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Defence responses in the xylem of *Ulmus americana* cultivars after inoculation with *Ophiostoma novo-ulmi*

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Abstract

Earlier, it has been shown that cultivars of American elm (*Ulmus americana*) can differ in their susceptibility to Dutch elm disease (DED) and in their ability to compartmentalize infection. To gain a better understanding of how certain factors of compartmentalization influence disease susceptibility, histological and histochemical studies were performed on five cultivars of American elm and two wild-type seedling populations. There were a variety of differences in barrier zone formation and barrier zone characteristics among the cultivars which may help explain variability in resistance to DED. Timing of barrier zone production may be one factor that helps determine whether a tree survives infection. At 20 days postinoculation (DPI) in 2015, "New Harmony," which had one of the highest mean disease severity ratings (DSR), was the only cultivar to have no barrier zones present in the samples examined. Barrier zones were present in all trees examined in 2016 for the two cultivars with the highest mean DSR, with many of the trees at 100% permanent wilt at 90 DPI, providing evidence that the formation of barrier zones does not ensure the tree will survive infection. When examining stem sections of these cultivars from 2016 for autofluorescence under blue light, which is indicative of phenolic compounds, they displayed significantly less autofluorescence than "Valley Forge," which had the lowest DSR. Another important finding from this work is that despite having weak or discontinuous barrier zones, cultivars can still have relatively low DSR. "Prairie Expedition" and "Princeton" had multiple samples which had barrier zones which were breached or circumvented. When a barrier zone was breached, these cultivars often formed a subsequent barrier zone. Findings from these examinations help illustrate the complex nature of compartmentalization in American elm and how a variety of factors are affecting disease resistance.

1 | INTRODUCTION

Every year, countless American elms (*Ulmus americana* L.) succumb to Dutch elm disease (DED) following infection with the fungus, *Ophiostoma ulmi* (Buism.) Melin & Nannf. or *O. novo-ulmi* Braiser. Despite the seemingly endless losses, there are American elm genotypes that show resistance to DED (Beier, Held, Giblin, Cavender-Bares & Blanchette, 2017; Townsend, Bentz & Douglass, 2005; Townsend, Bentz & Johnson, 1995). Scientists have long searched for the mechanisms responsible for this increased resistance. While differences in anatomical features of some elms have been examined (Elgersma, 1970; McNabb, Heybroek & Macdonald, 1970; Sinclair, Zahand & Melching, 1975a), much of the previous research has focused on host responses that are induced following infection (e.g., Aoun, Rioux, Simard & Bernier, 2009; Et-Touil, Rioux, Mathieu & Bernier, 2005; Rioux & Ouellette, 1991; Shigo & Tippett, 1981;

Sinclair, Zahand & Melching, 1975b). As many of these previous studies only examined susceptible elms or a small number of putative resistant genotypes and many new resistant elms are currently available, further investigation of resistant elms is needed. By comparing the histological responses of multiple putatively resistant cultivars to susceptible cultivars, greater insight into what makes certain genotypes more resistant can be realized. If the tree defence mechanisms responsible for resistance can be determined, it could aid in both plant selection and disease management recommendations.

The availability of multiple American elm cultivars, with varying levels of resistance to DED, has provided the opportunity to do a thorough comparison of the various mechanisms of resistance that may be operative. It has been shown that American elm cultivars can differ in their ability to compartmentalize infection (Beier et al., 2017). While studies conducted by Beier et al. (2017) provided a macroscopic view of differences in compartmentalization, histological assessments were warranted to further discern differences among cultivars.

Compartmentalization is a process involving the isolation of pathogens within a tree, limiting their systemic spread (Shigo, 1984; Shigo & Marx, 1977). While not always successful (Santamour, 1987; Shigo, 1984), effective compartmentalization has been associated with increased resistance to multiple vascular diseases (Jacobi & MacDonald, 1980; Tainter & Fraedrich, 1986; Tippett & Shigo, 1981; Yamada, Ichihara & Hori, 2003). The process of compartmentalization can be divided into two distinct parts, reaction zone formation and barrier zone formation (Shigo, 1984). Reaction zones involve chemical barriers which are formed in xylem present at the time of wounding, while barrier zones are rows of cells formed de novo by the cambium after wounding (Shigo, 1984; Yamada, 2001). Previous studies have shown barrier zones are associated with trees surviving DED (Banfield, 1968; Buisman, 1935; Et-Touil et al., 2005; Rioux & Ouellette, 1991; Shigo & Tippett, 1981). Due to the likely importance of barrier zones as a mechanism of resistance in American elm, barrier zone formation and changes in xylary characteristics were the focus of this investigation.

By examining cultivars with a range of disease susceptibility, the role of barrier zones as a potential resistance mechanism can be substantiated. The objectives of this study were to (a) determine whether differences occur in the timing of barrier zone formation among cultivars with differing levels of resistance to DED, (b) examine whether barrier zone continuity differs between cultivars, (c) compare barrier zone characteristics of different cultivars and how barrier zones relate to conducting xylem vessels and (d) determine whether there are differences in the production of phenolic compounds in resistant and susceptible genotypes.

2 | MATERIALS AND METHODS

2.1 | Plant material

To examine differences in barrier zone formation, five *Ulmus americana* cultivars ("Brandon," "New Harmony," "Prairie Expedition," "Princeton" and "Valley Forge") and two populations of wild-type seedlings were used. Wild-type seedlings originated from Ontario, Canada and Tennessee, USA, and hereafter are referred to as wild-type CA and wild-type US, respectively. Trees were purchased from commercial nurseries and were planted in a nursery field at the University of Minnesota, St. Paul campus during the summer of 2014. Five-hundred and fifty-four trees were planted: 97 "Brandon," 47 "New Harmony," 88 "Prairie Expedition," 81 "Princeton," 82 "Valley Forge," 84 wild-type CA and 75 wild-type US. This planting included extra trees in the event that some trees did not survive transplanting. Trees were spaced 0.9 m apart within rows and 3 m apart between rows. "New Harmony," "Princeton," "Valley Forge" and wild-type US were randomly assigned within a large plot in the field. Wild-type CA replicates were planted next to wild-type US replicates. "Brandon" and "Prairie Expedition" were randomly assigned to the last row of the previously described plot and a neighbouring plot. Trees inoculated in 2015 were 3 years old, while those inoculated in 2016 were 4 years old. All trees received water as needed during the growing season. In addition, trees were fertilized every 3 months during the growing season with 4.9 ml of Osmocote® Plus (15-9-12) (Everris NA Inc., Dublin, OH).

2.2 | Inoculations and disease severity ratings

Methods of inoculum preparation and inoculation have been described previously (Beier et al., 2017). In brief, an isolate of *Ophiostoma novo-ulmi* with known pathogenicity, collected from Minnesota, was used for inoculations. A drill was used to make a single small hole (2.38 mm wide by 4 mm deep) in the tree 0.5 m above the ground. Tape was wrapped tightly around the drill bit to maintain a consistent depth. Immediately following drilling, 25 µl of an *O. novo-ulmi* spore suspension (1×10^6 spores/ml) or sterile water was injected into the hole using a micropipette and the wound was subsequently wrapped with Parafilm M® (Bemis Co., Inc., Neenah, WI) to avoid desiccation. Trees inoculated with *O. novo-ulmi* are hereafter referred to as inoculated, and those inoculated with sterile water are referred to as mock-inoculated. To more accurately determine disease susceptibility in the cultivars, additional samples, which were not used for histological assessments, were included in 2015. Trees in the 2015 trial were inoculated on May 28 (43 days after budbreak), while trees in the 2016 trial were inoculated on May 26 (40 days after budbreak). Disease severity ratings (DSR) were made at 5, 10, 15, 20, 40 and 90 days postinoculation (DPI) based on the percentage of the crown exhibiting permanent wilt using a 1–12 disease severity scale: 1 = 0%, 2 = 1%–9%, 3 = 10%–19%, 4 = 20%–29%, 5 = 30%–39%, 6 = 40%–49%, 7 = 50%–59%, 8 = 60%–69%, 9 = 70%–79%, 10 = 80%–89%, 11 = 90%–99% and 12 = 100%.

2.3 | Sample collection, dye ascent and recovery of *Ophiostoma novo-ulmi*

For the 2015 trial, trees were destructively harvested at 5, 10, 15, 20, 40 and 90 DPI. A total of 425 trees were harvested over the

course of the trial. More specifically, at each time point, except for 15 DPI, six inoculated trees and six mock-inoculated trees were harvested for all cultivars, except for "New Harmony" and wild-type US. For "New Harmony," only three trees for each of the inoculated and mock-inoculated groups were harvested and at 90 DPI only two mock-inoculated trees were processed. In addition, at 90 DPI only three trees were harvested for each of the inoculated and mock-inoculated groups for the wild-type US, due to limited plant material. At 15 DPI, only three inoculated trees and three mock-inoculated trees were harvested for each cultivar.

At the time of harvesting, half of the trees were placed in a safranin O solution and the remaining trees were left unstained. The process of staining the trees with safranin O is described in Beier et al. (2017). In brief, cut trees were placed in 10 mM KCl with 0.1% w/v safranin O for 18–24 hr. As "New Harmony" and wild-type US (at 90 DPI) had fewer trees than the remaining cultivars, all trees were treated with safranin O. Additionally, since fewer trees were harvested at 15 DPI for all cultivars, all trees were treated with safranin O. Trees treated with safranin O are hereafter referred to as stained trees, while those which were not treated are referred to as unstained trees. The trees used for the safranin O treatment in 2015 were cut 30 cm below the site of inoculation. To avoid cavitation, the stems were placed into a shallow tub of water and subsequently cut. For unstained trees, a 20-cm sample, centred on the inoculation site, was cut and immediately placed on ice and subsequently stored at −20°C. For both stained and unstained trees, a 5-cm segment was collected 10 cm above the inoculation site and immediately placed on ice and stored at −20°C. In addition for the stained trees, a 2-cm segment was collected from 30 cm above the inoculation site and they were processed as described above.

For the 2016 trial, a total of 41 trees were harvested, five inoculated trees and one mock-inoculated tree were harvested at 90 DPI for each cultivar, except for "Valley Forge," which had four inoculated trees. Trees were cut at 10 and 15 cm above the inoculation site. The portion of the stem below the 10-cm cut was processed in the same manner as the unstained trees in 2015, while the portion of the stem above the 15-cm cut was placed into safranin O in the same manner as the stained trees in 2015. As described previously, the 5-cm segment at 10 cm above the inoculation site and the 2-cm segment at 30 cm above the inoculation site was immediately placed on ice and stored at −20°C. Since the trees in 2016 were too large to be cut underwater, harvesting was performed predawn to reduce the likelihood of cavitation.

To determine the presence of *Ophiostoma novo-ulmi*, the stem segments collected from 10 cm above the site of inoculation were used. The bark was removed from the portion of the stem that was 10–11 cm above the inoculation site, and four pieces (approximately 3 mm^3) were cut from the xylem. Pieces were collected from areas showing vascular discoloration. If no vascular discoloration was present, samples were collected every 90° around the circumference of the xylem. Segments were also plated onto a selective media for *Ophiostoma* described by Harrington (1981) and were monitored for 2–4 weeks. The fungus was identified using morphological characteristics.

Additional studies, which are further described below, were conducted on the trees. For each substudy, the time point(s) assessed, the trees examined, and the location within the tree is summarized in Table 1.

2.4 | Examination of timing of barrier zone formation

Samples from unstained and stained trees from 2015 which were fungal inoculated were assessed for presence and continuity of barrier zones at 5, 10, 15 and 20 DPI to determine the timing of barrier zone formation. Later, time points were not examined due to the large number of trees that died after 20 DPI. In addition, mock-inoculated trees were examined at 20 DPI. A small number of both the inoculated and mock-inoculated "Prairie Expedition" and "Brandon" samples had modified axial parenchyma and fibres surrounding the first cells formed in the early wood, which could not be distinguished from barrier zone cells. It was determined these were most likely formed prior to inoculation; therefore, they were not included in the analysis.

To examine the timing of barrier zone formation, transverse sections from 11 to 12 cm above the inoculation site were used. Thick cross sections (approximately 2 cm) of the main stem, which had been stored at −20°C, were quartered and thin freehand transverse sections, approximately 50 μm thick, were made using a high-profile microtome blade so the entire circumference of the most recent annual ring could be assessed. Sections were mounted in water, and the original position of sections was noted so the length of continuous barrier zones could be assessed. Each sample was examined using a Nikon E600 microscope (Nikon Instruments Inc., Melville, NY) at 200× to determine whether barrier zone formation had occurred and whether barrier zones were continuous tangentially for more than 1500 μm of the section. Images of barrier zones were taken at 100× using a Nikon DS-Ri1 camera (Nikon Instruments Inc., Melville, NY) mounted on a Nikon Eclipse Ni-U microscope (Nikon Instruments Inc., Melville, NY). Barrier zones were identified by examining the shape of cells, which generally appear more flattened than typical fibre and axillary parenchyma cells (Shigo & Tippett, 1981). In addition, barrier zone cells are often darker in appearance due to the accumulation of various tree defence compounds (Rioux & Ouellette, 1991).

2.5 | Examination of barrier zone presence and continuity

Barrier zone presence and continuity were examined at 90 DPI in stained trees for the 2015 trial and all trees for the 2016 trial. Thick transverse sections (approximately 1.5 mm thick) from 30 to 31 cm above the inoculation site, which had been allowed to air dry, were assessed. The thick transverse sections were resurfaced on one side using a high-profile microtome blade. Transverse sections were

TABLE 1 Summary of substudies conducted, including time point(s) examined, trees examined and the location of the tissue examined

Substudy	Time point(s) examined	Trees examined	Location above the inoculation site
Disease severity rating	5, 10, 15, 20, 40, and 90 DPI	2015 trial unstained and stained; 2016 trial	NA
Recovery of *Ophiostoma novo-ulmi*	5, 10, 15, 20, 40 and 90 DPI	2015 trial unstained and stained; 2016 trial	10–11 cm
Timing of barrier zone formation	5, 10, 15 and 20 DPI[a]	2015 trial unstained and stained	11–12 cm
Barrier zone presence and continuity	90 DPI	2015 trial stained; 2016 Trial	30–31 cm
Barrier zone characteristics and conducting xylem vessels	90 DPI	2015 trial stained; 2016 trial[b]	30–31 cm
Histochemical assessment of barrier zones	90 DPI	2015 trial unstained; 2016 trial[c]	9–10 cm

Notes. DPI: Days postinoculation.
[a]When examining timing of barrier zone formation, mock-inoculated samples were only examined at 20 DPI. [b]Since none of the mock-inoculated trees examined had barrier zones present at 30–31 cm above the inoculation site for both the 2015 and 2016 trial, they were not included in this substudy. [c]One mock-inoculated replicate was examined for each cultivar for both the 2015 and 2016 trial.

examined at 200× using a Nikon E600 microscope with supplemental top lighting. Each sample was assessed for whether a barrier zone was present and whether a barrier zone was completely continuous around the entire circumference of the stem.

2.6 | Examination of barrier zone characteristics and conducting xylem vessels

To determine the differences in barrier zone formation and characteristics between the cultivars and to gain a better understanding of the relationship between barrier zones and functional xylem vessels, the trees used in the previous section (2.5) were further assessed at 30–31 cm above the inoculation site. None of the mock-inoculated trees had barrier zones present, so no further examination was performed. Freehand sections, approximately 40 μm thick, were made using a high-profile microtome blade at the side of inoculation and the side opposite of inoculation. A wet mount was made and was immediately viewed and photographed at 40–100× using a Nikon DXM 1200F camera (Nikon Instruments Inc., Melville, NY) mounted on a Nikon E600 microscope or a Nikon DS-Ri1 camera mounted on a Nikon Eclipse Ni-U microscope. As many of the annual rings were larger than the field of view for the digital documenting system, multiple images of the same section were merged using the photomerge feature in Photoshop™ (Adobe Systems Inc., San Jose, CA) or the scan large image feature in Nikon Elements Advanced Research (Nikon Instruments Inc., Melville, NY). Z-stacking was performed as necessary using Photoshop™ or Nikon Elements Advanced Research.

For analysis, an image of the current annual ring was cropped to be 0.5 mm wide tangentially with the length including the annual ring radially. The image was cropped from the middle of the larger image or from the area not damaged during sectioning in the larger image. The presence of vessels stained red by the safranin O allowed for the determination of whether xylem vessels were still conducting. Sections were assessed for a number of variables including the presence of barrier zones, the number of barrier zones formed, whether new typical fibre and axial parenchyma cells (cells which did not appear flattened) were formed distally of the barrier zone, whether any xylem vessels within the barrier zone were still conducting, whether xylem vessels directly formed before or after the barrier zone were still conducting, and whether a barrier zone had been breached. A barrier zone was considered breached if xylem vessels, formed directly after the completion of the barrier zone, were nonconducting. If barrier zones were present, the mean thickness was determined by averaging the thickness of the barrier zone at both sides of the cropped image. In the event that the barrier zone was not continuous across the section, only one measurement was used.

2.7 | Histochemical assessment of barrier zones

Histochemical observations were performed on trees harvested at 90 DPI. For unstained trees from the 2015 trial, all inoculated trees and one mock-inoculated tree for each cultivar were assessed. "New Harmony" and wild-type US were not assessed because no samples were available. Unstained segments from all trees from the

2016 trial were used. A cross section (approximately 2 mm thick) of the main stem at 9–10 cm above the inoculation site was made using a band saw. A small piece (approximately 1 mm wide) of the most recent annual ring from the side of inoculation was placed in 100% TFM™ tissue freezing medium (Electron Microscopy Sciences, Hatfield, Pennsylvania) for approximately 16 hr at 1–4°C. Transverse sections, 20 μm thick, were made with an IEC Minotome ® cryostat (International Equipment Co., Needham Heights, MA) at −20°C. Sections were rinsed with water to remove residual TFM™ tissue freezing medium. To determine the localization of phenolic compounds and more specifically suberin and lignin, sections were either left unstained or stained with Phloroglucinol-HCl (Phl-HCl) or Sudan Black B (SBB) and viewed under blue light. According to Biggs (1984, 1985), Phl-HCl staining can be used to quench autofluorescence of lignin to allow for visualization of suberized tissues. In addition, localization of lignin accumulation can be determined by quenching suberin through the use of SBB staining (Biggs, 1984). Preparations of Phl-HCl and SBB were made according to Jensen (1962). Sections were stained with Phl-HCl for 10 min and then immediately viewed under blue light. For SBB staining, sections were immersed in 50% EtOH for 3 min, followed by staining with SBB for 10 min, and were subsequently differentiated in 50% EtOH for 1 min before being viewed under blue light.

To examine the sections for autofluorescence, a Nikon DAPI filter cube (Nikon Instruments Inc., Melville, NY) was used: excitation filter (340–380 nm), dichromatic mirror (400 nm) and barrier filter (435–485 nm). For image capture, a Nikon DS-Qi1 monochrome camera (Nikon Instruments Inc., Melville, NY) attached to a Nikon Eclipse Ni-U microscope was used. To account for uneven illumination, flat field correction was used. Background illumination intensity was subtracted from images using ImageJ (Schneider, Rasband & Eliceiri, 2012).

Nikon Elements Advanced Research was used to calculate the mean intensity of pixels. For determining intensity of samples, images were cropped to 500 × 500 μm, with the bottom of the cropped image starting at 50 μm into the first barrier zone. When a barrier zone was not present, the bottom of the cropped image started at the most recently produced portion of the xylem. Some samples in 2015 were too small for a 500 × 500 μm cropping, so the cropping size was reduced for those samples. In order to help differentiate differences in intensity, images were converted to intensity heat maps using Nikon Elements Advanced Research and were converted to RGB.

2.8 | Statistical analysis

Analysis was performed using the statistical package R version 3.2.2 (R Development Core Team, Vienna, Austria). Because disease severity was measured on an ordinal scale, data were analysed with the nonparametric Kruskal–Wallis rank sum test followed by Dunn's multiple comparisons test with a Benjamini and Hochberg (1995) *p*-value adjustment. Due to the small sample size of data collected from the 2015 trial, statistical analysis was only performed on data from the timing of barrier zone formation and autofluorescence intensity. Pairwise comparisons for contingency tables were made using Fisher's exact test. As data within particular cultivars for barrier zone thickness and autofluorescence intensity were not normally distributed, the Kruskal–Wallis rank sum test followed by Dunn's multiple comparisons test with a Benjamini and Hochberg (1995) *p*-value adjustment was used.

3 | RESULTS

3.1 | Disease severity and recovery of *Ophiostoma novo-ulmi*

Findings on DSR at all time points were previously reported in Beier et al. (2017) Table 1. A summary of the DSR at 90 DPI is presented in Table 2. At 90 DPI in both 2015 and 2016, all inoculated trees had a DSR greater than 1. No mock-inoculated trees displayed foliar symptoms at any time point for either trial (DSR = 1).

At 90 DPI for the 2015 trial, the pathogen was isolated from 86% of the trees inoculated, while in the 2016 trial at 90 DPI, the pathogen was isolated from 74% of the trees inoculated. Yeast and bacteria grew from some cultured samples and their presence apparently hindered the ability to isolate the pathogen in most samples where *Ophiostoma novo-ulmi* was not reisolated. The pathogen was isolated from one mock-inoculated control, a wild-type US replicate at 10 DPI. Although the pathogen was isolated, the tree did not display any external symptoms at the time of harvest.

3.2 | Timing of barrier zone formation

Barrier zone formation was first detected in the inoculated trees at 10 DPI for "Prairie Expedition," "Valley Forge," wild-type US and

TABLE 2 Disease severity ratings (1–12 scale) of *Ulmus americana* cultivars inoculated with *Ophiostoma novo-ulmi* at 90 days postinoculation for 2015 and 2016

	Disease severity (Mean ± *SE*)[a,b]	
	Year	
Cultivar	2015	2016
"Brandon"	6.9 ± 0.8 (*n* = 9) a	11.8 ± 0.2 (*n* = 5) c
"New Harmony"	12.0 ± 0.0 (*n* = 6) bc	11.2 ± 0.8 (*n* = 5) c
"Prairie Expedition"	5.7 ± 1.0 (*n* = 9) a	5.6 ± 0.9 (*n* = 5) ab
"Princeton"	12.0 ± 0.0 (*n* = 9) c	6.2 ± 1.5 (*n* = 5) abc
"Valley Forge"	8.7 ± 0.9 (*n* = 9) ab	3.3 ± 0.3 (*n* = 4) a
Wild-type CA	10.7 ± 0.7 (*n* = 9) bc	9.6 ± 1.0 (*n* = 5) bc
Wild-type US	9.3 ± 0.8 (*n* = 6) abc	6.8 ± 1.2 (*n* = 5) abc

Notes. [a]Disease severity ratings based on the percentage of the crown exhibiting permanent wilt were made on a 1–12 scale: 1 = 0%, 2 = 1%–9%, 3 = 10%–19%, 4 = 20%–29%, 5 = 30%–39%, 6 = 40%–49%, 7 = 50%–59%, 8 = 60%–69%, 9 = 70%–79%, 10 = 80%–89%, 11 = 90%–99% and 12 = 100%. [b]Groups containing the same letter within a column are not significantly different according to Dunn's multiple comparison test with a Benjamini and Hochberg *p*-value adjustment (α = 0.05).

TABLE 3 Frequency (expressed in the number of trees) of barrier zone presence and tangential continuity in *Ulmus americana* cultivars inoculated with *Ophiostoma novo-ulmi* examined at 5, 10, 15 and 20 DPI. Observations were made on sections 11–12 cm above the inoculation site

		Barrier zone present			Barrier zone continuity		
DPI	Cultivar[a]	Yes	No	Sig.[b]	<1,500 µm	>1,500 µm	Sig.
5	"Prairie Expedition"	0	5	a	–	–	–
	"Brandon"	0	6	a	–	–	–
	"Valley Forge"	0	6	a	–	–	–
	Wild-type US	0	6	a	–	–	–
	Wild-type CA	0	6	a	–	–	–
	"New Harmony"	0	3	a	–	–	–
	"Princeton"	0	6	a	–	–	–
10	"Prairie Expedition"	1	4	a	1	0	a
	"Brandon"	0	5	a	–	–	–
	"Valley Forge"	3	3	a	3	0	a
	Wild-type US	2	4	a	2	0	a
	Wild-type CA	1	5	a	1	0	a
	"New Harmony"	0	3	a	–	–	–
	"Princeton"	0	6	a	–	–	–
15	"Prairie Expedition"	2	0	a	2	0	a
	"Brandon"	2	0	a	0	2	a
	"Valley Forge"	3	0	a	0	3	a
	Wild-type US	1	2	a	1	0	a
	Wild-type CA	1	2	a	1	0	a
	"New Harmony"	1	2	a	1	0	a
	"Princeton"	3	0	a	3	0	a
20	"Prairie Expedition"	6	0	a	1	5	a
	"Brandon"	6	0	a	0	6	a
	"Valley Forge"	6	0	a	0	6	a
	Wild-type US	5	1	a	3	2	a
	Wild-type CA	5	1	a	3	2	a
	"New Harmony"	0	3	b	–	–	–
	"Princeton"	5	1	a	0	5	a

Notes. DPI: days postinoculation.
[a]Cultivars are ordered from lowest mean disease severity rating at 90 DPI in 2015 to highest.
[b]Groups containing the same letter within a column at the same time point are not statistically different according to Fisher's exact test ($\alpha = 0.05$).

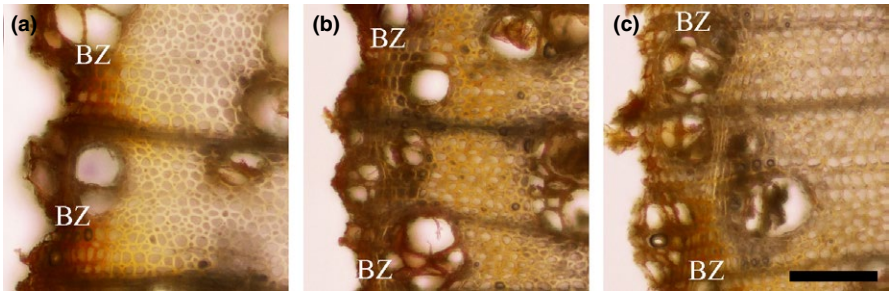

FIGURE 1 Barrier zone formation in *Ulmus americana* "Valley Forge" at multiple time points following inoculation with *Ophiostoma novo-ulmi* (bar = 100 µm). For all micrographs, the left side of the micrograph was distal of the earlywood vessels. Transverse sections were made 11–12 cm above the inoculation site. (a) 10 days postinoculation (DPI). (b) 15 DPI. (c) 20 DPI. BZ = barrier zone

wild-type CA (Table 3). "Valley Forge" had the highest proportion (0.5) of trees with barrier zones forming at 10 DPI; however, the difference compared with all other cultivars was not found to be significant ($p > 0.05$). Representative images displaying the progression of barrier zone formation in "Valley Forge" are shown in Figure 1. At 10 DPI, barrier zones were frequently only a few cells thick radially and were not very continuous tangentially (Table 3 and Figure 1a). By 15 DPI, all cultivars had at least one replicate with a barrier zone beginning to form (Table 3). All but "New Harmony" had barrier zones present at 20 DPI, and the differences between "New Harmony" and all other cultivars were found to be significant ($p < 0.05$). At 20 DPI, the three cultivars with the lowest disease severity rating, "Prairie Expedition," "Brandon" and "Valley Forge," had barrier zones present in all replicates examined (Table 3). In addition, nearly all of their barrier zones were continuous tangentially for over 1500 μm (Table 3). Although barrier zones were found in many of the trees examined at 20 DPI, only three of 33 were completely formed, noted by the return to typical fibre and axial parenchyma cells distally of the barrier zone. For mock-inoculated trees, none of the cultivars had any barrier zones present at 20 DPI (data not shown).

3.3 | Barrier zone presence and continuity

In 2015, for the inoculated trees at 90 DPI, all cultivars had barrier zones in at least two of three trees examined at 30–31 cm above the inoculation site (Table 4). The three cultivars with the lowest disease severity rating had barrier zones present in all samples. While "Prairie Expedition" and "Valley Forge" had barrier zones present in all trees examined, none of the barrier zones were completely continuous around the entire stem (Table 4). "Brandon," "Princeton," wild-type US and wild-type CA had at least one of the trees examined with a barrier zone continuous around the entire stem (Table 4). However, a barrier zone around the entire circumference of the stem did not ensure the tree would survive infection, as all of the "Princeton" trees were at 100% permanent crown wilt at 90 DPI (Table 2). None of the mock-inoculated trees for any of the cultivars had barrier zones present at 90 DPI (data not shown).

For the inoculated trees in 2016, barrier zones were present in all trees examined for all cultivars, except for wild-type CA, which had three of five trees with barrier zones present at 90 DPI. The two trees of wild-type CA without a barrier zone formed were at 100% permanent wilt at 90 DPI. There were considerable differences between the cultivars in whether the barrier zones were continuous around the stem (Table 4). In particular, the two cultivars with the lowest DSR, "Valley Forge" and "Prairie Expedition," had no barrier zones that were continuous around the entire circumference of the stem. Wild-type US and wild-type CA (when barrier zones were present) had continuous barrier zones around the entire circumference of the stem, and the differences between the wild-type trees compared with "Valley Forge" and "Prairie Expedition" were found to be significant ($p < 0.05$). At 90 DPI, no mock-inoculated trees had barrier zones present (data not shown).

3.4 | Barrier zone characteristics and conducting xylem vessels

Barrier zones frequently separated areas of nonconducting xylem from areas of conducting xylem (Figures 2b and 3a). Xylem was seldom conducting directly proximally of the barrier zones; this was only observed eight (9.5%) instances of 84 (Figure 3b). In 2015 and 2016, for both the side of inoculation and the side opposite of inoculation, there was sap conduction in at least part of the barrier zone for 55% of the barrier zones examined. When typical fibre and axial parenchyma (not flattened in appearance) were formed distally of the barrier zone, 71% had conducting xylem directly distally of the barrier zone.

There were observed differences among many of the inoculated cultivars for barrier zone formation and barrier zone characteristics in both 2015 and 2016 (Tables 5 and 6). In 2015, the two cultivars with the lowest disease severity rating had barrier zones present in all samples from both the side of inoculation and the side opposite of inoculation (Table 5). In addition, in the sections examined, there were no instances where barrier zones were breached, as indicated by nonconducting tissue directly after the barrier zone. "New Harmony," which was tied for the highest disease severity rating in 2015, had no barrier zones present in the sections from the side of inoculation and only one sample had a barrier zone present in the side opposite of wounding. In an interesting manner, "Princeton," which also had a mean DSR of 12 in 2015, had a barrier zone present on the side of inoculation in all trees examined and two of the three trees had barrier zones present on the side opposite of inoculation. Due to the small sample sizes, statistical testing could not be performed to determine whether differences were statistically significant. In general, cultivars with higher DSR had higher proportions of samples that lacked formation of typical fibre and axial parenchyma cells (cells which did not appear flattened) distally of the barrier zone (Table 5). In regard to the thickness of the barrier zones, there were no distinct differences between the cultivars with the lowest disease severity and those with the highest (Table 5). In an interesting manner, wild-type US, which was intermediate in its disease severity, had barrier zones over 100 μm thicker than all other cultivars (Table 5).

When comparing 2016 to 2015, there were some notable changes in the variables observed. "New Harmony," which had no barrier zones formed on the side of inoculation in 2015, had a barrier zone present in all sections examined on the side of inoculation in 2016, despite having a similar mean disease severity rating in both years (Tables 5 and 6). While not observed in 2015, in 2016 some cultivars had multiple barrier zones present within a section (Figure 2a). This occurred most frequently in "Prairie Expedition" and "Princeton" on the side of inoculation (Table 6). When examining the first barrier zone formed, these cultivars also had a higher proportion of barrier zones which were breached (Table 6, Figure 3c). In regard to whether a barrier zone was breached on the side of inoculation, the difference between "Prairie Expedition" and "Princeton" compared with "Valley Forge" and wild-type US was found to be significant ($p < 0.05$). While the cultivars with the lowest DSR had barrier

TABLE 4 Frequency (expressed in the number of trees) of barrier zone presence and tangential continuity in *Ulmus americana* cultivars inoculated with *Ophiostoma novo-ulmi* at 90 days postinoculation for 2015 and 2016. Observations were made on sections collected at 30–31 cm above the inoculation site

		Barrier zone present			Barrier zone[a]		
Year	Cultivar[b]	Yes	No	Sig.[c]	Continuous	Discontinuous	Sig.
2015	"Prairie Expedition"	3	0		0	3	
	"Brandon"	3	0		2	1	
	"Valley Forge"	3	0		0	3	
	Wild-type US	2	1		2	0	
	Wild-type CA	2	1		1	1	
	"New Harmony"	2	1		0	2	
	"Princeton"	3	0		1	2	
2016	"Valley Forge"	4	0	a	0	4	a
	"Prairie Expedition"	5	0	a	0	5	a
	"Princeton"	5	0	a	1	4	ab
	Wild-type US	5	0	a	5	0	c
	Wild-type CA	3	2	a	3	0	bc
	"New Harmony"	5	0	a	1	4	ab
	"Brandon"	5	0	a	2	3	abc

Notes. [a]Barrier zones were considered continuous if they were completely continuous around the entire circumference of the stem. [b]Cultivars are ordered from lowest mean disease severity rating to highest at 90 days postinoculation for 2015 and 2016. [c]Groups containing the same letter within the same column are not statistically different according to Fisher's exact test ($\alpha = 0.05$).

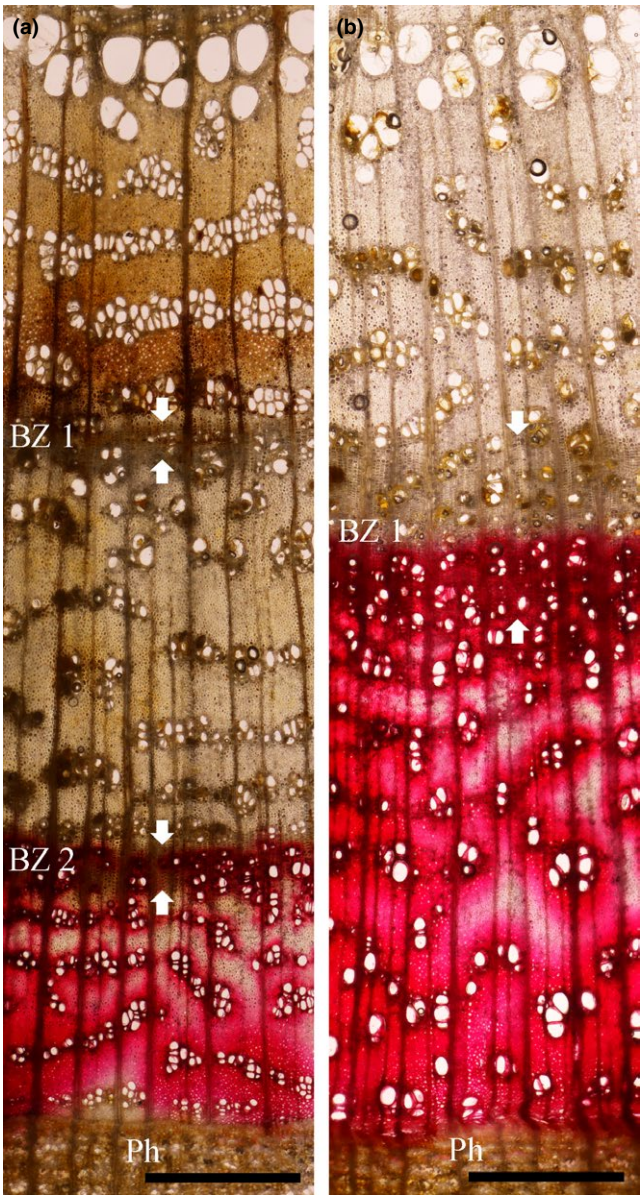

FIGURE 2 Representative transverse sections showing barrier zones and functional xylem vessels in *Ulmus americana* "Prairie Expedition" (a) and wild-type US (b) at 90 days postinoculation (bar = 500 μm). The sections display the most recent annual ring. Samples were collected during the 2016 trial. Transverse sections were made 30–31 cm above the inoculation site. Trees were stained with safranin O to indicate conducting (stained red) and nonconducting (not stained) areas of xylem. (a) "Prairie Expedition" with two barrier zones formed. The first barrier zone was breached and the second barrier zone is separating nonconducting tissue from conducting tissue. (b) Wild-type US with a single barrier zone separating nonconducting tissue from conducting tissue. Note the size difference compared with the barrier zones formed in "Prairie Expedition." White arrows indicate the start and end of barrier zones. BZ 1 = first barrier zone formed; BZ 2 = second barrier zone formed; Ph = phloem

zones present in all sections examined on the side of inoculation, interestingly, "Valley Forge," which had the lowest mean disease severity rating in 2016, had only one of four sections with barrier zones present on the side opposite of inoculation (Table 6). The difference between "Valley Forge" compared with "Prairie Expedition," "Princeton" and wild-type US for the proportion of samples with barrier zones present on the side opposite of inoculation was found to be significant ($p < 0.05$). Some trends observed in 2015 were also present in 2016. As was found previously, cultivars with higher mean DSR tended to have higher frequencies of samples that lacked formation of typical fibre and axial parenchyma cells (cells which did not appear flattened) distally of the barrier zone (Tables 5 and 6). As in 2015, wild-type US had the greatest mean thickness of any cultivar at 429.4 μm for the side of inoculation. The only significant difference between cultivars for barrier zone thickness was for wild-type US compared with "Prairie Expedition" and "Princeton" on the side opposite of inoculation ($p < 0.05$).

3.5 | Histochemical assessment of barrier zones

When examining unstained sections under blue light, all cultivars receiving fungal inoculation, except for "Prairie Expedition," displayed considerably less autofluorescence in 2015 compared with 2016 (Table 7). The autofluorescence of unstained samples viewed under blue light using a Nikon DAPI filter cube (excitation filter: 340–380 nm, dichromatic mirror: 400 nm and barrier filter: 435–485 nm) is indicative of phenolic compounds. For inoculated trees from the 2015 trial, there were no significant differences between the cultivars for intensity of autofluorescence. There was little autofluorescence detectable in the mock-inoculated trees (Table 7).

There was considerably more variation among the cultivars in 2016 for autofluorescence intensity of unstained samples from inoculated trees (Table 7). "Valley Forge," which had the lowest disease severity rating in 2016, had the highest mean intensity at 65.2 arbitrary units (a.u.). The next closest group was wild-type US at 42.7 a.u. There was a significant difference between "Valley Forge" and the two cultivars with the highest DSR in 2016 at 90 DPI, "Brandon" and "New Harmony," for mean intensity ($p < 0.05$). Similar to samples examined from 2015, little autofluorescence was present in mock-inoculated trees from 2016 (Table 7 and Figure 4h).

Autofluorescence of unstained samples, indicative of phenolics, was generally most intense on the inner side of the barrier zone (Figure 4a–g). Despite having very thick barrier zones, both wild-type US and wild-type CA showed little autofluorescence within much of the barrier zone (Figure 4d,e). In general, autofluorescence was diffused in the tissue formed before the barrier zone (Figure 4a–g); however, there were some instances when the autofluorescence was more highly localized. When barrier zones were found at the end of the annual ring, they frequently had areas within them with no autofluorescence, suggesting xylem cells were not fully matured at the time of harvest (Figure 4f,g).

Autofluorescence of sections stained with Phloroglucinol-HCl (Phl-HCl), which is indicative of suberin (Biggs, 1984, 1985), was generally quite faint relative to that of unstained sections, suggesting lignin and lignin-like compounds accounted for a vast majority of the autofluorescence observed in the unstained sections

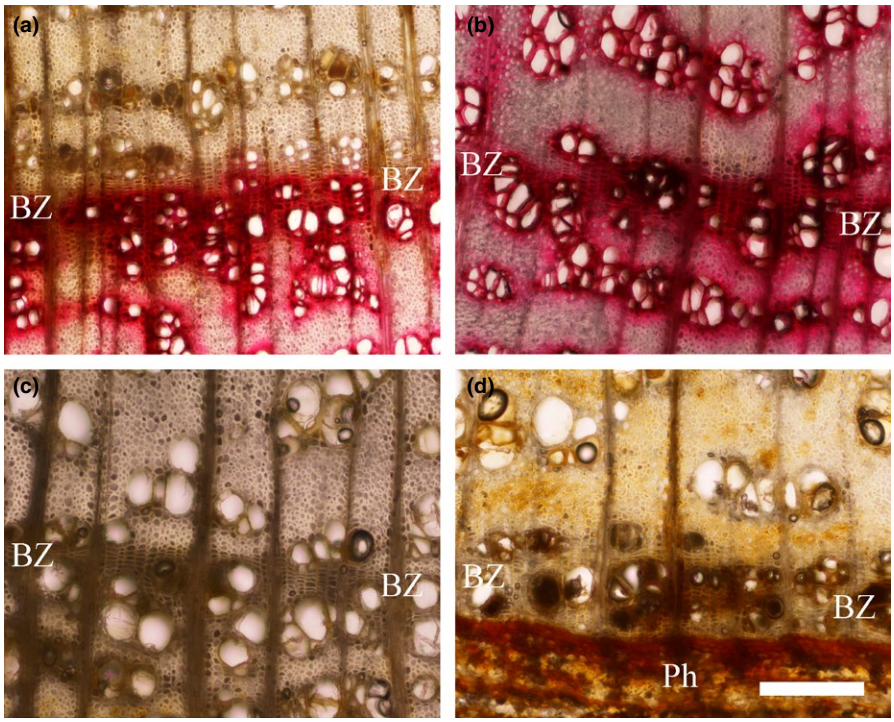

FIGURE 3 Transverse sections showing barrier zone characteristics and functional xylem vessels in *Ulmus americana* cultivars inoculated with *Ophiostoma novo-ulmi* at 90 days postinoculation (bar = 200 μm for all micrographs). For all micrographs, the bottom side of the micrograph was distal of the earlywood vessels. Samples were collected during the 2016 trial. Transverse sections were made 30–31 cm above the inoculation site. Trees were stained with safranin O to indicate conducting (stained red) and nonconducting (not stained) areas of xylem. (a) Barrier zone formed in "Prairie Expedition" separating nonconducting tissue from conducting tissue. Note many of the vessels within the barrier zone are conducting. (b) Barrier zone formed in "Valley Forge" with conducting tissue on both sides of the barrier zone. (c) Barrier zone formed in "Princeton" which has been breached, as indicated by the lack of conducting tissue formed directly distally of the barrier zone. (d) A barrier zone ending the annual ring in "New Harmony." BZ = barrier zone formed; Ph = phloem

(Figure 5b,e,h,k). This was supported by the observations on autofluorescence of sections treated with Sudan Black B (SBB), which quenches suberin (Biggs, 1984) (Figure 5c,f,i,l). When comparing autofluorescence of unstained sections to those stained with SBB, there was little difference in the intensity for most samples examined (Figure 5). The cultivar with the most consistent presence of autofluorescence following Phl-HCl staining was "Valley Forge" in 2016 (Figure 5b).

4 | DISCUSSION

The differences observed in barrier zone characteristics found in this study helps to elucidate potential mechanisms responsible for the variation in resistance to DED. Based on findings from these investigations, there are likely a variety of factors related to compartmentalization that are impacting disease resistance in American elm cultivars and multiple strategies appear to be utilized to survive infection. While some cultivars exhibit highly effective compartmentalization, other cultivars, such as "New Harmony," display weak compartmentalization (Beier et al., 2017). This variation in compartmentalization effectiveness among genotypes has been observed previously in other tree species, and evidence has been provided that it is likely under genetic control (Garrett, Randall, Shigo & Shortle, 1979; Santamour, 1979; Shigo, Shortle & Garrett, 1977). More specifically in *Ulmus*, evidence has been presented that resistance to DED is a heritable trait (Solla, Lopex-Almansa, Martin & Gil, 2014; Venturas, Lopez, Martin, Gasco & Gil, 2014). While genetics likely play a major role in compartmentalization, it has been demonstrated that other variables, such as available energy levels (Wargo, 1977) and the type or aggressiveness of the pathogen (Blanchette, 1982; Bonsen, Scheffer & Elgersma, 1985; Deflorio, Franz, Fink & Schwarze, 2009), can also impact compartmentalization.

It has been speculated that barrier zones, which form more rapidly following inoculation, may be responsible for more effective compartmentalization (Rioux & Ouellette, 1991). When examining when barrier zone formation takes place, it was observed at 20 DPI that one of the most susceptible cultivars, "New Harmony," had no barrier zones present, which was significantly different when compared to all other cultivars (Table 3). This delay in barrier zone formation may be contributing to its susceptibility. In some of the other cultivars, barrier zone formation was first detected at 10 DPI, which is considerably earlier than was found in previous studies on *Ulmus* species (Bonsen et al., 1985; Rioux & Ouellette, 1991). Rioux and Ouellette (1991) found barrier zones were first detected at 22 DPI in *Ulmus americana*, and the average time to barrier zone formation was

TABLE 5 Frequency (expressed in the number of trees) of barrier zone (BZ) presence and barrier zone characteristics in *Ulmus americana* cultivars inoculated with *Ophiostoma novo-ulmi* at 90 days postinoculation in 2015. Observations were made on sections of the most recent annual ring from the side of inoculation and from the side opposite of inoculation at 30–31 cm above the inoculation site. Images of the sections were cropped to be 0.5 mm wide tangentially with the length including the annual ring radially

		BZ present		End in BZ[a]		BZ breached[b]		BZ Thickness (µm)
Location	Cultivar[c]	Yes	No	Yes	No	Yes	No	Mean ± *SE* (*n*)
Side of inoculation	"Prairie Expedition"	3	0	0	3	0	3	131.2 ± 10.3 (*n* = 3)
	"Brandon"	3	0	1	2	0	2	137.7 ± 41.1 (*n* = 3)
	"Valley Forge"	3	0	1	2	0	2	100.2 ± 32.2 (*n* = 3)
	Wild-type US	2	1	1	1	1	0	254.9 ± 21.8 (*n* = 2)
	Wild-type CA	2	1	1	1	1	0	93.2 ± 8.1 (*n* = 2)
	"New Harmony"	0	3	–	–	–	–	–
	"Princeton"	3	0	3	0	–	–	146.2 ± 43.1 (*n* = 3)
Side opposite of inoculation	"Prairie Expedition"	3	0	0	3	0	3	113.3 ± 57.9 (*n* = 3)
	"Brandon"	3	0	1	2	0	2	129.2 ± 42.1 (*n* = 3)
	"Valley Forge"	1	2	0	1	0	1	95.6 (*n* = 1)
	Wild-type US	2	1	1	1	0	1	303.8 ± 70.6 (*n* = 2)
	Wild-type CA	1	2	1	0	–	–	126.2 (*n* = 1)
	"New Harmony"	1	2	1	0	–	–	106.4 (*n* = 1)
	"Princeton"	2	1	2	0	–	–	106.2 ± 68.1 (*n* = 2)

Notes. [a]End in BZ = End in barrier zone; if a barrier zone was present, did the barrier zone lack typical forming fibre and axial parenchyma cells, which did not appear flattened, directly distal of it. [b]BZ Breached = Barrier zone breached; if a barrier zone was present, were xylem vessels formed directly distally of the barrier zone nonconducting as indicated by a lack of staining with safranin O. [c]Cultivars are ordered from lowest mean disease severity rating at 90 days postinoculation in 2015 to highest.

TABLE 6 Frequency (expressed in the number of trees) of barrier zone (BZ) presence and barrier zone characteristics in *Ulmus americana* cultivars inoculated with *Ophiostoma novo-ulmi* at 90 days postinoculation in 2016. Observations were made on sections of the most recent annual ring from the side of inoculation and from the side opposite of inoculation at 30–31 cm above the inoculation site. Images of the sections were cropped to be 0.5 mm wide tangentially with the length including the annual ring radially

		BZ present			End in BZ 1[a]			BZ 1 breached[b]			BZ 1 thickness (µm)		BZ 2 thickness (µm)	BZ 3 thickness (µm)
Location	Cultivar[c]	Yes	No	Sig.[d]	Yes	No	Sig.	Yes	No	Sig.	Mean ± *SE*	Sig.	Mean ± *SE*	Mean ± *SE*
Side of inoculation	"Valley Forge"	4	0	a	0	4	c	0	4	b	139.0 ± 18.3 (*n* = 4)	a	–	–
	"Prairie Expedition"	5	0	a	0	5	c	4	1	a	179.7 ± 48.5 (*n* = 5)	a	93.9 ± 28.6 (*n* = 3)	–
	"Princeton"	5	0	a	0	5	c	4	1	a	204.1 ± 38.0 (*n* = 5)	a	97.8 ± 20.8 (*n* = 4)	–
	Wild-type US	5	0	a	1	4	bc	0	4	b	429.4 ± 46.6 (*n* = 5)	a	–	–
	Wild-type CA	3	2	a	2	1	abc	0	1	ab	279.1 ± 22.6 (*n* = 3)	a	–	–
	"New Harmony"	5	0	a	4	1	ab	1	0	ab	147.2 ± 28.2 (*n* = 5)	a	–	–
	"Brandon"	4	1	a	4	0	a	–	–	–	186.1 ± 88.6 (*n* = 4)	a	–	–
Opposite side of inoculation	"Valley Forge"	1	3	b	0	1	ab	0	1	a	123.3 (*n* = 1)	–	–	–
	"Prairie Expedition"	5	0	a	0	5	b	2	3	a	80.0 ± 16.9 (*n* = 5)	a	48.7 ± 7.4 (*n* = 2)	225.3 (*n* = 1)
	"Princeton"	5	0	a	1	4	ab	2	2	a	89.8 ± 21.7 (*n* = 5)	a	89.2 ± 27.9 (*n* = 2)	–
	Wild-type US	5	0	a	1	4	ab	0	4	a	337.6 ± 42.2 (*n* = 5)	b	115.0 (*n* = 1)	–
	Wild-type CA	3	2	ab	2	1	ab	0	1	a	225.5 ± 91.0 (*n* = 3)	ab	34.6 (*n* = 1)	–
	"New Harmony"	3	2	ab	3	0	a	–	–	–	82.4 ± 12.4 (*n* = 3)	ab	–	–
	"Brandon"	2	3	ab	2	0	a	–	–	–	258.6 ± 33.3 (*n* = 2)	ab	–	–

Notes. [a]End in BZ 1 = End in barrier zone 1; if a barrier zone was present, did the barrier zone lack typical forming fibre and axial parenchyma cells, which did not appear flattened, directly distal of it. [b]BZ 1 Breached = Barrier zone 1 breached; if a barrier zone was present, were xylem vessels formed directly distally of the barrier zone nonconducting as indicated by a lack of staining with safranin O. [c]Cultivars are ordered from lowest mean disease severity rating at 90 days postinoculation in 2016 to highest. [d]For BZ Present, End in BZ 1 and BZ 1 Breached, groups containing the same letter in a column within the same location are not statistically different according to Fisher's exact test ($\alpha = 0.05$). For barrier zone thickness, groups containing the same letter in a column within the same location are not significantly different according to Dunn's multiple comparison test with a Benjamini and Hochberg *p*-value adjustment ($\alpha = 0.05$).

TABLE 7 Intensity of autofluorescence of unstained sections of *Ulmus americana* cultivars inoculated with *Ophiostoma novo-ulmi* (inoculated) or sterile water (mock-inoculated) viewed under blue light excitation. Transverse sections were made 9–10 cm above the inoculation site from samples collected at 90 days postinoculation in both 2015 and 2016

	Intensity (arbitrary units) (Mean ± *SE*)[a,b]			
	Inoculated		Mock-inoculated	
Cultivar	2015	2016	2015	2016
"Brandon"	20.1 ± 0.9 (*n* = 3) a	30.0 ± 7.0 (*n* = 5) a	9.0 (*n* = 1)	13.4 (*n* = 1)
"New Harmony"	–	22.7 ± 4.1 (*n* = 5) a	–	5.9 (*n* = 1)
"Prairie Expedition"	32.3 ± 5.1 (*n* = 3) a	36.6 ± 5.8 (*n* = 5) ab	10.9 (*n* = 1)	11.0 (*n* = 1)
"Princeton"	19.2 ± 1.2 (*n* = 3) a	40.2 ± 1.8 (*n* = 5) ab	12.9 (*n* = 1)	6.7 (*n* = 1)
"Valley Forge"	32.3 ± 12.1 (*n* = 3) a	65.2 ± 4.0 (*n* = 4) b	16.2 (*n* = 1)	6.6 (*n* = 1)
Wild-type CA	26.7 ± 6.4 (*n* = 3) a	40.3 ± 10.8 (*n* = 5) ab	18.3 (*n* = 1)	6.1 (*n* = 1)
Wild-type US	–	42.7 ± 8.0 (*n* = 5) ab	–	8.4 (*n* = 1)

Notes. [a]For determining mean intensity, images of transverse sections were cropped to 500 × 500 μm, with the bottom of the cropped image stating at 50 μm into the first barrier zone. When a barrier zone was not present, the bottom of the cropped image started at the most recently produced xylem. Some samples in 2015 were too small for a 500 × 500 μm cropping, so the cropping size was reduced for those samples. [b]Groups containing the same letter within a column are not significantly different according to Dunn's multiple comparison test with a Benjamini and Hochberg *p*-value adjustment (α = 0.05).

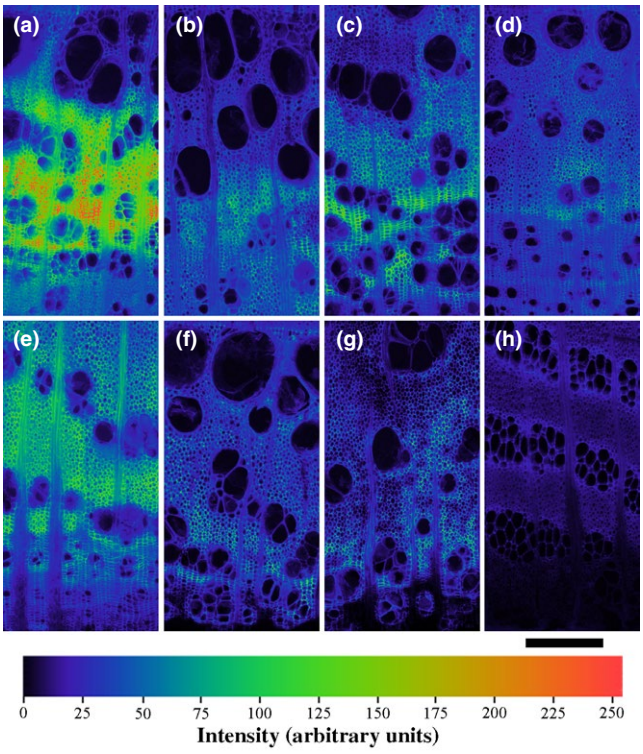

FIGURE 4 Representative images of autofluorescence from inoculated (a-g) and mock-inoculated (h) *Ulmus americana* cultivars at 90 days postinoculation (DPI) under blue light excitation. Samples were from the 2016 trial. For all micrographs, the bottom side of the micrograph was distal of the earlywood vessels. Transverse sections were made 9–10 cm above the inoculation site on the side of inoculation. Cultivars were ordered from lowest mean disease severity rating (a) at 90 DPI to highest (g). (a) "Valley Forge." (b) "Prairie Expedition." (c) "Princeton." (d) Wild-type US. (e) Wild-type CA. (f) "New Harmony." (g) "Brandon." (h) "Valley Forge." Bar = 250 μm

30 DPI. There are potential explanations for the differences between these studies. First, Rioux and Ouellette (1991) examined branches and annual shoots instead of the main stem, which may have contributed to the differences in the timing of barrier zone formation. When Shigo and Tippett (1981) examined small branches of dying American elm trees, no barrier zones were found; however, in trunks and larger branches, barrier zones were more frequently observed. Second, Rioux and Ouellette (1991) do not disclose whether the cultivar is putatively resistant or susceptible. If the speed at which a tree forms barrier zones is truly an effective resistance mechanism, highly susceptible trees would likely form barrier zones slower than resistant cultivars.

While barrier zones have been frequently reported in American elms surviving DED (Banfield, 1968; Buisman, 1935; Et-Touil et al., 2005; Rioux & Ouellette, 1991; Shigo & Tippett, 1981), their presence does not ensure the tree will survive. For 2016, all trees examined for the two cultivars in our study with the highest disease severity rating had barrier zones present and many of the trees were dead at 90 DPI (Table 4). A trend observed in these cultivars was a lack of typical fibre and axial parenchyma cells (cells which did not appear flattened) distally of the barrier zone (Table 6, Figure 3d). This has also been reported in other investigations on elm (Bonsen et al., 1985; Buisman, 1935; Shigo & Tippett, 1981). There are a variety of possible explanations for why the trees died when a barrier zone had been produced; potentially, the simplest explanation is that the fungus was able to advance into the barrier zone and cause cavitation in the remaining vessels resulting in the tree dying due to a lack of water. The study reported here as well as others have observed barrier zones which were breached in *Ulmus* species (Banfield, 1968; Buisman, 1935). In addition, the two cultivars with the highest DSR also had the lowest mean intensities of autofluorescence under blue light excitation, suggesting reduced phenolic

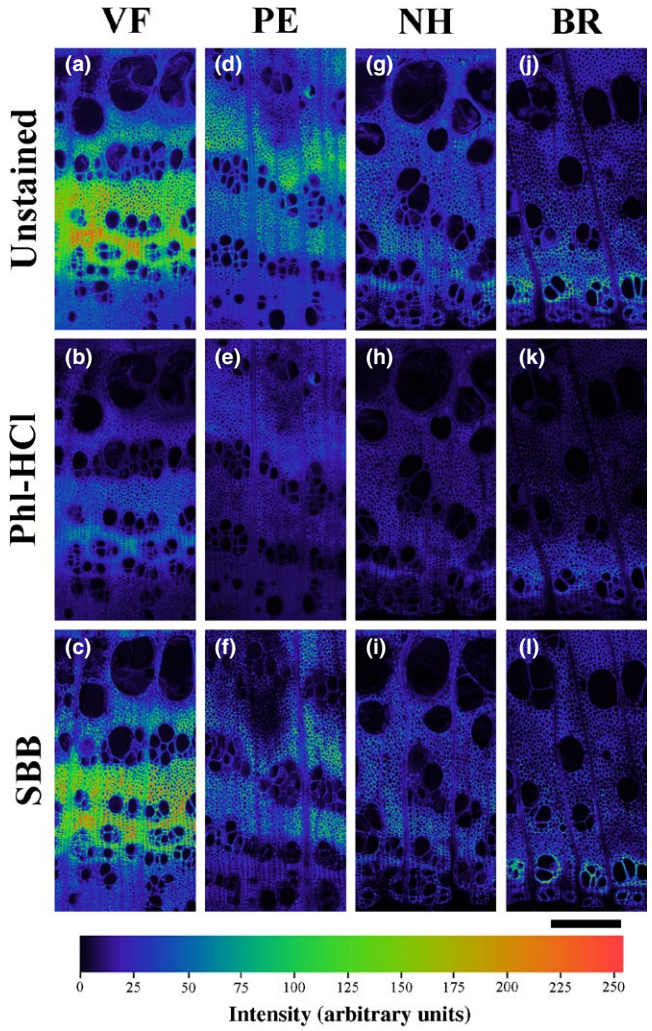

FIGURE 5 Representative images of autofluorescence of inoculated *Ulmus americana* cultivars at 90 days postinoculation (DPI) under blue light excitation using different staining methods. Samples were from the 2016 trial. For all micrographs, the bottom side of the micrograph was distal of the earlywood vessels. Transverse sections were made 9–10 cm above the inoculation site on the side of inoculation. The two cultivars with the lowest mean disease severity rating at 90 DPI ("Valley Forge" [VF] and "Prairie Expedition" [PE]) and the two cultivars with the highest mean disease severity rating ("New Harmony" [NH] and "Brandon" [BR]) are represented. Sections were either left unstained, stained with Phloroglucinol-HCl (Phl-HCl), or stained with Sudan Black B (SBB). (a–c) "Valley Forge." (d–f) "Prairie Expedition." (g–i) "New Harmony." (j–l) "Brandon." Bar = 250 μm

accumulation compared with the other cultivars (Table 7). A reduced phenolic content may have allowed for easier colonization by the pathogen. Moore (1978) demonstrated that extracts from barrier zones found in sweetgum trees (*Liquidambar styraciflua* L.) were capable of inhibiting fungal growth. Another potential explanation is that new tissue could not be generated quickly enough to maintain adequate sap transport. While a majority of samples examined had some xylem vessels conducting within the barrier zone, many did not. In addition, when examining the timing of barrier zone formation, it was often noted that xylem vessels formed in the barrier zone were not immediately conducting (data not shown). No samples were examined from below the inoculation site on the main stem. It is possible that there may have been areas lacking barrier zones which were entirely colonized and had no remaining conducting tissue, which would render all tissue above it nonconducting.

Some cultivars did have continuous barrier zones around the entire circumference of the stem in 2016; however, the two cultivars with the lowest disease severity rating, "Valley Forge" and "Prairie Expedition" had no barrier zones that were completely continuous (Table 4). When examining branches of American elm, Rioux and Ouellette (1991) reported that barrier zones were rarely continuous around the entire stem. "Valley Forge" generally displayed an effective reaction zone and barrier zone, which allowed for sap-conducting xylem to be maintained around much of the stem (Beier et al., 2017). The per cent of sap-conducting area in the most recent annual ring was at least three times larger in "Valley Forge" compared with all other cultivars in 2016 at 90 DPI (Beier et al., 2017). In three of the four samples of "Valley Forge" examined in 2016 at 90 DPI, barrier zones were only present around approximately 20%–30% of the circumference of the stem (data not shown). While "Prairie Expedition" did not effectively limit tangential spread, when the most recent growth of the annual ring was examined, it had 100% of the circumference conducting (Beier et al., 2017). Barrier zones in "Prairie Expedition" were generally found around the entire stem, but they were not continuous. In an interesting manner, in many of the samples examined in 2016 for "Prairie Expedition," barrier zones were present which had been breached, as was evident by the lack of conducting tissue formed directly after it. Often a new barrier zone was formed (Table 6), which maintained sap conduction in the newly developed xylem (Figure 2a). The formation of multiple barrier zones within the same year has been observed in other pathosystems (Blanchette, 1982). In 2016, only wild-type US had barrier zones continuous around the entire circumference of the stem for all samples examined. In addition, these barrier zones were considerably thicker than that of "Prairie Expedition" or "Valley Forge." In a study on different hardwood species and Douglas-fir, Deflorio et al. (2009) found barrier zone thickness was highly affected by the genotype inoculated. When examining the side where the inoculation was made and the side opposite of inoculation on the wild-type US trees in 2016, there were no instances when the barrier zone was breached. However, when the entire ring was assessed, there were two instances when the barrier zone was breached (data not shown). Despite having a very effective barrier zone, wild-type US had a slightly higher disease severity rating than "Prairie Expedition," although it was not significant ($p > 0.05$). The formation of barrier zones is believed to be a very energy intensive process (Tippett & Shigo, 1981). This energy cost likely came at the expense of new foliar growth. By producing a reflush of new growth, the proportion of the crown showing symptoms would have been reduced, resulting in a lower disease severity rating. It is unclear whether "Prairie Expedition" would have been more likely to have crossing over of the pathogen in the next year

as compared with the wild-type US. More studies are necessary to determine the long-term effectiveness of these different strategies of compartmentalization.

An accumulation of phenolic compounds has been associated with barrier zones in many tree species (Pearce & Rutherford, 1981; Pearce & Woodward, 1986; Rioux & Ouellette, 1991). When examining unstained barrier zones under blue light excitation in inoculated cultivars, there was often an increase in the amount of autofluorescence compared to the control samples. These areas of heightened autofluorescence were indicative of phenolics. These findings support those of previous studies in *Ulmus* species, where inoculated trees showed greater amounts of phenolics compared with controls (Martin, Solla, Woodward & Gil, 2005; Rioux & Ouellette, 1991). In addition, there was often a diffuse pattern of autofluorescence extending inward from the start of the barrier zone. This was also observed by Rioux and Ouellette (1991) when they examined American elm infected with *Ophiostoma ulmi*. Areas specific to suberin have also been associated with barrier zones (Pearce & Rutherford, 1981). In histochemical studies on *Ulmus*, areas indicative of suberin have been found in some barrier zones (Et-Touil et al., 2005; Rioux & Ouellette, 1991). When examining autofluorescence of sections treated with Phl-HCl, which quenches the fluorescence of lignin, but not suberin (Biggs, 1984), it was determined that autofluorescence was quite faint in most samples examined (Figure 5b,e,h,k). This was supported by the lack of a reduction in autofluorescence for sections treated with SBB (Figure 5c,f,i,l). In an interesting manner, Et-Touil et al. (2005) found that the aggressiveness of the *Ophiostoma novo-ulmi* isolate used influenced the amount of suberin detected in inoculated hybrid elms. When the more virulent isolate was used, no suberized areas were observed (Et-Touil et al., 2005). Martin, Solla, Domingues, Coimbra and Gil (2008) found that *Ulmus minor* trees treated with exogenous phenol had increased amounts of suberin in twig samples compared with controls. They suggested that this increase in suberin may have been one of the factors contributing to the reduced foliar symptoms following inoculation with *O. novo-ulmi* compared to the controls (Martin et al., 2008). In 2016, "Valley Forge" most consistently displayed areas indicative of suberin (Figure 5b). This increased amount of suberin may have aided in the effectiveness of barrier zones, not allowing them to be breached by the fungus. In a study on oak, Pearce and Rutherford (1981) found suberized areas of the barrier zone were the only areas not exhibiting extensive degradation by the decay fungus, *Stereum gausapatum* (Fr.) Fr., in in vitro examinations.

All cultivars, except for "Brandon," had lower DSR in 2016 (4-year-old plant material) compared with 2015 (3-year-old plant material) (Table 2). These findings are contrary to the findings of Solla, Martin, Ouellette and Gil (2005) in *Ulmus minor*, where 3-year-old trees showed significantly less foliar symptoms following inoculation compared with 4-year-old trees. When all cultivars were considered, there was a smaller proportion of samples with barrier zones present and a higher proportion of annual rings ending in barrier zones at 90 DPI in 2015 compared with 2016 (Tables 4–6). In addition, in the 2015 trial, the intensity of autofluorescence observed under blue light excitation for inoculated trees was less for all cultivars examined compared with 2016 (Table 7). A potential explanation for the differences between the years for the variables measured may be related to available energy reserves. Transplanting can result in significantly reduced growth (Watson, 1987, 2005; Watson, Himelick & Smiley, 1986), which could greatly reduce energy reserves. As discussed previously, barrier zone formation is believed to be a very energy intensive process (Tippett & Shigo, 1981). Smaller stem sizes in 2015 may have also contributed to the differences between the two trials. In 2015, the decrease in conductive tissue around the circumference of the stem was quite rapid (Beier et al., 2017). In larger stems, it would likely take longer for the pathogen to colonize the entire stem, which would allow for a longer period of time for barrier zones to form before conduction around the entire stem ceases. In a study by Bonsen et al. (1985), it was observed that when American elm was inoculated with an aggressive strain of *Ophiostoma ulmi*, small branches lacked barrier zones, while main stems developed barrier zones. When investigating the timing of barrier zone formation, it was shown that "New Harmony" did not produce barrier zones at the same rate as other cultivars (Table 3). The size of the stems may help explain why no barrier zones were observed on the side of inoculation for inoculated "New Harmony" in 2015, while in 2016, all samples examined had barrier zones on the side of wounding (Tables 5 & 6). In addition, the number of annual rings may have played a role in the difference in DSR. In a host study on *Verticillium nonalfalfae* by Kasson, O'Neal and Davis (2015), they speculated that the number of tree rings which are actively transporting sap may have influenced resistance to the disease between different species. All of the mock-inoculated trees in 2016 at 90 DPI had at least some conducting tissue, as indicated by the safranin O staining, in three annual rings or more (data not shown). Other factors may have been contributed to the differences in DSR observed between 2015 and 2016. While the timing of inoculation was very similar in 2015 and 2016, there are differences in weather conditions between years, which can affect development of the tree. Numerous studies have shown that the timing of inoculation can have an impact on symptom development in *Ulmus* inoculated with *O. novo-ulmi* or *O. ulmi* (Pomerleau, 1965; Smalley, 1963; Smalley & Guries, 1993; Smalley & Kais, 1966; Smalley & Lester, 1983; Takai & Kondo, 1979). In addition, environmental factors such as water availability (Solla & Gil, 2002b), amount of light and temperature (Sutherland, Pearson & Braiser, 1997) have been shown to influence disease expression in elm.

One potential limitation to these studies is that the inoculations were carried out artificially into the main stem. Some scientists have used branch inoculations to simulate bark beetle feeding (Ouellette, 1962; Smalley & Kais, 1966; Smalley & Lester, 1983), which is the primary method the pathogen is transmitted aboveground. Takai, Kondo and Thomas (1979) caged naturally contaminated bark beetles around the stem of *Ulmus americana* to infect the trees with *Ophiostoma ulmi*. Due to the small size of the trees and the variability in branch sizes, the main stem was selected to maintain consistency. In a study on *U. americana*, Smalley and Kais (1966) found that 10–20

inch dbh trees showed greater foliar symptoms when inoculated in the main stem compared with 1-year-old branches from the upper crown. Rioux and Ouellette (1991) noted that vessel occlusion was more frequently observed in artificial inoculations compared with naturally infected trees. In addition, barrier zones were found more frequently over occluded vessels in naturally infected trees compared with artificially inoculated trees (Rioux and Ouellette (1991). It would be advantageous in future experiments to also examine trees of these cultivars which were naturally infected with *Ophiostoma novo-ulmi* to determine histological and histochemical differences between natural and artificial inoculation.

Resistance to DED is highly complex and while evidence is presented that factors involving compartmentalization are likely critical in determining disease resistance, there are a variety of other host factors also involved. Certain anatomical characteristics have been found to be associated with resistant trees, such as smaller vessel diameters (Elgersma, 1970; McNabb et al., 1970; Pope, 1943; Sinclair et al., 1975a; Venturas et al., 2014). It is speculated that smaller vessels can be occluded more rapidly with tyloses, gums, and/or gels than larger vessels, which would help slow the spread of the pathogen (Elgersma, 1970; McNabb et al., 1970; Sinclair et al., 1975b; Solla & Gil, 2002a). While the formation of vessel occlusions is a part of the reaction zone during compartmentalization, differences in vessel occlusions, such as tylose formation, were not investigated. Rapid formation of tyloses has been associated with resistance in *Ulmus* x *hollandica* Mill. (Elgersma, 1973). It has also been shown in other vascular wilt pathosystems that faster coating of vessel walls and/or formation of vascular inclusions are associated with more resistant genotypes (Beckman, Elgersma & MacHardy, 1972; Shi, Mueller & Beckman, 1992). Ploidy level could also be affecting resistance. While most American elms are tetraploid, diploid and triploid specimens have been identified (Whittemore & Olsen, 2011). In a study examining the triploid American elm "Jefferson," Sherald, Santamour, Hajela, Hajela and Sticklen (1994) found "Jefferson" had higher levels of resistance to DED compared with wild-type American elms. Ploidy levels have been shown to have an impact on xylem characteristics in other plant species (Hao, Lucero, Sanderson, Zacharias & Holbrook, 2013; Maherali, Walden & Husband, 2009), which could help explain the differences in resistance. Pathogenesis-related proteins may also be playing a role in resistance to DED. When comparing gene expression in susceptible and resistant elms, Sherif, Shukla, Murch, Bernier and Saxena (2016) found significantly higher expression of both PR4 and PR5b at 96 hr postinoculation in the resistant genotype.

In summary, this work identified different factors relating to compartmentalization that appear to make up a potential component of resistance to DED. While some cultivars we studied exhibited different strategies of compartmentalization, allowing them to survive during the year of inoculation, it is unclear whether these strategies are sustainable over time. Older plant material of these cultivars should be examined to determine whether trends found in young trees are similar to those in older material. In addition, multiple year studies should be conducted to determine the extent of the pathogen crossing over into the new annual growth ring. If it is determined that the different strategies utilized by these cultivars do not result in crossing over of the pathogen, it could have significant implications on disease management. Instead of removing infected trees in the landscape at the first symptoms of DED, certain cultivars could be left, because of their ability to compartmentalize infection. If particular strategies are not effective at limiting the spread of infection to new growth in subsequent years, scientists working on breeding and selection could use histological examination to determine which genotypes should be advanced. Information from this study provides a framework for other genotypes to be evaluated for their ability to successfully compartmentalize DED infections.

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