CONSIGLIO NAZIONALE DELLE RICERCHE

Istituto per la Protezione Sostenibile delle Piante, UOS Bari

UNIVERSITÀ DEGLI STUDI DI BARI ALDO MORO

Dipartimento di Scienze del Suolo, della Pianta e degli Alimenti

CRSFA

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

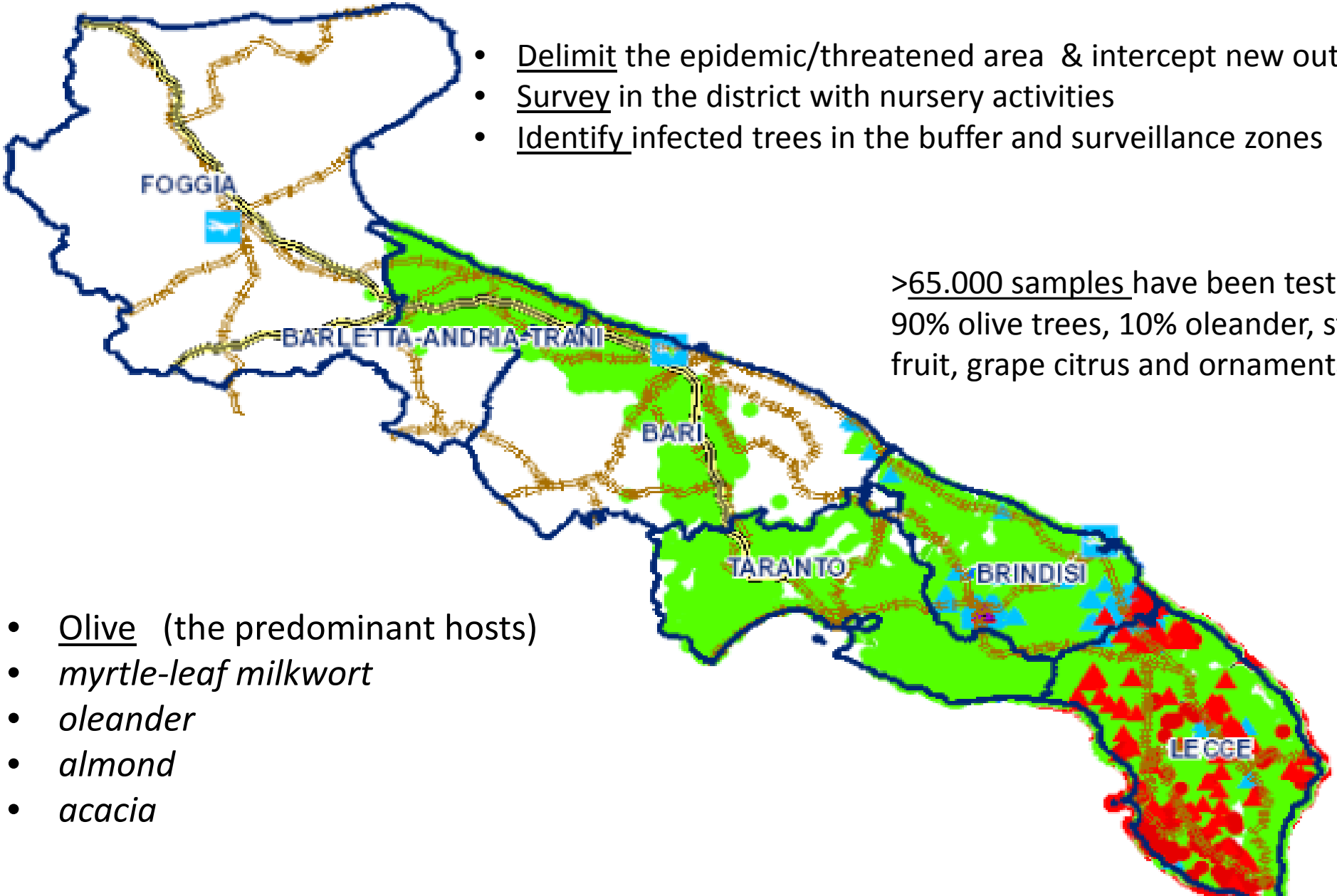
CIHEAM IAM BARI

Diagnostic tools and protocols currently adopted in Apulia for large scale field monitoring of *Xylella fastidiosa*

Maria Saponari, Giuliana Loconsole, Oriana Potere, Donato Boscia

Regional Monitoring Program: scope and target host plants

- Delimit the epidemic/threatened area & intercept new outbreak(s)
- Survey in the district with nursery activities
- Identify infected trees in the buffer and surveillance zones

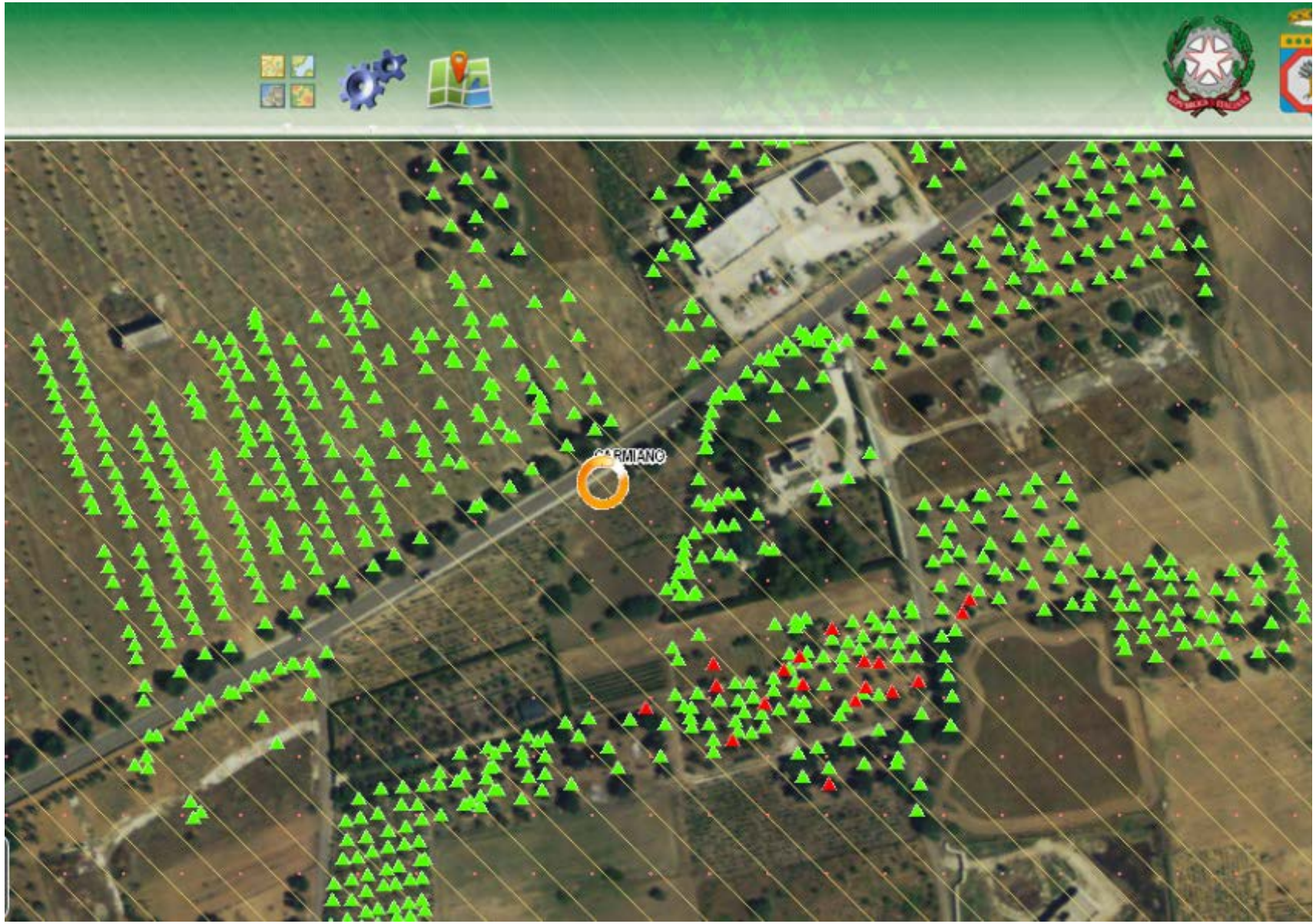


>65.000 samples have been tested
90% olive trees, 10% oleander, stone fruit, grape citrus and ornamentals

- Olive (the predominant hosts)
- *myrtle-leaf milkwort*
- *oleander*
- *almond*
- *acacia*

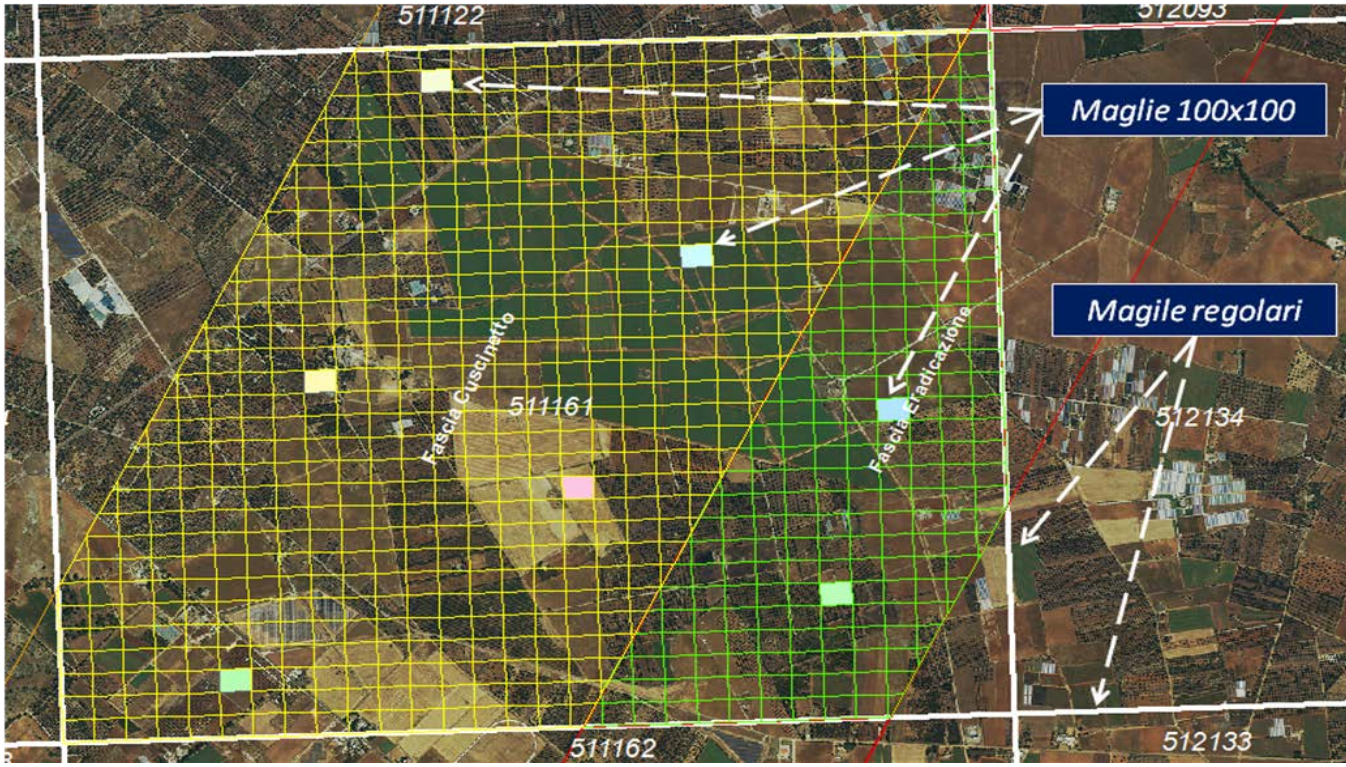
Close-up of the map

Survey in the surveillance area (single tree/plant of the specified host)



ELEMENTS characterizing an effective monitoring program

- Knowledge of the host range
- Knowledge of the symptoms associated to the infections
- Sites to be prioritized for monitoring
- Criteria for sampling
- Seasonal fluctuation of the bacterial population
- Laboratory tests for processing large scale number of samples



Sampling: the experience gathered upon the esthablishement of the bacterium under the Mediterranean conditions
(climate, hosts, isolate introduced, etc.)

- Major susceptible crops and host species affected show symptoms, while few susceptible hostm plants appear to be symptomless

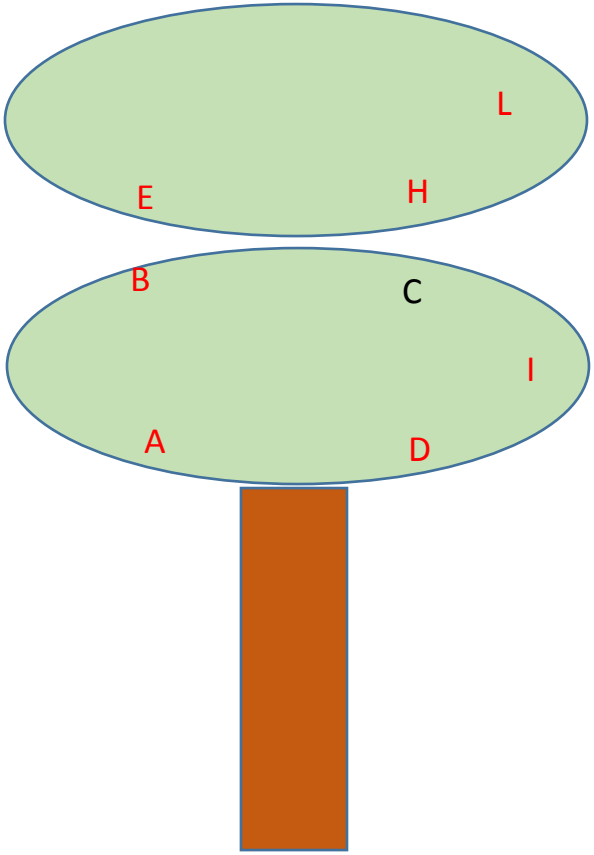
VISUAL INSPECTIONS IS A CRITICAL ASPECT

- Host species consisting in deciduos trees: the bacterium is undetectable in the leaves (qPCR/ELISA) in spring and early summer; in the wood it is detectable thought the entire year.

- Host species consisting in evergreen trees/shrubs: the bacterium is detectable in the leaves (qPCR/ELISA) through the entire year (referred to the specific conditions in the contaminated area); isolations from olive failed only in late summer; isolation from other susceptible hosts failed in late summer and in the winter season

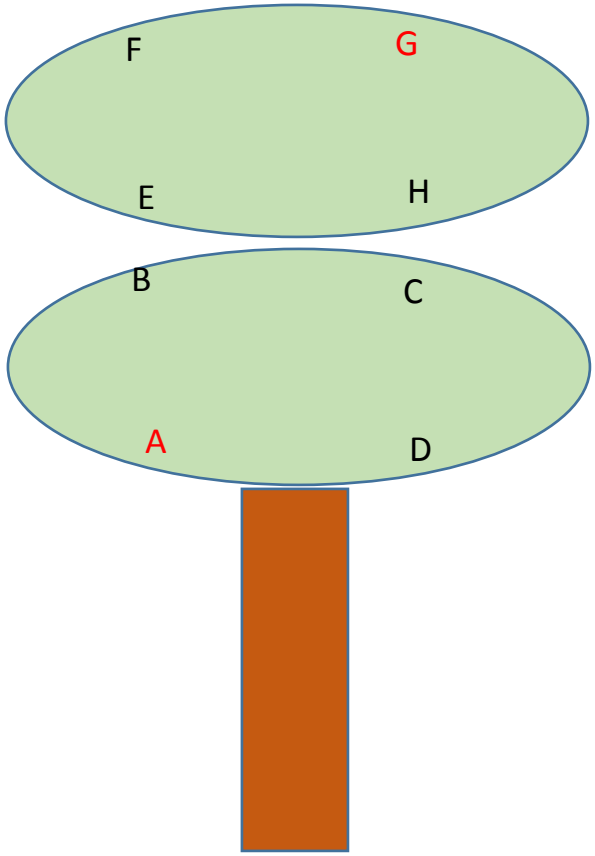
PERIOD FOR SAMPLING – RELATED TO THE DIAGNOSTIC TESTS

Sampling from symptomatic trees



7/8 subsamples tested positive qPCR/ELISA

Sampling from trees showing mild symptoms



2/8 sub-samples tested positive qPCR/ELISA

Correlation symptoms – Xf detection

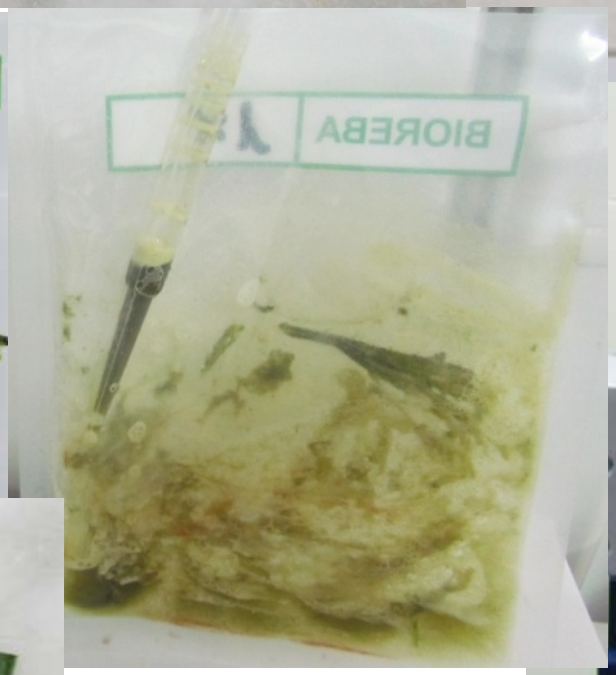
Young portions

Mature cuttings

Diagnostic approaches tested for the CoDiRO strain

- *ELISA*
- *DTBIA*
- *PCR*
- *qPCR*
- *LAMP*

SAMPLE PREPARATION for DNA purification and ELISA/LAMP sap preparation



Advantage: process 0,5-1gr of tissue

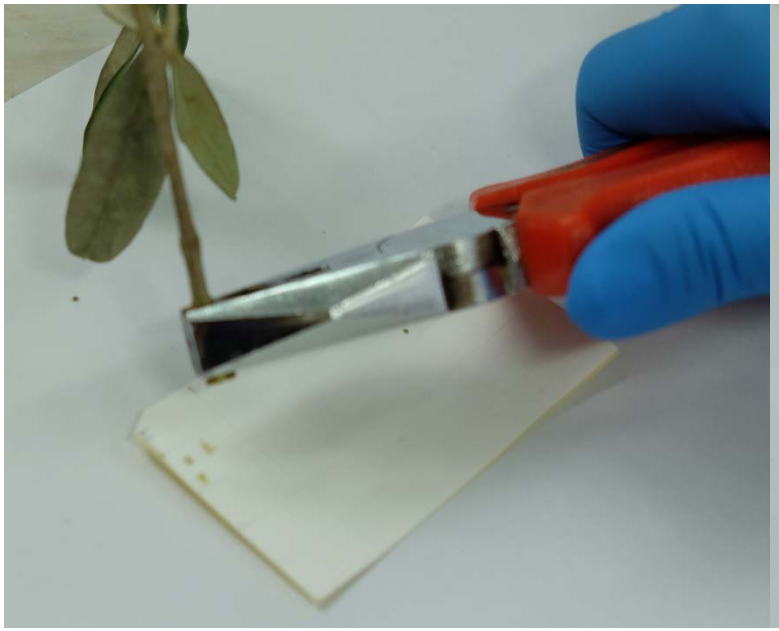
Representativeness of the *sample*

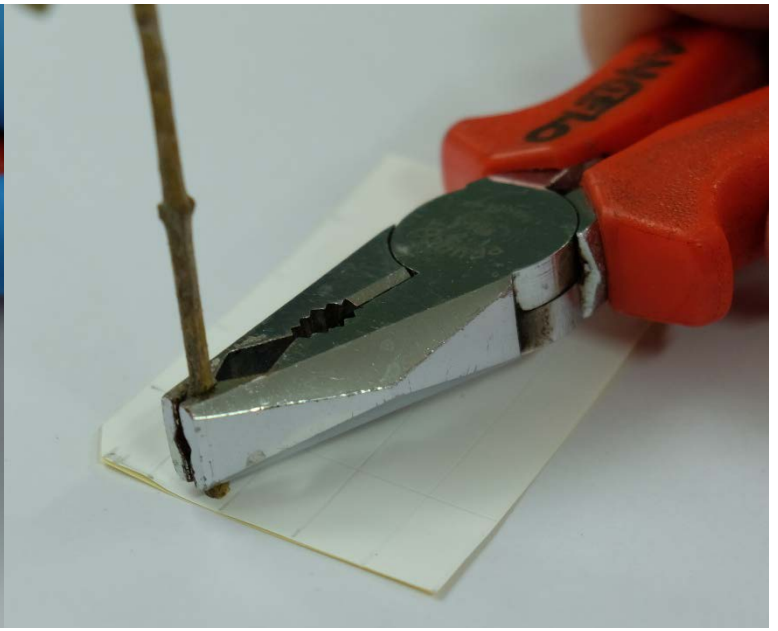
Update on the implementations developed by the Apulian research groups:

Serological approaches

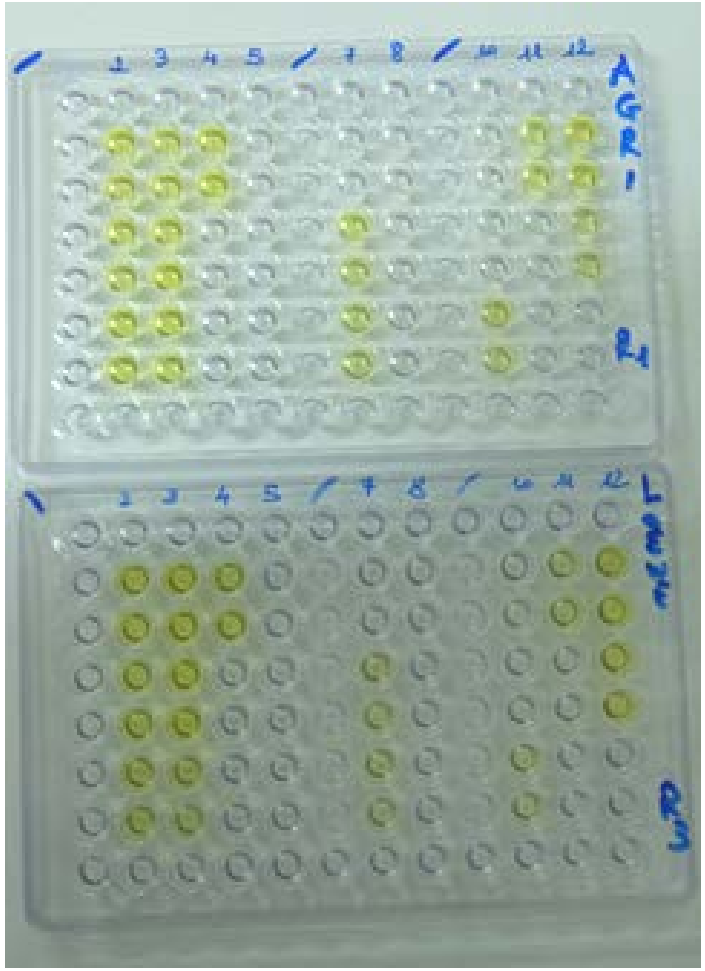
- *Antisera specific to the CoDiRO*

- *DTBIA*

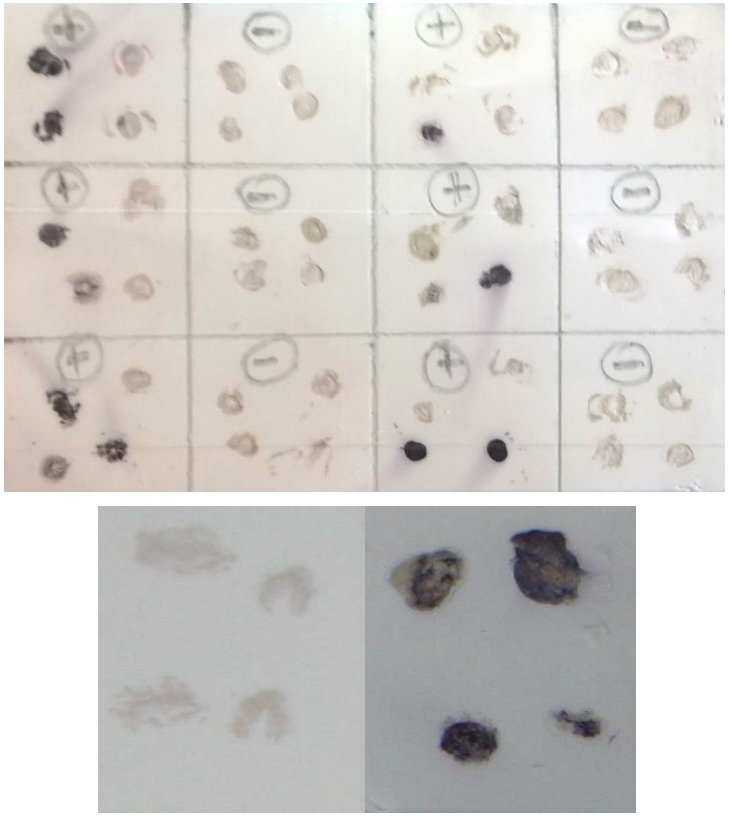




ELISA (LOEWE & AGRITEST)

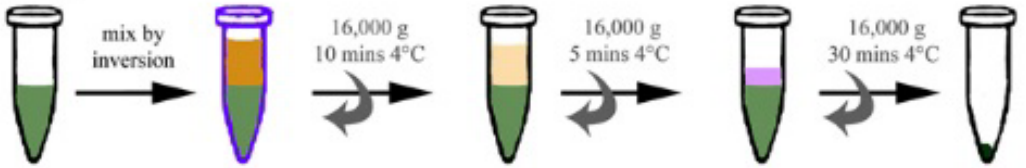


DTBIA

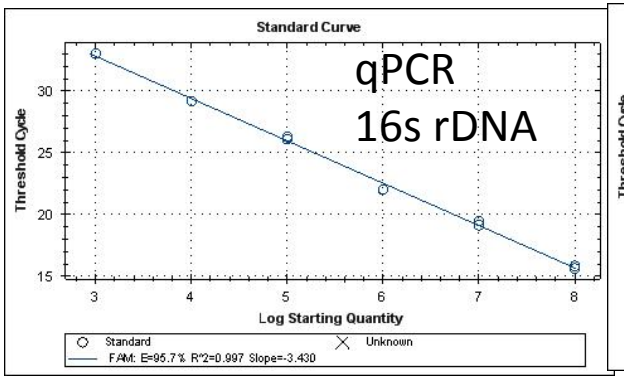


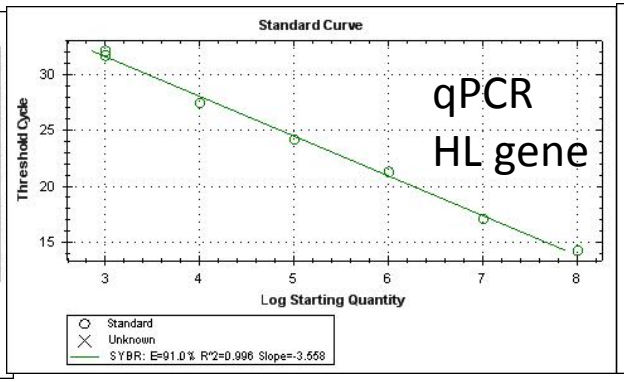
LAMP ASSAYS: no extraction

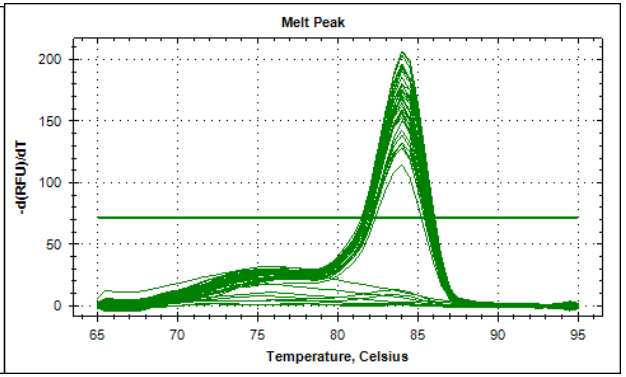
qPCR assays



CTAB extraction ➔ Isopropanol precipitation ➔ Purified DNA







Analytical sensitivity and interlaboratory validations

- ILV – every six months the laboratories involved

Panel of blind samples: - field infected samples of olive and oleander
- artificially spiked samples (quercus, citrus, almond and grape)

TESTS	OLIVE	OLEANDER	GRAPES	ALMOND	CITRUS	OAK
ELISA	PA= 100% NA=100%	PA= 100% NA=100%	PA= 100% NA=100%	PA= 100% NA=100%	PA= 100% NA=100%	PA= 100% NA=100%
PCR (RST31/33)	PA= 80% NA=100%	PA= 100% NA=100%	PA= 100% NA=100%	PA= 100% NA=100%	PA= 100% NA=100%	PA= 100% NA=100%
qPCR	PA= 100% NA=100%	PA= 100% NA=100%	PA= 100% NA=100%	PA= 100% NA=100%	PA= 100% NA=100%	PA= 100% NA=100%
DTBIA	PA= 100% NA=100%					
LAMP	PA= 100% NA=100%	PA= 100% NA=100%				

ELISA & qPCR on different matrices

Matrices (3 spiked samples)	Harper et al., 2010 Cq values	ELISA OD_{405} values
OLIVE	14.57 – 15.26 (ΔCq 0.69)	2.829 – 3.021
ALMOND	15.27 – 15.56 (ΔCq 0.29)	2.559 - 2.963
OAK	14.89 – 15.28 (ΔCq 0.49)	2.734 – 3.062
GRAPE	14.55 – 14.94 (ΔCq 0.39)	2.578 – 2.923
CITRUS	14.87 – 14.91 (ΔCq 0.04)	2.938 – 3.060

Field samples (reaction background can occur)

Notes

qPCR: **In 2 out 5 laboratories, negative samples produced Cq 35-38**

DTBIA: **Produced reliable results but their interpretation could be a limiting factor**

LAMP: **False negative if using only small piece/sample**

Alternatively crude sap of purified total nucleic acid can be used to overcome such erratic reaction

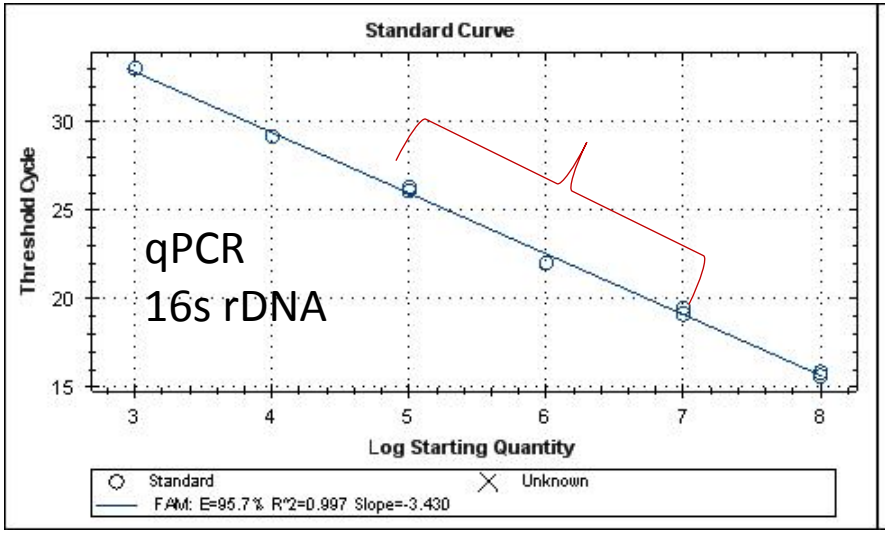
[Conc] & range for field samples

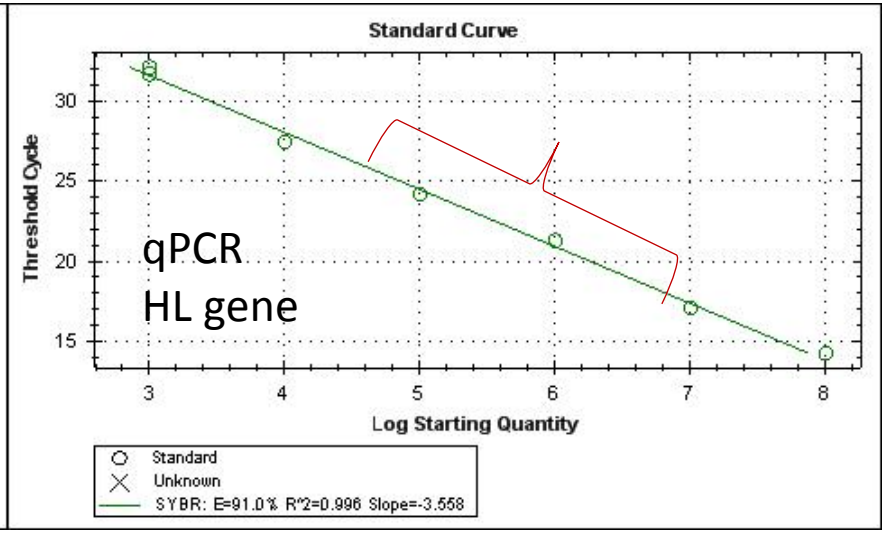
Analytical sensitivity

Technique	CONCENTRATION – CFU/ml OF PLANT EXTRACT						
	10^7	10^6	10^5	10^4	10^3	10^2	10
ELISA Average (Agritest/Loewe)	Positive **3,345**	Positive **3,360**	Positive **2,801**	Positive **0,403**	Negative 0,186	Negative 0,025	Negative 0,023
PCR	Positive	Positive	Positive	Positive	[illegible]	Negative	Negative
qPCR	Positive	Positive	Positive	Positive	Positive	Positive	Negative
LAMP Enbiotech	Positive	Positive	Positive	Positive	Positive	Positive	Negative

Suspected tree

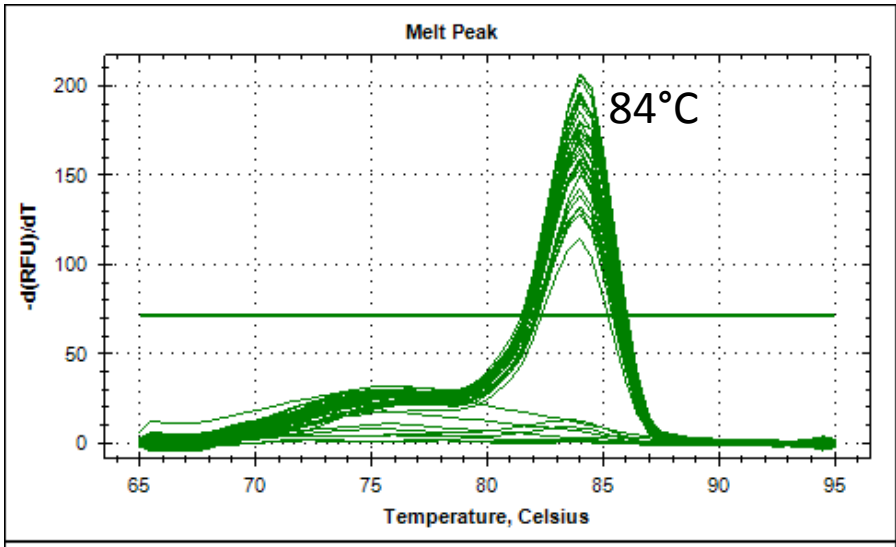
Positive

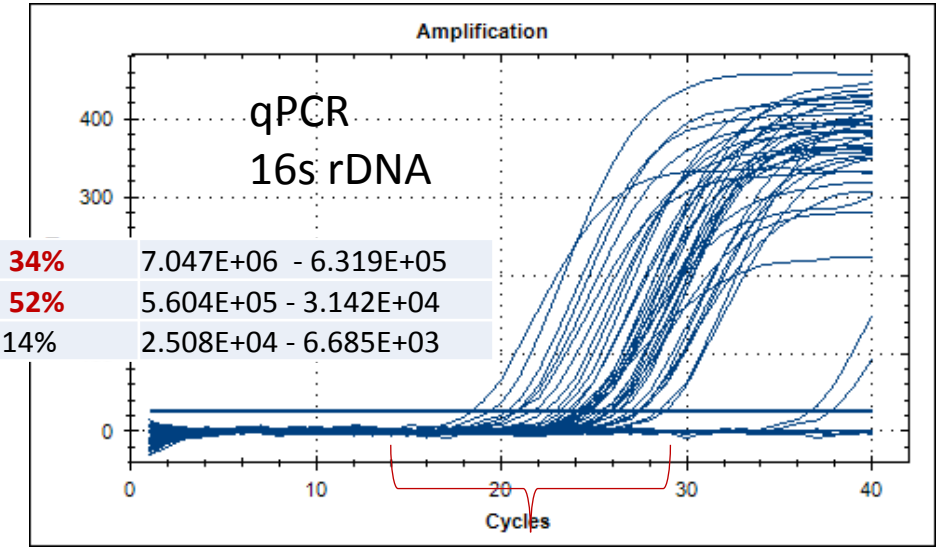




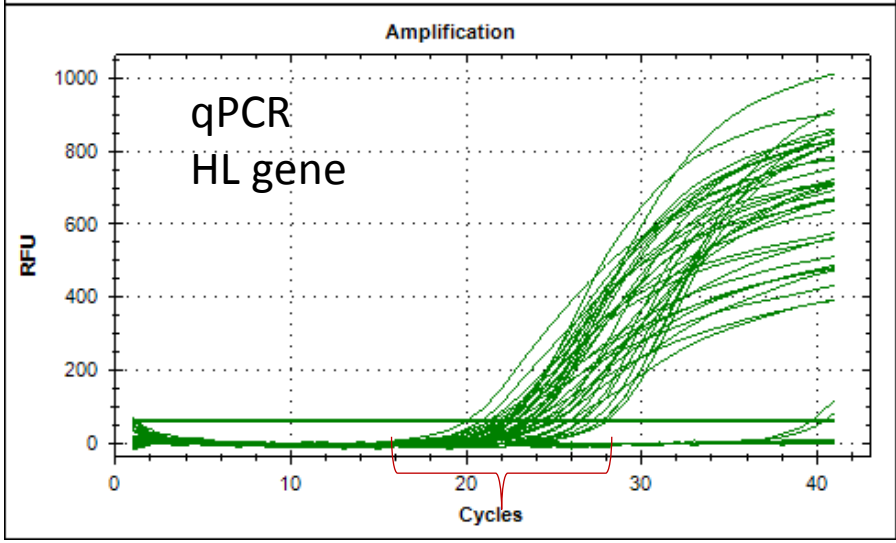
Validation of serological and molecular tests in the field in new the outbreaks (Brindisi province)

ELISA	Average of the OD_{405}
POSITIVE and SUSPECTED SAMPLES (ca. 75%)	57% **(>1,000)** 43% **(0,400 - <1,000)** 20% **(0,100- < 0,400)**
NEGATIVE SAMPLES (ca. 25%)	---





Cq < 24	**34%**	7.047E+06 - 6.319E+05
Cq 24-29	**52%**	5.604E+05 - 3.142E+04
Cq 29-32	14%	2.508E+04 - 6.685E+03



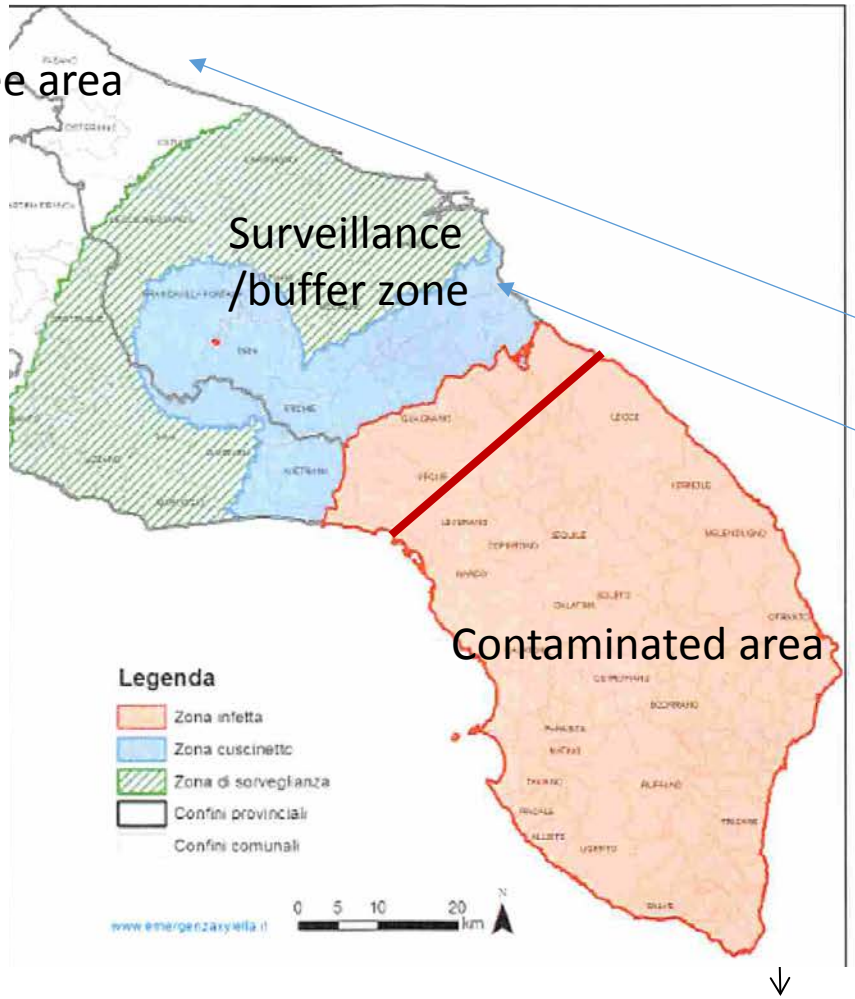
Test performed on October 15th

Validation of serological and molecular tests in the field in new the outbreaks

GIVEN A PANEL OF ca. 900 FIELD SAMPLES CATEGORIZED BY ELISA TEST AS

Positive/suspected (700 SAMPLES)		**Negative (200 samples)**	
qPCR positive	97%	qPCR negative	98%
ELISA false positive	3%	ELISA false negative	2% (qPCR positive)
PCR RST31/33	94%	nd	
DTBIA	71%	nd	
LAMP*	56%	nd	

* Protocol Enbiotech



Large scale monitoring
VISUAL INSPECTIONS + ELISA TESTS

Sample negative

Sample(s) positive or suspected

1% Sent for qPCR confirmation (Xf-free area)
5% sent for qPCR confirmation (Surveilance/Buffer zone)

Sent for qPCR confirmation

Negative

Positive

Negative

Positive

Plant/tree to be re-sampled

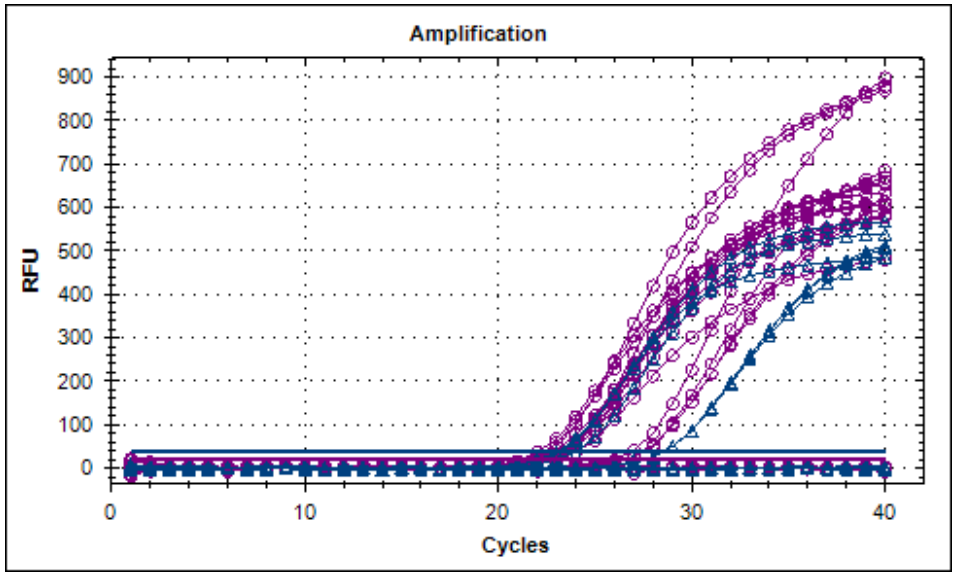
Sample is confirmed positive based on 2 different procedures

For samples that consistently give Cq values >32, it is suggested to proceed with an additional sampling

Ongoing researches:

1) Multiplex qPCR (target Xf gene [16s rDNA gene] and DNA plant internal control – COX)

10-fold serial dilutions (Xf)	Singleplex		Multiplex	
	Cox- CY5	Xf-FAM	Cox-CY5	Xf-FAM
1.000E+08	18.16	18.13	18.18	17.44
1.000E+07	18.95	21.83	18.63	21.50
1.000E+06	18.74	25.54	18.21	24.64
1.000E+05	20.01	28.00	19.03	28.85
1.000E+04	19.11	32.54	19.60	32.34
1.000E+03	18.02	35.36	18.03	35.52
1.000E+02	18.19	37.40	17.99	37.15



Li et al., 2006

Almond, Cherry
Citrus
Grape
Oleander
Acacia saligna
Myrtus and Rosmarinus
Euphorbia terracina
Rhamnus alaternus
Eremofila maculata
Grevillea juperina
Lavandula stoechas
Westringia glabra
Cistus creticus
Asparagus acutifolius
Carissa macrocarpa
Laurus nobilis
Myoporim spp.
Dodonea viscosa purpurea

Cq 16.77 – 19.45

2) Automatization of the extraction procedure

DNeasy *mericon* Food Kit (Modified CTAB procedure)

- **0,5-1gr of tissue**
- **1 spin column**

+

Qiacube

DNeasy *mericon* Food Kit (Modified CTAB procedure)

- *0,5-1gr of tissue*
- *1 spin column*

Parameters	ng/µl	ng/µl
	CTAB	*Mericon*
[DNA]	100-800	18-30
A260/208	>1,8	>2
Cq* Xf	21.68 - 28.62	**16.37 - 25.42**
Cq COX	15.53 - 25.66	18.06 - 20.28

*working on the detection limit

Evaluation of the qPCR master mix

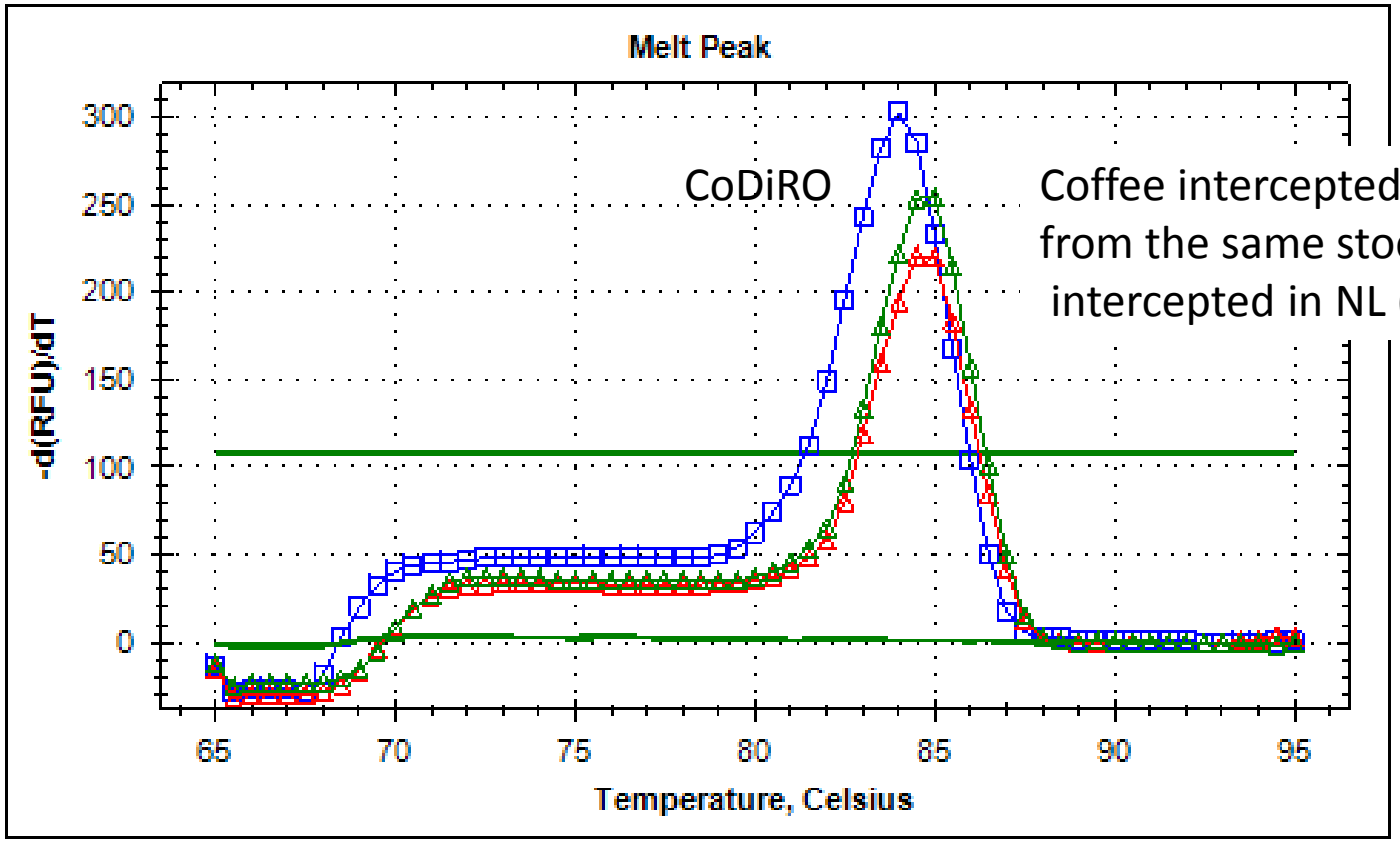
Master mix «A» and «B»

Master mix	Cherry		Oleander		Coffee		Almond		Olive	
	Undilulted	Diluted	Undilulted	Diluted	Undilulted	Diluted	Undilulted	Diluted	Undilulted	Diluted
«A»	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
«B»*	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(+)	(+)	(+)

* Negative control Cq $\geq$36

qPCR HL gene – Sybr green + melt curve analysis

Proved to be a useful tool for the preliminary isolate/strain discrimination



ISOLATION for the CODIRO: BCYE MEDIUM

Surface sterilization: 2% NaOCl – 70% ETOH

1) **Isolation**
2) **Triple cloning**
   (no more than 3 passages)

Cuttings: olive, oleander, cherry, almond, polygala, westringia

Bacterial suspension in PBS/Glycerol for storage

Questions from the Plant Health Service:

- Sampling and tests to be used in the nurseries on propagating material
- Sampling scheme in the field (in the xf-free areas)

THANKS FOR YOUR ATTENTION

maria.saponari@ipsp.cnr.it