A national test performance study (TPS) on the detection of *Xylella fastidiosa*: preliminary results

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CNR – Istituto per la Protezione Sostenibile delle Piante, Bari

ASPROPI (MIPAAF agreement)

CREA-PAV: organization of a test performance study (TPS) for the national validation of official protocols for bacteria, fungi, viruses, phytoplasma

- selection of the main important pests in cooperation with the Italian Plant Protection Services
- Constitution of working groups: expression of an 'interest in participation' to the Phytosanitary Services
- June 2015: establishment of the working group for *Xylella fastidiosa*

ASPROPI- *Xylella fastidiosa* working group

- CRA-PAV: Stefania Loreti
- CNR Bari: Maria Saponari
- PPS Lombardia: Francesca Gaffuri
- PPS Toscana: Domenico Rizzo
- PPS Liguria: Moreno Guelfi
- PPS Veneto: Alberto Saccardi
- PPS Emilia Romagna: Stefano Boncompagni
- PPS Trentino Alto Adige: Valeria Gualandri
- UNIMI: Paola Casati

ITL Organization

1. **Performing a PRETEST:**

- confirm/verify the stability of samples
- estabilish the analytical sensitivity of each method for the selection of the bacterial contamination of samples to be used in the final TPS and the methods to be tested in the TPS
- estabilish the repeatibility (possibly other performance criteria…)

2. **Performing the TPS with a large number of participant laboratories to detect the reproducibility of the selected methods**

ITL Organization:

1. PRETEST

3 labs

- CREA Centro di ricerca per la Patologia Vegetale
- CNR - Istituto per la Protezione Sostenibile delle Piante
- Plant Protection service of Lombardy

16 samples

Controls:
NTC
Healthy olive extract
Infected olive extract

Two series of olive extracts spiked with ten fold dilution of *Xylella fastidiosa* CODiRo strain suspensions (devitalized)

- 10^7CFU/ml
- 10^6 CFU/ml
- 10^5 CFU/ml
- 10^4 CFU/ml
- 10^3 CFU/ml
- 10^2 CFU/ml
- 10 CFU/ml
- Healthy extract

x 2

1. Direct analyses on crude extracts
2. Crude extracts to be subjected to DNA extraction (C-TAB method)

Olive extracts (prepared by CNR-IPSP)

1. PRETEST

Methods

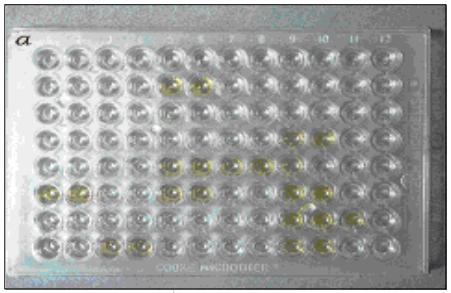
1. ELISA (Agritest)
2. ELISA (Loewe)
3. LAMP-PCR on crude extract (kit Enbiotech)
4. End-point PCR (Minsavage *et al*., 1994)
5. Real-time PCR (Francis *et al*., 2006)
6. Real-time PCR (Harper *et al*., 2010)
7. Real-time PCR (Kit Qualiplante)
8. LAMP-PCR on total DNA (kit Qualiplante)
9. LAMP-PCR on total DNA (kit Enbiotech)

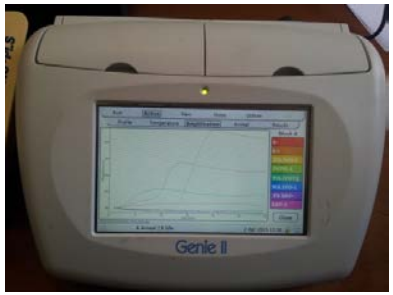
\+ Real-time PCR internal control (COX)

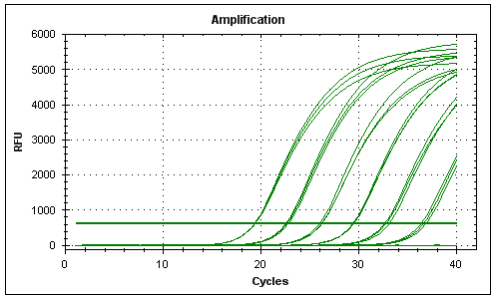
Crude extracts

Total DNA (DNA extraction by C-TAB method)

Methods reported in the ITL validation manual of May 2015 (M. Saponari)







1. PRETEST

➢ The produced data are **PRELIMINARY** and need to be extended and confirmed by TPS involving a higher number of labs and also compared with results performed on a high number of naturally infected samples

➢ These data are obtained by spiking samples with devitalized bacterial cells because is strictly prohibited the movement of infected material out of infected areas

1. Preliminary results

	Crude extracts			Total DNA					
* Two participating laboratories	ELISA-agritest *	ELISA-loewe	LAMP-PCR crude Enbiotech	PCR RST 31-33	rtPCR Qualiplant DNA	rtPCR Harper	rtPCR Francis	LAMP-PCR Qualiplant DNA	LAMP-PCR Enbiotech DNA*
Diagn sensitivity	53%	44%	64%	51%	98%	89%	100%	84%	83%
Analytical sensitivity	E4-5	E5	E3-4	E4-5	E1	E2	E1	E2-3	E3

E = 10^

Diagnostic sensitivity (proportion of infected samples giving positive result):

- **using DNA: higher sensitivity for real-time PCR and lower for end-point PCR (Minsavage *et al.*, 1994) to 100% for rt-PCR% (Francis *et al.*, 2004)**
- **using crude extract the most sensitive was the Enbiotech LAMP-PCR (62%) with respect the ELISA (44-53%)**

Analytical sensitivity:

- **10^1-2 CFU/ml for real-time PCR,**
- **10^2-3 CFU/ml for LAMP-PCR,**
- **10^4-5 CFU/ml for end-point PCR**

} Total DNA

- **10^4-5 CFU/ml for ELISA**
- **10^3-4 CFU/ml for LAMP-PCR**

} Crude extract

1. Preliminary results

	Crude extracts			Total DNA					
* Two participating laboratories	ELISA-agritest *	ELISA-loewe	LAMP-PCR crude Enbiotech	PCR RST 31-33	rtPCR Qualiplant DNA	rtPCR Harper	rtPCR Francis	LAMP-PCR Qualiplant DNA	LAMP-PCR Enbiotech DNA*
Diagn specificity	100%	100%	100%	100%	82%	92%	83%	92%	88%

Diagnostic specificity (affected by false positive (PD) results):

this value was low for the most sensitive methods: real time and LAMP PCR.
This can depend on a contamination of one of the healthy samples – that resulted positive in two labs – during the sample spiking/ liophylization step or in the laboratories activities (although the negative controls always produced negative results)

1. Preliminary results

Repeatibility: level of agreement between 5 replicates of a sample under the same condition

100% repeatibility for the tested methods : ELISA Kit Loewe, real-time PCR (Harper *et al.*, 2010 and Francis *et al.*, 2006), end-point PCR (Minsavage *et al.*, 1994) with ten-fold diluition DNA (80% with DNA extracts without dilution)

- **the internal control (*cox* gene) by real-time PCR was 100% for all performance criteria**

1. Preliminary results

Crude extracts | Total DNA

	ELISA-agritest*	ELISA-loewe	LAMP-PCR Enbiotech	PCR RST 31-33	rtPCR Qualiplante DNA	rtPCR Harper	rtPCR Francis	LAMP-PCR Qualiplante DNA	LAMP-PCR Enbiotech DNA*
Diagnostic sensitivity	53%	44%	64%	51%	98%	89%	100%	84%	83%
Diagnostic specificity	100%	100%	100%	100%	82%	92%	83%	92%	88%
Relative accuracy	63%	56%	71%	61%	95%	89%	96%	86%	84%
Reproducibility	90%	97%	93%	93%	97%	90%	100%	90%	100%

Accuracy: the closeness of agreement between a test result and the accepted reference value (or the expected response from reference material)

- **using crude extract the sensitivity was, as expected, lower then using DNA (with the exception of end-point PCR)**
- **molecular methods showed lower specificity probably due to a contamination…**
- **real-time PCR gave better performance with respect LAMP-PCR**

Exclusivity: capacity of a method to not give false positive results with non-target strains

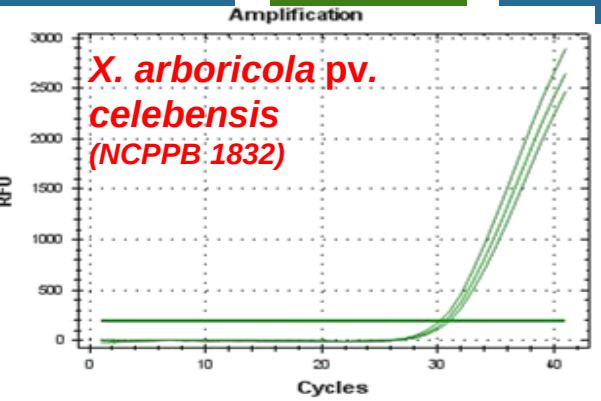
- **DNA extracted from bacterial cultures of different genera and species were checked by real-time PCR methods**

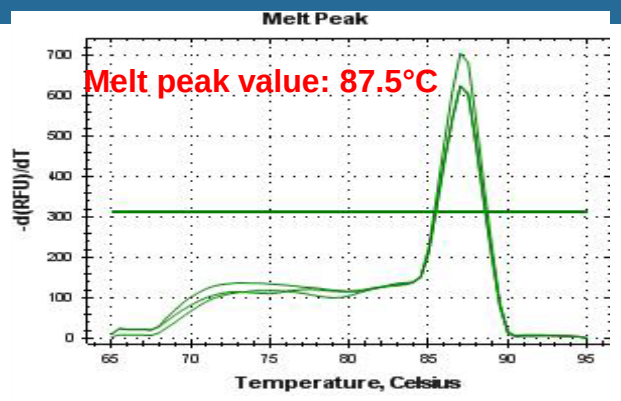
36 non-target bacterial strains :

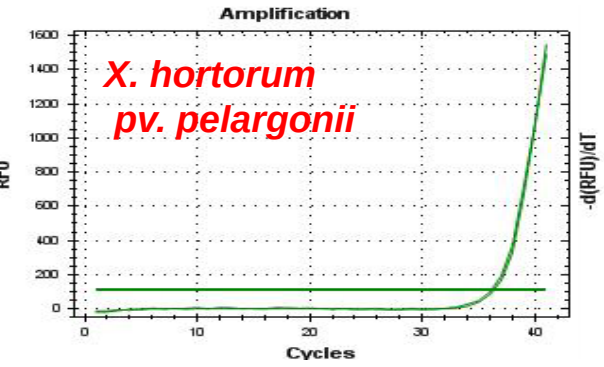
Xanthomonas arboricola* pvs *juglandis, pruni, corylina, fragariae, celebensis
X. campestris* pvs *campestris, populi
X. axonopodis* pv. *citri
X. hortorum pv. pelargonii
Pseudomonas savastanoi* pv. *savastanoi
P. marginalis
P. syringae pv. syringae
Brenneria rubrifaciens, B. quercina, B. salicis, B. populi
Pantoea stewartii, P. agglomerans
Erwinia amylovora
Agrobacterium tumefaciens,
Rhizobium vitis

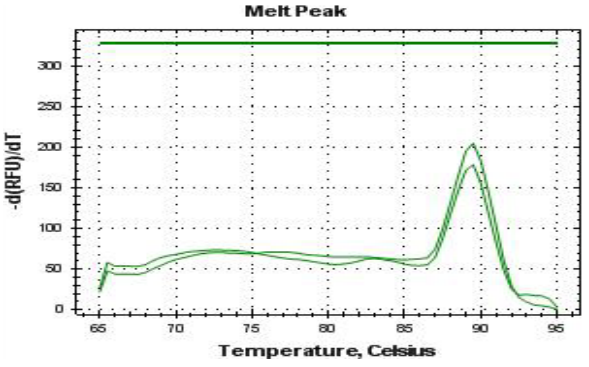
About 180 samples of olive, oleander, *Spartium, P. spumarius* collected in Latium region were checked during september-october (all negative) by real-time PCR

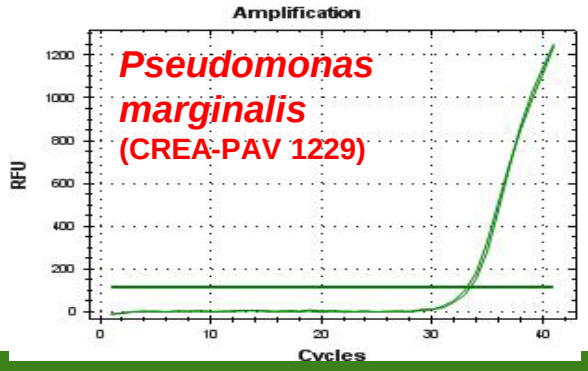
Analytical specificity: Real-time PCR

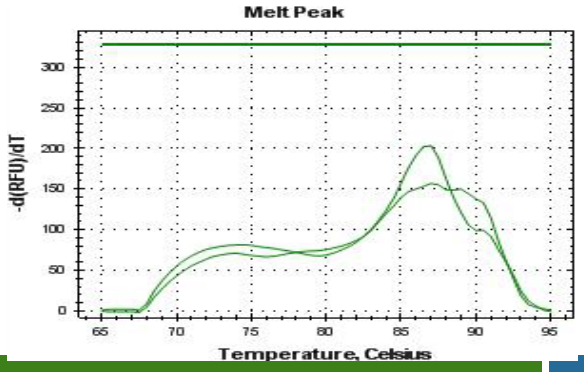


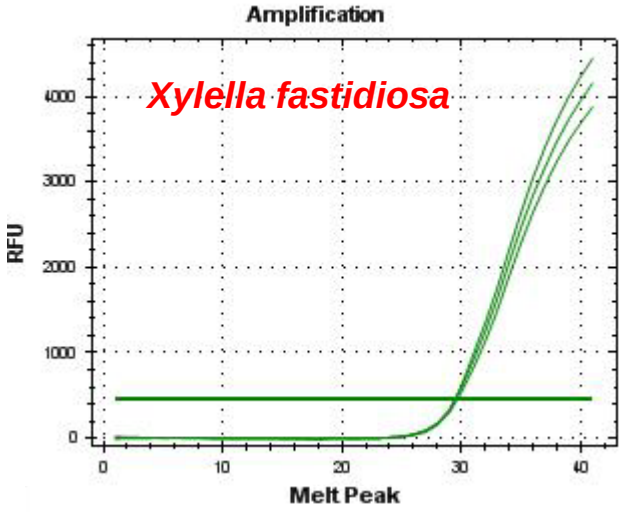


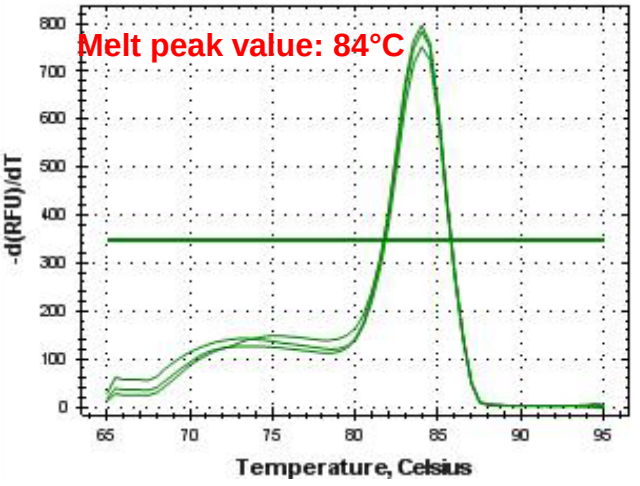






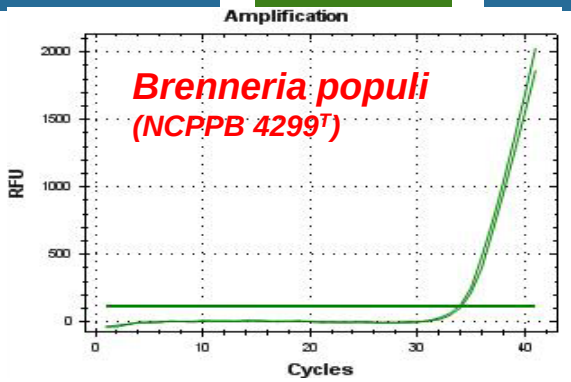


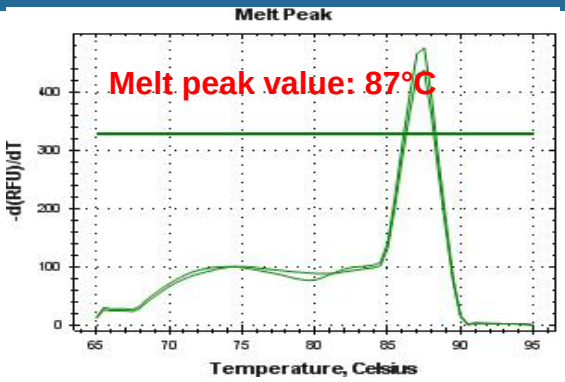


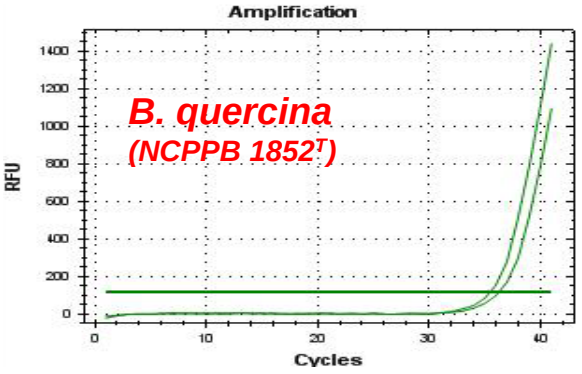


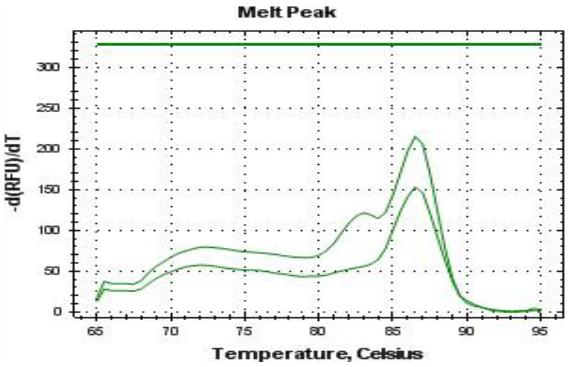
Analysis: Real Time PCR:
Method: Francis *et al*., 2006
Bacterial DNA (50/100 ng/µl)
1µl *per* PCR reaction

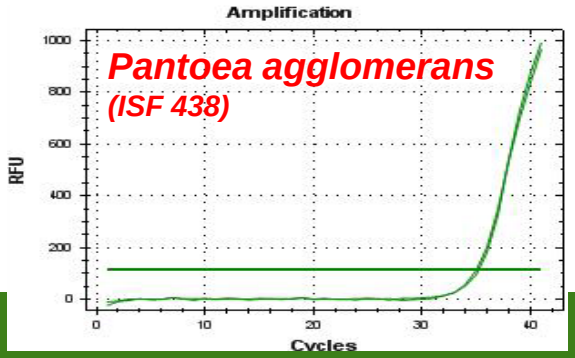
Analytical specificity: real-time PCR

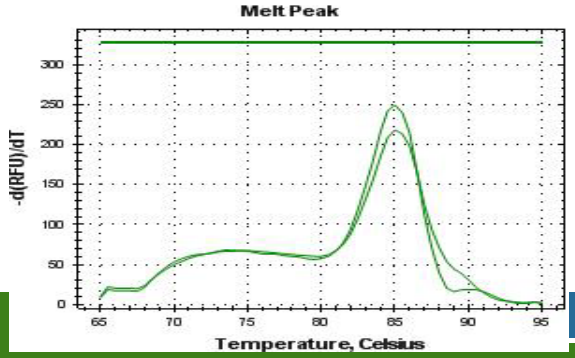


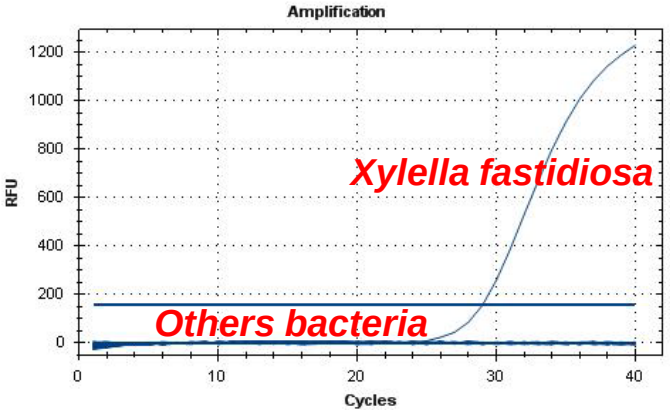












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Bacterial DNA (50/100 ng/µl)
1µl *per* PCR reaction

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Method: Francis *et al.*,2006
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1µl *per* PCR reaction

2. TEST PERFORMANCE STUDY: participants

- PPS Piemonte
- PPS Friuli Venezia Giulia
- PPS Lombardia
- PPS Toscana
- PPS Veneto
- PPS Emilia Romagna
- PPS Trentino Alto Adige
- PPS Marche
- SELGE
- CIHEAM-IAMB
- CRSFA
- CRA FSO
- CRA VIT
- UNI FI
- UNI BO
- UNI VT
- Centro di Sperimentazione Agraria e Forestale, Laimburg
- CAV (Faenza)

20 laboratories will partecipate to the TPS

Critical issues

- **Sample type to be prepared for a TPS (interdiction to move infected material or *Xylella fastidiosa* bacterial strains)**

- **Necessity to produce data for validation of isolation of *Xylella fastidiosa***

- **DNA extraction methods (kits are too much expensive for several Italian PPS)**

- **Consider the necessity to test by molecular methods either the extracted DNA and their decimal dilution: inhibition problems**

Methods to be used as preliminary screening

- For a preliminary SCREENING in a large scale monitoring of infected areas (symptomatic samples) the most suitable method is ELISA: LAMP PCR more sensitive, but too expensive?

- Screening of symptomless material or symptomatic samples in a pest-free area: more reliable real-time PCR or LAMP PCR

- Heterogeneity of expertise in Italian PPS: not all labs can perform real-time PCR or LAMP PCR or have the expertise…

THANK YOU FOR YOUR ATTENTION!

Acknowledgment:

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Vanessa Modesti, Simone Lucchesi, Nicoletta Pucci

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