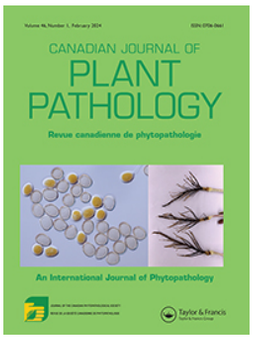

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Biosurveillance of oak wilt disease in Canadian areas at risk

MARIE-KRYSTEL GAUTHIER[1], ÉMILIE BOURGAULT[1], AMÉLIE POTVIN[1], GUILLAUME J. BILODEAU [2], SVEN GUSTAVSSON[3], SHARON REED[4], PIERRE THERRIEN[5], ÉVELYNE BARRETTE[5] AND PHILIPPE TANGUAY [1]

[1]*Natural Resources Canada, Canadian Forest Service, Laurentian Forestry Centre, Québec, QC, Canada*
[2]*Canadian Food Inspection Agency, Ottawa laboratory Fallowfield, Ottawa plant laboratory, Ottawa, ON, Canada*
[3]*Quebec Wood Export Bureau (QWEB), Softwood and Value-Added Softwood Lumber, Québec, QC, Canada*
[4]*Ontario Ministry of Natural Resources and Forestry, Ontario Forest Research Institute, Sault Ste Marie, ON, Canada*
[5]*Direction de la protection des forêts, Ministère des Ressources naturelles et des Forêts du Québec, Québec, QC, Canada*

(Accepted 17 September 2023)

Abstract: Biosurveillance of invasive species is critical for protecting native ecosystems and limiting economic losses. Early detection of pathogens through qPCR methods has recently shown great promise and can potentially slow the spread of devastating diseases. For instance, oak wilt, a disease caused by the fungus *Bretziella fagacearum*, can kill mature trees within weeks of infection. Originally contained in the USA, oak wilt has finally made its way into Canada, where it was recently observed for the first time in June 2023. This study has laid the foundations for a biosurveillance monitoring programme of *B. fagacearum* in Eastern Canada. From 2019–2021, insect vectors were baited and captured in Lindgren traps in various locations of interest, namely sawmills importing oak logs from the USA, forested areas containing mature oak trees and strategic sites along the border between the two countries. Insect vectors and collection fluids were analysed with our qPCR detection test for the presence of *B. fagacearum*. As a positive control to validate this method, we included traps in a known centre of oak wilt infection in Michigan (USA). Our analysis showed only one positive site at the border between Ontario (CA) and the USA, even though oak wilt has never been observed there. This result confirms that DNA from *B. fagacearum* can be detected with this method even before the appearance of symptomatic trees, which could be crucial in the current containment efforts in Ontario (CA).

Keywords: Biosurveillance, *Bretziella fagacearum*, molecular detection, oak wilt

Résumé: La biosurveillance des espèces invasives est essentielle pour préserver les écosystèmes indigènes et limiter les pertes économiques. La détection précoce des agents pathogènes à l'aide des techniques de PCR quantitative s'est révélée récemment très prometteuse et peut ralentir la propagation de maladies dévastatrices. Par exemple, le flétrissement du chêne, une maladie causée par *Bretziella fagacearum*, peut tuer des arbres matures quelques semaines après l'éclosion de l'infection. Initialement confiné aux États-Unis (É.-U.), le flétrissement du chêne a finalement atteint le Canada où il a été observé pour la première fois en juin 2023. Cette étude a établi les bases d'un programme de biosurveillance de *B. fagacearum* dans l'est du Canada. De 2019 à 2021, des insectes vecteurs ont été appâtés et capturés dans des pièges Lindgren à différents endroits d'intérêt, particulièrement dans des scieries important des billes de chêne des États-Unis, des chênaies matures et des sites stratégiques longeant la frontière entre les deux pays. Les insectes vecteurs et les fluides de collecte ont été analysés avec notre test de détection par PCR quantitative pour révéler la présence de *B. fagacearum*. En tant que témoins positifs pour valider cette méthode, nous avons inclus des pièges provenant d'un site de l'État du Michigan reconnu comme foyer du flétrissement du chêne. Notre analyse a révélé un seul site d'infection à la frontière de l'Ontario, au Canada, et des États-Unis, bien que le flétrissement du chêne n'y eût jamais été observé auparavant. Ce résultat confirme que l'ADN de *B. fagacearum* peut être détecté par cette méthode avant même que les arbres affichent des symptômes, ce qui serait crucial dans le cadre des efforts actuels d'endiguement de la maladie déployés en Ontario.

Mots clés: Biosurveillance, *Bretziella fagacearum*, détection moléculaire, flétrissement du chêne

Correspondence to: Philippe Tanguay. Email: Philippe.Tanguay@nrcan-rncan.gc.ca

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Introduction

Invasive species, accidentally introduced through foreign trade, are transforming North American forests. Diseases such as chestnut blight and beech bark disease have changed the composition and structure of forests, altering whole ecosystems and the services they provide (McCormick and Platt 1980; Diamond et al. 2000; Hancock et al. 2008; Garnas et al. 2011; Paap et al. 2020). Further alterations to forests are expected as climate change progresses, removing the environmental barriers that have prevented some invasive species from expanding their range (Paradis et al. 2008; Smith et al. 2012; King et al. 2021). Invasive species have an estimated mean cost of US$26.8 billion annually (Diagne et al. 2021). Detection of invasive species populations before they are too large to eradicate is critical for protecting native ecosystems and limiting economic losses.

Biosurveillance generally focuses on the protection of agricultural and natural resources through the gathering of data and information on animal and plant health pests (Quinlan et al. 2015; Hamelin and Roe 2020; Caton et al. 2022). Early detection of introduced organisms, specifically pathogens, is the most common activity related to biosurveillance (Kean et al. 2015). It can be used to prevent the establishment of invasive, non-native pathogens and monitor disease progression (Bilodeau et al. 2019). For instance, the eradication of the Asian longhorned beetle in Toronto, Canada, was made possible only because of an efficient early detection followed by a rapid and aggressive response to the invasion (Smith et al. 2009; Fournier and Turgeon 2017). Biosurveillance also serves to identify potential sources and pathways used by plant pathogens and their most common variants (Potgieter et al. 2020; Kirtane et al. 2022). Early detection and timely management interventions are key components to a successful eradication or containment operation, generating fewer economic and environmental costs (Luchi et al. 2020).

The rapid and accurate identification of biological samples collected during surveys or inspections is an essential first step in biosurveillance (Hamelin and Roe 2020; Chandelier et al. 2021). Early surveillance through a qPCR detection assay, for instance, can potentially prevent pathogen dispersal into new territories or hosts via plant material, soil, water or airborne pathways. This has been shown with various microorganisms such as *Phytophthora* (Migliorini et al. 2015) and *Ceratocystis platani* (Tremblay et al. 2019), and many qPCR assays have also been optimized to target fungal spores collected from insect vectors (Lamarche et al. 2015). Tremblay et al. (2018, 2019). used qPCR and metabarcoding tools on insect trap fluids to detect forest pathogens from insect traps. Biosurveillance could also detect latent fungal pathogens during asymptomatic phases (Luchi et al. 2016). As a last resort, it can support containment and eradication operations once the disease is already established or reaches outbreak levels (Luchi et al. 2020).

Oak wilt is an example of a disease that can be fought or at least contained through biosurveillance. It is a vascular disease caused by the fungus *Bretziella fagacearum* that primarily kills red oaks (*Quercus*, *Lobatae*) (Henry 1944; Bretz 1953; Beer et al. 2017). When the pathogen penetrates and colonizes the sapwood, it disrupts the xylem vessels, blocking nutrients and water flow, which may lead to the death of mature trees within weeks (French and Stienstra 1980; Gibbs and French 1980). Oak wilt can spread via a few ways: there can be underground transmission to nearby trees through naturally occurring root grafts, but also above ground transmission by sap beetles (mostly *Nitidulidae* species such as *Colopterus truncatus* and *Carpophilus sayi*) that feed on bulging fungal mats and carry spores up to 600 m in a single year (Shelstad et al. 1991; Cease and Juzwik 2001). Movement of contaminated logs and firewood between geographical locations also contributes to the spread of oak wilt (Juzwik et al. 2008).

The disease can be found in midwestern and eastern states as well as Texas in the USA (EFSA Panel on Plant Health et al. 2020), where it was potentially introduced many years ago (Juzwik et al. 2008). However, there has been a constant gradual spread of the disease, with an increasing number of infected counties being reported in states near the Canada–US border, sometimes as close as 500 metres from the border (Jensen-Tracy et al. 2009; Michigan Department of Natural Resources 2022). In June 2023, the Canadian Food Inspection Agency (CFIA) reported the first official detection of oak wilt in Canada, in Niagara Falls, Ontario. Although only a few trees harboured symptoms, the disease has since also been observed in the township of Springwater, Ontario, which is a great cause of concern (Government of Canada 2023). The CFIA is currently surveying both areas and is developing an eradication plan for oak wilt (North American Plant Protection Organization 2023).

Within this context, biosurveillance appears to be an appropriate tool to monitor the situation and prevent further disruptions to native ecosystems. Detecting the pathogen using molecular techniques before the appearance of any disease symptoms could likely limit the establishment of oak wilt in Canada. Otherwise, this invasion could lead to a large-scale mortality of urban

trees, the devastation of natural ecosystems and could cripple the oak log industry, with estimated economic losses of CAD$400 million (Pedlar et al. 2020). The emerald ash borer, as a comparison, was not detected during early stages of invasion and is now considered one of the most costly and destructive invasive forest pests in North America (Kovacs et al. 2011; Herms and McCullough 2014).

The objective of this study was to establish the premises of biosurveillance monitoring of *B. fagacearum* in Eastern Canada. To include spores naturally carried by insects but also the human-assisted movement of infected wood, locations close to the US–Canada border, forested campground sites as well as wooded areas near or within sawmills importing oak logs from the USA were investigated. This study presents biosurveillance data ranging from 2019–2021 where insects were collected from traps and analyzed with the qPCR TaqMan detection test for *B. fagacearum* developed in our laboratory.

Materials and methods

Lindgren funnel traps positioning and baiting

To assess potential incursions of *B. fagacearum* from 2019–2021, critical geographical sites were carefully selected in the province of Quebec (Canada), Ontario (Canada) and Michigan (USA – positive control), where Lindgren funnel traps (Distributions Solida, Quebec, CA) were set up (Fig. 1A). Specific woodland areas, located within the oak range of Canada in the southern part of the province of Quebec, were chosen because of their proximity to either sawmills processing US imported oak logs or campground sites where travel and associated firewood transport are a known risk of pest movement (Koch et al. 2012). Some traps were placed directly in the sawmills' backyards, with their participation, close to piles of imported oak logs. Other wooded sites were included along the Canada–US border in Southern Ontario, adjacent to infected US states. Locations with ongoing oak wilt outbreaks in Michigan were also included as a positive control for this study. A description of all sites, their geographical location and year investigated are listed in Table 1.

At each designated site in the province of Quebec, a single Lindgren funnel trap was suspended from the lower branch of an available nearby tree so that collection cups were approximately one metre above the ground. In Ontario and Michigan, three traps were set up at each site. Each trap was hung on a pole which was then placed within 10 m of an oak tree, with collection cups 1 m above the ground. Figure 1A explains how the Lindgren traps were baited for this study. Some traps were baited with specific pheromones designed to attract *Carpophilus sayi* and *Colopterus truncatus* (pheromones Trece, Distributions Solida, Quebec, CA). Those traps also contained 20 g of wholewheat bread dough inoculated with baker's yeast (450 g of wholewheat flour, 15 mL of sugar, 300 mL of warm water and a 7 g package of dry active yeast) in order to enhance the effect of the pheromones added (Lin and Phelan 1991). Insects were collected in a 500 mL container filled with propylene glycol (Laboratoire Mat, Quebec, CA). The remaining traps were equipped with a generic type of lure, Ultra High Release (UHR) alpha-pinene lure (Synergy Semiochemical Corp), that attracts wood-boring and forest insects. For those traps, insects were collected in a 500 mL container filled with UHR ethanol (Synergy Semiochemical Corp.). The propylene glycol, ethanol and lures (bread dough and pheromones) were replaced every 4 weeks in the province of Quebec (from May to September), and every 2 weeks in Ontario and Michigan (from May to June or July). The liquid samples containing the trapped insects were stored at 4°C until processed.

Sample preparation (insect and preservation fluid)

Figure 1B details the sample preparation method used here. Once in the laboratory, samples were passed through a stainless steel sieve (24 mesh size) to separate the trapped insects potentially carrying *B. fagacearum* spores from the collection fluid. Without any further type of sorting, all insects were quickly rinsed with cold sterile water and transferred to a Falcon tube, which was then lyophilized for 5 days at −50°C in a FreeZone 2.5 Liter freeze-dryer (Labconco, Kansas City, MO, USA). Next, dried insect samples were ground in liquid nitrogen and divided into powdered aliquots of 250 mg. To investigate whether spores carried by insects could have been left behind in the collection fluid of the traps, 10 mL of the filtered propylene glycol or ethanol solution was also collected and diluted with 40 mL of cold sterile distilled water. Samples were then centrifuged at 4700 rpm for 15 min at 12°C and pellets were transferred to 2 mL microtubes for two subsequent washes with 1.5 mL of cold sterile distilled water.

DNA extraction

For both insects and collection fluid samples, DNA was extracted using the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA) with a few modifications to the

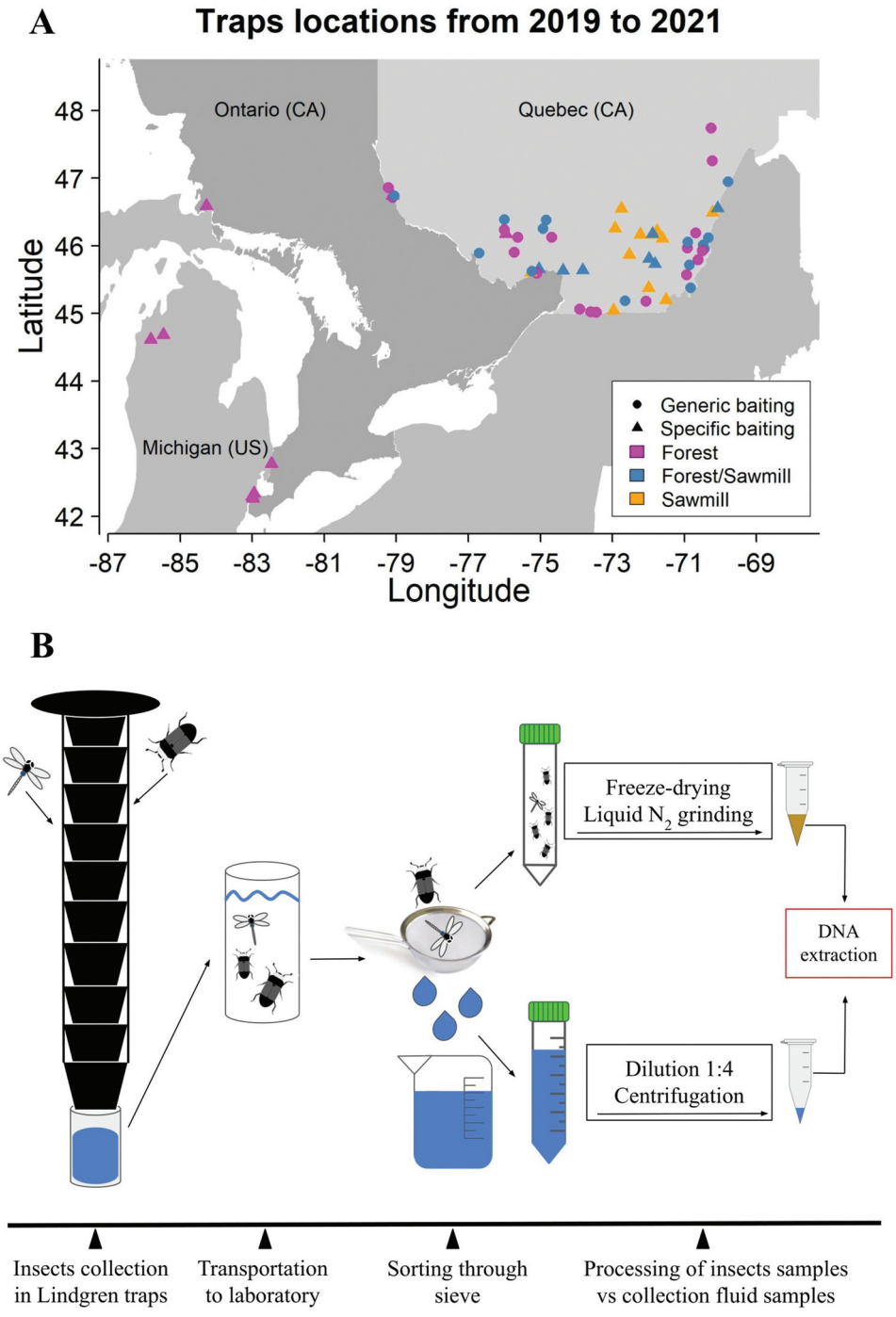

Fig. 1 Sampling method for this study. (A) Geographical map showing all sites selected, the environment type in which the traps were set up, as well as baiting type (generic or specific to *B. fagacearum*) for each location. (B) Processing of samples from the Lindgren traps in the laboratory. Both insects (without sorting) and collection fluid samples were processed through DNA extraction and submitted to the qPCR TaqMan detection assay for the presence of *B. fagacearum*.

manufacturer's protocol. For insects, 180 µL of ATL buffer and 20 µL of proteinase K were added to the 250 mg powder aliquots. Tubes were then incubated at 56°C and vortexed for 10 s every 10 min for 2 h, and finally left overnight at 56°C without shaking. The next day, tubes were centrifuged at 20 000 g for 10 min, and the supernatant was transferred onto a QIAcube Connect (Qiagen, Valencia, CA, USA) for automated extraction according

Table 1. Description of all sites selected for this study and their geographical coordinates.

Site	Province/ Country	Baiting type[a]	Environment[b]	Latitude	Longitude	Year(s) investigated
L'Annonciation	Quebec/CA	Generic	Forest/sawmill	46.385659	−74.84485	2019
Armstrong	Quebec/CA	Generic	Forest/sawmill	45.95998	−70.44933	2019
Baie des sables	Quebec/CA	Generic	Forest	45.5756562	−70.94595	2020
Bouchette	Quebec/CA	Specific	Forest	46.174408	−75.96348	2020, 2021
Lac Campion	Quebec/CA	Generic	Forest	46.1278538	−75.63625	2020
Clermont	Quebec/CA	Generic	Forest	47.739685	−70.26466	2019
La Conception	Quebec/CA	Generic	Forest	46.1303728	−74.68523	2020
Danville	Quebec/CA	Specific	Forest/sawmill	45.8076067	−71.98818	2020, 2021
Daveluyville	Quebec/CA	Specific	Sawmill	46.16761	−72.22799	2019, 2021
Bowman	Quebec/CA	Generic	Forest	45.90716	−75.72824	2020
Saint-Jean-Port-Joli	Quebec/CA	Generic	Forest	47.2565807	−70.22951	2020
Drummondville	Quebec/CA	Specific	Sawmill	45.868836	−72.52917	2019, 2021
Saint-Gédéon-de-Beauce	Quebec/CA	Generic	Forest	45.795538	−70.62793	2020
Fort-Coulonge	Quebec/CA	Generic	Forest/sawmill	45.8916693	−76.70693	2019
La Guadeloupe	Quebec/CA	Generic	Forest	45.9716267	−70.91837	2020
Hemingford	Quebec/CA	Generic	Forest	45.0238213	−73.6078	2020
Lachute	Quebec/CA	Specific	Sawmill	45.634623	−74.37017	2021
Lachute/Station 25 133	Quebec/CA	Specific	Forest/sawmill	45.635102	−74.37127	2020, 2021
Lac-Drolet	Quebec/CA	Generic	Forest/sawmill	45.7202487	−70.85994	2019
Louiseville	Quebec/CA	Specific	Sawmill	46.257124	−72.92437	2019, 2021
Pointe-Antoine, Ch. Lynn	Quebec/CA	Generic	Forest	46.8649547	−79.22158	2020
Maniwaki	Quebec/CA	Generic	Forest/sawmill	46.3916825	−76.00739	2019
La Minerve	Quebec/CA	Generic	Forest/sawmill	46.2576005	−74.93388	2019
Papineauville	Quebec/CA	Specific	Forest/sawmill	45.6568072	−75.03379	2020, 2021
Messines	Quebec/CA	Generic	Forest	46.2377187	−75.99944	2020
Plessisville	Quebec/CA	Specific	Sawmill	46.217852	−71.76156	2019, 2021
Plaisance	Quebec/CA	Generic	Forest	45.5991945	−75.10676	2020
Princeville	Quebec/CA	Specific	Sawmill	46.163807	−71.88136	2021
Princeville/Station 25 173	Quebec/CA	Specific	Forest/sawmill	46.1775673	−71.88672	2020, 2021
Notre-Dame-des-Pins	Quebec/CA	Generic	Forest	46.1898395	−70.69187	2020
Saint-Antoine-Abbe	Quebec/CA	Generic	Forest	45.0670838	−73.91044	2020
Sainte-Camille-de Bellechasse	Quebec/CA	Specific	Sawmill	46.491026	−70.2185	2021
Sherbrooke	Quebec/CA	Specific	Sawmill	45.376659	−71.99532	2019
Stanstead	Quebec/CA	Generic	Forest	45.1824715	−72.07182	2019
St-Armand	Quebec/CA	Specific	Sawmill	45.045575	−72.96758	2019, 2021
Saint-Bernard-de-Lacolle	Quebec/CA	Generic	Forest	45.0179437	−73.44887	2020
St-Côme	Quebec/CA	Generic	Forest/sawmill	46.0142583	−70.48681	2019
St-Éphrem-de-Tring	Quebec/CA	Generic	Forest/sawmill	46.0553533	−70.91152	2019
St-Ferdinand	Quebec/CA	Specific	Sawmill	46.105713	−71.60997	2019
Saint-Just-de-Bretenières	Quebec/CA	Specific	Forest/sawmill	46.554107	−70.07731	2020, 2021
St-Malo	Quebec/CA	Specific	Sawmill	45.199765	−71.50825	2019, 2021
St-Pamphile	Quebec/CA	Generic	Forest/sawmill	46.9515527	−69.79238	2019
Sainte-Thérèse	Quebec/CA	Specific	Forest/sawmill	45.6371077	−73.82635	2020, 2021
St-Théophile	Quebec/CA	Generic	Forest	45.9312017	−70.50233	2019
St-Zacharie	Quebec/CA	Generic	Forest/sawmill	46.120175	−70.33606	2019
Shawinigan	Quebec/CA	Specific	Sawmill	46.547186	−72.750460	2019
Témiscaming/Station 25 168	Quebec/CA	Generic	Forest	46.7179713	−79.10426	2020
Témiscaming/Station 25 169	Quebec/CA	Specific	Forest	46.7423542	−79.10789	2020, 2021
Témiscaming/Chemin Kipawa	Quebec/CA	Generic	Forest/sawmill	46.7417978	−79.05789	2019
Thurso	Quebec/CA	Specific	Sawmill	45.604269	−75.25299	2021
Thurso/Stations 14 374–14376	Quebec/CA	Generic	Forest/sawmill	45.6243268	−75.23803	2019
West-Brome	Quebec/CA	Generic	Forest/sawmill	45.1879525	−72.65204	2019
Woburn	Quebec/CA	Generic	Forest/sawmill	45.3800817	−70.8343283	2019
Wotton	Quebec/CA	Specific	Forest/sawmill	45.73237	−71.8150948	2020, 2021
Sault-Ste-Marie	Ontario/CA	Specific	Forest	46.589463	−84.27938	2019, 2020
St. Clair, Sarnia	Ontario/CA	Specific	Forest	42.77445	−82.46324	2019, 2020, 2021
Windsor, Devonwood	Ontario/CA	Specific	Forest	42.262802	−82.981628	2019, 2021
Windsor, Kiwanis Park	Ontario/CA	Specific	Forest	42.33862664	−82.95036878	2020

(*Continued*)

Table 1. (Continued.)

Site	Province/ Country	Baiting type[a]	Environment[b]	Latitude	Longitude	Year(s) investigated
Windsor, Optimist Memorial Park	Ontario/CA	Specific	Forest	42.29092891	−82.9983703	2020
East Bay Township	Michigan/USA	Specific	Forest	44.681387	−85.46602	2019
Green Lake Township	Michigan/USA	Specific	Forest	44.614468	−85.81547	2020

[a]**Specific baiting**: traps containing *Carpophilus sayi* and *Colopterus truncatus* pheromones and bread dough, insects collected in propylene glycol. **Generic baiting**: traps containing UHR alpha-pinene lure, insects collected in UHR ethanol.
[b]**Environment types**: **forest** = site located in a wooded area/camping site, **sawmill** = site where traps were placed inside a participating sawmill's backyard, **forest/sawmill** = wooded area located in close proximity to a sawmill.

to the QIAamp DNA mini tissue standard V1 protocol. For collection fluid samples, a 3 mm tungsten bead was first added to each tube containing a spore pellet. Tubes were then frozen in liquid nitrogen and loaded on a Tissue Lyser Lab Vibration Mill Mixer (Retsch MM300, Newtown, PA, USA) for two runs of 90 s at 30 Hz separated by a 10-min incubation at room temperature. Proteinase K and ATL buffer were then added to each sample as described above. Tubes were incubated at 56°C and vortexed for 10 s every 10 minfor 2 h, and left overnight at 56°C without shaking. The next day, tubes were quickly spun down and the whole sample, minus the tungsten bead, was transferred onto the QIAcube Connect extraction robot, following the QIAamp DNA mini tissue standard V1 protocol.

qPCR TaqMan *B. fagacearum* assay

The presence of *B. fagacearum* in all sample types was tested with our specific qPCR TaqMan assay published in 2022 (Bourgault et al. 2022). The assay targets the internal transcribed spacer (ITS) multicopy gene of *B. fagacearum* for more sensitivity. Primer and probe sequences, experimental details as well as running conditions are listed in Bourgault et al. (2022). A single sample was equivalent to the content of one trap at a given time-point, which was run in triplicate. Briefly, when all three Cts detected by qPCR for a given sample were below 34.831 (the average value associated with the limit of detection (LOD) of the qPCR test), that sample was considered positive for the presence of *B. fagacearum*.

Positive amplicons obtained by qPCR in 2019 were confirmed by Sanger sequencing using the forward and reverse primers, and the reads obtained were aligned with a target sequence of *B. fagacearum*.

Statistical analysis

Analysis was conducted using R Core Team, version 4.0.5 (R Core Team 2021). All maps were generated with the *sf* package. The beanplots and beeswarm plots depicting Michigan samples were created with *beanplot* and *beeswarm* packages. For beanplots, Ct values from all traps per location were grouped per time-point, for each sample type. Samples where no Ct could be detected are not shown in the graphs. Each sample represents a single trap from which both insects and collection fluid (2019) or just insects (2020) were investigated separately at a given time.

Results

A total of 623 samples were analyzed from 59 locations over the span of 3 years (2019–2021). As mentioned above, insects from the traps were not counted nor identified before processing.

Detection of B. fagacearum *in a known infection centre*

In order to validate our sampling method, samples collected in Michigan, a known centre of active oak wilt outbreak, were processed and analyzed first. Figure 2 shows a beanplot comparing results obtained from insect and collection fluid samples in 2019, and from insect samples only in 2020.

Among the four capture periods investigated in Michigan, a trend shows the first 2 weeks of June as the best detection window for *B. fagacearum*, where Cts are at their lowest. This also coincides with emergence peaks reported for the main vectoring sap beetles in northeastern USA (Ambourn et al. 2005). Captures made earlier in May showed detection with higher Cts or very often no detection at all.

Furthermore, in 2019, for a given trap sample, detection was better achieved from the insect subsample than from the corresponding collection fluid subsample. On average, the Ct was lower by a value of 4.2 for the insect subsample than for the collection fluid subsample, meaning that there was roughly 18 times ($2^{4.2}$) more *B. fagacearum* DNA extracted from the insects than

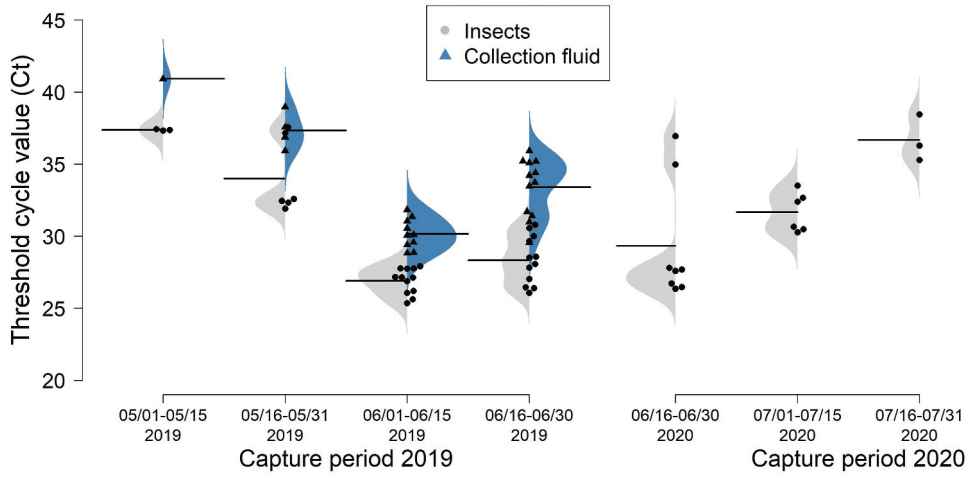

Fig. 2 Detection of *B. fagacearum* in samples from Michigan in 2019 and 2020. Beanplot combined with a beeswarm of all Cts obtained from the qPCR detection assay comparing 'insects' and 'collection fluid' subsamples across time. Data shows that a detection peak occurs around the month of June, which correlates with the life cycle of insect vectors. In 2019, 'insects' sample types also yielded better detection results than 'collection fluids' sample types, which is why the latter method was dropped in 2020. The right panel shows results from 'insects' samples in 2020, which confirms the tendency observed in 2019.

from the collection fluid. Hence, the collection fluid method was abandoned in subsequent years since insect subsamples were shown to increase the chance of detecting *B. fagacearum*. In the year 2020, due to restrictions in place related to the COVID-19 pandemic, the trapping system setup and subsequent harvesting of samples were delayed to late June till early August. As shown in Fig. 2B, this resulted in us nearly missing the detection window estimated to be in early June. When looking at the insect samples that year, a similar pattern emerges, confirming a higher detection rate in June that decreases over time as summer progresses. There was no detection of *B. fagacearum* at all in August 2020.

Detection of B. fagacearum *in Canada*

Our analysis of Canadian samples for the presence of *B. fagacearum* revealed only one positive location among all sites investigated (Fig. 3). The sole positive Canadian site was St. Clair in Ontario, where three traps were set up within 600 metres from the US border. Similar to results from Michigan, quantification levels, and therefore positivity of samples, varied in time. Positive samples in St. Clair occurred during the first 2 weeks of June 2019 (from a single trap at one time-point, Ct of 31.99 ± 0.03) and the last 2 weeks of May 2021 (from a single trap at one time-point, Ct of 32.80 ± 0.07). The rest of both summers yielded either higher or no Cts at all. Sequence analysis of amplicons obtained in 2019 from that site showed a perfect homology to the expected target sequence of *B. fagacearum*. The year 2020 showed no positive detection at all in Canada. It should be noted that this result cannot confirm whether the DNA was obtained from a viable spore, since the propylene glycol used in the traps is deleterious to spore viability.

The other Canadian sites, namely the sawmill backyards and forested areas near them, campground sites and other locations close to the US-Canada border (Windsor and Sault-Sainte-Marie, Ontario), all were found to be negative from 2019–2021 (Fig. 3).

Discussion

The aim of this study was to establish the basis of a biosurveillance programme through an early detection of *B. fagacearum* to prevent an outbreak of oak wilt in Canada, but also to assist in the current monitoring of oak wilt following its first detection in the country. Indeed, we were able to detect the pathogen DNA in St. Clair, Ontario, a site located 600 metres from a county where oak wilt was previously reported in Michigan (USDA Forest Services 2022). The positive detections obtained by qPCR on trapped insects were timely in 2019 and 2021. They have also both been confirmed by metabarcoding using high-throughput sequencing (data in preparation). Following this find, the CFIA subsequently expanded their oak wilt survey in Southern Ontario.

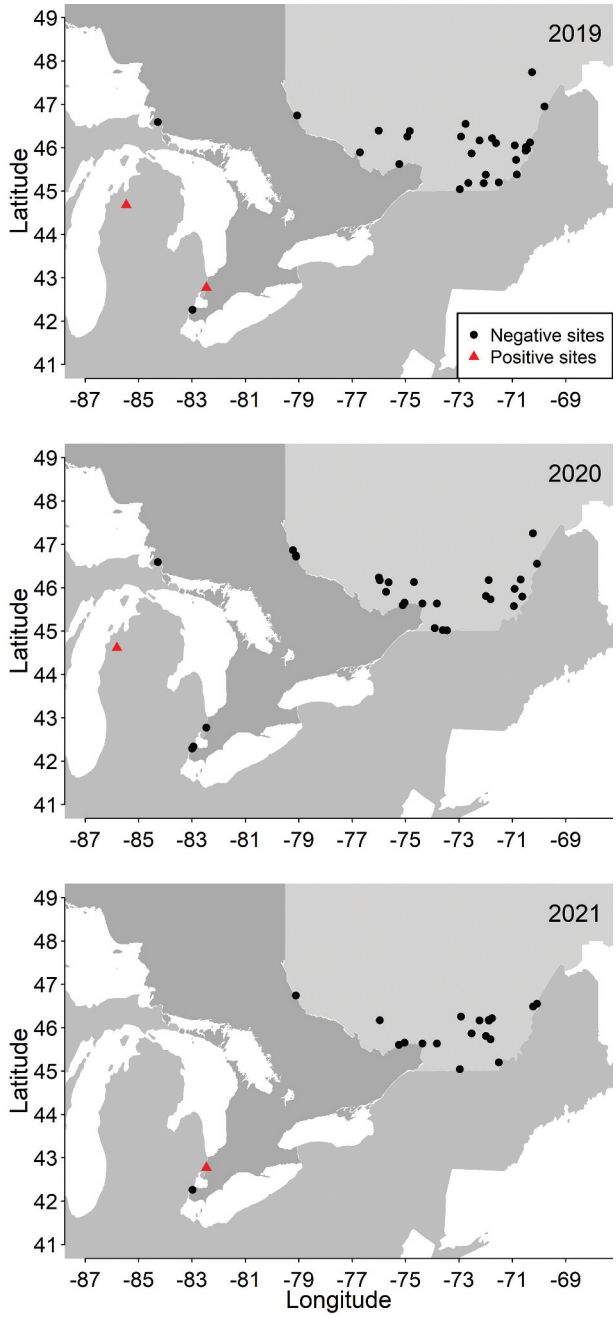

Fig. 3 Detection of *B. fagacearum*. Locations of positive and negative sites in the USA and Southeastern Canada from 2019–2021. Sites highlighted in red triangles were classified as positive when all Cts from one time-point respected the detection limit settings of the qPCR TaqMan detection assay. Only the Michigan (USA) (2019–2020) and the St. Clair (CA; 2019 and 2021) sites were positive.

Although we demonstrated in Michigan that *B. fagacearum* can be detected in trees when infection is apparent, biosurveillance requires detection to be possible even before symptoms appear. In fact, the methodology used here enables detection in advance of the disease progression front, since vectors are flying and could be carried by winds. The detection at St. Clair confirms this premise, as oak wilt has not yet been reported in that area, even though DNA from *B. fagacearum* was detected. As a comparison, it was shown that during the peak of sporulation, spores of *Hymenoscyphus fraxineus* were detected up to 50–100 km ahead of the disease front, confirming the presence of the pathogen before any visible symptoms (Grosdidier et al. 2018). Furthermore, molecular detection of plant pathogens through qPCR has already been shown to be reliable in various situations. One of many examples is a qPCR assay that was used to detect the fungus *Rhizoctonia solani* from soil and lettuce tissue samples (Wallon et al. 2020). Other methods, such as a quantitative nested PCR assay, have been used to detect the fungus *Diplodia corticola* from trapped insects (Bérubé et al. 2018; Muñoz-Adalia et al. 2022). Baroja et al. (2022) also developed a qPCR assay to detect the pine processionary *Thaumetopoea pityocampa*, but instead of using insect traps to capture the moth itself, they targeted the assay to its predator by studying bat faeces.

In addition to detection purposes, the quantitative data obtained using our method on positive samples from a known infection centre allows us to explore complex biological questions resulting from interactions between the pathogen, insect vectors, host and environment. We are only beginning to estimate when spore loads on insect vectors reach their peak, but also how this correlates with the emergence of vectoring insects. Interestingly, spore loads did vary across time, with positivity peaking around June in Michigan, and late May or early June in St. Clair. McMullen et al. (1960) established long ago that both insect vectors (*Ca. sayi* and *Co. truncatus*) are usually more abundant in May and early June, even though that study was conducted in Minnesota. *Carpophilus sayi* has indeed been previously found to be more abundant in oak wilt mats during spring than autumn (Cervenka et al. 2001). Results also seem to agree with the frequency of *Co. truncatus* that usually peaks between April and June (Kyhl et al. 2002). Ambourn et al. (2005) analyzed the monthly abundance of both species over 2 years, but also the proportion of insects contaminated with *B. fagacearum* in neighbouring Minnesota. After peaking from April to June, contaminated insect populations decreased as the season progressed, although quantification was done by growing dilutions of homogenized insects on potato dextrose agar instead of qPCR (Ambourn et al. 2005).

A similar and more recent study by McLaughlin et al. (2022) conducted in New York state from 2019–2021 used a nested PCR method developed by Yang and Juzwik (2017) to detect the oak wilt pathogen from individually sorted trapped insects. While the fungus was successfully detected from nitidulids trapped in previously treated oak wilt infection centres as well as early detection sites, the timing of positive collections showed no consistency from year to year nor within the same season.

In contrast, this study is the first where insects collected from traps were not sorted nor identified before DNA extraction. This modification considerably accelerates sample processing and eliminates the need for a skilled entomologist to sort insects beforehand. Sorting the content of the traps remains the method of choice in most laboratories as shown by other oak wilt studies (McLaughlin et al. 2022). A preliminary test run in our lab compared detection results using our qPCR method on individually sorted insect vectors and whole content of Lindgren traps. While the Cts obtained for individual insects were much lower, detection of the pathogen was still reliable from the whole trap samples (data not shown). We feel that the method used in this study, where insects are not sorted beforehand, offers benefits that outweigh disadvantages. While being less sensitive, it is still more cost-efficient, time-saving and is reliable when detecting *B. fagacearum*. Nonetheless, a ring trial currently underway between our laboratory and four others will focus on comparing the efficiency of our qPCR detection method with other published methods on various sample types (in preparation).

This method also suggests that *B. fagacearum* spores stay associated with the nitidulids, either by ingestion when insects feed on bulging fungal mats, or simply by adhering to their exoskeleton while doing so. Spores do not seem to be completely washed away in the collection fluid of the traps. Results from 2019 indicate that detection of insect samples yielded better results than sieved collection fluid samples. Since the latter necessitated considerable work, we decided not to include the analysis of collection fluids in subsequent years.

Despite all this, we feel that the present methodology could be enhanced to further reduce the risk of establishment of oak wilt in Canada. To facilitate the transition of this study into a completely functional biosurveillance programme, the trapping system should first be expanded to include more sites along the US-Canada border in Ontario and Manitoba, at the very least. Obviously, locations surrounding the recent oak wilt detections in Niagara Falls and Springwater in Ontario should be included. Other baiting techniques could also be explored, such as the use of a saturated salt solution instead of propylene glycol or ethanol, which is very affordable, non-toxic and non-flammable (Young et al. 2021). This early warning method also promotes awareness and communication with citizens, encouraging them to be more alert should symptoms of the disease appear at the border.

Work is also already underway in our laboratory to combine this qPCR TaqMan-specific assay with metabarcoding, a high-throughput sequencing method, in order to detect a wider variety of fungal pathogens and other pests that may lurk in the samples and otherwise go unnoticed (Bilodeau et al. 2021). This could also evaluate the current limitations of the insect trap method used here. Whole genome amplification and Nanopore MinION sequencing have already been used to identify plant pathogens and pests such as insects, bacteria, fungi and nematodes (Frey et al. 2022), although not through a biosurveillance programme. These methods are gaining in popularity due to their broad investigation range. Indeed, adding markers for insects could allow for identification of potential new vectors. If done regularly, this would enhance and complete our oak wilt biosurveillance programme by preventing further introduction of the pathogen or vectors we never thought of suspecting before. As an example, metabarcoding has successfully detected invasive pests at the Canadian border, using the same Lindgren traps, but replacing propylene glycol and ethanol with a saturated salt solution (Tremblay et al. 2019; Milián-García et al. 2021). Portable tools and tests are also currently being evaluated to see if fast and sensitive detection of the oak wilt pathogen can be achieved at sampling locations (Bourgault et al. 2022).

In conclusion, this study demonstrates that our monitoring approach, from insect baiting in strategically chosen trap locations to the qPCR detection of *B. fagacearum*, is efficient and reliable. It also shows that a sound biosurveillance network monitoring oak wilt is possible in Canada. Our method has been validated with positive controls from Michigan and gives satisfactory results. We also report the detection of *B. fagacearum* DNA in Ontario, close to the US border. With the recent and shocking announcement of the first official oak wilt detection in Canada, we hope this method can be used to help prevent the establishment and spread of this disease in the country.

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No potential conflict of interest was reported by the author(s).

ORCID

Guillaume J. Bilodeau http://orcid.org/0000-0003-1766-229X

Philippe Tanguay http://orcid.org/0000-0002-6247-0521

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