EUPHRESCO Final Report

Project Title (Acronym)
Development and validation of innovative diagnostic tools for the detection of fire blight (*Erwinia amylovora*) (ERWINDECT)

Project Duration:

Start date:	01/11/08
End date:	31/10/09

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Introduction

The project titled `Development and validation of innovative diagnostic tools for the detection of fire blight (*Erwinia amylovora*) (ERWINDECT)´ was a pilot project in the frame of the EUPHRESCO project funded by EU FP6 ERA-NET. The project ran one year from 1st November 2008 to 31st October 2009. The funding mode was a Virtual Common Pot, via a competitive call. Each funder only paid for the participation of their own national researchers. The total funding of the project was €231,074.
The most important aspect of EUPHRESCO was the increase of the cooperation between research institutions. For the subject `fire blight´ a research network has already been established as a result of former research projects (e.g. the EU project DIAGPRO). However, the networking has been extended for this pilot project.
Another aim of the pilot project was the testing of a new funding mode and a new form of organisation of international research activities. Both aims were successfully achieved during this project.
The research aims of the project were the development of methods to source-track *E. amylovora* considering sampling protocols and molecular identification to the strain level and the validation of recently published diagnostic screen tests and recently revised diagnostic protocols by ring testing.

Objectives of the project

The objectives of the project are

- development of methods to source-track *E. amylovora* considering sampling protocols and molecular identification to the strain level.
- Ring-testing trials to validate new test methods for application for:
  i. detection of *E. amylovora* in asymptomatic plants in laboratories
  ii. detection of *E. amylovora* in symptomatic plants on-site or in laboratories

Expected Benefits of the project

Quarantine laboratories of several countries facing fire blight epidemics. These laboratories will benefit since the use of validated methods improves the quality of the laboratory and validated diagnostic protocols are a major requirement in accredited laboratories. Validation of methods is also a contribution to the harmonization of diagnostic protocols in Europe and external. The ability to determine the source of pathogen inoculum will improve the quarantine and sanitation measures to control fire blight.

Plant Protection Service will benefit, since results are quickly available and decisions on phytosanitary measures would be reached earlier (especially in situations of imminent danger, e.g. in protected zones with a sudden disease outbreak).

Fruit growers and nurseries will profit, since the availability of a reliable screening method would allow an economically feasible testing of plants intended for planting and would facilitate the development of certification schemes.

Summary

Fire blight is a devastating disease of apples and pears and related ornamental and crop plants. *Erwinia amylovora*, causal agent of fire blight, is a quarantine bacterium in Europe and the long-term control of fire blight requires eradication of inoculum reservoirs.

One aim of the project was to develop and validate methods for strain level genotyping and pathogen source tracking. The purpose of this aim is to discriminate *Erwinia amylovora* strains from different geographical regions in order to identify inoculum sources. Three different approaches were used to achieve this aim: Sequencing of the genome of several *E. amylovora* strains, development of VNTR protocols and analysis of the plasmid content and distribution in *E. amylovora* strains.

14 different *E. amylovora* strains were sequenced in the project which is far beyond the expected goal. Based on the analysis of genome sequence data several genomic markers were identified and evaluated for potential application in strain genotyping, including genomic rearrangements, VNTRs (Variable Number Tandem Repeat) and plasmid content. VNTRs and the plasmid content were determined to have the highest potential for application in pathogen source tracking. High-throughput PCR methods based on VNTR-system and plasmid typing methods were developed and validated for source tracking. The development of sampling procedures to effectively determine inoculum sources at local level could not be formalised within the tight time schedule of the work plan. A survey was conducted to estimate the distribution of plasmids in European strains using duplex PCR. Significant deviations were identified in 1.534 screened *E. amylovora* strains. Prevalence of the plasmid pEI70 ranged from 0% up to 92.8%. A screening of Spanish strains revealed that the plasmid pEA29 is most often present (85%). The presence of both plasmids (pEA29 and pEI70) is detected in 12% of tested strains. The total absence of both plasmids is very rare (0.7%).

Another aim of this project was to validate recently published diagnostic screen tests and recently revised diagnostic protocols by ring testing. Newly available methods such as new PCR assay and real time PCR are reviewed with respect to their specificity, sensitivity and performance. A further topic was the validation of simple techniques that can be confidently performed without specific training on-site (quick tests).

In preliminary studies eight different DNA extraction methods were compared on 11 different host materials. Three protocols were selected for further ring testing. It could be shown that the matrix has an influence on the reliability of an extraction method and that the limit of detection is improved after enrichment during the sample preparation. Four novel PCR assays were also shown to be suitable for detection of low level of *E. amylovora* and were assessed for further ring testing.

Testing of three real time PCR assays (Ams, ITS and plasmid assay) revealed differences within the performance criteria (the analytical sensitivity, specificity and accuracy). Ams assay proved to be the most accurate in detection of *E. amylovora* compared to ITS and plasmid assays. Compared with nested PCR the Ams assay showed higher sensitivity and as additional benefit a much easier interpretation of results in samples a low target concentration. The modification of the assay allows the use of this real time PCR in field.

The final results of ring testing of newly available methods showed that Isolation, conventional PCR assays according Llop 2000, Taylor 2001, Stöger 2006 and Obradovic 2007 and real time PCR assay (Ams assay according Pirc 2009) with the tested DNA extraction protocols can be advised for the analysis of asymptomatic and symptomatic plant material. Based in these results another ring test coordinated by Partner 2 is being organized including

at least ten laboratories from the EU, USA, New Zealand, Morocco and Russia to evaluate a larger number of samples and some combination of techniques in four continents.

Detection of *E. amylovora* in asymptomatic plants was a further topic of this project. Real time PCR (Ams assay) and a serological quick test `Ea AgriStrip´ were tested for detecting *E. amylovora* in asymptomatic flowers. The results demonstrate that flower monitoring of asymptomatic pathogen populations is principally possible with both methods, but the Ams assay is more sensitive than the serological quick test. `Ea AgriStrip´ was evaluated for sensitivity and specificity in the field and it could be shown that this test is most notable for detection of the pathogen in symptomatic plant material. The ring testing of serological quick tests (`Ea AgriStrip´ and `Pocket Diagnostic Kit´) in laboratories are concordant with the study in the field. The sensitivity, the specificity and accuracy of both tests do not differ significantly. Both quick tests can be advised for the analysis of symptomatic material in laboratories and on-site, but not for latent infections.

Work package 1

Project Management and Co-ordination

Work package Objectives

To manage and coordinate the project in all issues which are not covered by national funders rules especially reporting and communication including decision making and meetings
To communicate the projects aims and outputs for the wider benefit of non-participating European countries and organisations.
To disseminate results obtained within the project stakeholders.

Participants

Project partner 1 and all partners where relevant

Meetings

- Kickoff meeting was held in Lubijana (Slovenia) on 9th Dec 2008.

Short minutes of the kickoff meeting

- Visitation of the NIB-laboratories
- Introduction of all partners
- Overview of the project (management and topics)
- Project administration (processing of reporting, appointment of a date for the final meeting, discussion about cooperation agreement, additional information about samples shipment)
- Discussion about the work plan of the different work packages
- Organisation of the ring testing trials (testing different DNA extraction methods are carried out between February to April. The comparative PCR studies will start after the DNA extraction ring testing. The protocols for these studies should be sent out in July, the final ring test should be started in September. In this ring testing trial DNA from symptomatic and asymptomatic plant material from task 3.2.1 will be tested with all published PCR methods except Scorpion PCR. The repetition of the ring test at different times will be done by one laboratory).

- The final meeting was held in Vienna (Austria) on 23 th Oct 2009.

Short minutes of the final meeting

- Visitation of the AGES-laboratories
- Introduction of the fire blight situation in Austria
- Presentation of the outcomes of the different work packages
- Discussion about the results
- Project coordination (time schedule for the final report, organisation of the final ring test, sample shipment)

Reports

A progress report was sent to EUPHRESCO in June 2009.

Work package 2

Development of methods to source-track *E. amylovora* considering sampling protocols and molecular identification to the strain level

WP 2 Objectives

Current molecular diversity techniques like AFLP technique can distinguish strains from different geographical regions, but have not been evaluated for applications to discriminate between strains at the local level (e.g., within an orchard, or an orchard and surrounding environs level). Novel genomics-based methods (e.g. VNTR systems and differences in the T3S effectors) will be evaluated as a potentially useful tool to enable discrimination of strains from putative inoculum sources and affected objects within a small geographic area.
Specifically, methods that will allow determination of whether a strain infecting an orchard is the same as a strain from a nearby *Crataegus* sp. or landscape (old growth) pome fruit tree, and thus identify the inoculum source.
Molecular variations in the pathogenicity system of *E. amylovora* especially differences in the T3SS effectors of the bacteria may have a strong impact on the adaptation of the bacteria to its host. The pathogenicity of *E. amylovora* has been under intensive investigation for twenty years. The major pathogenicity factors are nowadays well known in several reference strains. They are all carried by the bacterial chromosome. Pathogenicity relies mainly on a type III secretion system (T3SS, previously named Hrp system) allowing the secretion in the plant apoplast or the injection into the plant cells of proteinaceous effectors (Barny et al, 1990). These effectors are able to modify plant metabolism, to supply the bacteria with nutriments, and finally to cause plant cell death. In *E. amylovora* twelve T3SS secreted proteins have been identified so far (Nissinen et al. 2007). This limited number contrasts with the great number of such proteins identified in other plant Proteobacteria (more than 40 in *Pseudomonas syringae pv. syringae* as an example) but could increase with the annotation results of the sequence of *E. amylovora*. Two of them, HrpN and DspA/E, are essential pathogenicity factors (Wei et al. 1992, Gaudriault et al. 1997) and are involved in cell death through the induction of an oxidative burst (Venisse et al. 2003). This is another approach for the development of strain specific markers to trace back infections. A preliminary work from INRA (unpublished data), aimed at phenotyping a collection of strains isolated from various host species of *Maloideae* and from *Prunus*, as well as from different geographical origins, revealed for the first time in *E. amylovora* pathosystem a differential pathogenicity of some strains on particular genotypes belonging to different host species.

Plasmids have been found in most *E. amylovora* strains, being pEA29 previously considered as universal in this species. However, the previous work developed by partner 2 allowed the detection of strains without such plasmid but containing a previously undescribed plasmid called pEI70. As plasmids can also be used as epidemiological markers, after confirmation of their stability, the determination of the plasmid content of different strains by duplex PCR gives an overview of the distribution of plasmids in European strains.

The aims of the work packages were

To sequence the genome of an European *E. amylovora* strain
To develop and evaluate usefulness of selected molecular markers for discrimination of *E. amylovora* at strain level i.e. VNTR system for *E. amylovora*
To develop novel genomics-based methods (e.g. VNTR system for *E. amylovora*)
To evaluate existing and new developed methods such as AFLP, SSR typing and VNTR for differentiation of *E. amylovora* at the strain level
To analyse the plasmid content and distribution in European strains by duplex PCR
To develop sampling methods to effectively determine inoculum sources at the local level (e.g., sample orchards and surrounding environments)

WP2 Participants

WP coordinator: Partner 3
Other participants: Partners 1, 2, 4, 5, 6

WP2 Tasks

Task 2.1: Sequence the genome of an European *E. amylovora* strain
Task 2.2: Develop and evaluate usefulness of selected molecular markers for discrimination of *E. amylovora* at strain level (i.e., VNTR system for *E. amylovora*)
Task 2.3: Analyse the plasmid content and the distribution in European strains by duplex PCR
Task 2.4: Develop sampling methods to effectively determine inoculum sources at the local level (e.g., sample orchards and surrounding environments)

WP2 Methods and Results

All goals of WP2 were successfully achieved beyond the expected goals of the project. Complete genomes of 14 *E. amylovora* strains were sequenced. Several genomic markers were identified and evaluated for potential application in strain genotyping, including genomic rearrangements, VNTRs and plasmid content. VNTRs were determined to have the highest potential for application in pathogen source tracking. High-throughput PCR methods were developed and validated using global collections of *E. amylovora* strains. The development of sampling procedures to effectively determine inoculum sources at local level could not be formalised within the tight time schedule of the project.
Plasmid typing methods were an additional source tracking approach. A simple duplex PCR method for screening E.amylovora strains for content of plasmid pEA29 and pE170 was developed. Prevalence of pE170 in 1.534 screened *E. amylovora* strains ranged from 0% up to 92.8%. The development of sampling procedures to effectively determine inoculum sources at local level could not be formalised within the work plan.

Task 2.1 Sequence the genome of an European *E. amylovora* strain

Our Task was to sequence and annotate the complete genome of the first European strain of *E. amylovora.* We accomplished this by completing the genome sequence of the model strain CFBP 1430 isolated in 1972 in France. This has been submitted for the first publication of an

E. amylovora genome, and accepted with revision (Fig. 2.1.1). In addition to this, Partner 3 has completed the sequencing of a further 6 *E. amylovora* strains and Partner 5 has completed the sequencing of 8 *E. amylovora* strains from Europe and Canada (Table 2.1.1).

Figure 2.1.1: Complete genome of *E. amylovora* strain CFBP 1430 showing the chromosome (A) and plasmid pEA29 (B).

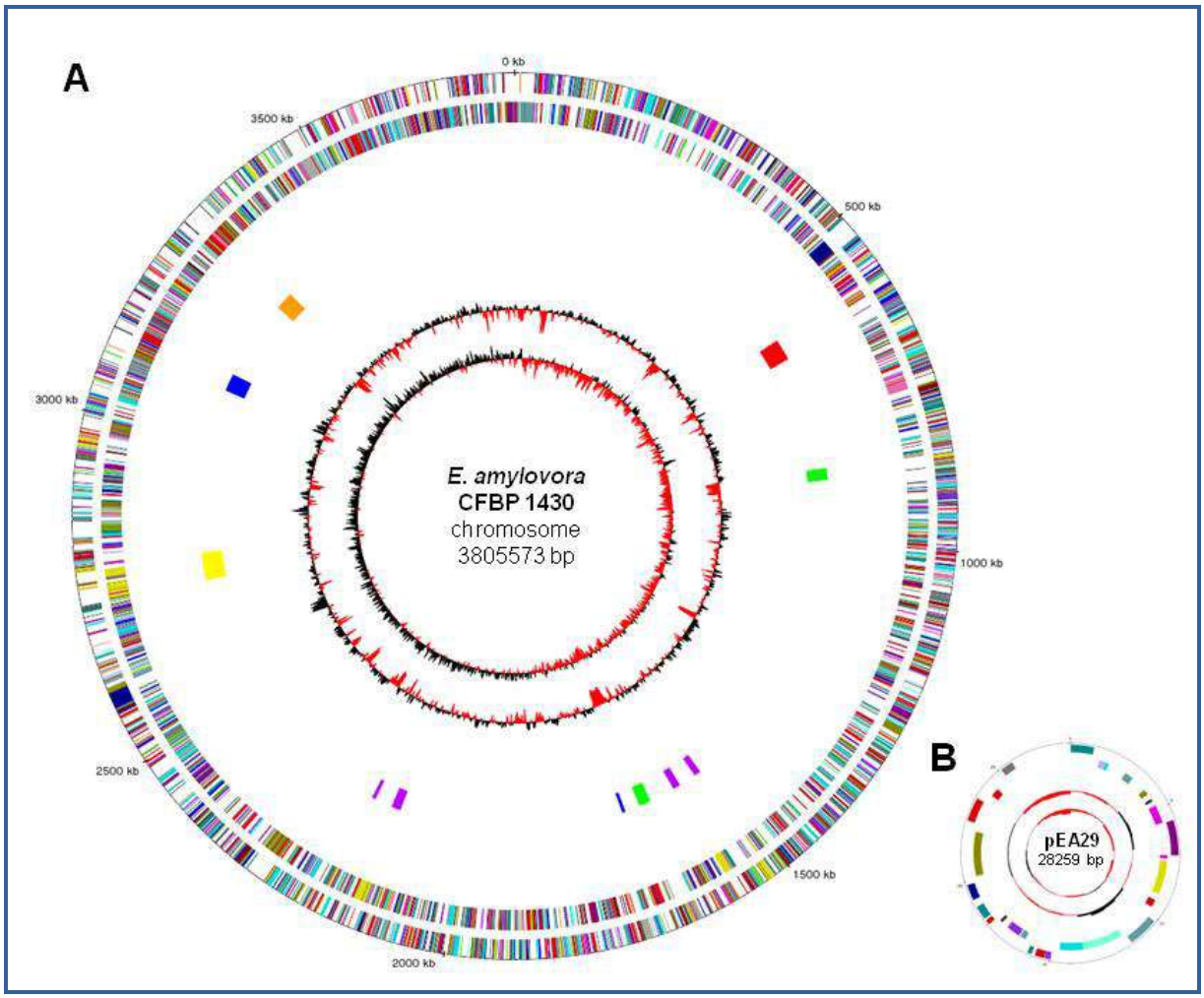


Table 2.1.1: Genome statistics for *E. amylovora* strains sequenced in the ERWINDECT project.

Strain	Origin	Isolation year	Host	Sequencing platform	Status
Ea273	New York, USA	1971	*Malus domestica*	Sanger	Finished, unpublished
CFBP1430	Lille, France	1972	*Crataegus sp.*	Illumina	Finished
CFBP1232T	United Kingdom	1959	*Pyrus communis*	Illumina	Assembly in progress
ACW56400	Fribourg, Switzerland	2007	*Pyrus communis*	Illumina	Assembly in progress
UPN527	Navarra, Spain	1997	*Malus domestica*	Illumina	Assembly in progress
01SFR-BO	Ravenna, Italy	1991	*Sorbus sp.*	Illumina	Assembly in progress
CFBP1197	UK	1959	*Crataegus* sp	454-FLX	First assembly
CFBP1367	France Nord	1972	*Crataegus oxyacantha*	454-FLX	First assembly
CFBP2301	France Haute Savoie Saint Julien en Genevois	1981	*Pyracantha* sp	454-FLX	First assembly
CFBP3020	The Netherlands	1981	*Pyrus communis* cv. Clapp´s Favourite	454-FLX	First assembly
CFBP3043	UK	1964	*Pyracantha augustifolia*	454-FLX	First assembly
CFBP3792	USA Parma Idaho	ND	*Prunus salicina*	454-FLX	First assembly
CFBP7159	France	1994	*Sorbus aucuparia*	454-FLX	First assembly
CFBP7161	France	1996	*Sorbus aucuparia*	454-FLX	First assembly

Task 2.2 Develop and evaluate usefulness of selected molecular markers for discrimination of *E. amylovora* at strain level (i.e., VNTR system for *E. amylovora*)

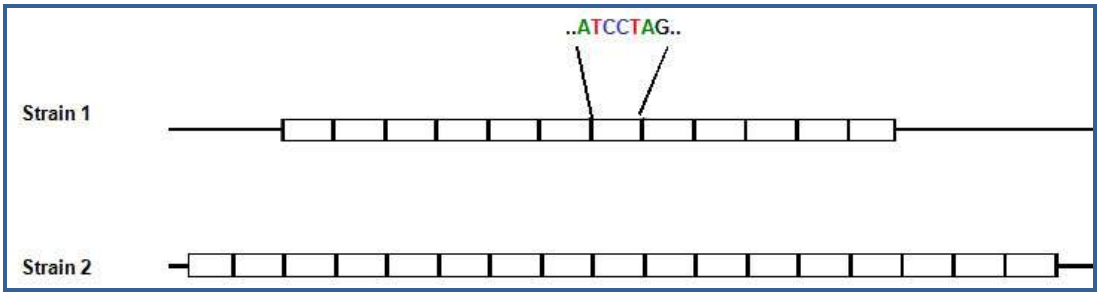
Methods of analyzing genetic diversity have been previously used in studies of *E. amylovora*, most notably pulsed field gel electrophoresis and AFLP. Any method based on fingerprinting, such as PFGE, is difficult to standardize (between laboratories, over time within a single laboratory) and is particularly subject to faulty calls. Sequence based methods are therefore preferable.

Levels of genetic diversity are sufficiently high in most microbial taxa to facilitate medium-resolution of genetic structure based on sequencing of just a few housekeeping genes. However, *E. amylovora* has unusually low level diversity when such sequencing approaches are applied, with insufficient polymorphism in these single loci among strains.

The extensive, and more-than-anticipated, genomic data obtained in Task 2.1 revealed new genetic markers that enable higher discriminatory power at the strain level. These have been characterized and validated against a large *E. amylovora* strain collection. A high-throughput method was developed for strain-level genotyping and pathogen source-tracking.

VNTR stands for **v**ariable **n**umber of **t**andem **r**epeats and describes repeated sequences that can be repeated more or less extensively in different strains (Fig. 2.2.1). Number of repeats is determined and reported as a number providing easy comparison between laboratories and enabling collection of results over years. The most useful VNTR loci are very similar between closely related strains, but are also sufficiently variable so that unrelated strains are extremely unlikely to have the same number of repeats. Analyses of VNTRs often at several loci (MLVA = multiple loci VNTR analysis) has proven extremely useful in clinical microbiology for genotyping and source-tracking in outbreak monitoring.

Figure 2.2.1 A Variable Number Tandem Repeat (or VNTR) is a location in a genome where a short nucleotide sequence organized as a tandem repeat. In the figure below, the rectangular blocks represent each of the repeated DNA sequences at a particular VNTR location. The repeats are tandem, they are clustered together and oriented in the same direction. Strains can contain different numbers of repeats sometimes leading to different biological properties.



Sequences with variable number of tandem repeats (VNTR) were identified throughout the genome of *E. amylovora*. We detected potential VNTRs in genome Ea273 through analysis program available at http://minisatellites.u-psud.fr/ASPSamp/base_ms/bact.php, observing that in comparison to some other bacterial pathogens of plants, the *E. amylovora* Ea273 genome harbours a relatively standard number of tandem repeat arrays (Tab 2.2.1). The chromosome contains 2155 tandem repeats arrays (566/Mb interval) with all repeated sequences representing less than 0.1 % of genome.

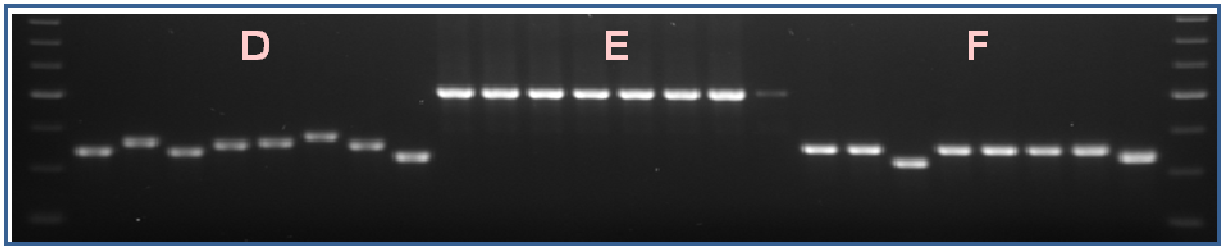
Table 2.2.1: Comparison of variable number of repeats sequences in several plant pathogenic bacteria as identified by http://minisatellites.u-psud.fr/ASPSamp/base_ms/bact.php. Settings for analysis were minimal total length = 10, minimum copy number = 2 and % match =100.

Bacterial strain	Genome size	Number of repeats	Repeat arrays per Mbp
Pseudomonas syringae phaseo. 1448A/Race6	5.9	2439	411
Pseudomonas fluorescens PfO-1	6.4	2719	422
Erwinia carotovora subsp. atroseptica SCRI1043	5.1	2204	435
Pseudomonas syringae B728a	6.1	2711	444
Pseudomonas putida F1	6.0	2880	483
Agrobacterium tumefaciens C58 Cereon	4.9	2535	516
Pseudomonas aeruginosa PA01	6.3	3398	542
Candidatus Phytoplasma mali	0.6	335	557
Ea273	**3.8**	**2155**	**566**
Acidovorax_avenae_subsp_citrulli_AAC00_1	5.4	3095	578
Xylella fastidiosa 9a5c	2.7	1561	583
Agrobacterium tumefaciens str. C58 Wash.U	4.9	2898	590
Xanthomonas campestris vesicatoria 85-10	5.2	3154	609
Xanthomonas campestris 8004	5.1	3139	610
Candidatus Phytoplasma australiense	0.9	687	782
Ralstonia solanacearum GMI1000	3.7	4621	1244

Variable number of tandem repeats were also detected in genome of CFBP 1430. For analysis of variable number of tandem repeats several loci were selected. PCR primers were developed and validated (Fig. 2.2.2) for discrimination of strains at the international and national levels using genotypically-representative collections of *E. amylovora* strains.

A total of 16 tandem repeat regions that met the selection criteria set in program JSTRING were detected in the genome sequence of strain CFBP 1430. For 10 of these repeat regions PCR primers were designed targeting flanking sequences and PCR amplification products generated for a panel of isolates. All loci showed variable polymorphism however, at 4 of these loci, sequence variation was insufficient to discriminate between the geographically distinct strains (also different using other methods) and were not further evaluated. Selected VNTRs were used to type a panel of 35 strains from geographically different locations. Used in combination, they provided high resolution and identified individual genotypes.

Figure 2.2.2: An example of PCR amplification of three VNTR loci (D, E, F) in eight strains of *E. amylovora* from geographically distinct locations. Different number of repeats results in different size of PCR product. VNTR D has a much higher resolution than VNTR systems F and E.

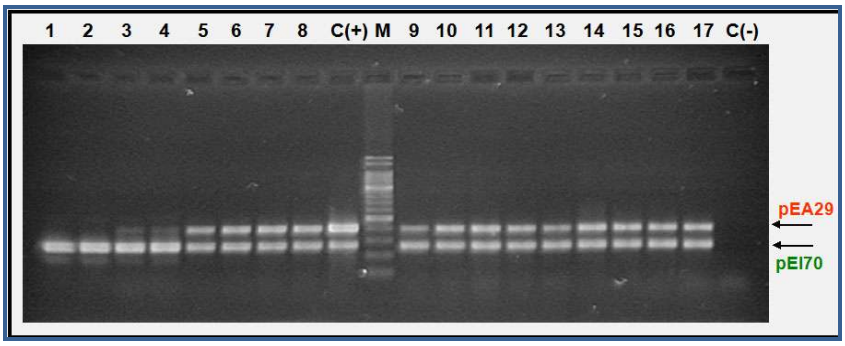


The evaluation of existing methods such as AFLP and SSR typing for differentiation of *E. amylovora* at the strain level was a subtask of this work package. Partner 2 has evaluated AFLP and SSR for differentiating recently isolated Spanish strains of *E. amylovora*, concluding that AFLP is more efficient that SSR using two microsatelites, for strain differentiation. These results confirm similar previous results published by Donat *et al*. (2007).
This methodology could be compared with the recently developed VNTR protocols in the near future, to conclude about their comparative efficiency for distinguishing strains at the European level and to source track recent fire blight outbreaks.

Task 2.3 Analyse the plasmid content and the distribution in European strains by duplex PCR

Plasmid-typing methods were developed and validated for *E. amylovora* genotypes in Europe as an additional source-tracking approach. Two plasmids were characterized and PCR primers developed for each (Fig. 2.3.1).

Figure 2.3.1: A simple duplex-PCR method was developed for screening *E. amylovora* strains for content of plasmid pEA29 and the newly described plasmid pEI70.



A total of 1'534 strains from Partners 3, 2, 1, 4 and 5 and from other countries were screened to determine the geographic distribution of the newly described plasmid pEI70. Prevalence of this new plasmid ranged from 0% up to 92.8%, with suspected origin in Europe (Fig. 2.3.2). In regions with low prevalence of pEI70, plasmid characterization may offer some level of strain genotypic discrimination. However, the maximum power is 2 (with or without), and thus decreased its utility in countries with moderate to high prevalence. Table 2.3.1 shows the plasmid content of 142 Spanish strains.

Table 2.3.1.: Presence of plasmids in Spanish strains (isolated from 1995 to 2007)

Plasmid content	%
pEA29	85,3
pEI70	2
pEA29 + pEI70	12
Absence: pEA29 and pEI70	0,7

Table 2.3.2: Plasmids of *E. amylovora* strains from European countries and from other countries. Dot letters indicate ERWINDECT Partner countries.

Country	% strains with:			
	pEA29	pEI70	pEA29+pEI70	Absence
Ireland	2/14	0	12/14	0
United Kingdom	6/8	0	2/8	0
Italy	2/5	0	3/5	0
The Netherlands	3/5	0	2/5	0
Bulgaria	300/300	0	0	0
Belgium	6/72		66/72	0
Slovenia	**ND**		**245/350**	**ND**
Poland	5/120		7/120	ND
Czech Republic	1/2	0	1/2	0
Germany	12/12	0	0	0
Austria	**25/25**	**0**	**0**	**0**
Hungary	10/10	0	0	0
Greece	15/15	0	0	0
France	**42**	**2**	**2**	**0**
Spain	**142**	**2**	**17**	**1**
Switzerland	**247**	**13**	**13**	**0**
United States*	117	0	0	0
Canada *	36	0	0	0
Lebanon	6	0	0	0
Morocco	8/17	0	9/17	0

The strains from USA were analized by Virginia Stockwell, Covallis, Oregon and those from Canada by Antonet Svircev, Ontario, Canada.

Task 2.4 Develop sampling methods to effectively determine inoculum sources at the local level (e.g., sample orchards and surrounding environments)

Formalized sampling procedures were not achieved during this project. However, discussions were conducted between partners in order to begin designing such procedures. Within a location, multiple strains not just one isolate per case should be collected. The value of existing collections in Switzerland, Slovenia, Austria and Spain from different outbreaks across regions and years was identified.

Implications for stakeholders:

Phytosanitary authorities: Determination of the source of pathogen inoculum is a major emerging issue with phytosanitary control of fire blight. Design of recommendations and regulations has thus far relied on many epidemiological assumptions. For example, it is assumed that infected old-growth cider trees (or native host plants such as hawthorne) are the source of new outbreaks in adjacent nurseries or orchards. These results in perhaps unnecessary felling of old trees (with significant cultural and ecological value) or perhaps unwarranted planting bans (such as removal and prohibition of alternate hosts).

Fruit producers / NGOs: Our new source-tracking methods, with further development/validation still needed, will for the first-time enable regulatory stakeholders to develop a more scientifically-based fire blight management strategy. These methods will clarify the risk of old-growth trees and native alternate host species to commercial orchards/nurseries. This will benefit fruit producer stakeholders in terms of optimizing protection of their property from fire blight. It will also benefit rural and environmental interest-group stakeholders in terms of promoting the co-existence of old trees/native species with commercial orchards/nurseries resulting in a better rural landscape.

Scientists and fruit breeders: Our new genomic insights will have a major impact on fire blight pathogen research for scientist stakeholders. Specifically, genome analysis is anticipated to reveal novel details on virulence determinants and host-pathogen interactions. This information can then be applied to improved fire blight resistance breeding.

Further research needed and continued collaboration:
Additional complete genome sequences of *E. amylovora* strains and related *Erwinia* species will facilitate further discovery of differential genetic sequences that will be useful for improvement of our strain-typing and source-tracking method. Partner 2 plans to conduct such further sequencing work with input from other partners.

Partner 5 has further determined differential phenotypes of the strains sequenced in this project in plant assays. A continuing collaboration has been formalised between Partners 3 and 5 with the aim of concluding a comparative genomics analysis of all sequenced *E. amylovora* strains. The anticipated result of this further cooperation in 2010 is to achieve novel insights into the host-pathogen genetic determinants.

VNTR analysis is a practical, relatively inexpensive method for genotyping of *E. amylovora*. Partners 3 and 4 continue to collaborate in setting up a high-throughput method based on amplification of VNTR regions using fluorescently labelled primers followed by fragment analysis of products by capillary electrophoresis. A further analysis of strains from different geographic levels is planned for winter 2009/2010 specifically strains isolated from single orchards and their surroundings to research importance of 'Hochstamm' trees in infections

and hopefully providing answers to long standing questions on possible coexistence of such trees with extensive fruit tree production. A scientist exchange between Partners 3 and 4 has begun to complete the application of VNTR genotyping of strain collections from epidemics in Switzerland and Slovenia.

Determination of the plasmid content of *E. amylovora* has provided very new and interesting results because the simultaneous analysis of the presence or absence of pEA29 (previously considered as a universal plasmid in *E. amylovora*, and of pEI70, has shown the interest of using the presence or absence of the pEA29 and/or pEI70 plasmids as epidemiological markers. It is surprising that the pEI70 has been found in ten European countries and at least in Spain, is being currently used for following the origin of the *E. amylovora* inoculum.

Work package 3: Ring-testing trials to validate new test methods for application for the detection of *E. amylovora* in asymptomatic plants in laboratories

WP3 Objectives

Ring test determination of the sensitivity and specificity of the different techniques selected. When an assay is applied to a population to detect *E. amylovora* in asymptomatic plants the diagnostic sensitivity becomes relevant. This is the proportion of infected samples (true positives) that gives a positive result in an assay. It is the ability to obtain the target organism in a processed sample. In contrast the analytical sensitivity of an assay (true negatives) is the ability to detect a low concentration of the target. Understanding the different meanings of these terms is important for a properly interpreting of diagnostic results and in the following for pest management.

The aims of the work package were

To compare different method procedures / assays of the detection scheme of the EPPO protocol for their diagnostic sensitivity
To validate new test methods for the determination of asymptomatic infections by ring-testing and to compare this tools with the methodology of the EPPO diagnostic protocol
To develop protocols based on Real time PCR and other new methods for detection of *E. amylovora* in asymptomatic plants.

WP 3 Participants

WP coordinator: partner 1 and partner 2. Other participants: partner 3, 4, 6

WP 3 Tasks

Task 3.1. Testing the diagnostic sensitivity of different method procedures for asymptomatic infections
Task 3.2. Testing the analytical specificity and sensitivity of newly available methods for the detection of *E. amylovora* in asymptomatic plants

WP 3 Methods and Results

All goals of WP3 were successfully achieved. Eight different DNA extraction methods were compared on 11 different host materials. Three protocols were selected for further ring testing. It could be shown that the matrix has an influence on the reliability of an extraction method and that the limit of detection is improved after enrichment during the sample preparation. Three novel PCR assays were shown to be suitable for detection of low level of *E. amylovora* and were assessed for further ring testing.
Real time PCR (Ams assay) and serological kit Ea AgriStrip have been further tested for detecting *E. amylovora* in asymptomatic flowers. The results demonstrate that flower monitoring of asymptomatic pathogen populations is possible.

Task 3.1. Testing the diagnostic sensitivity of different method procedures for asymptomatic infections

The objective of this subtask was to compare different method procedures of the detection scheme of the EPPO diagnostic protocol for their reliability to detect *E. amylovora* in asymptomatic samples. A comparison of the sensitivity of the different available procedures was included in WP 3 (see ring test, Task 4.2). Latent infections were simulated by spiking of host species with low levels of *E. amylovora* (below 10^6 cells/ml).

The integrated methodology advised at the EPPO protocol and other methods under study in this project was also compared for the detection of *E. amylovora* in asymptomatic fruits from fire blight affected trees. This methodology was previously applied to the analysis of inoculated apple fruits by Ordax *et al.* (2009b). Recently, we have demonstrated that *E. amylovora* adopts the 'viable but not culturable' (VBNC) state in the calyx of inoculated mature apples without showing symptoms, remaining the pathogen undetectable by isolation but being able to recover culturability and pathogenicity (Ordax *et al*, 2009b). This indicates that lack of presence of bacteria in the isolation plates is not an evidence of the pathogen absence as suggested by Ordax et al.(2006) and that such analyses require the use of several techniques.
Asymptomatic fruits were collected from trees naturally affected by fire blight. Fruits were washed in batches of five, in antioxidant maceration buffer (AB) in plastic bags. The EPPO diagnostic protocol (2004) was applied with some modifications. Then, washings were collected in sterile tubes and processed as follows: aliquots were directly plated on CCT and KBCu media (Ordax *et al.*, 2009a); 1 ml was enriched in CCT broth (Ishimaru and Klos, 1984) and afterwards aliquots from direct enrichments and also from their dilutions 1/10 were plated on CCT medium; 1ml was frozen after boiling for DNA extraction protocol [Llop *et al.*, 1999] and subsequent molecular detection by a chromosomal PCR for *E. amylovora* (Taylor *et al.*, 2001). The remaining extract was stored at -20ºC with 30% glycerol for further studies. In case *E. amylovora*-like colonies arose on plates, they were also analyzed by PCR to be confirmed. The results obtained confirmed the presence of *E. amylovora* in asymptomatic fruits and advice the use of this protocol for the analyses of other types of asymptomatic material.

A comparison of a new serology-based Ea AgriStrip test and real time PCR was conducted in Switzerland. Partner 3 working with industry developed a novel lateral-flow immunographic test strip (Ea AgriStrip) that is now commercially available (Fig. 4.1.2.1, WP4 below). Partner 4 has developed a new real time PCR assay (Pirc et al. 2009). These tests were applied for the first time for the purpose of flower monitoring in this project. The ability of these methods to detect asymptomatic populations of *E. amylovora* in apple flowers was conducted in collaboration of Partners 3 and 4 (see Task 3.2.3).

Task 3.2. Testing the analytical specificity and sensitivity of newly available methods for the detection of *E. amylovora* in asymptomatic plants

The analytical sensitivity and specificity of new test methods were compared with methods of the EPPO diagnostic protocol that will be further validated in a ring test with 10 laboratories that partner 2 is currently organizing for February 2010. Plant material of different hosts (spiked with low bacterial populations and/or with latent infections) are tested with the methodology of the EPPO protocol and compared with real time-PCR and enrichment-real time-PCR using different sets of primers and other techniques.

Task 3.2.1 Testing different DNA-extraction methods

Different DNA extraction methods were compared on tissues of different hosts and after enrichment. This preliminary study was performed by partners 6, 4 and 1.

Methods

Eight different DNA extraction methods have been compared on 11 different host materials (with and without enrichment). Two different PCR methods (nested PCR and real time PCR) have been used for evaluation of these methods. The detailed protocols and results are provided in annex 1.

Following DNA extraction protocols were used for this preliminary study.

P 1 – Isopropanol (Llop et al, 1999)
P2 – Easy-DNA kit Invitrogen (Pastrik et al, 2000)
P3 - RedeExtract-N-Amp ™ Plant PCR kit (Stöger et al, 2006)
P4 – Kit Ultra Clean ™ 15-MO BIO (Manceau et al, 2005)
P5 – DNA extraction Taylor (Taylor et al ,2001)
P6 – Kit Mycrosynth
P7 – Magnetic beads DNA extraction (Pirc et al., 2009)
P8 - DNeasy Plant Mini Kit (Qiagen)

Statistical analysis of the test data

Performance criteria of alternative method were assessed with accordances (A) and deviations (D) between expected results and obtained results when P (positive) N (negative):

Relative accuracy : AC = 100(PA+NA)/(NA+PA+PD+ND)

Relative sensibility : SE = 100PA/(ND + PA)

Relative specificity: SP = 100NA/(NA+PD)

Limit of detection and repeatability intra-laboratory: calculated as indicated annex 2 with the statistical table.

n		10			20			30			40		
q (%)		99	95	90	99	95	90	99	95	90	99	95	90
Given A for	□ □¡	1	2	3	1	3	5	2	4	7	2	5	9
	□ □ ¸□	0	1	2	1	3	4	1	4	6	1	4	7
	□ ¸ □0¸	0	1	2	1	2	4	1	3	5	1	4	6

Table : Given A for q value depending on number of samples and risk □

n : number of samples analyzed with repetition
q : assessed rate accords between pairs or accords with results of reference
A : number of maximum of deviations on n samples in order not discarding hypothesis that alternative method has a rate of accords close to q at risk □
□ : risk to discard a good method

Evaluation by PCR

Selected DNA extraction methods can differ considerably in the suitability for the type of sample, depending on its chemical composition, location of bacteria and target DNA in bacterial cells (plasmid versus chromosomal DNA). Two different PCR methods (nested PCR and real time PCR) have been used for evaluation of these methods. The PCR adopted for the detection of the target is the nested PCR (Llop et al, 2000) and real time PCR according Pirc et al. 2009.

Results

Comparison of different DNA extraction methods without enrichment

Expected results were considered all positive, they all are spiked asymptomatic samples excepted N0 (non-contaminated sample). The protocol giving the best sensibility was protocol n°5 (Taylor), then REDEDExtract modified – FR (different of REDEDExtract modified – AU). The reference method Isopropanol showed a lower sensibility. There was a problem of specificity on Taylor and Isopropanol protocols probably due to contamination of level N0. The performance criteria are shown in Table 3.2.1.1.

The Austrian results of protocol 1 and 3 varied from those obtained in the French laboratory. It can be explained by the application by AGES of centrifugation of 1mL of extract and suspension of the pellet in buffer in order to get the same amount of DNA. This preparation concentrate DNA but also inhibitors.

The protocol 6 did not reveal any satisfying results neither with undiluted samples nor with dilutions.

Table 3.2.1.1.: Performance criteria of different method assessed in comparison with expected results without enrichment.

		AC: Relative accuracy	SP: Relative specificity	SE: Relative sensibility
Protocol 5: Taylor		74,2%	90,9%	70,9%
Protocol 3: REDEExtract-FR		63,6%	100,0%	56,4%
Protocol 1: Llop-FR		59,1%	90,9%	52,7%
Protocol 2: Kit invitrogen		56,8%	100,0%	48,2%
Protocol 1: Llop-AU		44,2%	100,0%	33,0%
Protocol 3: REDEExtract-AU		42,5%	100,0%	31,0%
Protocol 4: Kit ultraclean		39,4%	100,0%	27,3%
Protocol 6: Kit microsynth	Pure	20,5%	100,0%	4,5%
	1/10	25,0%	100,0%	10,0%
	1/100	26,5%	95,5%	12,7%
	1/11000	27,3%	100,0%	12,7%

Comparison of different DNA extraction methods with enrichment

With enrichment, sensibility was better (see limit of detection below), but specificity was less good for French laboratory which got positives for the non-contaminated level (N0). Performance criteria are shown in Table 3.2.1.2. It could be explained by a contamination problem which could have been enhanced during enrichment. Partner 1 sent results with no positives for this level but it was after re iteration of extraction twice or three times. Actually, they got also positive results at level N0.

It could be assumed that a contamination happened at LNPV on asymptomatic twigs during their splitting and before sending them to the others laboratories (AGES – NIB).

Table 3.2.1.2.: Performance criteria of different method assessed in comparison with expected results with enrichment.

		AC: Relative accuracy	SP: Relative specificity	SE: Relative sensibility
Protocol 5: Taylor		93,9%	63,6%	100,0%
Protocol 2: Kit invitrogen		94,7%	72,7%	99,1%
Protocol 1: Llop-FR		93,9%	68,1%	99,1%
Protocol 3: REDEExtract-FR		97,0%	95,4%	81,1%
Protocol 4: Kit ultraclean		95,4%	100,0%	78,8%
Protocol 3: REDEExtract-AU		68,3%	100,0%	62,0%
Protocol 1: Llop-AU		64,2%	100,0%	57,0%
Protocol 6: Kit microsynth	Pure	90,1%	81,8%	92,7%
	1/10	94,7%	86,4%	96,4%
	1/100	87,1%	86,4%	87,3%
	1/11000	68,9%	100,0%	62,7%

Evaluation of the DNA extraction methods by PCR

Two different PCR methods (nested PCR and real time PCR) have been used for evaluation of these methods.

Assessment of performance criteria

Real time PCR provided better performance criteria but classification of methods didn't change (Table 3.2.1.3). Specificity was less good because of positives for the non-contaminated level (N0). Specificity calculated is relative, based on the non contaminated level, the real specificity of a method is assessed for the entire method (including PCR) with different strains.

Table 3.2.1.3.: Performance criteria of different methods assessed in comparison with expected results without enrichment and real time PCR.

	AC: Relative accuracy	SP: Relative specificity	SE: Relative sensibility
Protocol 5: Taylor	88,6%	81,8%	90,0%
Protocol 3: REDEExtract-FR	85,6%	63,6%	90,0%
Protocol 1: Llop-FR	81,8%	59,1%	86,4%
Protocol 2: Kit invitrogen	63,6%	27,3%	70,9%
Protocol 4: Kit ultraclean	45,5%	90,9%	36,4

Assessment of limit of detection

The best limit of detection without enrichment at the contamination level N3 (around 10^4 cfu/mL) was obtained with protocols P5 (Taylor) and with FR-modified protocol P3 (RedeExtract Stöger) (Table 3.2.1.4). Followed by the limit of detection N4 around 10^5 cfu/mL for the protocols, P1 (Isopropanol – Llop, 1999)-FR, P2 (Easy DNA kit Invitrogen – Pastrik) and n°3 (RedeExtract Stöger)-AU. AGES and LNPV obtained the best LOD with P3 compared to P1.
Protocol P4 gave a less good limit of detection (N5-10^6 cfu/mL) and results for protocol P6 could not permit to obtain a limit of detection at least equal to N6. It could be more than 10^6 cfu/mL.

Table 3.2.1.4: Limit of detection without enrichment assessed by using conventional PCR (Llop, 2000).

Without enrichment		P1	P 1AU	P 2	P 3	P 3AU	P 4	P 5
N3	90%							
	95%				X			
	99%							X
N4	90%	X			X	X		
	95%			X				
	99%							X
N5	90%					X	X	
	95%	X		X				
	99%		X		X			X

The limit of detection was improved of one dilution with Real time PCR (Table 3.2.1.5). The best limit was obtained for protocol P5 (Taylor), protocol P3 (modified RedeExtract Stöger), protocol P2 (Easy DNA kit Invitrogen – Pastrik) and protocol P1 (Isopropanol – Llop, 1999) around 10^3 cfu/mL, followed by protocol P4 with a limit of detection N5 (around 10^6 cfu/mL).

The limit of detection was good, but for two species (Hawthorne and Quince) there was no detection at all. It could be due to a bad DNA extraction for these matrices with protocols P2 and P4 or the presence of a high quantity of inhibitors for PCR.

Table 3.2.1.5: Limit of detection without enrichment assessed by using real time PCR (Pirc et al. 2009).

Without enrichment		**P1**	**P2**	**P3**	**P4**	**P5**
N1= 10^2cfu/mL	90%					
	95%					
	99%					
N2=	90%	**X**				
10^3 cfu/mL	95%		**X**	**X**		
	99%					**X**
N3 =	90%					
10^4 cfu/mL	95%					
	99%	X	X	X		X
N4 =	90%					
10^5 cfu/mL	95%					
	99%	X	X	X		X
N5 =	90%					
10^6 cfu/mL	95%					
	99%	X	X	X	X	X

The limit of detection with enrichment was improved to N1 (10^2 cfu/mL) for all protocols performed by FR: P1, P2, P3, P4, P5 and P6 most of them with 99% of chance not to mistake (Table 3.2.1.6.). But the contamination should be taken in account regarding the results of N0. For AGES, the limit of detection was improved of two levels (from N5 to N3), for P1 and of one level for P3 (from N4 to N3) (table 21 and 23).

Table 3.2.1.6.: Limit of detection with enrichment assessed by using real time PCR (Pirc et al. 2009).

With enrichment		P 1	P 1AU	P 2	P 3	P 3AU	P 4	P 5	P 6 SM	P 6 D1	P 6 D2	P 6 D3
N1	90%										X	
	95%						X					
	99%	X		X	X			X	X	X		
N2	90%											
	95%								X		X	
	99%	X		X	X		X	X		X		
N3	90%											X
	95%											
N4	90%											
	95%								X			X
	99%	X		X	X		X	X		X	X	
N5	90%											
	95%											
	99%	X		X	X		X	X	X	X	X	X

Assessment of repeatability

Results showed that laboratories obtained a good repeatability for protocols P5, P2, P3 and P1. The repeatability was generally good at the limit of detection with or without enrichment. The repeatability is better by using real time PCR for assessment.
(Table 3.2.1.7, Table 3.2.1.8 and Table 3.2.1.9).

The accuracy was improved and obtained till level N2 for P5 (with 99% of probability of accordance between reference results and results obtained), P3 (95%) and P1 (90%). Results for P2 and P4 were not accurate.

Table 3.2.1.7.: Assessment of repeatability without enrichment by using conventional PCR.

Without enrichment	N0	N1	N2	N3	N4	N5
protocol 1-FR	99%	99%	90%	99%	95%	**99%**
protocol 1-AU	99%	<90%	<90%	<90%	<90%	<90%
protocol 2-FR	99%	99%	95%	95%	**99%**	99%
protocol 3-FR	99%	99%	99%	**95%**	**90%**	99%
protocol 3-AU	99%	99%	99%	95%	90%	**90%**
protocol 4-FR	99%	99%	99%	99%	99%	**90%**
protocole 5-FR	95%	99%	<90%	**99%**	99%	99%
protocol 6 SM-FR	99%	99%	99%	99%	99%	99%
protocol 6 1/10-FR	99%	99%	99%	99%	99%	99%
protocol 6 1/100-FR	99%	99%	99%	99%	99%	<90%
protocol 6 1/1000-FR	99%	99%	99%	99%	95%	<90%

Table 3.2.1.8.: Assessment of repeatability with enrichment by using conventional PCR.

With enrichment	N0	N1	N2	N3	N4	N5
protocol 1-FR	99%	**99%**	99%	99%	99%	99%
protocol 1-AU	95%	99%	<90%	90%	**95%**	99%
protocol 2-FR	95%	**99%**	99%	99%	99%	99%
protocol 3-FR	99%	**90%**	99%	99%	99%	99%
protocol 3-AU	99%	99%	99%	**95%**	99%	99%
protocol 4-FR	99%	**99%**	99%	99%	99%	99%
protocole 5-FR	99%	**99%**	99%	99%	99%	99%
protocol 6 SM-FR	95%	**99%**	95%	99%	95%	99%
protocol 6 1/10-FR	90%	**99%**	99%	99%	99%	99%
protocol 6 1/100-FR	99%	90%	95%	99%	99%	99%
protocol 6 1/1000-FR	99%	90%	99%	90%	99%	99%

Table 3.2.1.9.: Assessment of repeatability without enrichment by using Real time PCR.

Without enrichment Real time PCR	N0	N1	N2	N3	N4	N5
protocol 1	90%	95%	90%	**99%**	**99%**	**99%**
protocol 2	99%	95%	95%	**99%**	**99%**	**99%**
protocol 3	99%	99%	99%	**95%**	**90%**	**99%**
protocol 4	99%	99%	99%	99%	99%	**90%**
protocol 5	95%	99%	<90%	**99%**	99%	99%

Assessment of accuracy

With enrichment, some results obtained for N0 were not accurate because of false positive (see explanation above). For all level of contamination and with enrichment, samples were detected positive with all protocols–FR. For protocols-AU and protocol P6 with dilutions 1/100 and 1/1000, accuracy was not improved with enrichment (Table 3.2.1.10.).

Table 3.2.1.10.: Assessment of accuracy with enrichment by using conventional PCR.

With enrichment	N0	N1	N2	N3	N4	N5
protocol 1-FR	No accurate	Accurate 99%	Accurate 99%	Accurate 99%	Accurate 99%	Accurate 99%
protocol 1-AU	Accurate 99%	No accurate	No accurate	No accurate	No accurate	Accurate 99%
protocol 2-FR	No accurate	Accurate 99%	Accurate 99%	Accurate 99%	Accurate 99%	Accurate 99%
protocol 3-FR	Accurate 99%	Accurate 95%	Accurate 99%	Accurate 99%	Accurate 99%	Accurate 99%
protocol 3-AU	Accurate 99%	No accurate	No accurate	No accurate	Accurate 90%	Accurate 99%
protocol 4-FR	Accurate 99%	Accurate 90%	Accurate 95%	Accurate 99%	Accurate 99%	Accurate 99%
protocole 5-FR	No accurate	Accurate 99%	Accurate 99%	Accurate 99%	Accurate 99%	Accurate 99%
protocol 6 SM-FR	Accurate 90%	Accurate 95%	Accurate 95%	Accurate 99%	Accurate 95%	Accurate 99%
protocol 6 1/10-FR	Accurate 95%	Accurate 95%	Accurate 99%	Accurate 99%	Accurate 99%	Accurate 99%
protocol 6 1/100-FR	Accurate 95%	No accurate	No accurate	Accurate 99%	Accurate 99%	Accurate 99%
protocol 6 1/1000-FR	Accurate 99%	No accurate	No accurate	No accurate	Juste 90%	Accurate 99%

The accuracy was improved by using Real time PCR and obtained till level N2 for P5 (with 99% of probability of accordance between reference results and results obtained), P3 (95%) and P1 (90%). Results for P2 and P4 were not accurate (Table 3.2.1.11)

Table 3.2.1.11.: Assessment of accuracy without enrichment by using real time PCR.

Without enrichment Real Time PCR	N0	N1	N2	N3	N4	N5
protocol 1	No accurate	No accurate	Accurate 90%	Accurate 99%	Accurate 99%	Accurate 99%
protocol 2	No accurate	No accurate	No accurate	No accurate	No accurate	No accurate
protocol 3	No accurate	No accurate	Juste 95%	Accurate 99%	Accurate 99%	Accurate 99%
protocol 4	Accurate 99%	No accurate	No accurate	No accurate	No accurate	Accurate 90%
protocole 5	No accurate	No accurate	Accurate 99%	Accurate 99%	Accurate 99%	Accurate 99%

Additional to the statistical analysis of the data a breakdown of the advantages and disadvantages of the different DNA extraction methods for the practical application in a laboratory was carried out (Table 3.2.1.12.).

Table 3.2.1.12.: .Application profile of different DNA extraction methods for the practical handling in a laboratory.

Extraction method	Relative cost	Processing time (h:mm)[a]		Sample volume (µL)	Volume recovered (µL)	Additional reagents/ equipment (not supplied)	Equipment	Experience required	Auto-mation possible
		min	max						
Kit Ultra Clean TM 15-MO BIOMO BIO	high	01:00	02:30	1000	50	Ultra clean Tm1 Kit MO BIO	heat block centrifuge	medium (pelleting and final pellet drying)	no
Kit Mycrosynth	low	00:30	01:00	200	500	Kit Mycrosinth		low	yes
Easy-DNA kit Invitrogen	high	02:00	02:30	100	100	Kit Easy DNA kit	heat block centrifuge	medium (pelleting and final pellet drying)	no
RedeExtract -N-Amp ™ Plant PCR kit	medium	00:35	01:30	100	100 but 1/5 or 1/150	Kit REDE Extract	heat block	low	yes
QuickPick				100	100[b]		heat block centrifuge apparatus for magnetic beads collection (pen or more high-throughput machine)	low	yes with special aparatus
Simple isopropanol	medium	02:30	03:00	1000	100		heat block centrifuge	medium (pelleting and final pellet drying)	no
Taylor	low	00:20	01:15	200	700			low	yes
DNeasy Plant Mini Kit	high			100	100	Kit Dneasy Plant	heat block centrifuge	low	yes vacuum pump needed

a The minimum processing time reflects extraction of single samples, whereas the maximum times reflect extractions of 12 samples each
b Low indicating there are no critical steps, medium several critical points, high indicates a procedure for which people need training and experience to perform it well. Indicate critical step.
c only part of lyzate goes trough the whole procedure

In conclusion the results of the preliminary study permitted to assume that protocols P5 (DNA extraction Taylor according Taylor et al. 2001), P3 (RedeExtract-N-Amp ™ Plant PCR kit according Stöger et al. 2006) and P1 (Isopropanol according Llop et al, 1999) could be assessed further through a ring test (see Task 4.3.1.).

Task 3.2.2 Testing conventional PCR

A different set of newly developed primers (e.g. target other than pEA29) for the detection of *E. amylovora* in asymptomatic plants were compared with commonly used primers.

The sensitivity of different conventional PCR assays (Bereswill 1992; Llop 2000; Stöger 2006; Obradovic 2007; Obradovic modificated by Gottsberger; Taylor 2001) was analysed with different concentrations of the Austrian reference strain 295/93 (=CFBP 6449) by partner 1.

Table 3.2.2.1: Sensitivity of different conventional PCR assays.

	E.amylovora [cfu/µL]	PCR according Bereswill 1992	PCR according Llop 2000	PCR according Stöger 2006	PCR according Obradovic 2007	Obradovic - modificated by Gottsberger	PCR according Taylor-Guilford 2001
Standard 1	200 000	+	+	+	+	+	+
Standard 2	20 000	+	+	+	+	+	+
Standard 3	2 000	+	+	+	+	+	+
Standard 4	200	+	+	+	+	+	+
Standard 5	20		+	+		+	+
Standard 5	20		+	+		+	+
Standard 5	20		+	+	+	+	+
Standard 6	2					+	+
Standard 6	2			+		+	+
Standard 6	2			+	+	+	+
Standard 7	0,2			+			+
Standard 7	0,2			+			+
Standard 7	0,2			+			

The preliminary assay showed that three PCR methods are suitable for detection of low *E. amylovora* concentration; Stöger 2006, Obradovic 2007 modificated by Gottsberger and Taylor and Guilford 2001 (Table 3.2.2.1)

Three PCR methods (Stöger 2006, Obradovic 2007 modificated by Gottsberger and Taylor and Guilford 2001) were selected for further ring testing (see Task 4.1.3.)

Task 3.2.3. Testing real time PCR

The objective of the task was to test real time PCR to allow fast, specific detection of low concentrations of *E. amylovora*. In the European diagnostic protocol (EPPO, 2004), enrichment of *E. amylovora* in liquid CCT and King's B media is suggested prior to further analysis for selected symptomatic material (i.e. advanced necrosis, or samples treated with copper or antibiotics), and for latent testing, due to low numbers of bacteria in these samples and possible VBNC state (EPPO, 2004; Ordax et al., 2006).

Real time PCR was used for detection of *E. amylovora* in enriched plant samples and for direct detection of low concentrations of *E. amylovora* in asymptomatic flower samples.

The ability of liquid CCT and King's B media to support the growth of *E. amylovora* in the presence of other organisms and plant material was determined by real time PCR Ams, novel

real time PCR assay developed by Partner 4 (Pirc et al., 2009). The strength of the assay is not only its highs sensitivity and reliability, but also its ability to quantify *E. amylovora* before and after enrichment.

Fig: Sensitivity and false negative rates of isolation on media CCT from enriched asymptomatic samples with low starting concentration of *E. amylovora* compared to real time PCR (Ams assay), initial concentrations at LOD level ($\approx 10^3$ cells/ml). Ams real time PCR was used for quantification as the target is likely to be present in one copy compared to several (and variable) target copies in case of ITS real time PCR and plasmid pEA29. Samples were analyzed directly, without DNA extraction step (Pirc et al., 2008).

		real-time PCR (Ams assay)		
		KB enrichment	CCT enrichment	KB & CCT enrichment
		Ea pos	Ea pos	Ea pos
isolation on media (CCT)	Ea pos	**15**	**10**	**19**
	Ea neg	**20**	**25**	**16**
Sensitivity		*0.43*	*0.29*	*0.54*
False neg. rate		*0.57*	*0.71*	*0.46*

From initial concentrations at LOD level ($\approx 10^3$ cells/ml) *E. amylovora* cells multiplied in all symptomless samples to a variable degree, with final concentrations ranging from 10^5 to 10^9 cells/ml. However, isolation of *E. amylovora* from enriched samples proved difficult. Despite using five 10-fold dilutions for plating to avoid problems with overgrowth by other bacteria (an increase from three 10-fold dilutions suggested by EPPO, 2004), target bacteria could be isolated in pure culture mainly from samples with at least 10^7 *E. amylovora* cells/mL of enriched extract. False negative rate was high, from 0.46 when extracts were enriched in both King's B and selective CCT media, to 0.71 when only CCT enrichment was used. Results suggest that real time PCR is a reliable, fast and sensitive method of choice for detection of low concentrations of *E. amylovora*.

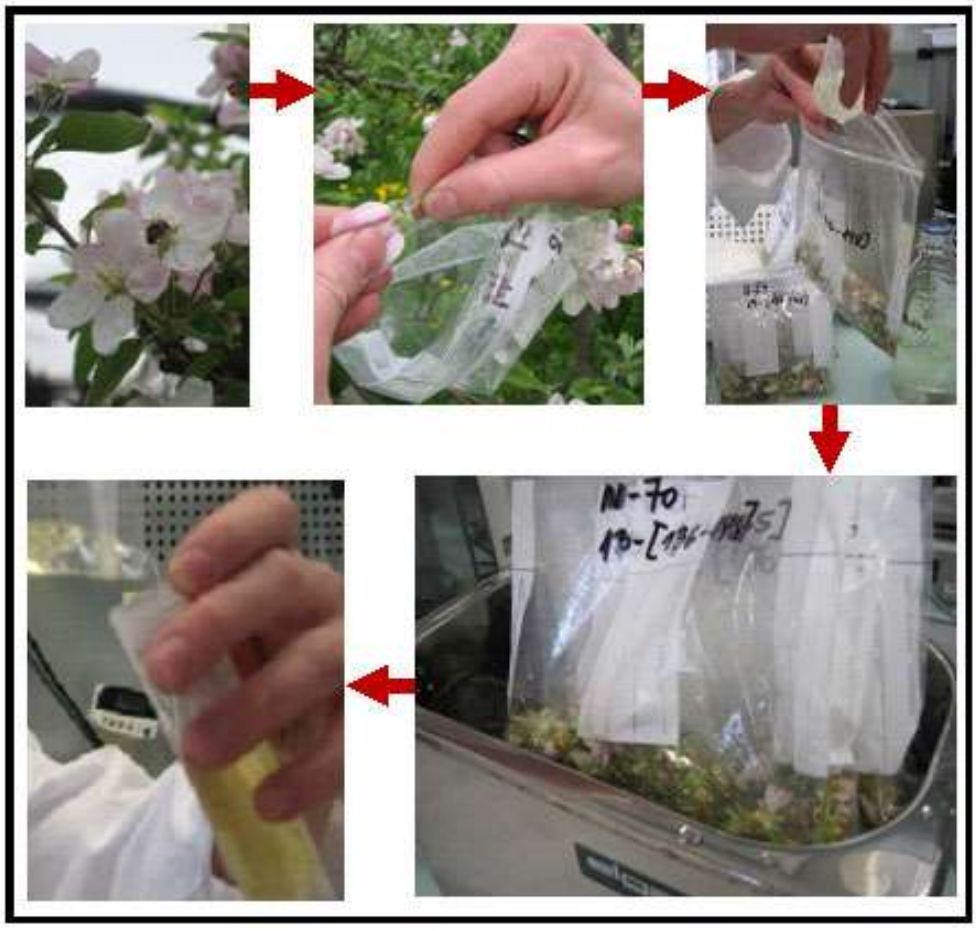
The Ams assay and serological kit Ea AgriStrip have been further tested for detecting *E. amylovora* in asymptomatic flowers for the first time in this project in collaboration of Partners 3 and 4. *E. amylovora* arrives on flower stigmas (via water splash, vectors such as honeybees) and then grows asymptomatically before entering the flower tissues and beginning infection. Population growth, and risk of infection, is correlated with environmental conditions (e.g., temperature, moisture). Forecasting using models (e.g., MaryBlyt) that process environmental conditions have been developed to predict fire blight infection risk in orchards. Models are an important tool for disease management, with application of antibiotics, biocontrol products, and sanitation resources timed based on forecasting predictions. However, currently there are no simple methods to determine pathogen presence in orchards during the flowering period. Thus this key variable used in forecasting, and therefore farmer decision-making, is just assumed. Effective methods to accurately determine pathogen population presence in flowers, flower monitoring, would be a major advance in fire blight forecasting, and would result in more judicious application of control measures – reducing farmer costs, and environmental risks with unnecessary antibiotic applications.

In 2008/2009 apple orchards were sampled during the flowering period (Fig. 3.1.1). Results from intensive sampling of 4 commercial apple orchards showed that real time PCR was considerably more sensitive than Ea AgriStrip but *E. amylovora* could be detected in several

samples using both methods. Further optimization of the sampling protocols is needed before this monitoring method can be effectively implemented.

The results demonstrate that flower monitoring of asymptomatic pathogen populations is possible. This has potentially significant importance for reducing unnecessary application of antibiotic and expensive alternate control products, which will benefit fruit growers (cost savings) and minimize potential public health/environmental risks.

Figure 3.1.1: Flower monitoring procedure involves collection of 2-3 day old open flowers, washing with buffer in a plastic bag, sonicating to remove bacteria from stigmas, and then decanting the sample supernatant. Centrifugation prior to analysis increases detection power.



Conclusions

The techniques evaluated in the above indicated tasks of the Work package 3 allowed the partners to select the techniques to be validated in a ring test described in Work package 4, because due to the time constraints it was considered most convenient to perform only one ring test for asymptomatic and symptomatic plant material

Implications for stakeholders

Interest groups / Fruit producers

The accurate determination of pathogen presence in flowers could be an important tool for the prediction of fire blight infection risk. The development of methods which are suitable to detect a low level of *E. amylovora* in the field during the flowering period are an improvement of fire blight forecasting. The determination of the presence of the pathogen in the field could therefore increase the reliability of risk announcements and as a result reducing farmer costs and environmental risks with unnecessary antibiotic applications.

Work package 4, Ring-testing trials to validate new test methods for application for the detection of *E. amylovora* in symptomatic plants on-site or in laboratories

Work package Objectives

The objective of the work package 4 was to evaluate suitability of novel methods, specifically serological kits and real time PCR assays for detection of *E. amylovora* in symptomatic plants on-site or/and in laboratories. All objectives were successfully achieved.

Participants

WP coordinator: partner 4. Other participants: partners 1, 2, 3, 4, 6

WP4 Methods and Results

Since publication of EPPO Standard PM 7/20 in 2004 (EPPO Bulletin 2004) detailing diagnostic methods, a combination of which gives desired level of diagnostic accuracy, two trends can be discerned: improvements made in simple techniques that can be confidently performed without specific training on-site (pregnancy test-like kits) and development of sophisticated molecular biology based techniques that provide increased specificity or sensitivity such as PCR (ref) and real time PCR (ref).

The objective of this work package was to test two methods representative of these developments, serological quick test developed by Partner 3 in collaboration with industrial partner and real time PCR method developed by Partner 4 (Pirc et al., 2009). Both methods were extensively evaluated and tested in the final ring-test.

Task 4.1. Testing the analytical specificity and sensitivity of newly available methods for the detection of *E. amylovora* in symptomatic plant on-site and in laboratories

Task 4.1.1 Testing real time PCR

The objective of this task was to determine analytical specificity and sensitivity of real time PCR assays.

To determine specificity of newly developed real time PCR assays targeting Ams gene and ITS region (Pirc et al., 2009) in comparison to existing real time PCR assay (Salm & Geider, 2004), relevant bacterial strains were collected among project partners to represent both diversity of *E. amylovora* and other bacteria present in the same plant material. Trough testing of 187 *E. amylovora* strains and 23 other bacteria, in addition to published results all three real time PCR assays were found to be specific for detection of *E. amylovora*, and results were in accordance with the characteristics of the target regions (Fig. 4.1.1.1).

Ams assay proved to be the most accurate in identification of *E. amylovora* compared to ITS and plasmid assays (Fig 4.1.1.1), displaying high sensitivity and specificity and no known false positive or negative reactions, detectiong all tested *E. amylovora* strains irrespective of their plasmid profile. This improves reliability of diagnosis by also detecting strains that do not posses pEA29, a common target of current PCR based diagnostic assays (e.i. Llop et al., 2000; Salm & Geider, 2004).

The ITS assay detects strains with the 139 bp sequence on rRNA operons that seems to be lacking from Rubus strains as reported previously (McGhee *et al.*, 2002) but is present in *Erwinia* spp. strains from Japan (Hokkaido), most likely reflecting their taxonomic proximity to *E. amylovora*. The ITS assay thus has a potential to be used as a broad-range test detecting both *E. amylovora* and *Erwinia* spp. causing blight of pears in Asia.

pEA29 real-time PCR		sanitary status		
		disease	no disease	total
Result of technique	pos	right **340**	false positive **0**	340
	neg	false negative **2**	right **94**	96
	total	342	94	436
prevalence		*0.78*		
Sensitivity		*0.99*		
Specificity		*1.00*		
false positive rate		*0.00*		
false negative rate		*0.01*		

Ams real-time PCR		sanitary status		
		disease	no disease	total
Result of technique	pos	right **421**	false positive **0**	421
	neg	false negative **0**	right **91**	91
	total	421	91	512
prevalence		*0.82*		
Sensitivity		*1.00*		
Specificity		*1.00*		
false positive rate		*0.00*		
false negative rate		*0.00*		

ITS real-time PCR		sanitary status		
		disease	no disease	total
Result of technique	pos	right **371**	false positive **3**	374
	neg	false negative **1**	right **91**	92
	total	372	94	466
prevalence		*0.80*		
Sensitivity		*1.00*		
Specificity		*0.97*		
false positive rate		*0.03*		
false negative rate		*0.00*		

Fig. 4.1.1.1: Contingency data for real time PCR as identification test. Data is based on Pirc et al. (2009) and results of this project and includes *E. amylovora* strains from different hosts and geographic origin and other strains (*Dickeya* spp., *Enterobacter pyrinus, Erwinia billingiae, Erwinia pyrifoliae, Erwinia tasmaniensis, Erwinia sp.* isolated from necrotic blossoms in *Spain, Erwinia spp.* isolated from pears in Hokkaido*, Pseudomonas syringae, Pseudomonas spp.* positive or negative in hypersensitive reaction on tobacco, biocontrol strains and environmental isolates of *Pantoea agglomerans,* 24 unidentified strains isolated from necrotic plant tissues that tested negative on *E. amylovora)* and 14 DNA isolated from non-infected plant material.

Sensitivity of real time PCR Ams assay was determined in combination with several DNA extraction methods ranging in their complexity in collaboration of Partners 1, 4 and 6 (see Annex 1 for details on extraction methods).

Several DNA extractions allowed reliable detection of low concentrations of *E. amylovora*: REDExtract-N-AmpT Plant PCR Kit (Sigma; Stöger, 2006), QuickPick™ SML Plant DNA Kit (Bio-Nobile; Pirc et al., 2009), isopropanol protocol (Llop et al., 1999), modified Taylor protocol and EasyDNA (Invitrogen). Some differences were seen at concentrations below 10^3 cfu/mL however, this differences may be due to stochastic effect and may not reflect characteristics of DNA extraction methods (Fig. 4.1.1.2).

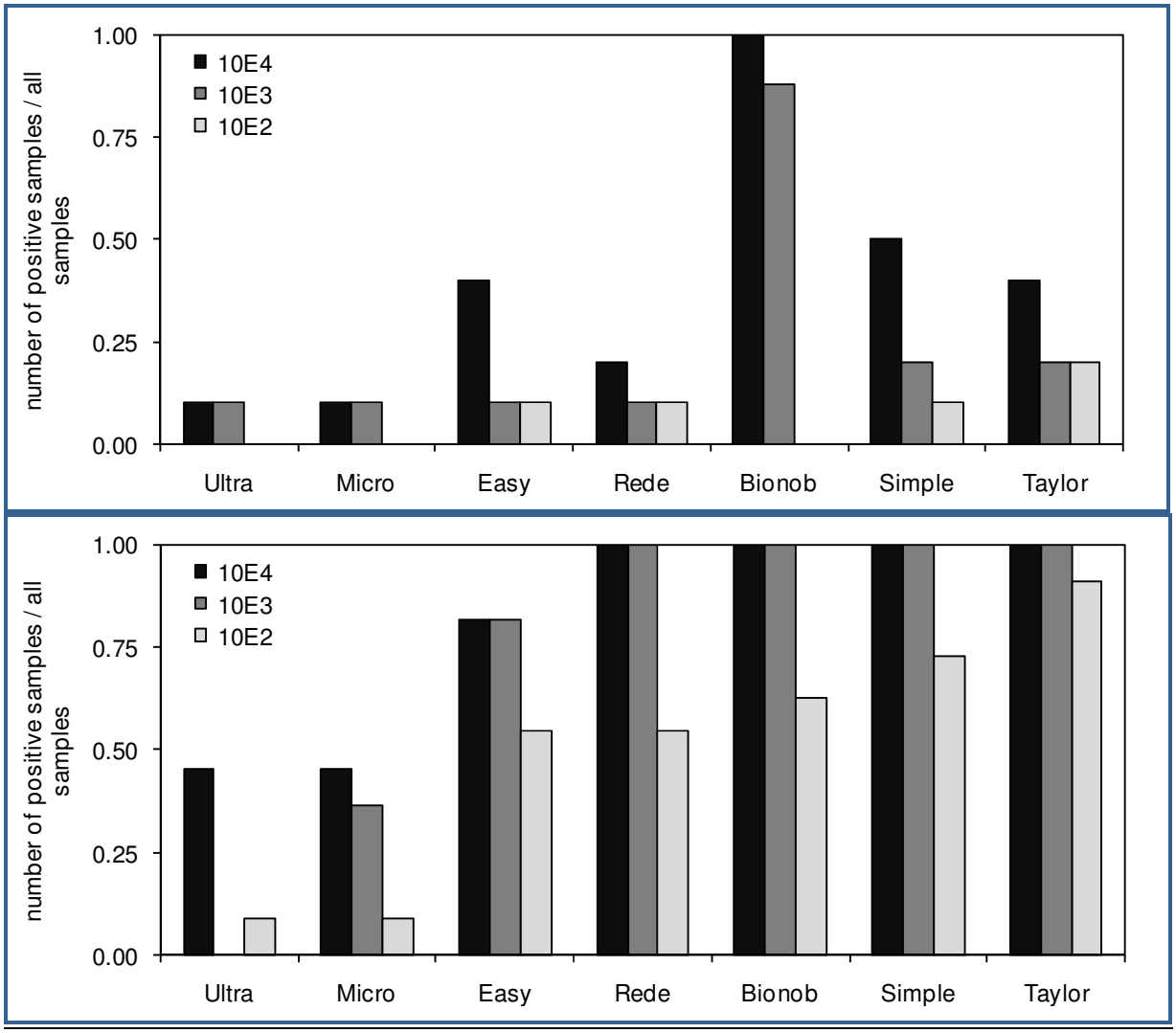


Fig. 4.1.1.2: Ratio of samples with positive result in nested PCR (top) and real time PCR Ams assay (bottom) depends on target concentration and DNA extraction method. DNA extraction methods are described in Annex 1: Ultra = ultra clean ™15 - MO BIO (Manceau, 2006); Micro = Microsynth, Easy = Easy DNA-Extraction Kit (Invitrogen; Pastrik, 2000); Rede = REDExtract-N-AmpT Plant PCR Kit (Sigma; Stöger, 2006); Bionob = QuickPick™ SML Plant DNA Kit (Bio-Nobile; Pirc et al., 2009), simple = isopropanol protocol (Llop et al., 1999), Taylor = modified Taylor protocol. Data for Bionob extraction is based on one DNA extraction and minimum 3 real time PCR reactions per each of 8 samples (apple, pear, Cotoneaster, quince, medlar, service, hawthorn, firethorn); data for all other DNA extractions is based on two DNA extractions analyzed in one real time PCR reaction from each of the 11 samples from a different set of samples (hawthorn, quince (young and old plant material), Cotoneaster, two pear samples, two apple sample, medlar, pyracantha).

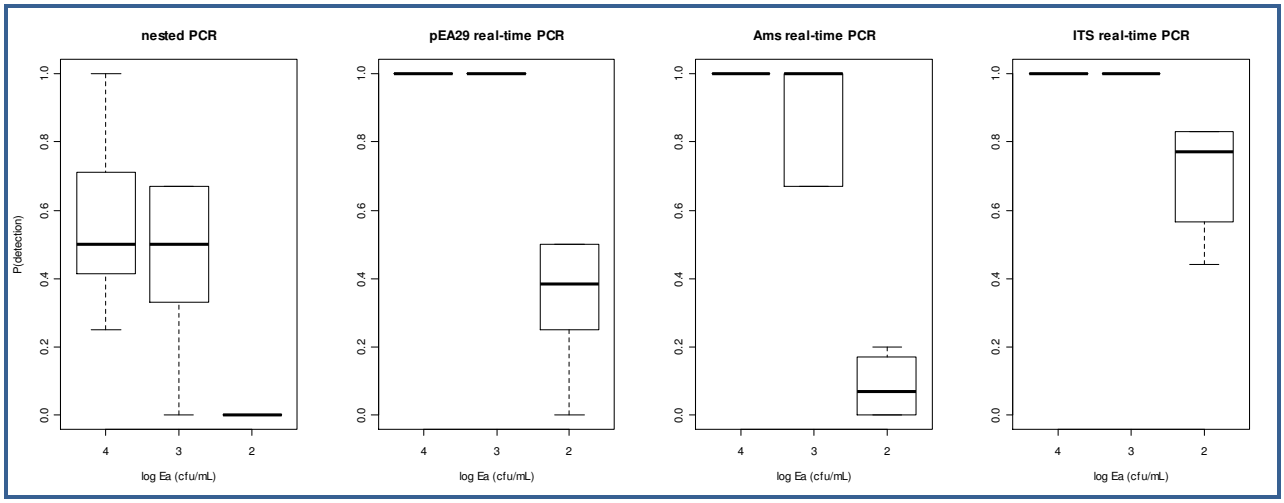


Fig. 4.1.1.3 : Boxplot of detection probability with nested or real time PCR from various host plants (pear, apple, Cotoneaster, quince, medlar, service tree, hawthorn, firethorn). DNA was extracted from 100 uL using magnetic beads based DNA extraction kit (Bionobile Plant Kit).

Real time PCR (Ams assay) was compared to nested PCR (Llop et al., 2000) which is highly sensitive and part of a recommended protocol of EPPO (EPPO, 2004). When starting with the same amount of material, all real time PCR showed higher sensitivity (Fig. 4.1.1.3) with the additional benefit of a much easier interpretation of the results in samples with low concentrations of target bacteria. In nested PCR, especially with lower concentrations, often weak bands were difficult to interpret and the repeatability of parallel reactions was low. At the current state of technology real time PCR methods are also more ammenable to high-throughput testing.

Real time PCR assay was adapted to a faster PCR protocol that can be run on a portable thermocycler apparatus. This allows for real time PCR to be used outside of the laboratory environment (Fig. 4.1.1.4)

Fig. 4.1.1.4: SmartCycler (Cepheid), a portable thermocycler allowing the use of real time PCR in the field, at ports, other entry points and other points of interest. Using a modified real time PCR Ams assay, results can be obtained in less than 40 minutes with sensitivity comparable to laboratory conditions.

Real time PCR proved to be a sensitive and specific method for *E. amylovora* detection.

Task 4.1.2 Testing serological kits

The objective of this task was to determine analytical specificity and sensitivity of serological kits for detection of *E. amylovora*.

A new serological test, Ea AgriStrip, was developed and validated during this project in collaboration of Partner 3 with a small-medium size industry partner (Fig. 4.1.2.1). This serological test is simple to use in the field and delivers rapid results within 15 minutes (Fig. 4.1.2.2).

Figure 4.1.2.1: A new fire blight diagnostic test based on lateral-flow immunography is now commercially available from BIOREBA AG, Switzerland.

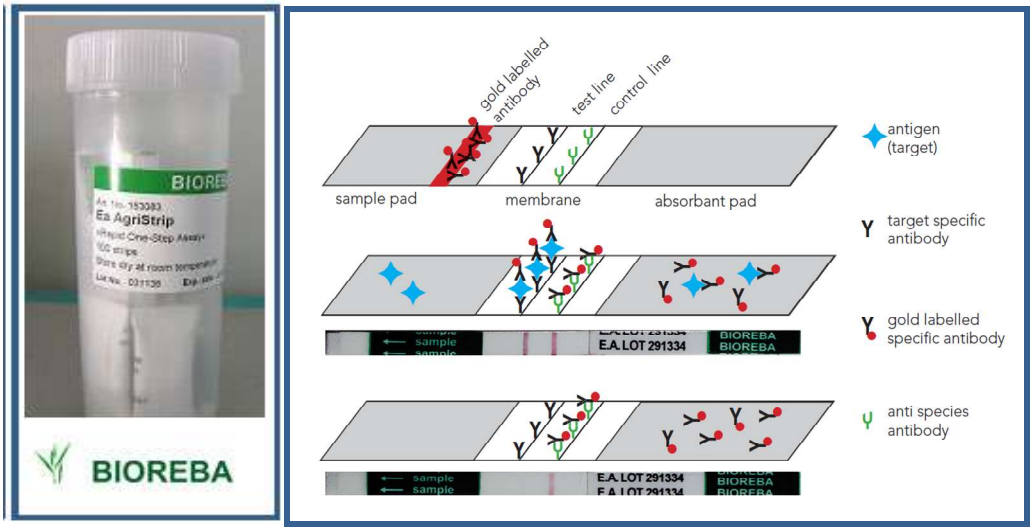


Figure 4.1.2.2: Plant sampling directly in the orchard. Suspicious plant material such as latent cankers (shown) are placed in an extraction bag with buffer and macerated. A few drops (2-3 drops correspond to approximately 100 µl) of suspension is placed in a cuvette or other support vessel and then an Ea AgriStrip is placed with just the lower end immersed in sample suspension. After 15 minutes, results can be read (2 pink lines develop in positive samples, only 1, upper line, develops in negative samples) directly in the field without any need to send samples to a laboratory.

Ea AgriStrip was evaluated for sensitivity and specificity, and validated for diagnostic reliability in the laboratory and in the field. Using plant samples spiked with different concentrations of *E. amylovora,* the test was found to have a reliable detection limit of 10^5 cfu per ml of extracted plant sample making it suitable for detection of *E. amylovora* in symptomatic plant material. A wide-range of related bacteria and species commonly co-isolated from fire blight diagnostic samples was used to determine specificity. Only the closely-related fire blight species, *Erwinia pyrifoliae*, gave a cross-reaction. These characteristics make the test comparable to another commercially available serological kit, Pocket Diagnostics (Forsite Diagnostics, UK).

The test was validated for identification of isolates on semi-selective media in the diagnostic laboratory and directly from symptomatic plant samples submitted by phytosanitary inspectors. Comparison of Ea AgriStrip with standard plating on semi-selective agar media and nested-PCR methods was performed in parallel with 201 plant samples from various fire blight host species submitted by plant inspectors to a diagnostic laboratory. Ea AgriStrip delivered more reliable diagnosis compared to plating, and slightly lower detection compared to nested-PCR (Fig. 4.1.2.3). However, the serological test required considerably less training to use compared to PCR and delivered faster diagnosis compared to plating or PCR.

A small-scale comparison of Ea AgriStrip performance in combination with various extraction buffers was done by IVIA: Results indicate that various buffers may be used without affecting the sensitivity of the Ea AgriStrip test. However, this should be checked and confirmed for individual buffers. Addition of a detergent such as Tween20 is necessary for satisfactory chromatography.

Figure 4.1.2.3: Comparison of serological, plating and PCR methods in parallel on naturally infected plant samples.

	Plant samples positive / total samples		
Host plant	**Ea AgriStrip**	**Plating**	**Nested-PCR**
Apple	95 / 141	79 / 141	98 / 141
Pear	28 / 49	18 / 49	36 / 49
Quince	3 / 3	3 / 3	3 / 3
Other hosts*	5 / 8	5 / 8	5 / 8
All hosts	131 / 201	105 / 201	142 / 201
Time required for diagnosis	10-15 Minutes	2-3 Days	5 Hours

*Other hosts sampled include *Crataegus*, *Sorbus*, *Cotoneaster*, *Salicifolius* spp.

Partner 3 organized two training courses and ring-trials with plant inspection officers in Switzerland (Fig. 4.1.2.4). Inspectors collected suspect plant samples and processed these directly in the field. Inspector feedback indicated a very high acceptance of sampling kits, simplicity of the test, and most importantly enhanced power of plant inspectors to provide extension information to farmers and rapidly implement fire blight control measures. One important application of the serological test was detection of latent cankers in orchards (Gersbach et al., 2009). Inspectors identified this as facilitating earlier sanitation of infected orchards while trees were still dormant. Thus reservoirs of primary inoculums could be removed before the spring growing season started.

Figure 4.1.2.4: A training course to use the new fire blight diagnostic test strip Ea AgriStrip was organized for plant inspectors including laboratory and theoretical modules.

Task 4.1.3 Ring test evaluation of the selected optimized techniques

The ring test of the selected techniques with the participation of the partners of the project was organized after a previous selection by the different partners of the methods to evaluate. The consensus protocols were designed with the input of partners 1, 2, 3, 4 and 6. The selected techniques were those previously evaluated by partners 1, 4 and 6. The samples were prepared by partner 2 and sent to the four other partners (1, 3, 4 and 6). They were five healthy blind samples (samples 6, 7, 8, 9 and 10) and pear shoots to prepare five spiked samples (samples 1, 2, 3, 4 and 5 with respectively 10, 10^2, 10^3, 10^5 and 10^6 cfu of *E. amylovora* per ml of extract). A positive *E. amylovora* control was also sent to all of them, as well as two serological kits, buffers, primers and other necessary reagents. The use of the same mix for PCR was also advised. The detailed protocol send to all the partners is in the Annex 2. Details about the real time PCR ring test are given in task 4.3.2.

The aim of this ring test was to check reliability of the selected assays for detection of a range of concentrations of *Erwinia amylovora.* Classical PCR tests were done in combination with several DNA extractions. Additionally, real time PCR was tested on both plant material and DNA to allow direct comparison of real time PCR results without the influence of DNA extraction and balancing the use of different real time PCR instruments.

The following tests were selected for ring-testing:

- Isolation on King's B media
- Serological quick test Ea AgriStrip (Bioreba)
- Serological quick-test Pocket Diagnostic Kit for *Erwinia amylovora*
- Isopropanol DNA extraction with nested PCR (Llop et al., 2000)
- Modified Taylor DNA extraction with nested PCR (Llop et al., 2000)
- Modified Taylor DNA extraction with Taylor PCR (Taylor et al., 2001)
- Modified Taylor DNA extraction with Obradović PCR (Obradović et al., 2007)
- Redextract DNA extraction with Stöger PCR (Stöger et al., 2006)

Modified Taylor DNA extraction with real time PCR Ams assay (Pirc et al., 2009)Real time PCR Ams assay (Pirc et al., 2009) tested on DNA (extracted using QuickPick Bionobile DNA extraction)The five project partners have taken part in the ring-test, performing all or selected methods.

Preliminary assays to select plant samples for the ring test

Before organizing the ring test, preliminary experiments were performed by partner 2 to select the most appropriate samples and reagents. Different samples from two pear cultivars (Blanquilla and Conference), apple and loquat from the IVIA greenhouses were evaluated with the different PCR protocols and DNA extraction methods to verify their suitability for the ring test purposes. One assay was performed with Blanquilla pear and the other hosts (Table 4.1.3.1) and three repetitions with pear Conference (Table 4.1.3.2), with more or less similar sensitivity results for the protocols of PCR assayed, and in any case all the negative samples were positive. Then the selected samples were considered appropriate for being prepared for the ring test.

Table 4.1.3.1.: Spiked pear samples cultivar Blanquilla, apple and loquat: evaluation of the four PCR protocols, with several *E. amylovora* concentrations and three DNA extraction methods

PCR protocols	DNA extraction	cfu/ml extract						
		10^5	**10^4**	**10^3**	**10^2**	**10**	**1**	**0**
		Spiked samples with positive amplification/Nº of analysed samples*						
PCR Llop	Isop.	1/1	1/1	1/1	0/1	0/1	0/1	0/1
	Taylor	1/1	1/1	1/1	1/1	0/1	0/1	0/1
PCR Stöger	RedEx	1/1	1/1	0/1	0/1	0/1	0/1	0/1
PCR Taylor	Taylor	1/1	1/1	0/1	0/1	0/1	0/1	0/1

* One pear sample, one apple sample, one loquat sample.

Table 4.1.3.2.: Spiked pear samples pear cultivar Conference: evaluation of the four PCR protocols, with several *E. amylovora* concentrations and three DNA extraction methods

PCR protocol	DNA extraction	cfu/ml pear extract							
		10^6	10^5	10^4	10^3	10^2	10	1	0
		Spiked samples with positive amplification/Nº of analysed samples*							
PCR Llop	Isop.	3/3	3/3	3/3	2/3	0/3	0/3	0/3	0/3
	Taylor	3/3	3/3	3/3	1/3	0/3	0/3	0/3	0/3
PCR Stöger	RedEx	3/3	3/3	3/3	1/3	0/3	0/3	0/3	0/3
PCR Taylor	Isop.	3/3	3/3	3/3	3/3	0/3	0/3	0/3	0/3
	RedEx	3/3	3/3	3/3	2/3	0/3	0/3	0/3	0/3
	Taylor	3/3	3/3	3/3	1/3	0/3	0/3	0/3	0/3
PCR Obradovic	Isop.	2/3	2/3	2/3	0/3	0/3	0/3	0/3	0/3
	RedEx	2/3	2/3	2/3	0/3	0/3	0/3	0/3	0/3
	Taylor	2/3	2/3	2/3	1/3	0/3	0/3	0/3	0/3

* Three replications with samples of cv. Conference.

The selected detection methods were also compared in artificially inoculated pear shoots prior to the ring test to evaluate their sensitivity and to compare different DNA extraction methods and PCR protocols.

Table 4.1.3.3.: Comparison of different methods for diagnostic on inoculated pear samples.

	ISOLATION Result (+ or -)			LLOP PCR			TAYLOR PCR		
	KB	NAS	CCT	Extraction TAYLOR	Extraction REDEX	Extraction ISOP	Extraction TAYLOR	Extraction REDEX	Extraction SOP
Stem	+	+	+	+	+	+	+	+	+
Leaf	+	+	+	+	+	+	+	+	+
Stalk	+	+	+	+	+	+	+	+	+
Not in-oculated	-	-	-	-	-	-	-	-	-

The results of this small experiment showed that for inoculated pear samples, all the methods selected for analysis of asymptomatic samples were also working with those showing symptoms. Then, due to time constraints and difficulties in obtaining naturally infected or inoculated samples with similar *E. amylovora* populations, only spiked samples were prepared for the final ring test. We consider that samples with 10^6 and 10^5 cfu/ml extract (samples 5 and 4) can be considered as having similar *E. amylovora* number than those of naturally infected samples

Preliminary experiments were also performed to evaluate the possibility of using the same buffer for the serological kits and for the other techniques as indicated in the Table 4.1.3.3 After some unexpected results the use of the kit buffer was advised in the ring test.

Table 4.1.3.4.: Spiked pear samples: Evaluation of the serological lateral flow device tests and the isolation on Kings B agar with respective confirmation of the suspected colonies. Number of Labs (x) with positive result compared to total results (y) (x/y). Test procedures marked in red = not reliable

		S5	S4	S3	S2	S1	S0		
Test	**Procedure**	**10^6**	**10^5**	**10^3**	**10^2**	**10**	**0**	**+**	**NTC**
Agristrip	Antiox	1/3	1/3	1/3	1/3	1/3	1/3	2/3	0/2
	Kit Buffer	5/5	1/5	0/5	1/5	0/5	0/5	5/5	0/3
Diagpocket	Antiox	3/3	0/3	0/3	1/3	0/3	0/3	3/3	0/2
	Kit Buffer	4/4	1/4	0/4	0/4	0/4	0/4	4/4	0/3
Isolation	Kings B	5/5	5/5	5/5	5/5	3/5	0/5	5/5	0/2

The preliminary assays also included several small experiments to compare different types of mix for the PCR protocols and comparison of Taq polymerase. A final comparison of the mix prepared with reagents from Biotools and the Sigma Readymix is presented in Table 4.3.1.4.

Table 4.3.1.4.: Comparison of cocktails for PCR prepared either with reagents from Biotools or Sigma Readymix in the four evaluated PCR protocols with several *E. amylovora* concentrations (spiked pear samples)

		cfu/ml				
PCR	**Reagents**	**10^6**	**10^5**	**10^4**	**10^3**	**0**
PCR	**Biotools**	+	+	+	+	-
Llop	**Sigma**	+	+	+	+	-
PCR	**Biotools**	+	+	+	w +	-
Taylor	**Sigma**	+	+	+	-	-
PCR	**Biotools**	+	+	+	-	-
Stöger	**Sigma**	+	+	-	-	-
PCR	**Biotools**	-	-	-	-	-
Obradovic	**Sigma**	+	+	+	-	-

It shows that the results were sometimes different between them, showing interactions with the protocols. However, as only one Taq polymerase should be used by all the labs in the ring test, the one from Sigma was selected.

Final preparation of plant samples for the ring test

Plant material of the several selected host plants was collected from the IVIA greenhouses and sent out fresh. The original plant material was tested at IVIA using the methods above indicated in the tables to confirm that they do not contain *E. amylovora*. From these material plant extracts were prepared in the different labs and some of them spiked with a known concentration of *E. amylovora* strain CFBP 1430 and analyzed in ring-test.

Preparation of DNA and material for ring-testing real time PCR

DNA samples were prepared with an apple plant extract that has been previously tested and shown to be negative using isolation on media and real time PCR. Plant extract was mixed with either buffer or *E. amylovora* suspensions, DNA isolated with QuickPick Plant kit (Bionobile, Turku, Finland).

4.3.2 Results of the ring test and analysis of the results

The detailed results obtained in the different labs are shown in annex 3.

For every technique the following parameters are shown:

- Sensitivity = true positives/(true positives + false negatives)
- Specificity = true negatives/(true negatives + false positives)
- Positive Predictive Value = true positives/(true positives + false positives)
- Negative Predictive Value = true negatives/(true negatives + false negatives)
- Accuracy = (true positives + true negatives)/total samples

The following tables summarize the values for true positives/negatives and false positives/negatives, for sensitivity, specificity, positive and negative predictive value and accuracy.

Isolation of *E. amylovora* on King B medium

Table 4.3.2.1.: Isolation: Values for true positives/negatives and false positives/negatives.

	True positives (TP)	True negatives (TN)	False positives (FP)	False negatives (FN)	Total samples/ Number of testers
Isolation	23	30	0	2	55/5

Table 4.3.2.2.: Isolation: Values for sensitivity, specificity, positive and negative predictive value and accuracy.

	Sensitivity	Specificity	Positive predictive value (PPV)	Negative predictive value (NPV)	Accuracy
Isolation	0.92	1	1	0.94	0.96

Serological quick-tests

Table 4.3.2.3.: Serological quick tests: Values for true positives/negatives and false positives/negatives.

	True positives (TP)	True negatives (TN)	False positives (FP)	False negatives (FN)	Total samples/ Number of testers
Pocket Diagnostic with kit buffer	6	23	1	15	44/4
Agristrip with kit buffer	5	30	0	18	55/5

Table 4.3.2.4.: Serological quick tests: Values for sensitivity, specificity, positive and negative predictive value and accuracy.

	Sensitivity	Specificity	Positive predictive value (PPV)	Negative predictive value (NPV)	Accuracy
Pocket Diagnostic with kit buffer	0.29	0.96	0.86	0.61	0.61
Agristrip with kit buffer	0.22	1.0	1.0	0.63	0.64

Conventional PCR combined with different DNA extraction methods

Table 4.3.2.5.: Different PCR assays: Values for true positives/negatives and false positives/negatives.

	True positives (TP)	True negatives (TN)	False positives (FP)	False negatives (FN)	Total samples/ Number of testers
Talyor PCR with extraction Taylor (undiluted)	9	24	0	11	44/4
Talyor PCR with extraction Taylor (1:10)	4	18	0	11	33/3
Obradovic PCR with extraction Talyor(undiluted)	7	18	0	8	33/3
Obradovic PCR with extraction Taylor (1:10)	4	18	0	11	33/3
StögerPCR with extraction Taylor (undiluted)	7	18	0	8	33/3
Stöger PCR with extraction Taylor (1:10)	5	18	0	10	33/3
Llop PCR with extraction Isopropanol (undiluted)	9	13	5	6	33/3
Llop PCR with extraction Isopropanol (1:10)	9	16	1	6	33/3
Llop PCR with extraction Talyor(undiluted)	13	29	1	12	55/5
Llop PCR with extraction Taylor (1:10)	5	18	0	10	33/3

Table 4.3.2.6 shows the same data as Table 4.3.2.3, but with different performance criteria.

Table 4.3.2.6.: Sensitivity, specificity and positive and negative predictive value for different PCR assays combined with different DNA extraction methods.

	Sensitivity	Specificity	Positive predictive value (PPV)	Negative predictive value (NPV)
Talyor PCR with extraction Taylor (undiluted)	0.45	1.0	1.0	0.68
Talyor PCR with extraction Taylor (1:10)	0.27	1.0	1.0	0.62
Obradovic PCR with extraction Talyor(undiluted)	0.47	1.0	1.0	0.69
Obradovic PCR with extraction Taylor (1:10)	0.27	1.0	1.0	0.62
StögerPCR with extraction Taylor (undiluted)	0.47	1.0	1.0	0.69
Stöger PCR with extraction Taylor (1:10)	0.33	1.0	1.0	0.64
Llop PCR with extraction Isopropanol (undiluted)	0.60	0.72	0.64	0.68
Llop PCR with extraction Isopropanol (1:10)	0.60	0.94	0.90	0.73
Llop PCR with extraction Talyor(undiluted)	0.52	0.97	0.93	0.71
Llop PCR with extraction Taylor (1:10)	0.33	1.0	1.0	0.64

The following table (Table 4.3.2.7) summarizes the accuracy results obtained for the different techniques in the ring test.

Table 4.3.2.7.: Final results of accuracy of the techniques analysed in the ring test.

Techniques	**Accuracy**
Isolation	0.96
Agristrip with kit buffer	0.66
Diagpocket with kit buffer	0.64
Llop PCR with extraction isopropanol (undiluted)	0.67
Llop PCR with extraction isopropanol (1:10)	0.76
Llop PCR with extraction Taylor (undiluted)	0.76
Llop PCR with extraction Taylor (1:10)	0.70
Taylor PCR with extraction Taylor (undiluted)	0.75
Taylor PCR with extraction Taylor (1:10)	0.67
Obradovic PCR with extraction Taylor (undiluted)	0.76
Obradovic PCR with extraction Taylor (1:10)	0.67
Stöger PCR with extraction Redextract (undiluted)	0.76
Stöger PCR with extraction Redextract (1:10)	0.70

We consider that among the assayed techniques: isolation, Llop PCR, Taylor PCR, Obradovic PCR and Stöger PCR with the tested DNA extraction protocols can be advised for the analysis of asymptomatic and symptomatic plant material, taking into account their sensitivity, specificity and accuracy in the analyses of the five labs participating in the ring test. However the serological reagents, Ea Agristrip and Pocket Diagnostic are more advised for the analysis of symptomatic material, due to their low sensitivity.
Related to PCR assays, the dilution 1:10 of the samples was convenient to improve the accuracy of the isopropanol extraction but was not efficient in the case of Taylor or Redextract protocols. The accuracy of the different combination of protocols was variable between a minimum of 0.67 and a maximum of 0.76 that is considered acceptable for being suggested for new EPPO and IPPC protocols. The Taylor extraction method combined with the different amplification protocols was giving satisfactory results.

Then, taking into account the results obtained in the different labs we consider that all the methods assayed (isolation, Agristrip, Diagpocket, Llop PCR, Taylor PCR, Stöger PCR and Obradovic PCR) have been accurate enough to be adviced to be used also with symptomatic samples. They have been validated for this type of samples in the five laboratories participating in the ring test (Partners 1, 2, 3, 4 and 6).
Based in these results another ring test coordinated by Partner 2 is being organized including at least ten laboratories from the EU, USA, New Zealand, Morocco and Russia to evaluate a larger number of samples and some combination of techniques in four continents

Real time PCR ring-test results

Real time PCR is a relatively new method in routine diagnostics and although several ring-tests were organized in recent years for real time PCR detection of plant pathogens, little data on critical points is available.

During this ring-test, attempts were made to standardize the procedure as much as possible by providing all the chemicals (2xTaqMan Universal Master Mix (Applied Biosystems); primers and probes synthesized by MWG, molecular-biology grade water as no template control) and giving detailed instructions on preparation of real time PCR, reaction conditions and analysis. Invariably ring-testers have different real time PCR instruments that often require different reaction volumes (Table 4.2.3.6) and enable different temperature ramp rates. Different reaction volumes were used simply by down- or up-scaling reactions from tested 10 uL. Temperature ramp rate were set to be similar to Applied Biosystems 9700 thermal cyclers ('9600 emulation' on some cycler or regular). Faster ramp rates and with that faster PCR cycling are possible on some instruments that were used however, the specific reaction mixture composition and enzymes are needed for the assay to work well under such conditions (see i.e. SmartCycler protocol under Task 4.1.1).

Table 4.3.2.6: Instruments and PCR conditions used by different partners in real time PCR analyses.

lab	1	2	3	4
instrument	ABI PRISM® 7500 FAST	Light Cycler 480 (Roche)	Eppendorf Mastercycler epgradient 4	ABI 7900 HT Sequence Detection System
reaction volume*	20 µl	10 µl	20 µl	10 µl
software	7500 Software v2.0.2	1.5.0 SP1	realplex 2.0	SDS version 2.3
cycling conditions	2 min at 50 °C (UNG activation step), 10 minutes at 95 °C, 40-45 cycles of 15 sec at 95 °C and 1 min at 60 °C			
temperature ramp rate	standard**	4,4 (°C/s)	1,62 °C/s heat, 1,215 °C/s cool (27%)	standard**
automatic treshold set at	NA***	0,6642	250	NA
manual treshold set at	0.08	NA	NA	0.10
automatic baseline	yes	0,6642	yes	yes
manual baseline set to from	NA	NA	NA	NA

* 10 uL reactions contained 1 uL water, 5 uL 2xTaqMan Universal Master Mix (Applied Biosystems), 2 uL primers and probe mixture (for final concentrations of 900 nM each primer and 200 nM probe) and 2 uL sample DNA. For 20 uL reactions all components were used in twice the volumes.
** The sample ramp rates for the 7500 and 7900 instruments in Standard Mode are: 1.6 °C/sec up and 1.6 °C/sec down.
*** NA = not applicable

As it was not clear how much the different instruments will influence the results, the ring test was composed of two parts:

1. Testing of the DNA isolated by Partner 4 (magnetic beads based DNA extraction, QuickPick Plant Kit, Bionobile, Turku).

2. Each partner prepared their own non-spiked and spiked samples using plant material sent out by Partner 2 and bacterial suspension of *E. amylovora* strain CFBP 1430. DNA from this material was isolated using modified Taylor DNA extraction. Four project partners have sent the results of real time PCR (Table 4.3.2.7).

Tab 4.2.3.7.: Real time PCR results (threshold values, Ct or Cp) obtained for samples of DNA and calculated characteristics of the Ams assay on individual instruments. DNA was isolated from plant apple tissue using QuickPick Plant kit (Bionobile, Turku).

DNA sample ID	*E. amylovora* [cfu/mL extract]*	preliminary testing		lab 1						lab 2			lab 3			lab 4		
				real-time PCR 1			real-time PCR 2			real-time PCR 1			real-time PCR 1			real-time PCR 1		
NTC**	0	neg**	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
DNA1	0	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
DNA2	10^3	34,82	35,33	34,91	34,70	35,10	34,60	34,70	33,68	35,00	35,00	35,00	35,56	36,04	36,31	34,92	neg	36,69
DNA3	10^4	31,91	32,43	31,73	31,58	32,39	31,45	31,81	31,83	33,47	33,55	33,23	31,98	33,12	32,69	32,18	32,80	32,30
DNA4	10^5	29,21	29,23	28,23	28,18	28,12	28,21	28,05	28,24	30,55	30,78	30,65	28,95	29,03	29,22	28,97	28,34	28,78
DNA5	10^5			28,10	28,47	28,38	28,28	28,21	28,43	30,01	29,85	29,71	28,61	29,16	29,06			
DNA6	10^6	25,99	25,96	25,11	25,10	25,04	24,91	24,91	24,93	27,47	27,03	27,25	25,51	25,69	25,76	25,33	25,42	25,48
slope		-3,0		-3,3			-3,2			-2,6			-3,5			-3,5		
efficiency		1,14		1,00			1,07			1,40			0,95			0,93		
theoretical 1 copy detection		36,8		37,8			37,3			36,9			38,9			37,8		

* actual concentration as determined by plating: $10E^x$ corresponds to 1.8 x $10E^x$
** NTC = no template control, molecular biology water added to reactions instead of DNA
*** neg = no signal (40 or 45 cycles performed)

Results of the four laboratories were in high accordance. No template control and DNA extracted from *E. amylovora* negative plant material gave negative results in all laboratories. The lowest concentration of target bacteria tested, corresponding to 1.8 x 10E3 cfu/mL plant extract, was positive in all cases with minimum two out of three parallel reactions positive. Higher concentrations of *E. amylovora* were detected in all cases.

This corresponds to specificity and sensitivity values of 1 with 0 rates of false positive and false negative results.

In addition, despite using different PCR cycling instruments and different software for signal acquisition and analysis, Ct values, efficiency of amplification and theoretical cycle threshold value for one copy detection were comparable in most cases (Fig 4.3.2.1). Laboratory 2 results are deviating slightly in this characteristics which is most likely due to higher temperature ramp rates used (Tab. Table 4.3.2.6)..This is an important observation as the presence of various instruments for real time PCR is a reality in both research and routine laboratories across Europe and consequently all these different instruments are used in routine analyses, ring-testing and research.

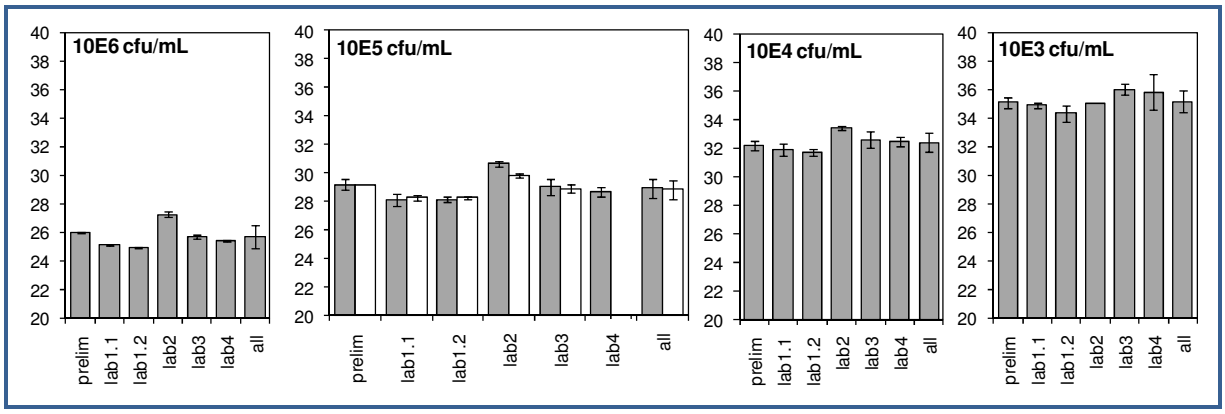


Fig 4.3.2.1: Variability of determined Ct values in DNA samples. Laboratories were using instruments and run reactions under conditions described in Table 4.3.2.6. Two samples had 10E5 *E. amylovora* cfu/mL and are shown separately (lab 4 did not test the second sample). Error bars represent standard deviation of average threshold values listed in Table 4.3.2.6.

Tab. 4.3.2.8: Results of real time PCR analysis of spiked plant material. Plant material provided by Partner 2 was sent out to ring-test partners who prepared plant extracts and spiked them with bacterial suspensions of *E. amylovora*. DNA was extracted according to modified Taylor extraction method and each sample analysed in one real time PCR reaction.

plant material	*E. amylovora* [cfu/mL extract]*	lab 1	lab 2	lab 3	lab 4	pos / all reactions
pyracantha	0	neg	neg	neg	neg	0/4
quince	0	neg	neg	neg	neg	0/4
apple	0	neg	neg	37.23	neg	1/4
loquat	0	33.72	neg	38.28	neg	2/4
pear	0	37.44	neg	neg	neg	1/4
no plant material	1000000	24.67	25.68	26.05	27.02	4/4
pear	0	neg	neg	neg	neg	0/4
	10	neg	neg	neg	neg	0/4
	100	neg	neg	neg	neg	0/4
	1000	35.70	35.00	36.52	37.06	4/4
	100000	31.28	30.89	31.04	30.91	4/4
	1000000	27.84	27.19	27.68	27.65	4/4

Sensitivity in spiked pear sample was 10E3 cfu/ml, as expected from preliminary studies and theoretical limits of real time PCR method.

There were some unexpected positive signals detected in supposedly negative samples. Due to growing conditions of the original plant material it is unlikely that it was infected and the positive results are likely due to contamination of samples during preparation. Still, apart from supposedly negative samples of apple, loquat and one pear samples, which gave positive results at least in one case, all controls had adequate results supporting the hypothesis that a critical step was preparation of spiked samples, a step that is usually separated from testing of samples by time, space or both. Also, as not all ring-test partners have experience of routinely using real time PCR, preparation of samples or other steps may not have been optimally carried out with respect to preventing contaminations.

On spiked plant samples, prepared by ring-test partners, real time PCR had sensitivity of 0.67 and specificity of 0.83.

False positive rate was 0.17, most likely due to contaminations during preparation of spiked samples especially as in one case also classical PCR was positive. It is generally accepted that the rooms for preparation of samples, extraction of DNA and real time PCR reaction preparation should be separated this could not be guaranteed by all ring-test partners. Based on experience of using real time PCR in routine analyses it is expected that by using proper procedure to minimize the risk of cross-contaminations the level of false positive results can be greatly reduced.

False negative rate was 0.33, due to samples with concentrations below theoretical and practical sensitivity of the method. While symptomatic fire-blight samples with such low concentrations are not expected this the sensitivity can be further increased by analyzing a bigger volume of sample DNA (more real time PCR reactions).

Main conclusions, discussion of results and their reliability

Several methods, both simple-to-use and more complex, became available for detection of *E. amylovora* in recent years. Two methods representing both groups of methods, a serological kit and a real time PCR assay, were extensively evaluated in the frame of this project, assessing their pros and cons, resulting in harmonized protocols for fire blight detection. Both were also included in ring-testing among project partners of negative and positive plant material, comparing them to existing methods.

Serological kits are very easy to use and are therefore a useful tool in the hands of non-laboratory personnel, such as phytosanitary inspectors and orchard growers, thus extending diagnostic expertise and enabling evidence-supported decisions beyond and outside laboratory environment. Not requiring extensive training they are suitable for quick technology transfer and have been readily accepted during training courses for testing of symptomatic plant material or infected blossoms, when bacterial concentrations are high. Their acceptance is especially important as their use in the orchards can facilitate rapid measurements needed to prevent spread of fire blight.

Real time PCR assays on the other hand are at the edge of current technology used in diagnostic laboratories and as we have shown, provide higher sensitivity, specificity and reliability of pathogen detection in combination with several DNA extraction methods based on isopropanol extraction, silica columns or magnetic beads. Real time PCR assays are especially useful when analysis of a larger number of samples is required in short time. High reliability of detection can be further improved by combining several assays with different targets and by quantification. While essentially suitable for and benefiting laboratories, we have modified the Ams real time PCR assay to allow it being performed using portable machines in the field or at other points of interest with the same sensitivity as in the laboratory.

Ring-testing of serological kits, conventional PCR and real time PCR has confirmed results of preliminary testing. In addition, critical points of both have been identified. A substitution of serological kit buffers with antioxidant maceration buffer has been attempted with the aim to combine preparation of samples for enrichment ELISA and serological kits. However, the results of comparison showed that antioxidant maceration buffer without modification, is not suitable for chromatography. In real time PCR the critical step seems to be its high sensitivity making this method particularly sensitive to contaminations. It should be noted however, that with experience of regular use of real time PCR and careful measures to prevent contaminations, this risk can be greatly reduced.
With serological kits, conventional and real time PCR, it has been observed that reliability of results is directly correlated to experience of the ring-test partners with that particular method. If ring-test partners lack the experience needed for reliable performance of a method, the ring-test results will favour simpler, more robust methods and great caution is needed in interpretation of results. This present certain difficulty for systematic ring-testing of methods as it is often difficult or even impossible to find partners with both the experience and the means to take part in ring-testing.

Although having different characteristics, both, serological kit and real time PCR assay, were found suitable for their purpose and providing significant improvements in the ease and reliability of fire blight detection scheme and inspection procedures for import/export plant material. Results benefit research laboratories, plant protection service and fruit growers. The project has provided a useful exchange of experience and knowledge among quarantine

laboratories of several counties facing fire blight epidemics and has enabled incorporation of reliable, state-of-the-art diagnostic methods in current fire-blight diagnostic protocols.

Through its support of an argumentative choice of diagnostic tests the project has significantly contributed to the good quality work.

Implications for stakeholders

Phytosanitary authorities: Novel methods available for detection of *E. amylovora* have been evaluated and can be incorporated in the existing diagnostic schemes, both to complement and increase reliability of diagnosis in the laboratory (real time PCR) and to provide alternative, extensively evaluated and easy to use tools for fire-blight detection in orchards by serological kits. On-site detection enables rapid, evidence-supported decision making.

Fruit producers / NGOs: Serological test that can be used without extensive training give the producers additional tool in preventing fire blight spread.

Scientists and fruit breeders: Results have identified critical steps in the methods that can be further improved and potential new developments. Real time PCR has proven as a valuable tool for detection of low concentrations of *E. amylovora* and for their quantification making it suitable for epidemiological studies on its own, in combination with existing methods (isolation on media) or newly developed methods i.e. VNTR analysis.

Further research needed and continued collaboration

It is expected that further progress will be made in improving existing or developing new methods of fire-blight detection. Promising detection methods include performance of PCR and real time PCR at constant temperatures eliminating the need for specialized equipment and novel ways of visualization of reaction products. Results of sequencing project and diversity analysis of the target pathogen, *E. amylovora*, performed in WP2 of this project that is continuing in collaboration of Partners 3 and 4, will no doubt play a significant role in the new developments.

A continuous, systematic support of comparison studies, validations and ring-testing is urgently needed to guarantee argumentative selections of methods best fit for purpose.

PROJECT OUTPUT

Output WP2
Meeting talks and posters (total 7)
International Congress for Molecular Plant-Microbe Interactions (Quebec City, Canada, July 2009) 3rd FEMS Congress of European Microbiology (Götheborg, Sweden, June 2009)
Biotech2009 (Wädenswil, Switzerland, July 2009)
Annual Meeting of the Swiss Phytiatry Society (Geneva, Switzerland, September 2009)
ProkaGenomiks2009 (Göttingen, Germany, October 2009)
Fire blight 5-Country Annual Meeting (Vaduz, Liechtenstein, December 2008)

Publications (total 3)
Smits et al. 2009. Complete genome sequence of the fire blight pathogen *Erwinia amylovora* CFBP 1430. *Accepted with revision.*
Llop et al. Plasmid diversity of *Erwinia amylovora. In final preparation for submission end 2009/early 2010.*
Rezzonico et al. *Erwinia amylovora* CRISPR regions and genotypic diversity. *In final preparation for submission end 2009/early 2010.*

Output WP3.1
Meeting talks and posters (total 3)
Biotech2009 (Wädenswil, Switzerland, July 2009)
Annual Meeting of the Swiss Phytiatry Society (Geneva, Switzerland, September 2009)
Fire blight 5-Country Annual Meeting (Bavendorf, Germany, December 2009)

Output WP3.2
Meeting talks/posters (total 1)
Pirc M et al. 2009. Development of real- time PCR for detection of *Erwinia amylovora* and its use in diagnostics. In: Macek J (ed). Abstracts of 9th meeting of Slovenian Plant protection Society*, Nova Gorica, 4.-5. marec 2009*. Ljubljana: DVRS, 2009, pp. 215-218.

Publications (total 1)
Pirc M et al. 2009. Improved fireblight diagnostics using quantitative real time PCR detection of *Erwinia amylovora* chromosomal DNA. Plant Pathology, 58:872-881.

Output WP4

Output WP4.1.1
Meeting talks/posters (total 7)
Pirc M et al. 2009. Development of real- time PCR for detection of *Erwinia amylovora* and its use in diagnostics. In: Macek J (ed). Abstracts of 9th meeting of Slovenian Plant protection Society*, Nova Gorica, 4.-5. marec 2009*. Ljubljana: DVRS, 2009, pp. 215-218.
Fire blight 5-Country Annual Meeting (Vaduz, Liechtenstein, December 2008)
International Plant Diagnostics Congress (Orlando, Florida, December 2009)
Bobev, S.G., Maes, M., Crepel, C., van Vaerenbergh, J., Llop, P., López, M.M. 2009 Fire blight in Bulgaria: studies on the pathogen *Erwinia amylovora* and its epidemiology COST Action 864 meeting Working groups 1 and 3, Valencia, Spain.
Santander R.D., Catalá-Senent J., Ferrer I., Ordax M., Marco-Noales E., López M.M., E.G. Biosca. 2009. Efecto de distintos desinfectantes en la supervivencia de *Erwinia amylovora* en agua. *III Reunión del grupo "Microbiología de Plantas" de la Sociedad Española de Microbiología (SEM). Granada, España.*

Ordax, M., Biosca, E.G., Wimalajeewa, S.C., López*, M.M., Marco-Noales, E**.** Induction of the viable but non-culturable (VBNC) state in *Erwinia amylovora* in mature apple fruit calyces. COST Action 864 meeting Working groups 1 and 3, Valencia, Spain.
Palacio-Bielsa, A., Cambra, M. A., Roselló, M.,Gorris, M.T., Peñalver, J., Montesinos, E., López, M.M. Efficiency of the EPPO protocol for preventing the introduction and dissemination of Erwinia in two Spanish areas COST Action 864 meeting Working groups 1 and 3, Valencia, Spain.

Publications (total 1)
Pirc M et al. 2009. Improved fireblight diagnostics using quantitative real time PCR detection of *Erwinia amylovora* chromosomal DNA. Plant Pathology, 58:872-881.

Output WP4.1.2
Meeting talks/posters (total 2)
Fire blight 5-Country Annual Meeting (Vaduz, Liechtenstein, December 2008)
International Plant Diagnostics Congress (Orlando, Florida, December 2009)

Publications (total 4)
Duffy B. 2009. Schweizerische Zeitschrift für Obst- und Weinbau, 9/09:16-17.
Gersbach K., A. Braun-Kiewnick, B. Duffy. 2009. Schweizerische Zeitschrift für Obst- und Weinbau, 9/09:17-18.
Braun-Kiewnick A., J. Vogelsanger, B. Schoch, L. Franck, E. Holliger, B. Duffy, D. Altenbach, T. Oberhänsli, W. Bitterlin. 2009. Schweizerische Zeitschrift für Obst- und Weinbau, 14/09:7-10.
Braun-Kiewnick et al. Development and validation of a novel immunological test for fire blight diagnosis in the laboratory and field. *Submitted by end 2009.*

Output WP4.1.2
Meeting talks/posters (total 2)

Publications (total 6)
Duffy B. 2009. Schweizerische Zeitschrift für Obst- und Weinbau, 9/09:16-17.
Gersbach K., A. Braun-Kiewnick, B. Duffy. 2009. Schweizerische Zeitschrift für Obst- und Weinbau, 9/09:17-18.
Braun-Kiewnick A., J. Vogelsanger, B. Schoch, L. Franck, E. Holliger, B. Duffy, D. Altenbach, T. Oberhänsli, W. Bitterlin. 2009. Schweizerische Zeitschrift für Obst- und Weinbau, 14/09:7-10.
Braun-Kiewnick et al. Development and validation of a novel immunological test for fire blight diagnosis in the laboratory and field. *Submitted by end 2009.*
Pirc, M., M. Ravnikar, J. Tomlinson, T. Dreo. 2009. Improved fireblight diagnostics using quantitative real time PCR detection of *Erwinia amylovora* chromosomal DNA. Plant Pathology, 58:872-881.
Ordax, M., Biosca, E.G., López, M.M., Marco-Noales**, E.** 2009. The addition of copper sulphate to a non-selective medium improves the recovery of plant associated bacteria: *Erwinia amylovora* as a model. In: A. Mendez-Vilas (ed.), Current Research Topics in Applied Microbiology and Microbial Biotechnology. World Scientific Publishing Co. ISBN: 978-981-283-754-7.
López, M. M., Peñalver J., Roselló M., Gorris M.T., Cambra, M., Marco-Noales, E., González - Biosca E., Palacio Bielsa A.,Llop, P**.** *Erwinia amylovora:* características generales. Métodos de diagnóstico de la enfermedad e identificación de E. amylovora. In: El fuego bacteriano (*Erwinia amylovora*) y su situación en España. Ministerio de Agricultura, Pesca y Alimentación (MAPA). Dirección General de Agricultura. (in press)

Technical meetings

López M.M. Protocols utilised in the Laboratory of Reference for Plant Pathogenic bacteria. Meeting of the Plant Protection Services Diagnostic Laboratories. Reus, Spain.

Conferences to growers

Llop,P. 2008. Fruit tree bacteriosis . JORNADAS IVIA . "FRUTICULTURA 2008". Transference Technology Conference in Fruit trees. Moncada, Valencia, Spain.
López M.M**.** 2009. Prevention of the introduction of *Erwinia amylovora* in loquat in the Valencian Community. Callosa d'Ensarria, Spain.

Participation in Technical courses

López M.M**.**2009. Plant pathogenic bacteria of fruit trees. FECOAV course on Fruit trees cultivation. Valencia (2 h).
López M.M**.** 2009. Inspection and detection of plant quarantine bacteria .Course for inspectors of the Inspection Services of the different Spanish regions. Madrid (2 h).
López M.M**.** 2009. Inspection and detection of plant quarantine bacteria. Course for inspectors of the Inspection Services of the different Spanish regions. Valencia (2 h).

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Pirc, M., M. Ravnikar, J. Tomlinson, T. Dreo., (2009). Improved fireblight diagnostics using quantitative real time PCR detection of *Erwinia amylovora* chromosomal DNA. Plant Pathology, 58:872-881.

Obradovic D., Balaz J., Kevresan S., (2007). Detection of *Erwinia amylovora* by Novel Chromosomal Polymerase Chain Reaction Primers. Microbiology 76 (6), 748-756.

Ordax, M., Biosca, E.G., López, M.M. and Marco-Noales, E. (2009a). The addition of copper sulphate to a non-selective medium improves the recovery of plant associated bacteria: *Erwinia amylovora* as a model, p. 92-96. *In* A. Mendez-Vilas (ed.), Current Research Topics in Applied Microbiology and Microbial Biotechnology. World Scientific Publishing Co. Pte. Ltd, Singapore.

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Annex 1: Comparative study for DNA extraction from samples of plant hosts for detection of *Erwinia amylovora.*

Annex 3: Results of the final ring testing

Annex 1 and 3 are enclosed as separate documents.

Annex 2: Protocol: Ring test on diagnosis and detection of *Erwinia amylovora*

Protocol: Ring test on diagnosis and detection of *Erwinia amylovora*

1. SAMPLE PREPARATION

The plant samples are prepared with a general procedure valid for isolation, serological tests and PCR analysis.

Provided plant material: healthy pear shoots to prepare spiked samples and blind samples. Prepare first the blind samples and then the spiked samples to avoid contaminations.

a) Spiked samples:

Prepare a macerate of healthy pear shoots (provided). Cut in some pieces the shoots, giving 0.1 g of material per sample (the material should include the apex, the leaves, the stems…), until having six samples. Put into 6 plastic bags (provided) with a heavy net and add to each bag 4.5 ml of the antioxidant maceration buffer (provided to prepare 200 ml, in case you need to repeat something).

Add to each bag 0.5 ml of serial dilutions 10^7, 10^6, 10^4, 10^3 and 10^2 cfu/ml of suspensions of 48-72h of strain 1430 (provided) of *E. amylovora* in PBS (provided). A culture provided on tube (one tube with a culture 48 h old and another of the same day of sending). According to the day of receiving the samples, prepare the suspension with this last tube (if *E. amylovora* has grown) or use the strain for preparing a new tube and a suspension of 48 h culture. Use PBS 10mM (phosphate buffer saline, pH 7.2: $Na_2HPO_4.12H_2O$, 2.7 g; $NaH_2PO_4.2H_2O$, 0.4 g; Na Cl 8.0 g, distilled water 1l) as negative control. Use an initial 10^9 cfu/ml suspension in PBS 10mM (OD 1.0 at 600 nm) to prepare the serial suspensions that you will add to the pear macerates (spiked samples).

Sample 5 = 0.5 ml 10^7 cfu/ml *E. amylovora* 1430 in the 4.5 ml macerate .
Sample 4 = 0.5 ml 10^6 cfu/ml *E. amylovora* 1430 in the 4.5 ml macerate .
Sample 3 = 0.5 ml 10^4 cfu/ml *E. amylovora* 1430 in the 4.5 ml macerate .
Sample 2 = 0.5 ml 10^3 cfu/ml *E. amylovora* 1430 in the 4.5 ml macerate .
Sample 1 = 0.5 ml 10^2 cfu/ml *E. amylovora* 1430 in the 4.5 ml macerate .
Sample 0 = 0.5 ml PBS in the 4.5 ml macerate .

Proceed with further processing of samples.

The suspension of 10^6 cfu/ml *E. amylovora* will be treated at 100 ºC for 10 min , frozen and used as positive control for the amplification.

b) Blind samples:

The provided samples (6 to 10) are prepared according to the following:

Cotoneaster (6), quince (7), apple (8), loquat (9) and pear (10). Take a piece of the shoot, including leaves, from the apex to the distance necessary to have 0.1 g of material. Put into the plastic bag (provided), add 4.5 ml of the antioxidant buffer and proceed with further processing.

c) Further processing of samples:

Let stand the samples in maceration at least 5 min. Slightly crush the plant material with a hammer or specific apparatus in the plastic bag.

Let stand the spiked samples and the blind samples on ice again for five minutes. Transfer each macerate to three sterile Eppendorf tubes by decantation (1.5ml/tube). Store two tubes of each sample at –20ºC for subsequent PCR analysis and keep the remaining tubes on ice.

The same day of the maceration of the samples you should perform the isolation and the serological tests. The PCR and real time PCR analysis can be performed at your earliest convenience using the Eppendorf tubes stored at –20ºC. The sample 0 is used as negative control for PCR assays.

2. ISOLATION

The macerate samples are plated on King's B medium. Prepare 1:10 and 1:100 dilutions of each macerate in PBS 10 mM. Pipette 50 µl of the diluted and undiluted macerates onto separate plates. A set of three plates of each medium (for undiluted and diluted samples) is used for each macerate. Start with the 1:100 dilution and proceed to the undiluted macerate. Use sterile loops and carefully spread the pipetted volumes by triple streaking over the media. Incubate the plates at 25ºC for 72 h., final reading is about 72 h. Obtain pure cultures from one to three individual suspect colonies of each sample (from sample 0 to 10) after incubation on King's B medium. Identify presumptive colonies of *Erwinia amylovora* by PCR or by two others tests (IF, inoculation, etc), according to the EPPO protocol.

Positive control:

Plate for counting the 10^3 and 10^4 cfu/ml dilution of the strain 1430 of *Erwinia amylovora* to verify the cfu/ml of the suspension and to compare the aspect and to know the number of the colonies. Count the *E. amylovora*-like colonies and make the calculations of the real cfu/ml of the suspensions used.

Colonies of *E. amylovora* on King's B are creamy white, circular intending to spread and non-fluorescent under UV light.

3. SEROLOGICAL KITS

Follow the instructions of the Agristrip kit and the Diagnostic Pocket. Take the appropriate amount of each of the samples from 0 to 10, prepared in the antioxidant buffer after crushing them and before plating, and follow the instructions of the kits. You do not need to use the buffers of the kits because we have confirmed that the sensitivity is the same with the antioxidant buffer.

4. DNA EXTRACTION PROTOCOLS

4.1. Purification of DNA for PCR using isopropanol protocol (Llop, 1999) – EPPO

Take directly 1 ml of each macerate (from samples 0 to 10)

Centrifuge the macerates at 10000g for 5 min at room temperature. The supernatant is discarded, and the pellet resuspended in 500 µl of extraction buffer (provided) (200 mM Tris HCl pH 7.5; 250 mM NaCl; 25 mM EDTA; 0.5% SDS; 2% PVP) and shaken for 1 h at room temperature.

Centrifuge the tubes at 4000g for 5 min at room temperature. Take 450 µl of the supernatant without disturbing the pellet and place it into a new Eppendorf tube. Add the same volume of isopropanol (provided) , invert and leave for 30min-1 h at room temperature.

Centrifuge at 10000g for 5 min, discard supernatant and dry on bench. If there is still a coloured precipitate (brown or green) at the bottom of the tubes, carefully take it while discarding the supernatant, thus obtaining a cleaner DNA. Normally, the DNA sticks at the wall of the Eppendorf tube more than at the bottom.

If it is not possible, and the pellet still remains when the tube is dry, resuspend the pellet in 200 µl of water, centrifuge again for 2 min at full speed this suspension of DNA plus pellet, and take the supernatant into a new tube. Store the supernatant at – 20ºC until use.

4.2. DNA Extraction by REDExtract-N-AmpT Plant PCR Kit (Stöger, 2006).

Take 100 µL of extracted plant material sample prepared according to the current protocol into an eppendorf tube. The REDExtract kit is provided.

Add 150 µl of extraction solution (kit) (supplemented with 0,1 % (v/v) Triton X-100 (provided), and 0,05 % (v/v) Nonidet NP-40 Igepal (provided).

Incubate at 95°C for 30 min on heating block.

Transfer 50 µl of extract to a new tube and dilute it with 50 µl of the dilution buffer (kit) (provided).

A 1:30 (v/v) dilution of the extract is prepared with a 1:1 mixture of extraction:dilution buffer.

Store the extract at -18 °C until use.

Perform PCR with the quantity required according PCR protocol.

4.3. Modified DNA purification from Taylor et al.,(2001).

Mix 200µL of extract in 500 µl of plant extraction buffer (provided)

NaCl :	8,19 g	140 mM
KCl :	3,73 g	50 mM
Tween 20 :	0,5 g	0,05%
PVP 10 :	20 g	2%
BSA :	4 g	0,4 %
Water :		1 liter

Incubate for 15 min at room temperature. Store at -20 °C until use.

5. PCR PROTOCOLS

All the primers are provided. You need to ask for the Sigma Readymix. For handling of the samples, use gloves. The preparation of reaction mixture and addition of DNA to reaction mixture should be done in separate places and away from amplified DNA. Use suitable pipette tips and plastic-ware (DNAse free). In all PCR protocols use as a positive control 1µl of the suspension 10^6 cfu/ml of *E. amylovora* strain1430 that you keep frozen. The ultrapure water and the extraction from sample 0 should be used as negative controls.

5.1. Conventional PCR according to Taylor *et al.,* (2001)

Primers (provided)

G1-F: 5‘-CCTGCATAAATCACCGCTGACAGCTCAATG-3‘
G2-R: 5‘-GCTACCACTGATCGCTCGAATCAAATCGGC-3‘

Mastermix

Reagent	Volume [µl]
Ultrapure water	7µl
Sigma Readymix	10µl
G1-F [10pmol/µl]	1µl
G2-R [10pmol/µl]	1µl

PCR-Mix

Mastermix: 19µl
Sample-DNA: 1µl

Cycling conditions

PCR-steps	Cycling	Temperature (°C)	Time
initial denaturation	1	95°C	3min.
denaturation	**35**	94°C	20sec.
primer-annealing		60°C	20sec.
extension		72°C	1min.
final extension	1	72°C	5min.
cooling	1	15°C	∞

Analysis

Sample volume for gel analysis: 20µl
Expected product size: 187 bp

Electrophoresis of PCR products

Prepare 2 % agarose gel in TAE buffer 0.5 X. Place ca. 3 µl droplets of loading buffer on parafilm, mix 20 µl of PCR product by gentle aspiration with the pipette before loading.

Load wells of gel and include positive and negative controls. Include DNA marker 100 pb in the first well of the gel.

Run the gel for 20 min at 120 V (medium gel tray: 15x10 cm) or 40 min at 160 V (big gel tray or electrophoresis tank: 15x25 cm).

Soak the gel in ethidium bromide solution for 20 minutes.

Visualise the amplified DNA fragments by UV transillumination.

5.2. Conventional PCR according Gottsberger adapted from Obradovic *et al.* 2007

Primers (provided)

FER1-F: 5`-AGCAGCAATTAATGGCAAGTATAGTCA-3`
rgER2R: 5`-AAAAGAGACATCTGGATTCAGACAAT-3`

Mastermix

Reagent	Volume [µl]
Ultrapure water	7µl
Sigma Readymix	10µl
FER1-F [10pmol/µl]	1µl
rgER2R [10pmol/µl]	1µl

PCR-Mix

Mastermix: 19µl
Sample-DNA: 1µl

Cycling conditions

PCR-steps	Cycling	Temperature (°C)	Time
initial denaturation	1	94°C	3min.
denaturation	**41**	94°C	10sec.
primer-annealing		60°C	10sec.
extension		72°C	30sec.
final extension	1	72°C	5min.
cooling	1	15°C	∞

Analysis

Sample volume for gel analysis: 20µl
Expected product size: 458 bp

Electrophoresis of PCR products

Prepare 1.5 % agarose gel in TAE buffer 0.5 X. Place ca. 3 µl droplets of loading buffer on parafilm, mix 20 µl of PCR product by gentle aspiration with the pipette before loading.

Load wells of gel and include positive and negative controls. Include DNA marker 100 pb in the first well of the gel.

Run the gel for 20 min at 120 V (medium gel tray: 15x10 cm) or 40 min at 160 V (big gel tray or electrophoresis tank: 15x25 cm).

Soak the gel in ethidium bromide solution for 20 minutes.

Visualise the amplified DNA fragments by UV transillumination.

5.3. Conventional PCR according Stöger *et al.* 2006

Primers (provided)

PEANT 1: 5´- TAT CCC TAA AAA CCT CAG TGC-3‘
PEANT 2: 5´- GCA ACC TTG TGC CCT TTA-3‘

Mastermix

Reagent	Volume [µl]
Ultrapure water	7µl
Sigma Readymix	10µl
PEANT 1-F [10pmol/µl]	1µl
PEANT 2-R [10pmol/µl]	1µl

PCR-Mix

Mastermix: 19µl
Sample-DNA: 1µl

Cycling conditions

PCR-steps	Cycling	Temperature (°C)	Time
initial denaturation	1	95°C	5min.
denaturation	**35**	95°C	15sec.
primer-annealing		58°C	30sec.
extension		72°C	45sec.
final extension	1	72°C	5min.
cooling	1	15°C	∞

Analysis

Sample volume for gel analysis: 20µl
Expected product size: 391 bp

Electrophoresis of PCR products

Prepare 1.5 % agarose gel in TAE buffer 0.5 X. Place ca. 3 µl droplets of loading buffer on parafilm, mix 20 µl of PCR product by gentle aspiration with the pipette before loading.

Load wells of gel and include positive and negative controls. Include DNA marker 100 pb in the first well of the gel.

Run the gel for 20 min at 120 V (medium gel tray: 15x10 cm) or 40 min at 160 V (big gel tray or electrophoresis tank: 15x25 cm).

Soak the gel in ethidium bromide solution for 20 minutes.

Visualise the amplified DNA fragments by UV transillumination.

5.4. Nested PCR (Llop *et al*, 2000)

The nested-PCR in a single tube (Llop *et al*, 2000) uses two sets of primers placed at the same time, and due to the different annealing temperatures the two PCR reactions are performed consecutively.

External primers (provided)

AJ75: 5' CGT ATT CAC GGC TTC GCA GAT
AJ76: 5' ACC CGC CAG GAT AGT CGC ATA

Internal primers (provided)

PEANT1: 5' TAT CCC TAA AAA CCT CAG TGC
PEANT2: 5' GCA ACC TTG TGC CCT TTA

Reagent	Volume [µl]
Ultrapure water	7.94 µl
Sigma Readymix	10 µl
AJ 75 [0.1 pmol/µl]	0.13 µl
AJ 76 [0.1 pmol/µl]	0.13 µl
PEANT1 [10 pmol/µl]	0.4 µl
PEANT2 [10 pmol/µl]	0.4 µl

Mastermix: 19µl
Sample-DNA: 1µl

Cycling conditions

PCR-steps	Cycling	Temperature (°C)	Time
initial denaturation	1	94°C	4 min.
primer-annealing	**25**	94°C	30 sec.
extension		72°C	1 min.
second denaturation	1	94°C	4 min.
denaturation	**40**	94°C	30s ec.
primer-annealing		56°C	30 sec.
extension		72°C	45 sec.
final extension	1	72°C	10 min.
cooling	1	15°C	∞

Analysis

Sample volume for gel analysis: 20µl
Expected product size: 391 bp

Electrophoresis of PCR products

Prepare 1.5 % agarose gel in TAE buffer 0.5 X. Place ca. 3 µl droplets of loading buffer on parafilm, mix 20 µl of PCR product by gentle aspiration with the pipette before loading.

Load wells of gel and include positive and negative controls. Include DNA marker 100 pb in the first well of the gel.

Run the gel for 20 min at 120 V (medium gel tray: 15x10 cm) or 40 min at 160 V (big gel tray or electrophoresis tank: 15x25 cm).

Soak the gel in ethidium bromide solution for 20 minutes.

Visualise the amplified DNA fragments by UV transillumination