Final Report

Project title (FLADO-VIGILANT)
Development of efficient methods and identification of barcodes for discriminating Grapevine flavescence dorée sensu-stricto from other related phytoplasmas and investigation of potential correlation between taxonomic identity and grapevine, alders and hazelnut plant hosts

Project duration:

Start date:	2021-05-01
End date:	2023-04-30

Contents

1. Research consortium partners

Coordinator – Partner 1	
Organisation	National Institute of Biology (NIB)
Name of contact (incl. Title)	Dr Nataša Mehle (coordinator), Zala Kogej (PhD student)
Postal address	Department of Biotechnology and Systems Biology Večna pot 111, SI-1000 Ljubljana, Slovenia
E-mail	natasa.mehle@nib.si, zala.kogej@nib.si
Phone	+ 386 (0)59 23 28 08, +386 (0)68 127 522

Partner 2	
Organisation	French Agency for Food, Environmental and Occupational Health & Safety (ANSES)
Name of contact (incl. Title)	Dr Marianne Loiseau
Postal address	Laboratoire de la santé des végétaux, 7 rue Jean Dixméras, 49044 Angers cedex O1, France
E-mail	marianne.loiseau@anses.fr
Phone	+332 41 20 74 59

Partner 3	
Organisation	National Research Institute for Agriculture, Food and Environment, Department of Plant Health and Environment (INRAE)
Name of contact (incl. Title)	Dr Xavier Foissac (phD/HDR), Dr Sylvie Malembic-Maher (PhD)
Postal address	71 avenue Edouard Bourlaux CS20032, 33882 Villenave d'Ornon, France
E-mail	xavier.foissac@inrae.fr, sylvie.malembic-maher@inrae.fr
Phone	+33 55712 2356 or +33 55712 2360

Partner 4	
Organisation	Julius Kühn-Institut - Institute for Plant Protection in Fruit Crops and Viticulture (JKI)
Name of contact (incl. Title)	Dr. Michael Maixner, Kerstin Zikeli
Postal address	Geilweilerhof, 76833 Siebeldingen, Germany
E-mail	michael.maixner@julius-kuehn.de, kerstin.zikeli@julius-kuehn.de
Phone	+49 6345 41 213 or +49 6531 956 483 and +49 6221 86805 53

Partner 5	
Organisation	Animal and Plant Health Inspection Service (APHIS)
Name of contact (incl. Title)	Dr Stefano Costanzo
Postal address	BARC-East, Bldg. 580 9901 Powder Mill Rd., Laurel, MD 20708, U.S.A.
E-mail	stefano.costanzo@usda.gov
Phone	+1 301 313-9268

Partner 6	
Organisation	Council for Agricultural Research and Economics (CREA) - Research Centre for Plant Protection and Certification
Name of contact (incl. Title)	Dr Luca Ferretti
Postal address	Via. C. G. Bertero 22 - 00156 Rome (Italy)
E-mail	luca.ferretti@crea.gov.it
Phone	+ 39 0682070223

Partner 7	
Organisation	Instituto Nacional de Investigação Agrária e Veterinária, INIAV,IP
Name of contact (incl. Title)	Dr Esmeraldina Sousa
Postal address	Av. República, Quinta Marquês. 2780-157.Oeiras, Portugal
E-mail	esmeraldina.sousa@iniav.pt
Phone	+351 214403500

Partner 8	
Organisation	Oklahoma State University. Institute for Biosecurity & Microbial Forensics
Name of contact (incl. Title)	Dr. Francisco Ochoa Corona, Dr. Andres Espindola, Dr. Marcos Ribeiro Junior.
Postal address	Oklahoma State University, 127 NRC, Stillwater OK 74078-3003. U.S.A.
E-mail	ochoaco@okstate.edu, andres.espindola@okstate.edu
Phone	+1 4057449946, +1 4057443749, first two authors respectively

Partner 9	
Organisation	DIENSTLEISTUNGSZENTRUM LÄNDLICHER RAUM RHEINPFALZ (DLR), Institut für Phytomedizin (Institute of Plant Protection)
Name of contact (incl. Title)	Dr Thierry Wetzel
Postal address	Breitenweg 71, 67435 Neustadt a. d. Weinstraße, Germany
E-mail	thierry.wetzel@dlr.rlp.de
Phone	+49 6321 671-393

Partner 10	
Organisation	University of Milan
Name of contact (incl. Title)	Dr Fabio Quaglino
Postal address	Via Celoria 2, 20133 Milan, Italy
E-mail	fabio.quaglino@unimi.it
Phone	+39 02 50316789

Partner 11	
Organisation	Università di Catania
Name of contact (incl. Title)	Prof. ssa Matilde Tessitori
Postal address	Via Santa Sofia, 100 – I95123 Catania, Italy
E-mail	matilde.tessitori@unict.it
Phone	+39 3666290335/+39 0957147256

Partner 12	
Organisation	Agroscope
Name of contact (incl. Title)	Dr. Christophe Debonneville
Postal address	Route de Duillier 50, Case postale 1012, CH-1260 Nyon, Switzerland
E-mail	christophe.debonneville@agroscope.admin.ch
Phone	+41 58 484 95 91

Partner 13	
Organisation	CREA-VE Council for Agricultural Research and Economics - Research Centre for Viticulture and Enology
Name of contact (incl. Title)	Dr Elisa Angelini
Postal address	Viale XXVIII aprile 26, I-31015 Conegliano (TV), Italy
E-mail	elisa.angelini@crea.gov.it
Phone	+39-0438-439171

2. Short project report

2.1. Short executive summary

This research project focused on ways to distinguish Grapevine flavescence dorée sensu-stricto (GFD) from other related phytoplasmas and to investigate a possible relationship between taxonomic identity and the host plants grapevine, alder, and hazelnut.
Samples of hazelnuts were collected in different regions of Europe to investigate the presence and prevalence of phytoplasmas. Phytoplasma 16SrV was detected in asymptomatic wild hazelnuts in France, Germany, and Italy and in symptomatic cultivated hazelnuts in Slovenia and central Italy. In addition, other phytoplasma species, such as *'Candidatus* Phytoplasma fragariae' and 16SrIX, were identified. To investigate possible insect vectors of 16SrV phytoplasma associated with hazelnuts, an extensive search was conducted, and samples of leafhoppers were collected. It was found that infection rates of leafhoppers collected on hazelnuts were significantly lower than those previously found on alders. Special attention was paid to *Orientus ishidae*, and preliminary transmission experiments were conducted to better understand their role.
Another objective of the study was to determine the diversity of 16SrV phytoplasma strains. Different genotypes were detected in grapevine, hazelnut, and alder in different countries. Some genotypes were found in both grapevine and hazelnut, suggesting a possible host crossover. In addition, new genotypes were discovered in hazelnuts and alders, expanding our knowledge of phytoplasma strains.
In an effort to improve diagnostic methods, several tests were evaluated to distinguish GFD phytoplasmas from other 16SrV phytoplasmas, and three tests (two nested-PCRs followed by nucleotide sequence analysis and one real-time PCR) were extensively validated in a test performance study. In addition, a prototype HTS-bioinformatics pipeline based on the original EDNA concept has been developed but needs further testing and validation.
The results of this project contribute to a more complete understanding of the epidemiological cycle of 16SrV phytoplasmas and may help in the development of effective strategies to control the disease. Further research in this area will provide valuable insights into the complex interactions between phytoplasmas, hosts, and vectors.

2.2. Project aims

The objective of this research project was to investigate epidemics caused by phytoplasmas belonging to the 16SrV group and to unravel the correlation between taxonomic identity and the host plants grapevine, alder, and hazelnut. The objectives of the project were to develop a reliable test to distinguish between Grapevine flavescence dorée (GFD) phytoplasma sensu stricto and other 16SrV phytoplasmas, determine the occurrence and geographic distribution of hazelnut trees infected with GFD-related isolates, identifying potential vectors associated with hazelnut and evaluating their role in transmission between hazelnut and grapevine, and collecting and comparing sequences of 16SrV phytoplasmas infecting grapevine, hazelnut, alder, Spartium, and leafhopper.

Several work packages were designed to achieve these objectives. Work package 2 focused on sampling hazelnuts in different countries and collecting symptomatic and asymptomatic shrubs to determine the occurrence and geographic distribution of phytoplasmas, especially GFD-related isolates, in hazelnuts. The collected samples were analysed for phytoplasma in general and for 16SrV in particular using different assays. The aim of this work was to gain a better understanding of the infection status of hazelnut orchards and wild hazelnut shrubs in different countries.

Work package 3 aimed to identify potential vectors associated with hazelnut and evaluate their role in phytoplasma transmission between hazelnut and grapevine. Leafhoppers collected from hazelnut orchards and wild shrubs were identified and the collected insect samples were analysed for the presence of 16SrV phytoplasmas using specific assays. The purpose of this study was to determine whether hazelnuts serve as hosts for phytoplasmas and whether certain leafhopper species, such as *Orientus ishidae*, play a role in the transmission of phytoplasmas between hazelnut and grapevine.

Work package 4 focused on obtaining genome sequences of several 16SrV phytoplasmas, including those infecting grapevine, hazelnut, alder, spartium, and leafhopper. Both Sanger sequencing of PCR products and high-throughput sequencing (HTS) were used to obtain the sequences. The goal was to compare sequences obtained from different sources and to identify differences between phytoplasmas from different hosts. This analysis contributes to a better understanding of the epidemiology of the disease.

Work package 5 focused on improving diagnostic tests to distinguish GFD phytoplasmas from other 16SrV phytoplasmas. The objective was to evaluate different tests and propose the best protocol for efficient discrimination. In addition, a test performance study (TPS) was organized to validate the selected tests for discrimination of GFD phytoplasma. This work package focused on improving the specificity and reliability of the tests to enable accurate identification of GFD phytoplasma strains. The goal was to provide valuable tools for early detection and prevention of GFD phytoplasma outbreaks, contributing to effective disease control in European vineyards.

Work package 6 focused on improving the identification of phytoplasmas using Microbe Finder (MiFi©). The objective was to use this user-friendly online platform that uses e-probes to rapidly detect and characterize specific plant pathogens in metagenomic datasets for phytoplasma detection. The focus was on streamlining the identification process and eliminating bioinformatics challenges associated with high-throughput sequencing (HTS). Tasks included developing the e-probes dataset for phytoplasmas, organizing training on the use of the MiFi diagnostic system, and validating the HTS approach with MiFi software. The goal of this work package was to improve phytoplasma diagnostics and enable researchers to effectively identify and monitor phytoplasmas of local importance.

2.3. Description of the main activities

<u>WP1</u>

The coordinator of the project FLADO-VIGILANT prepared the project proposal in the first phase and coordinated with the other partners to prepare the final project description. Project progress was monitored through four reports. Online meetings were organised throughout the project:

- Kick-off meeting (18 May 2021, 16 participants)
- Mid-term meeting (13 April 2022, 18 participants)
- Final meeting (20 July 2023, 17 participants)

WP2
Samples of wild and cultivated hazelnuts were collected in different countries and locations. Symptoms were noted and samples were subsequently processed by molecular tests to confirm phytoplasma infection and to determine if 16SrV phytoplasma were present. We determined the occurrence and geographic distribution of phytoplasma-infected hazelnuts.

WP3
We collected and identified leafhoppers on hazelnuts, alders, and other trees (growing near vineyards or in forests). A collection of possible vectors was made, and the collected leafhoppers were examined for the presence of 16SrV phytoplasma.

WP4
The collected 16SrV-positive samples of hazelnut, alder, grapevine, and leafhopper were sequenced to detect differences among 16SrV phytoplasma isolates. The newly discovered genotypes were added to the European Nucleotide Archive. A draft genome of alder was generated and published in GenBank.

WP5
Several tests were evaluated for efficient discrimination of GFD phytoplasma from other 16SrV phytoplasmas. A test performance study was performed for selected tests.

WP6
Assembly of a prototype pipeline of HTS-bioinformatic based on the original EDNA concept to demonstrate the applicability of EDNA MiFi© to detect and distinguish phytoplasmas, including GFD sensu-stricto from other related phytoplasmas.

2.4. Main results (knowledge, tools, etc.)

To achieve the overall objective of contributing to the understanding of the broader epidemiological cycle of the 16SrV phytoplasma group, we investigated the infection status of hazelnuts. Hazelnut samples were collected from different regions of Europe. Samples were collected as shoot or root, and DNA was isolated from phloem tissue by different methods (e.g., CTAB, KingFisher) and analysed by real-time PCR or nested PCR assays. In Slovenia and central Italy, symptomatic hazelnut shrubs were observed and sampled in different orchards. In Slovenia, 16SrV phytoplasma was detected in 49 of 131 sampled shrubs (all 16SrV phytoplasma-positive shrubs showed symptoms of decline). In Slovenia, in addition to 16SrV phytoplasmas, '*Ca*. P. fragariae' and 16SrIX phytoplasma were also detected in hazelnuts showing symptoms of decline (Mehle et al., 2019). However, in Italy, only the presence of '*Ca*. P. fragariae' was detected in symptomatic hazelnut trees belonging to commercial hazelnut cultivars. Asymptomatic wild hazelnuts were sampled in France, Germany, Italy, Portugal, and Switzerland, and a total of 379 were analysed. In France, samples of wild hazelnuts were collected near different vineyards (without FD, with isolated cases of FD, or with FD outbreaks; in combination with the presence of 16SrV-infected alders or the absence of alders). Only two shrubs were positive for 16SrV phytoplasma, and they

were collected in close proximity to infected alder trees. A similar result came from Germany, where the only positive sample (out of 67 examined) was taken from a hazelnut shrub near an alder tree in the Palatine region. Additional 16SrV-positive hazelnuts were found in northern Italy (Lombardy), where 18 of 78 asymptomatic wild hazelnuts were positive and all were located near FD-infected vineyards. However, as in cultivated hazelnuts, 16SrV phytoplasmas were not only detected in wild hazelnuts - in Switzerland, '*Ca*. P. fragariae' was found in two asymptomatic samples (out of 40 examined). In Portugal, all 45 asymptomatic hazelnut shrubs examined were found to be free of phytoplasmas.

To identify all possible insect vectors associated with hazelnuts, we conducted a comprehensive vector search. Samples were taken on hazelnuts inside and outside orchards, near vineyards, near infected alders and in the forest. Most of the sampled specimens were leafhoppers (Cicadellidae: Deltocephalinae). Samples were collected with a sweep net, motorised device or yellow sticky traps, mainly in 2021 and 2022. Samples, consisting of single specimens or pooled, were analysed with different DNA isolation methods and tested for the presence of 16SrV phytoplasma. The results of the collected leafhoppers that tested positive for 16SrV phytoplasma are summarised in Table 1. We found that the infection rates of the tested leafhoppers collected from hazelnuts were significantly lower compared to those known from populations of the same species on *Alnus glutinosa*.

Table1: list of leafhoppers caught on hazelnuts in different countries, that were positive on 16SrV phytopasma

County (region)	Germany	Slovenia	France	Italy (central)	Italy (northern)	Detected *map* genotypes (analysed in different countries and caught on different plant hosts)
Hazelnut growth	wild	cultivated	wild	cultivated, wild	wild	
No. of collected specimen	3510	100	7475	63	712	
	number of 16SrV positive samples / number of analysed samples*					
Reptalus quinquecostat.	0/2					
Dictyophara europaea			0/1			
Agallia consobrina	0/4					
Aphrodes makarovi				0/1		
Iassus spp.			4/9			'*Ca.* P. ulmi', ND
Penestragania apicalis			0/1			
Acericerus spp.				0/2		
Macropsis sp	0/5		1/1	0/1		ND
Opsius stactogalus			0/2			

Evacanthus sp.			0/1			
Allygidius atomarius			17/38			M50 variant, '*Ca.* P. ulmi', ND
Allygidius detectus			6/11			'*Ca.* P. ulmi', ND
Allygidius sp.	0/1		1/1			ND
Allygus mixtus	1/3		0/6			
Allygus modestus	0/3		9/57			'*Ca.* P. ulmi', ND
Allygus sp.			1/4			ND
Anoplotettix horvathi	*0/42*					
Conosanus obsoletus				0/6		
Euscelidius schenckii				0/2		
Euscelidius sp.				0/8		
Euscelis lineolatus				0/3		
Fieberiella florii	4/344		1/52			M38, Ca. P. ulmi, ND
Hishimonus sp.			1/1			ND
Japananus hyalinus	0/1		1/8			ND
Lamprotettix nitidulus	5/86		1/16			M38, ND
Orientus ishidae	13/273	13/65	3/77		70/712	M6, M12, M38, M50, M51, M122, M160, ND
Phlogotettix cyclops			5/15			M38, M50 variant, ND
Placottettix taeniatifrons			1/4			M50

Psammotettix confinis				0/35		
Psammotettix sp.	*0/58*					
Scaphoideus titanus			3/71			M51, M54, ND
Synophropsis lauri	0/18		12/57			M38, M38 variant, M50, M50 variants, ND
Thamnotettix dilutior	1/3		3/41			ND

* Sample can be an individual specimen or a pool of up to 5 insects
ND – not determined

To better understand the role of *O. ishidae* in hazelnut phytoplasma infection, preliminary transmission assays were conducted in 2022. However, due to the exceptionally hot and dry conditions, only a few specimens of the leafhopper could be collected. Three hazelnut cuttings were exposed to 21 *O. ishidae* (10, 9 and 2 specimens per cutting) for seven days. Six weeks after inoculation, they were first tested for phytoplasma infection. Two surviving plants were tested again nine weeks and 11 months after inoculation. All tests were negative, although 57% of the leafhoppers (12/21) used for inoculation were infected. One cutting died within 7 weeks of inoculation and the others did not regrow after hibernation. The experiments will be continued in the future.

To better understand the diversity of 16SrV phytoplasma strains, we collected and compared sequences of different 16SrV isolates from different countries and different hosts. The nucleotide sequence analysis was based on the *map* gene and compared with a sequence database of all published and recorded map genotypes. This database was created by INRAE (Partner 3) and is available to others upon request. Until May 2023, this database included genotypes M1 to M162. In grapevine, we have detected a total of 11 different map genotypes:

- in France M38, M50, M50 variant and M54
- in Germany 6 different PGY-associated genotypes (M43 and M52 were the most common, each accounting for a quarter of the samples)
- in Italy M3, M50, M51, M54 and M121 variant
- in Switzerland M54 and
- in Slovenia M38, M50, M51, M54, M122 and M158.

Some of these genotypes found in grapevine were also discovered in hazelnut: M38 (Germany, Slovenia), M50 (Northern Italy, Slovenia), M51 (Northern Italy), M54 (Northern Italy), M122 (Slovenia) and a mixture of map-FD1 and AldYp genotypes (France). In Slovenia, we discovered some new genotypes previously found only in cultivated hazelnuts (M159-162) and a genotype previously found only in alders (M48). The presence of different genotypes in alders was investigated in France and Italy. In France, it was analysed in three regions: in Bordeaux, there were M50, M58, M47 and mixtures of genotypes with SNP signatures of map-FD1, map-FD2 and AldYp (representing 75% of the samples analysed), in Savoie, a mixture of 16SrV phytoplasma or by the M53 genotype (AldYp) and in Champagne, 75% were mixtures of AldYp SNP signatures or with M52 (AldYp). In Italy, the 16SrV phytoplasma strains M50 (10 trees), M51 (4), M38, M48, M58, M78, M116, M117 and M121 (each strain in one tree) were found in alders. The genotypes detected in the leafhoppers found on grapevines, hazelnuts or alders are listed in the last column of Table 1.

With the aim of finding a reliable test that can distinguish GFD phytoplasma from other 16SrV phytoplasmas, four different tests developed by INRAe in Bordeaux were evaluated under intra- and inter-laboratory conditions. For this purpose, Partner 2 organised the collection of biological material. The other partners provided isolates from targets or non-targets. Isolates

from non- targets were obtained outside the partnership through the generous contribution of F. Constable and B. Rodrigues (Australian Government). DNA isolates from the phytoplasma collection of the University of Bologna were also acquired to evaluate the specificity of the methods. During intra-laboratory evaluation, two nested PCRs (one targeting the *map* gene and the other targeting the *Vmp* gene) and two duplex real-time PCRs (targeting *VmpA* and *VmpB* genes) were tested and characterised. For the conventional PCRs in particular, some adjustments were made for routine use. At the same time, their performances were compared with the official French method (Pelletier et al., 2009). We noted that the VmpB target does not seem to be specific for GFD phytoplasmas (Vmp2/3 cluster). For this reason, the real-time PCR vmp-RK-A1B23 was dropped from the rest of the study. Partners 2 and 3 prepared three data sheets with protocols suitable for specific detection of GFD phytoplasmas in the EPPO PM7 appendix format. The three tests were: a nested-PCR 16SrV map adapted from Arnaud et al. (2007), followed by sequence analysis; a nested-PCR VmpA-R1 adapted from Rossi et al. (2019) and Malembic-Maher et al. (2020), followed by sequence analysis; and a real-time PCR Vmp-RK-A23B1 (unpublished - developed by INRAe in Bordeaux). An inter-laboratory study (test performance study) was conducted for a full evaluation of these tests. A panel of 90 target and non-target DNA extracts was sent to 11 participating laboratories from 9 different countries for a double-blind analysis. The results highlight the limitations of the reproducibility of nested-PCR assays followed by nucleotide sequence analysis by addressing critical steps such as the risks of contamination during nested-PCR, sequence quality and operator training in sequence analysis. The performance characteristics of real-time PCR are most interesting for the identification of GFD phytoplasma *sensu stricto*. However, unlike the other methods evaluated, it does not allow the specific identification of the other non-epidemic phytoplasmas that can be detected in grapevine.

To explore other detection methods for phytoplasma, we also used the Electronic-probes Diagnostic Nucleic-acid Analysis for phytoplasma (EDNA-Phytoplasma). The research was conducted by Partner 8 and the initial research phase consisted of building three databases consisting of 1) the sequences of the phytoplasma species to be targeted, 2) the sequences of other phytoplasma species that are not targeted, and 3) the host genome, in this case grapevine. A prototype HTS bioinformatics pipeline based on the original EDNA concept was developed and its functionality was demonstrated using unassembled HTS sequencing metagenome data from a grapevine sample infected with FD (provided by Partner 1). Subsequently, the metagenome was tested with the prototype of EDNA -phytoplasma using the MiFi© MiDetectTM platform and used as a demonstration during a one-day workshop (20 September 2022 (6 participants), 19 January 2023 (7 participants)). Further testing with additional metagenomic files is required to continue the validation process of the method.

2.5. Conclusions and recommendations to policy makers

The following conclusions were drawn on the basis of our findings:

- Infection status of hazelnuts: The investigation of hazelnut samples from different regions of Europe provided information on the occurrence and prevalence of phytoplasmas. In Slovenia, hazelnut decline was associated with 16SrV phytoplasmas, which were detected in about 50% of the sampled shrubs. Previous reports have linked decayed cultivated hazelnuts to infection with phytoplasmas of the 16SrXII ('Ca. P. fragariae'), 16SrIX, and 16SrV groups (Mehle *et al.*, 2019). In central Italy, 'Ca. P. fragariae' was found in symptomatic cultivated hazelnuts. However, the presence of phytoplasmas in wild hazelnuts was limited, as only a few positive samples were detected in France (2 samples with 16SrV phytoplasmas), Germany (1 sample with 16SrV phytoplasmas), Italy (18 samples with 16SrV phytoplasmas), and Switzerland (2 samples with 'Ca. P. fragariae'). However, in Switzerland, detection of 16SrV phytoplasmas in asymptomatic, non-cultivated

hazelnut shrubs from the forest near a GFD-infected vineyard was also previously reported (Casati *et al*., 2017).

- Insect vectors: A comprehensive search for insect vectors associated with hazelnuts revealed that leafhoppers, mainly from the family Cicadellidae (Deltocephalinae), were the most frequently collected specimens. However, infection rates of these leafhoppers with the 16SrV phytoplasma were significantly lower than in populations found on *Alnus glutinosa*. Preliminary transmission experiments with *O. ishidae* leafhoppers have been carried out, but further experiments are required.
- Diversity of 16SrV phytoplasma strains: Sequence analysis of different 16SrV isolates from different countries and hosts revealed the presence of multiple genotypes. In grapevine, 11 different map genotypes were identified, some of which were also found in hazelnut. In addition, new genotypes were discovered in cultivated hazelnuts. The occurrence of different genotypes in alders has also been observed in France and Italy.
- Diagnostic methods: Evaluation of different diagnostic methods to distinguish GFD phytoplasma from other 16SrV phytoplasmas showed that nested PCR assays (based on map and Vmpa-R1) followed by nucleotide sequence analysis have limitations in terms of false negative and false positive results. The duplex real-time PCR Vmp-RK-A23B1 showed promising performance in identifying GFD phytoplasma sensu stricto. However, the primers and probe designed for the identification of the Vmp I vectotype (phytoplasmas of the 16SrV group but not Flavescence dorée phytoplasma sensu stricto) are not specific for this vectotype. Therefore, only primers and probe targeting Vmp-RK-A of vectotypes II and III should be used in a simplex real-time PCR in a routine laboratory.
- The applicability of EDNA MiFi© for the detection and discrimination of GFD sensu-stricto from other related phytoplasmas of grapevine was demonstrated with a prototype of HTS - bioinformatics pipeline based on the original EDNA concept but requires further testing and validation.

Based on the project results, it is recommended to establish and strengthen surveillance programs to monitor the occurrence and prevalence of phytoplasmas, particularly 16SrV phytoplasmas, in hazelnut orchards across Europe. Regular sampling and testing should be conducted using reliable diagnostic methods.

2.6. Benefits from trans-national cooperation

Trans-national cooperation in the field of plant health and phytoplasma research can bring numerous benefits and contribute to the overall understanding and management of these diseases. The findings presented in the previous sections highlight several key areas where collaboration among researchers had a positive impact.

Firstly, studying the infection status of hazelnuts in different regions of Europe provided valuable insights into the occurrence and spread of phytoplasmas. This information is crucial for the development of effective control and management strategies. By sharing data and harmonising detection methods, collaboration can help establish comprehensive surveillance programmes that track the spread and impact of phytoplasmas on hazelnut crops. This collaborative approach increased knowledge of 16SrV phytoplasmas in cultivated and wild hazelnuts and reduced the risk of widespread outbreaks and economic losses.

Secondly, the identification of insect vectors associated with hazelnuts highlighted the importance of studying the ecological dynamics of phytoplasma transmission. By conducting a comprehensive search for vectors and assessing their infection rates, researchers gained a better understanding of transmission mechanisms and potential risk factors. The collaboration enabled the exchange of knowledge and expertise in vector biology and entomology and facilitated the development of targeted control measures that can disrupt the transmission cycle and reduce the spread of the disease.

Furthermore, the study of the diversity of 16SrV phytoplasma strains across different countries and hosts highlighted the need for a comprehensive and coordinated approach to strain characterisation and surveillance. Cross-national collaboration allowed the sharing of resources, samples and expertise, which lead to a more thorough and accurate assessment of phytoplasma genetic diversity. This knowledge is important for identifying potential sources of infection and tracking the movement of strains across borders.

In the area of diagnostic methods, transnational cooperation plays a crucial role in improving and standardising techniques for detecting phytoplasmas. The evaluation of different detection and identification assays and the development of the EDNA-Phytoplasma method demonstrated the potential for innovative approaches to enhance diagnostic capabilities. By sharing protocols, conducting TPS studies and aligning diagnostic procedures, researchers can ensure the accuracy, reliability and reproducibility of diagnostic methods.

In conclusion, trans-national cooperation in phytoplasma research brings numerous benefits for understanding, detecting and managing these pathogens. Through transboundary collaboration, researchers and policy makers can establish comprehensive surveillance programmes, identify important vectors, characterise the genetic diversity of phytoplasmas and improve diagnostic methods. These joint efforts improve the ability to respond effectively to outbreaks, limit economic losses and protect hazelnut crops and other susceptible plant species.

3. Publications

3.1. Published articles with the results of the project

- Cai W, Schyler Nunziata S.O., Srivastava S.K., Wilson T, Chambers N., Rivera Y., Nakhla M., and Costanzo S. Draft Genome Sequence Resource of AldY-WA1, a Phytoplasma Strain Associated with Alder Yellows of *Alnus rubra* in Washington, U.S.A. *Plant Disease* **2022**, 106,7: 1971-1973.
- Debonneville C, Mandelli L., Brodard J., Groux R., Roquis D., and Schumpp O. The Complete Genome of the "Flavescence Dorée" Phytoplasma Reveals Characteristics of Low Genome Plasticity. *Biology* **2022**, *11*, 953. https://doi.org/10.3390/biology11070953
- Gentili A., Donati L., Bertin S., Manglli A., and Ferretti L. First report of '*Candidatus* Phytoplasma fragariae' infecting hazelnut in Italy. *Plant Disease* **2022**. Published Online: 20 Mar 2022. https://doi.org/10.1094/PDIS-11-21-2566-PDN
- Kogej Zwitter Z., Seljak G., Jakomin T., Brodarič J., Vučurović A., Pedemay S., Salar P., Malembic-Maher S., Foissac X., and Mehle N. **2023**. Epidemiology of Flavescence dorée and hazelnut decline in Slovenia: geographical distribution and genetic diversity of the associated 16SrV phytoplasmas. Front. Plant Sci. 14:1217425. doi: 10.3389/fpls.2023.1217425

3.2. Other dissemination activities

The goals of the project were presented at the following meetings:

- Euphresco Community Network (15.3.2022, online) - Mehle N.
- 9th EPPO Panel on Diagnostics in Virology and Phytoplasmology (5.5.2022, Olomouc, Czech) - Mehle N.

The results achieved in the project were presented by various partners at various conferences, meetings:

- Oral presentation at 15th International Plant Virus Epidemiology Symposium (5-8.6.2022, Madrid, Spain) - Kogej Zwitter Z., Seljak G., Jakomin T., Brodarič J., Vučurović A., Ravnikar M., Kutnjak D., Mehle N: Investigation of the diversity of the destructive 16SrV phytoplasma group in grapevine, hazelnut and leafhoppers.
- Poster presentation at the IX Italian National Meeting on Viticulture (13-15.6.2022, Conegliano, Italy) - Belgeri E., Forte V., Filippin L., Spada A., Guadagnino S., Angelini E., 2. Ruolo di alcuni cicadellidi nella diffusione della flavescenza dorata in vigneti del Veneto (Role of some leafhoppers in the spreading of Flavescence dorée in Venetian vineyards).
- Partner 7 held a seminar with a field visit on 28 June of 2022 in Amares (Minho), to present the results to the viticulture sector (winegrowers and their associations and official inspectors)
- Oral presentation at Plants in Changing Environment: international conference of the Slovenian Society of Plant Biology (15-16.9.2022, Ljubljana, Slovenia) - Kogej Zwitter Z., Seljak G., Jakomin T., Brodarič J., Vučurović A,, Ravnikar M., Kutnjak D., Pedemay S., Salar P., Malembic-Maher S., Foissac X., Mehle N.: Epidemiology and diversity of 16SrV phytoplasma group infecting grapevine and hazelnut.
- Oral presentation at congress for Slovenian growers of hazelnuts (11.3.2023, Ljubljana, Slovenia) - Kogej Zwitter Z., Seljak G., Jakomin T., Brodarič J., Vučurović A., Mehle N.: Propadanje lesk v Sloveniji zaradi okužbe s fitoplazmo, sorodno povzročiteljici zlate trsne rumenice (decline of hazelnut in Slovenia due to infection with GFD-related phytoplasma)
- Oral presentation at technical consultation on „Grapevine flavescence dorée and Scaphoideus titanus"(10.5.2023, Zagreb, Croatia) - Mehle N., Kogej-Zwitter Z.: Genetic diversity of 16SrV phytoplasma infecting grapevines and hazelnuts in Slovenia

- Conference presentation of short article at 5th Meeting of the International Phytoplasmologist Working Group (21-25.5.2023, Muscat, Oman) - Kogej Zwitter Z., Jakoš N. Mehle, N.: Unravelling the puzzle of 16SrV phytoplasma in hazelnuts: A systematic study of sampling and detection.
- Conference presentation of short article at 5th Meeting of the International Phytoplasmologist Working Group (21-25.5.2023, Muscat, Oman) - Auriol A., Salar P., Pedemay S., Lusseau T., Desqué D., Lacaze D., Bocquart M., Levillain M., Bey J.S., Pienne P., Delame M., Doublet B., Riou I., Abidon C., Foissac X., Malembic-Maher S.: Origin of isolated cases of Flavescence dorée in North-East of France: search for reservoir plants and insect vectors in semi-natural habitats near vineyards.
- The project and achieved results were mentioned in several presentations of Partner 3 and 4 for winegrowers and the phytosanitary services

3.3. Other planned dissemination activities:

- The results of WP5 will be presented at the 10th meeting of the Panel on Diagnostics in Virology and Phytoplasmology (a revision of EPPO PM7/79 based on the results of WP5 is proposed)
- The results obtained by partner 7 (INIAV, PT) will be presented at 20th ICVG meeting

4. Open Euphresco data

None.