EUPHRESCO Final Report

Phytosanitary diagnostic, on-site detection and epidemiology tools for *Erwinia amylovora* (PHYTFIRE)

Overview of Work Packages

Work Packages (WP)	
No. of WP	**Title**
1	Project Management and Co-ordination
2	Methods training and ring testing
3	Flower monitoring
4	Vector monitoring
5	Asymptomatic infections and environmental fitness
6	Source Tracking

Project duration:

Start date:	01/01/12
End date:	31/12/13

Introduction

Fire blight is a devastating disease of apples, pears and related ornamental plants. *Erwinia amylovora*, the causal agent of the disease, is a quarantine bacterium in Europe and the long-term control of fire blight still requires the eradication of inoculum reservoirs. The project entitled `Phytosanitary diagnostic, on-site detection and epidemiology tools for *E. amylovora* (PHYTFIRE)´ was a three-year project in the frame of the EUPHRESCO-2 action funded by EU FP7 ERA-NET that ran nominally from January 2012 to December 2014, although the start and ending dates were not identical among the partners involved. The funding mode was a Virtual Common Pot, via a non-competitive call (i.e., each funder paid for the participation of its own national researchers) for a total amount of € 554,290.-

Objectives

The research aims of the project were the development, adaptation, application and dissemination of innovative phytosanitary tools to fill fundamental research gaps in pathogen detection and epidemiology in order to improve phytosanitary and control strategies against fire blight. Procedures for monitoring of pathogen populations in flowers, vectors and asymptomatic infections, and for pathogen genotyping were to be developed. Deliverables were detailed for each Work Package (WP) and included: (1) development of novel methods and protocols (e.g., SNP genotyping, specific PCR for 'new' *Erwinia* spp., etc.); (2) validation of the newly developed diagnostic protocols in a ring test; (3) adaptation of existing techniques for phytosanitary applications (e.g., source-tracking, LAMP-PCR, etc.); (4) training in fire blight diagnostics at several levels with the ultimate goal of capacity building within a pan-European network of structures to support PPO; (5) generation of new epidemiological data.

General benefits

The most important outcome of this EUPHRESCO-2 action was the intensification of the cooperation between research institutions and the dissemination of the research results to the widest possible stakeholder community. The website www.phytfire.org was developed to maximize both in-house dissemination and outreach. A network had already been established on the research topic "fire blight" as a result of former research projects (e.g., the EU FP6 project ErwinDect). The interconnection between existing partners has been strongly intensified herein and big emphasis was placed on the recruitment, during the action, of new associate partners from regions that were only recently confronted with fire blight, like East European and Central Asian countries. The latter are the center of origin of domesticated *Malus* species and still feature pristine forests of apple and pear trees that represent an extraordinary reservoir of genetic diversity that is now put threatened by the arrival of fire blight.

Key results and outputs

- An intensive lab training course on new and established method for *Erwinia* spp. detection and discrimination was organized at the start of the project for all the participating and associated partners;
- Further technology transfer dissemination of knowledge to diverse stakeholder groups was actively promoted at a national level by each of the involved partners (e.g., grower events, training exhibitions);
- New associated partners from East European and Central Asian countries were recruited during the project. These partners participated on a self-funded basis and were able to take advantage of the experience of the consortium to improve their knowledge on fire blight detection and epidemiology. In exchange they provided valuable information about the fire blight situation in their own countries and delivered biological material (i.e., strains) that enriched our resources for pathogen analysis;
- The project website (www.phytfire.org) was developed to provide a central resource for accessing information on fire blight, fire blight scientists, research advances and activities;
- Laboratory assays for the detection and discrimination of *E. amylovora* and related species (*E. pyrifoliae*, *E. piriflorinigrans*, *E. uzenensis*) were developed and tested in a ring trial;
- The use of MALDI-TOF MS was validated for rapid population analysis of *E. amylovora* and related species on flower samples;
- A LAMP-based diagnostic kit for onsite detection of *E. amylovora* was developed and tested and is available to all partners involved;
- A protocol with user-friendly advices and most suitable methods for *E. amylovora* detection in asymptomatic plant material was developed for diagnostic labs and phytosanitary inspection services;
- Population structure and epidemiological data were obtained by analyzing epidemic foci in Switzerland, Slovenia and the Netherlands using a VNTR high-throughput ("MLVA-6") method developed herein;

- A diversity analysis was performed to understand the dissemination routes originating from Europe and the forthcoming hazards represented by fire blight to the native apple germplasm in Central Asia;
- Outcomes of this project have been published as nine peer-reviewed journal articles, 24 meeting presentations, six trade or popular press articles and a number of official protocols, all freely downloadable at the project website www.phytfire.org.

Final Project Report (1/10/2012 – 31/08/2014)

Each WP involved two phases of participation level. Phase 1 will generally occupied the first year of the project. Phase 2 was generally started in the second year.

WP1 Project Management (Project Coordinator: B. Duffy; Vice-Coordinator: F. Rezzonico)

WP1.1 Research Consortium Coordination (F. Rezzonico, B. Duffy)

- The website www.phyfire.org was developed in order to serve as a platform for all the partners in the PHYTFIRE community to interconnect, share results and retrieve important information such as lab/diagnostic protocols or scientific literature.
- Particular emphasis was placed in the extension of the PHYTFIRE network to new partners in Eastern European and Central Asian countries that were only recently confronted with fire blight, with the aim to gather first-hand epidemiological data and to transfer the know-how present in the PHYTFIRE network to their diagnostic services. Beside the original core affiliates, new partners from Kazakhstan, Kyrgyzstan, Latvia, Serbia and Poland joined the consortium as active collaborators or observers.
- The attendance to the mid-term meeting as well as to the 13th ISHS International Fire Blight Workshop of several participants from the countries mentioned above was heavily facilitated by travel grants sponsored by the Swiss National Science Foundation (SNSF), which were requested by the PHYTFIRE project management that was heavily involved in the organization of both meetings.

WP1.2 Meetings and training courses (co-Leaders: H. Ozaktan, L. Cruz)

- The project kick-off meeting was organized by Prof. Dr. Leonor Cruz (INIAV) in Oeiras (Portugal), October 1st-4th, 2012. The meeting presented a multi-purpose program ranging from direct communication to local stakeholders, to planning and presenting the scientific tasks to be performed during the action and was capped by a two-day lab training course designed to disseminate the techniques for plant inspection diagnostics in the participating countries.
- A mid-term meeting was held on July 1st, 2013 at ETH Zürich (Switzerland) as a satellite event of the 13th ISHS International Fire Blight Workshop. The meeting was organized by the project coordinators and was centered on the presentation of Phase 1 research progress by the different partners, the planning of Phase 2 cooperative research and the preparation of dissemination and publications output.
- The final meeting was hosted from May 13th to May 15th, 2014 in Ürgüp (Turkey) by the group of Prof. Dr. Hatice Ozaktan. Principal topics were the exchange of Phase 2 results and their discussion, the preparation of scientific and technical publications and the underpinning of the project final report. Follow-up strategies for continued cooperation were outlined and ideas for new funding initiatives were collected. It is indeed firm intention of the vast majority of the partners to continue their collaboration beyond the framework of this Euphresco II program.

WP1.3 Technology Transfer and Dissemination (co-Leaders: U. Persen, M. Bergsma-Vlami)

- In order to maximize stakeholder access to results and project expertise, all protocols and publications on detection of *Erwinia amylovora* and closely pathogens were disseminated via the PHYTFIRE website. There are several pathogens related to *E. amylovora* that have been described from East Asia (*Erwinia pyrifoliae* and *Erwinia uzenensis*) and more recently from Spain (*Erwinia piriflorinigrans*), all causing symptoms similar to fire blight. Whether these occur more widely in Europe or pose an emerging phytosanitary threat is largely unstudied. This dearth of basic data is due to a lack of efficient

methods for detecting these bacteria in plant samples.

- In the frame of the project kick-off meeting held in Oeiras, a two-day laboratory-training course was prepared by the Spanish group leading WP2 with input from the other Phase 1 partners and the local organizers. A complete overview of the established quantitative and qualitative diagnostic techniques for *E. amylovora*, *E. pyrifoliae* and *E. piriflorinigrans* available at the beginning of the action was presented. All partners contributed to the training course with at least one person to learn the different methods and, if necessary, to further disseminate them within their countries.
- Following the first training above, ring tests of selected methods were developed, optimized and adapted during the PHYTFIRE project. The results were then returned by each participating partner to the coordinators of WP2, which posted the validated protocols on the PHYTFIRE website (www.phytfire.org).
- Linked to the 13th ISHS International Fire Blight Workshop (www.fireblight2013.org), Dr. Galiya Zharmukhamedova (Kazak Scientific Research Institute for Plant Protection and Quarantine), was invited to Agroscope by the PHYTFIRE project management and had the opportunity to visit the institute facilities to learn more about the standard diagnostic techniques usage to detect fire blight in Switzerland. This is important because *E. amylovora* was detected in Kazakhstan only recently and the country is believed to be the center of origin of *Malus sieversii*, the wild ancestor of *Malus* x *domestica..*
- During the last year of the action, also the research groups of Dr. Tinatin Doolotkeldieva (Kyrgyz-Turkish Manas University) and Dr. Inga Moročko-Bicevska (Latvia State Institute of Fruit-Growing) joined the action on a voluntary basis and taking advantage of the experience of the consortium to improve their knowledge on fire blight detection and epidemiology. In exchange, they provided valuable information about the fire blight situation in their own countries and delivered strains for analysis.

WP2 Methods training and ring testing (Lead: M.M. López)
Phase 1 partners: M.M. López, B. Duffy, R. Gottsberger
Phase 2 partners: H. Ozaktan, N. Ustun, L. Cruz, K. Kornev, L. Baranauskaite, I. Kibildiene, V. Gusina, K. Petrutis, H. Lasner, M. Gusina, E. Pochukaeva
Note: Phase 2 activities in WP2 will commence from the project start in parallel with Phase 1.

Objectives

Selection of optimized molecular and serological techniques for diagnosis, detection, and identification of pathogenic *Erwinia* (*E. amylovora, E. pyrifoliae, E. uzenensis*, and *E. piriflorinigrans*). Provide protocols to be used in a ring test system. Offer basic training to establish competence in all partner countries for fire blight diagnostics.

Phase 1:

- Evaluation of *E. pyrifoliae* diagnostic and identification assays, genomics-informed design detection assays for *E. piriflorinigrans* and *E. uzenensis.*
- Organization of defined scale ring tests of selected methods developed, optimized, or adapted in the PHYTFIRE project.

Results

- Assessment of different protocols for detection of *E. pyrifoliae* has been performed, and the ones showing better sensitivity and specificity results have been included in the protocols for ring testing. New PCR systems for *E. piriflorinigrans* (conventional and real time) have been designed based on sequences from plasmid pEPIR37 and the sensitivity and specificity has been evaluated. In addition, the protocols have been validated in a survey and published in Applied and Environmental Microbiology.
- Sequencing of the ITS region of *E. uzenensis* strains has been performed. Sequence comparisons analyses by CLUSTALw were completed and a consensus sequence obtained. From this sequence, primers and probe were designed for a sensitive and specific real time PCR protocol. This protocol has been tested for its sensitivity and specificity with good results and a publication will be prepared.
- Protocols for detection and methods for plant samples preparation have been established and sent to

the partners for practicing and using in the ring tests.

- Lab training with participants from the different countries participating in the project was performed in Portugal, at the Instituto Nacional de Investigação Agrária e Veterinária, I.P. in October 2012. Comparison of the different PCR protocols for *E. amylovora* as well as for *E. pyrifoliae*, and *E. piriflorinigrans* were assayed and results discussed in a final meeting.
- A ring test of selected methods developed, optimized, or adapted in the PHYTFIRE project has been performed. A set of eleven blind pure cultures and molecular reagents were sent from IVIA to 8 participant laboratories (from EU countries and Russia). Protocols for performing the ring tests were provided to the participants and the data were analyzed. The obtained results are shown in the Tables 2.1-2.3.

Table 2.1: Ring test results for *E. amylovora* detection by PCR

COUNTRIES	Real-time PCR (Pirc *et al*) *E. amylovora*	Conventional-PCR (Gottsberger *et al*) *E. amylovora*
AUSTRIA	Positive results with Eamy and also with Epyr, Epfn, Euzn (no Ct available)	OK, but positive result with Epyr (no Ct available)
ESTONIA	OK (Ct 15,6)	NP
LITHUANIA	OK (Ct 16.9)	OK (Ct 20.5)
PORTUGAL	OK (Ct 16.0)	OK (Ct 14.7)
RUSSIA	NP	OK (Ct 20.7)
TURKEY	OK (Ct 18.5)	NP
SPAIN	Positive results with Eamy (Ct 16) and with Epyr (Ct 28), E uze (Ct 28)	OK (Ct 17.9)

NP not performed; Eamy: *E. amylovora*; Epyr: *E. pyrifoliae*, Epfn: *E. piriflorinigrans*; Euzn: *E. uzenensis*
OK Amplification with *E. amylovora* only
Positive results: (lowest Ct with *E. amylovora)*

Table 2.2: Ring test results for *E. pyrifoliae* detection by PCR

COUNTRIES	Real-time PCR (Wensing et al) *E. pyrifoliae*	Conventional-PCR (Wensing et al) *E. pyrifoliae*
AUSTRIA	Positive results with Epyr and also with Eamy, Euzn (no Ct available)	OK
ESTONIA	Positive results with Epyr (Ct 20.9) and also with Eamy, Epfn, Euzn (Ct higher than 38)	OK
LITHUANIA	OK (Ct 22.9)	NP
PORTUGAL	Positive results with Epyr and also with Euzn (Ct 35.6)	OK
RUSSIA	Positive results with Epyr and also with Eamy (Ct 33), Epfn, (Ct 34) Euzn (Ct 29)	OK
TURKEY	NP	OK
SPAIN	Positive results with Epyr and also with Eamy (Ct 27.9), Euzn (Ct 32)	NP

NP not performed; Eamy: *E. amylovora*; Epyr: *E. pyrifoliae*, Epfn: *E. piriflorinigrans*; Euzn: *E. uzenensis*
OK Amplification with *E. pyrifoliae* only
Positive results: (lowest Ct shown with *E. pyrifoliae)*

Table 2.3: Ring test results for *E. piriflorinigrans* and *E. uzenensis* detection by PCR

COUNTRIES	Real-time PCR (Barbé et al) *E. piriflorinigrans*	Conventional-PCR (Barbé et al) *E. piriflorinigrans*
AUSTRIA	Positive results with Epfn and also with Epyr (no Ct available)	Positive results with Epfn and also with Eamy, Epyr, Euzn (no Ct available)
ESTONIA	Not working	Positive results with Epfn and also with Eamy, Epyr, Euzn (no Ct available)
LITHUANIA	Positive results with Epfn and also with Eamy (Ct 34.7), Epyr (Ct 32.7), Euzn (Ct 31.0)	NP
PORTUGAL	OK (Ct 15.2)	OK
RUSSIA	Not working	OK

TURKEY	NP	Positive results with Epfn and also with Epyr, Euzn
SPAIN	OK(Ct 20.7)	OK

NP not performed; Eamy: *E. amylovora*; Epyr: *E. pyrifoliae*, Epfn: *E. piriflorinigrans*; Euzn: *E. uzenensis*
OK Amplification with *E. piriflorinigrans* or *E. uzenensis* only
Positive results: (lowest Ct shown with the target species)

The results showed the accuracy of the different protocols and the robustness of some of them. The lack of amplification in two labs with the real-time PCR protocol for *E. piriflorinigrans* could be due to stability problems of some reagents during transport. The obtained data also show that some PCR protocols were not specific for their target but the use of integrated protocols as indicated in the EPPO scheme for identification (EPPO, 2013) will avoid the false positives that could be obtained when using only one method for identification.

Deliverables, dissemination and training output

- Evaluation of recently developed protocols for identification of *E. amylovora* and *E. pyrifoliae*
- Development and validation of a new protocol for diagnosis and identification of *E. piriflorinigrans* (Barbé et al., 2014)
- Peer reviewed paper on the genome of *E. piriflorinigrans* CFBP 5888^T in cooperation with partner 1 (Smits et al., 2013).
- Set up a new protocol for identification of *E. uzenensis*
- Development and validation of a new protocol for diagnosis and identification of *E. amylovora* (Bühlmann et al., 2013)
- Ring test and training courses to the EUPHRESCO/PHYTFIRE partners
- Final version of the EPPO protocol for *E. amylovora* (EPPO, 2013)
- Final version of the IPPC protocols for *E. amylovora* (in phase previous of country consultation)

WP3 Flower monitoring. (Lead: A. Wensing)
Phase 1 partner: A. Wensing, T. Leichtfried
Phase 2 partners: F. Rezzonico, H. Ozaktan, N. Ustun, T. Leichtfried

Objectives

Evaluate specific detection protocols for *Erwinia* species for routine analysis of field samples. Develop a suitable method for comprehensive monitoring of culturable microbes in flower population.

Approach

Population monitoring: Protein fingerprinting by MALDI-TOF allows fast identification of non-specifically selected microorganisms (Sauer et al., 2008). Compared to DNA and sequencing-based methods, MALDI-TOF ID targets only cultivable organisms but is very cost efficient and suitable for high-throughput. The technique is applicable for analysis of mixed communities when individual colonies can be obtained (Wensing et al., 2012). We have used MALDI-TOF ID to investigate composition of bacterial communities on flowers during EPPO standard trials for fire-blight control.

Results

Different sample preparations for MALDI-TOF ID were compared and a draft protocol suitable for analysis of high sample numbers was developed. Briefly, individual flowers/fruitlets were collected in 2ml sample tubes or bags and extracted with 1ml sterile water under vigorous shaking for 10min. Plant material was removed and wash samples adjusted to 15% glycerol and stored at -80°C for subsequent analysis.

Aliquots of the wash sample were processed for MALDI-TOF ID in a stepwise analysis:

- 5µl drop as mixed sample

- dilution for analysis of single colonies (2-3 steps for enrichment cultures, 10-fold dilution), separation of 5µl drop for analysis of single colonies
- Sample preparation for MALDI-TOF by “lysis on target”
- Spectra comparison against Bruker database, sample MSPs and via Biotyper 3 “mixed culture modus”
- verification of MALDI-TOF identification by PCR and qPCR analysis of selected samples
- verification by 16S rDNA analysis of selected isolates
- Reprocessing of Bruker raw spectra with the open-source package MALDIquant
- start of a spectra database for project partners with the free online platform BIOSPEAN

To examine the procedure on field samples from a controlled fire blight environment, a test series was collected in the experimental orchard in Kirschgartshausen, DE. Samples from trees that were subjected to a) three (2013) or four (2014) variants of antagonist treatment and b) antagonist treatment plus artificial inoculation with *E. amylovora* were collected. Samples of non-inoculated trees were included for comparison. In two subsequent years, a number of 400 individual samples were collected and processed.

A first assessment of population composition was performed by analysis of mixed bacterial colonies from 5µl wash sample. This “mixed sample” approach gave a surprisingly fast overview on dominant cultivable genus for a large proportion of samples. Instead of generating only mixed and unidentifiable spectra, direct analysis resulted mostly in trustworthy score values against reference data-base similar to those obtained by single colony analysis. Less than 5% of samples did not generate spectra with the “lysis on target” procedure. In very few cases, two equivalent species scores were obtained. Presence of both species could be confirmed by single colony analysis. About 2/3 of the samples could be identified to genus (60%) or species level (40%) against the default Bruker biotyper reference data base (V3). Independent repetition of mixed culture analysis gave identical species/genus identifications for the repeated sample, indicating an analysis of dominant cultivable species per sample rather than a random result.

Some mixed samples gave weak score values for related *Erwinia* species not commonly reported in Europe, such as *E. pyrifoliae*. PCR analysis according to the protocols developed in WP2 has been applied to those samples and did not confirm species. Between closely related species, low score values should be confirmed by pure culture analysis against reference spectra generated under the same experimental conditions.

Flower wash samples were analyzed by PCR and qPCR for the fire blight pathogen *E. amylovora* and the antagonistic *E. tasmaniensis* and results compared to mixed culture analysis by MALDI-TOF identification. The qPCR analysis confirmed positive cPCR results in more than 95% of samples. Population density of *E. tasmaniensis* leveled about 10^5-10^6 per flower for positive samples. Number of samples with *E. tasmaniensis* as dominant species detected by MALDI-TOF ID was significantly lower, especially for the later time-points.

Aside of the artificial applied *E. tasmaniensis* and *E. amylovora* or another applied antagonist, *B. amyloliquefaciens,* a number of different *Pseudomonas* species, *Pantoea agglomerans* or yeasts like *Metschnikowia* or *Aureobasidium* were detected as domineering species. Both antagonist (*E. tasmaniensis* and *B. amyloliquefaciens*) were also observed on neighboring, non-inoculated, demonstrating a spread after application. Neither antagonist persisted at high frequency for more than 4 weeks post application.

One bottleneck within this working package has been the close restrictions manufacturers keep on MALDI-TOF data analysis. The default analysis software from both, Bruker and Shimadzu, usually stores and processes spectra data in a proprietary data format. No simple exchange between different systems is offered, group work is not easily possible and data export options are very limited. During the last section of this project we evaluated the use of two separate non-commercial software packages to circumvent these limitations.

The R-based packages “MALDIquant” and “MALDIquantForeign” by S. Gibb provide import, export and spectra preprocessing options and allow creating “reference spectra” by merging individual test runs (Gibb and Strimmer, 2012). The online tool BIOSPEAN (Raus and Šebela, 2013) offers the possibility to compare identity of various spectra, create user databases containing own references and share these spectra with groups of colleagues. A reference database for the PHYTFIRE project partners has been started.

Figure 3.1 shows a comparison of an *E. tasmaniensis* user spectrum processed with MALDIquant (A) against the peak list selected for this spectrum in BIOSPEAN (B). Panel (C) shows the same raw spectra processed to an "MSP" merged peak list processed with the Bruker biotyper software (upper half) and the default reference pattern for the same isolate (*E. tasmaniensis* Et1/99) provided by the Bruker database.

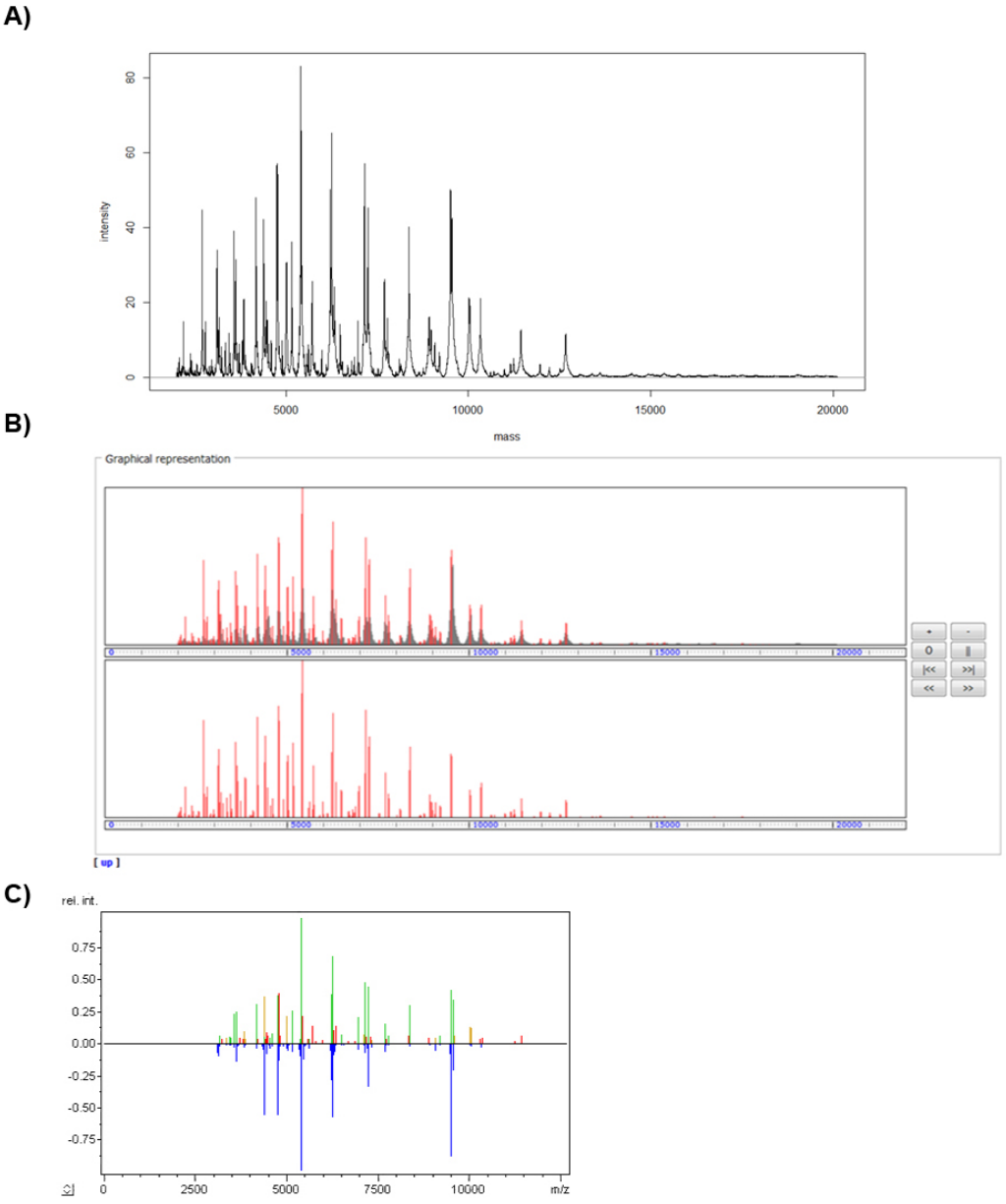

Fig. 3.1: Eleven individual spectra for *E. tasmaniensis* Et1/99 were processed to obtain one "reference spectrum" by the free R packages "MALDIquant" and "MALDIquantForeign" (A). The resulting spectrum was processed and saved as peak-list for comparison with the free online tool BIOSPEAN (B). The same eleven raw spectra were processed and analyzed with the Bruker biotyper software (C, upper half) and compared to the corresponding reference pattern (C, lower half). While the default peak picking and peak weighting differ between the software suites, the freeware tools gave satisfying identification of the *E. amylovora* related species tested within this project.

Conclusions

Direct analysis of mixed cultures by MALDI-TOF ID provides a fast and high-throughput approach for monitoring flower inhabitants after treatments for fire blight control. The method can be adopted for a fast assessment on "dominant cultivable species" by mixed sample analysis or, for a broader overview, on population composition by analysis of multiple single colonies. As this method will give an evaluable profile for most species its main drawback is its limitation to cultivable cells. The use of freely available, non-commercial software for spectra processing, analysis and even data sharing will greatly facilitate the further application of MALDI-ID by plant protection services.

Deliverables, dissemination and training output

- Step-by-step Sampling protocol (available at http://www.phytfire.org/MALDI_SampleAnalysis.pdf)
- Step-by-step Data analysis protocol (http://www.phytfire.org/MALDI_DataAnalysis.pdf)
- Implementation of a database of MALDI-TOF MS spectra for project partners on the free online platform BIOSPEAN (http://software.cr-hana.upol.cz/biospean/login.php)
- Wensing, A., M. Gernold and K. Geider (2012). Detection of *Erwinia* species from the apple and pear flora by mass spectroscopy of whole cells and with novel PCR primers. J. Appl. Microbiol. 112: 147-158.

References

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Raus M., Šebela M. (2013) BIOSPEAN: A freeware tool for processing spectra from MALDI intact cell/spore mass spectrometry. J. Proteomics Bioinform. 6: 282-287.

Sauer S., Freiwald A., Maier T., Kube M., Reinhardt R., Kostrzewa M., Geider K. (2008) Classification and identification of bacteria by mass spectrometry and computational analysis. PLoS ONE. 3: e2843.

Wensing, A., M. Gernold and K. Geider (2012). Detection of Erwinia species from the apple and pear flora by mass spectroscopy of whole cells and with novel PCR primers. J Appl Microbiol. 112: 147-158.

WP4 Vector monitoring. (Leaders: H. Halbwirth and C. Gosch)
Phase 1 partners: H. Halbwirth, C. Gosch, K. Stich, U. Persen
Phase 2 partners: E. Marco-Noales, M. M. López, R. Moosbeckhofer, R. Gottsberger

Objectives and Tasks

Blue ^{Ea}LAMP (Gosch et al. 2011; 2012), a novel colorimetric diagnostic assay for possible on-site detection of *E. amylovora* was recently developed at the VUT and is based on the loop-mediated amplification of specific DNA (LAMP).

A bee- and flower-monitoring was done in commercial orchards between 2012 and 2014 in four orchards in Austria and Switzerland. The aim was to gain qualitative and quantitative information about the abundance of *Erwinia amylovora* during bloom.

During the project both methods should be combined and evaluated for the integration into routine fire blight prognosis strategies. The specific aims were:

- Development of a protocol for semi-quantification of *E. amylovora* titres using Blue ^{Ea}LAMP and scaled color plates.
- Determination of the *E. amylovora* inoculum status (determined via beehive mounted collection devices combined with the Blue ^{Ea}LAMP method) and implementation into the prognosis model Maryblyt.
- Determination of stability and appropriate storage conditions for Blue ^{Ea}LAMP method.
- Provision of an easy-to-use detection kit consisting of a honeybee mediated sampling device and the Blue ^{Ea}LAMP with a step-by-step protocol.
- Dissemination of results via scientific and popular science publication or workshops.

Methods

Bee monitoring
2-5 honeybee colonies per test site were equipped with two foil lined tube collectors each. Sampling of monitoring tubes and replacement by new tubes with fresh foils took place daily. Inlays were kept at -18°C until lab processing. The surface was then washed in PBS buffer followed by centrifugation. Analyses for *E.amylovora* were carried out by qPCR (detection limit = 200 cfu/inlay, = 1 cfu/µl). (Gottsberger 2010)

Flower monitoring
100 randomly selected flowers per orchard were sampled daily. They were suspended in PBS buffer, surface washed by shaking and centrifuged. Analyses for *E. amylovora* were carried out by qPCR (detection limit = 80 cfu/flower, = 1 cfu/µl) (Gottsberger 2010)

Blue ^{Ea}LAMP
Blue ^{Ea}LAMP was performed according to Gosch et al. (2012).

Bst DNA Polymerase, Large Fragment (New England Biolabs, M0275), *Bst* 2.0 DNA Polymerase, Large Fragment (New England Biolabs, M0537) and *Bst* 2.0 WarmStart DNA Polymerase, Large Fragment (New England Biolabs, M0538) were compared.

The influence of the following chemicals on the performance of the Blue ^{Ea}LAMP was tested by directly adding the chemicals and by taking washing buffer of sprayed and washed single flowers. 10 µl sample were added to the Blue ^{Ea}LAMP reaction mixture.

Table 4.1: influence of plant protection products on the performance of the Blue ^{Ea}LAMP, tested by directly adding the chemicals and by taking washing buffer of sprayed and washed single flowers. 10 µl sample were added to the Blue ^{Ea}LAMP reaction mixture.

No.	Name	concentration of orchard applications	physical condition
1	Urea	0,5 %	WG
2	Delan WG	0,06 %	WG
3	Netzschwefel	0,5 %	WP
4	Dirigol N	5-20 g/100 l	WP
5	Syllit	0,14 %	L
6	Cuprofor	0,3 %	L
7	Regulex	0,05 %	L
8	Agro-N-Fluid	2 %	L
9	Schwefelkalk	1,2 %	L

WG: water dispersible granules, WP: wettable powder, L: liquid

Storage of the Blue ^{Ea}LAMP detection kit was tested at room temperature, 4°C and -20 °C. For the use of the Blue ^{Ea}LAMP detection kit for field trials the kit was stored at -20°C and the flower monitoring system was used.

Results and Discussion

Bee monitoring
E. amylovora was detected and quantified within the flight range of the bees as well as on flowers in orchards. The sampling system with tube collectors did not disturb the bee´s activities in any way. The data that were gained show a clear correlation between the findings of the pathogen via bee monitoring and the occurrence of fire blight symptoms in the particular orchards.
Fire blight occurred in orchards where the number of detected bacteria on tube collector inlays exceeded the limit of detection considerably.
For the reliable integration of the method into a fire blight prognosis model further trials under natural conditions and with various bacterial inoculum are required to achieve a validated correlation between the results of the bee monitoring and the occurrence of fire blight.

Table 4.2: Prognosis model indicates infection risk and single findings of *E. amylovora* on flowers and collector tube inlays at the limit of detection. No fire blight symptoms occurred.

date	CDH18	infection risk		cfu / flower	cfu /inlay		
					hive 1	hive 2	hive 3
10.04.12	0	L					
11.04.12	0	L					
12.04.12	0	M					
13.04.12	0	M					
14.04.12	0	M					
15.04.12	0	L					
16.04.12	0	L	open blossoms	0			
17.04.12	0	L		0			
18.04.12	0	M		0	0	0	0
19.04.12	0	M		0	0	0	0
20.04.12	0	M		80	0	0	0
21.04.12	0	M		0	0	0	0
22.04.12	0	M		0	200	0	0
23.04.12	0	M		0	0	0	0
24.04.12	0	M		0	0	0	0
25.04.12	0	M		0	0	0	0
26.04.12	30,1	L		0	0	0	0
27.04.12	82,4	M		0	0	0	0
28.04.12	144,7	HW-		0	0	0	0
29.04.12	221,4	HW-		0	0	0	0
30.04.12	257,8	HW-		0	0	0	0
01.05.12	281	HW-		0	0	0	0
02.05.12	226,9	HW-		0	0	0	0
03.05.12	235	I		0	0	0	0
04.05.12	112,4	HT-		0	0	0	0
05.05.12	84,5	M		0	0	0	0
06.05.12	101,2	L		0	0	0	0
07.05.12	29,7	M		0	0	0	0
08.05.12	40,8	M		0	0	0	0
09.05.12	73,1	H		0	0	0	0
10.05.12	98,1	H		0	0	0	0

Table 4.3: Prognosis model indicates no conditions for infection. Findings of *E. amylovora* on collector tube inlays predominantly above the limit of detection. Fire blight symptoms were observed on flowers.

				hive 1				hive 2			
				collector 1		collector 2		collector 1		collector 2	
date	CDH18	Risiko		cfu/µl	cfu/inlay	cfu/µl	cfu/inlay	cfu/µl	cfu/inlay	cfu/µl	cfu/inlay
10.04.14	0	L		0	0	0	0	0	0	0	0
11.04.14	7,6	L		0	0	0	0	0	0	0	0
12.04.14	15,5	L		0	0	0	0	0	0	1	200
13.04.14	16,4	L		0	0	0	0	0	0	0	0
14.04.14	10,9	L		0	0	0	0	0	0	0	0
15.04.14	5,5	L		0	0	1,74	348	0	0	0	0
16.04.14	0	L		0	0	0	0	0	0	0	0
17.04.14	0	L		0	0	0	0	0	0	0	0
18.04.14	0	M	open blossoms	0	0	0	0	0	0	0	0
19.04.14	0	M		0	0	0	0	0	0	0	0
20.04.14	0	M		0	0	0	0	0	0	0	0
21.04.14	0	M		1,78	356	0	0	0	0	0	0
22.04.14	2,8	M		0	0	1,68	336	1	200	0	0
23.04.14	17,6	L		0	0	0	0	0	0	0	0
24.04.14	38	L		1,13	226	4,96	992	1,34	268	0	0
25.04.14	57,2	M		12,5	2500	7,92	1584	1	200	1	200
26.04.14	38,1	M		0	0	0	0	0	0	0	0
27.04.14	18,1	M		0	0	0	0	1	200	0	0
28.04.14	0	M		0	0	0	0	0	0	0	0
29.04.14	0	M		0	0	0	0	0	0	0	0
30.04.14	0	M		0	0	0	0	0	0	0	0
01.05.14	0	M		0	0	0	0	0	0	0	0
02.05.14	0	M		0	0	0	0	0	0	0	0
03.05.14	0	M		0	0	0	0	0	0	0	0
04.05.14	0	M		0	0	0	0	0	0	0	0
05.05.14				1	200	0	0	0	0	0	0
06.05.14				0	0	0	0	0	0	0	0
07.05.14				0	0	0	0	0	0	0	0
08.05.14				0	0	0	0	0	0	0	0
09.05.14				1	200			0	0	0	0
10.05.14				0	0	0	0	0	0	0	0

Blue ^{Ea}LAMP

(A) *Bst* DNA polymerases; storage and stability

All three *Bst* DNA Polymerases had a similar performance in the Blue ^{Ea}LAMP but the reaction buffer of the *Bst* 2.0 und *Bst* 2.0 WarmStart DNA polymerases led to a slight initial colour shift of the hydroxynaphthol blue. Also storage of the Blue ^{Ea}LAMP kit was best, when the "normal" *Bst* DNA polymerase was used. Storage of the kit at -20 °C over the experimental time of 43 days was possible without decreasing performance.

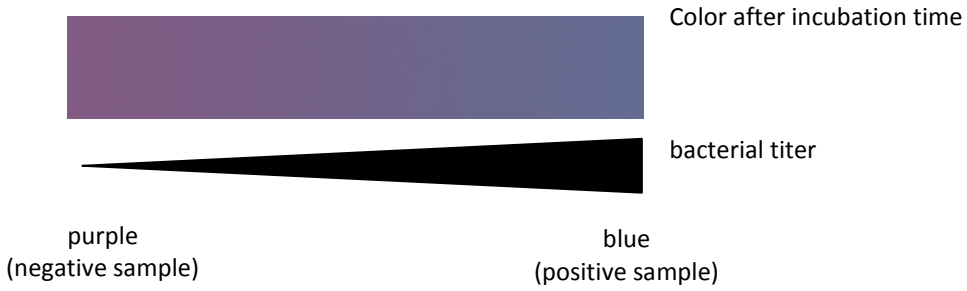
(B) Influence of chemicals

The chemicals Syllit, Cuprofor, Agro-N-Fluid and Lime sulfur caused an initial colour shift of the hydroxynaphthol blue already before incubation and also no amplification was found via the agarose gel (with 10 µl sample in 50 µl reaction volume). After incubation, Delan and Sulfur showed no color shift from violet to blue. Delan, Sulfur, Syllit, Cufprofor und Lime sulfur inhibited the Blue ^{Ea}LAMP reaction at the used concentrations, which were several times higher than expected in orchard samples. Therefor in a second experiment single flowers were sprayed with the chemicals and washed, whereas the washing buffer was used afterwards in the Blue ^{Ea}LAMP reaction to simulate a more realistic situation of the concentrations of the chemicals in orchard samples. In this experiment, only Cuprofor influenced the colour shift from violet to

blue but did not inhibit the amplification itself. Therefore, sample volume was reduced to the originally used 2 µl/50 µl reaction volume instead of 10 µl/50 µl reaction volume and no initial colour shift occurred.

(C) Step-by-Step protocol
A Step-by-Step Protocol for the Blue ^{Ea}LAMP *Erwinia amylovora* detection kit is provided on the PhytFire website: http://www.phytfire.org/Blue_EaLAMP_DetectionKit.pdf

(D) Semi-quantitative determination of *E. amylovora* titres
The semi-quantitative determination of bacterial titer via scaled color plate revealed to be difficult and is not applicable to date, mainly because the discrimination of color nuances under field conditions was found by the to be difficult by the test persons.



(E) Field trials
Tyrol 2013
Initial colour shift was observed with some samples when 10 µl sample in 50 µl reaction volume were used. No colour shift was observed with 2 µl sample in 50 µl reaction volume.

Haidegg 2013
No Blue ^{Ea}LAMP positive samples were detected.

AGES 2013
19 samples were taken and examined with different detection methods. Blue ^{Ea}LAMP gave satisfying results compared to the other field method with only one false negative sample (like the second field assay Agristrip)

Table 4.4: Evaluation of orchard samples "AGES 2013" with different detection methods.

Sample No.	PCR	Blue EaLAMP	qPCR	Agri Strip	cfu/µl	cfu/µl
704/13	+	+	+	+	24,188	241,880
705/13	+	+	+	+	1,805	18,050
706/13	+	+	+	(+)	1,116	11,160
707/13	+	+	+	+	4,284	42,840
708/13	+	+	+	+	2,719	27,190
722/13	-	-	-	-	0	0
723/13	-	-	-	-	0	0
724/13	-	-	-	-	0	0
725/13	-	-	-	-	0	0
726/13	-	-	-	-	0	0
727/13	-	-	-	-	0	0
748/13	+	+	+	+	6,964	69,640
750/13	+	+	+	(+)	808	8,080
658/13	+	+	+	(+)	1,933	19,330

659/13	**+**	**-**	**+**	**+**	**1,281**	**12,810**
661/13	**+**	**+**	**+**	**+**	**8,378**	**83,780**
642/13	**+**	**(+)**	**+**	**+**	**6,095**	**60,950**
635/13	**+**	**(+)**	**+**	**+**	**9,703**	**97,030**
330/13	**((+))**	**+**	**+**	**-**	**332**	**3,320**
Dilution	**1**	**1:10**	**1:100**	**1:10**	**1:100**	**1:10**

AGES 2012, 2013
78 samples were examinated in a blinded study with the Blue ^{Ea}LAMP. Bacterial titres (determined by qPCR) of the positive samples (see table) were very low between 1 and 57,3 cfu/µl. Most of the samples with higher titres were detected by Blue ^{Ea}LAMP but also one false positive (No 52; classified as "likely positive")

Table 4.5: Evaluation of orchard samples "AGES 2012, 2013" with Blue ^{Ea}LAMP compared to qPCR.

AGES-Nr	TU-Nr.	year	HNB/Gel(+/-)	qPCR AGES cfu/µl
35	1	2012	-	1,28
60	2	2012	-	1
67	3	2012	-	1
91	13	2012	-	1
278	23	2012	-	1
314	24	2012	-	1
538	25	2012	-	1,18
685	26	2012	-	1,9
701	27	2012	+	57,3
722	28	2012	-	4,82
723	29	2012	+	13,3
724	30	2012	+	11
725	31	2012	+	13,4
726	32	2012	-	1,54
729	33	2012	(+) possibly positiv	1
732	34	2012	-	1,9
772	35	2012	+	15,9
773	36	2012	+	14,4
780	37	2012	-	12,1
789	38	2012	+	2,94
801	39	2012	-	3,8
B212/13	48	2013	-	1
B213/13	49	2013	(+) likely positive	15,7
B214/13	50	2013	-	2,45
B215/13	51	2013	-	3,07
B216/13	52	2013	(+) likely positive	-
B267/13	68	2013	-	2,58
B268/13	69	2013	-	1,51
B348/13	73	2013	-	2,17

Tirol 2014
No Blue ^{Ea}LAMP positive samples were detected.

Haidegg 2014
Samples from 30 flower samplings (100 flowers/sample) and 20 foils (2 per hieve each) from bee hive mounted collection devices (bee-monitoring) were investigated in a blinded study (see tables below). Bacterial titres (determined by qPCR) of the positive samples (see table) were very low with both sampling methods. Results from the retained flower samples were consistent concerning Blue ^{Ea}LAMP and qPCR (positive sample No 5). However, on-site evaluation of Blue ^{Ea}LAMP by the extension worker showed additional positive samples. Regarding the foil samples from the bee-monitoring, the Blue ^{Ea}LAMP detected not all of the qPCR-positive samples. This is due to very low bacterial titres between 0.14 and 1.82 bacteria/µl, which is below the detection limit of the Blue ^{Ea}LAMP of 20-25 bacteria/µl.

Table 4.6: Evaluation of flower samples "Haidegg 2014" with Blue ^{Ea}LAMP compared to qPCR.

No.	On-site evaluation Haidegg (Blue ^{Ea}LAMP)	retained samples (Blue ^{Ea}LAMP)	retained samples (qPCR)	bacteria/µl sample (for Blue ^{Ea}LAMP)
1	+	-	-	-
2	-	-	-	-
3	-	-	-	-
4	-	-	-	-
5	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	+	-	-	-
9	-	-	-	-
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	+	-	-	-
15	-	-	-	-
16	-	-	-	-
17	+	+	+	1.296
18	-	-	-	-
19	-	-	-	-
20	-	-	-	-
21	-	-	-	-
22	-	-	-	-
23	-	-	-	-
24	-	-	-	-
25	-	-	-	-
26	-	-	-	-
27	-	-	-	-
28	-	?(+)	-	-
29	-	?(+)	-	-
30	+	-	-	-

Table 4.7: Evaluation of bee monitoring samples "Haidegg 2014" with Blue EaLAMP compared to qPCR.

Nr.	Blue ^{Ea}LAMP (TU)	qPCR (AGES)	bacteria/µl sample (for Blue ^{Ea}LAMP)
1A	-	-	-
1B	-	-	-
2A	?+	+	0,16
2B	?(+)	+	0,14
3A	?+	+	0,68
3B	-	-	-
4A	-	+	1,67
4B	-	+	1,28
5A	-	-	-
5B	-	-	-
6A	-	+	0,26
6B	-	-	-
7A	-	-	-
7B	-	-	-
8A	-	+	0,48
8B	?(+)	+	0,22
9A	?+	+	1,82
9B	?+	+	0,95
10A	-	+	0,53
10B	-	+	0,30

Results with orchard samples showed that methods like qPCR which use concentrated (15 – 25fold by centrifugation) samples can detect samples with very low bacterial titres compared to Blue ^{Ea}LAMP which uses directly an aliquot of the sample (washing water of flowers or foil). The definition of a threshold level (bacterial titre) at which an infection actually occurs would be necessary to decide if the detection limit of 20-25 bacteria/µl of the Blue ^{Ea}LAMP is sufficient for the use in fire blight prognosis models. The low infection rates and the low bacterial titres found in samples within the last years suggest that the threshold level is beyond the detection limit of the Blue ^{Ea}LAMP.

Bernburg 2014 (performed by A. Kusterer, LLFG)
Blue ^{Ea}LAMP positive flower samples were found in quince orchards which showed fire blight symptoms the years 2012 and 2013. These results were consistent with those obtained by Agri Strip assay.

(F) Conclusions Blue ^{Ea}LAMP

The Blue ^{Ea}LAMP detection kit including the *Bst* DNA Polymerase can be stored at -20°C at least over the flowering period of pome fruit trees.

2 µl sample in 50 µl assay volume is optimal for avoiding interferences or inhibition in Blue ^{Ea}LAMP assays.

The performance of the Blue ^{Ea}LAMP is comparable with other (more expensive or time consuming) methods available to date.

The semi-quantitative determination of bacterial titer via scaled colour plate revealed to be difficult and is not applicable to date.

Extension workers were able to handle and perform Blue ^{Ea}LAMP assays. No specially trained lab personal was needed.

Results of Blue ^{Ea}LAMP assays maybe could support extension workers in fire blight infection risk prognosis.

Prototypes of the Blue ^{Ea}LAMP *Erwinia amylovora* detection kit are available for research institutions at cost price at the Vienna University of Technology.

After further evaluation – mainly with samples from years with heavy fire blight infections which are not available do date – the Blue ^{Ea}LAMP method could be integrated step by step into prognosis models. However, for the reliable integration of any *E. amylovora* detection method (Blue ^{Ea}LAMP, qPCR,...) into fire blight prognosis models the determination of a threshold value (bacterial titre at which fire blight symptoms occur) will be needed by additional experiments.

References

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Gosch C, Gottsberger RA, Stich K, Fischer TC (2012). Blue ^{Ea}LAMP - a specific and sensitive method for visual detection of genomic *Erwinia amylovora* DNA. European Journal of Plant Pathology 134, 835-845.

Gottsberger, R.A. (2010), Development and evaluation of a real-time PCR assay targeting chromosomal DNA of *Erwinia amylovora*. Letters in Applied Microbiology, 51: 285–292.

WP5 Asymptomatic infections and environmental fitness. (Lead: E. Marco-Noales)
Phase 1 partners: E. Marco-Noales, M.M. López
Phase 2 partners: L. Cruz, M. M. López, E. Marco-Noales, L. Baranauskaite, I. Kibildiene, V. Gusina, K. Petrutis, H. Lasner, M. Gusina, E. Pochukaeva

Objectives

Evaluate the presence and the physiological status of *E. amylovora* in asymptomatic plant material or under stress conditions: optimization of detection methods, implications in fire blight epidemiology and phytosanitary decision-making.

Approach

Perform surveys to determine epidemiological significance of asymptomatic infection and/or survival strategies of *E. amylovora*. To this end an EPPO survey coordinated by the WP5 leader and performed at the EU level provided information on the methodology used in the different countries. Besides, different sets of plant material from naturally and severely affected trees by fire blight (as a control) and asymptomatic plant material coming from close asymptomatic trees and/or orchards with fire blight story, have been harvested and analyzed by cultural (using several semi-selective media) and molecular methods.

Determine the number of samples of asymptomatic plant material which can be analyzed without inhibition of the techniques employed. To this end, different sets of plant material inoculated with the pathogen were analyzed by cultural, serological and molecular methods.

Determine the genes involved in the response of *E. amylovora* to copper stress.

Deliverables, dissemination and training output

- Optimized protocol for detection of *E. amylovora* in asymptomatic plant material published in the EPPO Bulletin and is in final step of evaluation at the IPPC, FAO.
- Survival ability of *E. amylovora* under adverse conditions that can promote non-detection by conventional methods evaluated.
- Genes involved in the response of *E. amylovora* against adverse conditions determined and manuscript in preparation.

Survey on the procedures implemented in laboratories for the analysis of asymptomatic plants for the detection and identification of *E. amylovora*

A survey coordinated by EPPO was organized on the sampling and testing procedures for asymptomatic material. The questions were designed to provide information about the methodology and results obtained in different countries, and the questionnaire was sent to all the NPPO of the EPPO region. The questionnaire was developed by the WP5 leaders, and posted online on April 2012. The answers are summarized below:

• Sampling procedure for testing:

- 6 laboratories (Belgium, the Netherlands, Portugal, Slovakia, Spain and United Kingdom) perform both single plant analyses and composite samples.
- 4 laboratories (Austria, Bulgaria, the Czech Republic and Switzerland) perform single plant analyses only.
- 3 laboratories (France, Lithuania and Slovenia) perform composite sample analysis only.
- The Estonian partner also performed analyses with spiked and bulk samples with pear, apple and hawthorn but the results were not conclusive.

Table 5.1: Sampling procedure for testing

Nb of bulked samples	3	4	5	10	30	100
Country of the respondent	Belgium	Portugal	Spain	France	United Kingdom	Lithuania, The Netherlands, Slovakia, Slovenia

• Protocol used for testing:

- - Four laboratories follow PM 7/20 (2004) for testing.
- - Nine laboratories also perform other tests.

Table 5.2: Techniques used for testing asymptomatic samples

Technique	Nb of labs	Reference
Isolation	8	Austria, France, the Netherlands, Portugal, Spain, United Kingdom, Bulgaria, Czech Republic
Enrichment isolation	9	Austria, Portugal, Slovenia, Spain, Czech Republic, Belgium, Lithuania, The Netherlands, Slovakia
Immunofluorescence	7	Czech Republic, France, United Kingdom, Bulgaria, Lithuania, The Netherlands, Slovakia
Enrichment-DASI-ELISA	1	Spain
PCR	7	Czech Republic, Spain, Austria, Belgium, The Netherlands, Portugal, Slovakia
Enrichment-PCR	4	Portugal, Lithuania, Slovakia, Spain
Real-time PCR	6	Austria, Lithuania, Slovakia, Spain
Bioassay	8	Austria, Belgium, Portugal, Bulgaria, Czech Republic, Lithuania, The Netherlands, Slovakia
Other	3	Belgium, Czech Republic, The Netherlands

Table 5.3: Number of positive *E. amylovora* tests/ Number of tests performed per year

Country of the respondent	2007	2008	2009	2010	2011
Austria	2095/3345	2369/4495	2567/3918	2145/5839	1220/4936
Belgium	0/0	0/0	3/97	0/139	0/184
Bulgaria	73/491	6/360	2/569	1/349	0/224
Czech Republic	4/31	7/53	12/68	13/155	17/141
France	0/62	0/50	0/179	0/211	0/220
Lithuania	9/334	3/273	1/173	0/130	0/2
Netherlands	1/640	1/680	2/652	12/646	6/660
Portugal	0/200	0/200	0/0	11/26	7/112
Slovakia	18/203	7/162	1/45	10/68	3/99
Slovenia	1/27	1/30	0/30	0/34	0/52
Spain	0/1376	0/2385	0/5115	4/2529	39/6164
Switzerland	1/1	1/1	1/1	1/1	1/1
United Kingdom	3/83	21/172	0/53	1/38	5/113

The results of the survey have demonstrated that the EPPO protocol for *E. amylovora* (2004) was appropriate for detecting the target in asymptomatic samples. However, it was observed that the concept of "asymptomatic plants" was not clear for all the participants, and some of them included data about asymptomatic parts of symptomatic plants.

Determination of the number of samples of asymptomatic plant material which can be analyzed without inhibition of the techniques employed

To this end, different sets of plant material inoculated with the pathogen were analyzed by cultural, serological and molecular methods.

A protocol has been sent to all the participants in order to evaluate the sensitivity of the different techniques in all the laboratories of the different partners, as well as the possibility to detect *E. amylovora* in asymptomatic samples of 3, 5 and 10 plants.

• Spiked samples

Spiked samples with different *E. amylovora* known concentrations have been prepared to analyze the individual samples using the techniques of the EPPO protocol, and select the positive samples for preparing with them new samples simulating 3, 5 or 10 analyzed plants. Results from Spain, Lithuania and Estonia are available.

The sensitivity of the detection was 10^2 cfu/ml in a sample consisting of 1, 3, 5 or 10 plants for all techniques after enrichment. Relatively similar PCR results were obtained by the partner from Lithuania with apple samples. Consequently, the EPPO 2013 protocol has been validated for asymptomatic pear samples, and as much as 10 samples can be analyzed together with acceptable sensitivity.

Survival ability of *E. amylovora* under adverse conditions

The survival ability of *E. amylovora* under stress conditions was evaluated in the area of cultivations of

loquat in Alicante, Spain, where about thousand hectares of loquat are concentrated. In such area *E. amylovora* was found on pear and other hosts, but not yet on loquat. The work has been performed in close cooperation with the two growers associations of the area, Cooperativa Ruchey and Cooperativa de Altea.

Table 5.4: Analysis of asymptomatic loquat samples from a Spanish area where *E. amylovora* was detected

	November '13	January '14	March '14	
Nº analyzed samples	Plot 1-142 Blossoms Plot 2-122 Blossoms	Plot 1-61 Blossoms 61 Fruits 61 Shoots	Plot 3-30 Fruits-shoots Plot 4-39 Fruits-shoots	516
Nº E-ELISA +	3 3	0 0 0	0 0	6
Nº real time PCR+	21 4	ND ND ND	2 (1fruit-1shoot) 0	27
Nº Taylor PCR+	6 1	ND ND	ND ND	7
Isolation	0	0	0	0

The results are shown in Table 5.4 and were negative isolation in winter 2014, following positive detection by ELISA, conventional and real-time PCR in November 2013. Probably the negative isolation is consequence of intensive copper treatments that could induce the Viable But Not Cultivable (VBNC) state and is currently under investigation.

Genes involved in the response of *E. amylovora* to copper stress

The differential expression of *E. amylovora* genes in the presence or absence of copper has been studied, using microarray hybridization transcriptomic technology. Source RNA was isolated from exponential grown cells after $CuSO_4$ 0.5mM shock exposure for five minutes. Under these experimental conditions, 44 genes were differentially expressed. The induced genes were distributed in the functional categories of transport, stress, movement, and metabolism, besides a conserved protein and some genes of unknown function. . To validate gene expression patterns observed in the microarray analysis, selected genes were analyzed for expression profile by qRT-PCR, and 23 up-regulated genes were confirmed. Then, some genes were selected for mutational analysis; the mutants exhibiting the most severe decrease in growth by copper effect were complemented and then assayed to prove restoration of normal phenotype. A manuscript is in preparation with the obtained results.

WP6 Source Tracking (Lead: F. Rezzonico)
Phase 1 partners: F. Rezzonico, M. Bergsma-Vlami
Phase 2 partners: E. Marco-Noales, M.M. López, L. Cruz, T. Dreo, M. Ravnikar, L. Baranauskaité, I. Kibildiené, H. Ozaktan, N. Ustun, N.Drenova, I. Moročko-Bicevska, T. Doolotkeldieva, G. Zharmukhamedova

Objectives

Develop high-resolution, high-throughput genotyping methods for fingerprinting isolates within local/regional epidemic foci, here referred to as source tracking.

Approach

Recent genomics-based genotyping methods based on variable number of tandem repeats (VNTR) (Dreo *et al*., 2011) and clustered regularly interspaced short palindromic repeats (CRISPR) (Rezzonico *et al.*, 2011) were further developed for source-tracking applications and the number of tested isolates was significantly enlarged.

The development Phase 1 has utilized existing collections and new field isolations involving Phase 1 partners. Emphasis was placed on intensive sampling of epidemic foci: (a) same host across a region, (b) proximal hosts and/or objects (e.g., nursery, orchard, garden adjacent or near each other). The first goal

was to assess the diversity of *E. amylovora* isolates using appropriate typing methods (VNTRs, RAPD's, CRISPRs). A global collection of 833 isolates was tested using Multi-Locus VNTRs Analysis (MLVA), with particular focus to analyses at national scale in Slovenia, Switzerland and, in a separate study, in the Netherlands, where the technique was compared to RAPD analysis on additional 22+1 strains.

Following the expansion of the PHYTFIRE community to Eastern European and Central Asian countries and the recent appearance of *E. amylovora* in the latter region, which is recognized as the center of origin of domesticated apple and pear species, an epidemiological study based on CRISPRs diversity was initiated with the aim of understanding the dissemination routes that led to arrival of fire blight to Central Russia, Kazakhstan and Kyrgyzstan and the potential forthcoming hazards to the native germplasm that is present in the forests and orchards of these countries.

Results

- Population structure and epidemiological insights obtained by analyzing epidemic foci in Switzerland, Slovenia and the Netherlands using a VNTR high-throughput ("MLVA-6") method.
- SI/CH: MLVA-6 allowed the distinction of 227 haplotypes among a collection of 833 isolates of worldwide origin. Three geographically separated groups were recognized among global isolates using Bayesian clustering methods. Analysis of regional outbreaks confirmed presence of diverse haplotypes, but also high representation of certain haplotypes during outbreaks. We failed to detect significant diversity of haplotypes within outbreaks between years suggesting that multiple introductions of *E. amylovora* occur in orchard ecosystems. Once introduced, they remain active within geographic regions and one or several of the underlying genotypes become dominant during an outbreak.
- NL: five different (closely related) typing groups were found within the 23 Dutch strains (all isolated in 2012, except for control strain PD 269) by combining two different sets of RAPDs primers. Higher variability was found using the MLVA-6 multiplex with respect to RAPDs, even if this is often limited to certain VNTRs. Among the two loci on the plasmid, locus A (region in plasmid pEA29 with an amplicon 450-550 bp) was more discriminative than locus B. Locus F on the chromosome did not result in any variation among the tested set. While it is possible that additional VNTRs might help to improve discrimination, it is also conceivable that the analysis reflects the actual situation present in the Netherlands where, despite being an early site of fire blight in Europe, the climate conditions are not very favorable for the development of the disease, thus resulting in a relative slow spread and evolution.
- Genotyping approaches based on SNP analysis and CRISPRs were enhanced through inclusion of new isolates to fill current knowledge gaps in history and worldwide dispersal of fire blight, in particular pertaining to the recent spread in Eastern Europe and Central Asia.
- The deletion of a spacer duplication in CRISPR repeat region 1 (CRR1) was identified and determined to have appeared in Europe about twenty years after the first detection of *E. amylovora*, either by modification of the existing genotype or, more likely, by a second independent introduction from the North America. All European isolates so far analyzed are either identical to or can traced back to one of these two CRR1 genotypes (denominated "D" and "A", respectively), which thus represent the two ancestral populations in the Old World. With respect to CRRs, a significant increase in genetic diversity among European strains was apparent only in the last decade.
- The CRR1 duplication was targeted to design a PCR assay (**Fig 6.1**), which is useful to swiftly identify to which of the two European ancestral populations an isolate can be traced back. This circumvented the tedious sequencing work needed to exactly characterize the CRISPR regions and allowed to build, together with data obtained from whole genome sequencing, a streamlined model of the dissemination routes of fire blight toward Central Asia, which suggests that the strains responsible for the recent outbreaks in Kazakhstan and Kyrgyzstan are derived from CRR1 genotype A and share a common ancestry with the population that is present in Caucasus region and around the Black Sea (**Fig 6.2**).

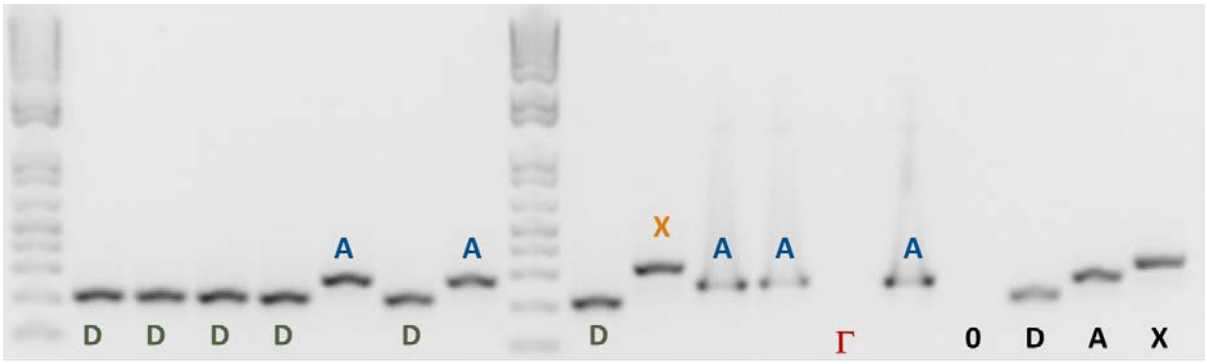

Fig. 6.1. PCR amplification of the partial CRR1 containing the discriminating spacer (Rezzonico et al., 2011) in *E. amylovora* isolates from Turkey, Iran, the Caucasus region and Central Asia. **A:** discriminating spacer duplicated, strain derived from the primeval European genotype; **D:** single discriminating spacer, strain derived from the second CRR genotype appeared in Europe; **X:** discriminating spacer triplicated, strain derived from CRR1 genotype A by additional duplication of discriminating spacer; **Γ:** extensive deletion of region containing discriminating spacer eliminates primer annealing site and leads no PCR amplification; **0:** negative control of PCR.

Fig. 6.2. Geographical distribution of *E. amylovora* isolates derived from the ancestral European CRR1 genotypes A (blue) and D (green). Bacteria isolated in Central Asia are derived from the CRR1 genotype A, which corresponds to the one found in the Caucasus region, and are clearly distinct from the CRR1 genotype D which is peculiar to Northeastern Europe. The region between Ukraine and the Ural mountains lies at the crossroad between the two different CRR1 genotypes.

Dissemination and training output

- Optimized protocol for MLVA-6 multiplex was transferred from lead of WP6 to Phase-1 partner in the Netherlands, where it was effectively implemented on local isolates without the need of the planned on-site training.
- Upon request, the optimized protocol for MLVA-6 multiplex was transferred to Nataliya Drenova (All-Russian Plant Quarantine Centre), where it was employed to investigate recent epidemics in the

Russian Federation and neighboring countries.
- Epidemiological data were delivered to phase-2 partners in return for the strains supplied for this study.

Deliverables, dissemination and training output

- Development of a genotyping method based on VNTR analysis that was optimized using a high-throughput multiplex ("MLVA-6").
- A study about phylogeography and population structure of *E. amylovora* in Switzerland and Slovenia that was published in Environmental Microbiology (Bühlmann et al., 2014).
- An epidemiological study on the introduction of *E. amylovora* in Central Asia (manuscript in preparation).
- Genome/SNPs data for a total of 140 strains of *E. amylovora* (Mann et al., 2013, Bühlmann et al., 2014, Smits et al., unpublished).

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Dreo T., Ravnikar M., Frey J.E., Smits T.H.M., Duffy B. (2011) *In silico* analysis of variable number of tandem repeats in *Erwinia amylovora* genome. Acta Hort. 896: 115-118.

Mann R.A., Smits T.H.M., Bühlmann A., Blom J., Goesmann A., Frey J.E., Plummer K.M.; Beer S.V.; Luck J., Duffy B., Rodoni B. (2013). Comparative genomics of 12 strains of *Erwinia amylovora* identifies a pan-genome with a large conserved core. PLoS ONE, 8 e55644.

Rezzonico F., Smits T.H.M., Duffy B. (2011) Diversity and functionality of CRISPR regions in fire blight pathogen *Erwinia amylovora*. Appl Environ Microbiol 77: 3819-3829.

Project output (not including presentations at PhytFire internal meetings)

Output WP2 (Methods training and ring testing)

Peer-reviewed papers

1. Barbé S., Bertolini E., Roselló M., Llop M., López M.M. (2014) Conventional and real-time PCR to detect *Erwinia piriflorinigrans* allow its distinction from the fire blight pathogen *Erwinia amylovora*. Appl. Environ. Microbiol. 80: 2390-2398.
2. Smits T.H.M., Rezzonico F., López M.M., Blom J., Goesmann A., Frey J.E., Duffy B. (2013) Phylogenetic position and virulence apparatus of the pear flower necrosis pathogen *Erwinia piriflorinigrans* CFBP 5888^{T} as assessed by comparative genomics. Sys. Appl. Microbiol. 36:449-456. **[jointly with WP6]**

Abstracts/Presentations in meetings

1. López M.M. Routine diagnosis of *Erwinia amylovora* and validation by EU official laboratories. Oeiras (Portugal), October 1st, 2012.
2. López M. M. Bacteriosis en frutales de pepita. Foro INIA Adaptación al cambio climático en la producción frutícola de hueso y pepita. Lleida (Spain), May 16th, 2013.
3. Barbé S., Bertolini E., Roselló M., Llop P., López M.M. Real - time and conventional PCR allow detection of *Erwinia piriflorinigrans*, a new pathogenic species causing necrosis of pear blossoms and its distinction from the fire blight pathogen, *Erwinia amylovora*. 13th ISHS International Fire Blight Workshop, Zürich (Switzerland), July 2nd-5th, 2013.
4. Barbé S., García F., Conesa A., Cock P.J.A., Pritchard L., Hedley P.E., Morris J.A., López M.M., Llop P. Role of Erwinia amylovora plasmids pEA29 and pEI70 on the expression of chromosomal genes during symptoms development. 13th ISHS International Fire Blight Workshop, Zürich (Switzerland), July 2nd-5th, 2013.

Training courses

1. Training course to the EUPHRESCO/PHYTFIRE partners. Oeiras (Portugal), October 3rd-4th, 2012.

Documents/Diagnostic protocols *(available at www.phytfire.org)*

1. EPPO protocol for *E. amylovora* (2013). PM 7/20 (2)* *Erwinia amylovora*. Bulletin OEPP/EPPO Bulletin 43:21-45.
2. IPPC protocol for *E. amylovora* (in phase of country consultation)

Output WP3 (Flower monitoring)

Abstracts/Presentations in meetings

1. Wensing A. PHYTFIRE Projekt - MALDI-TOF Diagnostik zur Analyse von Blütenbesiedlung. 35. Jahrestagung der Deutsche Phytomedizinische Gesellschaft (Arbeitsgruppe Phytopathologie). Berlin (Germany), Sept. 4th-5th, 2014.
2. Wensing A. MALDI-TOF Analyse der Blütenbesiedlung. Feuerbrand Fünfländertreffen, Nals (Italy), Nov. 19th-20th, 2014.

Documents/Protocols *(available at www.phytfire.org)*

1. Sampling protocol for MALDI ID
2. Data analysis protocol for MALDI ID
3. Sample database in BIOSPEAN

Output WP4 (Vector monitoring)

Peer-reviewed papers

1. Gosch C., Gottsberger R.A., Stich K., Fischer T.C. (2012) Blue EaLAMP - a specific and sensitive method for visual detection of genomic *Erwinia amylovora* DNA. Eur. J. Plant Pathol. 134:835-845.

Abstracts/Presentations in meetings

1. Stich K., Gosch C., Fischer C.T. Blue EaLAMP - Der Schritt in die Praxis. Feuerbrand Fünfländertreffen, Sankt Gallen (Switzerland), November 8th-9th, 2012.
2. Persen U., Moosbeckhofer R., Gottsberger R.A. Bienenmonitoring 2012. Feuerbrand Fünfländertreffen, Sankt Gallen (Switzerland), November 8th-9th, 2012.
3. Persen U., Moosbeckhofer R., Gottsberger R.A., Reizenstein H. Monitoring the presence of *Erwinia amylovora* in apple orchards by using honey bees. 13th ISHS International Fire Blight Workshop, Zürich (Switzerland), July 2nd-5th, 2013.

4. Gosch C., Stich K., Fischer C.T. Blue ^{Ea}LAMP - aktuelle Ergebnisse aus dem Freilandeinsatz. Feuerbrand Fünfländertreffen, Immenstaad (Germany), November 18th-19th, 2013.
5. Persen U., Moosbeckhofer R., Gottsberger R.A. Bienenmonitoring 2013. Feuerbrand Fünfländertreffen, Immenstaad (Germany), November 18th-19th, 2013
6. Persen U., Moosbeckhofer R., Gottsberger R.A. Honigbienen als Helfer bei der Feuerbrandprognose. 17. Feuerbrand Round-Table AGES, Vienna (Austria), December 5th, 2013.
7. Gosch C., Stich K., Fischer C.T. Blue ^{Ea}LAMP - aktuelle Ergebnisse aus dem Freilandeinsatz. 17. Feuerbrand Round-Table AGES, Vienna (Austria), December 5th, 2013.
8. Persen U., Moosbeckhofer R., Gottsberger R.A. Honigbienen als Helfer bei der Feuerbrandprognose. 54. Österreichische Pflanzenschutztage, Ossiach, November 27th, 2013.

Documents/Protocols *(available at www.phytfire.org)*

1. Blue ^{Ea}LAMP detection kit description
2. Blue ^{Ea}LAMP setup and implementation
3. Sampling protocol for pome fruit flowers

Output WP5 (Asymptomatic infections and environmental fitness)

Peer-reviewed papers

1. Águila-Clares, *et al*. Use of copper against plant pathogenic bacteria: new prospects for an old product. Plant Disease (in preparation).
2. Águila-Clares, *et al*. Genetics of the mechanisms developed by *Erwinia amylovora* after a copper shock. Molecular Plant Pathology (in preparation).

Unrefereed papers/Popular press

3. Roselló M., Gamón Vila M., Ferrer A., Dalmau Sorlí V., Palacio-Bielsa A. , López. M.M. (2014) Prevención del fuego bacteriano en plantaciones de níspero (Eriobotrya japonica) en la Comunitat Valenciana. Phytoma España, 259: 20-27.

Abstracts/Presentations in meetings

1. López M.M. Asymptomatic infections of *Erwinia amylovora* and environmental fitness. Oeiras (Portugal), October 1st, 2012.
2. Águila-Clares B., Marco-Noales E., López M.M., Sundin G.W. How does *Erwinia amylovora* face up to stress by copper? 13th ISHS International Fire Blight Workshop, Zürich (Switzerland), July 2nd-5th, 2013.
3. Águila-Clares B., Marco-Noales E., López M.M., Sundin G.W. Análisis transcriptómico de la bacteria *Erwinia amylovora* en respuesta al estrés por cobre. XXIV Congreso de Microbiología SEM, Barcelona (Spain), July 10th-13th, 2013.
4. Llop P. El fuego bacteriano en frutales. Ayuntamiento de Alborache, Alborache (Spain), December 16th, 2013.
5. López M.M. Prevención del fuego bacteriano en níspero. Jornada Técnica sobre el fuego bacteriano (Grupo Cajamar). Callosa d'en Sarrià (Spain), November 26th, 2014.
6. López M.M., Montesinos E. Conocer la biología y epidemiología de *Erwinia amylovora* a nivel local, para reducir sus poblaciones y mejorar el control del fuego bacteriano. Smart Fruit Congress, Barcelona (Spain), February 3rd, 2014.

Documents/Diagnostic protocols *(available at www.phytfire.org)*

1. Advices for detection of *E. amylovora* in asymptomatic samples.
2. Protocol describing the suitable methods for *E. amylovora* detection in asymptomatic plant material to be evaluated.
3. El fuego bacteriano de las rosáceas. Generalitat Valenciana. Conselleria de Agricultura, Pesca, Alimentación y Agua. Spain
4. Encuesta epidemiológica sobre el fuego bacteriano. Generalitat Valenciana. Conselleria de Agricultura, Pesca, Alimentación y Agua. Spain

Output WP6 (Source Tracking)

Peer-reviewed papers

1. Malnoy M., Martens S., Norelli J.L., Barny M.-A., Sundin G.W., Smits T.H.M., Duffy B. (2012) Fire blight: applied genomic insights of the pathogen and host. Annu. Rev. Phytopathol. 50:475-494.
2. Bühlmann A., Pothier J.F., Rezzonico F., Smits T.H.M., Andreou M., Boonham N., Duffy B., Frey J.E. (2013) *Erwinia amylovora* loop-mediated isothermal amplification (LAMP) assay for rapid pathogen detection and on-site diagnosis of fire blight. J. Microbiol. Meth. 92:332-339.

3. Smits T.H.M., Guerrero-Prieto V.M., Hernández-Escarcega G., Blom J., Goesmann A., Rezzonico F., Duffy B., Stockwell V.O. (2014) Whole-genome sequencing of *Erwinia amylovora* strains from Mexico detects single nucleotide polymorphisms in rpsL conferring streptomycin resistance and in the avrRpt2 effector altering host interactions. Gen. Announc. 2:e01229-13.
4. Bühlmann A., Dreo T., Rezzonico F., Pothier J.F., Smits T.H.M., Ravnikar M., Frey J.E., Duffy B. (2014) Phylogeography and population structure of the biologically invasive phytopathogen *Erwinia amylovora* inferred using minisatellites. Environm. Microbiol. 16:2112-2125.
5. Ismail E., Blom J., Bultreys A., Ivanovic M., Obradovic A., van Doorn J., Bergsma-Vlami M., Maes M., Willems A., Duffy B., Stockwell V., Smits T.H.M. , Puławska J. (2014) A novel plasmid pEA68 of *Erwinia amylovora* and the description of a new family of plasmids. Arch. Microbiol. (DOI 10.1007/s00203-014-1028-5).
6. Bergsma-Vlami M., van de Vossenberg B.T.L.H., Tjou-Tam-Sin N.N.A., Gorkink-Smits P.P.M.A., Landman N.M., van de Bilt J.L.J., Wenneker M., Pham K.T.K., van Vaerenbergh J. (2014) First report of a pathogenic *Erwinia* sp. 'assigned to the *E. pyrifoliae* taxon' on strawberry in the Netherlands, Plant disease (submitted).

Unrefereed papers/Popular press

1. Drenova N.V., Isin M.M., Dzhaimurzina A.A., Zharmukhamedova G.A., Aitkulov A.K. Bacterial fire blight in the Republic of Kazakhstan. Plant health. Research and practice (Russian-English journal), March 1st, 2013 3:39-48.
2. Rezzonico F., Duffy B. (2013) Thirteenth Int'l Workshop on Fire Blight. Chronica Horticulturae, 53(4):40-41.
3. Wenneker M., van Laarhoven A., Bergsma-Vlami M. (2014) Nieuw bacterieziekte in aardbei. Groenten en Fruit, March 2014, pp. 40-41.

Abstracts/Presentations in meetings

1. Rezzonico F. Source tracking and epidemiology of fire blight: exploiting the unexplored diversity of *E. amylovora*. Oeiras (Portugal), October 1st, 2012.
2. Kharchenko A., Kuznetsova A., Balandina M., Erohova M., Kulakova J., Kvashnina N., Shneider E., Drenova N. Distribution, characteristics and diagnostic methods for fire blight (*Erwinia amylovora*) in the Russian Federation. 13th ISHS International Fire Blight Workshop, Zürich (Switzerland), July 2nd-5th, 2013.
3. Djaimurzina A., Umiralieva Z., Zharmukhamedova G., Born Y., Bühlmann A., Rezzonico F. Detection of the causative agent of fire blight - Erwinia amylovora (Burrill) Winslow et al. - in the southeast of Kazakhstan. 13th ISHS International Fire Blight Workshop, Zürich (Switzerland), July 2nd-5th, 2013.
4. Pflüger V., Rezzonico F., Pothier J., Smits T.H.M., Duffy B. MALDI - TOF as tool to distinguish species in *Erwinia* and related genera. 13th ISHS International Fire Blight Workshop, Zürich (Switzerland), July 2nd-5th, 2013.
5. Bergsma-Vlami M., Tjou-Tam-Sin N.N.A., van de Bilt J.L.J., Wenneker M., Pham K.T.K., van Vaerenbergh J. (2014). Nieuwe bacteriële ziekteverwekker van aardbei. Abstract for the Royal Netherlands Society of Plant Pathology (workgroup Phytobacteriology).
6. Bergsma-Vlami M. A new *Erwinia* sp. pathogenic on strawberry. Presentation at EPPO Panel meeting on Diagnostics in Bacteriology, Wageningen (The Netherlands) 18th-21st March, 2014.
7. Wenneker M.,Bergsma-Vlami M. *Erwinia pyrifoliae* as a new pathogen on strawberry in the Netherlands. Proceedings IOBC 8th Meeting Working Group "Integrated Plant Protection in Fruit Crops", Pergine Valsugana (Italy), 25th-28th May, 2014.

Documents/Diagnostic protocols *(available at www.phytfire.org)*

1. VNTR-6 protocol using labeled or unlabeled primers.
2. List of known *E. amylovora* allele profiles.

Administrative details and partner roles

Partners (* Early Stage Researcher; # female)	**Research contributions**
1. Dr. Brion Duffy (ACW, CH) Tel.: +41 44 783 6111 E-Mail: duffy@acw.admin.ch	WP1 Lead Project Coordinator Contributed to WP2 as phase 1 partner involved in genomics-informed diagnostic assay development.
Dr. Fabio Rezzonico* (ACW, CH) Tel.: +41 44 783 6331 E-Mail: fabio.rezzonico@acw.admin.ch	WP6 Lead Project Vice-coordinator Shared and analyzed strains from different partners for source tracking.
2. Dr. Ulrike Persen# (AGES, AT) Tel.: +43 50 5 553 3342 E-Mail: ulrike.persen@ages.at	WP1 co-Lead for Technology Transfer Contributed to WP4 as phase 1 partner. Inoculum monitoring (using flowers and honeybees), evaluation of fire blight symptoms in orchards.
Dr. Richard Gottsberger (AGES, AT) Tel.: +43 50 5 55 33343 E-Mail: richard.gottsberger@ages.at	WP2 co-Lead Contributed to WP4 as phase 2 partner. Method development and evaluation and training in WP2, including testing Austrian strains of the AGES collection. Field sample testing with qPCR in WP4.
DI Thomas Leichtfried (AGES, AT) Tel.: +43 50 5 55 33351 E-Mail: thomas.leichtfried@ages.at	Contributed to WP3 as phase 1 partner. Test of Austrian isolates of *E.amylovora* and closely related taxa via MALDI-TOF. Build-up of a database and exchange of data files with other partners.
Dr. Rudolf Moosbeckhofer (AGES, AT) Tel.: +43 50 5 55 33121 E-Mail: rudolf.moosbeckhofer@ages.at	WP4 Contributed to WP4 as phase 2 partner. Inoculum monitoring (using flowers and honeybees).
Msc. Helga Reisenzein# (AGES, AT) Tel.: +43 50 5 55 33340 E-Mail: helga.reisenzein@ages.at	WP6 Contributed to WP6 as phase 2 partner. Participation in training event. Validation of source-tracking method and analysis of Austrian field isolates.
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5. Prof. Dr. Hatice Ozaktan# (EGE, TR) Tel.: +90 232 388 4000/1410 E-Mail: hatice.ozaktan@ege.edu.tr	WP1 co-Lead for Project Meetings Contributed to WP2, WP3 and WP6 as phase 2 partner. Application and evaluation of new protocols for diagnosis and detection of *E. amylovora*. Application of qPCR technique for monitoring of *E. amylovora* in flower population.
Dr. Nursen Ustun# (MARA-GDAR-BPPRS-TR) Tel:+90 232 388 00 30/108 E-Mail: nursen_ustun@yahoo.com	Contributed to WP2, WP3 and WP6 as phase 2 partner. Application and evaluation of new protocols for diagnosis and detection of *E. amylovora*. Application of qPCR technique for monitoring of *E. amylovora* in flower population.
6. Dr. Annette Wensing*, # (JKI, DE) Tel.: +49 6221 868 0510 E-Mail: Annette.Wensing@jki.bund.de	WP3 Lead

7. Dr. Heidrun Halbwirth[#] (TUW, AT) Tel.: +43 1 588 011 66559 E-Mail: hhalb@mail.zserv.tuwien.ac.at	WP4 Lead Developed a user-friendly "diagnostic kit" for *E. amylovora* based on the Blue ^{Ea}LAMP method, established an easy orchard sample collection method via vectors such as honeybees, evaluated the system under orchard conditions
Dr. Christian Gosch* (TUW, AT) Tel.: +43 1 588 011 66654 E-Mail: chgosch@yahoo.com	WP4 co-Lead Developed a user-friendly "diagnostic kit" for *E. amylovora* based on the Blue ^{Ea}LAMP method, established an easy orchard sample collection method via vectors such as honeybees, evaluated the system under orchard conditions
Prof. Dr. Karl Stich (TUW, AT) Tel.: +43 1 588 011 66003 E-Mail: kstich@mail.zserv.tuwien.ac.at	Contribute to WP 4 as phase 1 partner Developed a user-friendly "diagnostic kit" for *E. amylovora* based on the Blue ^{Ea}LAMP method, established an easy orchard sample collection method via vectors such as honeybees, evaluated the system under orchard conditions, provided protocols for testing the Blue ^{Ea}LAMP method for ring-test trials in WP 2
8. Dr. Maria M. López[#] (IVIA, ES) Tel.: +34 96 342 4076 E-Mail: mlopez@ivia.es	WP2 Lead, WP5 Co-Lead Contributed to WP4, WP5, and WP6 as phase 2 partner. Diagnosis and detection methods for *E. amylovora.*
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Dr. Konstantin Kornev* (FGU VNIIKR, RU) Tel.: +7-495 785 7613 E-Mail: konstantin.kornev@gmail.com	Contributed to WP2 as phase 2 partner.
10. Dr. Olga Bokshan[#] (Uzhgorod Univ., UA) E-Mail: carantin@carantin.uzhgorod.ua	Signed original project proposal but was unable to participate due to lack of national funding
Dr. Iuliia Bunduk*,[#] (UkrNDSKR, UA) E-Mail: ukrndskr@gmail.com	Signed original project proposal but was unable to participate due to lack of national funding
11. Dr. Laima Baranauskaite[#] (LT) Tel.: +370-5 2 616801 E-Mail: laima.baranauskaite@vatzum.lt	Contributed to WP2 and WP5 as phase 2 partner.
Dr. Ilona Kibildiene[#] (PhRL, LT) Tel.: + 370 5 261 1802 E-Mail: ilona.kibildiene@vatzum.lt	Contributed to WP2, WP5 and WP6 as phase 2 partner. Diagnostic and identification assays for *Erwinia amylovora*, ring test and/or training courses; bacterium detection in asymptomatic plant tissues.
12. Karme Petrutis[#]; Valentina Gusina[#] , Helena Lasner[#] , Maria Gusina[#] , Ekaterina Pochukaeva[#] (ARC, EE) Tel.: + 372 6729161 / 77 E-Mail: karme.petrutis@pmk.agri.ee; valentina.gusina @pmk.agri.ee; helena.lasner@pmk.agri.ee; maria.gusina@pmk.agri.ee; ekaterina.pochukaeva@ pmk.agri.ee	Contribute to WP2 and WP5 as phase 2 partner. Laboratory ringtests

Co-operators that joined activities during project	
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