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## 2<sup>ND</sup> EUROPEAN CONFERENCE ON XYLELLA FASTIDIOSA: HOW RESEARCH CAN SUPPORT SOLUTIONS

Ajaccio 29<sup>th</sup> - 30<sup>th</sup> October 2019



2<sup>nd</sup> European  
conference on  
*Xylella  
fastidiosa*  
2019

# OPTIMIZATION OF SAMPLING AND TESTING PROCEDURES FOR DETECTING XYLELLA FASTIDIOSA IN LARGE LOTS OF PLANT FOR PLANTING AND NURSERY STOCKS

**Loconsole G.<sup>1</sup>, Zicca S.<sup>2</sup>, Altamura G.<sup>2</sup>, Manco L.<sup>1</sup>, El Hatib O.<sup>3</sup>, Potere O.<sup>1</sup>, Susca L.<sup>1</sup>, Elicio V.<sup>4</sup>,  
Trisciuzzi N.<sup>5</sup>, Boscia D.<sup>2</sup>, Savino V.N.<sup>1</sup>, Saponari M.<sup>2</sup>**

<sup>1</sup>Dipartimento di Scienze del Suolo della Pianta e degli Alimenti, Università degli Studi di Bari Aldo Moro, Italy

<sup>2</sup>Istituto per la Protezione Sostenibile delle Piante, Italy

<sup>3</sup>Mediterranean Agronomic Institute of Bari-CIHEAM, Valenzano (BA), Italy

<sup>4</sup>Agritest S.r.l. Valenzano (BA), Italy

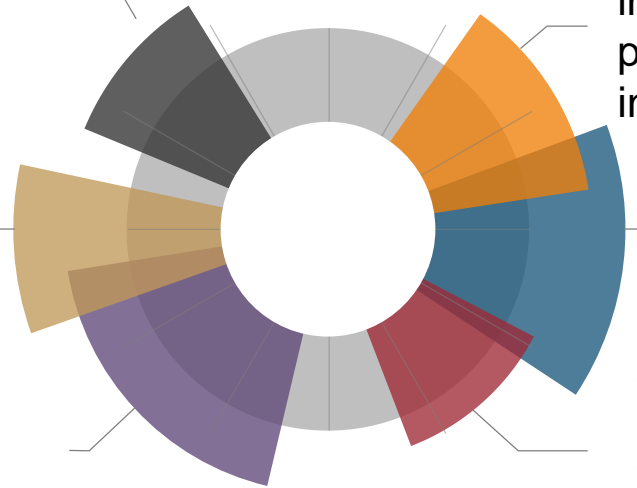
<sup>5</sup>Centro di Ricerca, Formazione e Sperimentazione in Agricoltura "Basile Caramia" (CRSFA), Locorotondo (BA), Italy



Polyphagous, colonizes 563 host species (EFSA, 2018)

Different subspecies and genotypes, extensive list of susceptible species, spittlebugs vector in EU/Med

Often detected on plants for planting imported into or moved between EU countries, particularly on coffee (*Coffea* spp.). (updating on EUROPHYT)



Most relevant pathway for introduction of *X. fastidiosa*: the importation of plants for planting and infectious insects (vectors)

Plants for planting considered a high risk of pest introduction:

- the pest can survive and multiply on living hosts
- once at destination can transfer to a suitable host in appropriate conditions, if plants are grown outdoors.

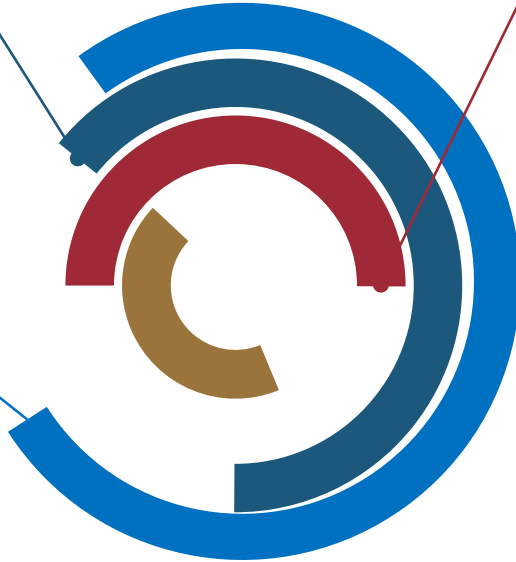
# Phytosanitary measures in the EU



- Regulated as a quarantine in EU
- Decision (EU) 2015/789 and amendments



- monitoring programs mandatory in the EU countries



Strict regulations [Decision (EU) 2015/789, amended by Decisions (EU) 2017/2352 and 2018/1511] for over 300 plant species

## INSPECTION :

- of import at plant consignments
- At production places
- For movement inside and out demarcated areas.

*Coffea*, *Lavandula dentata* L., *Nerium oleander* L., *Olea europaea* L., *Polygala myrtifolia* L., *Prunus dulcis* (Mill.)

Moved/introduced in the EU if grown in a site subjected to annual official inspection and sampling, taking into account:

- the technical guidelines for the survey of *Xylella fastidiosa* (Commission website)
- The international standards (ISPM-31) to confirm the absence of the specified organism, using a sampling scheme able to identify with 99% reliability the level of presence of infected plants of 5%
- the specified organism shall be screened by one test
- prior to its first movement out of its production site, lot of plants of *Polygala myrtifolia* L. shall be subjected to official visual inspection and sampling

## MOVEMENT OF SPECIFIED PLANTS FROM THE DEMARCATED AREA

using a sampling scheme able to identify with 99% reliability a level of presence of infected plants of 1% in accordance with international standards, and targeting symptomatic plants, as well as asymptomatic plants in the proximity of the symptomatic ones

**Table 1. Table of minimum sample sizes for 95% and 99% confidence levels at varying levels of detection according to lot size, hypergeometric distribution**

Number of units in lot	P = 95% (confidence level)						P = 99% (confidence level)				
	% level of detection × efficacy of detection						% level of detection × efficacy of detection				
	5	2	1	0.5	0.1	5	2	1	0.5	0.1	
25	24*	-	-	-	-	25	-	-	-	-	
50	39*	48	-	-	-	45*	50	-	-	-	
100	45	78	95	-	-	59	90	99	-	-	
200	51	105	155	190	-	73	136	180	198	-	
300	54	117	189	285*	-	78	160	235	297*	-	
400	55	124	211	311	-	81	174	273	360	-	
500	56	129	225	388*	-	83	183	300	450*	-	
600	56	132	235	379	-	84	190	321	470	-	
700	57	134	243	442*	-	85	195	336	549*	-	
800	57	136	249	421	-	85	199	349	546	-	
900	57	137	254	474*	-	86	202	359	615*	-	
1 000	57	138	258	450	950	86	204	368	601	990	
2 000	58	143	277	517	1553	88	216	410	737	1800	
3 000	58	145	284	542	1895	89	220	425	792	2353	
4 000	58	146	288	556	2108	89	222	433	821	2735	
5 000	59	147	290	564	2253	89	223	438	840	3009	
6 000	59	147	291	569	2358	90	224	442	852	3214	
7 000	59	147	292	573	2437	90	225	444	861	3373	
8 000	59	147	293	576	2498	90	225	446	868	3500	
60 000	59	149	298	595	2921	90	228	457	912	4431	
70 000	59	149	298	596	2932	90	228	457	913	4455	
80 000	59	149	298	596	2939	90	228	457	914	4473	
90 000	59	149	298	596	2945	90	228	458	915	4488	
100 000	59	149	298	596	2950	90	228	458	915	4499	
200 000+	59	149	298	597	2972	90	228	458	917	4551	

**SAMPLE SIZE** to be inspected and tested

# SAMPLING FOR LABORATORY TESTING

How to perform  
sampling on  
asymptomatic plants :  
minimum n. of  
leaves/cuttings collected  
from the consignments

**N. leaves/unit ?**

How to process large  
amount of materials  
collected from the  
sample unit

**How many  
leaves/shoot/cuttings  
I can pool in the lab ?**



**Xf detection at plant consignments, place of  
production and nursery**

## **COMPOSITE SAMPLES**

**1**

**Practice when a  
large number of  
samples have to be  
selected to satisfy  
sample size  
requirements  
keeping the number  
of diagnostic tests  
affordable**

**2**

**EPPO protocol until 2018**

only for olive with  
CTAB and coffee with  
Quick pick (Loconsole  
et al., 2014; Bergsma-  
Vlami et al., 2017)

Up to 10 g (per sample  
or Subsample)

**3**

Necessity to  
have more  
data



# Objective

## Composite samples for *Xf*: validation of sampling and diagnostic procedures

Plant species selected: Portions (single leaves or pieces of stems)

Composite samples:  
by pooling at different ratio



**Olive:**  
leaf petioles



**Polygala:**  
vegetative shoots



**Oleander:**  
leaf midribs



**Lavender:**  
vegetative shoots



**Cherry:**  
xylem tissue scraped

N. portions (1,2,3..) of naturally *Xf*-infected plant tissues (subsp pauca ST53)

+

*N. portions of Xf-free materials*

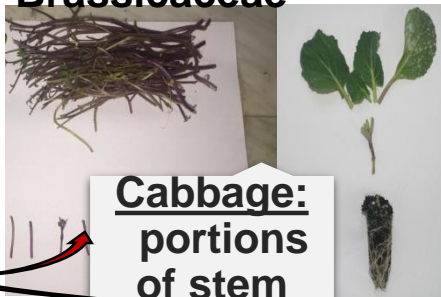
**Pool of 20 gr/sample**



**Stem of inf periwinkle**

+

**Brassicaceae**



**Cabbage:**  
portions of stem

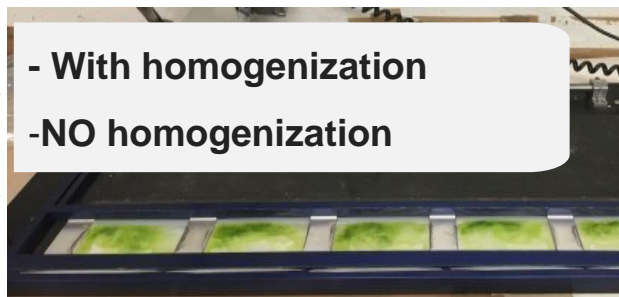
**Pool 40 gr/sample**

non-host plants of the ST53-strain

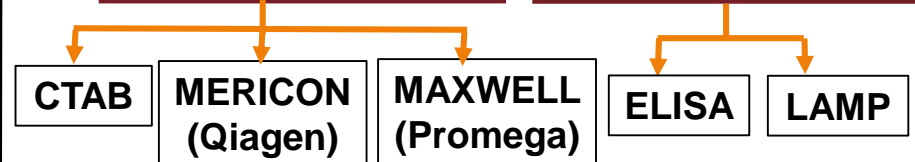
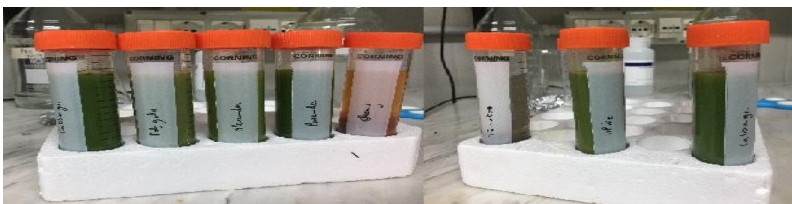
**Solanaceae**



**Tomato:**  
portions of stem



PBS (sodium metabisulfite)  
 - 1:1.5 (w:v) for unligified herbaceous plant materials  
 - 1:3 (w:v) for woody plant materials



qPCR (Harper et al. 2010)

At least 3 replicates/test

# Results

**Composite sample for *Xylella fastidiosa*: best pools woody plant materials**

Species	N. infected portions/ N. <i>Xf</i> -free portions	Type of tissues recovered	Pool (20 g)	Diagnostic sensitivity %		
				qPCR	LAMP	ELISA
<b>Olive</b>	4/approx. 800-900	Leaf midribs or petioles or leaf basal part	corresp. to <b>200-225 sampled plants</b>	100 by CTAB Mericon Maxwell	100	100
<b>Oleander</b>	2 / approx. 200	Leaf petioles	20 g corresp. to <b>100 sampled plants</b>		100	100
<b>Polygala</b>	2 / approx. 250	2cm of vegetative shoots	20 g corresp. to <b>125 sampled plants</b>		100	100
<b>Lavender</b>	2 / approx 200	2-3cm of vegetative shoots	20 g corresp. to <b>90-100 sampled plants</b>		100**	100
<b>Cherry</b>	2 / approx. 200	0.1 g of xylem tissue	Up to 20 g appr. <b>100 sampled plants</b>		0	0

- 1) Inconsistent results without homogenization step and using 1 infected portion
- 2) Tests performed on Polygala leaves repeatedly failed
- 3) \*\*\*4/8 replicates negative in the 1st run, when diluted 1:3 positive

reaction similar to signal of negative control

## Composite sample for *Xylella fastidiosa*: best pools herbaceous materials

Species	N. of infected portions/N. of <i>Xf</i> -free portions	Type of tissues recovered	Pool	Diagnostic sensitivity %		
				qPCR	LAMP	ELISA
<b>Tomato</b>	1 stem of infected periwinkle / N. 200 stem of tomato	1,5-2 cm of stem	40 g corresp. <b>200 sampled plants</b>	100	86	57
<b>Cabbage</b>	1 stem of infected periwinkle / N. 200 stem of cabbage	1,5-2 cm of stem	Up to 40 g appr. <b>200 sampled plants</b>	100	37.5	37.5

- 1) Inconsistent results without homogenization step
- 2) qPCR generated 100% of diagnostic sensitivity independently by the extraction methods used
- 3) both LAMP and ELISA tests produced lower values of diagnostic sensitivity which was drastically low (37.5) for cabbage composite samples

Host species	Range of Cq (min-max)			ELISA Range of OD (405nm) 120 (min-max)
	CTAB	MERICON	MAXWELL	
Olive	(26.20 - 31.04)	(30.10 - 31.10)	(29.40 - 31.18)	(0.212-0.394)
Oleander	(24.03 - 28.38)	(26.21 - 27.71)	(24.38 - 26.09)	(0.493-1.248)
Polygala	(25.55 - 27.03)	(27.81 - 30.17)	(28.51 - 29.03)	(0.256-0.913)
Lavender	(32.04 - 32.56)	(29.96 - 30.14)	(28.40 - 30.89)	(0.307-1.004)
Cherry	(28.17 - 30.84)	(31.03 - 32.94)	(31.11 - 33.08)	-
Herbaceous host (Tomato)	(27.43 - 28.42)	(26.66 - 30.23)	(28.85 - 30.02)	(0.174-0.308)
Herbaceous host (Cabbage)	(26.25 - 31.69)	(28.40 - 31.41)	(27.13 - 30.02)	(0.217-0.590)

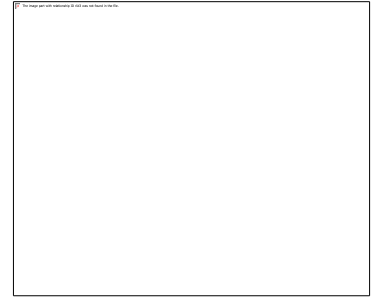
**Cq values** in an optimal range

- higher for lavender CTAB extracts and cherry Mericon and Maxwell extracts
- negative control not produced any amplification curve (N/A)

**OD values** in an optimal range

- low for olive and herbaceous hosts even if considered positive in accordance to PM 7/101 (1) for ELISA test

# Testing composite samples – small amount



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USING CONVENTIONAL EXTRACTION METHODS  
AND PROTOCOLS, EPPO 7/24 (4)

At least 6 independent replicates

# Olive



Xf infected leaves

.....+



healthy leaves

We increased the number of infected leaves: **2-4 infected leaves** mixed with a variable n. of healthy leaves

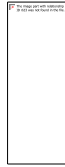
Composite samples				Diagnostic sensitivity		
Infected leaves		TOT. pool with healthy leaves	~gr (leaf pool)	Mericon +qPCR (Harper et al. 2010)	Lamp Enbiotech on sap	ELISA
2	+	10	0.2	83%	67%	67%
		20	0.5	92%	83%	83%
		30	0.8	78%	78%	67%
4	+	20	0.6	100%	100%	100%
		40	1.0	89%	100%	89%

Using 4 infected leaves best values of diagnostic sensitivity

Equal to pool 10 plants



# Oleander



- **Poor results** testing **1 infected leaf** mixed with a variable n. of healthy leaves
- increased the number of **infected leaves**

Composite samples				Diagnostic sensitivity		
Infected leaves		TOT. pool with healthy leaves	~gr (leaf pool)	Mericon +qPCR (Harper et al. 2010)	Lamp Enbiotech on sap	ELISA
2	+	16	1.7	100%	100%	89%
3		24	2.2	89%	100%	89%

**Equal to pool 8 plants**

Increasing the number of leaves, inhibitors could affect the assays , mainly qPCR

# Polygala myrtifolia



- **Poor results** testing **2 infected leaves** mixed with a variable n. of healthy leaves
- increased the number of **infected leaves**

Composite samples				Diagnostic sensitivity		
Infected leaves		TOT. pool with healthy leaves	~gr (leaf pool)	Mericon +qPCR (Harper et al. 2010)	Lamp Enbiotech on sap	ELISA
4	+	32	0.5	83%	83%	83%
6		48	1.0	100%	100%	100%

Using 6 infected leaves best values of diagnostic sensitivity

Equal to pool 8 plants

# Cherry dormant material



parts of infected scraped xylem

+



parts of healthy scraped xylem

Composite samples				Diagnostic sensitivity		
parts of infected xylem	+	parts of healthy xylem	~gr (xylem pool)	Mericon +qPCR (Harper et al. 2010)	Lamp Enbiotech on sap	ELISA
2		8	1.0	100%	83%	100%
4		16	2.0	100%	83%	100%

## Equal to pool 5 plants

Increasing the amount of xylem , inhibitors could further affect LAMP assay

## STANDARD DIAGNOSTIC PROCEDURES – SMALL TISSUE AMOUNT

Host specie	Minimum n. of leaves /xylem parts to be collected	N. Plants that can be pooled	maximum n. of units
Olive	4	10	40
Oleander	3	8	24
Polygala myrtifolia	6	8	48
Cherry	4	5	20

# Conclusions

01

Sensitive and reliable to correctly detect *Xf*

02

Homogenization is a crucial step to successfully assess the presence of the bacterium

03

Guidance for sampling at consignments/ place of prod./ mvmt of “specified plants” produced in nurseries in “demarcated areas”, it is included in EPPO PM7/24 (4), 2019.

04

Guidelines:

- Type of tissue to be processed
- Min n. of plant portions collected from the single unit of the lot
- Max n. of units pooled and processed (up to ~100/200 plants) according to the different host species
- App. weight of lab composite samples
- Highest-performance tests according to host species

**Future activities:-Produce more data on composite samples for DORMANT MATERIAL**

**- Organize interlaboratory validation at EU level**

# Thank you.....

Dipartimento di Scienze del Suolo, della Pianta e  
degli Alimenti - Di.S.S.P.A.



Stefania Zicca



Giuseppe  
Altamura



Donato Boscia



Maria Saponari

 AGRITEST  
PLANT HEALTH MANAGEMENT



Lilia Formica



Vito Elicio



Centro di Ricerca  
Sperimentazione e Formazione  
in Agricoltura **Basile Caramia**

Nico Trisciuzzi



CIHEAM  
IAM BARI



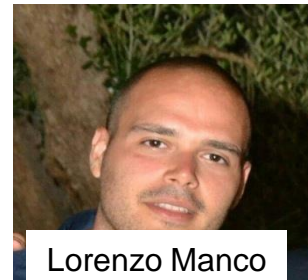
EL Hatib  
Oumaima



Giuliana  
Loconsole



Potere Oriana



Lorenzo Manco



Leonardo Susca



Vito Nicola  
Savino