Intra and inter-laboratory evaluation of molecular methods for detection of *Xylella fastidiosa*

Plant health laboratory

Bruno Legendre, Valérie Olivier, Dimitri Molusson, Stelly Mississipi, Anaïs Couton, Margot Hervouet, Marie Moisson, **Françoise Poliakoff**

Anses - Plant health laboratory – Angers – France

AEP-OEPP Copenhagen October 2015

How to face the Emergence of *X. fastidiosa* in Europe: a reliable early detection method

Need to rely on diagnosis methods with performant detection threshold to detect latent infections

Selection, evaluation and validation of molecular tools:

➢ First step : selection based on scientific publication

- PCR Firrao & Bazzi, 1994
- PCR Pooler & Hartung, 1995
- PCR Minsavage *et al.*,1994

- Real–Time PCR Harper *et al.,* 2010, erratum 2013

- LAMP Harper *et al.,* 2010

Evaluation of methods

➢ Second step: Assay on pure culture of strains (2012)

Inclusivity

Capacity of a method to detect all the target strains

• 15 strains of *X. fastidiosa* – 4 subspecies

Results :
100% for all the methods

Exclusivity

Capacity of the method to not give false positive results with non-target strains

• 29 non-target strains :

Genetical proximity 16 *Xanthomonas* spp.

Same host plants 1 *Xylophilus ampelinus*
1 *Ca. Liberibacter asiaticus*
1 *Ca. L. africanus*
6 *Coffea* spp. saprophytes and 4 *Citrus sinensis* saprophytes

Results :
100% for all the methods

Detection threshold

Enumeration by microscopy IF
DNA extraction by thermal lysis

Best results : **Real-Time PCR Harper > PCR Minsavage**

Third step: Evaluation on spiked plant samples (2013)

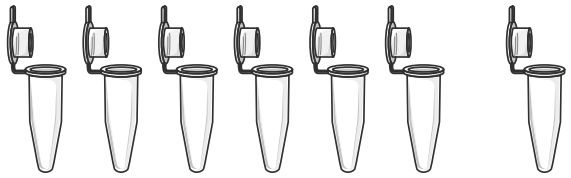
•5 plant species: 2 *Coffea* sp., grapevine, sweet orange and peach tree

Petioles and midribs → Crushing with Qiagen lysis buffer 1g/5mL

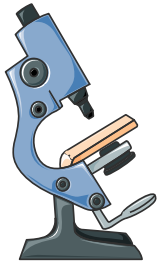
•4 *X.fastidiosa* strains

Tenfold dilution of bacterial suspension

Spiking with 5 µL / tube + 395 µL /tube



(bact/mL)	1,25 .10^5	1,25 .10^4	1,25 .10^3	1,25 .10^2	1,25 .10^1	1,25 .10^0	NEG



Enumeration by microspcopy IF

X 3 assays / pair plant / strain
X 3 dilution series / assay

= 63 samples per plant species

DNA extraction with DNeasy Plant Mini Kit® (Qiagen)

↓

Amplification

Third step: Evaluation on spiked plant samples (2013)

Definition:

•Diagnosis sensitivity: proportion of infected sample giving a positive result

•Repeatability: level of agreement between replicates of a sample tested under the same conditions

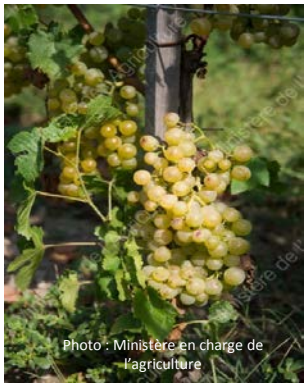
➡ Best results with the Real-Time PCR (Harper *et al.*, 2010)

Criteria	Real-Time PCR Harper *et al.*, 2010	End point PCR Minsavage *et al.*, 1994
Diagnosis sensitivity (%)	77 to 97	65 to 82
Repeatability (%)	93 to 98	80 to 98
Detection threshold* (bact./g plant tissues)	5.10^2 to 5.10^3	5.10^2 to 5.10^4

➡ But variability in the results according to plant matrices

Fourth step: Inter-laboratory evaluation (2014)

- **7 participating laboratories** (France, Italy, UK, New-Zealand, Netherland)
- **5 spiked matrices**



Samples	Plant and strain	Concentration (bact./mL)	Expected result
1	Coffee (Coffea arabica)	3,4E+04	Positif
2		3,4E+03	Positif
3	X. f. subsp. pauca* CFBP8072	3,4E+02	Positif
4		0 (matrice saine)	Négatif
5	Olive tree (Olea europaea)	2,8E+06	Positif
6		2,8E+05	Positif
7		2,8E+04	Positif
8	X. f. subsp. multiplex ATCC35871 (CFBP8173)	0 (matrice saine)	Négatif
9	Grapevine (Vitis vinifera)	1,1E+06	Positif
10		1,1E+05	Positif
11		1,1E+04	Positif
12	X. f. subsp. fastidiosa ATCC35879 (CFBP7970)	0 (matrice saine)	Négatif
13	Orange tree (Citrus sinensis)	3,1E+03	Positif
14		3,1E+02	Positif
15		3,1E+01	Positif
16	X. f. subsp. pauca* CFBP8072	0 (matrice saine)	Négatif
17	Peach tree (Prunus persica)	2,8E+04	Positif
18		2,8E+03	Positif
19		2,8E+02	Positif
20	X. f. subsp. multiplex ATCC35871 (CFBP8173)	0 (matrice saine)	Négatif

Fourth step: Inter-laboratory evaluation (2014)

➡ Confirmation of the performances of the method

Performance criteria	DNeasy® extraction + Real-time PCR Harper *et al.*, 2010
Diagnostic sensitivity	97%
Specificity	100%
Repeatibility	91%
Reproducibility	84%
Limit of detection (with detection probability of 100%)	Depending of matrices: ➢ **orange tree: 3.10^2 bact/mL** ➢ coffee tree: 3.10^4 bact/mL ➢ peach tree: 3.10^4 bact/mL ➢ **olive tree: 3.10^5 bact/mL** ➢ **grapevine: 1.10^6 bact/mL**

Necessity to improve the limit of detection on complex matrices

anses

Comparison with an alternative extraction method: QuickPick™ SML Plant DNA (Bio-Nobile) vs DNeasy®

Results

Performance criteria (%)	DNeasy® Plant mini kit Qiagen	QuickPick™ + Robot (BioSprint 15 = KingFisher™ mL)	QuickPick™ + magnets (manual protocol)
Sensitivity	52,8	80,6	79,2
Specificity	100	100	100
Repeatability	97,8	97,8	96,6
Limit of detection	Orange≈ 10^2 bact./mL Grapevine≈10^6bact./mL Olive≈10^5 bact./mL	Orange≈ 10^2 bact./mL Grapevine≈10^3bact./mL Olive≈10^5 bact./mL	

→ Improvement of the limit of detection and sensistivity

Detection and identification of *Xylella fastidiosa*

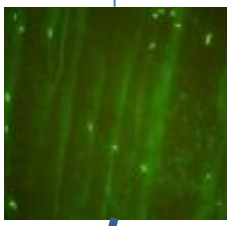
0,5 -1g of symptomatic or asymptomatic samples grounding in water

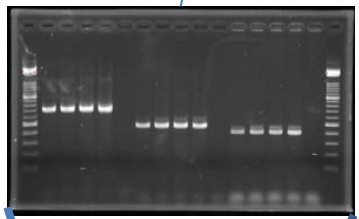
2X250 mL for DNA extraction
2X Rt-PCR (Harper et al, 2010-2013)

Positive

Negative

- PCR (Hernandez-Martinez et al 2007)
- (Pooler & Hartung 1995)





=

Xf detected and confirmed

Xf not detected

Xf subsp Y detected

Xf detected subsp indetermined

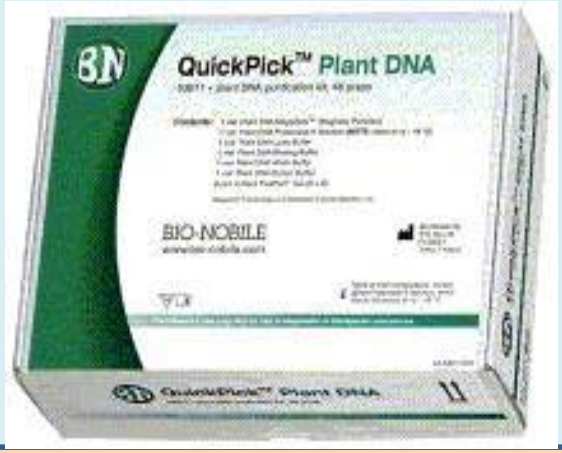
DNA extraction

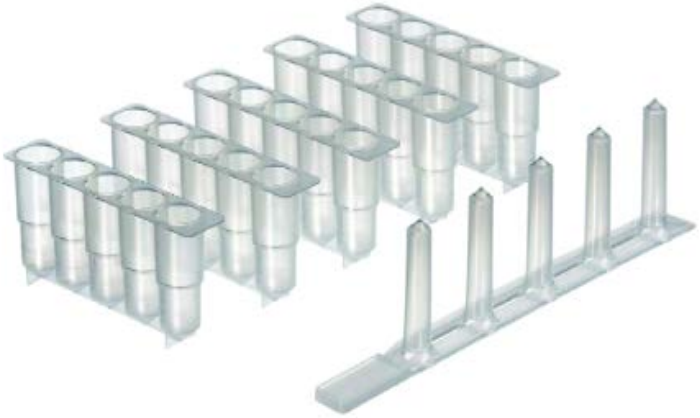
Automat :

BioSprint15 (Qiagen) = KingFisher™ mL(Thermo)
+ 5tubes strips and 5rods cover(Qiagen / Thermo)

Commercial kit :

Quick Pick™ SML Plant DNA (Bio-Nobile)



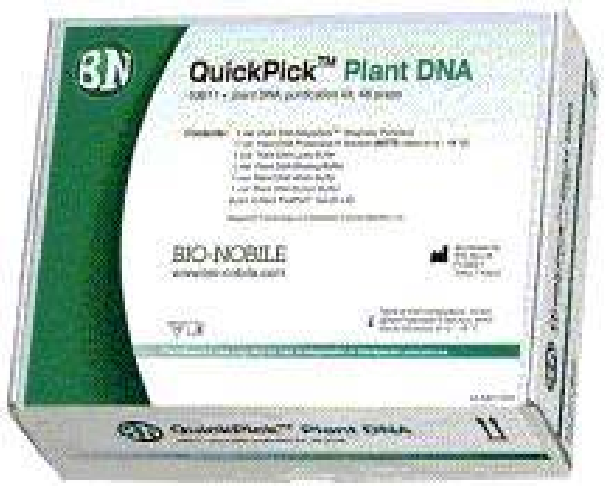


Manual:

Magnetic strip DynaMag™-2 (Invitrogen)

QuickPick™ SML Plant DNA (Bio-Nobile)

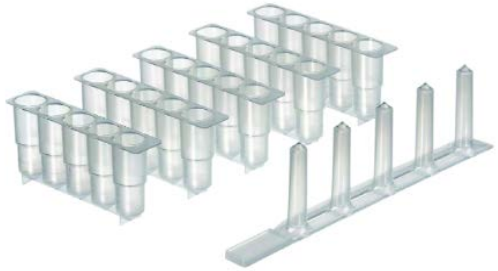
Lysis buffer+ Protéinase K + Pellet
75 µL 5 µL



Binding buffer (C) + Magnetic beads (D)
125 µL 5 µL

Washing buffer (E)

Elution buffer (F)



Cupule 1	Cupule 2	Cupule 3	Cupule 4	Cupule 5
C + D	E (250 µL)	E (250 µL)	E (250 µL)	F (50 µL)

DNA

DNA amplification with real-time PCR

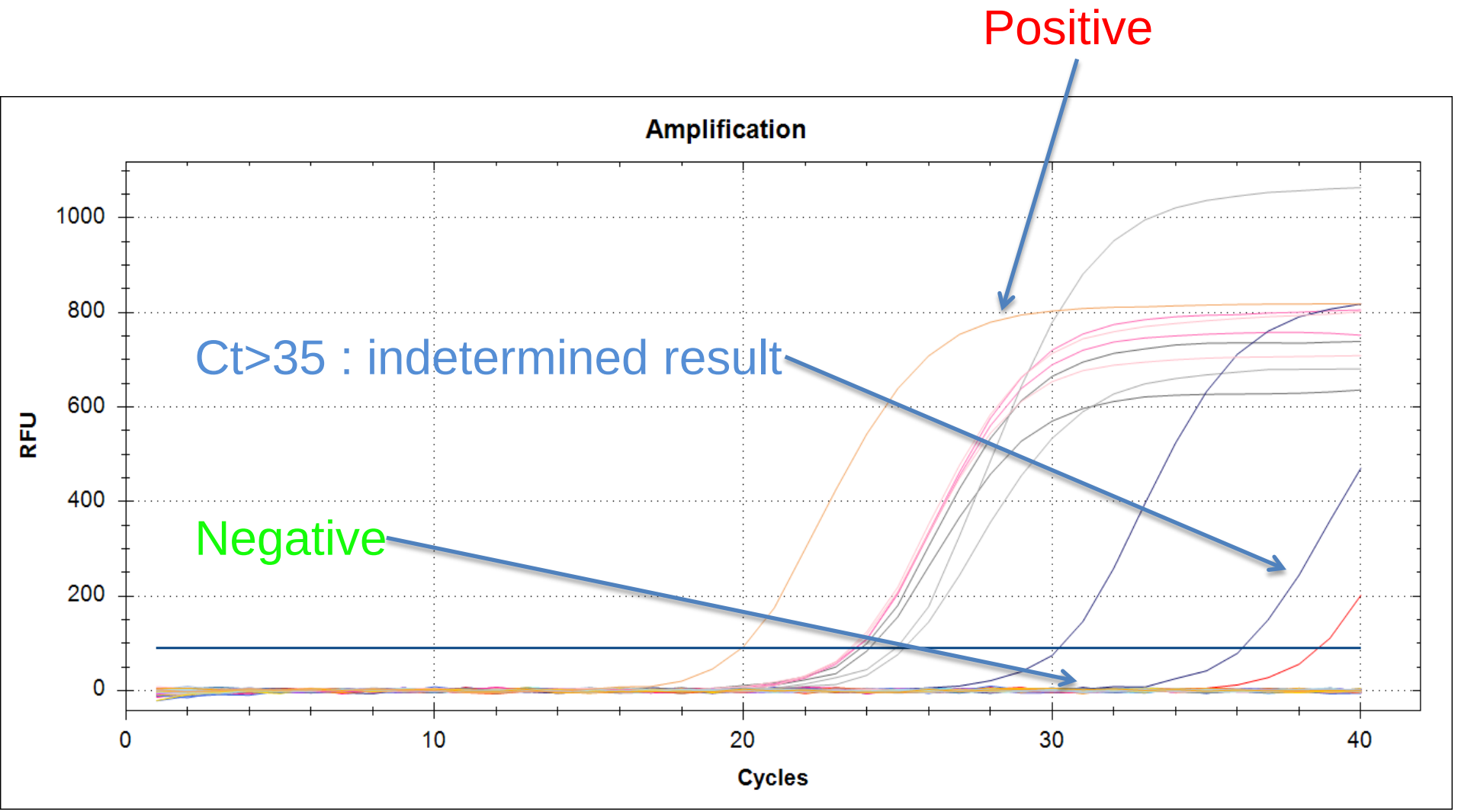
Primers, probe and PCR Cycles according to Harper et al., 2013

Pre-incubation		50°C	2min
		95°C	10min
Number of cycles :	**40**		
	denaturation	94°C	10s
	hybridization/ elongation	62°C	40s

Thermocyclers

7500 Fast (Applied Biosystem) / CFX 96 (Bio Rad)

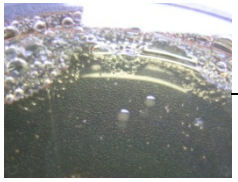
Real-time PCR results

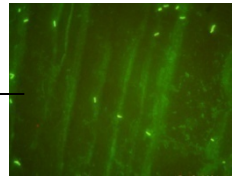


Confirmation of presence of *Xylella fastidiosa*

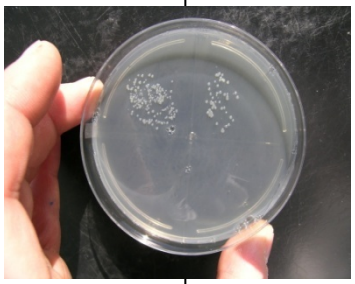
Dilaceration of petioles and veins

Isolation by plating on mPWG





IF (antiserum INRA/LSV)



Typical colonies

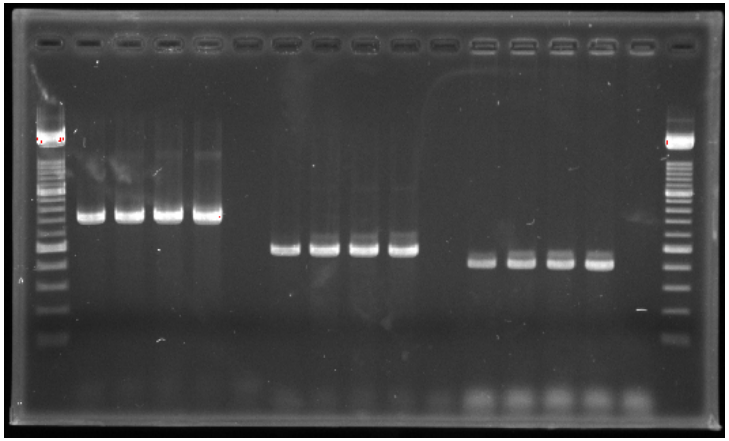
Typical cells

Confirmation of the species X fastidiosa:
PCR (Minsavage *et al*. 1994)

Identifcation of the subspecies , phylogeny
PCR multiplexe (Hernandez-Martinez *et al.,* 2007)
and/or (Pooler *&* Hartung 1995)
MLSA (7 housekeeping genes)

Genome sequencing

RST31/RST33 **272-1-int/272-2-int** **XF1-F/XF6-R**



Conclusion

- The most performant method for early detection of *Xylella fastidiosa* in various matrices :

  DNA extraction with QuickPick™ (Bio-Nobile)
  + Real-Time PCR (Harper *et al*., 2010-2013)

- Some matrices are complex for PCR (olive, oak…)
- PCR multiplex (Hernandez Martinez et al , 2007) and PCR (Pooler and Hartung, 1995) for subsp identification.

Thanks to Anses LSV:
Bruno Legendre,
Valérie Olivier,
Stelly Mississipi,
Dimitri Molusson,
Corinne Audusseau,
Christèle Dousset,
Sandrine Paillard,
Christelle François,
Carène Rivoal

Thanks to IRHS-INRA - Emersys
Marie-Agnès Jacques
Nicolas Denancé

Thank you for your attention