

# Fungitoxicity of Sulfuryl Fluoride to *Ceratocystis fagacearum* In Vitro and in Wilted Red Oak Log Sections

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## ABSTRACT

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The European Union requires imported red oak logs to be fumigated with methyl bromide to eradicate the oak wilt fungus, *Ceratocystis fagacearum*. The restriction of methyl bromide would threaten the export of oak, as other fumigants have not been evaluated. Sulfuryl fluoride penetrates wood and has been used extensively for insect control, but fungicidal effects are unknown. The effect of sulfuryl fluoride exposure on 10-day-old *C. fagacearum* cultures was tested in a closed circuit fumigation chamber at concentrations of 16, 40, 60, 80, 100, and 120 g/m<sup>3</sup> for 24 and 48 h. Rates of 80 g/m<sup>3</sup> for 48 h and 120 g/m<sup>3</sup> for 24 h rendered cultures unable to grow. When stained with Janus Green B, both mycelia and conidia were nonviable. End-sealed log sections from wilted red oak trees were fumigated at 160, 220, and 280 g/m<sup>3</sup> for 72 h. Eradication of *C. fagacearum* from red oak log sections was achieved after exposure to 280 g/m<sup>3</sup> for 72 h. (concentration × time product = 18,530 g h/m<sup>3</sup>). *Graphium*, *Verticillium*, and *Paecilomyces* spp. (also isolated initially) were eradicated after the 18,530 g h/m<sup>3</sup> treatment. This is the first report of fungitoxicity of sulfuryl fluoride.

Additional keywords: *Quercus rubra*

Concern about the introduction of *Ceratocystis fagacearum* (T.W. Bretz) J. Hunt, causal organism of oak wilt, into Europe prompted the European Union to place restrictions on the importation of red oak (*Quercus rubra* L.) logs (1). Currently, methyl bromide (MB) fumigation is an effective eradication treatment (9,10,21). Since 1986, logs have been treated with MB, allowing for continued export of oak (21). The potential restriction of MB for phytosanitary uses threatens this segment of the export industry, as the efficacy of other fumigants to eradicate *C. fagacearum* has not been evaluated for raw wood materials, particularly logs with intact bark.

Sulfuryl fluoride (Vikane, DowElanco, Indianapolis, IN) has been used to control wood-destroying insects in structures for over 35 years (5,7,15,23,24). Fungitoxicity of sulfuryl fluoride (SF) has not been reported. Among the properties that make SF an excellent fumigant are its low boiling point (-55.2°C) and high vapor pressure (18 kg/cm<sup>2</sup> at 25°C) (7). SF has been shown to penetrate a variety of wood matrices more rapidly than MB (19).

The objectives of this study were to: (i) determine the approximate concentration × time (CT) factor required to eradicate *C.*

*fagacearum* in vitro, and (ii) determine the CT factor required to eradicate *C. fagacearum* from naturally infected northern red oak log pieces.

## MATERIALS AND METHODS

**Fumigation.** Fumigation chambers consisted of large diameter Pyrex desiccators (250 mm i.d., ca. 9 liters), each fitted with a support plate, a propeller attached to a magnetic stir bar, and a no. 8 neoprene stopper with three holes. A 12-cm-long glass tube was inserted into one hole with 4 cm extending into the chamber. A 4-mm NMR septum was inserted into the outer end of the tube and folded over to provide an airtight seal to serve as an injection port. A second glass tube (18 cm long, 10 cm within chamber) was inserted into the stopper for sampling gas levels during fumigation. Teflon TFE tubing (two 30-cm sections connected by a Pyrex no. 7282 stopcock) connected the sampling tube to the intake port of a thermal conductivity gas analyzer (Fumiscopes Model D, Key Chemical & Equipment Co., Clearwater, FL). A third glass tube (10 cm long, 2 cm within chamber), connected similarly to the exhaust port of the analyzer, returned gas to the chamber. Since SF is a toxic, colorless, odorless gas with a TLV of 5 ppm, extreme caution was taken to prevent gas leakage. All glass/neoprene and glass/Teflon contact points were secured with epoxy adhesive, silicone sealant, and a covering of Strip-Calk (3M Co., St. Paul, MN). Lids were sealed to desiccator bodies with high-vacuum grease.

SF was made available for introduction into the chamber by a transfer of gas from a commercial dip tube cylinder to a stainless steel tubeless cylinder (500 cm<sup>3</sup>, Whitey Co., Highland Heights, OH) as described by Scheffrahn et al. (20). Regulated gas was transferred into a 14-cm-diameter vinyl "playground" ball fitted with 10-cm glass tube (6 mm i.d.) in the air valve. A 6-mm NMR septum was inserted into the glass tube after gas delivery. No gas leakage was detected over time from the gas holding ball. Exact amounts of SF were transferred from the holding ball to the chamber using a gastight syringe. Gas was mixed as it was injected into the chamber and for 15 min afterward using the magnetic propeller.

**Culture studies.** Two *C. fagacearum* isolates (recovered from infected red oak in the St. Paul area) were used in the study. Isolates were maintained in petri dishes on potato-dextrose agar (PDA) (Difco). Ten-day-old cultures were placed on the support plate inside the fumigation chamber, and the covers were removed. Four cultures (two per isolate) were used in each fumigation. Cultures were fumigated with SF (Vikane, commercial grade, 99%) at concentrations of 16, 40, 60, 80, 100, and 120 g/m<sup>3</sup> of chamber space for 24 and 48 h. Fumigations were conducted at ambient temperature (21 to 23°C). Gas concentrations were initially assessed using a Fumiscopes Model D thermal conductivity gas analyzer calibrated for SF. During 24-h fumigations, gas concentrations were verified at 12 h and at termination. During 48-h fumigations, gas concentrations were verified at 12 and 30 h and at termination. Each concentration × time (CT) treatment was conducted three separate times.

Glass petri dish covers were immediately placed on the cultures at the end of each fumigation. Cultures were removed after a 15-s gas dispersment period. Twenty-five 6-mm mycelial plugs were removed from the periphery of each of the four fumigated cultures, transferred to fresh PDA plates (five plugs per plate), and monitored for mycelial growth at 3, 7, 14, and 21 days. Fifty plugs were assessed per isolate per individual fumigation (100 total). Isolations from control cultures (equal age, treated identically except equal quantity of ambient air substituted for SF) were taken at the same time (20 per isolate). The viability of fumigated cultures that displayed no mycelial growth after 14 days was assessed using Janus Green B

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vital staining. Five 6-mm plugs were removed and placed in 25 ml of Janus Green B stain solution (0.1% Janus Green B in 0.2 M sodium acetate acid buffer, pH 4.6) (14). After 30 min, plugs were removed and rinsed in 0.18 M KOH. Mycelium was teased from the agar plugs and observed microscopically. The viability of conidia from cultures unable to grow also was assessed. Mycelium adjacent to the inoculation point was removed, placed in 2-ml of sterile distilled water in a screw-top vial, and agitated for 30 s. After the presence of conidia was verified microscopically, 100  $\mu$ l of solution was spread across the surface of a PDA plate and observed for growth for 14 days.

**Log studies.** Two naturally infected red oak trees (12 to 15 cm diameter) that were symptomatic 3 to 4 months earlier (more than 75% of foliage wilted) were felled in October 1994 and cut into approximately 1-m sections. The log sections were end-coated with paraffin wax solution to prevent desiccation and stored in a walk-in cooler at 4°C to maintain *C. fagacearum* viability. Log sections were removed from the cooler and allowed to warm to ambient temperature (approximately 22°C) over a 3-day period. Segments (25 cm) were cut from the logs, and 5-cm cross sections were then removed from both ends. The sapwood of both cross sections was sampled in the 5-mm zone beneath the bark (25 points per section) and adjacent to the heartwood (25 points per section), as described by Schmidt et al. (22), to provide isolation attempts at 50 points around the circumference for *C. fagacearum* using Barnett's semiselective oak wilt medium (2). Resulting 15-cm log cross sections were then end-coated with water-emulsified asphalt and aluminum foil to ensure gas penetration through the bark rather than through the exposed ends. Sections were then placed into the fumigation chambers. Fumigation rates were 160, 220, and 280  $\text{g}/\text{m}^3$  for 72 h at ambient temperature (21 to 23°C). Gas concentrations were verified

every 12 h, and gas was applied as necessary to regain the initial air concentration that dropped, presumably due to log sorption. At the conclusion of a fumigation trial, the chamber was vented, and aluminum foil was removed to allow the sections to aerate. After 18 h of aeration, the fumigated log piece was cut into two 7.5-cm cross sections, and each section was again sampled for *C. fagacearum*. Three fumigations were done at each gas rate.

## RESULTS AND DISCUSSION

Mycelial growth percentages from fumigated cultures are shown in Table 1. Mycelial growth from plugs exposed to SF for 24 h ranged from 0 to 100% depending on the fumigant concentration. Complete absence of mycelial growth was achieved only at the 120  $\text{g}/\text{m}^3$  concentration. Vital staining with Janus Green B revealed that mycelia and conidia exposed at this rate were nonviable. In contrast, 100% of plugs removed from cultures fumigated at 16 and 40  $\text{g}/\text{m}^3$  for 24 h grew. At fumigation rates of 80 and 100  $\text{g}/\text{m}^3$  for 24 h, reductions in the percent of plugs with mycelial growth were found. Growth of nonfumigated cultures was 100% after 3 days. At 80  $\text{g}/\text{m}^3$ , growth was found from 38% of plugs removed from fumigated cultures (62% nonviable). At 100  $\text{g}/\text{m}^3$ , only 7% of the plugs displayed growth after 14 days (93% nonviable). At all fumigation rates, the percentage of plugs showing growth was nearly identical among cultures of the same isolate and between the two isolates.

Exposure of *C. fagacearum* to concentrations of 80  $\text{g}/\text{m}^3$  or higher for 48 h killed all cultures (Table 1). At 60  $\text{g}/\text{m}^3$  for 48 h, 98% of the plugs were killed. Although mycelial growth was noted in 71% of subcultures 14 days after fumigation at 40  $\text{g}/\text{m}^3$ , changes in culture morphology such as lack of characteristic pigmentation, slower growth rate, and appressed mycelium were noted. At both exposure times, a delay in the onset of growth was noted, with the exception of cultures fumigated at

16  $\text{g}/\text{m}^3$ . A reduction in mycelial growth (29% nonviable) was first observed using a concentration of 40  $\text{g}/\text{m}^3$ .

This is the first report of the fungitoxicity of SF. In insect studies, Meikle et al. (11) concluded that the toxicity of SF ( $\text{SO}_2\text{F}_2$ ) resulted from the inhibitory action of the fluoride ion on glycolysis and enzyme systems such as lipases or esterases that depend on the magnesium ion for their activity. They suggested that amino acids of the fumigated insect enter the TCA cycle at an abnormally high rate in an attempt to maintain an energy level sufficient for survival. The normal channels for maintaining the energy balance of the organism, (glycolysis, fatty acid metabolism, and oxidative phosphorylation) appeared to be blocked. This metabolic imbalance may delay insect mortality for several days following fumigation (15).

Since SF disrupts basic pathways in cell metabolism in insects (11,16), toxicity in fungi is assumed to occur through the same mechanism. The delay in mycelial growth observed in cultures fumigated at concentrations above 16  $\text{g}/\text{m}^3$  may have resulted from an initial disruption of carbon metabolism. The fumigant concentration and exposure time may have been sufficient to deplete energy levels of the fungal cells delaying hyphal elongation but not to permanently disrupt cell metabolism. The morphological abnormalities observed at 40  $\text{g}/\text{m}^3$  for 48 h suggest that this fumigation rate may have deleteriously affected the metabolism of *C. fagacearum*.

Busvine (3) first proposed the model  $C^n t = k$  (where  $C$  = concentration,  $t$  = time,  $n$  = toxicity index, and  $k$  = dosage for a specific mortality level) to describe the fumigant concentration, exposure time, and mortality relationship. The model states that the product of concentration and time (CT product) is a constant for a specific mortality level. This concept has been expounded by researchers working with insects (8,13,15,23), nematodes (4,25), and fungi (6,12). The current label for SF assumes that exposure time is as important as concentration (i.e.,  $n = 1$ ) regarding structural fumigation for insect eradication (5). The results of Stewart (23) suggest that this assumption is valid for SF and MB against the drywood termite, *Incisitermes minor* (Hagen).

The observation of growth at 60  $\text{g}/\text{m}^3$  for 48 h and the absence of growth at 120  $\text{g}/\text{m}^3$  for 24 h (equivalent CTs of 2,880  $\text{g h}/\text{m}^3$ ) suggests that a strict linear relationship does not exist with *C. fagacearum* (i.e.,  $n \neq 1$ ). In addition, differences in growth were found at CT product of 1,920  $\text{g h}/\text{m}^3$ . Gas concentrations of 80  $\text{g}/\text{m}^3$  for 24 h produced 38% growth, but concentrations of 40  $\text{g}/\text{m}^3$  for 48 h produced 71% growth. These findings are similar to those reported for MB by Munnecke et al. (12). In a study of soilborne plant pathogenic

**Table 1.** Mycelial growth percentages from *Ceratocystis fagacearum* cultures following fumigation with sulfuryl fluoride

Fumigant conc. ( $\text{g}/\text{m}^3$ )	Exposure time (h)	Conc. $\times$ time prod. ( $\text{g h}/\text{m}^3$ )	Growth percentage <sup>a</sup>		
			3 days <sup>b</sup>	7 days	14 days
16	24	384	100 <sup>c</sup>	100	100
16	48	768	100	100	100
40	24	960	71	100	100
40	48	1,920	51	71	71
60	24	1,440	53	99	99
60	48	2,880	0	1	2
80	24	1,920	2	35	38
80	48	3,840	0	0	0
100	24	2,400	0	6	7
100	48	4,800	0	0	0
120	24	2,880	0	0	0
120	48	5,760	0	0	0

<sup>a</sup> Percentage of mycelial plugs removed from fumigated cultures that showed mycelial growth after transfer to potato-dextrose agar (PDA).

<sup>b</sup> Days of plug incubation on PDA. No increases noted at 21 day observations.

<sup>c</sup> Grand mean of three fumigations (300 isolations total).

fungi, they reported the CT products sufficient to kill 90% of fungal propagules (LAD<sub>90</sub>) may vary depending on fumigant concentration for certain fungi such as *Rhizoctonia solani*, *Verticillium albo-atrum*, and *Sclerotium rolfsii*. In addition, sclerotia of *S. rolfsii* were relatively insensitive to MB at 21.2 g/m<sup>3</sup> (5,000 ppm) but sensitive at MB concentrations of 84 g/m<sup>3</sup> (20,000 ppm).

Stewart (23) reported that the dosage response curves of MB and SF were similar with respect to insecticidal activity against drywood termites. As a matter of comparison, the LAD<sub>99</sub> found in this study was approximately 30 times that of a typical drywood termite fumigation and three times that needed to eradicate eggs of wood boring beetles (18,24). The active gas exchange system found in target insects would mean that larger amounts of toxin are encountered over time compared to fungi, which have a passive gas exchange system (16). The lack of in vitro toxicity data for fungi other than *C. fagacearum* make direct rate comparisons between SF and other fumigants difficult. Early fumigation studies found that MB eradicated *C. fagacearum* in vitro (17). However, the fumigation rates were extremely high (1,000 g/m<sup>3</sup> for 72 h).

Caution must be used in comparing the relative toxicity of SF between exposed *C. fagacearum* cultures and infected logs. The results of the log cross section studies are given in Table 2. Reductions in total fungal and *C. fagacearum* isolation frequencies were found with each SF concentration using a 72-h exposure. At 160 g/m<sup>3</sup>, total fungal isolation was reduced from 93% in the nonfumigated sapwood to 27% after fumigation. A threefold reduction in *C. fagacearum* (45 to 15%) also was found. At 220 g/m<sup>3</sup>, total fungal isolation was reduced by fumigation from 87 to 12%, and *C. fagacearum* was reduced from 63 to 7%. No fungal growth was noted from log pieces fumigated at 280 g/m<sup>3</sup> for 72 h. Bacterial growth also was absent in pieces fumigated at this rate. In addition to the oak wilt fungus, *Verticillium*, *Paecilomyces*, and *Graphium* spp. were isolated from the sapwood of each control log. Barnett's media contains phenylalanine as the sole nitrogen source (2). Since *C. fagacearum* can utilize phenylalanine, it has a competitive advan-

tage in the medium. However, other fungi can grow at a reduced rate. Fumigation rates of 280 g/m<sup>3</sup> for 72 h eradicated the entire mycoflora of the red oak log. In addition, mycophagous nematodes were noted in 3% of the sapwood samples from the trees felled in October 1994. Nematodes were not noted after fumigation at any application rate.

MacDonald et al. (10) reported that fumigation with MB at 240 g/m<sup>3</sup> for 72 h eradicated *C. fagacearum* from red oak logs. This rate was close to that found to sterilize red oak sapwood using SF. Scheffrahn et al. (19) reported that SF penetrated red oak wood at a rate approximately three times that of MB. This favorable penetration ability would result in a higher accumulated dosage of the fumigant within the sapwood of the log. Differences in toxicity (if any) between MB and SF may be offset or enhanced by the ability to accumulate a higher fumigated dosage.

The current study suggests that SF possesses the properties to merit further attention for phytosanitation. No discoloration of sapwood was noticed due to SF in fumigated log sections. The next step in this research would be outdoor fumigations of commercial-sized logs under gastight tarpaulin to confirm eradication and note any changes in efficacy at lower temperatures common during winter log export.

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Table 2. Fungal isolation percentage from infected red oak log sections following fumigation with sulfuryl fluoride

Fumigant conc. (g/m <sup>3</sup> )	Exposure time (h)	Conc. × time prod. (g h/m <sup>3</sup> )	Percent growth from sapwood isolations <sup>a</sup>			
			Nonfumigated		Fumigated	
			Total <sup>b</sup>	<i>C. fagacearum</i>	Total	<i>C. fagacearum</i>
160	72	11,280	93 <sup>c</sup>	45	27	15
220	72	14,950	87	63	12	7
280	72	18,530	89	53	0	0

<sup>a</sup> Sapwood isolations were placed on Barnett's semiselective oak wilt medium (2).

<sup>b</sup> All sapwood isolations from which mycelium grew, regardless of genus.

<sup>c</sup> Grand mean of three fumigations (300 isolations total).