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FISH Illumination of the Oak Wilt Pathogen, *Bretziella fagacearum*, on Vectoring Insects and in the Red Oak Host Tissues

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ABSTRACT

Oak wilt, caused by *Bretziella fagacearum*, is a destructive vascular disease of oaks in North America, yet fine-scale spatial localisation of the pathogen in host tissues and on insect vectors remains poorly characterised. In this study, we developed and validated a species-specific fluorescence in situ hybridisation (FISH) probe targeting the 28S rDNA of *B. fagacearum* and evaluated its capacity to detect and localise the pathogen in naturally infected red oak (*Quercus rubra*) tissues and on sap beetle vectors (Nitidulidae). Probe specificity was confirmed through fluorescence in situ hybridisation (FISH) and confocal laser scanning microscopy (CLSM), which showed strong hybridisation to *B. fagacearum* and no detectable signal in closely related Ceratocystidaceae. FISH successfully visualised ascospores on *Carpophilus sayi* collected from sporulating mats, with consistent localisation on terminal abdominal sternites and occasionally on mesoventral grooves, legs and antennae. Within oak twig tissues, FISH detected *B. fagacearum* hyphae predominantly in vessel elements, fibres, and occasionally in axial and ray parenchyma. In infected petioles, hyphae occurred in xylem vessels, collenchyma, and parenchyma, demonstrating pathogen presence beyond midrib tissues. Overall, this study establishes CLSM–FISH as a powerful visualisation tool for investigating the host–pathogen–vector interactions of the oak wilt disease.

1 | Introduction

Oak wilt, a devastating vascular disease of oak trees (*Quercus* spp.), is caused by the fungal pathogen *Bretziella fagacearum* [Bretz] (formerly *Chalara quercina* [B.W. Henry], *Endoconidiophora fagacearum* [*Bretz*], *Thielaviopsis quercina* [(B.W. Henry) A.E. Paulin, T.C. Harr. & McNew] and *Ceratocystis fagacearum* [(Bretz) J. Hunt]). Since its first identification in the early 20th century in the United States, oak wilt has emerged as one of the most destructive tree diseases in North America, leading to widespread mortality in both red and white oak groups. The pathogen invades the xylem vessels, leading to rapid wilting, leaf discoloration, vascular dysfunction, and ultimately, tree death often within weeks in susceptible species (Henry et al. 1944; Henry and Riker 1947; Chahal et al. 2026).

Despite decades of research on the epidemiology and management of oak wilt (Juzwik et al. 2011), the fine-scale anatomical and cytological interactions between *B. fagacearum* and oak tissues remain incompletely understood. Previous studies have largely focused on macroscopic symptoms, disease spread mechanisms, and host resistance responses, whereas comparatively few have investigated the pathogen's behaviour

[Correction added on 6 April, after first online publication: The middle name of the author Karandeep Singh Chahal has been removed, and the name has been corrected to Karandeep Chahal.]

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and host responses at the microscopic level. Being able to precisely localise the pathogen within the host tissues and on the structures of the insect vectors would help to elucidate the pathogen–vector–host interactions, which may in turn inform more effective diagnostic, resistance breeding and management strategies.

Earlier studies using isolation and histological observations have shown that *B. fagacearum* colonises petioles, twigs and branches primarily through xylem vessels (Henry et al. 1944; Henry and Riker 1947; Young 1949). The pathogen can move radially via ray and axial parenchyma cells (Struckmeyer et al. 1958; Wilson 1961) and penetrate several annual rings in the sapwood (Jones and Bretz 1955). Cell wall erosion and cavity formation consistent with carbohydrase enzymes' activities have been reported (Sachs et al. 1970). In red oaks, extensive vascular anastomoses and small conidia facilitate rapid systemic longitudinal spread, whereas in white oaks, tyloses and gums appear to limit fungal dispersion within the host tree (Jacobi and MacDonald 1980).

Localised dispersal is mainly a result of underground pathogen movement through interconnected roots that have formed natural root grafts. Vectoring insects are responsible for long distance overland dispersion of the disease. Long distance dispersal also occurs through the movement of *B. fagacearum* killed firewood. *B. fagacearum* completes its life cycle by colonising the wood of recently killed host trees and forming reproductive structures in the form of fragrant mycelial mats (Chahal et al. 2026). These mats grow just below the bark, and upon maturity cause the bark to crack. Volatiles emitted by the mats at maturity attract a variety of insects. Native insects, in particular nitidulid beetles, will crawl and feed on the mat; the sticky spores of *B. fagacearum* will attach to the insects. New hosts bearing fresh wounds are attractive to sap feeding insects and may become infected when *B. fagacearum* infected insects visit the fresh wounds (Yount et al. 1955; Juzwik et al. 1998; Morris et al. 2024).

Although these major studies have provided fundamental knowledge of how *B. fagacearum* invades and colonises host tissues, most used conventional methods that offer limited spatial resolution and specificity. Fluorescence in situ hybridisation (FISH) is a molecular technique that uses fluorescent labelled probes to detect specific nucleic acid sequences directly within intact cells, enabling spatially resolved identification without cultivation. The first documented application of FISH to a living fungus was reported by Li et al. (1996), who visualised *Aureobasidium pullulans* on apple seedling using fluorescence microscopy. Since then, imaging approaches have evolved with confocal laser scanning microscopy (CLSM) coupled with FISH now enabling high-resolution, three-dimensional visualisation of microorganisms in their native environments. For example, Cardinale et al. (2018) applied CLSM-FISH to detect *Xylella fastidiosa* within xylem vessels of olives trees, revealing biofilm formation and occlusion of vascular tissues. Although fluorescent proteins allow live-cell imaging and tracking pathogen invasion in a unique specimen over time, this strategy relies on transfection of the gene encoding a fluorescent protein to cells of the microorganism of interest.

Given that no genetic transformation system is available yet for *B. fagacearum*, the use of CLSM–FISH was investigated in the present study. More specifically, we aimed to (1) develop and validate FISH probes for specific detection of *B. fagacearum* and (2) determine if this assay could be used to localise *B. fagacearum* within the host tissues and on the insect vectors.

2 | Materials and Methods

2.1 | Sampling Sites and Biological Material

Sap beetles were collected from sporulating mats on diseased oak trees in Michigan during May and June 2019 (Chahal 2024). The Nitidulidae species included in this study were *Epuraea corticina*, *Carpophilus sayi*, *Coleopterus semitectus* and *Glischrochilus sanguinolentus*, all recognised as vectors of *B. fagacearum* (Morris et al. 2024). As negative controls, *C. sayi* specimens were collected approximately 500 km away from any oak wilt infection center at Valero Les Écarts Park, Lévis, Québec (46°46′19.15″ N, 71°10′32.44″ W). These control insects were captured in 99.9% propylene glycol in May 2022 using Lindgren funnel traps baited with Trece aggregation pheromone lures (Distributions Solida, product no. 3013041) and bread dough.

Twigs and petioles from healthy and wilted oak trees were collected from seven locations in the northwest Lower Peninsula of Michigan during 2023 and 2024. Fifty symptomatic twig sections (1 cm long, 0.5–0.6 cm in diameter) were sampled from ten infected red oak trees (five twigs per tree), and twenty asymptomatic twig sections were collected from ten healthy red oak trees (two twigs per tree) (Chahal et al. 2026). Additionally, thirty petiole sections (1 cm long from the point of attachment) were obtained from symptomatic leaves of six infected red oak trees (five leaves per tree), and sixteen petiole sections were collected from asymptomatic leaves of eight healthy red oak trees (two leaves per tree) (Chahal et al. 2025).

The *B. fagacearum* isolate (BF220) originated from the Forest Pathology culture collection at Michigan State University (MSU). *Ophiostoma novo-ulmi* isolate (CR2023) and *Endoconidiophora resinifera* isolate (CFL2206) were obtained from the Laurentian Forestry Centre (LFC) culture collection. All isolates were maintained on 2% Difco malt extract agar. The *B. fagacearum* isolate was cultured at MSU, and agar plugs were fixed in Carnoy's solution, transferred to microtubes containing 100% ethanol, and shipped to the LFC for microscopic examination.

2.2 | Processing of Samples for Microscopic Observations

Insects, twig and petiole sections, as well as fungal mycelium plugs, were fixed overnight at 4°C in Carnoy's solution (chloroform:ethanol:glacial acetic acid, 6:3:1, v/v). Following fixation, samples were washed twice and preserved in absolute ethanol at room temperature until further processing. Insect specimens underwent a decolorisation step to reduce cuticular

autofluorescence and improve visualisation of fluorescent probes. Fixed insects were incubated in a 6% alcoholic hydrogen peroxide solution for 2 weeks, with the solution replaced every 3 days, as described by Koga et al. (2009). After bleaching, insects were thoroughly rinsed and stored in absolute ethanol at room temperature until use.

Twig sections approximately 80 μm thick were prepared using a sliding microtome (WSL Lab-Microtome, Swiss Federal Research Institute, Birmensdorf, Switzerland). Three serial sections were collected from each sample: one designated for qPCR analysis and two for microscopy. To minimise lignin-related autofluorescence and remove extractives that could interfere with imaging, sections were cleared using the ClearSee protocol developed by Ursache et al. (2018). The ClearSee solution consisted of 10% xylitol, 15% sodium deoxycholate, and 25% urea in water. Sections were placed in multiwell plates (Falcon 24-well Clear Flat Bottom) containing 1 mL of ClearSee solution, which was replaced every 2 days for 7 days. After clearing, sections were preserved in phosphate-buffered saline pH 7.4 (PBS) and stored at 4°C until use.

Petiole sections approximately 100–150 μm thick were prepared manually using a razor blade. Three serial sections were obtained from each petiole: one for qPCR analysis and two for microscopy. Sections designated for microscopy were processed immediately, whereas those intended for qPCR were stored at −20°C until DNA extraction.

2.3 | Fluorescence In Situ Hybridisation

Twig and petiole sections were pre-hybridised in 2 mL Eppendorf Safe-Lock microtubes for 10–30 min at 32°C on a nutator shaker set at 60 rpm in a buffer containing 20 mM Tris–HCl (pH 8.0), 0.9 M NaCl, and 0.01% sodium dodecyl sulphate. After pre-hybridisation, the buffer was discarded and replaced with a hybridisation solution composed of 20 mM Tris–HCl (pH 8.0), 0.9 M NaCl, 0.01% sodium dodecyl sulphate, and 30% formamide (Kliot et al. 2014), supplemented with 100 nM of the *B. fagacearum*-specific probe (Bfag 28S: 5′-/5ATTO647NN/ACT CGG GAC TAC AAA AGT AGC C-3′). In some specimens, the universal fungal probe PF1200 (Grünwald et al. 2010) (5′-/5ATTO488N/CTG GAC CTG GTG AGT TTC CC-3′) was also included at a concentration of 100 nM. Hybridisation was carried out overnight in the dark at 32°C on a nutator shaker operating at 60 rpm. Control samples underwent the same procedure using either water in place of the probe to assess background signal or a non-sense probe (Bfag 28S DECOY: 5′-5ATTO425N/CCG ATG AAA ACA TCA GGG CTC A-3′).

Following hybridisation, samples were washed in probe-free hybridisation buffer at 42°C for 30 min on a nutator shaker at 60 rpm, with two buffer changes during the wash. Twig and petiole sections were then mounted on microscopic slides using 2.5% 1,4-diazabicyclo-[2,2,2]-octane (DABCO; Johnson et al. 1982) antifading mounting medium and prepared for imaging. Dry insect samples were mounted on slides and stabilised under a cover glass fixed with dental wax. All samples were stored in the dark at 4°C until confocal microscopy observations.

2.4 | Microscopic Observations

CLSM observations were performed using an inverted IX83 confocal microscope coupled to a Fluoview FV1200 laser scanning unit (Olympus Corporation, Tokyo, Japan), equipped with UPLSAPO objectives of 4×/0.16, 10×2/0.4, 20×/0.75 and 30×/1.05 silicone oil immersion. Hybridised sections and control sections were imaged under identical acquisition parameters, and multiple fields of view were examined systematically for each sample to ensure representative observations. For preliminary validation of the PF1200 probe conjugated to Atto488, sections were excited using a 488 nm argon ion laser. For imaging mycelium hybridised with the Bfag 28S DECOY probe, excitation was performed using a 405 nm blue diode laser, with emission collected using the SDM 490 filter. For counterstaining of oak tissue and insects hybridised with the *B. fagacearum* Atto647N probe, a 559 nm green-yellow diode and a 635 nm red diode laser were employed. A DM405/488/559/635 dichroic mirror was used in combination with emission filters SDM560 for 488 nm and SDM640 for 559 nm. For 635 nm excitation, emissions were detected within the BA655–755 range, as no specific bandpass filter was available.

Image acquisition was carried out using the Olympus FV1200 system, and image processing was performed with Olympus cellSens Dimension software. Extended Focal Imaging was applied to generate two-dimensional images by combining focal planes from Z-stacks, filtering out-of-focus structures, and enhancing sharpness. Deconvolution processing with cellSens Dimension software was used to improve resolution and contrast. Final micrograph editing and optimisation were completed using Adobe Photoshop Elements 11 (Adobe Systems, San Jose, CA, USA).

2.5 | DNA Extraction and qPCR Assays

Mycelium from fungal cultures, whole insects previously processed and observed by CLSM–FISH, as well as twig and petiole sections, were transferred to 2 mL Eppendorf Safe-Lock microtubes containing a tungsten bead and disrupted in a MixerMill at 26 Hz for two 1-min cycles. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and eluted in 50 μL of 10 mmol/L Tris–HCl (pH 8.0). Prior to extraction, all samples were spiked with 1 μL of 10 pg. lambda bacteriophage DNA (NEB, Ipswich, MA, USA) as an internal control. Detection assays for *B. fagacearum* and the lambda internal standard were performed in duplex using oligonucleotides, reagents, and thermocycling conditions described by Bourgault et al. (2022) and Rutledge and Stewart (2008), with the Bfag and CAL probes conjugated to FAM and Cy5 fluorophores, respectively. Positive qPCR detection of *B. fagacearum* was defined as all technical replicates of a biological sample exhibiting a C_t value below 35.17, corresponding to two copies of the target DNA, which represents the LOD95% determined experimentally in the study describing the *B. fagacearum* qPCR assay (Bourgault et al. 2022). To resolve PCR enzymatic inhibition observed in four samples where qPCR results of the lambda DNA internal extraction and amplification control was undetermined, qPCR was repeated using a fivefold dilution of the extracted DNA. The C_t values of the Bfag and CAL qPCR assays for each sample examined in this study are presented in Table S1.

3 | Results

3.1 | Design and Validation of FISH Probes

The Bfag 28S FISH probe was designed to target a region within the *B. fagacearum* 28S rDNA sequence that contains single nucleotide polymorphisms (SNPs) distinguishing it from closely related species. Specifically, the probe binds to a segment featuring at least two SNPs that differentiate *B. fagacearum* from the nearest known Ceratocystidaceae species (de Beer et al. 2017; Figure S1). Consistent with the in silico design, the Bfag 28S probe demonstrated specific hybridisation to *B. fagacearum* cells (Figure 1). No fluorescence signal was detected when the probe was applied to other fungal species including the closely related *E. resinifera*. In contrast, the panfungal FISH probe PF1200 (Grünwald et al. 2010) successfully hybridised with all tested fungi and co-localised with the Bfag 28S probe in *B. fagacearum* cells.

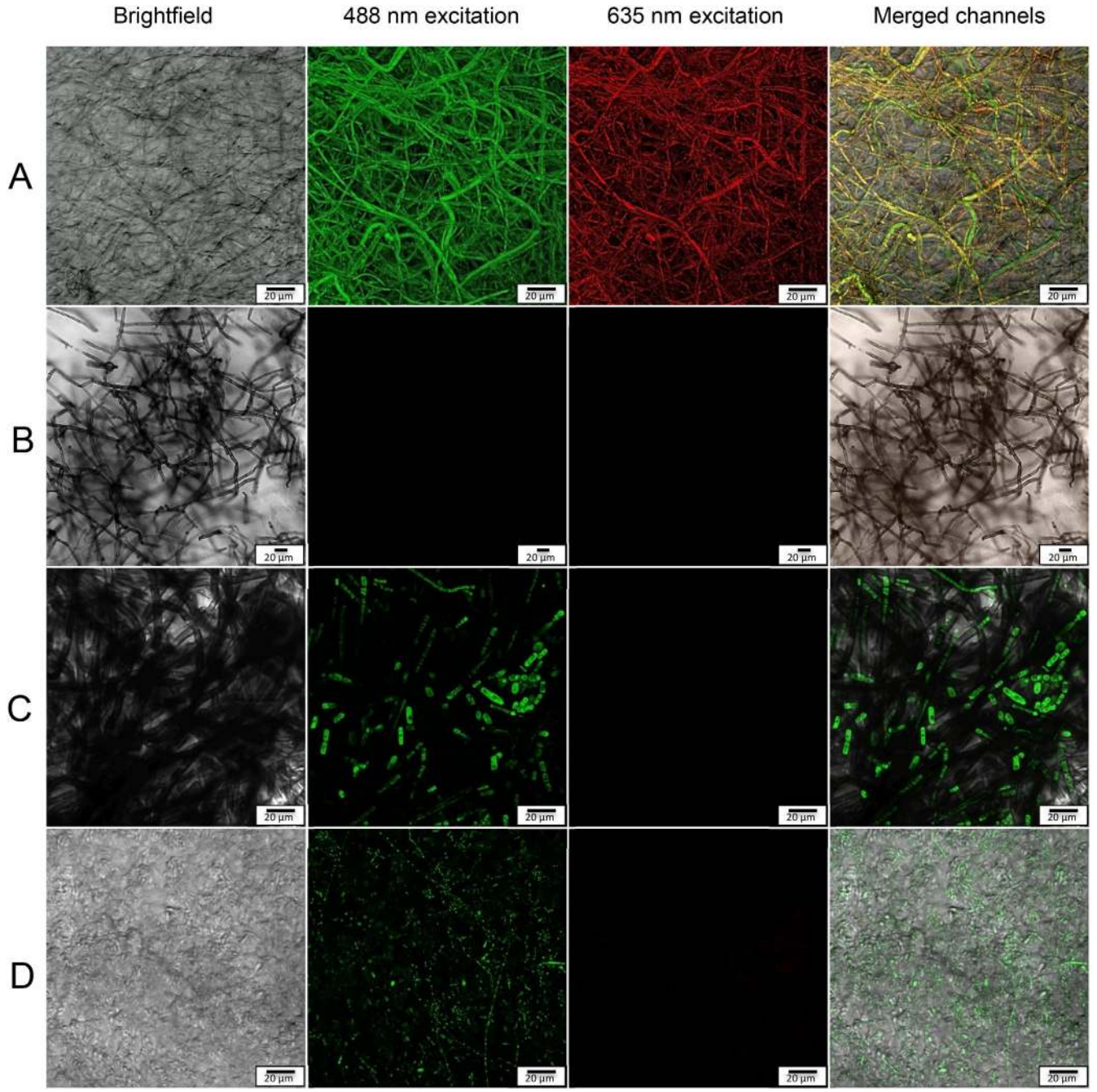


FIGURE 1 | Specificity of the *Bretziella fagacearum* Bfag 28S FISH probe. CLSM images. (A) *Bretziella fagacearum* BF220 mycelium hybridised with the universal fungal probe PF1200 conjugated with Atto488 nm and the *B. fagacearum* specific (Bfag 28S) probes conjugated with Atto647N nm. (B) *Bretziella fagacearum* BF220 mycelium unlabelled (negative control). (C) *Endoconidiophora resinifera* CFL2206 mycelium hybridised with PF1200 and Bfag 28S probes. (D) *Ophiostoma novo-ulmi* CR2023 mycelium hybridised with PF1200 and Bfag 28S probes. The last column represents a fusion of the brightfield (column 1), 488 nm excitation (column 2) and the 635 nm excitation (column 3) channels.

3.2 | Detection on Sap Beetles

The FISH technique revealed the presence of *B. fagacearum* on the exoskeleton of sap beetles collected directly from fungal mats in Michigan. Among the species examined, the highest concentration of ellipsoidal ascospores was observed on the terminal abdominal sternite in the ventral view of *C. sayi* (Figure 2A–D). Additional spores were detected on the hairy tarsal setae of the second pair of legs and within the adjacent mesoventral grooves (Figure 2E), covering these surface interstices. No FISH signal was detected in

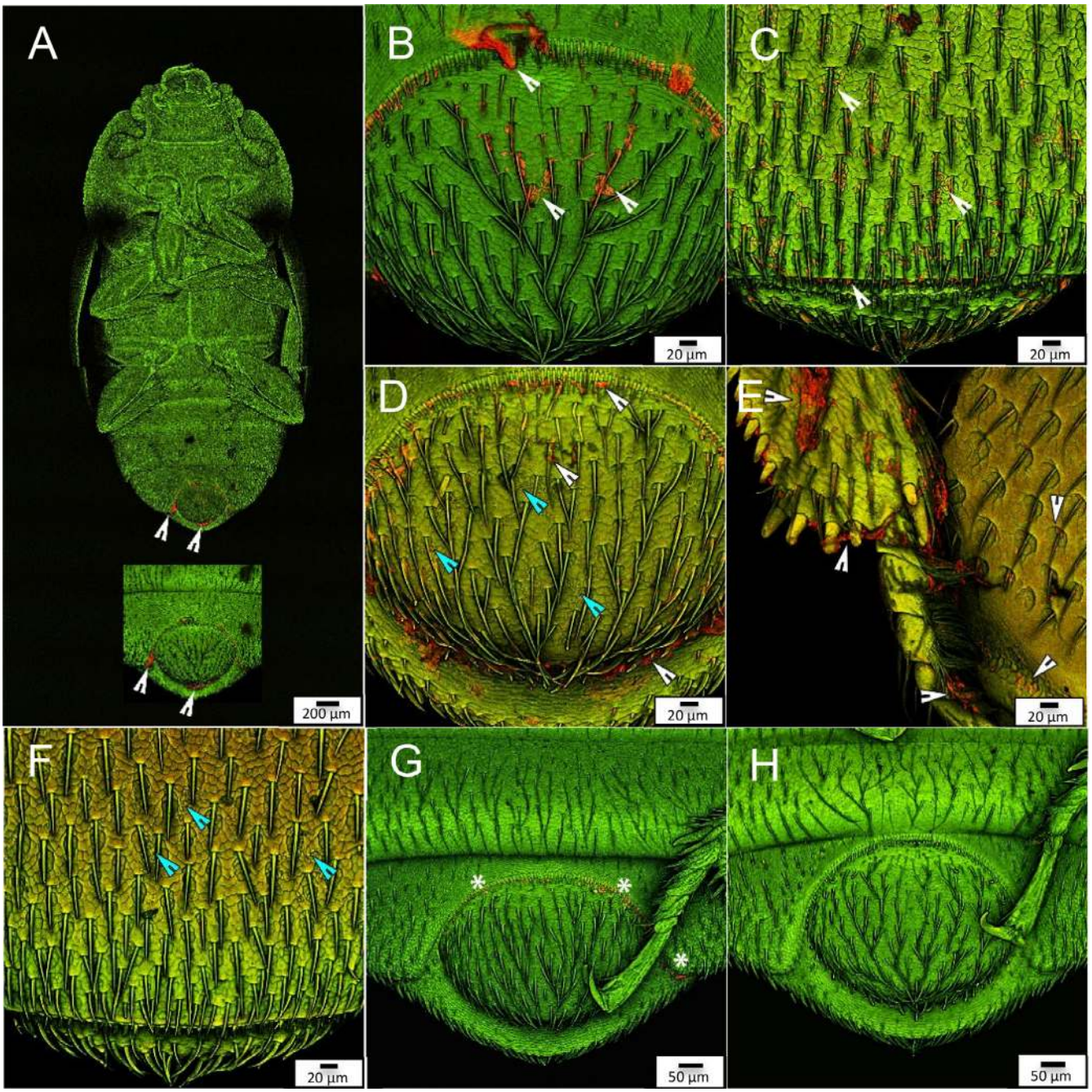


FIGURE 2 | CLSM micrographs showing specificity of FISH signal in *Carpophilus sayi*. (A) Whole male specimen (ventral view; stitched from two fields) from Michigan site showing strong red FISH localised sternite signal. Inset: Closer view of the sternite. (B) Same specimen as in (A), shown at higher magnification with clearly visible ascospores. (C) Female V-shaped sternite with visible abdominal cavities and hybridisation red signal. (D) Male sternite showing both hybridised ascospores in red (confirmed by FISH) and non-hybridised faintly green spore-like structures (morphologically similar with *Bretziella fagacearum* spores but not confirmed; ton-sur-ton with the sternite). (E) Hairy tarsal setae and mesoventral groove of female with localised red fluorescence signal from clustered ascospores. (F) Infected control (from fungal mat, no probe), showing no fluorescence, but with morphological distinguishable faintly green spore-like structures (non-hybridised). (G) Québec specimen (negative control) showing localised sternite fluorescence (false positive). (H) Same Québec specimen as in (G) after supplementary washes, showing complete loss of red fluorescence signal, confirming that the initial signal represented non-specific binding. White arrowheads indicate hybridised ascospores (red signal); turquoise arrowheads indicate non-hybridised ascospores; white asterisks indicate false signals.

E. corticina, *C. semitectus* or *G. sanguinolentus*. For each species, two specimens were examined—one hybridised with the Bfag 28S probe and one processed as a no-probe control—and all hybridised specimens were FISH negative. qPCR analysis indicated that *G. sanguinolentus* and *E. corticina* were both negative, whereas in *C. semitectus*, the no-probe control tested positive by qPCR while the hybridised specimen was negative.

In *C. sayi*, qPCR detected *B. fagacearum* in all 13 specimens hybridised with the Bfag 28S probe (Table S1). Among these, 10 individuals (five females and five males) exhibited clear FISH signals associated with fungal structures in the terminal abdominal regions, particularly the discoidal sternite in males (Figure 2A,B,D) and the V-shaped region near the ovipositor in females (Figure 2C). In one specimen, isolated FISH signals were also observed on the antennae and thoracic segments. No FISH signal was detected in control beetles, including infected individuals processed without probe and negative controls collected in Québec (Figure 2F–H), where all specimens tested negative for *B. fagacearum* by qPCR. Two Québec control specimens initially showed non-specific fluorescence with the Bfag 28S probe, but this signal disappeared after additional rinsing, confirming that it did not represent true hybridisation (Figure 2G).

3.3 | Detection in Red Oak Tissues

In a subset of 10 symptomatic twigs analysed, fungal structures were detected in all samples and consistently colocalized with a positive FISH signal for *B. fagacearum* (10/10), supporting the interpretation of specific hybridisation despite background fluorescence observed in some no-probe controls (5/6). Hyphae were found almost exclusively within the xylem, predominantly in vessel elements (10/10) and fibres (7/10) (Figure 3B,C). Axial parenchyma cells contained hyphae in half of the samples, whereas parenchyma ray cells (3/10), vascular cambium (1/10) and bark tissues (1/10) rarely exhibited fungal structures. Hyphae were detected not only in the most recent outer growth ring but also in older xylem rings, extending up to approximately 4 years in some samples (Figure 3A). In certain fields of view from symptomatic control twigs processed without probe, background green autofluorescence of fungal hyphae inside vessels was visible (Figure 3D).

In a subset of six asymptomatic twig samples, no visible fungal structures were detected (Figure 3F). However, five of six samples exhibited a red fluorescent signal in the presence of the Bfag 28S probe that was not associated with any fungal

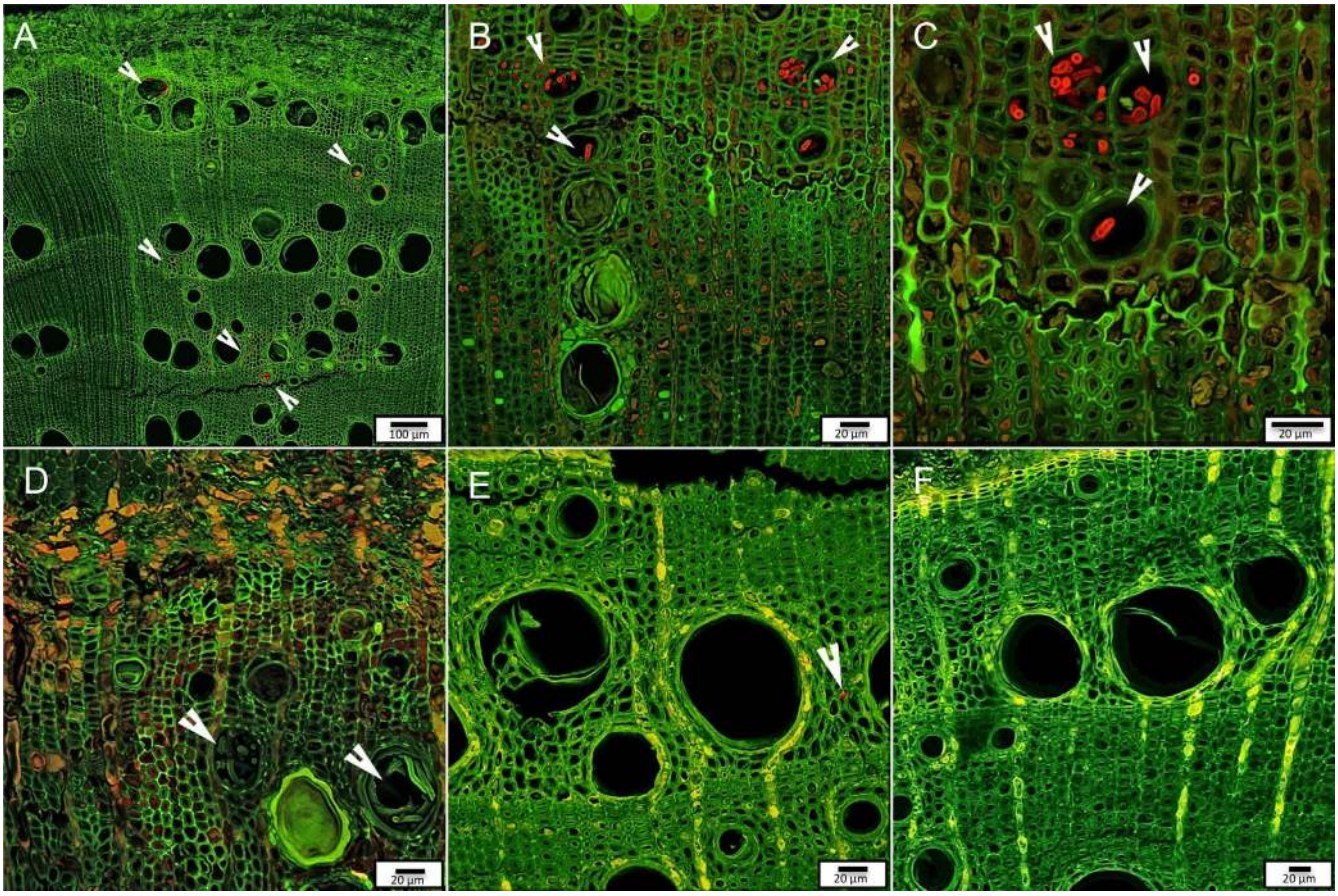


FIGURE 3 | CLSM–FISH detection of *Bretziella fagacearum* in symptomatic and asymptomatic oak twigs. (A) Symptomatic twig showing FISH positive red signal. Fungal hyphae detected in xylem rings up to 4 years old. (B) Detailed view of the xylem showing hyphae within vessels, fibres and axial parenchyma cells. (C) Same field of view as image B at higher magnification showing clearly hyphal morphology confirming the FISH positive signal. (D) Symptomatic control without probe (negative FISH), showing background autofluorescence and visible hyphae within vessels. (E) Asymptomatic twig hybridised with probe showing weak, diffuse red background signal without visible fungal structures. (F) Asymptomatic twig without probe and no background red fluorescence. White arrowheads indicate fungal hyphae.

morphological features, indicating background autofluorescence or non-specific probe binding (Figure 3E). In Figure 3E, the red fluorescence highlighted by the arrowhead represents a localised background signal rather than a fungal hypha, which is why the co-occurrence of fluorescence and identifiable fungal morphology is required to confirm a true Bfag positive signal.

In four of the subsets of the six symptomatic petiole samples analysed, *B. fagacearum* hyphae were consistently observed across multiple tissue types, primarily within xylem vessels (Figure 4B,C), but also in collenchyma and in vascular and cortical parenchyma cells. qPCR analysis was positive in four of the six samples; however, the results did not always correspond to the specimens that exhibited FISH signals. Two samples were negative by qPCR but positive by FISH, whereas two others were positive by qPCR but negative by FISH. In the symptomatic petiole sample processed without probe, no red fluorescence signal was detected (Figure 4D).

In a subset of the three asymptomatic petiole samples, all were negative by both FISH (Figure 4F) and qPCR. Nevertheless, in asymptomatic samples hybridised with the Bfag 28S probe, some crystalline structures displayed red fluorescence similar to that observed in symptomatic samples (Figure 4B), suggesting non-specific probe binding (Figure 4E). At low magnification, crystalline structures were visible throughout the petiole tissue, and these structures co-localised with the red FISH signal (Figure 4A), consistent with a potential non-specific probe interaction.

4 | Discussion

In this study, we demonstrated that FISH is an effective method for detecting *B. fagacearum* within its host tree and on its insect vectors. The Bfag 28S FISH probe exhibited high specificity for the oak wilt pathogen, with no hybridisation observed in any non-target fungi tested.

FISH enabled precise localisation of *B. fagacearum* propagules on distinct anatomical structures of vectoring insects. Although the number of specimens examined was limited, the only propagule type detected on *C. sayi* was ascospores. In contrast, Juzwik and French (1983) reported both conidia and ascospores on specimens of *Epuraea* spp., *Colopterus truncatus*, *G. sanguinolentus*, *G. quadrisignatus* and *G. fasciatus* using scanning electron

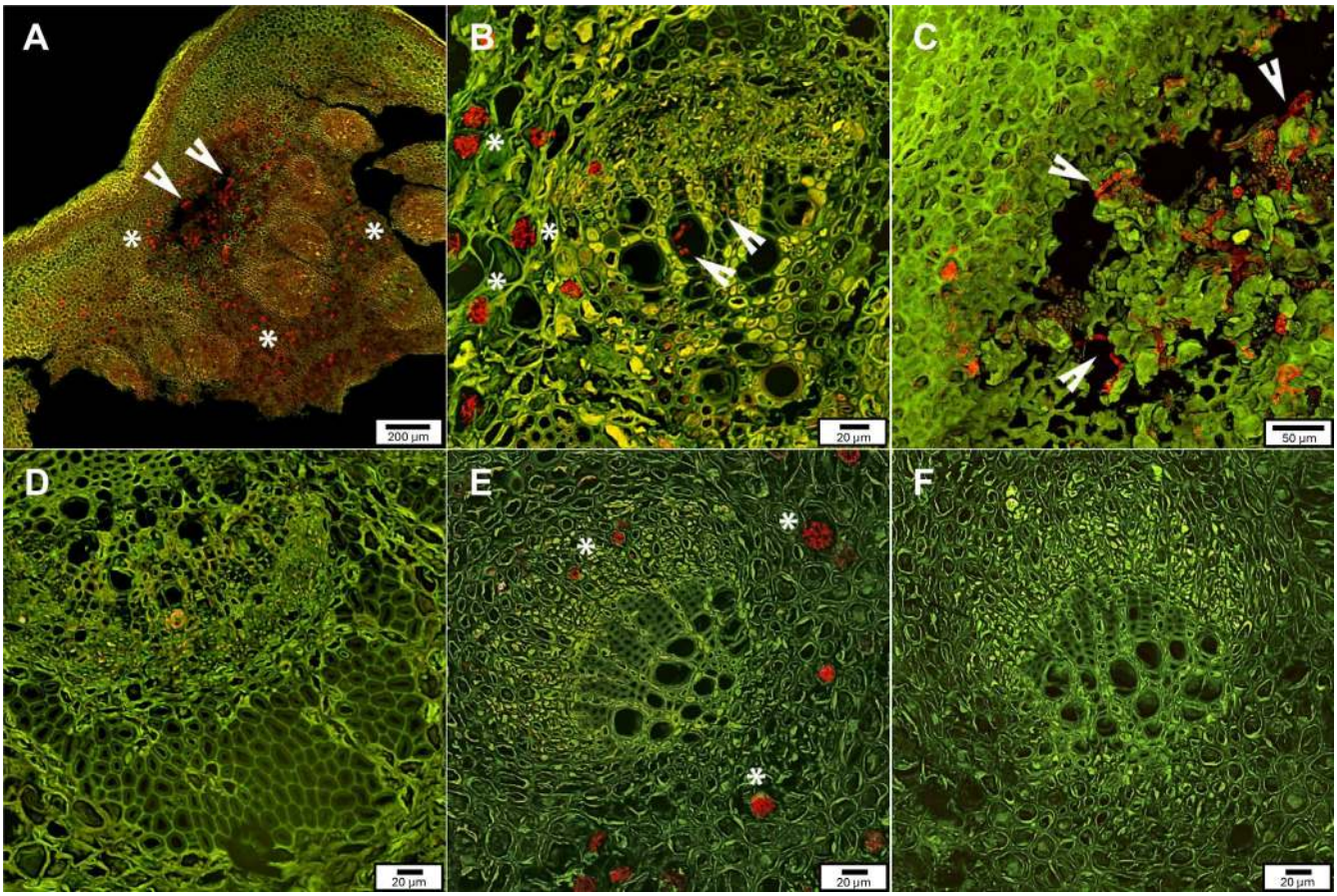


FIGURE 4 | CLSM–FISH detection of *Bretziella fagacearum* in symptomatic and asymptomatic oak petioles. (A) Low magnification view of a symptomatic petiole showing FISH positive red signal and the presence of crystals. Fungal hyphae are detected in the vascular tissues. (B) Detailed view of the xylem/phloem region showing hyphae within vessels and red signal crystals in peripheral parenchyma tissue. (C) Same field of view as image A at higher magnification showing clearly hyphal morphology confirming the FISH positive signal. (D) Symptomatic control without probe (negative FISH), showing background green autofluorescence. (E) Asymptomatic petiole hybridised with probe showing red background signal associated with crystal but without visible fungal structures. (F) Asymptomatic petiole without probe showing no red background fluorescence. White arrowheads indicate fungal hyphae; white asterisks indicate crystals.

microscopy (SEM). That study also documented a temporal shift in spore types on insect surfaces: conidia predominated early in the season, whereas ascospores were more frequent later. According to Juzwik and French (1983), this pattern reflects the progression of sporulation on fungal mats in infected trees. The late-May collection date of our insect samples likely explains the absence of conidia. Consistent with SEM observations by Juzwik and French (1983), we detected ascospores on the legs, in grooves between body segments, and in large numbers smeared across abdominal segments in ventral view.

Beyond confirming spore presence, FISH revealed a recurring localisation pattern at the terminal sternite suture. Although *C. sayi* lacks specialised mycangia, repeated detection of ascospores in this region suggests that this passive structural feature may facilitate spore retention, consistent with passive mycangial analogs in beetle–fungus associations (Whitney and Farris 1970; Lévieux et al. 1991; Six 2003).

Early investigations into oak wilt hypothesised that *B. fagacearum* could be disseminated via spores ingested by sap beetles (Bart and Griswold 1953; Jewell 1954, 1956; Yount et al. 1955). In contrast, SEM observations led Juzwik and French (1983) to propose that spores are primarily acquired passively and transported on external insect surfaces. To date, the relative contribution of ingested versus externally carried spores among different insect vectors has not been systematically quantified. Integrating molecular quantitative detection methods (Bourgault et al. 2022) with the FISH protocol described herein offers a promising approach to address this unresolved question.

This study provides the first documented visual evidence of *B. fagacearum* occurring outside leaf xylem. Young (1949) first reported the presence of *B. fagacearum* mycelium in microscopic sections of midribs and petioles. In our investigation of naturally infected leaves, *B. fagacearum* cells were consistently detected across multiple tissue types—primarily within xylem vessels, but also in collenchyma and in vascular and cortical parenchyma cells. These observations offer new insights into the pathogen's distribution beyond vessel elements and underscore tissue-specific colonisation patterns during disease progression.

In *Quercus rubra*, the vascular bundles of petioles are larger and exhibit thicker cell walls than those of other oak species. As noted by Berkley (1931), these bundles do not fracture along the same anatomical plane as adjacent cells during senescence, often resulting in the persistence of leaf traces and the formation of a partially developed abscission layer. This incomplete abscission may maintain vascular continuity between senescent leaves and stem tissues. Within the context of oak wilt, such partial compartmentalisation of infected tissues could facilitate pathogen advancement. Although this hypothesis remains to be validated, it offers a promising avenue for investigating the potential role of marcescence in disease dynamics.

The localisation of *B. fagacearum* in our twigs samples was predominantly in the functional xylem, consistent with previous studies, but our FISH assay also revealed the pathogen in vessels and parenchyma cells of annual rings preceding the formation of functional xylem (Figure 3A). This observation suggests that the pathogen may persist in older xylem (Henry and Riker 1947; Young 1949; Struckmeyer et al. 1958) or colonise these rings later in the infection process, as reported by Jones and Bretz (1955) when mycelial mats develop and trees become moribund or die. These findings contrast slightly with previous studies in which artificially inoculated red oaks succumbed within months of inoculation (Henry et al. 1944; Henry and Riker 1947), confining the fungus to the current annual growth ring. Using isolation-based detection, Henry and Riker (1947), and Jones and Bretz (1955) demonstrated that in naturally infected trees, the radial distribution of *B. fagacearum* extended to the second and tenth growth rings, respectively.

Our observations align with reports from other oak species considered more tolerant to oak wilt, where infections remain localised, allowing some trees to survive for several years post-infection. We noted that certain infected twigs exhibited vessels arranged in a radial pattern (data not shown), which may restrict lateral pathogen spread, unlike the extensive vascular anastomoses characteristic of red oaks (Jacobi and MacDonald 1980). By analogy with xylem compartmentalisation mechanisms that limit embolism propagation (Brodersen et al. 2013), this vascular architecture could slow the progression of *B. fagacearum*. These preliminary observations are consistent with Rioux and Blais (2023), who emphasised that vessel characteristics, including diameter and arrangement, may influence host–pathogen interactions during oak wilt development. Overall, these observations provide a foundation for future studies on structural defence mechanisms such as CODIT, previously described in oaks (Tainter and Fraedrich 1986), and support the hypothesis that anatomical traits contribute to disease resistance.

FISH is a powerful tool for plant pathogen detection, combining high specificity, spatial resolution, and the ability to detect non-culturable organisms directly in planta (Cardinale et al. 2008; Salgado-Salazar et al. 2018). It supports multiplexing through non-overlapping fluorescent probes, enabling discrimination of fungal species and detection of latent infections (Vági et al. 2014). In our study, we confirmed this multiplexing capability by pairing the universal probe PF1200 (Grünwald et al. 2010) with the Bfag 28S-specific probe.

Initial observations emphasised the difficulty of isolating *B. fagacearum* from wood heavily colonised by secondary fungi (Bretz and Morison 1953), a challenge that remains relevant for culture-based approaches despite the advent of molecular tools. Similar issues have been reported in other tree vascular wilt pathosystems, such as Dutch elm disease, where secondary fungi proliferate in compartmentalised tissues, complicating the isolation of *O. novo-ulmi* (Jürisoo et al. 2025). Furthermore, Jürisoo et al. (2025), showed that fungal community composition varies with host species and growth environment, adding another layer of complexity to culture-based isolation. FISH provides the unique ability to localise the pathogen within host tissues, enabling insights into interactions with secondary fungi.

Strong FISH signals were obtained even from insect specimens collected years earlier, stored at −20°C, and only fixed in Carnoy's solution prior to analysis. This suggests that nucleic acid targets can remain stable under prolonged storage, consistent with findings in histological samples, where archival duration influences but does not prevent RNA detection in FISH-based assays (Sari

et al. 2025). However, the occasional absence of hybridisation, particularly in spore-like structures resembling *B. fagacearum* ascospores (Figure 2D), may reflect technical limitations rather than true negatives. A plausible explanation is that these ascospores were embedded in a mucilaginous layer, which could act as a physical barrier to probe penetration, similar to limitations reported in tissues with abundant collagen and extracellular protein matrix (Sari et al. 2025). Similarly, structural barriers such as thick-walled or pigmented conidiogenous cells may also restrict probe access (de Beer et al. 2017), as suggested by our own observations of signal absence in structures resembling conidiogenous cells during protocol optimisation. Both scenarios highlight the need for tailored permeabilisation strategies to improve FISH performance when extracellular deposits or complex cell walls are present, either by combining surfactants and osmotic shock (Villa et al. 2009) or by enzymatic treatment (Cardinale et al. 2018). Although not applied in this study, incorporating a nuclear counterstain such as DAPI could provide an indirect assessment of cell integrity in cases where FISH signals are absent (Rickerts et al. 2011). These observations highlight the importance of considering specimen history, structural variability, and complementary treatments when interpreting FISH results.

Although FISH is highly informative for pathogen localisation, it has limitations. Its sensitivity is lower than PCR-based methods, requiring sufficient target abundance and prior sequence knowledge. Comparative studies evaluating qPCR and FISH side by side in plant pathology remain rare and are mainly limited to soil-borne pathogens (Milner et al. 2019), but they confirm the same trend observed in medical diagnostics: FISH is less sensitive than PCR but offers unique visualisation capabilities. For example, Milner et al. (2019) combined qPCR-based quantification of *Sclerotium rolfsii* DNA in soils with FISH smears to visualise hyphal abundance. The hybridisation signals were positively correlated with qPCR DNA levels, demonstrating that FISH provides reliable visual confirmation of molecular data while adding spatial context. Similarly, in the field of clinical diagnostics, FISH has shown practical advantages: da Silva et al. (2015) reported that FISH detected slightly more fungal infections than conventional microscopy (48 vs. 46 out of 122 samples) and provided results within approximately 5 h, compared to 20 h for PCR and 6 days for traditional culture methods. This difference in sensitivity, combined with the complementary nature of the information provided by each method, reinforces the idea that FISH should be used as a complement to qPCR rather than as a standalone diagnostic tool.

Autofluorescence interference, combined with potential cross-hybridisation or non-specific probe binding, represents a major challenge for accurate FISH interpretation. These factors can lead to false positives, especially in complex plant tissues rich in autofluorescent compounds and other molecules that may interact with FISH probes. In our study, birefringent crystals were observed within petiole tissues (Figure 4B,E). Although no autofluorescence was emitted in hybridisation controls without probe, a strong autofluorescent signal colocalized with the crystals when the FISH probe was included in the hybridisation buffer. These results suggest interaction between crystal molecules and the probe. As observed in other FISH studies of fungi in plant tissues (Rickerts et al. 2011; Tsui et al. 2011), non-specific signals may occur. To circumvent these artefacts and ascertain the *B. fagacearum* diagnostic in our samples, we complemented fluorescent signal interpretation with observation of the fungal structures. Optimisation of sample pre-treatment and hybridisation conditions for plant tissues and insects is ongoing in our laboratory.

Photobleaching is another consideration, but no significant signal loss was observed despite repeated scans and long acquisition times for thick insect and plant sections. FISH remains limited for quantitative analysis and for detecting targets located within thick, melanized, or otherwise poorly permeable structures where probe access is restricted.

Our comparison of petiole samples illustrates the sensitivity limitation: two samples were qPCR-negative but FISH-positive, suggesting that FISH may detect localised fungal structures even when DNA quantity is below the amplification threshold or unevenly distributed. Conversely, two samples were qPCR-positive but FISH-negative, which may reflect very low pathogen abundance or restricted probe accessibility. The lambda DNA recovery control was positive in all samples, confirming that qPCR-negative results were not due to extraction failure. These outcomes highlight the complementarity of FISH and qPCR for studying oak wilt epidemiology.

This study demonstrates the utility of FISH for detecting and visualising *B. fagacearum* in complex biological samples. The Bfag 28S FISH probe enables highly specific localisation of the pathogen within host structures and even on whole-mount sap beetle vectors. These findings advance our understanding of oak wilt pathology and provide a framework for future diagnostic and ecological studies.

Author Contributions

P.T. conceived the original idea. K.C. collected the field samples. M.B. performed the sample preparation and analyses. M.B. and P.T. wrote the manuscript draft. All authors discussed the results, provided critical feedback, and contributed to the final manuscript.

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Disclosure

This manuscript was drafted by the authors and subsequently refined using Microsoft Copilot, an AI-powered writing assistant, for language clarity and style improvement. Copilot was not used to generate scientific content, interpret data or draw conclusions.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** efp70070-sup-0001-FigureS1.xlsx. **Table S1:** efp70070-sup-0002-TableS1.xlsx.