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Foliar nematode, *Litylenchus crenatae* ssp. *mccannii*, population dynamics in leaves and buds of beech leaf disease-affected trees in Canada and the US

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Abstract

A foliar nematode, *Litylenchus crenatae* ssp. *mccannii*, is associated with beech leaf disease (BLD) symptoms. Information about the types of tissues parasitized and how nematode populations fluctuate in these tissues over time is needed to improve surveys as well as understand the nematodes role in BLD. During this study, the nematode was detected throughout the known range of BLD by researchers at both Canadian and US institutions using a modified pan method to extract nematodes. Monthly collections of symptomatic and asymptomatic leaves during the growing season (May–October), and leaves and buds between growing seasons (November–March), revealed that nematodes were present in all tissue types. Progressively larger numbers of nematodes were detected in symptomatic leaves from Ohio and Ontario, with the greatest detections at the end of the growing season. Smaller numbers of nematodes were detected in asymptomatic leaves from BLD-infected trees, typically at the end of the growing season. The nematode was detected overwintering in buds and detached leaves. The discovery of small numbers of nematodes in detached leaves at one location before BLD was detected indicates that nematodes may have been present before disease symptoms were expressed. Other nematodes, *Plectus* and *Aphelenchoides* spp., were infrequently detected in small numbers. Our findings support the involvement of the nematode in BLD and indicate that symptoms develop only when certain requirements, such as infection of buds, are met. We also found that the nematode can be reliably detected in buds and leaves using the modified pan extraction method.

KEYWORDS

American beech, Chinese beech, disease development, European beech, *Fagus engleriana*, *Fagus grandifolia*, *Fagus orientalis*, *Fagus sylvatica*, foliar nematode, *Litylenchus*, Oriental beech, symptoms

1 | INTRODUCTION

Beech leaf disease (BLD) is a newly described tree disease that occurs in United States and Canadian forests on American beech (*Fagus grandifolia* Ehrh.) and in plantings of European (*Fagus sylvatica* L.), Oriental (*Fagus orientalis* Lipsky.) and Chinese beech (*Fagus engleri* Seemen ex Diels.; Burke, Hoke, & Koch, 2020; Ewing, Hausman, Pogacnik, Slot, & Bonello, 2019). The disease was first reported in Lake County, Ohio (USA) in 2012 and is now known to occur in Pennsylvania, New York, Connecticut (USA) and Ontario (Canada; Ewing et al., 2019; Marra & LaMondia, 2020). Leaf symptoms include swelling and darkening of interveinal leaf tissues as well as chlorosis, tissue necrosis and leaf curling at more advanced stages of the disease (Figure 1). The first symptom is typically striped bands resulting from the darkening of interveinal tissues (Ewing et al., 2019). Severe infections in overstorey beech have been observed in the United States and Canada. In the United States, mortality of infected, understorey beech has been reported after several years of disease (Ewing et al., 2019).

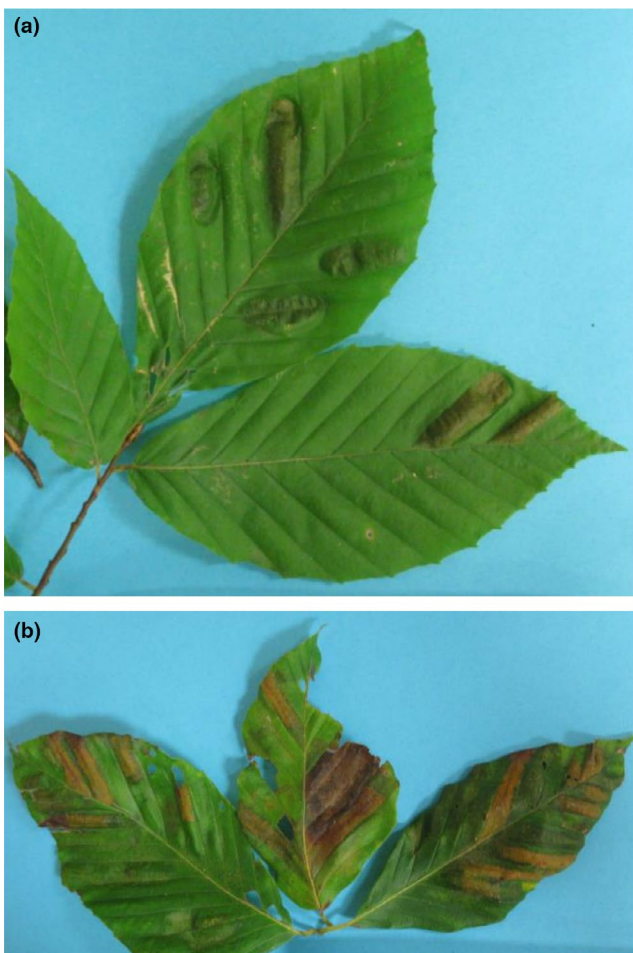


FIGURE 1 American beech leaves (*Fagus grandifolia* Ehrh.) with beech leaf disease symptoms. Bands of darkened, interveinal tissues are characteristic of low severity infections (a). Severe beech leaf disease symptoms include thickening of tissues, curling, chlorosis and necrosis (b)

Parasitism of American beech leaves and buds by the nematode, *Litylenchus crenatae* ssp. *mccannii* (Carta et al., 2020) is known to result in BLD (Carta et al., 2020). The mechanisms resulting in disease development and whether other organisms, such as the bacteria *Mucilaginbacter*, are needed for symptoms to develop are being investigated (Burke et al., 2020). *Litylenchus crenatae* Kanzaki, Ichihara, Aikawa, Ekino, and Masuya (2019) was first described from Japan, parasitizing and forming galls on leaves of Japanese beech (*Fagus crenata* Blume). Other anguinids known to cause similar leaf symptoms are *Ditylenchus leptosoma* Geraert & Choi, 1990 reported parasitizing *Carpinus laxiflora* (Siebold & Zucc.) Blume in Korea, *Subanguina chilensis* Vovlas, Troccoli & Moreno, 2000 reported parasitizing a *Nothofagus* species in Chile, and *Zeatylenchus pittosporum* Zhao, Davies, Alexander & Riley, 2013 reported parasitizing *Pittosporum* species in New Zealand (Li, Xu, & Zhao, 2017). *Litylenchus coprosma* Zhao, Davies, Alexander, and Riley (2011) has been reported parasitizing *Coprosma repens* A. Rich in New Zealand, but leaf symptoms are limited to small, rounded, chlorotic patches of leaf tissue.

Researchers have described some life-history characteristics of nematodes of the genus *Litylenchus*. Kanzaki et al. (2019) observed adult male and female *L. crenatae* in beech leaf mesophyll tissue, and Carta et al. (2020) observed female, juvenile and eggs of *L. crenatae* ssp. *mccannii* in buds of beech. Similarly, Zhao et al. (2011) reported the presence of *L. coprosma* eggs and adults in leaf mesophyll tissues and suggested that the species carries out its entire life cycle in the leaf. Zhao et al. (2011) did not find *L. coprosma* in soil under the tree, nor did they find evidence that the species could survive desiccation. They also found that overall population sizes of *L. coprosma* declined when leaves were stored at 5°C for 2 weeks, and the ratio of slender to semi-obese females shifted towards more semi-obese individuals (Zhao et al., 2011).

Here, we report the current detection of *L. crenatae* ssp. *mccannii* relative to the range of BLD. We also describe the population dynamics of the nematode in different types of tissues as they fluctuate throughout the year as a contribution to our understanding of BLD development and the life history of *Litylenchus* nematodes, with the goal of improving BLD surveys. In addition, we assessed if a simple, modified pan method could be used to detect nematodes in different types of tissues at different times of the year (Townshend, 1963; Zhen, Agudelo, & Gerard, 2012).

2 | METHODS

2.1 | Detecting *L. crenatae* ssp. *mccannii* in Canada and the United States

Confirmations of the nematode *L. crenatae* ssp. *mccannii* from BLD symptomatic samples were undertaken by taxonomists at Agriculture and Agri-Food Canada (AgCanada) and the USDA-ARS laboratory. Ontario nematode samples submitted to AgCanada by the Ministry of Natural Resources and Forestry (MNR) pathology

diagnostician were from an informal BLD survey by provincial forest health technicians between June 2018 and October 2019. All suspect BLD samples were sent for examination for *L. crenatae* ssp. *mccannii*. In the United States, BLD suspect samples were submitted to the USDA-ARS laboratory by state, university and arboretum staff, with no requirement that the nematode be confirmed for all locations with BLD symptoms.

2.2 | Detecting and assessing abundance of *L. crenatae* ssp. *mccannii* in beech tissues at select sites over a year

2.2.1 | Sampling during growing season (May–October)

In Ontario, three forested locations with BLD were identified in June 2018. These locations were within 46 km of one another and within 9 km of Lake Erie near Alymer, Port Stanley, and Welsingham in Elgin and Norfolk counties. We categorized these stands as *BLD easily detected* because BLD was spread throughout and symptoms were present in the under- and overstory. At each location, one to two symptomatic, understory beech trees were flagged for sampling from June to August. Before September, additional symptomatic trees were flagged at all three locations for a total of five trees per location. For each symptomatic beech tree, five asymptomatic and five symptomatic leaves were collected the second week of each month between June and October.

In June of 2018, one “control” site (no symptomatic trees) was identified 29 km north of London, Ontario. One understory beech tree was randomly selected, marked, and asymptomatic leaves were collected. Sampling was repeated the second week of July and again in August. As was done for sites with BLD, the number of trees flagged and sampled was increased to five before September. During this process, one tree with four BLD symptomatic leaves was found. These leaves were collected, and the presence of nematodes was confirmed by AgCanada. Additional survey of the London control site did not reveal any additional trees or leaves with BLD symptoms. A decision was made to continue sampling at the London, Ontario site, and to establish a fifth site 29 km to the southwest in Delaware, Ontario (Middlesex county) that had no detectable BLD symptoms. As at the other sites, five understory beech trees were randomly selected and tagged to sample asymptomatic leaves. The Delaware and London, Ontario, sites were categorized as *few to no trees with BLD symptoms*, with the understanding that BLD is likely present or developing in these stands, which are at the edge of the known range of the disease.

Sampling of symptomatic and asymptomatic leaves was repeated from May to August 2019. All sampling was performed as before, with five trees sampled at each location for a total of 15 trees. The 2019 locations with BLD were near Wallacetown, Alymer, and Fairground, Ontario, in Elgin and Norfolk counties. Like the stands sampled in 2018, BLD symptoms were easily detected at the 2019

sites, with BLD present throughout the stand in the under- and overstory. Asymptomatic leaves were also collected at three Ontario locations more than 80 km outside the known range of BLD, near the towns of Goderich, Bognor, and Peterborough in Huron, Grey and Peterborough counties.

In Ohio, between August and November 2018, BLD symptoms were detected in an experimental plantation at Holden Arboretum containing beech-scale resistant trees established in 2007 (Koch, Carey, Mason, & Nelson, 2010). The presence of *L. crenatae* ssp. *mccannii* in leaves of trees at the plantation was confirmed by the USDA-ARS laboratory in Beltsville, Maryland, using microscopic examination. One severely affected *F. grandifolia* tree, ID No. 2014-57-M, was chosen for biweekly sampling to quantify changes in nematode populations between August and November. Five leaves were sampled each time. Fourteen additional *Fagus grandifolia* trees with varying levels of symptoms were sampled on 8 October to quantify the numbers of nematodes present in samples taken from BLD-affected trees. Again, five leaves were collected from each tree.

2.2.2 | Sampling between growing seasons (October–March)

In Ontario, beech buds, attached leaves and detached leaves were collected to determine whether the nematode persisted in these tissue types over winter. Buds and attached and detached leaves were collected the second week of every month between November 2018 and March 2019 from the same 25 trees sampled between June and October. Five attached and five detached leaves were randomly selected per sample. Detached beech leaves were collected from the leaf litter directly below the sample tree. Twigs with buds were pruned from trees for a total of 5–10 buds per sample. No attempt was made to discern between symptomatic and asymptomatic leaves at BLD sites because September and October sampling had revealed *Litylenchus* nematodes in both types of leaves and it became increasingly difficult to differentiate between BLD symptoms and other types of damage.

Some symptomatic beech trees did not have enough attached leaves for collection in all five months. Collections from these trees were made until only a few leaves remained. The last leaves were removed during the March sampling to better understand the effects of the full cold period on nematode populations in attached leaves.

All samples collected during and after the growing season were bagged separately and placed on ice packs immediately after sampling. Nematode extractions and counting were performed by AgCanada and Holden Arboretum staff in 2018 and by MNRF staff in 2019.

2.2.3 | Extracting and identifying the nematode from leaves and buds

Nematodes were extracted using a quick, easy, modified pan method, also called a water-soaking method, as these are quick, easy,

and can be carried out in laboratories without equipment for DNA-based assays (Townshend, 1963; Zhen et al., 2012). In Ontario, three leaves were randomly selected from each sample. Each leaf was cut in half and nematodes were extracted from one of the halves. Each half leaf was cut into 1–2 mm wide strips, placed in a Petri dish, and submerged in 50 ml water overnight. Nematodes were left to settle naturally in the bottom of the tube for 3 hr, water was drawn off, and the entire 2 ml of remaining sample was placed in a gridded dish before counting a tenth of the cells.

In Ohio, the entire sample of five leaves was cut into 2 cm² pieces before soaking overnight at 22°C (Zhen et al., 2012). The sample was centrifuged at 1252 g for 3 min to form a nematode pellet. The nematodes were redistributed in 1 ml of water. Approximately 200 µl of the nematode suspension was removed and 200 µl of 100% ethanol was added to kill the nematodes before counting. Nematodes were counted using a Sedgewick rafter at a magnification of 4×, on an Olympus BH-2 microscope. The nematodes remaining in the sterile water were used for DNA extraction and sequence analysis.

In Ohio, DNA extracted from leaf tissue, using a bead beating phenol–chloroform extraction (Burke, Smemo, López-Gutiérrez, & DeForest, 2012) followed by precipitation in 20% polyethylene glycol 8,000 with 2.5 M NaCl, was used to confirm nematode identity. We used specific primers, forward TW81 (5'-GTTCCGTAGGTGAACCTGC-3') and reverse primer 5.8MS (5'-GGCGCAATGTGCATTCGA-3'), to amplify the ITS region from nematodes (Subbotin, Maafi, & Moens, 2003; Vovlas, Subbotin, Troccoli, Liébanas, & Castillo, 2008). Amplification reactions were performed in an MJ Research PTC-200 thermal cycler (Bio-Rad Laboratories, Inc.). An initial denaturation for 2 min at 94 °C was followed by 35 cycles of denaturation for 30 s at 94 °C, annealing for 45 s at 55 °C and extension for 3 min at 72 °C, with a final extension of 10 min at 72 °C (Esmaeili, Heydari, & Ye, 2017). Sequence accession numbers for nematodes recovered at Holden Arboretum were MN625146–MN625161.

2.3 | 3 Analysing nematode detection and abundance data

We used Fisher's exact test to determine whether the probability of detecting *L. crenatae* ssp. *mccannii* was similar in symptomatic and asymptomatic leaves at BLD sites during 2018 and 2019. The multiple comparisons form of the test was used to determine whether detections were more likely in September or October 2018 or any of the months sampled during 2019. We also used this test to determine whether the probability of detecting the nematode was similar in buds and attached and detached leaves, and if the probability of detecting *L. crenatae* ssp. *mccannii* was similar for all winter months. To determine which of the multiple comparisons were significant, we ran a Fisher's exact test for each comparison and to reduce the risk of type I error ($\alpha = 0.05$) applied Bonferroni's correction for multiple comparisons (MacDonald, 2014; MacDonald & Gardner, 2000).

To determine whether the abundance of nematodes differed in detached leaf and bud samples over time, we used a mixed

model with repeated measures (PROC GLIMMIX, SAS 9.4; SAS Institute) and the default distribution. Random factors were site and site × time (month of sampling) and site × treatment interactions. We removed the site by treatment factor from the random statement because its convergence status was zero. The covariance structure AR(1) had the smallest AIC score, and its use resulted in a positive definitive g matrix. The subject in the random statement was tree with site nested in tree. Fisher's least square means procedure was selected to compare among treatments, time and treatment × time interactions.

The same options as used for the GLIMMIX procedure were used to analyse differences in nematode abundance in symptomatic and asymptomatic leaves for May to August 2019 and the September to October 2018 timeframes.

3 | RESULTS

3.1 | Detections of *L. crenatae* ssp. *mccannii* in Canada and the United States

Ontario MNRF staff detected *L. crenatae* ssp. *mccannii* nematodes at all Ontario locations with BLD symptoms (Figure 2). The USDA-ARS laboratory staff extracted the nematode from symptomatic beech leaves submitted by state agents and professionals from Pennsylvania, Ohio, Connecticut and New York (Figure 2). In US samples, nematodes extracted from beech leaves over a period of 18–44 days ranged from 3,023 to 6,805 per leaf.

3.2 | Detection and abundance of *L. crenatae* ssp. *mccannii* in beech tissues at select sites over a year

3.2.1 | Growing season 2018—Ontario

The only nematodes detected in beech leaves (111 samples) during the 2018 growing season were *L. crenatae* ssp. *mccannii* and a *Plectus* species. Four individuals of the *Plectus* species occurred in one asymptomatic leaf sample collected in October from the Delaware site.

At the three sites with easily detectable BLD, *L. crenatae* ssp. *mccannii* occurred in symptomatic leaves between June and August. As the growing season progressed, the number of nematodes counted increased steadily from a few individuals to hundreds of individuals per leaf (Figure 3). Sample sizes were too small to determine whether trends were statistically significant. Between June and August, nematodes were not detected in asymptomatic leaves.

In the fall at sites with easily detected BLD, *L. crenatae* ssp. *mccannii* was detected less frequently in asymptomatic leaf samples than in symptomatic leaf samples (16 of 29 samples versus 26 of 30; $p = .0101$). The nematode was just as likely to be detected in samples collected in September (20 of 30 positive) as in October (22 of 29 positive; $p = .57$).

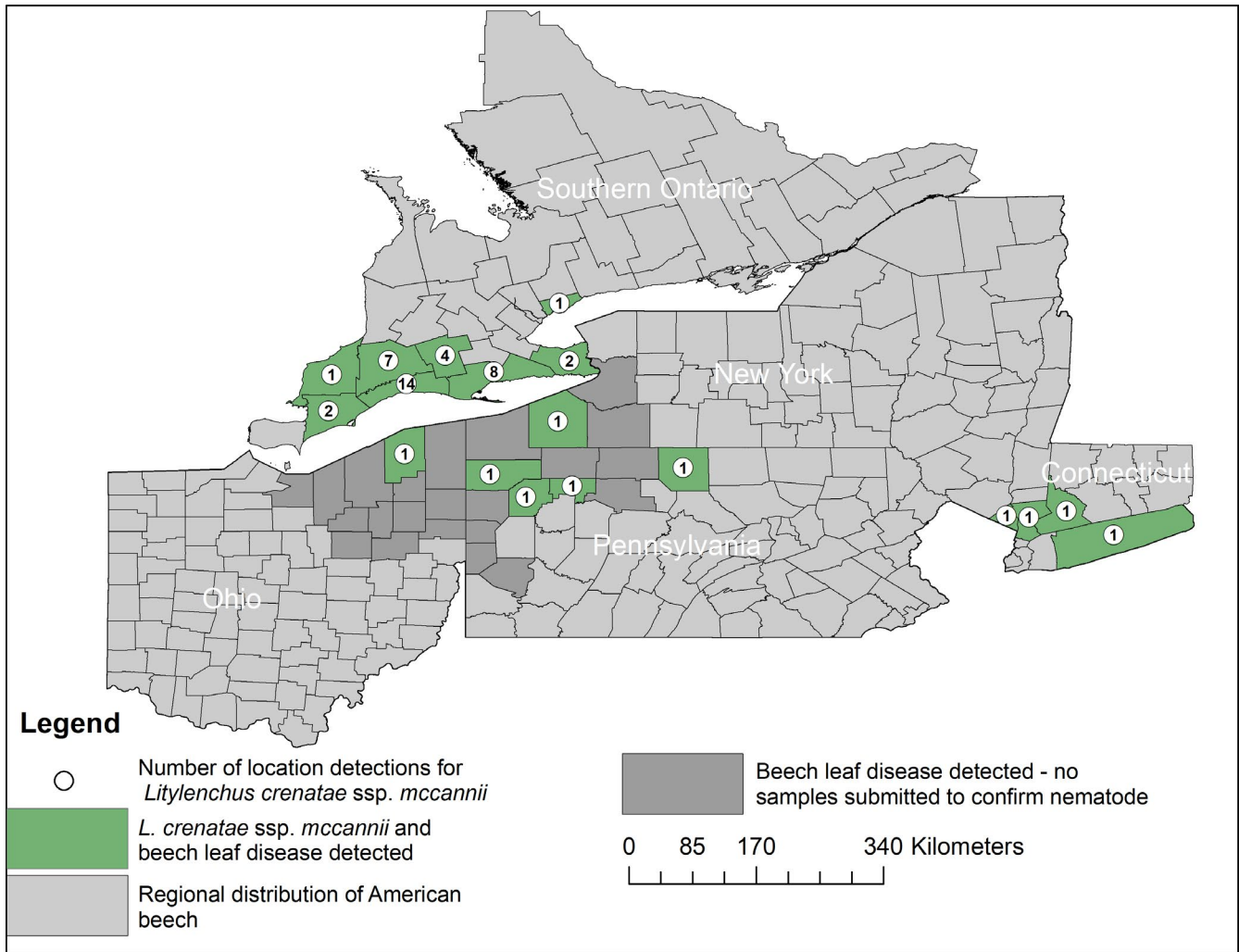


FIGURE 2 Number of locations where the nematode, *Litylenchus crenatae* ssp. *mccannii* (Carta et al., 2020), was detected in Canadian and US counties in the Great Lakes Region and along the Atlantic coast as well as the known range of beech leaf disease (BLD) as of October 1, 2019. BLD US county distribution in part from Martin, Volk, Macy, and Pognacik (2019). Samples were not submitted from outside the known range of beech leaf disease

At locations with easily detected BLD, numbers of nematodes counted in symptomatic leaf samples ranged from 0 to 33,000 and in asymptomatic samples from 0 to 100 per sample. Symptomatic leaf samples ($3,347 \pm 967$ SE) had more nematodes than asymptomatic leaf samples (7 ± 2.6 SE; $F_{(1,14)} = 9.31, p = .009$). September ($3,362 \pm 1,435$ SE) and October (775 ± 327 SE) samples had similar numbers of nematodes ($F_{(1,14)} = 3.80, p = .07$) in both types of leaf samples.

For the two locations with few to no trees with BLD, *L. crenatae* ssp. *mccannii* nematodes were only found in four symptomatic leaves collected from the London, Ontario, site in September when the study was being expanded. No additional symptomatic leaves were found at the London site in September or October, and no symptoms or nematodes were observed at the Delaware, Ontario, site during this period.

3.2.2 | Growing season 2018—Ohio

In Ohio, nematode populations in leaves from tree No. 2014-57-M increased in fall 2018 and then decreased as leaf senescence

occurred. Between 7 August and 19 September 2018, nematode populations ranged from 28 to 87 nematodes per gram of leaf tissue. Whole beech leaf weights typically ranged from 0.12 to 0.2 g. In early October, nematode populations began to rise, eventually reaching a peak of 4,589 nematodes per gram on October 31, 2018 (Figure 4). Within a week of peaking, nematode numbers decreased to 19 nematodes per gram of leaf tissue. Nematode populations in Ohio varied greatly among trees. Additional counts conducted on 8 October 2018 from 14 trees at the Holden site ranged from 0 to 19,531 nematodes per gram of leaf tissue (mean = $1,794 \pm 1,376$).

3.2.3 | Growing season 2019—Ontario

Sampling of symptomatic and asymptomatic leaves from 15 trees between May and August 2019 confirmed the trends observed during the same period in 2018. Except for a single *Plectus* sp. individual, *L. crenatae* ssp. *mccannii* was the only nematode detected in 120 samples taken from sites with BLD. No *L. crenatae* ssp. *mccannii*

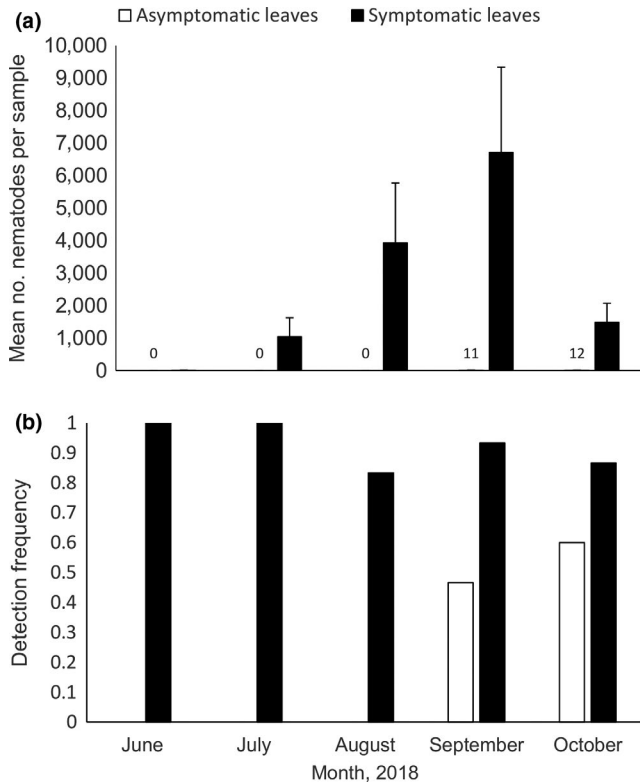


FIGURE 3 (a) Mean number of *Litylenchus crenatae* ssp. *mccannii* (Carta et al., 2020) nematodes per leaf sample (\pm SE) and (b) frequency of their detection from asymptomatic and symptomatic leaves between June and October 2018 from three locations with easily detectable beech leaf disease in southwestern Ontario ($n = 1$ –2 trees per site June through August and 5 trees per site in September and October)

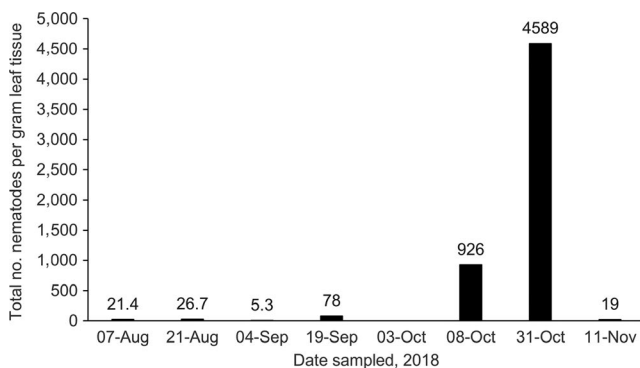


FIGURE 4 Total number of *Litylenchus crenatae* ssp. *mccannii* (Carta et al., 2020) per gram leaf tissue collected from *F. grandifolia* Ehrh. tree, ID No. 2014-57-M (Holden Arboretum) during fall 2018. Five leaves were sampled each time

individuals were detected at locations without detectable BLD. Only a *Plectus* sp. was detected in five of the 120 samples taken from the three sites with no detectable BLD.

At locations with easily detected BLD, numbers of *L. crenatae* ssp. *mccannii* increased from almost no immature or adult nematodes in May to $1,858 \pm 469$ SE nematodes per sample in August. Almost all nematodes were collected from symptomatic leaves (599 ± 153

SE), with very few observed from asymptomatic leaves ($<1 \pm 0.1$ SE; $F_{(1,14)} = 24.26$, $p = .0002$). The exception was four nematodes extracted from one asymptomatic leaf sample in June. The number of nematodes per symptomatic leaf sample increased from June to a maximum in August ($F_{(3,42)} = 13.71$, $p < .0001$; Figure 5). Numbers of nematodes collected from symptomatic leaves did not differ in May and June (Figure 5).

Litylenchus nematodes were more likely to be detected in samples taken from symptomatic trees in June, July and August than in those collected in May ($p = .002$ for May–June comparison, $p < .0001$ for all other comparisons with May; Figure 5). *Litylenchus crenatae* ssp. *mccannii* nematodes were equally likely to be detected in leaf samples from symptomatic trees in June, July and August ($p = 1.0$ all comparisons).

3.2.4 | Between growing seasons—Ontario

Between November 2018 and March 2019, we detected *L. crenatae* ssp. *mccannii* in buds and attached and detached leaves at locations with easily detectable BLD symptoms. Overall, we collected 75 buds, 60 attached and 75 detached leaf samples from these locations. The nematode was detected more often in buds and detached leaves than attached leaves ($p < .0001$) and was just as likely to be detected in buds as detached leaves (Figure 6). The nematode was detected more often in beech leaves and buds in November compared with

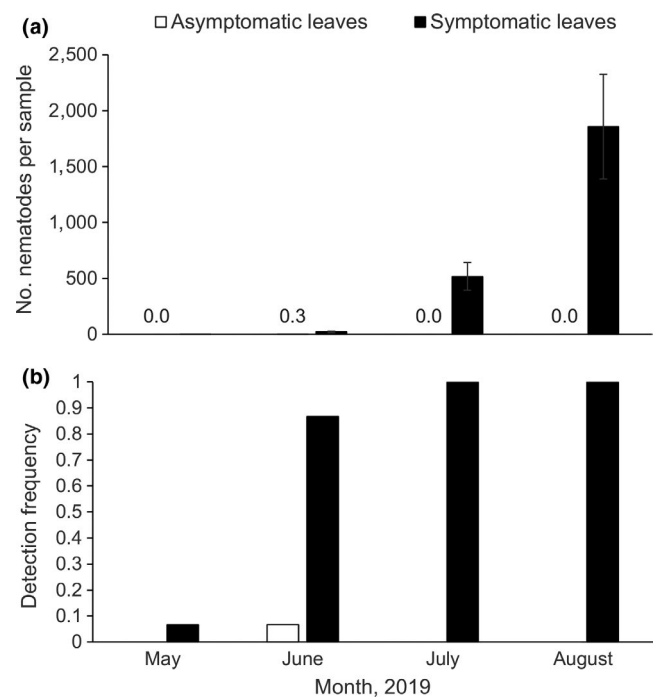


FIGURE 5 (a) Mean number of *Litylenchus crenatae* ssp. *mccannii* (Carta et al., 2020) nematodes per leaf sample (\pm SE) and (b) frequency of their detection from 15 trees with asymptomatic and symptomatic leaves between May and August 2019. Samples were taken from locations with easily detectable beech leaf disease in southwestern Ontario

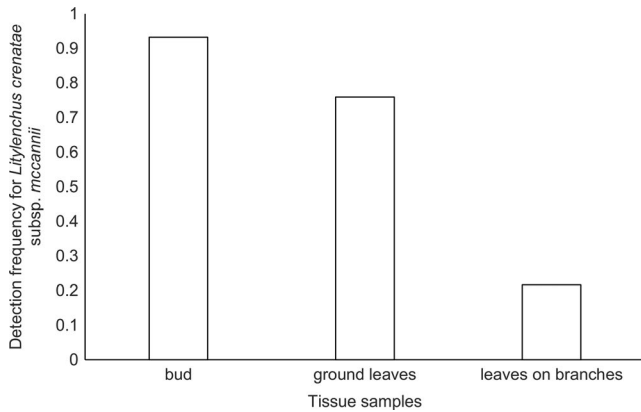


FIGURE 6 Frequency of detecting *Litylenchus crenatae* ssp. *mccannii* (Carta et al., 2020) in samples consisting of buds, attached leaves or detached leaves during late fall and winter months from three locations with easily detectable beech leaf disease between November 2018 and March 2019

December ($p = .0006$), February ($p = .0007$) and March ($p < .0001$; Figure 7).

During the winter, similar numbers of *L. crenatae* ssp. *mccannii* were found in buds (528 ± 131 SE) and detached leaves ($1,082 \pm 327$ SE) at sites with easily detected BLD ($F_{(1,70)} = 2.76$; $p = .10$). Attached leaves had few or no nematodes after the initial sample in November 2018. Abundance data for attached leaves were not analysed because too few leaves were available at some sample times.

The numbers of *L. crenatae* ssp. *mccannii* nematodes in samples changed between late fall and winter ($F_{(4,56)} = 3.89$, $p = .007$). More nematodes occurred in November samples ($2,105 \pm 810$ SE) than in any other month (contrast Dec. $p = .003$; Jan. $p = .008$, Feb. $p = .001$, Mar. $p = .004$). Despite the large number of nematodes detected in detached leaf samples in November ($3,367 \pm 1,466$ SE), the interaction between sample type and sampling month was not significant ($F_{(4,70)} = 2.38$, $p = .06$; Figure 8).

We also detected *L. crenatae* ssp. *mccannii* during the late fall and winter of 2018–19 at locations with few to no trees with detectable

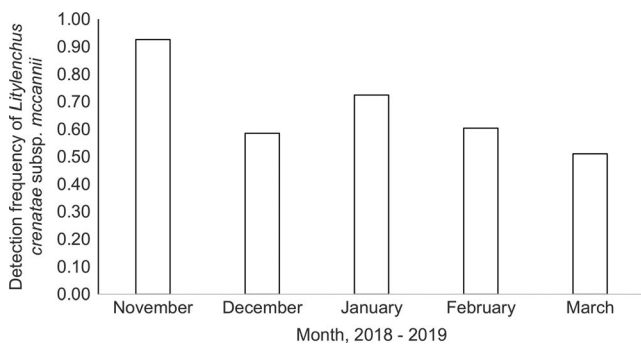


FIGURE 7 Frequency of detecting *Litylenchus crenatae* ssp. *mccannii* (Carta et al., 2020) in leaf and bud samples collected each month from three locations with easily detectable beech leaf disease between November 2018 and March 2019

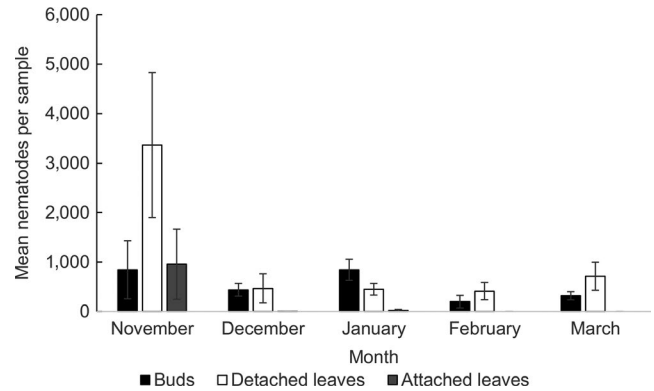


FIGURE 8 Mean number of *Litylenchus crenatae* ssp. *mccannii* (Carta et al., 2020; \pm SE) collected from buds, attached leaves or detached leaves from three locations with easily detectable beech leaf disease, between November 2018 and March 2019 ($n = 75$ bud, detached leaves, $n = 60$ for attached leaves)

BLD symptoms. Of the 50 detached leaf, 50 attached leaf, and 50 bud samples, *L. crenatae* ssp. *mccannii* was found in three detached leaf samples. These detections were from different trees and sites, and occurred in different months (November, December and January). One detection was at the London site where four BLD symptomatic leaves were found in September 2018 and the other two were at the Delaware site where no symptoms had been reported. All samples contained 20 or fewer *L. crenatae* ssp. *mccannii* individuals.

We detected two other genera of nematodes in beech leaves and buds during the winter, including eight occurrences of *Plectus* nematodes (in bud, attached and detached leaves) and two occurrences of *Aphelenchoides* nematodes (detached leaves). Ten or fewer individuals occurred in each of these samples. In total, ten of the 360 samples collected between growing seasons contained *Plectus* or *Aphelenchoides* nematodes.

4 | DISCUSSION

Litylenchus crenatae ssp. *mccannii* has been detected throughout the known range of BLD in Ontario, Connecticut, New York, Ohio and Pennsylvania. As of fall 2019, MNR staff had detected *L. crenatae* ssp. *mccannii* only at locations with BLD symptoms. Even at our initially BLD-free site, from which a small number of *L. crenatae* ssp. *mccannii* nematodes were collected in November 2018, a symptomatic leaf was detected in August 2019 after additional searches of the under- and overstory. BLD may have been newly establishing at this location, as small numbers of nematodes do occur in asymptomatic leaves at symptomatic sites. As of fall 2019, No *L. crenatae* ssp. *mccannii* nematodes had been detected at three Ontario sites more than 80 km north of the known BLD range.

In Ontario, *L. crenatae* ssp. *mccannii* nematodes were present in asymptomatic beech leaves but in much smaller numbers than in symptomatic leaves. The presence of pathogenic nematodes in asymptomatic tissues is not unusual. In general, successful infection

and symptom development result from the presence of the infective stage of a pathogenic nematode and host conditions that optimize nematode growth and reproduction. The nematode must have fulfilled all dormancy requirements. The foliar nematode, *Aphelenchoides fragariae* (Ritzema Bos, 1891) Christie, 1932 occurs in asymptomatic *Lantana* leaves in small numbers, typically 10 or less nematodes per gram fresh weight (Kohl et al. 2010). In the same study, symptomatic *Lantana* leaves were found to have more than 100 nematodes per gram fresh weight. For *A. fragariae*, these small populations in asymptomatic leaves resulted from recent dispersal events. This may be the case for *L. crenatae* ssp. *mccannii*, or it may be that disease development requires infection of buds or immature leaves or an additional disease agent (Burke et al., 2020; Carta et al., 2020).

Detections of *L. crenatae* ssp. *mccannii* in asymptomatic leaf samples from locations with severe BLD symptoms were higher during fall 2018 than previously. This difference could be the result of increased sampling after August 2018 but sampling during the 2019 growing season confirmed that asymptomatic leaf samples rarely had detectable levels of the nematode between May and August. Foliar nematodes are known to migrate in films of water or with the assistance of insects and other animals (Kohl, 2011). Although determining exactly when or how nematodes migrated to asymptomatic tissues is beyond the scope of this study, the data suggest that in Ontario populations, movement occurs before September.

Small numbers of the nematode were extracted from leaves early in the growing season. More nematodes may have been present in newly emerged leaves. Eggs of *L. crenatae* ssp. *mccannii* have been reported in beech buds and newly emerged leaves and may have been the dominant form at the time (Carta et al., 2020). Eggs were not included in this study because they are difficult to quantify and identify to species.

Populations increased over time and were highest in late summer and early fall, with hundreds to many thousands of nematodes present in leaf samples. Data from Holden Arboretum in Ohio confirmed increasing populations, and large numbers of nematodes were present in symptomatic leaves in fall. Many parasitic nematode species complete their life cycle in a few weeks to a month, resulting in rapid increases in population sizes (Kohl, 2011; Shurtleff & Averre III, 2000). In Ontario, the number of nematodes extracted from symptomatic leaves decreased between September and October. This decrease coincided with the start of leaf senescence and nighttime temperatures below 10°C in late September. Environment Canada data from a weather station in St. Thomas, Ontario (near the sample locations) indicated nighttime temperatures as low as 3.5°C on 5 October 2018, which may have decreased the quality of beech leaves as a food source.

Populations of nematodes varied by 10 to 100-fold among symptomatic leaf samples collected from trees in Ontario and Ohio. The current study did not differentiate between leaves with severe and slight symptoms. The few symptomatic leaf samples that did not yield *L. crenatae* ssp. *mccannii* nematodes in fall 2018 in Ontario were noted as severely affected by the disease. Tissues of severely

affected leaves are thickened, chlorotic, curled, and necrotic and may no longer be suitable for sustaining nematodes.

Litylenchus crenatae ssp. *mccannii* populations overwintered in leaves on the ground and in beech buds but did not survive in the desiccated leaves still attached to the tree. For most of the winter, detached leaves on the ground were protected from desiccation by snow and ice. Some *Aphelenchoides* species are also known to overwinter in buds and dehisced leaves (Jagdale & Grewal, 2006). The presence of nematodes in leaves on the ground throughout the winter indicates one potential pathway for local movement. Detached leaves may be dispersed by wind, introducing the nematode to new locations.

Numbers of nematodes extracted from beech buds and detached leaves were similar for all winter months. No significant decreases in extraction numbers were observed after a polar vortex in late January 2019. Temperatures reached as low as -26°C on 2 February 2019 in St. Thomas, Ontario. Detached leaves were protected by snow, but buds were exposed. Several freezing rain events that occurred during the winter did not seem to affect nematode counts.

One goal of the current study was to develop a better understanding of the type of tissue to sample using the modified pan method. The modified pan method was used successfully to detect nematodes in symptomatic leaves during the growing season except during May 2019. Late spring may be when adult nematode numbers are low, and eggs are the predominant form. Similar observations were made when surveying for BLD symptoms and *L. crenatae* ssp. *mccannii* throughout southern Ontario. *Litylenchus crenatae* ssp. *mccannii* was not detected in symptomatic samples collected in May and early June but when the same trees were resampled later in the growing season it was detected.

Detection frequencies for *L. crenatae* ssp. *mccannii* decreased after November; however, 50% or more of the samples collected in any month from BLD infected trees were positive for *L. crenatae* ssp. *mccannii*. These results suggest that the nematode can be detected by sampling buds and detached leaves during winter, but that larger sample sizes are needed to detect it reliably.

Other nematode species were extracted infrequently (17 of 711 samples) and in small numbers from beech buds and attached and detached leaves. Detections consisted of ten or fewer *Plectus* or *Aphelenchoides* nematodes per sample. This contrasts with numbers of *L. crenatae* ssp. *mccannii*, which were often 10 or 100-fold higher in symptomatic tissues collected between July and March. This difference in population numbers may be useful for diagnosticians determining which BLD suspect samples need to be further scrutinized.

In summary, *L. crenatae* ssp. *mccannii* has been detected from symptomatic American and European beech trees throughout the range of BLD. At six Ontario locations with BLD, it was consistently associated with symptomatic leaves. The nematode was also associated with symptomatic leaves at Holden Arboretum in Ohio. In both Ontario and Ohio, nematode populations increased in symptomatic tissues until the fall. In Ontario, the nematode overwinters in detached leaves and buds. In winter 2018–19 in Ontario, cold temperatures did

not seem to affect nematode populations. The nematode was present in small numbers in asymptomatic leaves at symptomatic sites, mainly during the fall when populations were very high in surrounding symptomatic leaves. At one location thought to be symptom free, the nematode was detected multiple times in asymptomatic leaves, but the site was later found to have one symptomatic leaf. Further research and monitoring are needed to determine how far the nematode *L. crenatae* ssp. *mccannii* occurs outside the range of BLD and if symptoms always develop at locations where it occurs.

The modified pan method was successfully used to detect nematodes in many different tissue types throughout the year. Late summer and early fall were the best times to extract live nematodes. Buds and detached leaves can be sampled in the winter, but to ensure successful detection, we suggest sampling more buds and leaves than used in this study.

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