EUPHRESCO Final Report

for Non-Competitive research projects

Please send the final report to all your project partners, to the NC topic coordinators and to the EUPHRESCO Secretariat (euphresco@fera.gsi.gov.uk).

EUPH05 Pantoea stewartii* ssp. *stewartii
Ring test on diagnostic methods for *Erwinia stewartii* ssp. *stewartii* (*Pantoea stewartii* ssp. *stewartii*)

Project Duration:

Start date:	**22nd February 2008**
End date:	**30th December 2009**

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2. Executive Summary

Introduction

The project titled ` **Ring test on diagnostic methods for *Erwinia stewartii* ssp. *stewartii* (*Pantoea stewartii* ssp. *stewartii*)** (EUPH05)´ was a pilot project in the frame of the EUPHRESCO project funded by EU FP6 ERA-NET. The project duration was one year from 22nd February 2008 to 31 st December 2009. The funding mode was a Non-Competitive one. The most important aspect of EUPHRESCO was the increase of the cooperation between research institutions. 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.

For the EU member states there is no harmonized diagnostic method for *Erwinia stewartii* ssp. *stewartii* (*Pantoea stewartii* ssp. *stewartii*). The EPPO protocol PM 7/60 (1) lists several tests for the first investigation and the following isolation/screening and identification. These tests haven't been tested in ring tests so far. For the preliminary examination, isolation, IF, ELISA and conventional PCR were proposed. The EPPO protocol contains no information on sensitivity, specificity and reproducibility. In addition, naturally infested seeds were not used for the development of these protocols. Newly available methods as real-time assays were also considered for inclusion in the ring test.

The research aims of the project were validation and standardisation of the diagnostic methods used by national plant protection services and other relevant laboratories, considering detection and identification methods by ring testing.

Objectives of the project

The objectives of the project was a ring test consisting of four parts:

1. Determination of the sensitivity, specificity and reproducibility of the different methods.
2. Comparison of different media for the isolation of the bacteria, and comparison of isolation with other tests, using artificially contaminated seed.
3. Test of the sensitivity, specificity and reproducibility of the results of methods used so far (IF and ELISA) and the PCR (with previously released primers) with naturally infected seed, or with artificially contaminated samples if naturally infected seeds are not available.
4. A key issue will be sample size in relation to levels and probability of detection: comparison of methods using replicates of 400 seeds will be done.

Expected Benefits of the project

Quarantine laboratories of EU countries and other external countries have to control imported maize seeds. These laboratories will benefit since the use of validated methods improves the quality system 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 within Europe and outside Europe. The ability to determine the pathogen will improve the quarantine and sanitation measures to prevent its introduction.

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).

Summary

Pantoea stewartii ssp. *stewartii* (Pss) is a bacterium responsible for serious crop losses within the world and especially in America to which it is indigenous. Its principal host is maize especially sweetcorn but also dent, flint, flour and popcorn cultivars. This bacterium is a quarantine bacterium in Europe.

The main mode of transmission is the insect vector, *Chaetocnema pulicularia*, but seed transmission also poses a potential risk of spreading of the bacteria..

For EU member states there is no harmonized diagnostic method for *Erwinia stewartii* ssp. *stewartii* (*Pantoea stewartii* ssp. *stewartii*). The EPPO protocol PM 7/60 (1) (2006) lists several tests for the first investigation and the following isolation/screening and identification. These tests have neither been evaluated nor tested in ring tests and no information on sensitivity, specificity and reproducibility were available. Real-time PCR or other new detection/identification techniques have not yet been included in this protocol. In addition, naturally infested seeds were not used to develop these protocols.

This project offered evaluations of detection and/or identification methods: isolation, Fatty Acid, IF, ELISA, pathogenicity test, conventional PCR and real time PCR through intra-laboratory work between 4 laboratories (WP1). Selected methods with best performance criteria were then evaluated through ring testing. These included immunofluorescence (IF) and both conventional and real time PCR. Standardisation of diagnostic methods for routine use by national plant protection services and other laboratories was then proposed.

In the framework of the interlaboratory study, standardized material (slides, inactivated seed soaks) were provided by the organiser to all participants in order to achieve comparable results in the different: ten positive samples at different concentration of Pss and 5 negative samples (one healthy and others contaminated by non-Pss bacteria). Furthermore, antisera, standard primer sets and dNTPs were also provided.

The participating laboratories obtained the samples anonymously with instruction for subsequent use. Strict guidelines for storage of the samples, timeframe of testing, specification of the methods and interpretation of the test results were prescribed.

One aim of the project was to validate currently recommended tests and to assess their performance criteria on target and non target strains. Sensitivity, specificity, detection threshold, repeatability and accuracy of the recommended pathogenicity test, isolation on Nutrient Broth Yeast extract medium (NBY), fatty acid profiling, IF, ELISA and PCR (Coplin, 2006) were all validated.

Another aim of this project was assessment of recently published or unpublished tests. Newly available methods included a new conventional PCR assay (Hufnagl-AGES) (Gottsberger *et al*., in preparation) and a real time PCR assay (Tambong, 2008), which were reviewed with respect to their specificity, sensitivity and performance.

15 target strains and 15 non target strains were selected to be used in the internal study by each partner.

In WP2 the most appropriate means of providing positive and negative samples for the ring test (WP3) were determined. Naturally contaminated samples are usually used to test extraction techniques and robustness of the methods in routine analyses conditions. Artificially contaminated samples are preffered for method evaluation as infection rates are more homogeneous and well quantified. As the bacterium *Pantoea stewartii* ssp. *stewartii* is not present in Europe, attempts were made to obtain contaminated seed lots from USA but were unsuccessful. Different inoculation methods to produce artificially contaminated seeds were also unsuccessful due to problems of homogeneity. It was therefore decided to produce artificially inoculated seed washings (soaks) for the ring test.

Work packages	**Objectives**	**Deliverables**	**Agenda**	**Comments**	**Degree of achievement**
WP1	Work on optimisation and validation of the existing protocols	Sensitivity, specificity and repeatability of the different protocols.	**December 2008 to June 2009**	Deliverables are available in this final project report. Additional work done after the meeting in Angers on new primers	Achieved
WP2	Supplying samples	Number of samples provided for the ring test.	**April to June 2009**	180 slides and 180 microtubes were sent.	Achieved
WP3	Ring testing	Test plan. Test report.	**From March to October 2009**	Test plan and samples were sent the 16th of June to the 5 participating labs. Test report was sent in October.	Achieved

The project achieved its aim to produce evaluation data of currently- used detection and identification methods for *Pantoea stewartii* ssp. *stewartii*. It has also provided useful data on new real time PCR methods (Tambong *et* al.).

The establishment of different performance criteria allowed prioritization of available detection and identification methods. This is important for accurate and efficient diagnosis and management of *Pantoea stewartii* ssp.*stewartii* within Europe.

The project has enabled the transfer to all participants of the Tambong real time PCR and IF with the Linaris antiserum as very accurate detection methods.

Some further work will be required to further test this method in analyses conditions with naturally infected seeds.

3. Report

Work package 1

Comparison of different detection /identification protocols described in EPPO protocol.

WP 1 Objectives

To test different bacteria extraction methods from naturally contaminated seeds.
To select reference target strains and non-target strains to be used by each partner in this work.
To determine for each detection or identification methods of the detection scheme of EPPO protocol and of new protocols, their validation parameters according to EPPO guidelines: relative sensitivity, relative specificity, detection threshold (diagnostic sensitivity) and repeatability.

WP 1 Participants

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

WP 1 Tasks

Task 1.1: Choosing an extraction protocol and testing samples size in relation to levels and probability of detection: comparison of methods using replicates of 400 seeds (partner 2).
Task 1.2: Comparing different media for isolation of the bacteria
Task 1.3: Comparing identification with other tests: IF, ELISA, PCR, Q-PCR, pathogenicity test.

WP 1 Method and results

Task 1.1: Choosing an extraction protocol and testing samples size in relation to levels and probability of detection: comparison of methods using replicates of 400 seeds

This task could not be implemented because of lack of naturally contaminated seeds samples. See WP2.

Task 1.2: Comparing different media for the isolation of the bacteria

Method

<u>Bacterial strains used</u>

The reference target strains and non-target strains used in this work are listed in **table 1**. Their host, origin, year of isolation and comments are also reported when available in this table. 15 target strains and 15 non target strains were used in the internal study.

Most strains were obtained from the ‘’Collection Française des bactéries Phytopathogènes’’ (CFBP), INRA, Angers, France and one of the *Pantoea stewartii* ssp. *stewartii* strain, was obtained from the National Collection of Plant Pathogenic Bacteria, Fera, York, United Kingdom.

All the target strains were plated on King B medium and verified by immunofluorescence before being used in the project. The strains were distributed to project participants by the project coordinator. Non target strains were plated either on King B or YPGA medium.

Référence	**Host**	**Origine**	**Year of isolation**	**Comments**
Pantoea stewartii subsp. *stewartii*				
CFBP 3167/ NCPPB 2295/ICMP 257ATCC 8199	Zea mays	USA	1970	LNPV 2004
CFBP1719/ ICPB SS104	Zea mays	USA		LNPV 2004
CFBP 2502 / NCPPB 449	Zea mays var rugosa	USA	1957	LNPV 2004 Mergaert 1993
CFBP 3157/ NCPPB 1553	Zea mays	USA	1963	LNPV 2004 Mergaert 1993
CFBP 3166 / ICMP 5930	Zea mays	USA	1975	LNPV 2004
CFBP 3393/ LMG 2716/ PDDCC 270	-	-	-	LNPV 2004 Mergaert 1993
CFBP 3394/ LMG 2717/ PDDCC 722	Coléoptère	USA	1954	LNPV 2004 Mergaert 1993
CFBP 3395/ LMG 2718/ ATCC 8200		USA		LNPV 2004 Mergaert 1993
CFBP 3396/ LMG 2719/ PDDCC 5929	Coléoptère	USA	1975	LNPV 2004 Mergaert 1993
CFBP 3445/ NCPPB 3379	Zea mays	USA	1985	LNPV 2004
CFBP 3517	Zea mays	USA		
CFBP 3168	Zea mays	USA	1957	
CFBP 3165	Zea mays	USA	1932	
CFBP 3169 NCPPB 3253	Zea mays	USA		
Clavibacter michiganensis subsp.michiganensis CFBP **4999 /** LNPV 30.31				Tambong 2007
Clavibacter michiganensis subsp. nebraskensis				
CFBP **2405 /** LNPV 10.17	*Zea mays*	USA	1971	Coplin 2002 - Tambong 2007
Curtobacterium flacumfasciens pv *flacumfasciens*				
CFBP **3456 /**LNPV 10.24	*Phaseolus vulgaris*	Hongrie	1957	Coplin 2002
Erwinia chrisanthemi pv.*zea*				
CFBP **2052**	*Zea mays*	USA	1970	Tambong 2007
Erwinia amylovora				
CFBP **1232**/ NCPPB 683/ ATCC 15580/ CCM 114	*Pyrus communis*	UK	1959	LNPV 2004
Erwinia carotovora subsp. *carotovora*				
CFBP **2046**	*Solanum tuberosum*	Danemark	1952	Coplin 2002
Erwinia carotovora subsp. *atroseptica*				
CFBP **1526**	*Solanum tuberosum*	UK	1957	Coplin 2002
Pantoea agglomerans				
CFBP **3845**/ ATCC 27155/ CIP 5751			1956	LNPV 2004
Pantoea ananas pv. *uredovora*				
CFBP **3171**	*Puccinia graminis*	USA	1954	Coplin 2002
Pseudomonas syringae pv. *syringae*				
CFBP **1392**	*Syringae vulgaris*	UK	1950	Coplin 2002 - Tambong 2007
Pseudomonas viridiflava				
CFBP **1141 /** LNPV 3.40	*Phaseolus vulgaris*	suisse	1927	Tambong 2007
Xanthomonas campestris pv. campestris				
CFBP **5251 /**NCPPB 528	*Brassicae oleracea gemmifera*	UK	1957	Coplin 2002 - Tambong 2007
Pantoea stewartii subsp.*indologenes*				
CFBP **3614**/ ICMP 77 / LMG 2632 / NCPPB 2280	*Setaria italica*	Inde	1995	Rangaswami G.
Pseudomonas syringae pv *lapsa*				
CFBP **1731**	*Zea* sp		1731	Bradbury
Pseudomonas corrugata				
CFBP **2431**				

Table 1 : bacterial reference strains used in the internal (WP1) and the external (WP3) studies

Task 1.3: Comparing identification tests: IF, ELISA, PCR, Q-PCR, pathogenicity test.

The major methods described in the EPPO protocol, especially those which are used in routine analyses in the EU countries, were evaluated in order to determine their main performance criteria: sensitivity, specificity and repeatability.

Moreover, the second year of the project, AGES primers for conventional PCR were tested in order to compare performance criteria with the currently –recommended primers of Coplin & Majerczak (2002).

Table 2 shows all the tested methods as well as the partners involved in each evaluation.

WP1 Tested methods	**Partners involved**
Pathogenicity test	FR
NBY medium	DE
Fatty acid profiles	UK
IF (Loewe / Linaris)	FR/UK/DE/TR
ELISA (AGDIA)	FR/UK/TR
PCR test (Coplin et. al.)	FR /TR
PCR test (Hufnagl-AGES)	DE/AU/FR
Q-PCR (Tambong et al.)	UK
Q-PCR (Thwaites)	UK

Table 2 : evaluated methods in WP1 (internal evaluation)

Pathogenicity test

In spring 2009, each of the 15 Pss strains were inoculated into two sweet corn plants in a climatic chamber in EPPO-recommended conditions (24-48h / 25-27℃ / 80-100% humidity) under which pathogenic strains would develop wilt symptoms. Isolation on media was performed from symptomatic plants and identification of typical isolates was carried out using IF.

Nutrient Broth Yeast extract medium (NBY)

15 target strains of *Pantoea stew*. ssp. *stewartii* and 15 non target strains were plated on NBY medium. The recipe of the medium was as follows (composition per 900 ml):
Agar ……..….12,0g
Nutrient broth…….8,0g
Yeast extract……..2,0g
K_2HPO_4……..2,0g
KH_2PO_4…….0,5g
Glucose solution…..50,0ml
Mg SO_4 $7H_2O$…….50,0ml

After incubation of the strains for 48 h at 25 ℃ (+/- 3℃) typical colonies were selected and identified by IF.

Bacterial suspensions were made in 10mM PB and were adjusted to an optical density of 0.15 till 0.16 at 600 nm. IF- test was performed (1000x magnification) and 40 microscopic fields were examined.

The antibody used was the monoclonal from Linaris Kat.-Nr. ASC 52000; Batch No A7403; dilution 1: 150 of ascites fluid and the conjugate was the Sigma anti mouse IGG; Nr. F9137; dilution 1:200.

Fatty acid profiles, IF, ELISA, PCR test (IG1/IG2, ES16/IG2, HRP, CPS)

The partners involved in either WP, followed the protocols described in the EPPO protocol (EPPO, 2004).

AGES- Hufnagl PCR (Gottsberger *et al.* in preparation)

Specific primers designed on the 16S rRNA by Peter Hufnagl at the AGES (Austria) were evaluated as an alternative method to detect/identify the bacteria.

Q PCRs

The primer and probe sequences were as follows:

Richard Thwaites (Fera) in-house assay:
stew1F: ttatccgcgcgttcgtaaa
stew1R: gggacgagagagaatggtgtttt
Probe: stew1: ccggccttcgcgatccagc

Tambong et al (2008) :
cps-RT74F: tgctgattttaagttttgcta
cps-177R: aagatgagcgaggtcaggata
Probe: cps-133: tcgggttcacgtctgtccaact

The thermal cycling conditions for both were an initial denaturation of templates at 95℃ for 15 min followed by 50 cycles at 95℃ for 1 s and 60℃ for 1 min.

Results

Pathogenicity test

Results were not reproducible at all. Some strains were highly aggressive whereas others were not clearly pathogenic. It was concluded that further testing with more recently isolated strains was required.

Other tests

Table 3 presents all results and the performance criteria obtained by the panel,

Methods	Detection threshold	Sensitivity	Specificity	Repeatability	Accuracy
NBY	[10^2 – 10^4]	100%	0%	100%	50%
IF (Loewe)	10^3	100%	96,2%	100%	98,2%
IF (Linaris)	10^3-10^4	100%	100%	100%	100%
ELISA (Agdia)	10^5- 10^6	100%	100%	100%	100%
Fatty acid profiles	-	93.3%	92.8%	Only one repeat	93,1%
PCR_ ES16/IG2 (Coplin)	10^4	62 %	*(100%)*	97%	80%
PCR _HRP (Coplin)	10^6	58,9%	*(100%)*	98%	78%
PCR _IG1/IG2 (Coplin)	-	-	-	-	-
PCR _CPS (Coplin)	-	-	-	-	-
Hufnagl (AGES)	10^2	100%	*(99%)*	-	-
PCR real time (Tambong)	10^4	100%	92.9%	100%	96,4 %
PCR real time in house (Fera)	10^4	100%	81%	98%	94 %

Table 3 : performance criteria of the main detection and identification methods

The best performance criteria are highlighted in yellow. As expected, the NBY medium which is a non-specific medium, gave false positive results with all non-target strains able to grow on it. However, the detection threshold was the most sensitive of all tests being estimated between 10^2 and 10^4 bacteria per milliliter. Further discrimination between target and non target using an serological or molecular identification technique is required.

IF gave two false positive results with *E. amylovora* and *P.corrugata* using the antibody of Loewe (one repeat). Using the antibody of Linaris the IF was 100 % sensitive and specific with a very good detection threshold. .

ELISA is 100% sensitive and 100% specific but the detection threshold is not very convenient for routine analyses, only for identification use.

Disappointing results were obtained using conventional PCR: HRP and ES16/IG2 primers gave some positive results but weren't very sensitive, respectively 30% and 29 %. Moreover, in one laboratory, HRP and ES16 / IG2 primers didn't give any results at all, reducing the reproductibility and robustness scores.

CPS and IG1/IG2 primers failed to give results in both France and Turkey, where they were evaluated.

All *Pss* isolates tested in WP1 were detected by the Hufnagl primers with a sensitivity of 100% and a detection threshold of 100 cells/ml. The specificity wasn't tested on the whole collection of non target strains but DNA from several *Pantoea stewartii* and other bacteria including *Pantoea stewartii* ssp. *indologenes* (Psi) had been tested by France and Austria. Only Psi was detected as a Pss, giving a 99% specificity which cannot be compared with the other specificity due to the incomplete test of the non target strains.

Regarding real time PCR, we obtained one false positive results with Tambong primers (*P. stewartii ssp. indologenes*) and several false positive results with in-house Fera primers (*P. stewartii ssp. indologenes*, *P. agglomerans*, *E. amylovora* (2 repeats)). Tambong primers seemed to be more accurate than those of the Fera method as they do not detect *Erwinia amylovora* as a *Pantoea stewartii* ssp. *stewartii*.

Regarding detection threshold, the best methods are isolation on media, IF (Loewe) and Hufnagl primers that allowed the detection of Pss with sensitivity between 100 and 1000 cells per ml.

Acceptable sensitivity (or inclusivity) was obtained by all methods except fatty acid profile and Coplin's PCR.

The best specificity was obtained with Linaris antiserum for IF and ELISA.

The **qualitative repeatability**, also known as the **accordance** according to the NF/EN/ISO 16140 standard, is the percentage of chance to obtain identical results (i.e. both positive and both negative) for two identical subsamples analysed by the same operator of the same laboratory, using the same material and consumables. All the methods showed good repeatability between 97% and 100%.

According to ISO 16140, the r**elative accuracy** is the proportion of agreement between the results obtained with a tested method and expected results on identical samples. The more a method is accurate, the less false positive or false negative results it has.

The final classification of accuracy of the methods (more accurate to less accurate) and detection theshold was as follows:

IF (Linaris)
ELISA
Real time PCR
Fatty acid profile
Conventional PCR

Work package 2

Providing samples

WP 2 Objectives

To get different contaminated seed lots to perform the ring test (WP3) with stable and homogeneous contamination levels.

WP 2 Participants

WP coordinator: Partner 1

WP 2 Tasks

Task 2.1: Active search of naturally infected seeds
Task 2.2: Production of artificially contaminated seeds
Task 2.3: Homogeneity tests and stability tests of the samples

WP 2 Methods and Results

Task 2.1: Active search of naturally infected seeds

To provide positive and negative samples for internal method evaluation and ring test, two different kinds of samples can be used: naturally contaminated samples and artificially contaminated samples. The first ones are mainly used to test extraction techniques and robustness of the methods in routine analyses conditions, and the second ones are used to evaluate the methods as they can be homogeneous and well determined regarding their infection rate.

Sources of naturally contaminated seed lots and negative seed lots of susceptible corn varieties were explored. However**,** due to the healthy status of EU against *Pantoea stewartii* ssp. *stewartii,* no naturally infected seeds were obtained. Although good contacts were established with Charles Block (USA), it was not possible to obtain naturally contaminated seed lots. However, healthy sweet corn seeds of a Pss-sensitive cultivar were received from USA and were used for pathogenicity tests.

Comparison of the different techniques for Pss detection was therefore done using artificially contaminated samples. Comparison of inoculation methods was conducted in the LNPV (Fr) from January to June 2009.

Task 2.2: Production of artificially contaminated seeds

Methods

Artificially contaminated samples:
Three techniques were tested: injection into imbibed seeds, contamination of seeds under vacuum and contamination of seed washings (soaks). Bacterial suspensions prepared from target and non target strains are presented in the table 4.

	Type of inoculation	Bacterial strains	Concentration	Volume of inoculation
Contamination of seeds	Injection	Pantoea stewartii subsp. stewartii CFBP 3167	10^8	3 ml
		Pantoea stewartii subsp. stewartii CFBP 3167	10^6	2 ml
		Pantoea stewartii subsp. stewartii CFBP 3167	10^3	2 ml
		Pantoea stewartii subsp. indologenes CFBP 3614 (Psi)	10^8	3 ml
		Saprophyte n°3	10^8	3 ml
	Contamination under vacuum	Pantoea stewartii subsp. stewartii CFBP 3167	10^6	12 ml
Contamination of soaks		Pantoea stewartii subsp. stewartii CFBP 3167	10^8	7 ml
		Pantoea stewartii subsp. stewartii CFBP 3169	10^6	7 ml
		Pantoea stewartii subsp. stewartii CFBP 3169	10^5	7 ml
		Pantoea stewartii subsp. stewartii CFBP 3169	10^4	7 ml
		Pantoea stewartii subsp. stewartii CFBP 3169	10^3	7 ml
		Pantoea stewartii subsp. indologenes CFBP 3614	10^8	7 ml
		Pseudomonas corrugata LNPV 2.29 (Pc)	10^8	7 ml

Table 4: bacterial suspension used in artificially contaminated samples

Saprophyte n°3 is a bacterium isolated from maize seeds for which morphological parameters were very close to those of *Pantoea stewartii* ssp. *stewartii* on King B medium.

60 maize seeds were soaked in sterile water over night at 4°C.

Injection in imbibed seeds

100 µl of the bacterial suspensions were injected into 50 seeds with a 1 ml needle. Seeds were put on absorbent paper under a laminar flow hood during 5 hours then put at 4°C over night.

Contamination under vacuum

The remaining 10 seeds were placed in 12 ml bacterial suspension of *Pantoea stewartii* ssp. *stewartii* under vacuum during 2 to 4 minutes while stirring.
Seeds were then drained in a filter and dried on absorbent paper under a laminar flow hood during 5 hours then put at + 4°C over night.

Seed lots were made with 5 artificially contaminated seeds added to 100 seeds. Each seed lot was tested by isolation on media after and by direct IF.

Contamination of soaks: 35g of healthy maize seeds (lot E2009.PL5.0.0121 from GNIS) were soaked with 70 ml (two times weight of seeds) of PBS over night at + 4°C. After contamination by a bacterial suspension to obtain several final concentration per group of seeds, seeds were then stirred at 200 rpm at room temperature for 10 to 15 minutes. 20 ml of the soak were centrifuged at 10 000 g for 10 minutes.

The 20 ml supernatant was discarded and 2 ml of saline solution was added to the pellet. The extracts were inactivated in a water bath at 70°C for 15 minutes and then placed at room temperature for gradual cooling.

Freeze-drying: Freeze-drying of Pss contaminated corn soaks were performed in November and were kept at room temperature. The methodology was the following:

1.Performing the extracts :

- Addition of 60 ml of sterile water per sample of 100 corn seeds; soaking overnight.
- Contamination of soaks by serial tenfold dilutions of a suspension of Pss (CFBP 3167) to obtain a final concentration from 10^5cfu/ml to 1 cfu/ml.
- No contamination of a seed soak (seeds-negative control)
- Negative control: water
- Positive control : strain in water (2 repeats)

2. Preparation of tubes for freeze-drying : For 1ml : 0.25ml of horse antiserum; 0.25 ml of nutrient broth; 0.5 ml of seed extracts.

3. Freeze-drying

Six different levels of freeze dryed infected soak were hydrated in King 's B broth and plated on YPGA and King 's B media.

Results

No reproducible results were obtained on the seeds (see tables 5 to 8).

The contamination was successful at a maximum of 60%, so it was difficult to determine whether a seed lot was contaminated or not. It was not possible to obtain homogeneously infected seed lots for the ring-test. All the seed lots were negative by isolation on media but positive by IF.

		Nb of contaminated seeds out of 10	
Type of inoculation	**Lots**	**IF**	**Isolation on media**
Injection	**Pss 10^3**	6	0
	Pss 10^6	10	6
	Pss 10^8	10	6
	Psi 10^8	-	10
	Sapro n°3 10^8	-	5
under vacuum	**Pss 10^6**	9	1

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Table 5 : Contamination of seeds : number of contaminated seeds out of 10

		Rate of bacteria per seed		Survival rate of bacteria
Type of inoculation	**Lots**	**IF**	**Isolation on media**	**Isolation on media / IF**
Injection	**Pss 10^3**	$4{,}2.10^3$	< 20	<0,5%
	Pss 10^6	$1{,}8.10^4$	72	0,40%
	Pss 10^8	2.10^5	5.10^4	25%
	Psi 10^8	-	740	-
	Sapro n°3 10^8	-	6.10^4	-
under vacuum	**Pss 10^6**	6.10^3	21	0,35%

Table 6 : Contamination of seeds : rate of bacteria per seed (en cfu/seeds)

The survival rate of Pss, 3 days after contamination was between 0.5 % and 25 %, which was too low. Moreover, the bacteria didn't survive at all over a longer period: no living bacteria were detected after one week, although dead cells could still be detected by IF.

	Inoculum (ufc / mL)	Expected result before soak overnight at 4°C	Testing of soak before overnight soak at 4°C		Testing of soak after overnight soak at 4°C		Testing of soak after centrifugation		Testing of soak after inactivation		Testing of soak after week + 1
			Isolation	IF	Isolation	IF	Isolation	IF	Isolation	IF	IF
Pc 2.29 à 10^8	$1,2.10^8$	$1,2.10^6$	$1,3.10^6$	0	$2,6.10^4$	0	$3,1.10^5$	0	0	0	0
Psi 3614 à 10^8	$5,7.10^8$	$5,7.10^5$	$7,9.10^6$	0	2.10^7	0	$3,2.10^8$	0	0	0	0
Pss 3167 à 10^8	$1,3.10^8$	$1,3.10^6$	$1,2.10^7$	$1,5.10^7$	$9,7.10^6$	$4,6.10^7$	$3,3.10^8$	$5,1.10^8$	$3,2.10^7$	5.10^7	TMTC
Pss 3169 à 10^6	$1,1.10^7$	$1,1.10^5$	$5,2.10^5$	$1,2.10^7$	$1,5.10^6$	$1,1.10^7$	$7,7.10^6$	$1,6.10^7$	0	TMTC	TMTC
Pss 3169 à 10^5	$1,1.10^6$	$1,1.10^4$	$6,9.10^4$	$2,3.10^6$	$1,5.10^6$	$1,2.10^6$	$2,4.10^6$	$7,7.10^5$	0	TMTC	TMTC
Pss 3169 à 10^4	$1,1.10^5$	$1,1.10^3$	$5,6.10^3$	$1,5.10^5$	$9,1.10^3$	$4,1.10^4$	$1,3.10^5$	$7,7.10^5$	0	$3,2.10^5$	4.10^4
Pss 3169 à 10^3	$1,1.10^4$	$1,1.10^2$	$1,0.10^3$	$1,3.10^4$	$1,0.10^3$	$3,8.10^4$	$1,0.10^4$	6.10^3	0	$2,1.10^3$	$2,7.10^3$

TMTC: too much saprophyte colonies to count

Table 7 : Contamination of soaks : rate if bacteria per soak (en cfu/mL)

Contaminated soak by freeze-drying assay

A good recovery of the bacteria was observed on media, better on King 's B than on YPGA.

SEED EXTRACTS	Theorical concentration	Obtained results	comment
A7	10^5cfu/mL	6.10^2cfu/mL	Problem of decreasing
A6	10^4cfu/mL	2.10^4cfu/mL	Conform results
A5	10^3cfu/mL	3.10^3cfu/mL	
A4	10^2cfu/mL	4.10^2cfu/mL	
A3	10^1cfu/mL	6.10^1cfu/mL	
A2	1cfu/mL	<20 cfu/mL	Below the detection threshold
A-	0	0	
NEG	0	0	
POS	+	+	

Table 8. Results of control by analyses after 5 months at room temperature

Comparison of methods to get contaminated seeds.

	Advantages	Disadvantages
Naturally infected seed lots	Possibility to test extraction techniques and robustness of the methods in routine analyses conditions	Very difficult to obtain
injection seed by seed	higher survival rate than vacuum a few days after contamination	it is difficult to calibrate it because of the manual intervention effect. Fastidious and time consuming process. No survival in time.
vacuum technique	useful to evaluate the methods as they can be homogeneous and well determined regarding their infection rate easy to contaminate a large number of seeds in homogeneous way	mortality of the Pss
Contamination of soaks	useful to evaluate the methods as they can be homogeneous and well determined regarding their infection rate	Need to inactivate the soaks : inconvenient for methods that detect living bacteria
freeze-drying assay of soak	useful to evaluate the methods as they can be homogeneous and well determined regarding their infection rate survival of the bacteria several months after contamination. Good recovery rate on non-selective media.	add one step in the samples making process for the organizer lab and increase the uncertainty of the homogeneity. The hydration is done in a broth and the components of this broth could interfere with the different detection techniques such as PCR or IF. This technique appears to be well adapted to isolation on non selective media only.

Table 9 : advantages and disadvantages of the different methods of providing contaminated seed lots

As the preliminary experiments showed that it was difficult to make homogeneously infected samples by artificially contaminated seeds and as the time left for the panel work was short, it was decided to provide artificially contaminated seed extracts (soaks) as samples for the ring test.

The main problem with artificially contaminated extract is that the analyses have to be carried out as soon as the samples arrive in the laboratories. As none of the three selected detection methods required the detection of living bacteria, it was decided that heat inactivation would be carried out after contamination of the extracts in order to ensure conservation of the original inoculum concentration.

Work package 3

Ring test

WP 3 Objectives

To get different contaminated seed lots to perform the ring test (WP3) with a stable and homogeneous contamination.

WP 3 Participants

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

WP 3 Tasks

Task 3.1: selection of techniques to be ring tested
Task 3.2: ring test evaluation of the selected techniques

WP 3 Methods and Results

Task 3.1: selection of techniques to be ring tested

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. The selected techniques were those previously evaluated by partners 1, 2 and 3. The samples were prepared by partner 1 and sent to the four other partners (2, 3, 4 and 5).

The aim of this ring test was to check reliability of the selected assays for detection of a range of concentrations of *Pantoea stewartii* ssp *stewartii.* Classical PCR tests were done in combination with several DNA extractions. Additionally, real-time PCR was tested.

The following tests were selected for ring-testing:

Tested methods	**Partners involved**
IF (Loewe)	FR/TR/AT
IF (Linaris)	UK/ DE
PCR test (Hufnagl's primers)	FR /TR/UK/DE/AT
Q-PCR (Tambong et al, 2008)	FR /TR/UK/DE/AT

Table 10: evaluated methods in WP3 (ring test)

Task 3.2: ring test evaluation of the selected techniques

Final preparation of seed samples for the ring test

Artificially contaminated seeds lots were prepared by partner one and send to the four other partners.

Methods

Samples and controls sent in WP3 are described in the tables 11 and 12.

Negative samples: 2 repeats / soak / lab The choice of no-target was defined by the evaluation data on specificity carried out by AGES, JKI, FERA and LNPV	Healthy seed soak (one seed soak) Artificial contamination with no-Pss: ✓ *P. stewartii* ssp. *indologenes* (one seed soak) ✓ *Pseudomonas corrugata* (one seed soak) ✓ corn seed saprophyte 3 (one seed soak) ✓ corn seed saprophyte 12 (one seed soak)
Positive samples: 2 repeats / soak / lab The choice of target concentrations was defined by the evaluation data on sensitivity carried out by AGES, JKI, FERA and LNPV	Ten samples were contaminated with different concentrations of the following strains of *P. stewartii* subsp *stewartii* : ✓ CFBP 3169 ✓ CFBP 3396 ✓ type-CFBP 3167
Number of samples per lab	Each sample was analysed with the 3 methods / lab. The labs received thirty slides and thirty inactivated seed extracts. They obtain 90 final results (30 IF, 30 regular PCR, 30 RT PCR).
Codification of subsamples	Each sample received a code for slide and a second code for extracts for PCR (see appendix 3).

Table 11: Positive and negative samples used in the ring test (WP3)

Technique to test	**Positive controls**	**Negative controls**
IF	IF Pss positive slides Provided by LNPV	1.IF Ewinia positive slides Provided by LNPV 2. Sterile water Included by each laboratory
All PCR	Pss DNA extract Provided by LNPV	Sterile water Included by each laboratory

Table 12: Positive and negative controls used in the ring test (WP3)

Five packages were sent to the 5 European laboratories including LNPV (France) on 16th of June.

All the results were received in August and September. The data analysis was conducted in October and the first draft of the final report was drafted in the beginning of November.

Results

Details of all data and full-analysis are in the ring test report (Collaborative study for detection of *Pantoea stewartii* ssp. *stewartii*). Only summarised results are presented below.

Sensitivity / specificity / accuracy

These performance criteria are presented in table 13.

Performance criteria (%)	IF	Reg PCR Hufnagl Without DNA extraction	RT PCR Tambong Without DNA extraction
Relative Sensitivity	82 *(92,5 Linaris; 75 Loewe)*	70	96
Relative Specificity	58 *(95 Linaris; 33 Loewe)*	76	78
Relative Accuracy	74 *(93 Linaris; 61 Loewe)*	72	90

Table 13: determination of relative sensitivity, specificity and accuracy:

The best sensitivity was obtained by the real-time PCR and the lowest by conventional PCR. It has to be considered that the Hufnagl-AGES protocol was written for identification test for bacterial colonies: 30 cycles of PCR were sufficient for that purpose. To increase the sensitivity, two laboratories performed extra analyses after 35 and 40 cycles. The ring test report data show an increase of the sensitivity (65% to 100%) and a decrease of the specificity (80 to 50%) at only one laboratory. The best results of specificity are obtained by both PCRs even if the cross-reactions with *Ps ssp. indologenes* are confirmed. The lowest specificity was obtained with the IF method, but a large difference between the two sources of antibodies was observed. Indeed, two laboratories used Linaris antisera and obtained 92,5% of sensitivity and 95% of specificity. On the contrary, 3 laboratories used Loewe antisera and obtained respectively 75% and 33% for these criteria. So, immunoflorescence with Linaris antiserum gave best results although these results were obtained in only 2 laboratories. Sensitivity was slightly lower than with real-time PCR and the specificity was better than that obtained with the PCR tests. Nevertheless, cross reactions with *P.corrugata* but not with *Ps ssp. Indologenes* were observed with IF.

In conclusion, the best relative accuracy was obtained with the real time PCR by the Tambong primers (90%) and the immunofluorescence with the Linaris antiserum (93%).

Detection threshold

The results of detection threshold presented in table 14 did not confirm completely the detection threshold previously obtained with pure strains. It could be explained by the presence of many bacteria and debris within the corn seed soak that obscures the reading of IF slides, and that PCR inhibitors were extracted from the seeds.

Detection threshold, for α = 1%	IF	Regular PCR Without DNA extraction	Real Time PCR Without DNA extraction
q = 99%	7.10^4	7.10^5	2.10^4
q = 95%	$<7.10^3$	7.10^4	$<7.10^3$

Table 14: determinations of the detection threshold depending of the methods

Accuracy

Table 15 shows accuracy results. Immunofluorescence with Linaris and real-time PCR with Tambong primers were accurate at 99% and at 95% (in one laboratory each for IF; in 2 laboratories each for RT-PCR). RT-PCR was accurate too at 90% in one laboratory. With the regular PCR and IF using Loewe antiserum, the accuracy was lower than 90%.

Number of labs	IF	Regular PCR Without DNA extraction	Real Time PCR Without DNA extraction
Accuracy at 99%	1lab (Linaris)	0	2 labs
Accuracy at 95%	1 lab (Linaris)	0	2 labs
Accuracy at 90%	0	0	1 lab
Accuracy <90%	3 labs (Loewe)	5 labs	0

Table 15: results of accuracy of methods used in each lab

Repeatability

The repeatability obtained by the different laboratories are presented in table 16.

Repeatability (%)	Code of lab	Protocols		
		IF	Regular PCR H	R-time PCR T
Per lab	Lab 1	97	97	90
	Lab 2	97	97	97
	Lab 3	80	93	100
	Lab 4	73	93	100
	Lab 5	83	95	97
Qualitative repeatability		**86%**	**95%**	**97%**

Table 16: results of qualitative repeatability (accordance) depending of the protocols, per lab and for all labs (global)

In this study, the best results of qualitative repeatability were obtained with the PCR methods. With IF, laboratories using Linaris antiserum gave best repeatability results (97% both) than laboratories using Loewe (80%, 73%, 83%).

Accordance

The best percentage of qualitative reproducibility was obtained firstly with the real-time PCR, secondly with IF and finally with regular PCR (see table 17). As high percentages for qualitative reproducibility supported the reliability of the protocol, real-time PCR with Tambong primers was concluded to be the most reliable protocol of this ring test.

Reproducibility (%)	Lab	Protocols		
		IF	Regular PCR H	R-time PCR T
Qualitative reproducibility		**65%**	**58%**	**87%**

Table 17: results of qualitative reproducibility (concordance) depending of the protocols:

Main conclusions, discussion of results and their reliability

WP1 : internal validation

For each method evaluated in the project most of the different performance criteria are now available: detection threshold, sensitivity, specificity, repeatability, accuracy, reproducibility.
As the results of internal validation showed that better performance criteria were obtained with IF, Hufnagl primers and real time PCR (Tambong et al.), it was decided to compare these three methods in the ring test.

Results for each individual performance criterion must be weighted against the others. For example, ELISA is very accurate but the detection threshold is insufficient. Knowledge about the performance criteria of the different methods will allow laboratories to choose one method from another depending on its intended use.

WP2 : samples providing

The only practical way to compare the different techniques of Pss detection was to obtain artificially contaminated samples. Preliminary experiments on homogeneity and stability showed that it was difficult to make homogeneous samples by artificially contaminating seeds. As time was limited, it was decided to provide artificially contaminated seed extracts as samples for the ring test.

WP3 : external validation

The collaborative study allowed the determination of performance criteria on artificially contaminated seed extracts and the comparison of 3 protocols. The best results of accuracy, repeatability and reproducibility were obtained by real-time PCR using the primers of Tambong *et al.* (2008).

Concerning specificity, the problem remains cross reaction with *Pantoea stewartii* ssp. *indologenes*. None of the tested methods was able to overcome this with the exception of IF with Linaris antiserum.

Immunofluorescence protocol gave very good results of accuracy (sensitivity and specificity) with the monoclonal antibodies from Linaris. Unfortunately, only 2 laboratories performed the ring test with this antiserum.
It would be interesting that the 3 other laboratories test again extracts of the ring test with Linaris antibodies. As we prepared more extracts than necessary, this possibility will be proposed to the different partners. On the contrary, the results obtained with the polyclonal Loewe antibodies were not satisfactory. This antiserum is not therefore recommended to detect *Pantoea stewartii* ssp*. stewartii*.

Detection of Pss with conventional PCR with the Hufnagl-AGES primers was less accurate but two improvements to this protocol were suggested. First, an increase of the number of PCR cycles could improve the sensitivity without decreasing too much the specificity, although it was confirmed in the ring test that *Pantoea stewartii* ssp. *indologenes* gave false-positive reactions with the Hufnagl primers. Another way would be to improve DNA extraction methods. Two labs obtained better results with DNA extraction than without in this ring test.

Unfortunately, methods for bacteria extraction from seeds could not be studied in this ring test due to the lack of naturally infected seed samples.

In conclusion, the results obtained with this collaborative study can improve the detection scheme of *Pantoea stewartii* ssp*. stewartii* from maize seeds. However it will be important to validate the different protocols on naturally contaminated samples.

Usability of results and future work

Main conclusions

The project achieved its aim to produce evaluation data of currently- used detection and identification methods for *Pantoea stewartii* ssp. *stewartii*. It has also provided useful data on new real time PCR methods (Tambong *et* al.).

The establishment of different performance criteria allowed prioritization of available detection and identification methods. This is important for accurate and efficient diagnosis and management of *Pantoea stewartii* ssp.*stewartii* within Europe.

The project has enabled the transfer to all participants of the Tambong real time PCR and IF with the Linaris antiserum as very accurate detection methods.

Some further work will be required to further test this method in analyses conditions with naturally infected seeds.

Implication for stakeholders

Phytosanitary authorities: Novel methods available for detection of *P.stewartii ssp. stewartii* 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). Before that, validation will have to be done on contaminated seeds.

Scientists: 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 *P. stewartii* and could be suitable for epidemiological studies in combination with existing methods (isolation on media).

Further research needed and continued collaboration:
It is expected that further progress will be made in improving existing or developing new methods on naturally infected seeds. In particular, Pss seed extraction step has not been evaluated in this study.

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 WP1
Meeting talks and posters (total 2)

Visage M., Paillard S., Olivier V, Poliakoff F., Soubelet H., 2009. Une coopération européenne pour la validation de protocoles de détection et d'identification de *Pantoea stewartii* subsp. *stewartii* sur semences de maïs. (poster) 9ème conférence internationale sur les maladies des plantes, AFPP, Tours, France, 8-9 décembre 2009.

Visage M., Paillard S., Olivier V, Poliakoff F., Soubelet H (2010) Evaluation interne des méthodes de détection de *Pantoea stewartii subsp. stewartii* sur semences de maïs par plusieurs pays européens. (poster) Rencontres plantes-bactéries d'Aussois. Janvier 2010.

Publications (total 0)

Output WP2
Meeting talks and posters (total 0)

Publications (total 1)

Gottsberger RA, Müller P, Soubelet H, Stöger A, Ruppitsch W, Hufnagl P. (in preparation) Detection and identification of *Pantoea stewartii* subsp. *stewartii* the bacterial wilt agent from maize seeds by conventional PCR. (working title)

Output WP3
Meeting talks and posters (total 1)

Olivier V, Visage M., Paillard S., Poliakoff F., Soubelet H. (2010) Evaluation de méthodes de détection du *Pantoea stewartii* subsp. *stewartii.* (meeting talk) Rencontres plantes-bactéries d'Aussois. Janvier 2010.

Publications (total 0)

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The project was led by **France** as project coordinator with the following countries as consortium partners: **Austria, Germany, Turkey and the United Kingdom**. In April/May, 2009, EUPHRESCO observer countries and non-EUPHRESCO countries were contracted to determine interest in participating. Several countries were contacted (Estonia, Greece, Hungary, Lithuania, Malta, Portugal). Only **Hungary** accepted to be associated in the project as an observer.
In the middle of the project one laboratory from The Netherlands announced its intention to be part of the ring test but due to the agenda deadline, unfortunately, this wasn't possible.

Data collection and diffusion

The discussions were mainly conducted by e-mail to collect information and points of view from all project participants, one meeting was organised in the middle of the project (April 2008) to take strategic decisions about the ring test: methods to be studied and time schedule. One global report with 6 appendixes was produced and distributed to enable the collection and return of data to the LNPV.