.8-m length) that were a minimum of 15.2-cm diameter (inside bark) and without visible defects were cut from the trees and transported to the study site at the University of Minnesota in St. Paul, Minnesota.

In July 2016, 12 healthy *Q. ellipsoidalis* (22.1- to 28.7-cm diameter at 1.5-m height) in North Branch, Minnesota, were selected and artificially inoculated with the oak wilt fungus. The tree selection criteria and inoculation procedure used for artificially inoculated trees in 2015 was followed for the trees selected in 2016. Study trees were felled in February 2017, cut into logs (1.8-m length), and transported to the University of Minnesota in St. Paul, Minnesota.

Log preparation and sampling.—For both years of the experiment, sections (22.5 cm) were removed from the ends of each log and pretreatment sample disks (7.5 cm) were then cut for biological evaluation. The remaining length and end diameters were measured and recorded for each of the naturally infected ($n = 14$ logs in 2016) and artificially inoculated logs ($n = 20$ logs in 2016, $n = 48$ logs in 2017). The maximum bark depth, minimum bark depth, and sapwood width were measured at six sample locations on each disk in 2017 (Table 1). Samples were also collected from disks for moisture content determination. The sapwood samples were oven-dried (approx. 103°C for 24 h) and the moisture content calculated on a dry weight basis.

The sample disks removed from logs prior to fumigation were used to assay for *B. fagacearum* presence. Bark was removed with a sterile chisel to expose the outer sapwood (one to three outermost annual rings) at eight locations (2.54 cm wide) on disks taken from logs in 2016. In 2017, the bark was removed at six locations to expose the outer sapwood alternating with six locations to expose the inner sapwood (approx. one to two annual rings outside of the heartwood). Four small (approx. 6.4 mm^2) wood chips were removed from discolored sapwood characteristic of *B. fagacearum* colonization and placed on Petri plates containing a semiselective growth medium (Barnett 1953). The plates were incubated at room temperature (approx. 21°C to 23°C) under ambient lighting and were observed for the presence of the fungus for a maximum of 21 days. Positive cultures were identified based on the occurrence of brown to olive-green colonies, fruity odor, presence of endoconidia, and presence of hyphae characteristic of the fungus on the medium (Barnett 1953).

Drill shavings for the detection of *B. fagacearum* DNA were collected from undisturbed portions of the same disk locations and DNA was extracted following the method of Banik et al. (2013). Amplification of DNA with nested polymerase chain reaction (PCR) and sequencing were carried out per Yang and Juzwik (2017) for detection of the fungus (regardless of viability).

Following fumigation, posttreatment disks were removed from the treated logs in the same manner as described previously. Sapwood samples were taken from posttreat-

Table 1.—Characteristics of logs from Quercus ellipsoidalis *trees that were naturally infected (*n = *14 logs in 2016) or artificially inoculated (*n = *20 logs in 2016,* n = *48 logs in 2017) prior to fumigation with sulfuryl fluoride and methyl bromide.*

	Mean measurement ± SE					
Year	Length (m)	Diameter (cm)	Max. bark depth[a] (cm)	Min. bark depth (cm)	Sapwood width (cm)	Sapwood moisture (%)
2016	1.20 ± 0.01	23.23 ± 0.57	—[b]	—	—	—
2017	1.20 ± 0.01	22.33 ± 0.36	0.89 ± 0.01	0.46 ± 0.01	0.73 ± 0.01	83.0 ± 1.0

[a] Measurements for maximum bark depth, minimum bark depth, sapwood width, and sapwood moisture were obtained from pretreatment sample disks. Maximum bark depth measured from outer bark to cambium and minimum bark depth measured from bottom of bark furrow to cambium.
[b] Maximum bark depth, minimum bark depth, sapwood width, and sapwood moisture content were not measured for the sample disks in 2016.

ment disks for attempted reisolation of *B. fagacearum,* and drill shavings were collected for molecular detection of *B. fagacearum* DNA.

Log fumigations.—Logs were end-sealed prior to fumigation by applying a thin coat of paraffin wax to the freshly exposed ends of each log. This was done for consistency with commercial logs, which are also typically end-sealed. Additionally, the log sections were shorter than commercial logs, so end grain penetration would allow for higher sorption rates and potentially greater efficacy of the fumigant.

In 2016, logs were randomly assigned to the following SF treatments: 240, 280, and 320 g/m^3 for 72 hours, and an untreated control group. The treatments were replicated three times, with two logs from artificially inoculated trees and one log from a naturally infected tree. The untreated logs were used as an indicator of fungus viability under ambient conditions over the length of the experiment. A randomized complete block design was used for the 2017 experiment with the following treatments: 128 and 240 g/m^3 SF for 96 hours, 240 g/m^3 MB for 72 hours, and an untreated control group. Each treatment was replicated three times, with four randomly chosen logs from artificially inoculated trees.

Stainless-steel sample cylinders (Swagelok Co., Solon, Ohio) were filled from source tanks containing either MB (99.8% Meth-o-Gas Q; Great Lakes Chemical Corp., Middlebury, Connecticut) or SF (99.8% ProFume; Douglas Products, Liberty, Missouri). The sample cylinders and logs were acclimated to the target fumigation temperature for ≥24 hours prior to treatment. Fumigations took place in steel chambers (665 L), with an average chamber load of 26.0 percent (± 0.92 SE) based on measurements of each log. The fumigation chambers were equipped with fans to ensure circulation of the fumigants and were housed in a climate-controlled unit set to 15.6°C. Using a vacuum pump, air was removed from the chambers creating a vacuum (70 to 100 mmHg) to allow room for gas introduction. Fumigant gas delivery was facilitated using plastic tubing connected to the sample cylinder and a tube fitting (0.64 cm; Swagelok, Co.) with septa on the fumigation chamber. The sample cylinder was placed on a scale and monitored as a predetermined amount of gas was delivered to the chamber to yield the desired concentrations. Fumigant concentrations were calculated using the ideal gas law to correct for atmospheric pressure and treatment temperature.

Gas samples were taken immediately after gas delivery and every four hours during treatment to confirm that the target fumigant concentration was attained and to monitor concentrations in the headspace using an Agilent 490 Micro Gas Chromatograph (GC; Agilent Technologies Inc., Santa Clara, California). Samples were pulled into the GC using a six-position stream selector valve through PEEK tubing (2.0 m long, 0.076 cm ID; Valco Instruments Inc., Houston, Texas) inserted into the septum of a tube fitting (0.64 cm; Swagelok Co.) on each chamber. The samples entered an unheated injection port on the GC and were transferred to a 10-m PoraPlot Q column (Agilent Technologies Inc.) held at 100°C. The column connected to a thermal conductivity detector set to 100°C and the carrier gas used was helium (30 psi). Output chromatograms were analyzed using OpenLAB EZChrom software (Agilent Technologies Inc.). Chromatographic peaks analyzed were specific for SF and MB, which had retention times of 0.23 and 1.04 minutes, respectively. Previous work has shown this method to work equally well for both fumigants. This allowed for a single GC instrument to be used for both treatments without switching methods between samples. Calibrations of the instrument's response to varying concentrations of methyl bromide and sulfuryl fluoride were used to determine the headspace concentrations during the fumigation. The concentration × time product (CT) was calculated for each of the fumigations (Table 2).

Objective 3: Oak block fumigations

Block sample preparation.—Healthy *Q. alba* and *Q. rubra* trees were felled from the University of Tennessee Arboretum in Oak Ridge, Tennessee, in 2016 and 2017. The trees were cut into large sections and transported to the University of Tennessee in Knoxville, Tennessee, where they were cut into small blocks (approx. 10.2 by 10.2 by 11.4 cm^3) with the bark intact on one side. The blocks were placed in plastic bags and stored in a freezer at –20°C until further sample preparation.

Moisture content was determined on a dry-weight basis by oven-drying cross-sectioned subsamples (1.3 cm) from

Table 2.—Parameters for sulfuryl fluoride (SF) and methyl bromide (MB) fumigation of red oak logs in fumigation chambers (665 L) maintained at 15.6°C.

Year	Fumigant	Applied concentration (g/m^3)	Exposure time (h)	Mean C × T product[a] ± SE (h × mg/L)
2016	SF	240	72	17,585 ± 864
		280	72	20,384 ± 913
		320	72	21,344 ± 193
2017	SF	128	96	11,786 ± 476
		240	96	22,488 ± 945
	MB	240	72	13,991 ± 1,518

[a] Concentration–time product (C × T) for fumigation replicates ($n = 3$).

each block at 103°C for 24 hours. The blocks were coated with microcrystalline wax on all sides except the bark face to facilitate gas diffusion through the bark to simulate the treatment of logs. A cavity was drilled into the waxed blocks on the side opposite the bark face using a drill bit (1 cm diameter) at various depths, which penetrated to either the sapwood or sapwood–heartwood boundary. The cavity was tightly secured to a tube fitting (0.64 cm; Swagelok Co.) on the chamber lid and further sealed to the lid by applying construction adhesive (Gorilla Glue Co., Cincinnati, Ohio) around the bottom edges of the block. Once the adhesive dried (24 h), microcrystalline wax was coated on top to completely seal the block (Fig. 1A). The chamber lids had three tube fittings (0.64 cm; Swagelok Co.) and septa as gas sampling ports. The tube fittings were secured to holes in the lid with polyurethane adhesive sealant (3M Company, Maplewood, Minnesota).

Block fumigations.—The chamber lids, with blocks attached, were placed over glass jars (10 L) for the fumigation trials (Fig. 1B). Vacuum grease (Dow Corning Corp., Midland, Michigan) was applied to a rubber gasket on the jar lip before placing the lid on top, creating a fully sealed chamber. A ball valve (Swagelok Co.) was attached to a tube fitting on the lid and a gas-tight syringe (1 L; Hamilton Co., Reno, Nevada) was used to remove air from the chamber. This created a slight vacuum (20 to 70 mmHg) in the chamber to allow room for fumigant introduction. Predetermined amounts of fumigant were drawn from Tedlar sampling bags (1 L; SKC Inc., Eighty Four, Pennsylvania) containing either MB (99.8%; Great Lakes Chemical Corp.) or SF (99.8%; Douglas Products). Fumigant dosing concentrations were calculated volumetrically using the ideal gas law, which accounted for atmospheric pressure and temperature.

Gas samples (100 μL) were taken periodically to monitor fumigant concentrations in the cavity and headspace by sampling from the ports on the chamber lid using a gas-tight syringe (100 μL; Hamilton Co.). Samples were manually injected into an Agilent 6890 GC (Agilent Technologies Inc.) for quantitative analysis of MB and SF (Table 3). A calibration of the instrument's response to varying concentrations of both fumigants was used to determine cavity and headspace concentrations during treatment. Peaks indicative of SF and MB were analyzed on output chromatograms using ChemStation software (Agilent Technologies Inc.).

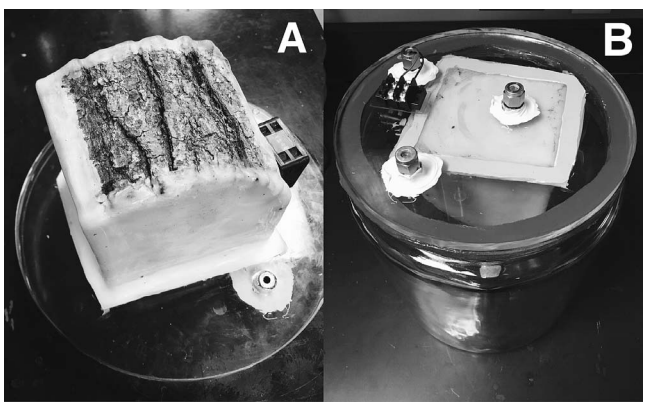

Figure 1.—Experimental setup for sulfuryl fluoride and methyl bromide oak block fumigations in 10-L glass jars. Blocks were coated with microcrystalline wax and sealed to the lid (A). Lids were equipped with gas sampling ports and secured on top of the fumigation chambers (B).

Table 3.—Agilent 6890 Gas Chromatograph (GC) parameters for quantitative analyses of sulfuryl fluoride (SF) and methyl bromide (MB) concentrations in fumigation chambers.

GC parameters	Sulfuryl fluoride	Methyl bromide
Inlet T (°C)	110	100
Split ratio	50:1[a]	1:1[b]
Column	GS-Gaspro (27 m × 320 μm)	GS-Q (30 m × 530 μm)
Oven T (°C)	100	100
Detector	Flame photometric (FPD)	Flame ionization (FID)
Detector T (°C)	175	250
Detector flows (mL/min) of air, hydrogen, helium	100, 60, 30	400, 40, 10
Run time (min)	3.0	9.0
Retention time (min)	2.1	6.2

[a] Split ratios were altered for measurements of low SF concentrations (<0.42 g/m^3) to 10:1.

[b] Splitless injections were performed for measurements of low MB concentrations (<0.19 g/m^3).

Two block fumigation experiments were conducted to characterize the diffusion and penetration of SF and MB through bark and into green oak blocks. In the first experiment, 10 *Q. alba* blocks were fumigated with either 128 g/m^3 SF or 240 g/m^3 MB for 15 days to monitor fumigant movement into the blocks and observe if equilibrium was attained between concentrations in the cavity (5 cm deep, 3.85 cm^3 volume) and headspace within this time period. In the second experiment, 47 oak blocks (*Q. alba* $n = 10$; *Q. rubra* $n = 37$) with varying cavity depths (4.5 to 9.0 cm, 3.46 to 6.92 cm^3 volume) were fumigated with 120 g/m^3 SF to determine when the gas was first detected in the cavity and if a relationship existed between diffusion distance and time.

Statistical analysis.—Generalized linear-effects models (Agresti 2002) were used to identify differences in the frequency of detected fungal colonization from pretreatment log disks. The model for the logs treated in 2016 has the following form:

$$Y_{ijk} \sim \text{Bernoulli}\,(16, P_{ijk})$$

$$\text{Logit}\,(Y_{ijk}) = \mu + I_i + \alpha_j + \gamma_{k(j)}$$

The model for the logs treated in 2017 has the following form:

$$Y_{ijkl} \sim \text{Bernoulli}\,(12, P_{ijkl})$$

$$\text{Logit}\,(Y_{ijkl}) = \mu + I_i + D_j + ID_{ij} + \alpha_k + \gamma_{l(k)}$$

where μ is the overall mean, I is the infection type, D is the sapwood depth, α is the random effect associated with tree number, and γ is the random effect associated with log number. A fixed effect for log position (not shown) was added to the 2017 pretreatment model to determine if the position of a log within tree had an effect on detected colonization.

Posttreatment data was examined by adding the achieved CT (fixed effect) and pretreatment isolation result (covar-

iate) to the existing models from 2016 and 2017 (posttreatment models not shown). All calculations were performed using R (version 1.0.14; R Core Team 2017). Estimated probabilities of detecting the fungus were conducted using the lsmeans package in R (Lenth 2016).

Spearman's rank correlation test was used to determine the relationship between SF and MB concentration in the headspace or cavity and time for the block fumigations. The same test was also used to determine if there was a correlation between SF diffusion distance and time in the blocks.

Results

Objectives 1 and 2: Oak log fumigation trials

Presence of B. fagacearum *in logs before fumigation*.—For both years of the experiment, *B. fagacearum* was isolated prior to fumigation from at least one of four wood chips plated from each subsample location from all logs (Figs. 2 and 3). Tissues sampled from logs cut from AI trees resulted in the highest frequencies of pathogen detection by isolation or nested PCR in 2016 (92% of isolation attempts and 80% of PCR attempts). Similar frequencies of pathogen detection were observed in tissues sampled from the outer sapwood of AI trees in 2017 (84% of isolation attempts and 89% of PCR attempts). Based on model estimates, the frequencies of pathogen isolation differed ($\alpha = 0.05$) by tree

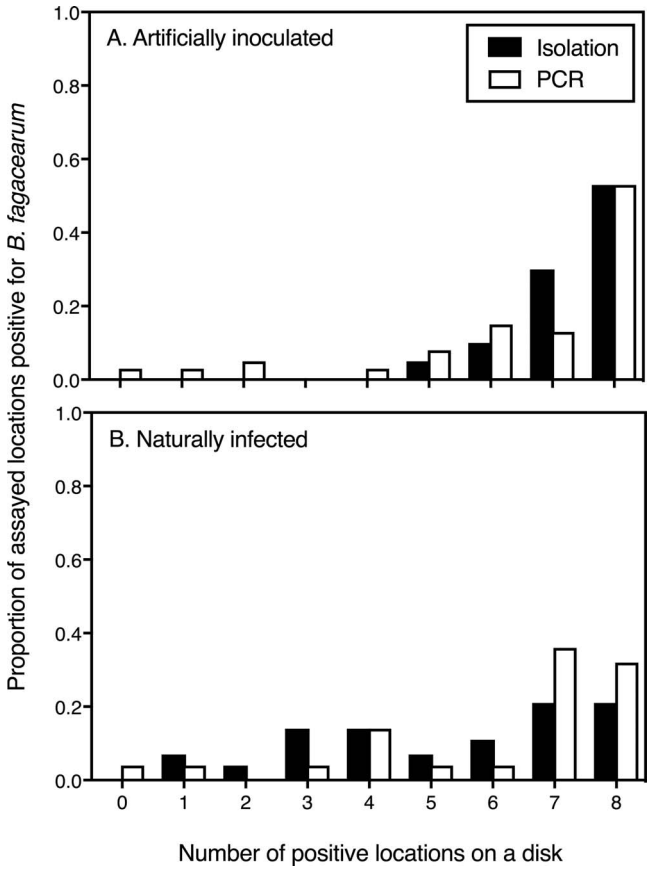

Figure 2.—Frequency distribution for the number of disk subsample locations yielding Bretziella fagacearum *per disk from artificially inoculated (A) and naturally infected (B) red oak trees using isolation techniques and nested polymerase chain reaction (PCR) prior to fumigation with sulfuryl fluoride in 2016.*

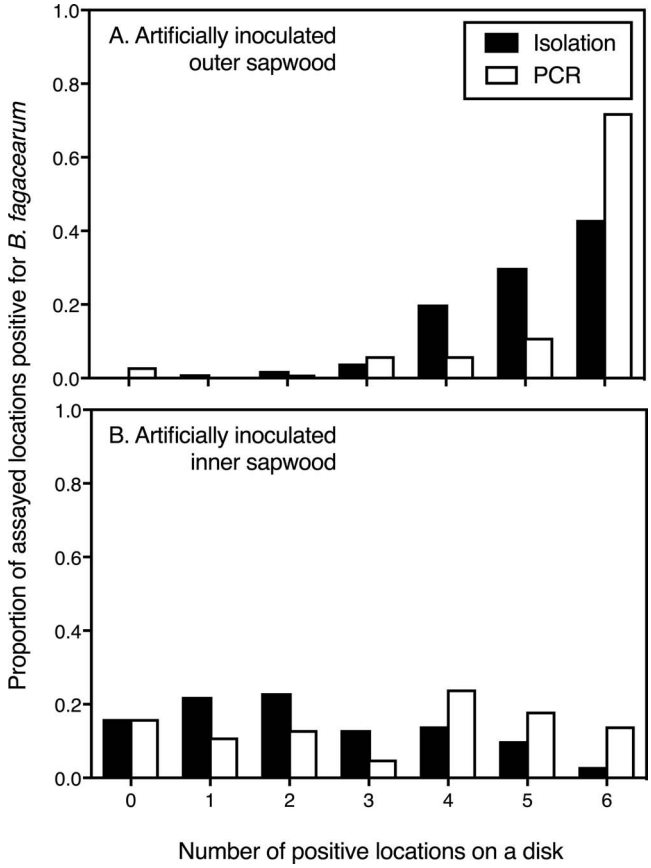

Figure 3.—Frequency distribution for the number of disk subsample locations yielding Bretziella fagacearum *per disk from the outer sapwood (A) and inner sapwood (B) of artificially inoculated red oak trees using isolation techniques and nested polymerase chain reaction (PCR) prior to fumigation with sulfuryl fluoride and methyl bromide in 2017.*

infection type and sapwood depth (Table 4). Differences in detection using nested PCR were observed only between sapwood depths. The estimated probabilities of detecting *B. fagacearum* were highest for samples that were taken from the outer sapwood of AI trees and lowest for samples that were taken from the inner sapwood region of AI trees (Table 5).

When a fixed effect for log number was included in the model for the 2017 fungus isolation data, a difference in pathogen presence was observed ($\alpha = 0.05$, $P = 0.0014$) based on the position of a log within AI trees. Mean occurrence of detected fungal colonization in AI trees was lowest in logs sampled closest to the root collar and highest in logs sampled from the uppermost height ($P = 0.0048$; Tukey's HSD).

Presence of B. fagacearum *in logs after fumigation*.—In 2016, viable *B. fagacearum* was isolated from four AI logs following treatment with 280 and 320 g/m^3 of SF and from one NI log following treatment with 320 g/m^3 SF (Table 6). In 2017, the pathogen was isolated from at least one log from each treatment group. Linear mixed-model analysis from both years indicate that achieved treatment (CT) had a significant effect ($\alpha = 0.05$) on killing the fungus (Table 7). Based on model results for 2016 data, logs from NI trees had a lower estimated probability of pathogen isolation following fumigation (0.005 ± 0.064 for NI logs; 0.056 ±

Table 4.—Coefficients of the generalized linear mixed-effects models fitted to the Bretziella fagacearum *isolation and nested polymerase chain reaction (PCR) data for locations of disks sampled prior to fumigation. Sample locations on disks were from logs obtained from artificially inoculated (AI) and naturally infected red oak trees in 2016 and from AI trees only in 2017. Only outer sapwood was sampled in 2016.*

Detection method	Year	Variable	Coefficient estimate	SE	*P* value
Isolation	2016[a]	Intercept	2.56	0.35	<0.01
		Natural infection	−1.09	0.48	<0.01
	2017[b]	Intercept	−0.56	0.23	0.02
		Outer sapwood	2.45	0.16	<0.01
Nested PCR	2016	Intercept	2.33	0.46	0.00
		Natural infection	−0.90	0.67	0.18
	2017	Intercept	0.13	0.25	0.62
		Outer Sapwood	2.83	0.22	<0.01

[a] Reference level for 2016 regression model result is artificial inoculation.
[b] Reference level for 2017 regression model result is inner sapwood.

0.048 for AI logs). Although not statistically tested, the rate of pathogen isolation did not appear to decrease over the duration of the experiments based on untreated control logs.

Objective 3: Oak block fumigations

Penetration and diffusion of MB and SF.—Q. alba blocks ($n = 10$) had an average moisture content of 66 percent. The cavities drilled were 5 cm deep, leaving approximately 5.2 cm of wood and bark for the fumigant to diffuse through before reaching the cavity. Generally, penetration of SF and MB through the bark and into the blocks was weak and concentrations in the cavity and headspace did not reach equilibrium over the 15-day experiment (Fig. 4). Figure 4 displays results from two *Q. alba* blocks fumigated with SF and MB, representing a general model of diffusion that was observed for all of the block fumigations. Equilibrium was established for only 1 of the 10 treated blocks, which was fumigated with SF. MB diffused through all blocks ($n = 5$) slowly, with an average first detectable presence of MB in the cavities at 1.93 days with an average concentration of 0.51 g/m^3 MB. Spearman's rank correlation test indicated a strong, negative correlation between headspace concentration and time ($P < 0.0001$, $r_s = -0.928$) and a moderate, positive correlation between cavity concentration and time ($P = 0.0002$, $r_s = 0.537$) for the blocks fumigated with MB. SF also diffused through the blocks ($n = 5$) slowly, with an average first detectable presence of SF in the cavities at 0.43 days and an average concentration of 22.3 g/m^3 SF. In blocks fumigated with SF, a weak, negative correlation was identified between headspace concentration and time ($P = 0.005$, $r_s = -0.346$) and a weak, positive correlation was identified between cavity concentration and time ($P = 0.0003$, $r_s = 0.387$). The average maximum concentration of MB and SF in the cavities of the fumigated blocks were 8.75 and 29.8 g/m^3, respectively.

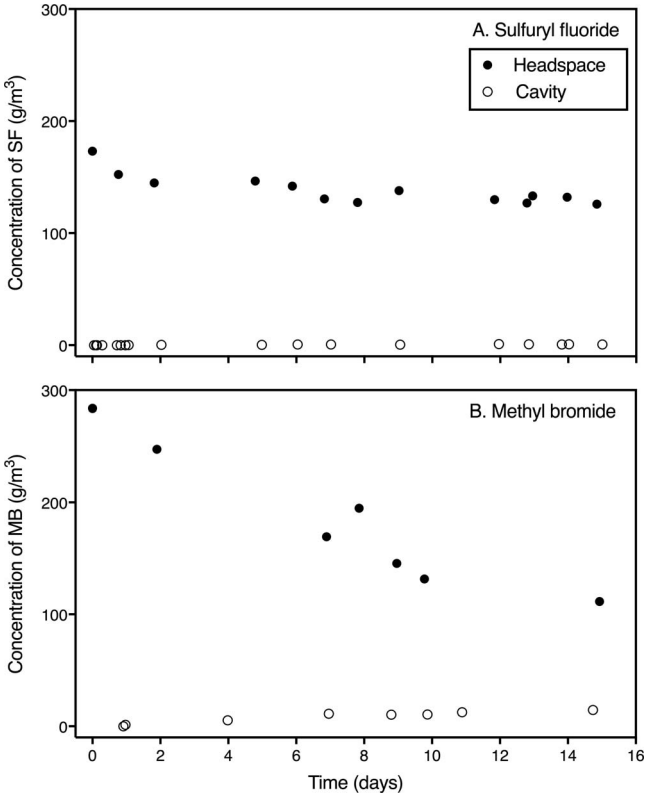

Figure 4.—Fumigant concentrations in cavity and headspace of two Quercus alba *blocks treated for 15 days with either 120 g/m^3 sulfuryl fluoride (SF; A) or 240 g/m^3 methyl bromide (MB; B). The first detectable presence of gas in the cavity was observed at 0.83 days using SF and 0.97 days using MB. Graphs represent a general model of diffusion observed from the block fumigations.*

SF diffusion distance and time relation.—The average moisture contents for the *Q. alba* ($n = 10$) and *Q. rubra* ($n =$

Table 5.—Estimated probabilities of Bretziella fagacearum *detection in pretreatment log disks by isolation and nested polymerase chain reaction (PCR) based on generalized linear mixed effects models of the interactions of infection type (naturally infected or artificially inoculated for 2016) and sapwood depth (for 2017).*

Year	Tree infection type	Sapwood location	Probability ± SE[a]			
			Isolation		Nested PCR	
			Estimated[b]	Actual[c]	Estimated	Actual
2016	Artificial	Outer	0.93 ± 0.02	0.92 ± 0.02	0.91 ± 0.04	0.83 ± 0.02
	Natural	Outer	0.68 ± 0.07	0.67 ± 0.03	0.81 ± 0.08	0.75 ± 0.02
2017	Artificial	Outer	0.87 ± 0.03	0.84 ± 0.02	0.95 ± 0.01	0.89 ± 0.01
		Inner	0.36 ± 0.05	0.38 ± 0.02	0.53 ± 0.06	0.53 ± 0.03

[a] Denotes standard error of the mean proportion of positive sample locations.
[b] Estimated probabilities based on logit transformation from model estimates.
[c] Actual probabilities based on calculated proportions from samples used in this study.

Table 6.—Number of subsample locations yielding Bretziella fagacearum *in culture from the sapwood of logs from artificially inoculated and naturally infected red oak trees following fumigation with sulfuryl fluoride and methyl bromide.*

Year	Inoculation type	No. of logs treated	Treatment (g/m^3)	Treatment time (h)	No. of positive logs	No. of positive sapwood locations	
						Outer	Inner
2016[a]	Artificial	5	0 (control)	72	5	71	—[b]
		4	SF 240	72	0	0	—
		6	SF 280	72	2	9	—
		5	SF 320	72	1	2	—
	Natural	2	0 (control)	72	2	5	—
		5	SF 240	72	0	0	—
		3	SF 280	72	0	0	—
		4	SF 320	72	1	1	—
2017	Artificial	12	0 (control)	96	12	117	62
		12	SF 128	96	5	20	8
		12	SF 240	96	5	9	5
		12	MeBr 240	72	4	4	3

[a] Data for 2016 represents results for $n = 16$ attempts/log and data for 2017 represents results for $n = 12$ attempts/log at each depth.
[b] Detection of *B. fagacearum* by isolation was not attempted at the innermost sapwood for disks sampled from logs treated in 2016.

37) blocks were 67 and 63 percent, respectively. The cavities were various depths, resulting in diffusion distances (distance from the bark to the cavity) ranging from 0.8 to 5.75 cm. There was a moderate, positive correlation between diffusion distance and time ($P < 0.001$, $r_s = 0.593$). At a diffusion distance of 5 to 6 cm, SF penetrated to the cavity either within 3 hours or after 20 hours (Fig. 5).

Discussion

In the 2016 experiments, *B. fagacearum* was not isolated from any logs treated with 240 g/m^3 SF; however, higher concentrations (280 and 320 g/m^3 SF) used the same year did not completely eradicate the pathogen. Based on these results, it was not possible to determine an efficacious SF treatment against *B. fagacearum*, because higher concentrations should have been at least equally capable of killing the pathogen. This prompted the decision to increase the SF treatment time from 72 to 96 hours in the 2017 trial and to also compare the results to fumigations using the current MB treatment schedule for oak logs. Despite the increase in SF treatment time and the addition of the MB treatment, all of the subsequent fumigation treatments were unsuccessful in completely eradicating *B. fagacearum* from the logs. The treatments greatly reduced the recovery of viable *B. fagacearum* in the logs; the total reduction in living fungus (posttreatment vs. pretreatment isolation) was >89 percent for all SF treatments. SF treatments at a rate of 240 g/m^3 for either 72 or 96 hours resulted in very similar levels of pathogen eradication as 240 g/m^3 MB for 72 hours (>96% reduction in pathogen viability for both fumigants).

Previous studies evaluating the efficacy of SF on logs infested with *B. fagacearum* have used wood material obtained from naturally infected red oak trees. In addition to naturally infested materials, this study included logs from trees that were artificially inoculated with the pathogen. Rates of fungus isolation before treatment were higher for logs obtained from artificially inoculated trees (92% in 2016; 84% for outer sapwood and 38% for inner sapwood in 2017) than for logs from trees with naturally occurring infection (67% in 2016). This indicates that artificially inoculated trees provide a more rigorous standard for

Table 7.—Coefficients of the generalized linear mixed-effects models fitted to the Bretziella fagacearum *isolation data for locations of disks sampled after fumigation. Sample locations on disks were from logs obtained from artificially inoculated (AI) and naturally infected red oak trees in 2016 and from AI trees only in 2017. Only outer sapwood was sampled in 2016. CT is concentration × time.*

Year	Variable	Coefficient estimate	SE	*P* value
2016[a]	Intercept	3.01	1.08	<0.01
	Natural infection	−5.18	1.62	<0.01
	CT	−6.59	1.11	<0.01
	Pretreatment	−0.01	0.63	0.99
	Natural infection × CT	3.17	1.79	0.08
2017[b]	Intercept	−0.79	0.46	0.09
	Outer sapwood	1.78	0.32	<0.01
	CT	−3.23	0.51	<0.01
	Pretreatment	0.29	0.11	0.28
	Outer sapwood × CT	−0.93	0.42	0.03

[a] Reference level for 2016 regression model result is artificial inoculation.
[b] Reference level for 2017 regression model result is inner sapwood.

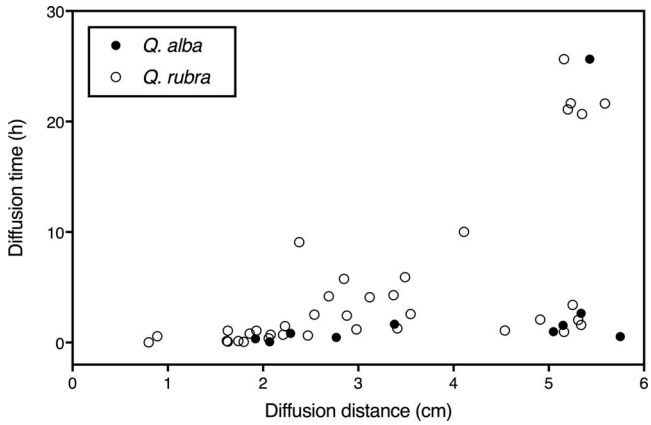

Figure 5.—Time (h) required for sulfuryl fluoride to diffuse through the bark and reach a cavity of varying distances (cm) in fumigated Quercus alba *(*n *= 11) and* Quercus rubra *blocks (*n *= 37).*

phytosanitary testing. The distribution of the fungus around the circumference of the sapwood was also more uniform in logs from AI trees. The higher level of pathogen colonization in AI trees led us to omit NI trees in the 2017 fumigation experiments. Schmidt et al. (1997) and MacDonald et al. (1985) discussed whether it could be concluded that fumigation treatment fully eradicated the fungus if pathogen recovery before treatment was low. Schmidt et al. reported no growth of the oak wilt fungus following SF fumigation (280 and 420 g/m^3 for 72 h); however, rates of pathogen isolation before treatment were lower (0% to 37.5% of isolation attempts) than the rates reported in this study, suggesting the logs were not well-colonized prior to treatment. Similarly, other studies have described low frequencies of *B. fagacearum* detection in samples taken from naturally infected trees (Jones 1973, Schmidt 1983).

Isolation of *B. fagacearum* on a semiselective oak wilt identification medium has been used in fumigation studies as a means of testing for viable fungal propagules (Schmidt et al. 1982, MacDonald et al. 1985, Woodward and Schmidt 1995, Schmidt et al. 1997). Successful detection of the oak wilt fungus using traditional isolation techniques is highly dependent on the condition of the sampled sapwood. Desiccation and presence of secondary fungi in decaying wood can make isolation of the fungus difficult in sapwood obtained from naturally occurring oak wilt infections (Bretz and Morrison 1953). The study presented here employed a dual assay using nested PCR and traditional isolation for determination of pathogen presence. Molecular methods have demonstrated the ability to detect the presence of *B. fagacearum* DNA in tissues, where isolation methods alone often produced a negative result (Yang and Juzwik 2017). The occurrence of samples that were positive for *B. fagacearum* DNA in logs after treatment in 2017 indicates that the fungus was present in many of the sampled regions (88% of outer sapwood and 48.8% of inner sapwood). Rates of detection were similar for isolation and nested PCR in logs from AI trees, but detection was greater in logs from NI trees using the molecular method, validating the use of PCR as a secondary detection method.

In order to help understand the general challenges of fumigating logs, experiments were conducted to investigate the movement of SF and MB into green wood blocks, which simulated the treatment of logs. SF and MB penetrated through the bark and diffused into the cavities slowly. In general, SF was detected in the drilled cavities faster and at higher concentrations than MB; however, results of correlation analyses suggested that the MB diffusion might have a more predictable relationship with time than SF. In vitro concentration–time combinations that are lethal to *B. fagacearum* have been documented for SF and MB (Liese and Ruetze 1985, Woodward and Schmidt 1995). Lethal fumigant concentrations were reached in one of the SF block fumigations and two of the MB block fumigations, but the lethal concentrations did not persist for the minimum time required to kill the pathogen. Similarly, CTs that were successful in killing *B. fagacearum* in colonized barley were not achieved during the SF block fumigations (Uzunovic et al. 2017). The slow penetration and accumulation of SF and MB in the sapwood region of the fumigated blocks may explain the variable, albeit reduced, viability of *B. fagacearum* that was observed in the log fumigations. Results from the block fumigations agree with findings from previous fumigant penetration and diffusion studies (Cross 1992, Scheffrahn et al. 1992, Rhatigan et al. 1998, Ren et al. 2011), which may help explain the observed failure of attaining complete eradication of the pathogen from the logs.

Generally, as the diffusion distance increased, the time required for SF to diffuse to the cavity also increased. A quadratic-like relationship between diffusion distance and time would be expected, based on diffusion laws and the kinetic molecular theory that assume a proportional relationship between diffusion time to distance squared. However, SF diffused through specified distances with extreme variability, yielding an inconsistent relationship between diffusion distance and time of SF into logs. The variable diffusion of SF into oak blocks may be attributed in part to the inherent variability of the wood structure (Hughes 1968, Scheffrahn et al. 1992). The presence of bark presents a physical barrier to fumigant penetration and the high moisture content reduces void space, thus limiting movement of fumigants and making diffusion into wet wood difficult (Cross 1992, Morrell 1995, Rhatigan et al. 1998, Hall et al. 2018). Considering the weak penetration of SF and MB into the blocks, it is reasonable to expect even slower diffusion of fumigants into logs, which have a higher average moisture content (83% ± 1.0 SE) compared with the blocks (*Q. alba* 67% ± 0.8 SE; *Q. rubra* 63% ± 0.5 SE). It is necessary to note that moisture contents reported in this study include heartwood and sapwood for the blocks and only sapwood for the logs. The sapwood may have a higher moisture content than the heartwood, but the difference is small for *Quercus* species (Denig et al. 2000).

Results of this study contrast with previous findings, where lower CT values of SF were capable of killing the fungus in logs. The log treatments presented in this study represent a rigorous test because of their high level of pathogen colonization, high sapwood moisture content, and the presence of intact bark. Uzunovic et al. (2017) suggested that an increase in CT may be required to obtain lethal concentrations of SF in fumigated logs. Results from both years of the log fumigations indicated that as CT products of SF increased, the likelihood of pathogen viability after treatment decreased. Higher CT products achieved by increasing treatment time or applied concentration could increase efficacy of the fumigations. However, results from block fumigations suggest that it may be difficult to precisely determine the time and dose required for SF to accumulate in the sapwood region where the fungus resides, especially considering variability among physical characteristics of the wood.

The phase-out of MB will generate a need for additional approaches to treat logs from diseased trees. Other alternative fumigants have been investigated, but are often complicated by matters related to chemical penetration into the wood (Tubajika and Barak 2011). There would be significant advantages to replacing the current MB schedule with SF; however, results from log fumigations demonstrated that full reliance on the toxicity of SF might not be sufficient to eradicate *B. fagacearum* in logs cut from diseased trees. Recently, a nonchemical alternative treatment (i.e., vacuum–steam process) was shown to kill *B. fagacearum* colonizing large diameter *Q. rubra* logs (Juzwik et al. 2019).

Results from this study also suggest that further investigation into the efficacy of the current treatment

schedule using MB is warranted using the presented method of fumigating artificially inoculated logs with high levels of infestation. Achieving 100 percent efficacy of treatments is often difficult and using probit-9 mortality as a standard may not be the best way to develop quarantine treatments because of limitations of this method (Schortemeyer et al. 2011). An integrated approach that combines multiple phytosanitary measures could be successful in mitigating the risk of spreading pathogens such as *B. fagacearum*. The duration of the saprophytic presence of viable *B. fagacearum* in logs has been reported to be dependent on sapwood moisture, and recovery of the fungus becomes increasingly difficult as time from harvest elapses (Bretz et al. 1955, Gibbs and French 1980). An effective systems approach could combine SF fumigation with practical measures (e.g., bark removal to promote sapwood drying, pest-free areas, harvesting guidelines) that reduce the likelihood of moving viable *B. fagacearum* in oak logs to negligible levels.

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