Final Report

For more information and guidance on completion and submission of the report contact the Euphresco Call Secretariat (bgiovani@euphresco.net).

Project title (Acronym)
Plant health status of *Fagus* spp.(FAGUSTAT) Euphresco topic 2020_a_334

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Contents

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1.1. Executive Summary

Beech Leaf Disease (BLD) was detected in Ohio, USA, in 2012, on American beech (*F. grandifolia*). Since then it has spread to 12 northeastern states and Canada (Ontario), in forests and landscaped areas. The symptoms of BLD include interveinal dark bands on the leaves, crinkling and irregularly thickened leaves and twig dieback; ultimately leading to decline and death of young trees within years. Beech Leaf Disease causes severe damage to stands of *F. grandifolia*, but also to European beech, *F. sylvatica*, the main beech species in Europe. A new nematode, *Litylenchus crenatae* subsp. *mccannii* (Carta *et al.,* 2020)*,* is now considered as the causal agent of BLD (Vieira et al., 2023) but it was suggested that other microorganisms also play a role in the disease (Burke *et al.*, 2020; Ewing *et al.*, 2021). Pathways of transmission between trees have not been elucidated yet; they might include windborne water, mites, insects, or birds. BLD has never been reported from Europe and could be a severe threat to *Fagus* spp. stands.

In this project, we made a first assessment of the presence/absence of BLD in Europe. Surveys were organised in Belgium, Ireland, the Netherlands, Romania, Slovenia and the UK. A fact sheet about BLD was made to inform people in the field and samples were collected using a common sampling strategy. Samples were taken during summer and fall (July-November) in 2021 and 2022, from beech trees showing symptoms similar to those caused by BLD. This resulted in 658 samples taken at 561 sites. More sites were visually inspected, but not all had trees showing BLD-like symptoms. Nematodes were extracted from leaves, buds and beechnuts, using the Baermann technique, with or without mistifier. Occasionally some non-plant parasitic nematodes were observed, but *Litylenchus crenatae* subsp. *mccannii* was never found.

This project also aimed to increase public awareness of BLD and encourage the reporting of symptoms. Therefore, people in the field (inspectors, foresters, botanic gardens, parks, arboreta, NPPO…), as well as the general public, were informed via leaflets, posters, presentations, newsletters, publications in specialized magazines, institutional websites or observation platforms.

Although the nematode was not found, the microbiome of symptomatic and healthy leaves was studied in Belgium and UK, using RNA-sequencing (metatranscriptomics) and metabarcoding. There were no clear differences compared with genera of bacteria and fungi reported from America. The drivers for the fungal and bacterial leaf microbiome, studied using barcoding, were mainly “location” and “environment” (nursery vs forest). Attempts were made to obtain inoculum of *L. crenatae* for the evaluation of the host status of European beech cultivars and to study the nematode’s behaviour under European climatic conditions (in confined conditions), but acquiring the nematode was very difficult. Moreover, attempts to culture the nematode on carrot disks, fungal mycelium, in beech plantlets and on beech callus failed. We engaged with BLD researchers in the USA and Canada through regular online meetings with BLD researchers, receiving valuable and updated information.

To assess the possibilities of entry from America or Asia, information was gathered on the origin of beech tree materials. As current legislation does not allow import, except for some cases which require phytosanitary certificates, entry of infected leaves is theoretically impossible. Imports with BLD, could have occurred some decades ago however, so vigilance for the presence of BLD is still appropriate. The obtained information will contribute to pest risk assessments for Europe and the development of appropriate measures for protecting *Fagus* spp. in Europe.

1.2. Project aims

Description of the work-plan

A large part of our project will be dedicated to a comprehensive survey on the health status of *Fagus* spp. by sampling trees and analysing samples for the presence of nematodes, especially *L. crenatae* and similar leaf nematodes. The risk posed by the movement of beech trees will be assessed by screening beech trees in nurseries and acquiring information on (inter)national traffic of beech seed, seedlings and plantlets. We will also raise public awareness by involving the public, botanical gardens and arboreta, through existing networks and using various ways of outreach. In-depth studies are planned to understand the biology of the nematode(s) involved and the epidemiology of BLD. We will determine the microbial community of beech leaves and buds as input for a broader European picture of beech leaf microbiomes that can be compared with those in America. Finally, we will exchange expertise, findings and experiences with international partners.

Activities set out for project were:

- **survey** *Fagus* spp. for symptoms of BLD in forests, parks, botanical gardens and nurseries, and create public **awareness** for the disease (in the participating countries)
- collect data for the assessment of the **risk** of introduction and spread of BLD in Europe
- study the **biology** of the nematode and the epidemiology of BLD under European climatic conditions (if found, or other under simulated conditions in the glasshouse)
- investigate the **microbial community** of leaves and buds to describe the microbiome associated with symptomatic and asymptomatic buds and leaves
- explore measures to curtail the disease in an **international** context

1.3. Description of the main activities

All partners were involved in surveying and public outreach (workpackages 2 and 5), while the activities in the other workpackages were for ILVO and Fera. See description activities per partner.

WP1. Project management and coordination (ILVO)

Predefined tasks

- Task 1.1-1.4: Project Management.

Description activities partners

ILVO: Monitor the project progress, sustain dissemination of the project’s activities and results, act as the intermediary between the project partners, EUPHRESCO, and broader public. Organise consortium meetings, elaborate minutes, submit periodic progress reports and a final report.

WP2. Survey for BLD and identification of paths of entry and spread (all partners)

Predefined tasks

- T2.1 Sampling leaves, buds and nuts from symptomatic and asymptomatic trees
- T2.2 Extraction and identification of nematodes from plant tissues
- T2.3 Identify the different pathways of introduction and spread of BLD

Description activities partners

T2.1. A common surveying and sampling protocol was drawn up, discussed and agreed upon by all partners. All partners surveyed during summers and early fall over 2 years. Surveying and sampling was usually performed by a different organisation than the one analysing the samples (details given in 1.4 Main results). In most countries, only leaves showing symptoms very similar to those of BLD were sampled and analysed. Note that the number of samples taken are an underestimation of the number of trees observed because samples were taken from trees showing symptoms similar to those of BLD (look-alike symptoms).

T2.2. Extraction of nematodes was performed using Baermann funnels, sometimes in a mistifier (NL, BE, RO), depending on the lab. ILVO compared this method with nematode extraction using zonal centrifugation.

T2.3. Different pathways of introduction were described in quick/mini PRAs, based on literature, current legislation, available data on tree stands, and a questionnaire about the sources of *Fagus* plants (BE). Samples from nurseries and plantlet stores were included in the survey, as well as seeds (beechnuts), to check for spread of nematodes via planting materials (T2.1).

Description activities partners
See below (1.4 Main results) for details per partner.

WP3. Study the biology and epidemiology of *L. crenatae* under European climatic conditions

Predefined tasks

- T3.1. Study the biology of L. crenatae during and between growing seasons
- T3.2. Study the biology of *L. crenatae* under controlled conditions

Description activities partners

The plan was to study the life cycle and population dynamics of the nematode under natural conditions, if found (T3.1). As this was not the case, specimen of *Litylenchus crenatae* subsp. *mccannii* were requested from the USA to study its biology in beech plants maintained under biosafety conditions in the glasshouse (T3.2) and shared between the 2 partners involved in this WP.

ILVO:
In 2021, nematodes were extracted from infected leaves and buds obtained from NY, USA, and used to set up cultures. Nematodes were inoculated on carrot discs, on mycelium of *Botrytis cinerea* in Petri dishes, on beech callus tissue and in 18 buds of 10 *F. sylvatica* plants. Cultures were maintained at appropriate temperatures and observed regularly. Fagus plants were transferred to 20 °C in spring to stimulate leaf emergence and monitored for BLD symptoms. In 2022 and 2023, exporting infected plant materials from the USA under biosafety regulations turned out to be too difficult and no new material was acquired before the end of the project.

Fera:
Litylenchus specimens were extracted from BLD symptomatic leaf material obtained from ILVO scientists in 2021. *F. sylvatica* saplings were inoculated with the nematode suspension following methodology discussed with USDA colleagues. Saplings were inoculated with the nematode suspension by cutting small notches at the base of buds, pipetting nematodes into the cut before covering with damp tissue and sealing with parafilm. Beech leaves from Fera cultures were regularly tested for the presence of *Litylenchus*. Further sapling cultures were established in 2023 with a *Litylenchus crenatae* population received from an outbreak location in Ohio, USA.

WP4. Study of the microbial community of symptomatic and asymptomatic leaves/buds

Predefined tasks

This WP was aimed at detecting microbes associated with *L. crenatae* (if found) and compare them with the known microbiome associated with infested leaves in America (Ewing *et al.,* 2021). Symptomatic and asymptomatic leaves and buds were distinguished to see if differences were due to the presence of particular organisms.

- T4.1. In-depth analysis of pooled samples using metagenomics and metatranscriptomics (untargeted approach)
- T4.2 Large-scale taxonomic screening using metabarcoding (targeted approach)

Description activities partners

ILVO:

There were not enough buds to make pooled samples, so the study was performed with leaves only. Three pools of leaf samples from different sites were made: (1) 30 samples of asymptomatic leaves; 2) 30 samples of symptomatic leaves and 3) one batch of leaves with *L. crenatae* (N.Y., USA). The organisms living in and on the leaves were studied using RNA-sequencing (metatranscriptomics) only, and not metagenomics (DNA-sequencing) because we did not find *L. crenatae* and therefore reduced this WP. RNA was extracted and sent for sequening on an Illumina NovaSeq instrument. RNA libraries were prepared using the NEBNext Ultra II RNA (directional) kit and plant rRNA was depleted using the QIAseq FastSelect plant kit.

After the second survey, where again no *L. crenatae* was found, the metabarcoding study was performed to look for the main drivers of the fungal and bacterial leaf microbiome of *Fagus* spp. We selected 120 samples from different years, different regions, different origins (forests, parks, nurseries) and different tissue types (leaves and buds). We also studied differences in microbiome between symptomatic and asymptomatic leaves. Therefore, from 20 trees, 3 symptomatic and 3 asymptomatic leaves were selected per tree and handled as biological replicates. DNA was extracted and used in two PCR reactions to target fungi and bacteria. Two specific regions (the 16S rDNA V5/V7 region and ITS1 regions) were chosen because they are known to have mismatches against plant chloroplast and mithochondrial DNA (abundantly present in leaf material). Purified amplicon pools were sent for library preparation (organised per research question) and Illumina sequencing. The resulting amplicon sequencing dataset was thoroughly analysed using appropriate software for bioinformatics workflow.

Fera:

Beech foliar samples with and without a range of BLD like symptoms were collected in the UK, with DNA sequencing based metabarcoding carried out to compare the fungal and bacterial populations of the leaves. Infected beech leaves obtained from the USA were also included. DNA was purified from samples using appropriate methods for the sample type. Amplicons for 16s (bacteria) and ITS (fungi) were generated using FERA's standard primer sets. Library preparation for all amplicons took place based on the Illumina protocol for 16S Metagenomic Sequencing Library Preparation Part # 15044223 Rev. B. Libraries were then sequenced on an Illumina MiSeq sequencer, using the MiSeq Reagent Kit V3. Bioinformatic analyses were performed using appropriate software for bioinformatics workflow. Bacterial and fungal biomarkers of BLD were identified by re-analysing data from Ewing et al., 2021 using the LEfSe algorithm. Normalised abundances (sum of the values to 1 million) of bacterial and fungal taxa were compared only between symptomatic and asymptomatic samples from US. Finally, the mean abundance of the taxa included in the list of biomarkers were compared between symptomatic samples from US with the samples collected in UK. Genus *Paraphaeosphaeria* was not included among the list of fungal biomarkers determined by LEfSe, but it was used in the subsequent comparison analysis.

WP5. Raise public awareness of BLD

Predefined tasks

- T5.1 Produce information and outreach materials

The aim was to provide stakeholders with simple and concise information on BLD (identification, origin, pathways of spread, damage symptoms, life cycle, host range, and phytosanitary measures), and to encourage people to report findings of BLD symptoms on beech. This comprised producing fact sheets, posters, slide presentations for information sessions directed at different target groups (foresters, traders-importers, inspectors, researchers, citizens, …) and exchange materials and information between the different partner countries.

Description activities partners

See below (1.4 Main results) for details per partner.

WP6. Exchange knowledge between European and American research consortia

Predefined tasks

- T6.1 Publications and international platforms

This task included exchange of information and the project results through emailing and at (online) meetings with international researchers in the field. Also, to publish results and present them at international fora.

ILVO:

- Participated in the online BLD Research Meetings (USA and Canada), organized by the Holden Arboretum (Ohio, USA). This informal working group exchanges all types of information on observations, ongoing and planned studies and allows for discussion and exchange of ideas and opinions.
- The wider international scientific community was informed about the FAGUSTAT project via posters and presentations.

Fera :

- Fera scientists participated in online BLD research meetings and organised a knowledge transfer meeting with USDA nematologists and Defra colleagues in support of the Defra BLD PRA.
- Fera scientists attended BLD workshops at the SON meeting, Ohio, 2022 and visited outbreak sites to improve understanding of symptom recognition and means of spread.

1.4. Main results

WP1. Project management and coordination (ILVO)

A kick-off meeting was organized, a common Dropbox space was created. In March 2022, a follow-up meeting (online) after the first survey enabled exchange of ideas and results. Most partners met during the ICN (International Congress of Nematology) in May 2022 and at the EPPO Panel on Diagnostics in Nematology in November 2022 where info was exchanged. Occasionally, emails were sent to keep partners informed on new publications or research findings (in America).

WP2. Survey for BLD and identification of paths of entry and spread (all partners)

<u>T2.1 Sampling leaves, buds and nuts from symptomatic and asymptomatic trees.</u>

<u>T2.2 Extraction and identification of nematodes from plant tissues</u>

<u>ILVO</u>:
Surveys were performed by ILVO in botanic gardens, OWSF in Walloon forests, CARAH and PCS in parks, forests and nurseries in the Hainaut province and Flanders, respectively. A total of 462 samples were collected (222 in 2021, 240 in 2022) from 101 sites. Samples were obtained from forests (283 samples), botanic gardens (72 samples), parks (5 samples), nurseries (79 samples), and private properties/gardens (23 samples), situated in 101 different sites across the country. The majority of the samples (426) consisted of leaves, 24 samples were beech buds from plantlets and seedlings in nurseries and plant stores, 12 samples were beechnuts. The most sampled tree species was *F. sylvatica*, comprising over 27 cultivars. Other species were *F. grandifolia, F. crenata, F. japonica, F. lucida, F. engleriana, F. orientalis, F. hayatae* and *F. longipetiolata*, mainly from arboreta.

Nematodes were extracted from 30 leaves/buds/nuts using Baermann funnels in a mistifier. Leaves were washed in the first year to remove saprophytic nematodes, but this practice was abandoned in the second survey. Very few nematode specimens were found, at most 1 or 2 per sample. They belonged to the genera *Aphelenchoides, Plectus, Panagrolaimus, Diplocapter, Prismatolaimus, Eudorylaimus, Laimaphelenchus, Chronogaster* and *Mesorhabditis*. None of the nematodes were plant-parasitic species. *Litylenchus crenatae* was not found.

Two nematode extraction methods were compared using leaf samples spiked with *Bursaphelenchus xylophilus*, similar to *L. crenatae*. Extraction from leaves cut in small pieces and placed on a Baermann funnel in a mistifier resulted in more nematodes than automated zonal centrifugation (AZC) of cut or blended leaves.

<u>NVWA:</u>
Surveys were performed by NVWA in parks and forests in 2020, 2021 and 2022 . A total of 276 inspections was performed (77, 99 and 100 inspections for each year), resulting in 16 samples of symptomatic leaves. Samples were extracted with the mistifier method. In 2020, only one sample was analysed, but in 2021 and 2022 there were 10 and 5 symptomatic samples, respectively. *L. crenatae* was not found. In 4 samples *Laimaphelenchus* sp. was found in low numbers (1-5 nematodes). *Laimaphelenchus* is a saprophagous nematode.

<u>SFI & KIS:</u>

SFI surveyed 40 locations in managed forests and sampled leaves from symptomatic beech trees. Altogether 16 samples from 15 locations were taken. Only *F. sylvatica* was sampled. All samples were delivered to KIS within 24 hours for further analysis. At KIS, nematode extraction was performed overnight (24 hours) using the Baerman funnel method. All 16 samples tested negative for *Litylenchus* sp. Furthermore, all analysed samples tested negative for the presence of any nematode taxon.

Fera:

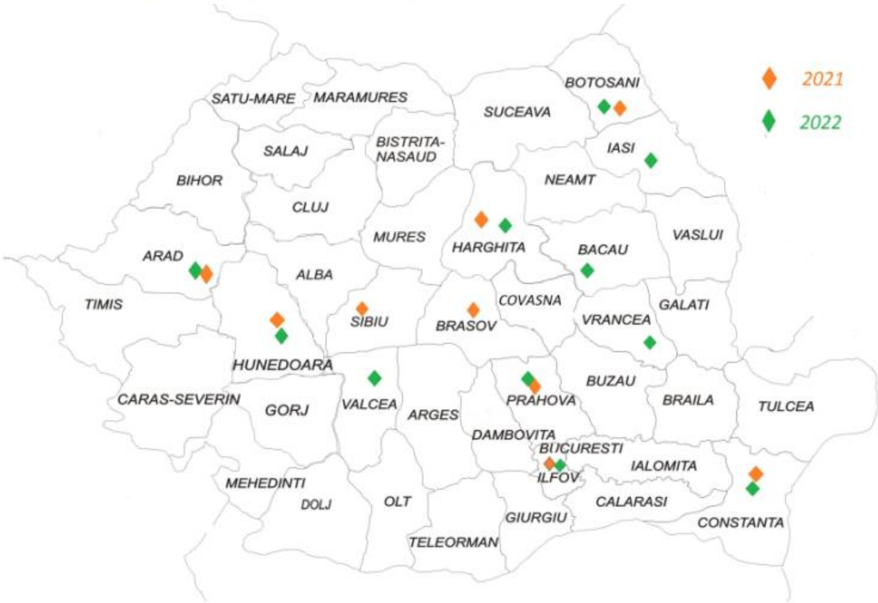
A targeted national survey (2021-22) of 100 trees from forests, parks and national gardens has been carried out by Plant Health and Seed Inspectorate (PHSI), Observatree lead volunteers and Fera scientists, focusing on *Fagus sylvatica* cultivars. *F. grandifolia* and *F. orientalis* exhibiting possible leaf symptoms.

Example of a *Fagus* sp., exhibiting 'look-alike' BLD symptoms, sampled as part of a targeted UK Y1 national survey.

Collected leaf material was processed using the Baermann funnel technique by cutting in addition to tearing the plant tissue, with a subsample retained and frozen for biome analysis. No *Litylenchus* were detected in the samples analysed. Specimens of *Laimaphelenchus deconincki*, *Aphelenchoides* sp. and *Tylaphelenchus* sp. were found.

NPA:

Surveys were performed by NPA in: forests (Bacău, Botoșani, Harghita, Ilfov, Sibiu counties); nurseries (Ilia - Hunedoara, Măneciu - Prahova, Glăjărie - Sibiu, Țarina - Vrancea); parks (Brașov, Bucharest, Constanța), natural reservation (Archisel - Arad county) (see map). A total of 45 samples were collected (20 in July – November 2021 and 25 in August – November 2022) from forests (24 samples), nurseries (6 samples), parks (13 samples), natural reservation (2 samples) located in 22 different sites from 13 counties across the country. The samples consisted of leaves (42 samples), beech buds from plantlets and seedlings in nurseries (2 samples), beech nuts (1 sample). The most sampled tree species was *Fagus sylvatica*. Nematodes were extracted using Baermann funnels and funnel spray method. Very few saprophytic nematodes specimens were found. *Litylenchus crenatae* was not found.



The distribution of sampled areas for *Fagus* spp. during 2021-2022 in Romania

<u>DAFM</u>:

Surveys for BLD were predominately carried out by the inspectors for the Irish competent authority and were incorporated into the annual forestry survey program. Trained laboratory staff performed further surveys of public greens and gardens. *Fagus sylvatica* was the most common species sampled. In total, surveys were performed at 22 sites throughout the county (see map below). A total of 19 samples consisting of leaves, buds and nuts (if available) were submitted to the Plant Health Laboratory for analysis. In addition to the sites surveyed above, visual checks were also carried out where beech was a component of a forest being surveyed for another tree species.

For samples submitted to the laboratory for analysis, nematodes were extracted using a Baermann funnel. In year one of the survey the nematode extract was collected after 48 h, replenished with fresh water and collected again after 72 h. As there was very little difference between the two extraction periods, in year two nematode extracts were collected after 72 h only. Nematodes, belonging to the genera *Aphelenchoides,* were recovered from only two samples analysed, with <5 nematodes per sample. All samples tested negative for *L. crenatae.*

The distribution of sampling areas for *Fagus* spp. during 2021-2022 in Ireland

T2.3 Identify the different pathways of introduction and spread of BLD

ILVO:

A questionnaire was sent to professionals in the landscaping business, parks and botanic gardens, inquiring about the origin of their *Fagus* planting materials in the last 10 years. Of the 50 respondents, almost all (98 %) indicated *F. sylvatica* as the only species they were dealing with. Most planting materials originated from Belgium. One (arboretum) reported importing *Fagus* species from Taiwan and Japan as seed. A literature study was performed and a PRA was drafted, focusing on Belgium/EU, to describe different possible pathways.

NVWA:

A quickscan was performed focusing on the Netherlands to determine possible pathways for introduction of *L. crenatae*. Additionally the CLIENT database of the NVWA was searched to check on imports of *Fagus* in 2019 and 2020. No *Fagus* was imported from the USA and Canada. In 2019, four imports of *Fagus crenata* from Japan and in 2020, four imports of *F. crenata* from Japan and one from China into the Netherlands were extracted from the database. These imports concerned bonsai plants, not intended for planting outdoors, which are excluded from the import prohibition. These data show that introduction of *L. crenatae* with imported Fagus seems unlikely.

NPA:

All planting materials in Romania originates in the country. Fagus trees cover about 2.100.000 ha from total forest stock (6.500.000 ha) (2019 data). There were no import cases of plant material of Fagus during the last 3 years. The planting material is obtained from seeds produced in Romania.

WP3. Study the biology and epidemiology of *L. crenatae* under European climatic conditions

T3.1 was not performed as no *L. crenatae* was found. However, its life cycle and biology of the nematode became more clear thanks to studies and observations in the USA and Canada (see WP6).

T3.2. Study the biology of *L. crenatae* under controlled conditions

ILVO:

Some leaves of the beech plants in the greenhouse, emerged from buds that had been inoculated with *L. crenatae,* showed cupping and necrotic spots. However, we found very few *L. crenatae* (13 juveniles and females) in these symptomatic leaves, probably survivors of the inoculum, unable to multiply in the leaves. The inoculum had also been used to set up cultures on carrot discs, mycelium of *Botrytis cinerea*, and callus of the beech nuts and buds. Nematodes did not reproduce on any of the inoculated substrates.

Fera:

Litylenchus specimens extracted from BLD symptomatic leaf material were morphologically confirmed as *L. crenatae* and material utilized for the development of molecular protocols at Fera.

Beech leaves from Fera cultures were regularly tested for the presence of *Litylenchus*. The trees exhibited certain minor symptoms of BLD, however we were unable to isolate any further *Litylenchus* specimens from subsequent leaf analysis. No results have been obtained yet with cultures established in 2023 with a *L. crenatae* population received from an outbreak location in Ohio, USA.

Photos: Fagus culture leaves infected with Litylenchus prior to nematode extraction (left). Cupping and necrotic spots on beech leaves emerged from buds inoculated with *Litylenchus crenatae* (right.)

WP4. Study of the microbial community of symptomatic and asymptomatic leaves and buds

<u>ILVO:</u>

<u>T4.1. Metatranscriptomics</u>

RNA-seq (metatranscriptomics) performed on 3 pools of leaves revealed a very similar composition of the asymptomatic (pool 1) and symptomatic (pool 2) pool: half of the sequences was plant-derived, and Fagus leaf material generally contained more fungi than bacteria. However, the Arthropoda group consisted of 1% of the sequences in the symptomatic pool, versus 0.5% of the sequences in the asymptomatic pool, suggesting that the Arthropods were relatively more abundant in the symptomatic pool. When zooming in, these sequences belonged to Lauxaniidae. These woodland flies could have contributed to symptoms in the sampled leaves. Also, in pool 2 (symptomatic), the viruses were dominated by an unknown Carlavirus (Quinvirinae), while in pool 1, there was a more diverse background of viruses. We wondered whether this virus could be responsible for the symptoms in many of the samples and developed PCR primers suited to detect the virus. Next, 30 asymptomatic and 50 symptomatic leaf samples that were previously sampled were chosen to cover an as wide range of sampling sites as possible. None of the 30 asymptomatic leaves showed a PCR band for the Carlavirus, while only 3 of the 50 symptomatic leaves did. Since these three samples were from different regions and different sampling years, we concluded that it is very unlikely that the Carlavirus is the cause of all observed symptoms. In pool 3 (leaves received from USA), 6% of the sequences were identified as *L. crenatae*, which was expected.

<u>T4.2. Large-scale taxonomic screening using metabarcoding</u>

Sufficient reads were obtained for both runs combined (for fungi using ITS1, and for bacteria using 16S V5V7) to capture the complete microbiome diversity of the leaves. Leaves from forest trees showed a significantly higher bacterial and fungal richness than leaves from trees in nurseries. All factors (tree species, tissue type, location and sampling year) had a significant influence on the fungal microbiome diversity. For the bacterial microbiome, only the tree species did not have a significant effect on the microbiome constitution. Location seems to have the largest effect on both the bacterial and the fungal composition of the microbiome. *Pseudomonas* and *Erwinia* were among the top 5 taxa in our leaf samples; these are among the bacterial genera reported by Ewing et al. (2021) that might be associated with BLD. For the fungi, Ewing et al. (2021) reported *Paraphaeosphaeria* in BLD -infected leaves, but this genus was only 0.03% of the total fungal community in our leaf samples. However, the related genus *Phaeosphaeria* was among our top 40 fungal genera. When comparing microbiomes of symptomatic and asymptomatic leaves of a same tree, we found that the main factor affecting the microbiome's constitution was the tree itself. The microbiome varied a lot between each of the 20 trees investigated, and it was impossible to pinpoint general genera that are more or less abundant in symptomatic leaves compared with asymptomatic leaves.

Fera:

T4.2. Large-scale taxonomic screening using metabarcoding

Our results determined 14 bacterial genera and 7 fungal genera to be significantly associated with BLD symptoms in the US data. The most abundant classes found in samples processed at Fera were Alphaproteobacteria, Actinobacteria and Gammaproteobacteria. There was a higher proportion of the Bacteroidia class and a substantial reduction of Actinabacteria class proportion in the only sample that presented clear symptoms of BLD (a positive sample from the US). However, more replicates should be sequenced to determine a significant association between this taxon and BLD symptoms. In addition, there was no significant association between the class Bacteroidia and any symptomatic condition in the outcomes of LEfSe. ITS data was mostly annotated to fungal taxa in both cases. However, each dataset was produced using different primer sets. Hence, observed differences between samples collected in UK and US would not be exempt from primer performance bias. Classes *Dothideomycetes, Leotiomycetes, Tremellomycetes* and *Sordariomycetes* were the most abundant in samples from UK. Again, the only sample with clear BLD symptoms (US positive control) showed unusual large proportion of class *Leotiomycetes* and lower proportion of class *Tremellomycetes*. However, there was no significant association between *Leotiomycetes* class and BLD symptoms determined by LEfSe in samples from US. Bacterial genera *Chryseobacterium* and, to a much lesser extent, *Stenotrophomonas* formed the few exceptions which were approximately 6-fold and 1.4-fold higher in UK sequenced than US sequenced data, respectively.

All taxa reported by Ewing et al. (2021) to be associated with symptomatic material (*Wolbachia, Pseudomonas, Paenibacillus* and *Erwinia* and the fungal taxa *Paraphaeosphaeria*) were found in UK leaves but at a lower abundance suggesting that if they are a requirement for nematode infection then they are already present in the UK samples, and this would not be a barrier to infection. A further analysis of the data from Ewing (2021) identified a number of other potential markers of BLD and most of these were also found in UK samples. This suggests that if there were a requirement for these microbial taxa for *Litylenchus* infection, this would not be a barrier to infection in the UK as they have been confirmed as present.

WP5. Raise public awareness of BLD

Predefined tasks

- T5.1 Produce information and outreach materials

ILVO:

Fact sheets and a presentation were drafted in English and shared with the project partners. Leaflets were produced in English, French, Dutch, and distributed at several occasions. In the first year, only professionals were informed about BLD. In the second year, when it was known that the disease was not present, a broader audience was informed. Distribution of outreach materials occurred via existing communication routes (newsletters, websites, meetings, workshops), and an online national monitoring and reporting alert systems (waarnemingen.be /observations.be). A poster was shown at 1 event, 2 presentations were given for stakeholders and a press release resulted in at least 6 articles on BLD in specialty publications. Information was made available on various websites. A few people reported diseased beech trees, but there were no pictures nor samples of trees with very typical BLD symptoms submitted by citizens, nurseries, arboreta nor plant health inspectors. Leaves with similar symptoms (look-alikes) were sent by one arboretum, but were negative. This observation was a result of the IPSN (International Plant Sentinel Network) collaboration with Belgian arboreta and botanic gardens.

NVWA

A factsheet describing biology and symptoms and an inspection and sampling protocol (in Dutch) was prepared for inspectors. Trainings in recognizing symptoms were given for inspectors of NVWA and of the inspection body Naktuinbouw. Presentations were given with general information and symptoms of BLD for several audiences: NPPO the Netherlands, Arbeitskreis Nematology Germany, Nematology working group the Netherlands. A short note was published in Gewasbescherming 2022, vol 53 nr 6.

SFII/KIS:

Public awareness was raised through a post with basic information on BLD on the Integrated Plant Protection Platform in Slovene language (www.ivr.si; https://www.ivr.si/wp-content/uploads/2020/07/Poster_DVRS_BLD_2022_za-net.pdf).

Further, the public was informed about BLD and the Fagustat project through posts on FaceBook and Twitter by the Agricultural institute of Slovenia (https://www.facebook.com/KISinstitut/posts/261734935709028, https://twitter.com/KISinstitut/status/1406951711663693828).

Several presentations were given. At one occasion, an abstract was published:
Gerič Stare B., Ogris N., Širca S. 2022. New plant disease – Beech leaf disease (BLD). 15th Slovenian conference on plant protection with international participation, 1.–2. March 2022, Portorož, Slovenia. Plant Protection Society of Slovenia

Fera:

A BLD factsheet was prepared to raise public awareness in the UK and to assist Observatree volunteers with symptom recognition prior to project surveillance activities. Several presentations were provided to Defra, APHA and industry workers, in addition to posters at scientific conferences.

Initial presentation by collaborators of Beech Leaf Disease and the Euphresco Fagustat project, at the 'UK Tree Health Citizen Science Network (THCSN)', 15th September 2021, Tree Health Centre, Yorkshire Arboretum.

Production of a sampling guide for the PHSI and citizen scientists to facilitate the Y1 National BLD survey.

A summary of Y1 survey work was presented by collaborators at the 'Observatree Lead Volunteer Day', 13th October, Tree Health Centre, Yorkshire Arboretum, and Observatree volunteer day, 21st October (online).

Presentation summarising BLD, the Euphresco project and the planned botanical gardens Y2 surveillance work at the BGCI 'European Arboretum and Botanical Gardens' meeting, 13th October (online).

Presentation provided to PHSI inspectors regarding BLD at the 2021 APHA Technical conference, January, York.

The Observatree BLD guide was drafted by UK collaborators and has been published on the website to assist with Y2 surveillance activities (https://www.observatree.org.uk/pests-and-diseases/priority-pests-and-diseases/beech-leaf-disease/)

Specialists at the BGCI presented a BLD poster at the IPSN meeting (WP2, WP5) and coordinated sampling strategies for the Y2 surveillance activities. A sampling guide and submission form has been produced.

A poster summarising the threat of beech leaf disease in the UK presented at the International Plant Health Conference: 21-23 September 2022, London.

NPA:

A Phytosanitary Sheet and Sampling Instructions were produced in Romanian and distributed to Phytosanitary County Office in order to help phytosanitary inspectors in detection of BLD.

DAFM:

A sampling guide containing information on the phytosanitary implications of *Litylenchus crenatae,* susceptible host plants, BLD symptoms, and sampling procedure was circulated to the Irish Forestry Service, who carried out the Beech surveys in Ireland.

BLD information was shared through several channels over the course of the project, including, agricultural workshops, newsletters and academic lectures and conferences. A BLD Fact sheet was prepared and shared on the Department of Agriculture, Food and the Marines (DAFM) website.

WP6. Exchange knowledge between European and American research consortia

Predefined tasks

- T6.1 Publications and international platforms

ILVO:

- Dr. Lynn Carta, was invited speaker at the 2nd annual workshop of the EURL for plant-parasitic nematodes, held online on 30/11/2021. This workshop is attended by all NRLs (national reference laboratories) of the EU.
- Two presentations were given at EPPO Panels.
- ILVO attended about 10 online meetings with the working group of BLD researchers in the USA. These meetings are a great source of information on this threatening disease.
- Two posters about FAGUSTAT were presented at international meetings of nematologists (SON meeting, online, 2021, and at the ICN, Antibes, France, 2022)

Fera:

- Fera scientists attended all BLD presentations and the BLD symposium at the SON meetings in 2022 (Anchorage, Alaska) and 2023 (Columbus, Ohio). During these meetings contact was made with USDA BLD specialists, and in-depth knowledge transfer regarding the status of BLD and its spread via vectors in both the UK and USA was exchanged.
- Following this contact Fera scientists arranged to visit a BLD outbreak site in Ohio to gain experience in the recognition of BLD symptoms in a forest setting. Positive control material was collected from beech trees for reference standards and culturing.
- Fera staff arranged for USDA BLD specialists to present current knowledge on BLD directly to the Defra pest risk analysis (PRA) group to demonstrate the importance of this pest and its risk to the UK through trade. This assisted with the production of a Defra UK PRA for *Litylenchus crenatae*.
- Fera scientists regularly attended the USA BLD researchers working group meetings, participating in discussions on pathways of spread, epidemiology, and Koch's postulates. These

meeting provided current information on the status of BLD in the USA and the evolving research areas. This information is routinely disseminated to Defra and other important government departments.

1.5. Conclusions and recommendations to policy makers

Conclusions

- ✓ Extensive surveys in 2021 and 2022 did not reveal the presence of *L. crenatae* in the six partner countries (Belgium, the Netherlands, Ireland, Romania, Slovenia and UK).
- ✓ Several beech leaves showed symptoms very similar to BLD but did not contain the nematode. These look-alike leaves were found in all countries.
- ✓ The extraction method that was used (Baermann funnel in mistifier) recovered more nematodes than a faster method based on centrifugal flotation**.**
- ✓ We were not able to establish cultures of *L. crenatae* subsp*. mccannii* on carrot disks, fungal mycelium nor beech callus. Neither did nematodes reproduce in inoculated beech buds in greenhouses. Hence, no cultured nematodes were available for studies of the life cycle of *L. crenata*e or testing the host status of European beech cultivars. There is no protocol for culturing *L. crenatae* to date and scientists rely on nematode specimen collected from infected trees (USA, Canada) for study.
- ✓ Following up on recent studies in the US we learned that nematodes survive in dormant buds. Import of *Fagus* plants, even dormant without leaves, could introduce *L. crenatae*.
- ✓ In Belgium, very few *Fagus* planting materials were imported from outside the country or even the EU in the last 10 years, based on the 50 responses from an anonymous survey.
- ✓ EU legislation prohibits import of *Fagus* plants from countries other than UK, because Fagus is considered a “high risk plant” (CELEX_32018R2019_EN_TXT, annex I), therefore chances for importing BLD into the EU via trade are low.
- ✓ From researchers in America we learnt that the large numbers of *L. crenatae mccannii* found in leaves and buds indicate that these plant parts are the main source of spread. Other pathways of nematodes, like soil, twigs, wood or roots, are not applicable to this nematode. We also note from their observations that *L. crenatae* nematodes are probably mostly spread by rain and wind, and occasionally by vectors such as arthropods and birds.
- ✓ Metatranscriptomics analysis confirmed nematode detection in infected leaf material from the USA. Fungi prevailed over bacteria in Belgian leaf pools. No significant differences in fungal and bacterial communities were found between asymptomatic and symptomatic leaves. Due to the significant variation in microbiomes among individual trees, it is not feasible to identify specific genera that are consistently more or less abundant in symptomatic leaves compared to asymptomatic leaves.
- ✓ The microbiome analysis of some symptomatic leaves in Belgium pointed to a Carla virus and woodland flies in Belgium, but we did not confirm symptoms due to the presence of these organisms.
- ✓ Both leaf microbiome analyses in Belgium and the UK revealed that genera reported by Ewing et al. (2021) to be associated with BLD (*Wolbachia, Erwinia, Pseudomonas, Paenibacillus*, and the fungus *Paraphaeosphaeria*) are also present on European beech leaves. If these genera are a requirement for nematode infection, then they are already present. It must be noted that these are common genera and more specific information on bacteria or fungi potentially involved in BLD is needed to draw conclusions.

- ✓ The information and knowledge gathered in this project will contribute to the pest risk analysis for *L. crenatus* in the EU and other countries of the EPPO region.
- ✓ While no confirmed BLD cases were reported, the project raised awareness and prompted citizens to report similar leaf symptoms. Overall, the project successfully engaged professionals and the public in BLD awareness. It is also important that tree nurseries and traders, owners or supervisors of gardens, arboreta, parks and forest pay attention to the origin of the trees and possible import of new organisms.

Recommendations

- ✓ Perform studies on survival of *L. crenatus* under European conditions and screen local Fagus cultivars for resistance. For this, easier import procedures for research on non-regulated organisms would be helpful.
- ✓ Beechnuts have only occasionally been investigated for the presence of nematodes. As no nematodes were found, seeds are not considered as a possible pathway. It would be reassuring if more seeds from beech stands with BLD (in the USA or Canada) were analysed to confirm this assumption.
- ✓ Pay attention to bud symptoms, next to leaf symptoms, when inspecting Fagus plant(let)s, especially to dormant buds as they can contain large numbers of *L. crenatae*.
- ✓ Survey from mid summer to late fall because nematode numbers in leaves increase during the growing season and are the highest in fall.

1.6. Benefits from trans-national cooperation

- ✓ The consortium of trans-national partners were able to share their expertise and exchange knowledge and experiences during online meetings and via several emails.
- ✓ Reduction of workload for partners is a great benefit. Working with a common fact sheet and sampling procedure reduced the time spent by each partner for looking up the information, acquire pictures, formatting. Similarly, sharing presentations, and posters enhanced spreading the word about BLD and raising public awareness. Also, information needed for pest risk analysis was shared, e.g. the legislation, the general knowledge about the nematode.
- ✓ A common sampling scheme and extraction method made it possible to compare results of all surveys. Although only 6 countries were involved, we have at least an idea of the presence/absence of BLD in Europe.
- ✓ Exchange of infected leaf materials between two partners (Fera and ILVO) engaged in performing tests with the *L. crenatae* made it possible for both to realize some of the work that was planned.
- ✓ In the partner countries, there is a great awareness for BLD now, both by nematologist and people involved in forests and parks. This awareness can spread further through to their own national and international contacts.
- ✓ A renewed partnership between the IPSN (International Plant Sentinel Network) and the Belgian arboreta and botanic gardens was created, extending beyond observations for BLD. This is promising for future collaboration in finding new pests and diseases in sentinel plants.
- ✓ Through the strengthened transnational network that was built, cooperation with a number of transnational partners will be continued after the closing date of the project.

2. Publications

The scientific publications linked to the project should be included in extenso *in the report, and clear reference to them should be given. It is recommended to contact the [Euphresco Secretariat] to explore the opportunities for dissemination via publication. The research consortium is responsible for the publication strategy for a given project.*

2.1. Article(s) for publication in the EPPO Bulletin

Publication in the EPPO Bulletin is not automatically granted and is dependent on the quality of the article. If an article is accepted, charges could apply to cover publication costs.

None.

2.2. Article for publication in the EPPO Reporting Service

Publication in the EPPO Reporting Service is not automatically granted and is dependent on a number of criteria, among which the novelty and the quality of the information proposed. Only references to the Euphresco project (and no reference to the individual scientists) will be given in the published article.

None.

2.3. Article(s) for publication in other journals

The research consortium is solely responsible for publishing project outputs in peer-reviewed scientific journals.

None.

3. Open Euphresco data

List here the research data types that the consortium makes open and provide the url to various databases. It is recommended to contact the [Euphresco Secretariat] to explore the opportunities for dissemination via open data.

None.

4. Bibliography

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