WP5
Asymptomatic infections of *E. amylovora* and environmental fitness

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WP5 Problem

- *E. amylovora* exhibits different **survival strategies** to face stress environmental or plant conditions
- Many aspects of its life cycle remain unclear
- Its ability to survive in plants and to produce **latent infections** can lead to false negative results
- It is essential for giving scientific support to phytosanitary decisions:
  - the determination of survival mechanisms displayed by this pathogen under different situations
  - their involvement in fire blight epidemiology
  - the optimization of protocols for *E. amylovora* detection in the environment and asymptomatic plant material

WP5 Objective

Evaluate the presence and the physiological status of *E. amylovora* in asymptomatic plant material or under stress conditions

- Optimization of detection methods
- Implications in fire blight epidemiology and phytosanitary decision-making

WP5 Approach

1. Perform surveys to determine epidemiological significance of asymptomatic infection and/or survival strategies of *E. amylovora*

- Harvest different sets of plant material from naturally and severely affected trees by fire blight (as a control) and asymptomatic plant material from close asymptomatic trees
- Analyze them by cultural and molecular methods

2. Asses differential expression of *E. amylovora* cells under stress conditions by high-throughput RNA-seq technologies to determine if survival strategies adopted by the pathogen have a genetic base

This part is under work

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1a. EPPO SURVEYS TO DETECT *E. amylovora* IN ASYMPTOMATIC PLANTS

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

- **Panel on Diagnostics in Bacteriology (Sofia, 2011):**
  survey organized on the sampling and testing procedures for asymptomatic material.
- **A questionnaire developed by IVIA, ES and posted online on April 2012.**

Sampling procedure for testing PM 7/20 (2004)

- **6 laboratories (Belgium, the Netherlands, Portugal, Slovakia, Spain and United Kingdom) perform both single plant analyses and composite samples.**
- **4 laboratories (Austria, Bulgaria, the Czech Republic and Switzerland) perform single plant analyses only.**
- **3 laboratories (France, Lithuania and Slovenia) perform composite sample analysis only.**

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

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Techniques used for testing asymptomatic samples

TECHNIQUE	Nº LABS	REFERENCE
Isolation	8	*Austria, France, the Netherlands, Portugal, Spain, United Kingdom, Bulgaria, Czech Republic*
Enrichment isolation	9	*Austria, Portugal, Slovenia, Spain, Czech Republic, Belgium, Lithuania, The Nethelands, Slovakia*
Immunofluorescence	7	*Czech Republic, France, United Kingdom, Bulgaria, Lithuania, The Netherlands, Slovakia*
Enrichment-DASI- ELISA	1	*Spain*
PCR	7	*Czech Republic, Spain, Austria, Belgium, The Netherlands, Portugal, Slovakia*
Enrichment-PCR	4	*Portugal, Lithuania, Slovakia, Spain*
Real-time PCR	6	*Austria, Lithuania, Slovakia, Spain*
Bioassay	7 +(1[2])	*Austria, Belgium, Portugal, Bulgaria, Czech Republic, Lithuania, The Netherlands, Slovakia*
Other	3	*Belgium, Czech Republic, The Netherlands*

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

Country of the respondent	2007	2008	2009	2010	2011
Austria	2095/3345	2369/4495	2567/3918	2145/5839	1220/4936
Belgium	0/0	0/0	3/97	0/139	0/184
Bulgaria	73/491	6/360	2/569	1/349	0/224
Czech Republic	4/31	7/53	12/68	13/155	17/141
France	0/62	0/50	0/179	0/211	0/220
Lithuania	54/334	3/273	1/173	0/130	0/2
Netherlands	1/640	1/680	2/652	12/646	6/660
Portugal	0/200	0/200	0/0	11/26	7/112
Slovakia	18/203	7/162	1/45	10/68	3/99
Slovenia	1/27	1/30	0/30	0/34	0/52
Spain	0/1376	0/2385	0/5115	4/2529	39/6164
Switzerland	1/1	1/1	1/1	1/1	1/1
United Kingdom	3/83	21/172	0/53	1/38	5/113

E. amylovora positive samples

Laboratory	Techniques utilised for screening	Type of sample analysed	Composition of sample	Nº of plants	Protocol used for extraction from plant material
Austria	PCR	blossom, shoots, buds, rootstocks, canker, bees	single		PM 7/20
Belgium	Isolation	buds	single & composite	3	200 buds are taken on each sample (100 twigs per sample or type plant).
Bulgaria	PM 7/20	blossom, shoots, buds, twigs leaves, green fruits, trunk	single		PM 7/20
Czech Republic	Isolation	shoots	single		PM 7/20
Lithuania	IF and PCR	blossom, shoots, twigs	single & composite	30	According to PM 7/20, appendix II.
Netherlands	Isolation	shoots	composite	100	modified version of PM 7/20
Portugal	Isolation, Nested PCR	blossom, shoots, buds	single & composite	4	PM 7/20
Slovakia	IF, PCR, Enrichment PCR	blossom, shoots, buds	single & composite	100	A. Extraction from blossom and buds (or single shoots samples). B. Extraction from shoots (composite samples of 100 plants).
Slovenia	Enrichment isolation, Enrichment-real time PCR	blossom, shoots	composite	100	EPPO 1992 and 2004 with minor modifications
Spain	PM 7/20	shoots	single & composite	5	PM 7/20,2004
Switzerland	Plating on KB/NSA, Immunoassay	blossom, shoots, buds, woody tissue, cankers	single		
United Kingdom	Isolation and IF	Twigs	single & composite	30	PM 7/20(1)

In several EPPO countries, *E. amylovora* has been detected in asymptomatic plants using different techniques

1 b. Protocol for evaluation of different numbers of samples for the analysis of asymptomatic plants for *E. amylovora*

Preparation and analyses of SPIKED SAMPLES

4 g healthy plant material in 200 ml of antioxidant buffer (EPPO 2013)

⇩

Add bacterial suspensions of *E. amylovora* strain to 25 ml extracts
(final concentrations: 10, 10^2, 10^3, 10^4, 10^5 and 10^6 cfu/ml)

⇩

Isolation and techniques of EPPO protocol
Keep 5 ml at - 20ºC, and add 30% glycerol to the remaining amount for bulk samples

⇩

Enrichment in King's B and CCT media

⇩

Isolation on CCT, ELISA, Real time-PCR or Conventional PCR

⇩

Calculate the sensitivity of the protocol in each country
(Individual samples)

Euphresco Phytosanitary ERA-NET

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Preparation and analyses of BULK SAMPLES

0.1 g of the same type of plant material from each 2, 4, 9 or 19 healthy plants of the same host (samples b1, b2, b3 or b4)

⇩

Add 6.5ml of each of the positive defrosted samples

⇩

Enrichment in King's B and CCT media

⇩

Isolation on CCT, ELISA, Real time-PCR or Conventional PCR

⇩

Calculate the sensitivity of the protocol with the 3, 5, 10 or 20 samples of plant material

SPIKED SAMPLES

Defrost samples that gave positive result

⇦

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Results

IVIA – Assay 1
Pear Conference

Spiked samples

		Neg	10^2	10^3	10^4	10^5	10^6	10^7	Ea control
Isolation		-	+	+	+	+	+	+	+
E-ELISA-DASI	KB	-	-	-	-	-	-	-	+
	CCT	-	+	+	+	+	+	+	+
rt-PCR (Pirc et al, 2009)		-	-	-	+	+	+	+	+

Sensitivity: 10^2 cfu/ml for isolation and ELISA DASI (after enrichment in CCT)

10^4 cfu/ml for real-time PCR

Bulk samples

b1: sample consisting in 3 plants ; b2: 5 plants; b3: 10 plants; b4: 20 plants

		10^2				10^3				10^4			
		b1	b2	b3	b4	b1	b2	b3	b4	b1	b2	b3	b4
Isolation	KB	+	+	+	ND	+	+	+	ND	+	+	+	ND
	CCT	+	+	+	-	+	+	+	+	+	+	+	+
E-ELISA-DASI	KB	+	-	+	ND	+	+	+	ND	+	+	+	ND
	CCT	+	+	+	-	+	+	+	+	+	+	+	+
rt-PCR (Pirc et al, 2009)	KB	+	+	+	ND	+	+	+	ND	+	+	+	ND
	CCT	+	+	+	+	+	+	+	+	+	+	+	+

Sensitivity: 10^2 cfu/ml in a sample consisting on 1, 3, 5 or 10 plants for all techniques(after enrichment)

Results

IVIA – Assay 2
Pear Blanquilla

Spiked samples

		Neg	10^1	10^2	10^3	10^4	10^5	10^6	10^7	Ea control
Isolation		-	+	+	+	+	+	+	+	ND
E-ELISA-DASI	KB	-	+	+	-	+	+	+	+	ND
	CCT	-	-	-	+	+	+	+	+	ND
rt-PCR (Pirc et al, 2009)		-	-	-	+	+	+	+	+	ND

Sensitivity: 10 cfu/ml for isolation and ELISA DASI (enrichment in KB)

10^3 cfu/ml for real-time PCR

Bulk samples

b1: sample consisting in 3 plants ; b2: 5 plants; b3: 10 plants; b4: 20 plants

		10^1				10^2				10^3			
		b1	b2	b3	b4	b1	b2	b3	b4	b1	b2	b3	b4
Isolation	KB	+	+	+		+	+	+		+	+	+	
	CCT	+	+	+		+	+	+		+	+	+	
E-ELISA DASI	KB	-	-	+		+	+	+		+	+	+	
	CCT	+	+	-		+	+	+		+	+	+	
rt-PCR (Pirc et al, 2009)	KB	+	+	+		+	+	+		+	+	+	
	CCT	+	+	-		+	+	+		+	+	+	

Sensitivity: 10 cfu/ml in a sample consisting on 1, 3, 5 or 10 plants for isolation, ELISA, DASI and rt-PCR (after enrichment in KB)

1.c. Analysis of asymptomatic loquat samples from a Spanish area where *E. amylovora* was detected

Cooperative work with Plant Protection Service of Comunidad Valenciana (Spain) and Cooperative Ruchey,callosa d'Ensarrià, Spain

Materials and Methods: OEPP Protocol

Results

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

Negative isolation in winter, but positive detection by ELISA, conventional and real-time PCR in November. Probably latent infections.

WP5 Deliverables

- Optimized protocol for detection of *E. amylovora* in asymptomatic plant material, based on the results obtained in the different countries.

- Survival ability of *E. amylovora* under adverse conditions that can promote non-detection by conventional methods evaluated.

- Genes involved in the response of *E. amylovora* against determined adverse conditions.

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WP5 Dissemination and training output

- Protocol with advices and the most suitable methods for detection of *E. amylovora* in asymptomatic plant material.

- A transcriptome of *E. amylovora* under copper stress:

*Águila-Clares, B., Marco-Noales, E., López, M.M., Sundin, G.W. 2013. How does *Erwinia amylovora* face up to stress by copper? 13th International Workshop in Fire Blight. Zurich.

*Águila-Clares, B., Marco-Noales, E., López, M.M., Sundin, G.W. 2013. Análisis transcriptómico de la bacteria *Erwinia amylovora* en respuesta al estrés por cobre. XXIV Congreso de Microbiología SEM. L'Hospitalet, Barcelona.

- Scientific publication in a peer-reviewed journal describing the survival ability of *E. amylovora* in asymptomatic plant material and an optimized protocol for its detection.

- Scientific publication in a peer-reviewed journal on the genetic basis of survival response of *E. amylovora* to certain stress conditions: Águila-Clares B, Marco-Noales E, Penyalver R, López MM, Sundin GW. Genetic systems operating in *Erwinia amylovora* after a copper shock: the role of *copA*. Manuscript in preparation for Molecular Plant Pathology.

- Article in a popular science magazine translating research in an accessible format for e.g., plant health inspectors, diagnostic laboratories, pome fruit growers, and extension workers: Roselló M, Gamón M, Ferrer A, Dalmau V, Palacio-Bielsa A, López MM. 2014. Prevención del fuego bacteriano en plantaciones de níspero (*Eriobotrya japonica*) en la Comunitat Valenciana. Phytoma 259: 1- 5.

Thanks for your attention!!