Euphresco

Final Report

Project title (Acronym)
The biology and epidemiology of '*Candidatus* Liberibacter solanacearum' and potato phytoplasmas and their contribution to risk management in potato and other crops (PhyLib II)

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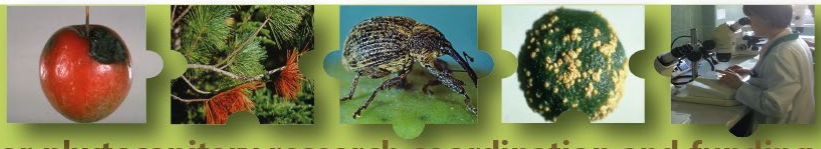
Contents



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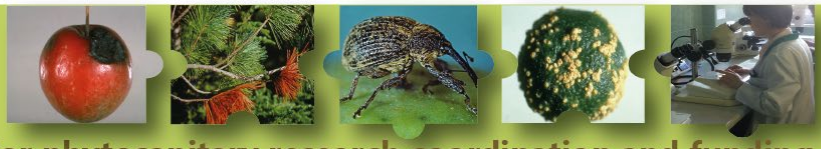

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2. Short project report

2.1. Executive Summary

PhyLib II is a consortium of 18 institutions from 14 countries and successor to the PhyLib consortium. The consortium collaborated on the project: 'The biology and epidemiology of '*Candidatus* Liberibacter solanacearum*'* (Lso) and potato phytoplasmas and their contribution to risk management in potato and other crops (PhyLib II)'. The aims of this project were to expand knowledge of emerging bacterial plant diseases associated with the presence of phytoplasmas and Lso and to survey their distribution, genetic diversity, epidemiology, insect vectors, and risk to crops worldwide. The consortium aimed to co-ordinate research on these crop pests and to provide a network of expertise to develop detection methods and a framework within which to share knowledge and compare the experiences of disease epidemiology across a wide range of geographical locations including North America (Canada), Northern Europe, the Mediterranean basin, Central Europe and Eastern Europe. The consortium developed and validated methods for the extraction of metagenomic DNA and total RNA and for the detection of bacterial DNA from complex matrices including plants, seeds and insect vectors. This involved testing current methodologies and developing novel approaches including High Throughput Sequencing (HTS)-based diagnostic techniques. Through field surveys carried out by the partners, our knowledge of the distribution and genetic diversity of phytoplasmas and Lso worldwide has improved. This includes the discovery of novel Lso haplotypes in different plants and insect vectors and the finding of Lso and phytoplasmas in areas where hitherto they were not known to be present. The consortium was effective in promoting exchange of materials between partners and sharing knowledge, enabling countries to facilitate testing for phytoplasmas and Lso. The consortium will build on the success of PhyLib II and the Euphresco project PhyLib III (17 institutes from 14 countries) has started in 2020.

2.2. Project Aims

'*Candidatus* Liberibacter solanacearum' and phytoplasmas are phloem restricted bacteria which have been implicated in emerging diseases of important crops in the Solanaceae and Apiaceae. These pathogenic bacteria are transmitted by insect vectors such as psyllids and leafhoppers. Knowledge on distribution, epidemiology and diversity of these bacteria in the Solanaceae and Apiaceae is limited and there are major areas of the biology of pathogens and vectors that are understudied. This project aimed to bridge some of these knowledge gaps and to provide a harmonised approach for research in this area with the following expected results:

- Identification of insect vectors involved in transmission of Lso and phytoplasmas
- Development and validation of tests for the detection of Lso and phytoplasmas
- Development of tests for the rapid identification of psyllid vectors of Lso
- Evaluation of suction trap networks as sentinel systems for the detection of psyllid vectors of Lso
- Field scale surveys for Lso and phytoplasmas across Europe
- Understanding the insect and plant host range of Lso and its diversity across Europe
- Development of a resource network to aid research on Lso and phytoplasmas
- Examining interactions between Lso and its plant hosts

The project's overall aim was to improve our knowledge of the epidemiology and distribution of Lso and phytoplasmas to help understand the potential and current impacts of these pathogens on agriculture. This work will lead to better monitoring and detection of these pathogens (and their insect vectors) and provides information on best practices regarding detecting these pathogens *in-situ*. Considerable advances have been made on understanding the distribution and genetic diversity of Lso which will inform better risk assessment and phytosanitary management.

2.3. Description of the main activities and results

2.3.1. Bacterial Collections

The project aimed to facilitate the exchange of reference material including infected plant matrices and pathogen and insect DNA. CFIA maintained a collection of Lso infected micro-propagated plants and made available Lso infected Solanaceous plant sap for diagnostic test development and validation. The consortium provided materials and expertise to Estonian institutes which led to the fast development of a testing system for screening of potato tubers in response to requirements for statutory testing put in place by the Agricultural Board in Estonia. The project was also an opportunity to exchange information on availability of material etc.

2.3.2. Bacterial Detection/ Diagnostics

2.3.2.1. Developing new diagnostic assays for detection and identification of Lso

CFIA investigated the potential of next generation sequencing technologies for the detection of Lso. Genomics and meta-transcriptomic sequences were obtained using Illumina MiSeq, HiSeq and NextSeq facilities, respectively. Bioinformatics pipelines and workflows allowed simultaneous detection and identification of potato zebra chip pathogen Lso and *Potato Virus S* in potato and tomato plants. The results were confirmed using conventional PCR and real-time PCR (for Lso), and real-time RT-PCR (for PVS) and electronic microscopy (for both). At the same time, a draft genome for Lso associated with potato Zebra Chip disease was also determined using a metagenomics approach. Comparative genomics analysis of this Lso haplotype A strain with the other seven genome sequences deposited at GenBank identified 11,128 SNPs (Single-Nucleotide Polymorphisms) or indels (inserts and deletions) for genome differentiations among Lso haplotype A, B, and C. Association of the signature SNPs and the functional genes or transcription factors with the virulence on individual host plants help unveil the pathogenicity characteristics. It is suspected that flagellin domain-containing protein may play a key role in virulence and pathogenicity of Lso. In a study external to this project Lso flagellin domain-containing protein elicited pattern triggered immunity (PTI) response and induced innate immune responses in *Nicotiana benthamiana* [1].

2.3.2.2. Evaluate and validate current molecular tests for the detection of Lso in plant tissues

Considerable effort was placed into harmonising the detection of Lso in plant tissues. Several test-performance studies were organised by members of the consortium on the detection of Lso from plant tissues. Three independent test-performance studies examined the optimal methods for Lso DNA extraction and detection with conventional and real-time PCR. Each test-

performance study concluded that the real-time PCR of Li *et al*., 2009 [2] was the most reliable and sensitive for detection Lso.
In the study performed in conjunction with the EU2020 POnTE project four different extraction methods were tested CTAB; DNeasy Plant mini kit (Qiagen); NucleoSpin Food (Macherey-Nagel); and NucleoMag Plant with MC1 buffer (Macherey-Nagel). CTAB and NucleoMag Plant Kit were recommended for extraction with the Li *et al*., 2009 [2] real-time PCR for the detection of Lso from different plant matrices.
EVPM and NIB organised ring-tests to identify the best diagnostic test to detect Lso and Li *et al*., 2009 [2] performed the best and is recommended for Lso detection. ANSES also organised a ring test in conjunction with the EU2020 POnTE project and the French CaLiso project to evaluate the different protocols included in the EPPO diagnostic protocol (to be published in 2020) to detect Lso.
AGES tested the conventional PCR method from Levy *et al*., 2011 [3] and two real-time PCR methods Li *et al*., 2009 [2] and Teresani *et al*., 2014 [4] with the Li *et al*., 2009 test performing the best.
A review paper by 7 consortium partners (AGES, ANSES, CREA, ILVO, SASA, UniBO) was produced discussing current PCR based methods for detection of Lso [5].
Evira developed and validated a detection method for Lso from potato tubers using KingFisher instrument with Bio-Nobile DNA extraction kit and qPCR methods based on Li *et al*., 2009 [2] and Teresani *et al*., 2014 [4]. See section 4 'Open Euphresco data' for more details. NIB validated a similar procedure and implemented routine monitoring of Lso in potato tubers (latent testing). To determine the analytical sensitivity of the method, synthetic DNA and naturally contaminated material, provided by SASA, were characterized with digital PCR.

2.3.2.3. Developing new diagnostic tests for the detection and identification of Lso in seeds

Conventional PCR methods for haplotyping Lso extracted from seed were tested and optimized for low titre samples by using a more sensitive hot start proof-reading polymerase (Bio X-Act Short Mix (Bioline)) and template volume was increased to 2 µl in a 25 µl reaction. All seed samples tested by AGES were haplotype D or E (7 lots of different companies tested, 57% positive). NIB detected Lso in carrots for the first time; the haplotype was determined in collaboration with AGES as D/E.

2.3.2.4. Evaluate and validate current molecular methods for detection of Lso in seed

The consortium evaluated methods for the extraction of Lso DNA from seeds. CREA developed an improved DNA extraction protocol to isolate Lso from carrot seed [6]. This protocol was tested and validated in 11 laboratories. This new extraction method coupled with the Li *et al*., 2009 [2] real-time PCR and Ravindran *et al*., 2011 [7] conventional PCR gave the most sensitive and reliable detection methods; with the real-time PCR outperforming the conventional PCR.

2.3.2.5. Evaluate current molecular methods for the detection of Lso in insect vectors

In conjunction with the EU2020 project "POnTE", a test performance study was conducted to validate the best methodology for the extraction and detection of Lso from three major Lso

vectors: *Bactericera cockerelli, Trioza apicalis* and *B. trigonica*. Three extraction protocols were evaluated, CTAB, TNES and QuickPick (Bio-Nobile) and two real-time PCR tests Li *et al*., 2009 [2] and Teresani *et al*., 2014 [4]. The Li *et al*., 2009 real-time PCR outperformed the Teresani *et al*., 2014 real-time PCR and was best coupled with the CTAB DNA extraction which provided 100% true positives. In conjunction with the POnTE project a non-destructive DNA extraction method was developed to extract DNA from psyllid hosts available at: https://www.ponteproject.eu/protocols-calsol/non-destructive-dna-extraction-psyllids/ . This protocol is also routinely used for detection of Lso from psyllid hosts with the Li *et al*., 2009 real-time PCR.

2.3.2.6. Evaluate and validate characterisation methods for haplotype determination

During the survey in Belgium, only two carrot plots were found infected with '*Ca*. L. solanacearum' [8], hence only limited infected tissue was available for the validation by the Belgian partner ILVO. In addition to the PCR test using the primer pair OI2c/OA2 efficiently amplifying a fragment from the 16S region (Jagoueix *et al*., 1996 [9]; Liefting *et al*., 2009 [10]), the characterisation methods described in Nelson *et al*., 2011 [11] using primers from Munyaneza *et al*., 2009 [12] (targeting rplJ/rplL); Hansen *et al*., 2008 [13] (targeting 16S-23S rRNA, ISR partial & 23S rRNA) as well as Ravindran *et al*., 2011 [7] (targeting Adk & flanking regions) were evaluated and validated. All tested PCR tests performed well and are suitable for haplotype determination. At both Belgian sites that were found infected with '*Ca*. L. solanacearum', haplotype D was found. The methods were also tested successfully on seeds (also confirming the presence of haplotype D).

2.3.2.7. Haplotyping Lso present in wild plants and in various vector species

Several novel haplotypes of Lso were found during this project by partners. In Finland a novel haplotype U was found in nettle and its psyllid host *Trioza urticae* [14]. A further novel haplotype was described from carrot and plants in plants of the Polygonaceae family [15]. Surveys of carrot fields and their associated wild plants and psyllids showed the presence of novel Lso haplotypes of Lso in two psyllid species outside the Triozidae family. This survey also showed the presence of haplotype U in *Urtica dioica* (nettle) and *T. urticae* in the UK and Germany. In the UK, wild Apiaceous host plants were shown to harbour Lso haplotype C and a small number of carrot plants were also positive for Lso haplotype C. In Finland MLST analysis of Lso C from carrot and cow parsley showed distinct strains associated with different host plants [14]. Surveys in France demonstrated the presence of Lso in wild plants of the Apiaceae family.

2.3.2.8. Developing new diagnostic tests for detection and identification of phytoplasma using HTS approaches

Preliminary work by SASA was performed using HTS technologies to detect phytoplasmas. Experiments using MinION flow cells to characterise bacterial and fungal diversity from 4 Lso vectors (*B. trigonica, B. nigricornis, T.apicalis,* and *T. urticae*) showed low levels of phytoplasma detection. Numbers of *Phytoplasma mali* sequences were highest in *B. trigonica* (average 266.5 sequences per specimen), the other species each had <35 sequence hits to phytoplasma. *Bactericera cockerelli* was not infected with phytoplasmas. This suggests

MinION technology could be a useful tool for detecting high and low levels of infection with bacterial plant pathogens. HTS and bioinformatics workflows were developed for simultaneous detection and identification of potato zebra chip pathogen Lso and *PVS* in potato and tomato plants at CFIA.

2.3.3. Bacterial Epidemiology

2.3.3.1. Lso surveillance by country (Austria, Estonia, Netherlands, Czech Republic, France, Russia, Spain and Finland)

To understand the distribution and genetic diversity of Lso across Europe several partners performed Lso screening of crops, wild plants and psyllids.

In France three large surveys were performed by ANSES in Apiaceae fields (in collaboration with POnTE and CaLiso projects) and by FN3PT in potato and carrot fields. LsoD and LsoE was observed in plants of the Apiaceae in several regions. Lso was detected in carrot, celery, chervil, fennel, parsley and parsnip, suggesting a wider host range within the Apiaceae than expected [16]. During the large surveys performed by FN3PT on Apiaceae crops, none of the potato plants tested during the surveys were positive for the presence of this bacterium. Using an MLST approach the presence of a genetic variant of Lso in carrots was found, but ribosomal RNA genes were not sequenced and the new genetic type was not fully characterised [17]. Moreover, field experiments mixing potato crops with carrot crops and set up in an area where both, Lso and *Bactericera trigonica* occurred, did not resulted in any transmission of Lso to potato crops.

In Scotland 11 carrot fields and surrounding vegetation were surveyed. Lso was found in a small number of Apiaceous hosts including *Anthriscus sylvestris* (cow parsley) and *Aegopodium podagraria* (ground elder). One site showed a small number of Lso positive carrot plants harbouring Lso haplotype C.

In Austria in 2015-2018, 69 plant samples and 605 psyllid samples were analysed. Lso was detected in carrot, celery, parsnip and parsley (haplotype C). No Lso was found in Solanaceous plants. *Trioza apicalis* was the main vector of Lso in the outbreak area.

In Estonia 23 carrot and parsnip samples showed one potential positive albeit with a high Ct 36. This sample was from carrot material grown in the field, but the result could not be confirmed and will require further testing to confirm if Lso is present. Of concern were batches of carrot seed that tested positive for Lso.

In Finland a ware potato survey was conducted by Evira in 2017–2018. 218 potato samples from retail stores around Finland and were all negative for Lso. In Finland in 2018, 97 symptomatic carrots and 65 parsnips grown near to the carrots, from two separate geographical regions, were analysed. In the western region, 96% of the sampled parsnips were infected with Lso haplotype C, like the nearby carrots, whereas in the eastern region the parsnips were infected with a novel Lso haplotype (suggested name H) [15], and carrots only showed low infection levels. The symptoms of the infected parsnips were mild.

In Belgium, a nation-wide survey initially did not reveal the presence of Lso, however in many carrot fields the presence of aster yellows phytoplasma (‘*Ca*. P. asteris’) was detected. However, an additional survey, specifically targeting Auchenorryncha (plant- and leafhoppers) in a selected number of carrot plots resulted in the detection of Lso in two carrot plots (both haplotype D) in the West-Flanders region [8].

In Slovenia, a survey identified only one sample of symptomatic carrots infected with Lso (first finding in this country). The haplotype was determined in collaboration with AGES as D/E
Further sampling of different weeds and potential Lso vectors on weeds are planned in the frame of the Euphresco project WEEDVECT and PhyLib III.

2.3.3.2. Survey of Lso resistant and tolerant potato and tomato varieties

To date, no Lso resistant varieties of potato have been found [18] while some tomato varieties such as Moneymaker were found tolerant to Lso haplotype A, but not to haplotype B [19]. Transcriptomics approaches were carried out at CFIA using HTS technologies to examine gene expression and plant interaction with Lso haplotypes A and B in tomato plants (cv Moneymaker). Overall, Lso haplotype B (n=4) strongly affected tomato plant's gene expression in extracellular components, cell walls, and apoplast, while haplotype A (n=7) has significantly less influence on gene expression of these pathways in comparison with healthy control (n=6). For instance, Lso haplotype B affects the carbohydrate metabolic process and cell wall components in tomato plants: β-galactosidase synthesis and related pathways were strongly altered in gene expression profiles while endochitinase and related genes were unusually expressed. The expression levels of selected gene sets need be validated using qPCR in further investigation. A linux-based workflow was established to identify Lso haplotypes.

2.3.3.3. Seed transmission study in carrots

A publication produced by UNIBO showed Lso haplotypes D and E were not detected in seedlings from infected seed, however there was evidence to suggest that phytoplasmas were transmitted from infected seed to seedlings [20]. Studies by AGES found Lso D and E on carrot seed but did not confirm that Lso could be transmitted to the seedling, and only haplotype C was found in the outbreak area where infected seed had been planted. In Finland, seeds were produced in a greenhouse from carrots infected with Lso haplotype C, and the seeds were Lso positive. However, all the seedlings grown from these seeds were Lso negative. While most evidence suggests that seed to seedling transmission of Lso is not a major source of infection in plants, the possibility of seed transmission in disease free regions cannot be completely ruled-out. In an experiment at CFIA, 15 carrot plants were germinated from seeds obtained from Europe in a containment facility. Plant material from the 15 plants was pooled and tested for Lso. At least one Lso positive seedling was present among the 15 plants. After carrot plants re-grew, three carrot plants were randomly collected and grafted onto three tomato plants. (cv. Moneymaker). The presence of Lso in carrot and graft-transmitted tomato plants were confirmed twice by PCR and by HTS analysis in two separate laboratories. However, the same batch of carrot seeds was substantially germinated and generated seedlings with no detectable Lso using PCR in the Summer and Fall.

2.3.3.4. Development and survival of Lso in cold storage conditions

The results of cold storage and field experiments suggest that Lso can survive in carrots over winter, under the condition that the roots stay alive. The results will be published soon.

2.3.3.5. Phytosanitary surveillance of phytoplasma

A survey for phytoplasma detection in Turkey was also conducted between 2013-2014 in four provinces of Turkey (Bolu, Kırşehir, Sivas and Yozgat). The only known phytoplasma

associated with “stolbur” of potato in Turkey is ‘*Candidatus* Phytoplasma solani’ (CPs); the main vector of which is *Hyalesthes obseletus* (Hemiptera: Cixiidae). Hemipteran fauna from the four provinces and 248 localities in Turkey were recorded and included 13 hopper species from 4 families (9 Cicadellidae; 2 Delphacidae; 1 Cixiidae; and 1 Tettigometridae). All specimens were tested for CPs were negative despite 2 known insect vectors of CPs being found (*H. obseletus* and *Macrosteles laevis*). Full results were reported at the 1st International Molecular Plant Protection Congress in Turkey 2019 [21]. PPCRI also completed surveys across 4 provinces in Turkey (403 weed samples) from 2017-2018. Plants were collected from 23 different families; no parasitic plants were found positive for phytoplasma. 10 positive samples from weeds were infected with “stolbur” phytoplasmas. Infected weed samples came from 8 field bindweed (*Convolvulus arvensis*); 1 mallow (*Malva neglecta*); and 1 redroot pigwheat (*Amaranthus retroflexus*). Surveillance has been continued in weed species and vectors under a project funded by PPCRI-GDAR under the Turkish Ministry of Agriculture and Forestry.

Selected symptomatic carrot and celery samples from the Austrian Lso outbreak area and some symptomatic celery samples from East Austria were tested for the presence of phytoplasma using the generic primers of Lorenz *et al*., 1995 [22]. Although no phytoplasmas were detected in the Lso outbreak area, three “stolbur” positive samples were detected from East Austria. No potential insect vectors in the “Stolbur” positive fields were tested.

Carrot samples which tested negative for Lso were tested for phytoplasmas by NIB. All samples with clear leaf reddening were positive with the universal phytoplasma test of Christensen *et al*. 2013 [23]. Phytoplasmas from the aster yellows (group 16SrI) and ‘*Ca*. Phytoplasma solani’ (group 16SrXII) groups were found, as well as mixed infections of the two were confirmed. Among the 16SrII group, the ‘Ca. P. solani’ there were several different tuf types [24].

2.3.4. Bacterial Control

2.3.4.1. Monitoring and implementation of Lso tests at import and export and post entry quarantine

Routine Lso monitoring was implemented in a number of countries (Austria, Czech Republic and Estonia), facilitated by the resources and expertise of the PhyLib II consortium.

2.3.4.2. Monitoring- Implementation of phytoplasma tests at import and export and post entry quarantine

CFIA carried out non-routine tests for identification of potato witches’ broom and purple top phytoplasmas using conventional and nested PCR. It is a non-routine test for the detection of potato plants infected by phytoplasmas.

2.3.5. Vector Collections

2.3.5.1. Develop database of DNA sequences and collection of psyllid voucher specimens

The ‘Psyllid DNA Database’ developed and curated at SASA during this project contains 76 species of psyllids that were characterised in either the ITS2 or CO1 gene regions, or both. This database now includes the major vectors of Lso which previously were not all available on public DNA databases, see section 4 ‘Open Euphresco data’ for accession numbers. Over 6,000 specimens from around the world including: Finland, France, Germany, Israel, Mexico,

New Zealand, Serbia, South Africa, Spain, Sweden, Tunisia, UK, and USA were tested. A non-destructive DNA extraction method was developed (see section 2.3.2.5) which allows the retention of voucher specimens. The DNA database will be uploaded to NCBI GenBank and EPPO Global Database once results are published (currently under review by the Journal). Voucher specimens will be retained for reference at SASA or donated to museum collections such as the NHM London. EVPM have developed a DNA database of CO1 sequences from psyllids from carrot fields using a destructive DNA extraction. Damage was observed on carrot and parsnip associated with respectively *Trioza viridula* and *Psila rosae* (carrot fly). Collaboration between taxonomists and molecular biologists has been essential to produce good quality identifications with high confidence of accurate identifications.

2.3.6. Vector Detection/ Diagnostics

2.3.6.1. Optimising DNA extraction techniques for vectors

Destructive methods were developed in conjunction with the POnTE and CaLiso projects and test performance studies were conducted by ANSES. All tested DNA extraction methods (CTAB, TNES and Quick-Pick (Bio-nobile)) were sufficient for the identification of psyllids. The non-destructive method was favoured to ensure a voucher specimen could be kept for subsequent morphological examination and verification of sequencing results. The non-destructive method used with the DNeasy blood and tissue kit (Qiagen) was able to efficiently extract psyllid DNA from all 76 species and extracted sufficient Lso DNA from infected psyllids for subsequent real-time PCR testing and haplotype identification. Currently non-destructive methods using DNeasy Blood and Tissue kit support 24 samples per extraction but theoretically can be scaled up to 48 or 96 samples. A destructive DNA extraction method was tested by AGES (QIAamp DNA Micro Kit, Qiagen) along with the real-time PCR Li *et al*., 2009. An optimized PCR was used to identify Lso haplotypes.

2.3.6.2. Combining morphological and molecular methods for identification of psyllid species

Real-time PCR TaqMan probe tests have been designed to detect the major and potential vectors of Lso: *B. cockerelli, B. nigricornis, B. tremblayi, B. trigonica,* and *T. apicalis.* This work was developed in conjunction with the POnTE project. All tests can detect up to 200 copies of ITS2 gene regions and can be used to detect single specimens of adults, immatures and eggs. These are the first species-specific molecular tools developed to rapidly identify these important crop pests. The tests have been submitted for publication and are currently under review.

2.3.6.3. Validation of molecular methods for identification of psyllid species

The tests mentioned in 2.3.6.2 were validated in inter- and intra-laboratory tests and were tested for reliability, reproducibility, repeatability, robustness and optimization of PCR conditions. Test for *B. cockerelli* is currently in press in Plos One with the title “A diagnostic real-time PCR assay for the rapid identification of the tomato-potato psyllid, *Bactericera cockerelli* (Šulc, 1909) and development of a psyllid barcoding database”. A link to this published article will be added to the PhyLib II webpage found in section 2.5. This test can detect adults, eggs and immatures of *B. cockerelli* up to 0.000001 ng DNA. For detection of *B.*

tremblayi, B. trigonica, B. nigricornis and *T. apicalis* real-time assay are in the final stages of validation. All real-time assay was tested against 73 different non-target psyllid species. Further validation is being performed to allow the use of real-time PCR module for the detection of *B. trigonica, B. tremblayi* and *B.* nigricornis in multiplex. Further information on validation of molecular methods for identification of psyllids is included in section 2.3.6.1. ANSES to add conclusions?

2.3.7. Vector Epidemiology – Lso & Phytoplasma

2.3.7.1. Lso vector surveillance – carrot and other Apiaceae

Extensive surveys were performed on carrot. Screening of commercially available carrot seeds and subsequent detection in seedlings were performed by UNIBO with negative results and haplotype D was only detected in seeds [20]. Lso haplotype D and E were first detected in seed lots from Italy in 2016 [25]. AGES confirmed that in the Austrian outbreak area *T. apicalis* is the main vector, Lso is established and yearly re-infection occur vectored by overwintering infected *T. apicalis.* EVPM also surveyed vectors as part of a survey and Lso positive *Trioza apicalis* was confirmed to be haplotype C by ANSES.

2.3.7.2. Lso Surveillance – Potato

Slovenia implemented routine monitoring of Lso in potato (latent testing); no Lso was detected in potato tubers.

2.3.7.3. Lso vector surveillance – General

The performance of suction trap networks was evaluated as sentinel systems for Lso detection from psyllid vectors. Psyllids from Finland, Germany, Spain, Sweden and the UK were collected, identified and tested for Lso. Suction trap data did not accurately represent the actual vector presence at a field level [26] but was useful in identifying previously unknown hosts of Lso [27,28]. These surveys have led to the finding of Lso present in *Trioza anthrisci* a psyllid species closely related to *T. apicalis*. The threat of *T. anthrisci* to agriculture requires further assessment. In Austria psyllids were collected from carrot and celery located in the region where Lso was first found in Austria. All psyllid specimens collected in Austria from 2016-2018 were *T. apicalis* with up to 60% infection by haplotype C. Females seemed to show higher infection rates than males. In Belgium, surveys were performed to monitor Auchenorrhyncha. During this survey Lso was found but it was not possible to extend the survey to include psyllid vectors.

2.3.7.4. Vector transmission of Lso to crop plants

T. anthrisci can complete its lifecycle on carrot plants and will feed and lay eggs. Low level transmission of Lso was observed after 4 weeks of introducing insects to carrot plants and infection levels increased after 12 weeks. No obvious Lso symptoms were observed in the infected carrots. In Finland, *T. anthrisci* adults were collected from flower stalks of its primary host, *Anthriscus sylvestris*, in the end of May at Jokioinen. The species were ensured by checking the shape of the subgenital plate (females) and parameres (males) under a stereo microscope. All together 19 psyllids were released to each cage (n=10), where they were offered one seedling of carrot and cow parsley. The living psyllids were removed after 7-day exposure. Carrots were transferred individually to empty and clean cages, and the corresponding cow parsleys left in the original cages. First adults were observed on cow

parsleys and carrots 6 weeks after the beginning of the exposure. On all the carrot where the psyllid eggs were observed (n=5) a new generation of *T. anthrisci* adults emerged. In one of the cages, cow parsley showing discoloration symptoms was detected Lso positive by cPCR and confirmed to be haplotype C by sequencing the PCR products from 16S, 50S, and 23S. The corresponding carrot exposed to *T. anthrisci* feeding in the same cage produced a weak amplicon with primer pair OA2/LsC2, yet, the infection by Lso could not be confirmed since the DNA concentration was too low for sequencing. These results suggest that one transmission event to carrot during exposure of *T. anthrisci* may have occurred.

2.3.8. Vector Control

2.3.8.1. Lso vectors – collect data on long-term monitoring strategies for psyllid species

See results in section 2.3.7.4. A survey of UK psyllids was performed using suction trap networks with the Insect Survey at Rothamsted Research [29].

2.3.8.2. Phytoplasma - collect data on long-term monitoring strategies for planthopper and leafhopper species

Continuous surveys are being performed in carrot and celery fields in Belgium. Data on the diversity and presence of Auchenorrhyncha in several carrot plots has been collected and can be found in the VECTACROP (Euphresco) project report https://zenodo.org/record/1341600#.XiB2v3dFynt available to Euphresco members. Further surveys will be performed as part of the PhyLib III project.

2.3.9. Literature Cited

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2.4. Conclusions and recommendations to policy makers

The phloem limited bacteria '*Candidatus* Liberibacter solanacearum' and phytoplasmas are emerging threats to agriculture. However, little is known about their distribution, transmission, interactions *etc*. with plants and insect hosts. This consortium was built to tackle some of the most important topics that are in need of study to help the understanding and control of these diseases. Multiple field scale surveys have been performed across Europe as part of the PhyLib II project and have given a better understanding of the distribution and genetic diversity of Lso and phytoplasmas. The impact of Lso and phytoplasmas appears to differ from region to region with some countries suffering from long-term infection, whilst other countries have discovered recent outbreaks. This project has improved the understanding of Lso haplotypes present in wild plants, crops and insect hosts across Europe and has uncovered previously unknown Lso haplotypes showing that the range of Lso and its diversity is much wider than previously known. It was also found that Lso can co-infect tomato plants with Potato Virus S (PVS). During this project Lso has been found in several new psyllid hosts and their impact on agriculture needs to be understood. For example, *T. anthrisci* is closely related to the carrot psyllid *T. apicalis* and is found to harbour a similar Lso haplotype. Furthermore, this species can feed, reproduce and transmit Lso to carrot. Its overall impact on carrot production however is not known. One of the major Lso vectors in Europe *T. apicalis* has been shown to play a role in Lso transmission in a wider range of countries than known before. Further research efforts should resolve to understand the impact of these newly discovered psyllid hosts and their unique Lso haplotypes. It may be that other crops could be at risk from this pathogen. Surveys such as those carried out in this project should continue to monitor Lso and phytoplasmas in wild plants, crops and their insect vectors.

The question of Lso transmission from seed to seedling and its impact on the spread of Lso and phytoplasmas was explored. Although results have been contradictory, some results suggest that seed transmission is a rare/ very unlikely event and support no transmission from seed to seedling. However, the lack of positive results found here should not be ignored as only a small number of transmission events from seed to seedling could potentially cause

outbreaks in areas where Lso is otherwise absent. It is most likely that the presence of an efficient vector is the most important aspect for Lso transmission in all these cases. For phytoplasmas, evidence has been found that seed to seedling transmission is possible but as for Lso, further study in this area is necessary to fully understand seed to seedling transmission and further work should be done to identify the insect vectors that may aid the spread of these bacteria.

This consortium placed effort and priority on developing and testing the best methodologies for extraction and detection of Lso and Phytoplasma DNA from multiple matrices. These recommendations should be implemented by future researchers that intend to study these pathosystems. The most robust and sensitive Lso detection method tested here was quantitative PCR by Li *et al*., 2009, which performed well in all intra- and inter-laboratory tests with most DNA matrices. For Lso extraction from plant matrices, CTAB and NucleoMag Plant Kit with MC1 buffer (Macherey-Nagel) are recommended.

Further work is needed to build on the work performed in this project and other projects on Lso and phytoplasmas (such as POnTE, WeedVect). The follow-on project PhyLib III intends to address the questions that have arisen from this successful collaboration and hopes to expand the understanding of Lso and phytoplasmas and to continue to inform risk assessments and management of the diseases associated with the presence of these pathogens.

2.5. Benefits from trans-national cooperation

The sharing and availability of Lso or phytoplasma infected control material and insect specimens was a major strength of this project. This material would be otherwise difficult or impossible for partners to obtain without collaboration within a global consortium. This network enabled the quick and efficient set-up of interlaboratory tests necessary to rigorously test many different methodologies which were necessary for the fulfilment of multiple work packages. These collaborations have led to essential recommendations for best practice in monitoring the presence of the pathogens associated with these important diseases which will enable quick response in the control of these diseases and a harmonized approach to continued collaborations. Through this collaboration expertise and knowledge were shared, leading to important discussions and understanding of these patho-systems. These knowledge exchanges include technical help, practical workshops (2 x molecular and morphological psyllid identification courses) and establishment of methodologies. The consortium also cooperated across different projects and facilitated the sharing of data and knowledge, drawing information from the POnTE project, CaLiso, Euphresco Weedvect and other government funded projects (the urls of the various projects are presented at the end of this section). This led to increased visibility of results from other studies and also led to larger collaborations and more cohesive work being performed. The consortium was particularly effective in helping to set-up Lso monitoring in response to requirements for statutory testing, which were put in place by the Agricultural Board in Estonia. The wide range of geographical locations covered by the consortium has given a wider perspective on Lso and phytoplasma and the differences in impact, transmission, vector pressure and agricultural practices. This leads to a more diverse and interesting discussion on results and interpretations of outcomes.

URLs of research projects of interest for the PhyLib II consortium:
CaLiso: https://www.anses.fr/fr/content/le-projet-caliso

PhyLib: https://www.sasa.gov.uk/content/phylib-end-project-meeting
PhyLib II: https://www.sasa.gov.uk/PHYLIBII
POnTE: https://www.ponteproject.eu/
WeedVect: https://zenodo.org/record/3246356#.XhcLIHd2uAh
VECTRACROP: https://zenodo.org/record/1341600#.XiB2v3dFynt

3. Publications

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

None.

3.2. Article for publication in the EPPO Reporting Service

None.

3.3. Article(s) for publication in other journals

The following publications were outputs from work performed in the PhyLib II project:

- Bell J, Greenslade AFC, Kenyon D, Sjölund J, Highet F, Carnegie M (2017). Detection and monitoring of psyllid vectors of "*Candidatus* Liberibacter solanacearum" in Scotland - Final report of project RRL/001/14
- Carminati G, Satta E, Paltrinieri S, Bertaccini A (2019). Simultaneous evaluation of '*Candidatus* Phytoplasma' and '*Candidatus* Liberibacter solanacearum' seed transmission in carrot. Phytopathogenic Mollicutes 9: 141 DOI:10.5958/2249-4677.2019.00071.9
- Haapalainen M, Wang J, Latvala S, Lehtonen M, Pirhonen M, Nissinen A (2018). Genetic variation of "*Candidatus* Liberibacter solanacearum" haplotype C and identification of a novel haplotype from *Trioza urticae* and stinging nettle. Phytopathology 108(8): 925-934
- Haapalainen M, Latvala S, Wickstrom A, Wang J, Pirhonen M, Nissinen AI (2020). A novel haplotype of '*Candidatus* Liberibacter solanacearum ' found in Apiaceae and Polygonaceae family plants. European Journal of Plant Pathology 156: 413-423 DOI: https://doi.org/10.1007/s10658-019-01890-0
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- Karahan A, Erdoğan P, Alkan M, Demir Ozden E, Altundağ Ş (2019). The study on vectors of Stolbur phytoplasma in potato growing areas of Bolu, Kırşehir, Sivas and Yozgat Provinces (Poster). 1st International Molecular Plant Protection Congress. Program and Abstract Book. 51 p., April 10-13, 2019, Adana, Turkey
- Mirmajlessi SM, Sjölund MJ, Mänd M, Loiseau M, Ilardi V, Haesaert G, *et al*. (2019).

PCR-based diagnostic methods for ‘ *Candidatus* Liberibacter solanacearum ’ – Review. Plant Protection Science 55: 229–242.

- Sjölund MJ, Arnsdorf YM, Carnegie M, Fornefeld E, Will T (2018). ‘*Candidatus* Liberibacter solanacearum’ detected in *Trioza urticae* using suction trap-based monitoring of psyllids in Germany. Journal of Plant Diseases and Protection 126: 89–92. DOI: 10.1007/s41348-018-0187-z
- Sjölund MJ, Clark M, Carnegie M, Greenslade AFC, Ouvrard D, Highet F, *et al*. (2017). First report of ’*Candidatus* Liberibacter solanacearum ’ in the United Kingdom in the psyllid *Trioza anthrisci*. New Disease Reports 36, 4 DOI: 10.5197/j.2044-0588.2017.036.004.

4. Open Euphresco data

Validation data and Standard Operating Procedures for detecting Lso from potato tubers using KingFisher instrument are available at EPPO Database of Diagnostic Expertise https://dc.eppo.int/validation_data/validationlist.

Psyllid barcoding data is available on GenBank under accession numbers: ITS2(MT038907-MT038996) and CO1 (MT027551-MT027599). https://www.ncbi.nlm.nih.gov/genbank/