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Fungi from Galleries of the Emerald Ash Borer Produce Cankers in Ash Trees

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Abstract: The emerald ash borer (EAB, *Agrilus planipennis*) is a devastating invasive pest that has killed millions of ash trees in the United States and Canada. EAB was discovered in the US in 2002 and first reported in Minnesota in 2009. It attacks ash trees that are native to the United States, including *Fraxinus americana* (white ash), *F. nigra* (black ash) and *F. pennsylvanica* (green ash). It also attacks *Chionanthus virginicus* (white fringe tree). Seven species of fungi isolated and identified only from EAB-infested trees in a previous study as having the potential to cause cankers were used to test their pathogenicity in *F. americana* (white ash). The fungi used were *Cytospora pruinosa*, *Diplodia mutila*, *Diplodia seriata*, *Paraconiothyrium brasiliense*, *Phaeoacremonium minimum*, *Phaeoacremonium scolyti*, and *Thyronectria aurigera*. Two field experiments that used *F. americana* used two inoculation methods: woodchip and agar plug inoculations. Results indicated that all of the fungi tested caused cankers in varying amounts, as compared to the controls. The largest cankers were caused by *D. mutila* (270 mm^2), *C. pruinosa* (169 mm^2), and *D. seriata* (69 mm^2). All fungi except for *T. aurigera* were re-isolated and sequenced to confirm Kochs' postulates. Canker-causing fungi found in association with EAB galleries have the potential to contribute to tree dieback and mortality.

Keywords: *Agrilus*; Ascomycota; invasive species; *Fraxinus*; fungi

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1. Introduction

The emerald ash borer (EAB) (*Agrilus planipennis*) is a destructive invasive pest that has killed hundreds of millions of ash trees (*Fraxinus* spp.), causing great economic and ecological damage. The larvae of the borer feed in the phloem of the tree and repeated attacks can girdle the stem [1,2]. This buprestid, with characteristic metallic-green coloration, is native to China and other Eastern Asian countries [1]. First detected in North America on ash trees in the southeastern part of Michigan in 2002 [3], it is thought to have entered the United States in the late 1990s in solid-wood shipping material from Asia [1]. Since its discovery in the United States, EAB has devastated ash trees from the Midwestern US to the East Coast. As of January 2021, EAB has been detected in 35 states, the District of Columbia, and five Canadian provinces [4]. It was first found in Minnesota in 2009 and is now located in 25 of 87 counties in Minnesota [5]. In its native range, the beetle is not known to cause severe problems to Asian ash trees [1]. Although the beetle attacks stressed ash native to China and the Russian Far East, serious outbreaks and tree damage are rarely seen in that country [3,6,7]. The ash species *F. chinensis*, *F. mandshurica*, and *F. rhychophylla* co-evolved with the beetle and likely developed defense mechanisms, while ash trees native to North America, *F. americana* (white ash), *F. nigra* (black ash), and *F. pennsylvanica* (green ash) did not, making these trees highly susceptible to attack by the beetle [7]. In addition to these ash species, *Chionanthus virginicus* (white fringe tree) is also susceptible [8]. Eggs are laid on the trunk or branches of trees and hatch in one-to-two weeks. Once the eggs hatch, the larvae bore serpentine-shaped galleries into the tree [9].

A recent paper by Held et al. [10] identified 1126 fungi isolated from the galleries of the EAB in logs from the main stem and branches collected from different regions of

Minnesota. Logs of ash trees without EAB were also sampled, and fungal pathogens were not isolated from trees without EAB [10]. The fungal isolates obtained from EAB logs were classified into three functional guilds: canker, wood degraders, and entomopathogenic fungi. Canker-causing fungi comprised 36% of all fungi that were isolated (403 isolates). The most abundant genera of fungi that may have the potential to cause cankers in trees were *Cytospora*, *Phaeoacremonium*, *Paraconiothyrium*, *Thyronectria*, and *Diplodia*. For most of these fungi, little to nothing is known about their pathogenicity in ash or how they may contribute to cambial lesions resulting in tree death. Since large numbers of these fungi were isolated from galleries of ash trees *F. americana*, *F. nigra* and *F. pennsylvanica*, their role in accelerating tree death was suspected. The objectives of this study were to determine the pathogenicity of seven fungal species that were isolated from EAB galleries in two field experiments by evaluating the extent of canker formation in ash trees, using two types of inoculum, fungal colonized wood or agar plugs.

2. Materials and Methods

2.1. Fungi Used for Inoculation

Seven fungal isolates were obtained from a previous study by the authors: *Cytospora pruinosa* (isolate number—EAB 67-4, GenBank number—MZ452627), *Diplodia mutila* (EAB 42-6, MZ452622), *Diplodia seriata* (EAB 64-12, MZ452623), *Paraconiothyrium brasiliense* (EAB 58-13, MZ452626), *Phaeoacremonium minimum* (EAB 66-10, MZ452624), *Phaeoacremonium scolyti* (EAB 64-22, MZ452625), and *Thyronectria aurigera* (EAB 45-20, MZ452628) were used for inoculations in field trials [10] (Figure 1). Isolates were identified by using sequences of the internal transcribed spacer region of rDNA (ITS1F and ITS4), and additional gene regions were needed to resolve four of the seven isolates. *Phaeoacremonium minimum* and *P. scolyti* were identified to species by using two additional genes: the ß-tubulin gene (TUB) and actin gene (ACT), using the primer pairs T1 [11], Bt2b [12], ACT-512F and ACT-783R [13]. *Diplodia mutila* and *Diplodia seriata* were identified to species by using two additional primer pairs: the elongation factor 1-alpha (EF1-α) primer pair EF1-728 and EF1-986 [13] and ß-tubulin gene primer pair Bt2a and Bt2b [12]. The BLASTn algorithm was used to compare sequences to known reference sequences of known species in GenBank [14]. All isolates used matched species from taxonomic studies or type species.

2.2. Field Trials

Autumn Purple®white ash (*Fraxinus americana* 'Junginger') trees were used in this study. Trees 2.5 m tall and approximately 2.5 to 3 cm in diameter were grown at the University of Minnesota experimental field plots in the spring of 2019. Three-year-old trees were used in the study. The bareroot trees were planted in two rows, with 60 trees in each row, spaced 1 m apart and numbered sequentially for a total of 120 trees. A root irrigation dripline was installed for weekly watering, and each tree was fertilized three times with Osmocote 12-7-18 to help with establishment of vigorous roots and shoots. The field trials were split into two studies of 56 trees. Eight treatments (including one control) were used with seven replications per treatment. The treatments included two inoculation methods consisting of the assay fungus growing on woodchips or on 1.5% Difco malt extract agar (15 g Difco Bacto agar and 15 g of Difco Bacto malt extract per liter of deionized water). Woodchips were made from birch tongue depressors and cut into 1 cm^2 squares, soaked in deionized water and autoclaved twice for 30 min each. Woodchips were then placed on two-week-old pure cultures of the assay fungi and grown for another two weeks. Agar plugs were made by using a 4 mm cork borer from the assay fungus growing on 1.5% Difco agar. SAS version 9.4 statistical software was used to randomize the treatments to the 56 trees. Each treatment consisted of one agar plug or one woodchip inoculated into a cut wound 1 cm in length. Agar plugs were inoculated 1.5 meters up from the root graft on the south-facing side of the tree and woodchips were inoculated 0.5 meters up from the agar-plug inoculation on the opposite side (north facing) of the tree. Each inoculated wound was wrapped in parafilm and covered with duct tape to protect the inoculation site,

maintain moisture, and to prevent contamination. Control wounds received a sterile wood chip or agar plug. The wounds that were made during the inoculations healed over with callus tissue after one-to-two months.

Figure 1. Pure cultures of fungi isolated from EAB galleries used for the study, top row form left to right: *Cytospora pruinosa* (EAB 67-4), *Diplodia mutila* (EAB 42-6), *Diplodia seriata* (EAB 64-12), and *Paraconiothyrium brasiliense* (EAB 58-13). Bottom row from left to right: *Phaeoacremonium minimum* (EAB 66-10), *Phaeoacremonium scolyti* (EAB 64-22), and *Thyronectria aurigera* (EAB 45-20).

The first experiment was initiated on 19 September 2019. The trees from this study were harvested 14 months later, on 3 November 2020. The second experiment was initiated on 30 April 2020, and the trees were harvest 8 months later, on 30 December 2020. Inoculated sections of the tree were brought into the laboratory, where the lesions on the stems were measured in millimeters, using a digital caliper. The size of the lesions was calculated by multiplying the length by width to get values for mm^2. This method of reporting lesion size is consistent with the existing literature [15,16]. Wood samples were taken from each lesion and placed into (M+) 1.5% Difco malt extract agar with 0.1 g streptomycin sulfate added after autoclaving (15 g Difco Bacto agar, 15 g of Difco Bacto malt extract, and 0.1 g streptomycin sulfate per liter deionized of water) to confirm that the inoculated fungus could be re-isolated. Fungi growing from the isolations were identified and confirmed by using microscopic identification and sequencing of the ITS region of rDNA.

Data were examined by using SAS version 9.4 statistical software [17]. Each treatment was compared to the control by using the Wilcoxon's Rank Sum test. A confidence level of $p < 0.05$ was used to determine the statistical significance. There were two trees in Experiment 1 and four trees in Experiment 2 that died from transplanting and not related to wounding or inoculations and were removed from the analyses.

3. Results

3.1. Experiment 1

During the 14-month period of the study, 33 of the 49 trees inoculated with the assay fungi by using agar plugs formed cankers. Thirty-six of the 49 trees inoculated by using fungal colonized wood chips formed cankers. Two of the seven control treatments using agar plug inoculation and three of seven control trees using the woodchip inoculation had very slight measurable lesions (Table 1). The median lesion area for both agar plug and woodchip for the controls was 0 mm^2 (Table 2). The largest median lesion area (270.8 mm^2) was found on trees inoculated with *D. mutila* by using agar plugs (Table 2) and was significantly greater than the controls ($p = 0.04$). Two other treatments also generated lesions significantly greater than the controls, including *D. mutila* woodchip treatments and *C. pruinosa* agar plug treatments. The median lesion size for *D. mutila* woodchip treatment was 141.6 mm^2 (Interquartile Range 37.9 to 176.9, $p = 0.03$). *Cytospora pruinosa* agar plug treatment had a median lesion area of 169.2 6 mm^2 (IQR 48.5 to 218.8, $p = 0.04$). None of the inoculated fungi used in this study was re-isolated from the controls. The fungi that were re-isolated and sequenced from the controls with dieback were ubiquitous fungi, including *Penicillium*, *Aspergillus*, and *Trichoderma* species. Canker-causing fungi were not isolated from the controls. The recovery rate for each fungus is listed in Table 3. Fungi recovered from lesions on inoculated trees were confirmed as the species used for inoculation, except for *T. aurigera*, which was unable to be re-isolated to confirm its identity.

Table 1. Number of trees with measurable lesions as compared to total number of trees used for each treatment in Experiments 1 and 2.

	Experiment 1		Experiment 2	
Fungus	**Agar Plug**	**Woodchip**	**Agar Plug**	**Woodchip**
Control	2/7	3/7	0/7	0/7
Cytospora pruinosa (EAB 67-4)	7/7	5/7	6/6 [a]	6/6 [a]
Diplodia mutila (EAB 42-6)	6/6 [a]	6/6 [a]	7/7	7/7
Diplodia seriata (EAB 64-12)	5/7	5/7	5/7	7/7
Paraconiothyrium brasiliense (EAB 58-13)	1/7	2/7	3/7	6/7
Phaeoacremonium minimum (EAB 66-10)	6/7	6/7	6/7	6/7
Phaeoacremonium scolyti (EAB 64-22)	4/7	6/7	4/6 [a]	4/6 [a]
Thyronectria aurigera (EAB 45-20)	4/7	6/7	6/7	6/7

[a] Some treatments had only 6 replications due to a few trees that died during transplanting and were excluded from analysis.

Table 2. Experiment 1 median canker area (mm^2) by treatment type and fungus. *p*-values were calculated by using Wilcoxon Rank Sum test. Treatments are being compared to the controls in each experiment. Significant results are shown in bold.

	Agar Plug			Wood Chip		
	Area (mm^2)			Area (mm^2)		
Fungus	**Median**	**Interquartile Range**	***p*-Value**	**Median**	**Interquartile Range**	***p*-Value**
Control	0	0–16.5	-	0	0–15.5	-
Cytospora pruinosa (EAB 67-4)	169.2	48.5–218.8	**0.04**	63.9	0–82.5	0.2
Diplodia mutila (EAB 42-6)	270.8	86.4–507.3	**0.02**	141.6	37.9–176.9	**0.03**
Diplodia seriata (EAB 64-12)	36.5	0–93.3	0.2	34.8	0–85.8	0.25
Paraconiothyrium brasiliense (EAB 58-13)	0	0–0	0.6	0	0–37.5	0.77
Phaeoacremonium minimum (EAB 66-10)	23.9	13.2–36.4	0.12	24.8	15.6–64.7	0.11
Phaeoacremonium scolyti (EAB 64-22)	42.3	0–62.0	0.37	31.5	24.8–43.0	0.11
Thyronectria aurigera (EAB 45-20)	63.8	0–75.6	0.37	59	9.7–74.8	0.17

Table 3. Recovery of fungi re-isolated for both treatments agar plug and woodchip from inoculated *F. americana* trees.

	Experiment 1			Experiment 2		
Fungus	**Agar Plug**	**Woodchip**	**Percent Recovery (Agar Plug, Woodchip)**	**Agar Plug**	**Woodchip**	**Percent Recovery (Agar Plug, Woodchip)**
Control	0/7	0/7	0, 0	0/7	0/7	0, 0
Cytospora pruinosa (EAB 67-4)	5/7	4/7	71, 57	6/6	6/6	100, 100
Diplodia mutila (EAB 42-6)	6/6	6/6	100, 100	7/7	7/7	100, 100
Diplodia seriata (EAB 64-12)	6/7	7/7	86, 100	7/7	7/7	100, 100
Paraconiothyrium Brasiliense (EAB 58-13)	7/7	7/7	100, 100	6/7	6/7	86, 86
Phaeoacremonium minimum (EAB 66-10)	7/7	7/7	100, 100	5/7	6/7	71, 86
Phaeoacremonium scolyti (EAB 64-22)	5/7	6/7	71, 86	5/6	5/6	71, 71
Thyronectria aurigera (EAB 45-20)	0/7	0/7	0, 0	0/7	0/7	0, 0

3.2. Experiment 2

During the 8-month study period, 37 of 49 trees treated with the assay fungi by using agar plug inoculation formed cankers. Forty-two of the 49 trees treated with fungal colonized woodchips formed cankers. None of the control wounds treated with a non-inoculated agar plug or woodchip formed lesions (Table 1). The largest median lesion area was caused by *D. mutila* inoculated with the fungus grown on agar (Table 4). The median area was 341.9 mm^2 (IQR 101.0 to 928.1); the difference between this treatment and the control was statistically significant ($p = 0.006$). All other treatments, except for inoculation with *P. brasiliense* by using an agar plug, were also significantly greater than the controls. Median lesion areas for the other treatments ranged from 0 to 72.6 mm^2 (Table 4 and Figure 2). Similar to Experiment 1, the re-isolated fungi from the control wounds yielded ubiquitous fungal species and no canker-causing fungi from the study were isolated. Again, except for *T. aurigera*, which was not re-isolated, all other canker fungi were isolated and identified as coming from their respective inoculated lesions (Table 3).

Table 4. Experiment 2 median canker area (mm^2) by treatment type and fungus. *p*-values were calculated by using the Wilcoxon Rank Sum test. Treatments are being compared to the controls in each experiment. Significant results are shown in bold.

	Agar Plug			**Wood Chip**		
	Area (mm^2)			**Area (mm^2)**		
Fungus	**Median**	**Interquartile Range**	***p*-Value**	**Median**	**Interquartile Range**	***p*-Value**
Control	0	0–0	-	0	0–0	-
Cytospora pruinosa (EAB 67-4)	72.6	38.5–121.6	**0.008**	74.4	70.5–96.0	**0.008**
Diplodia mutila (EAB 42-6)	341.9	101.0–928.1	**0.006**	323.5	233.2–402.5	**0.006**
Diplodia seriata (EAB 64-12)	69.9	0–77.4	**0.03**	74.5	63.3–99.8	**0.006**
Paraconiothyrium brasiliense (EAB 58-13)	0	0–29.8	0.1	59.2	27.3–70.0	**0.01**
Phaeoacremonium minimum (EAB 66-10)	35.8	30.4–50.9	**0.01**	32.6	21.0–107.5	**0.01**
Phaeoacremonium scolyti (EAB 64-22)	41.6	0–63.3	**0.04**	46.1	0–75.8	**0.04**
Thyronectria aurigera (EAB 45-20)	38.2	10.9–74.3	**0.01**	46.1	18.9–52.8	**0.01**

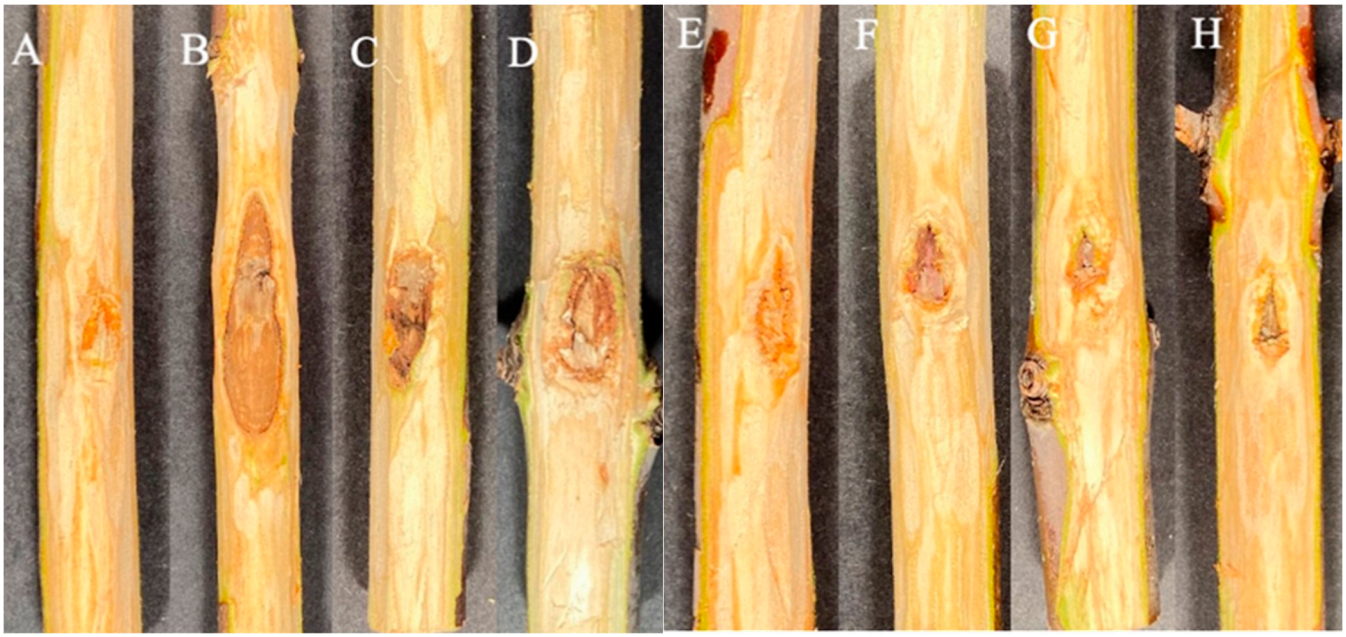

Figure 2. White ash stems from Experiment 2 to demonstrate the lesion size that is representative of the fungus and treatment. From left to right: (**A**) Control, (**B**) *Diplodia mutila* (EAB 42-6), (**C**) *Diplodia seriata* (EAB 64-12), (**D**) *Cytospora pruinosa* (EAB 67-4), (**E**) *Thyronectria aurigera* (EAB 45-20), (**F**) *Phaeoacremonium scolyti* (EAB 64-22), (**G**) *Phaeoacremonium minimum* (EAB 66-10), and (**H**) *Paraconiothyrium brasiliense* (EAB 58-13).

4. Discussion

All seven of the inoculated fungi evaluated in this study produced lesions on ash trees, in varying amounts, with the exception of *P. brasiliense* using agar plug inoculum, in Experiment 1. *Diplodia mutila*, *D. seriata*, and *C. pruinosa* caused the largest lesions that were most significantly different than the controls. This is consistent with previous studies that used similar fungal species that caused cankers after inoculation on other hardwood species [18–20]. Most fungi that may have the capacity to cause cankers that were isolated from *Fraxinus* spp. have not been reported to cause cankers in ash. Previous research by the authors found 134 isolations of *Cytospora* in a survey of fungi in EAB galleries in Minnesota, USA [10]. This fungus appears to be commonly associated with EAB galleries from all regions of Minnesota investigated. Other fungi, such as *Diplodia* species, also produced large cankers on the trees during the relatively short time they were tested. Inoculation results suggest that these fungi are likely contributing to the size of lesions associated with EAB galleries and may accelerate tree death. The results presented here suggest that canker fungi contribute to the decline and death of ash trees when EAB is present.

Previous results by the authors indicate that 36% of 1126 fungi isolated from EAB galleries in Minnesota were fungi that could be grouped into the guild of canker-causing fungi [10]. *Cytospora* species are well-known canker-causing fungi of woody plants and shrubs, including fruit trees and grapevines [21]. Kepley and Jacobi (2000) reported host specificity of *C. pruinosa* on (*F. pennsylvanica*) during artificial inoculations, using different *Cytospora* species on six hardwood hosts. *Cytospora pruinosa* and with *D. mutila* have been cited as contributing to the decline of *F. excelsior* and caused necrotic lesions on stems of this species [18]. Many *Diplodia* species are considered opportunistic pathogens and are common canker-causing fungi and known trunk diseases for a wide array of woody plants [22]. *Diplodia* species used in the study reported here caused the largest of cankers of all the fungi tested. *D. mutila* has been reported in China as causing branch cankers on *Ziziphus jujube* [23]. *Diplodia mutila* has also been identified as causing dieback on *F. excelsior* in Poland and dieback of English walnut in Chile [19,20]. *Diplodia seriata*, termed black rot (not to be confused with black rot of grapes by *Guignardia bidwellii*), is a major agent causing disease of fruit trees in the pomaceous family [24]. This fungus can attack branches

or limbs, causing cankers, and produces a reddish-brown coloration at the lesion [24]. In South Africa, *D. seriata* has been isolated from cankers on apple and pear trees, as well as cankers from pear trees in Turkey [25,26]. *Paraconiothyrium* is commonly found on the wood and leaves of *Prunus*, *Actinidia*, and *Laurus* species [27]. *Phaeoacremonium* species are known to cause wood staining within larval galleries. This has been observed in *F. pennsylvanica* in North Dakota, as well as *F. excelsior* in Sweden [28,29]. *Fraxinus excelsior* and *F. latifolia* are other known hosts of *Phaeoacremonium*, particularly *P. mortoniae. Phaeoacremonium* has been found on grape vines and ash trees in areas that surround vineyards in California [30]. The spores from the fungi are spread during rainfall and are present in vineyards and the surrounding ash trees throughout the growing season [30]. The genus *Thyronectria* is found throughout the world and has a wide range of host species. In the United states, *Thyronectria* has been reported to cause cankers on thornless honeylocust (*Gleditsia triacanthos* f. *inermis*), with canker size varying depending on drought stress and water availability [31–33]. While some dieback and small lesions occurred at the wound site for some of the control treatments in Experiment 1, no canker fungi were isolated from this area. We attribute the increased size of the wound to dieback that occurred after wounding due to desiccation. This type of dieback did not occur in control wounds in Experiment 2. This is likely due to the different environmental conditions during the spring inoculations, as compared to those made in the fall, which was a dryer time of year.

While EAB is most commonly reported on *F. pennsylvanica*, this study was performed by using *F. americana*. White ash is representative of other ash species, and similar results likely would occur on other species of ash. This is consistent with other findings from other studies, including studies of *F. excelsior* [18].

Our results suggest that six of the fungi tested, namely *C. pruinosa*, *D. mutila*, *D. seriata*, *P. brasiliense*, *P. minimum*, and *P. scolyti*, cause cankers and can be re-isolated, thus proving Koch's postulates. These fungi may work in concert with EAB to accelerate the rate of decline and death in ash trees. The fungi enter into the tree via wounds created by the adult insect and appear to be opportunistic fungi, taking advantage of the stressful situation occurring from EAB gallery formation. While the lesions observed in the study presented here occurred on young vigorously growing trees in our field studies, ash trees attacked by EAB would be under stress, and it is likely that these canker-causing fungi could cause larger lesions in stressed trees. The canker fungi tested appear to be opportunistic organisms that can take advantage of reduced host responses and contribute to larger zones of dead cambium around galleries and faster mortality. Trees would be less likely to recover from initial EAB attack if canker-causing fungi are also present, especially the species, such as *D. mutila*, *D. seriata* and *C. pruinosa*, that cause large lesions.

Ash is an important species in urban and forested parts of Minnesota and around the country. This study broadens our understanding of how canker fungi in association with EAB may cause more rapid ash mortality. The role of canker-causing fungi in ash attacked by EAB needs to be examined carefully, especially in the presence of the nation's most destructive invasive pest. The fungi associated with EAB galleries appear to have the potential to contribute to accelerated ash mortality, and these insect–fungal interactions deserve additional investigation.

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