

**European Research
on Emerging Plant Diseases
2nd Joint Annual Meeting**

**IMPLEMENTATION OF SAMPLING PROCEDURES FOR
TESTING COMPOSITE SAMPLES
FOR *XYLELLA FASTIDIOSA***

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Commission Implementing Decision (EU) 2018/1511 and 2017/2352, amending Implementing Decision (EU) 2015/789

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

Commission Implementing Decision (EU) 2018/1511 and 2017/2352, amending Implementing Decision (EU) 2015/789

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 or sampled

SAMPLING FOR LABORATORY TESTING

How to process
materials collected
from the sample unit

**? How many leaves
I can pool in the lab**

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

? N. leaves/unit



Testing single infected leaves

Olive

From infected field
symptomatic plant



CTAB extraction
+ qPCR (Harper et al., 2010)

Infected leaves (%)

98%

samples from
monitoring program
(containment area)



33-100%

Oleander

From infected field
symptomatic plant

Extraction by mericon kit
+ qPCR (Harper et al., 2010)



67%

Testing composite samples



A) CONVENTIONAL EXTRACTION METHODS
AND PROTOCOLS, EPPO 7/24 (3)

At least 6 independent replicates

Olive



1 Xf infected
leaf

+



healthy leaves

We started testing **1 infected leaf** mixed with a variable n. of healthy leaves

Poor results, as expected because not all leaves from an infected plants would be positive

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

Testing composite samples to process large sample size

B) USING HIGHER AMOUNT OF TISSUES, WITH CONCENTRATION STEPS IN THE PROTOCOL

At least 7 independent replicates

Olive

20gr ($\approx$ 800
petioles)



+

4 infected petioles



Homogenization
PBS (0.01M) 1:1.5 w:v



REP	Cq	RESULTS
A1	29.45	POS
A2	29.54	POS
A3	28.15	POS
A4	31.04	POS
A5	29.35	POS
A6	31.23	POS
A7	31.36	POS
A8	29.89	POS
A9	30.43	POS
A10	28.71	POS

in RT



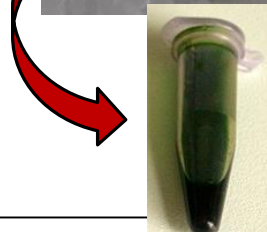
Pellet resuspended
in 1.5 ml PBS (0.01M)



25 min, 10.000g



sup



20 min, 17.000g

Pellet in buffer of
DNA extraction
(CTAB method) + qPCR
Harper et al., 2010)



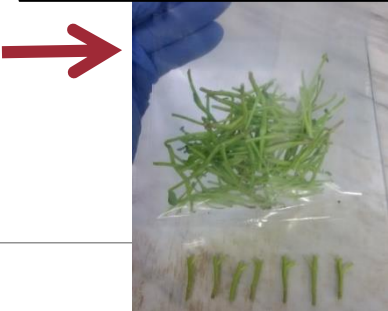
Equal to pool 200 plants

Cabbage

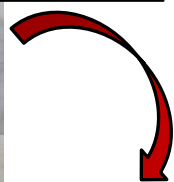


≈ 200 cabbage plants

40gr (≈ 200 stems,
size of 1,5-2cm)



4 infected petioles
of periwinkle



Homogenization
PBS (0.01M)
1:1.5 w:v

25 min, 10.000g



Pellet resuspended
in 1.5 ml PBS (0.01M)



5 min at 3000g



Pellet in buffer o
DNA extraction
(CTAB method) + qP
Harper et al., 2010



20 min,
17.000g

REP	Cq	RESULTS
A1	31.23	POS
A2	26.95	POS
A3	30.31	POS
A4	30.68	POS
A5	30.78	POS
A6	28.03	POS
A7	33.45	POS

Equal to pool 200 plants

Conclusion:

Preliminary important indications both for SAMPLING AND LABORATORY TESTING:

Composite sampling protocols satisfied the samples size requirements (large number of samples selected) using either **the standard extraction procedures** or the **protocol adapted for large tissue amount**:

- (i) minimum number of leaves/xylem parts to be collected from the individual plant of the **sample unit, ranged from 2 to 6** according to the host species, allowing **to pool up to 10 plants with diagnostic standard procedures**
- (ii) the maximum n. of units that can be pooled and processed

STANDARD DIAGNOSTIC PROCEDURES

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

Conclusion:

**Preliminary important indications both for
SAMPLING AND LABORATORY TESTING:**

PROTOCOL ADAPTED FOR LARGE TISSUE AMOUNT

Host species	Sample unit	Pool weight	Leaves-stem/plant
Olive (mature leaves, xylem)	N. 200 plants	20 gr	4 leaves/plant
Cabbage (herbaceous host)	N. 200 plants	40 gr	1 stem/plant

Future activities:

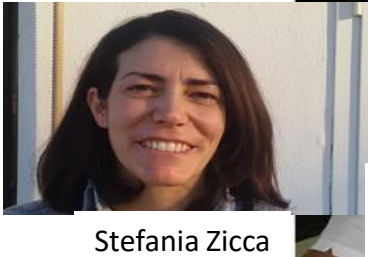
- Produce more data on testing composite samples for other host species using either the standard extraction procedures or the protocol adapted for large tissue amount
- Produce more data on testing composite samples for DORMANT MATERIAL

Thank you.....



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