Act.3.3 „Train laboratory staff on laboratory methods for diagnosing harmful organisms”

TWINNING BA/12/IB/AG 01

„FURTHER STRENGHTENING OF CAPACITIES OF PHYTOSANITARY SECTOR IN THE FIELDS OF PLANT PROTECTION PRODUCTS, PLANT HEALTH AND SEEDS AND SEEDLINGS, INCLUDING PHYTOSANITARY LABORATORIES AND PHYTOSANITARY INSPECTIONS”

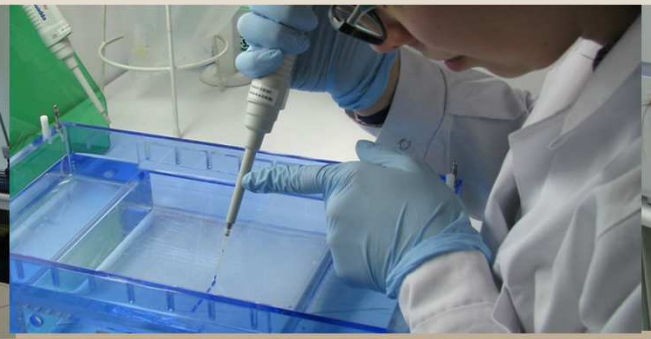
SARAJEVO 7-11th SEPTEMBER 2015

THE STATE PLANT HEALTH AND SEED INSPECTION SERVICE – THE NPPO IN POLAND

THE STATE PLANT HEALTH AND SEED INSPECTION SERVICE – THE NPPO IN POLAND

- MAIN INSPECTORATE OF PLANT HEALTH AND SEED INSPECTION – CENTRAL LABORATORY

  - phytosanitary diagnostics
  - analysis of plant protection products residues
  - GMOs analysis
  - The laboratory with a reference function


Quality Assurance

POLSKIE CENTRUM AKREDYTACJI
POLISH CENTRE FOR ACCREDITATION

PCA

Sygnatariusz EA MLA
EA MLA Signatory

CERTYFIKAT AKREDYTACJI
LABORATORIUM BADAWCZEGO
ACCREDITATION CERTIFICATE OF TESTING LABORATORY
Nr **AB 1205**

Potwierdza się, że: / This is to confirm that:

GŁÓWNY INSPEKTORAT OCHRONY ROŚLIN I NASIENNICTWA
ul. Jana Pawła II 11, 00-828 Warszawa
CENTRALNE LABORATORIUM
ul. Żwirki i Wigury 73, 87-100 Toruń

spełnia wymagania normy PN-EN ISO/IEC 17025:2005
meets requirements of the PN-EN ISO/IEC 17025:2005 standard

Akredytowana działalność jest określona w Zakresie Akredytacji Nr AB 1205
Accredited activity is defined in the Scope of Accreditation No AB 1205

Akredytacja pozostaje w mocy pod warunkiem przestrzegania wymagań jednostki akredytującej określonych w kontrakcie Nr AB 1205
This accreditation remains in force provided the Laboratory observes the requirements of Accreditation Body defined in the Contract No AB 1205

Certyfikat akredytacji ważny do dnia 12.07.2014 r.
The certificate of accreditation is valid until 12.07.2014

POLSKIE CENTRUM AKREDYTACJI PCA 1

ZASTĘPCA DYREKTORA
POLSKIEGO CENTRUM AKREDYTACJI
LUCYNA OLBORSKA

Warszawa, dnia 13 lipca 2010 roku

Quality assurance system implemented since 2010

ISO 17025
Partially flexible scope since 2013
Phytoplasmas since 2012
Plum Pox virus 2010
and a lot of different…

PCA
Polskie Centrum Akredytacji
BADANIA
AB 1205

ilac-MRA

Monday, September 7th

10.00-17.00 Meeting at Federal Institute for Agriculture with representatives of three laboratories

(FEDERAL INSTITUTE OF AGRICULTURE, FEDERAL AGRO-MEDITERRANEAN INSTITUTE AND AGRICULTURE INSTITUTE OF REPUBLIKA SRPSKA)

Theoretical training:

a) Presentation on viruses and phytoplasmas relevant for phytosanitary inspection and the methods of detection and diagnosis
b) Drafting of the methodologies proposed to be developed

Tuesday, September 8th

6

9.00-17.00 Meeting with staff at Federal institute of Agriculture

a) Training on viruses detection

b) Development of SOP on viruses detection

Wednesday, September 9th

9.00-17.00 Meeting with staff at Federal institute of Agriculture

a) Training on viruses detection

b) Development of SOP on viruses detection

Thursday, September 10th

8

9.00-17.00 Meeting with staff at Federal Institute of Agriculture

a) Training on phytoplasma detection

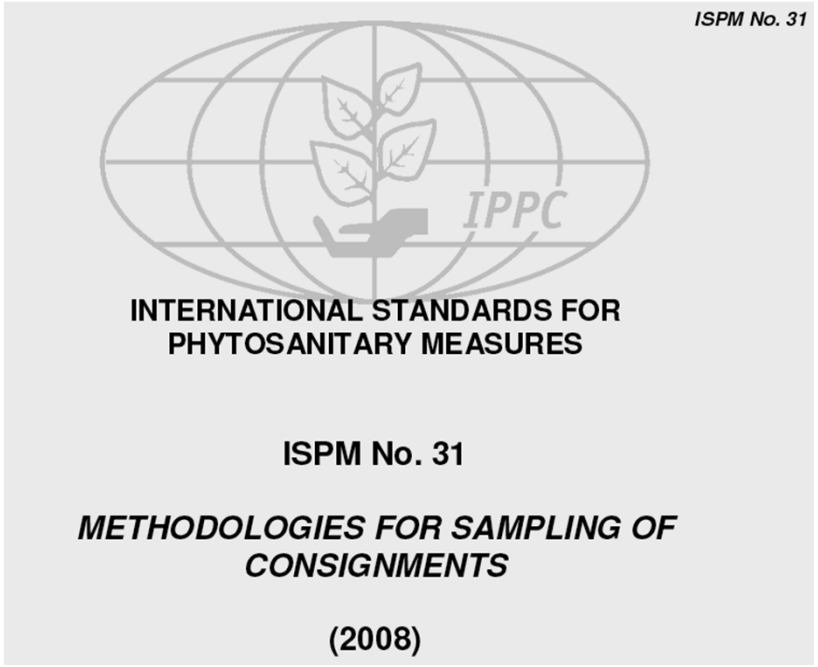
b) Development of SOP on phytoplasma detection

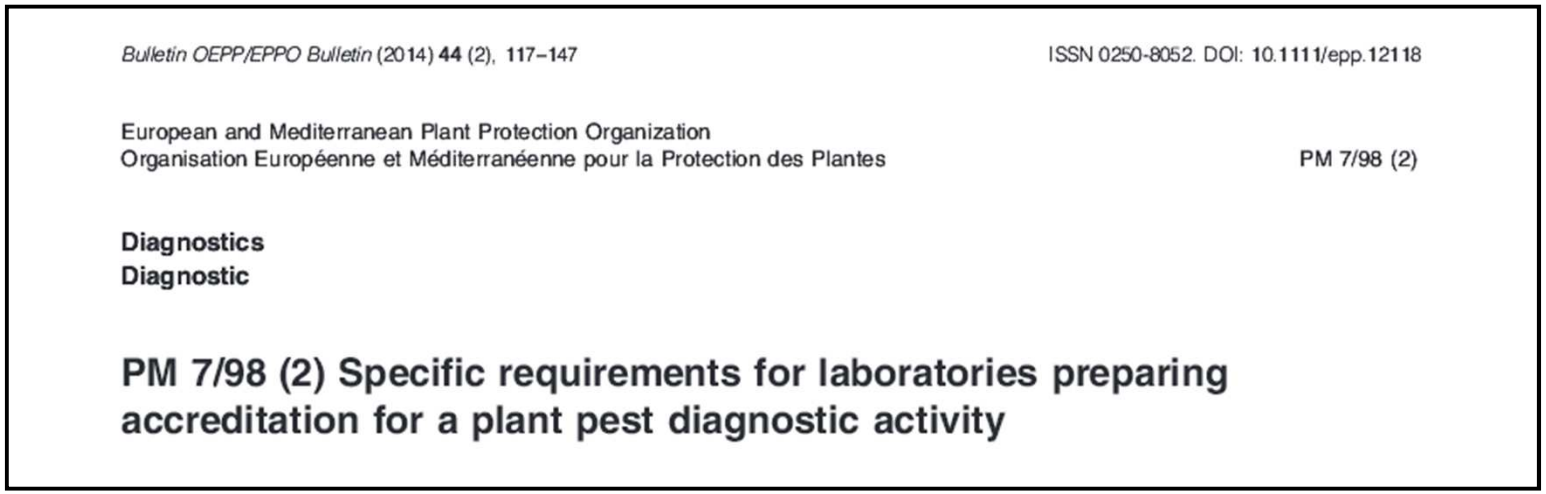
Friday, September 11th

9.00-12.00 Meeting with staff at Federal institute of Agriculture

a) Final discussion on the methods developed and their application

12.30-17.00 Debriefing meeting with RTA and PHPA representatives. Reporting


ISPM No. 31
IPPC
INTERNATIONAL STANDARDS FOR
PHYTOSANITARY MEASURES
ISPM No. 31
METHODOLOGIES FOR SAMPLING OF
CONSIGNMENTS
(2008)


Bulletin OEPP/EPPO Bulletin (2014) 44 (2), 117–147
ISSN 0250-8052. DOI: 10.1111/epp.12118
European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes
PM 7/98 (2)
Diagnostics
Diagnostic
PM 7/98 (2) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity

Diagnostics in practice:

EXPERIMENT

&

REACTION SETUP

Plum Pox Virus

PPV- symptoms leaves

- Symptoms appears on different parts of plant: leaves, petals, fruits and stones.
- Mild, light green discoloration, chlorotic spots, bands or rings, vein clearing or yellowing, or even leaf deformation.
- Flower symptoms can occur on petals (discoloration) of some peach varieties.

PPV- symptoms fruits and stones

- Chlorotic spots or lightly pigmented yellow rings or line patterns.
- Deformed or irregular in shape can develop brown or necrotic small areas.
- Internal browning of the flesh and reduced quality of fruits(can drops prematurely from the tree)
- Pale rings or spots on stones

Photos: EPPO standard PM 7/32 (1)

EPPO A2 List of pests recommended for regulation as quarantine pests

(version 2014-09)

Viruses and virus-like organisms			
Beet leaf curl virus	ds	-	pict
Beet necrotic yellow vein virus (Benyvirus)	ds	diag+fig	pict
Blueberry leaf mottle virus (Nepovirus)	ds	-	-
Blueberry scorch virus (Carlavirus)	(ds)	-	-
Chrysanthemum stunt viroid (Pospiviroid)	ds	diag	pict
Citrus tristeza virus (Closterovirus)	ds	diag	pict
Cucumber vein yellowing virus (Ipomovirus)	ds	diag	pict
Cucurbit yellow stunting disorder virus (Crinivirus)	ds	-	-
Impatiens necrotic spot virus (Tospovirus)	ds	diag	pict
Pepino mosaic virus (Potexvirus)	(ds)	diag	pict
Plum pox virus (Potyvirus)	ds	diag	pict
Potato spindle tuber viroid (Pospiviroid)	ds	diag	pict
Raspberry ringspot virus (Nepovirus)	ds	-	pict
Satsuma dwarf virus (Sadwavirus)	ds	-	pict
Squash leaf curl virus (Begomovirus)	ds	-	pict
Strawberry veinbanding virus (Caulimovirus)	ds	-	pict
Tobacco ringspot virus (Nepovirus)	ds	diag	pict
Tomato chlorosis virus (Crinivirus)	ds	diag	-
Tomato infectious chlorosis virus (Crinivirus)	ds	diag	-
Tomato ringspot virus (Nepovirus)	ds	diag	pict
Tomato spotted wilt virus (Tospovirus)	ds	diag	pict
Tomato yellow leaf curl virus (Begomovirus) and related viruses	ds	diag	pict

EPPO recommends its member countries to regulate the pests listed below as quarantine pests (A2 pests are locally present in the EPPO region). The EPPO A2 List is reviewed every year by the Working Party on Phytosanitary Regulations and approved by Council.

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

archives.eppo.int/EPPOStandards/diagnostics.htm

PM 7/32(1)	*Plum pox potyvirus* - under revision	-

International Plant Protection Convention
Protecting the world's plant resources from pests

https://www.ippc.int/en/core-activities/standards-setting/ispms/

ISPM 27 Annex 02 (2012)	DP 02: Plum pox virus	17 Jul 2012	show/hide Details

International Plant Protection Convention
Protecting the world's plant resources from pests

https://www.ippc.int/en/core-activities/standards-setting/ispms/

ISPM 27	Diagnostic protocols for regulated pests	28 Aug 2012	show/hide Details
ISPM 27 Annex 01 (2010)	DP 01: Thrips palmi Karny	15 Dec 2011	show/hide Details
ISPM 27 Annex 02 (2012)	DP 02: Plum pox virus	17 Jul 2012	show/hide Details
ISPM 27 Annex 03 (2012)	DP 03: Trogoderma granarium Everts	17 Jul 2012	show/hide Details
ISPM 27 Annex 04 (2014)	DP 04: Tilletia Indica Mitra	21 Apr 2015	show/hide Details
ISPM 27 Annex 05 (2014)	DP 05: Phyllosticta citricarpa (McAlpine) Aa on fruit	29 Aug 2014	show/hide Details
ISPM 27 Annex 06 (2014)	DP 6: Xanthomonas citri subsp. citri	02 Sep 2014	show/hide Details
ISPM 27 Annex 7 (2015)	DP 07: Potato spindle tuber viroid	18 Feb 2015	show/hide Details

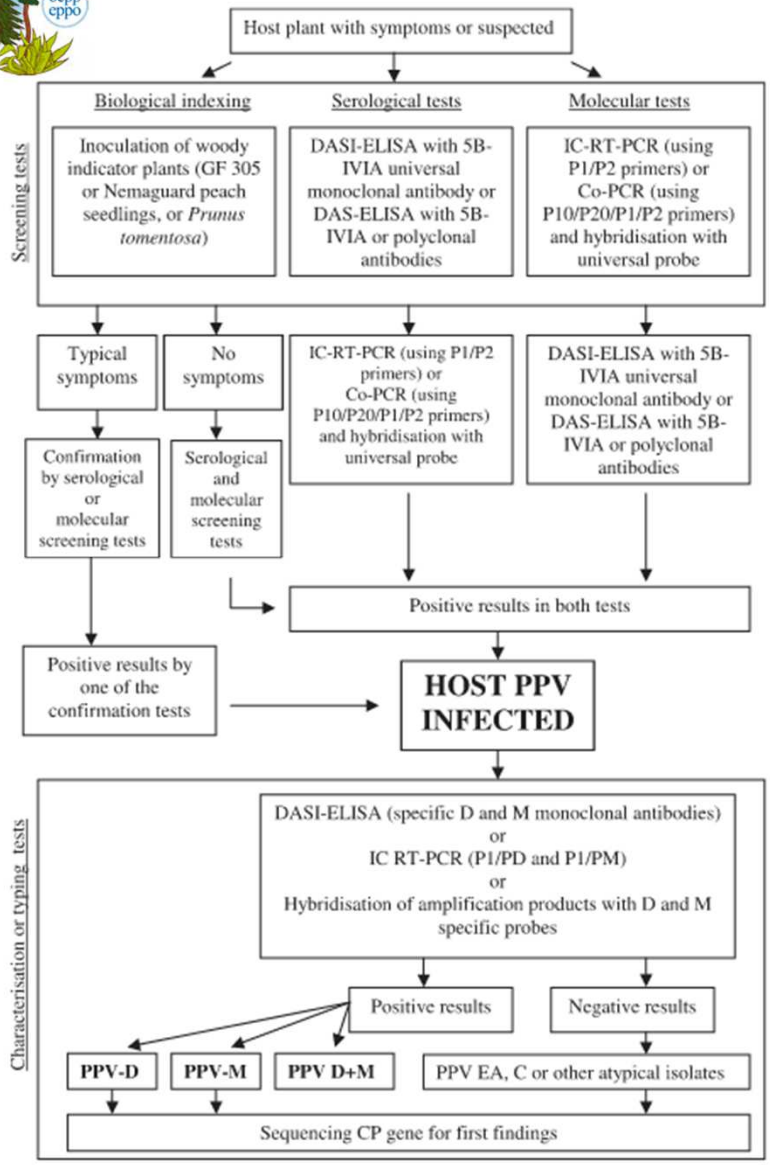


Fig. 3 Decision scheme for detection and identification of ***Plum pox potyvirus***.

Detection of PPV can be achieved using a biological, serological or molecular test; identification requires either a serological or molecular test. A serological or molecular test is the minimum requirement to detect and identify PPV (e.g. during routine diagnosis of a pest widely established in a country). In instances where the national plant protection organization (NPPO) requires additional confidence in the identification of PPV (e.g. detection in an area where the virus is not known to occur or detection in a consignment originating in a country where the pest is declared to be absent), further tests may be done. Where the initial identification was done using a molecular method, subsequent tests should use serological techniques and vice versa.

Further tests may also be done to identify the strain of PPV present

PPV- SAMPLING

General guidance on sampling methodologies is described in ISPM 31:2008 (*Methodologies for sampling of consignments*). Appropriate sample selection is critical for PPV detection. Sampling should take into account virus biology and local climatic conditions, in particular the weather conditions during the growing season. If typical symptoms are present, collect flowers, leaves or fruits showing symptoms. In symptomless plants, samples should be taken from at least one-year-old shoots with mature leaves or fully expanded leaves collected from the middle of each of the main branches (detection is not reliable in shoots less than one year old). Samples should be collected from at least four different sites (e.g. four branches or four leaves) in each plant; this is critical because of the uneven distribution of PPV. Sampling should not be done during months with the highest temperatures. Tests on samples collected in the autumn are less reliable than tests done on samples collected earlier in the spring. Plant material should preferably be collected from the internal parts of the tree canopy. In springtime, samples can be flowers, shoots with fully expanded leaves or fruits. In summer and autumn, mature leaves and the skin of mature fruits collected from the field or packing houses can be used for analysis. Flowers, leaves, shoots and fruit skin can be stored at 4 °C for not more than 10 days before processing. Fruits can be stored for one month at 4 °C before processing. In winter dormant buds or bark tissues from the basal part of twigs, shoots, or branches, or complete spurs can be selected.

ISPM 27 Annex 02 (2012)

In some circumstances (e.g. during the routine diagnosis of a pest widely established in a country) multiple plants may be tested simultaneously using a bulked sample derived from a number of plants. The decision to test individual or multiple plants depends on the virus concentration in the plants and the level of confidence required by the NPPO.

PM 7/32(1)

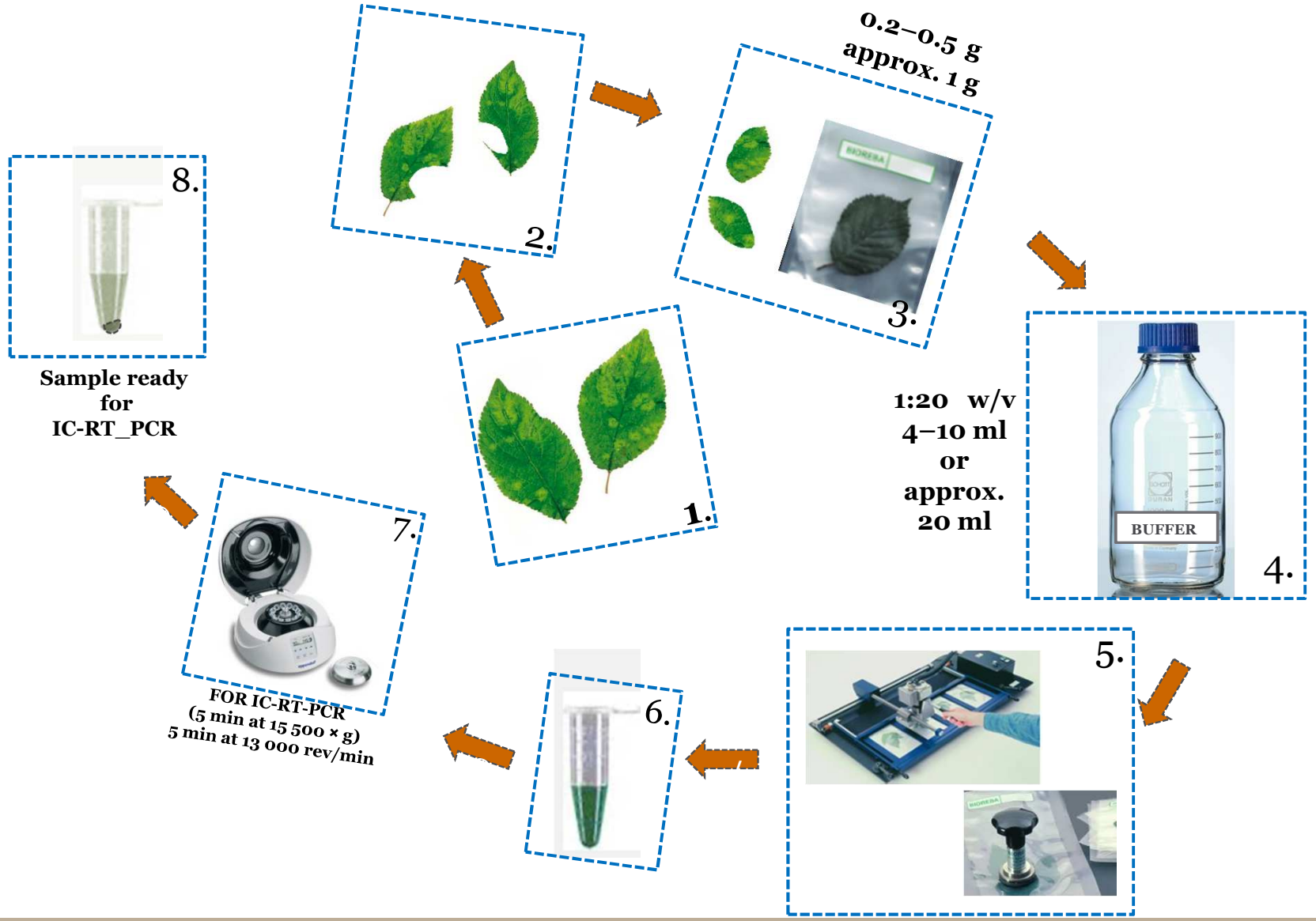
under revision

Sampling

Appropriate sample selection is critical for serological or molecular detection. If typical symptoms are present, symptomatic flowers, leaves or fruits should be collected. In symptomless plants, a standard sample should be taken of 5 shoots or 10 fully expanded leaves collected around the canopy of each individual tree from the middle of each scaffold branch, until the appearance of high temperatures at the beginning of summer.

Sampling from July to the beginning of September should be avoided in Mediterranean climates. Plant material should preferably be selected from the internal structure of the tree. Samples in spring can be flowers, young shoots or small fruits. Mature leaves can be collected for analysis in autumn. Samples can be stored at 4 °C for not more than 7 days before processing. Fruits can be stored for 1 month at 4 °C. Dormant buds or bark from shoots or branches can be selected in winter.

PPV Sample preparation

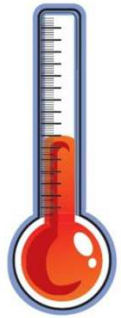


PPV IC-RT-PCR

1. Coat PCR tubes: prepare a dilution (1 µg/ml) of PPV specific antibody in carbonate buffer Dispense **100 µl** of the diluted antibodies into the tubes.

2. Incubate at **37 °C** for **3 h**

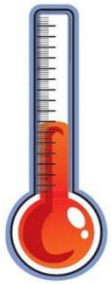
37°C



3. Wash tubes twice with **150 µl** of sterile washing buffer

4. Submit freshly prepared sample extract to an immunocapture phase for **2 h** on **ice or** alternatively **at 37** °C in coated tubes.

or 37°C



5. Wash tubes three times as before

6. Prepare the RT-PCR reaction mix and add directly to the coated PCR tubes. Perform the amplification and visualise products on a gel

PPV IC-RT-PCR MIX preparation

P1 (5′-ACC GAG ACC ACT ACA CTC CC-3′)
P2 (5′-CAG ACT ACA GCC TCG CCA GA-3′)

Wetzel *et al.* (1991)

Component	Concentration	Final Concentration	V per reaction	number of reactions	V per ..... reactions	identification
H_2O			17,735			
10x Taq Buffer with 15 mM $MgCl_2$	10x (1.5 mM $MgCl_2$)	1x	2,5			
dNTPs	10 mM	250 µM	0,625			
Triton X-100	4%	0,30%	2			
Primer P1	25 µM	1 µM	1			
Primer P2	25 µM	1 µM	1			
AMV	25 U/ µl	1 U	0,04			
TaqPol	5 U/µl	0,5 U	0,1			
Total			**25**			

*Pipetting loss: allow cca 10% more (e.g. if you have 10 reactions, preapare for 11)

Conditions for RT-PCR:
42°C for 45 min; 94°C for 2 min; 40 cycles (at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min); 72°C for 10 min; 4°C hold

Prepare 2% agarose gel in 0.5X TAE buffer
The P1/P2 primers produce a 243 base pair (bp) amplicon

PPV IC-RT-PCR

- Remember about positive and negative controls
- How about environmental control?
- WATCH OUT- **CONTAMINATION**!!!!

Diagnostics in practice:

EXPERIMENT

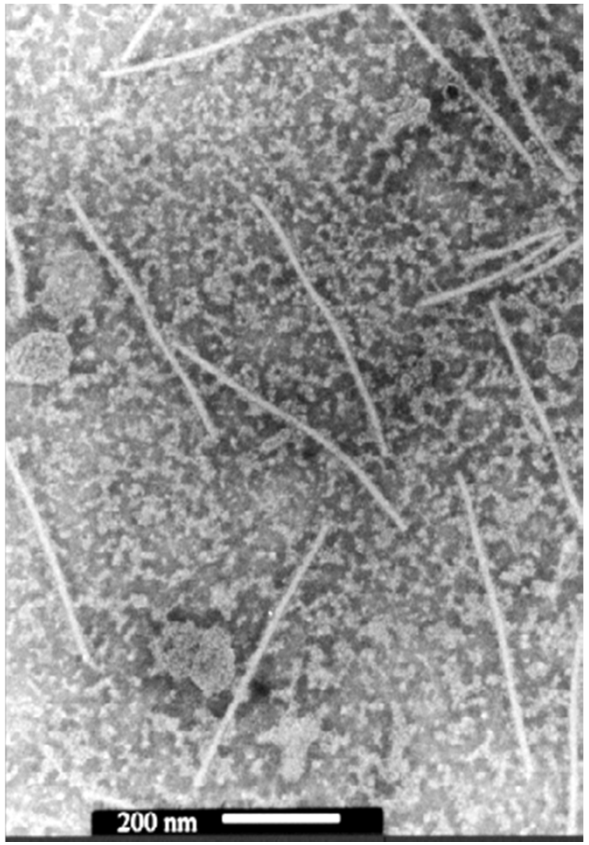
&

REACTION SETUP

Pepino Mosaic Virus

Pepino mosaic virus (PepMV)

- Genus: *Potexvirus*
- First findings in 1980 on Pepino (Peru), on tomatoe since 1999
- Found in Great Britain and the Netherlands- then in the rest of european countries

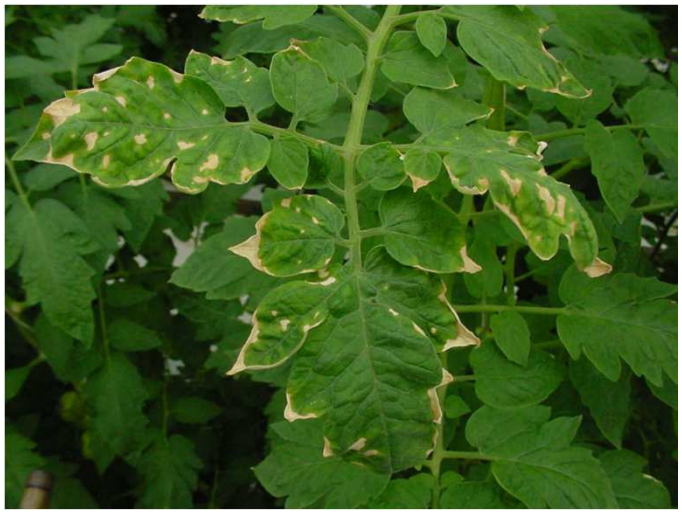


PepMV: early symptoms

- „Bubbles” on the top leaves of young plants. Plant looks a little like a nettle.

- Sometimes yellow mosaics

PepMV: late symptoms



- During the maturation of plants most of the symptoms disappear, they remain only a few yellow spots

- Burned leaves / wilting plants at high temperatures can also be associated with PepMV

PepMV: transmission

- Mechanical
- Vectors? Bees
- Plant-plant contact
- Seeds ?
- Soil

EPPO A2 List of pests recommended for regulation as quarantine pests

(version 2014-09)

Viruses and virus-like organisms			
Beet leaf curl virus	ds	-	pict
Beet necrotic yellow vein virus (Benyvirus)	ds	diag+fig	pict
Blueberry leaf mottle virus (Nepovirus)	ds	-	-
Blueberry scorch virus (Carlavirus)	(ds)	-	-
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Citrus tristeza virus (Closterovirus)	ds	diag	pict
Cucumber vein yellowing virus (Ipomovirus)	ds	diag	pict
Cucurbit yellow stunting disorder virus (Crinivirus)	ds	-	-
Impatiens necrotic spot virus (Tospovirus)	ds	diag	pict
Pepino mosaic virus (Potexvirus)	(ds)	diag	pict
Plum pox virus (Potyvirus)	ds	diag	pict
Potato spindle tuber viroid (Pospiviroid)	ds	diag	pict
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Strawberry veinbanding virus (Caulimovirus)	ds	-	pict
Tobacco ringspot virus (Nepovirus)	ds	diag	pict
Tomato chlorosis virus (Crinivirus)	ds	diag	-
Tomato infectious chlorosis virus (Crinivirus)	ds	diag	-
Tomato ringspot virus (Nepovirus)	ds	diag	pict
Tomato spotted wilt virus (Tospovirus)	ds	diag	pict
Tomato yellow leaf curl virus (Begomovirus) and related viruses	ds	diag	pict

A2 in 2012

EPPO recommends its member countries to regulate the pests listed below as quarantine pests (A2 pests are locally present in the EPPO region). The EPPO A2 List is reviewed every year by the Working Party on Phytosanitary Regulations and approved by Council.

PepMV EPPO Standard PM 7/113 (1)

Bulletin OEPP/EPPO Bulletin (2013) **43** (1), 94–104 ISSN 0250-8052. DOI: 10.1111/epp.12023

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

PM 7/113 (1)

Diagnostics
Diagnostic

PM 7/113 (1) Pepino mosaic virus

PepMV- EPPO PM 7/113 (1)

Courtesy: Dr Andrea Minuto, Centro di Saggio, CERSAA, Albenga (IT)

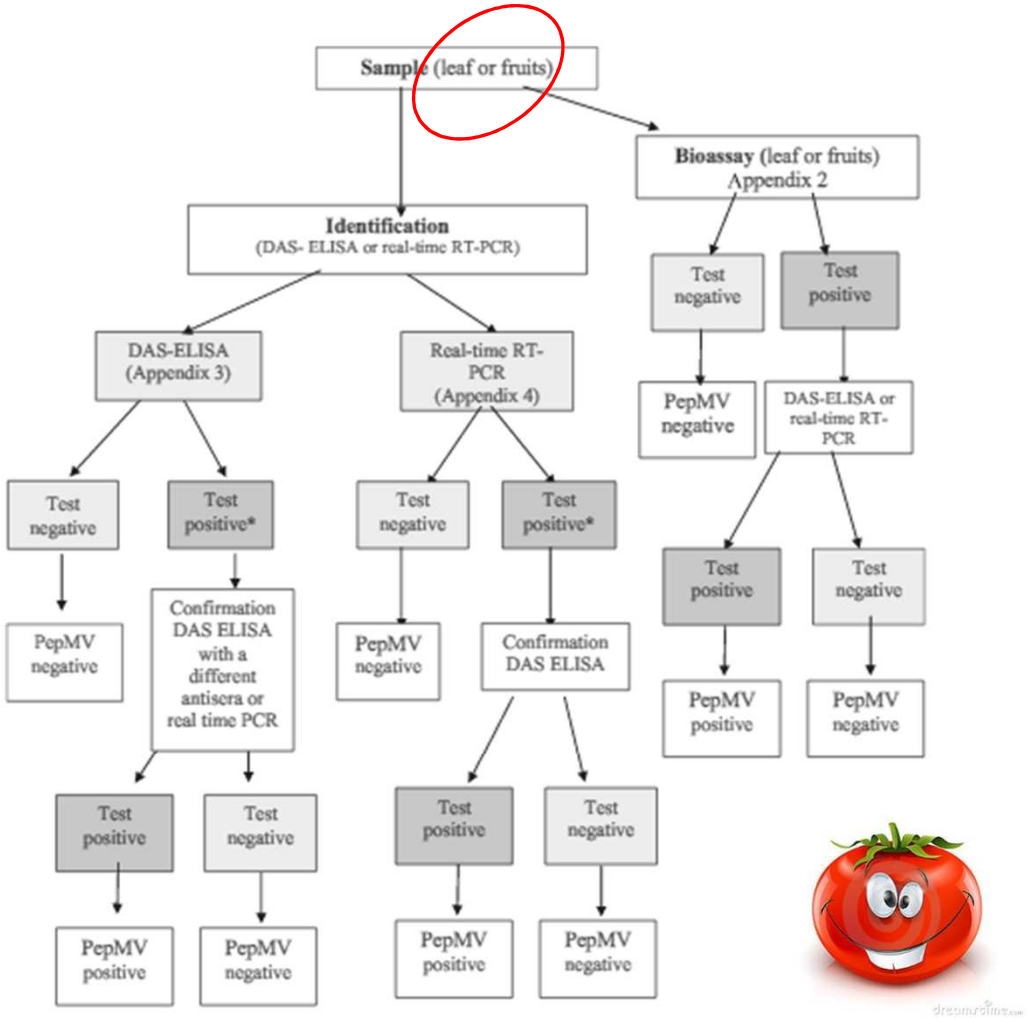
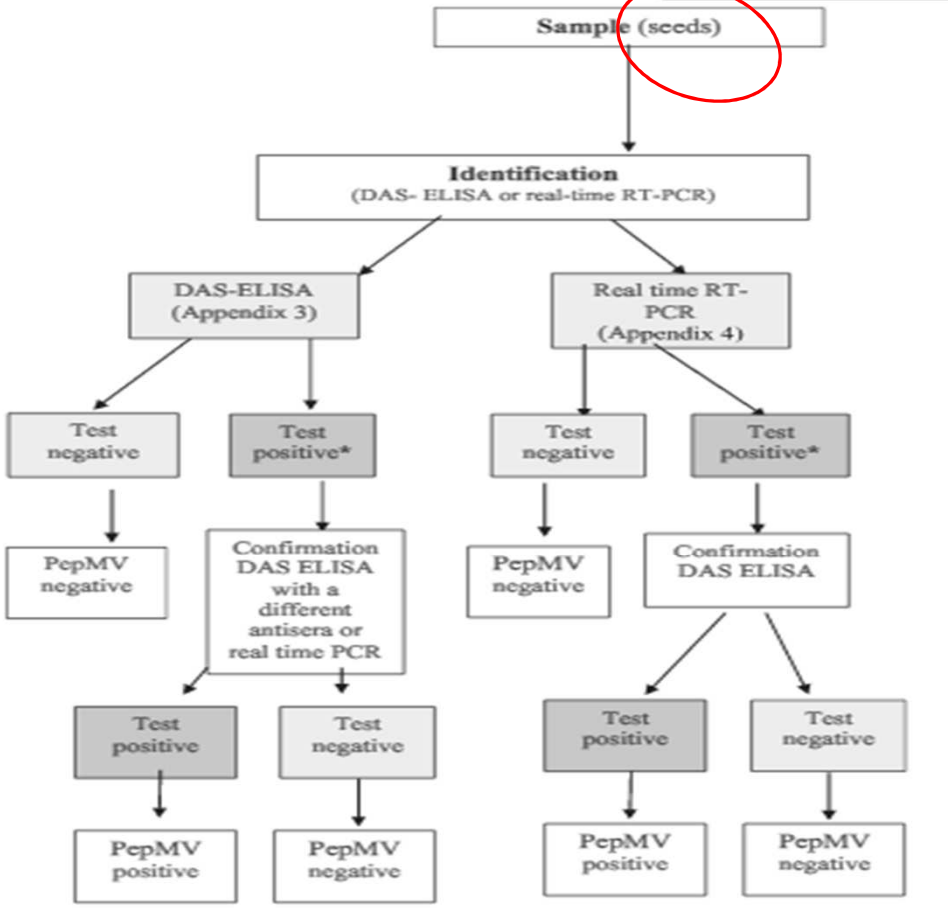


Fig. 1 Flow diagram for the detection and identification of PepMV on fruit or leaf samples. Sample (leaf or fruits). *In specific situations (see PM 7/76) a confirmatory test is required which should be different from that used for primary identification.

* In specific situations (see PM 7/76) a confirmatory test is required which should be different from that used for primary identification.

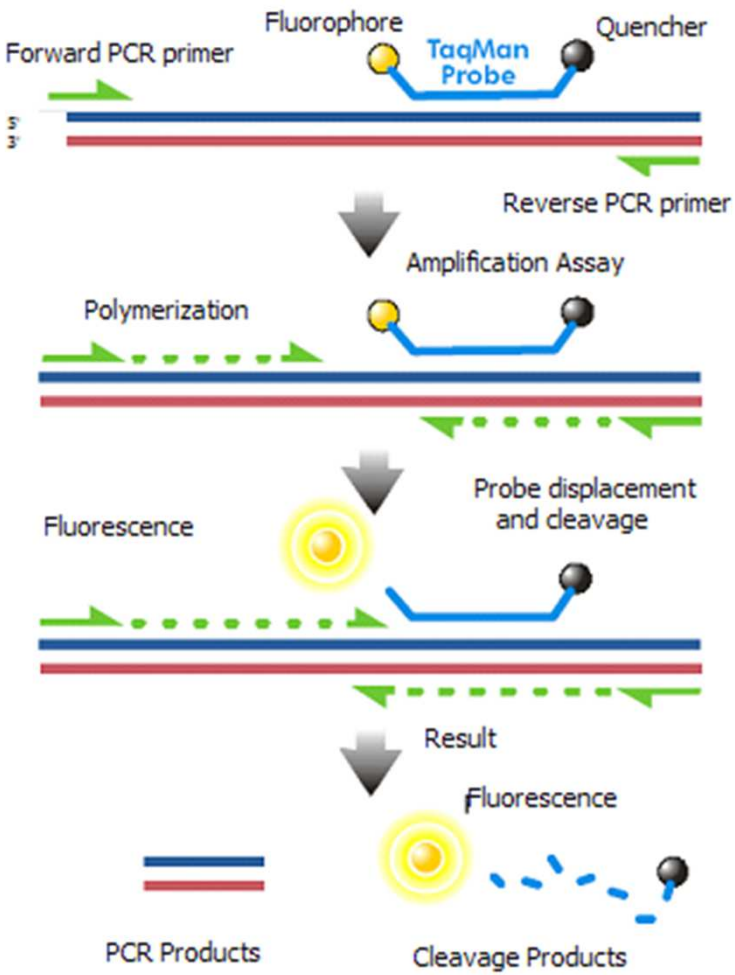
PepMV- EPPO PM 7/113 (1)



* In specific situations (see PM 7/76) a confirmatory test is required which should be different from that used for primary identification.

Fig. 2 Flow diagram for the detection and identification of PepMV on seeds. Sample (seeds). *In specific situations (see PM 7/76) a confirmatory test is required which should be different from that used for primary identification.

PepMV- TaqMan probe chemistry mechanism



Spectral Properties Table

Dye	Max. EX (nm)	Max. EM (nm)	Compatible Quencher
6-FAM™	494	515	BHQ-1, TAMRA
JOE™	520	548	BHQ-1, TAMRA
TET™	521	536	BHQ-1, TAMRA
Cal Fluor® Gold 540[1]	522	541	BHQ-1
HEX™[2]	535	555	BHQ-1, TAMRA
Cal Fluor Orange 560[2]	540	561	BHQ-1
TAMRA™	555	576	BHQ-2
Cyanine 3	550	570	BHQ-2
Quasar® 570[3]	548	566	BHQ-2
Cal Fluor Red 590[4]	565	588	BHQ-2
ROX™	573	602	BHQ-2
Texas Red®	583	603	BHQ-2
Cyanine 5	651	674	BHQ-3
Quasar 670[5]	647	667	BHQ-3
Cyanine 5.5	675	694	BHQ-3

[1]JOE/TET alternative
[2]VIC® alternative
[3]Cyanine 3 alternative
[4]TAMRA alternative
[5]Cyanine 5 alternative

PepMV- seed sampling

Sampling for seed testing

The recommended minimum sample size is 3000 seeds with a maximum sub-sample size of 250 seeds (ISHI-Veg Seed Health Testing Methods Reference manual http://www.worldseed.org/isf/ishi_vegetable.html). For small seed lots, smaller samples size may have to be tested (e.g. in France only 1000 seeds are sampled from lots smaller than 600 g).

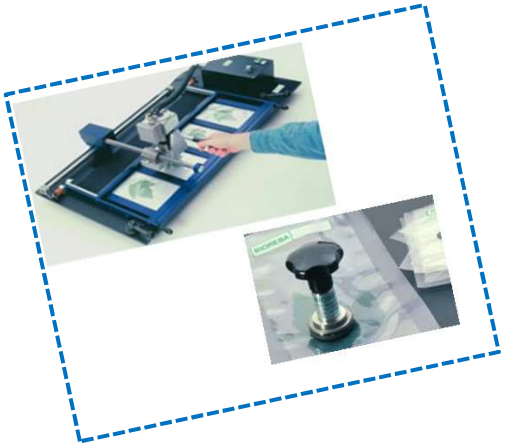
PepMV- seed extraction

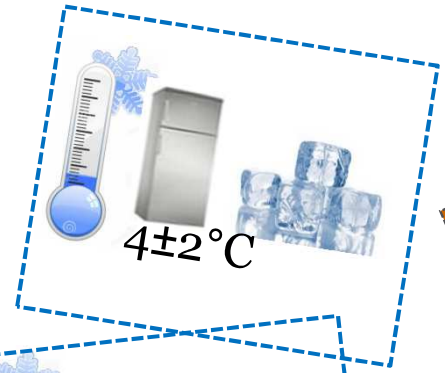
CLOSE!

250 seeds
+ 10 ml
0.1 M phosphate buffer

4±2°C

overnight





4±2°C

- 20±2°C

Or other methode of extraction RNA

PepMV- Q-bank information

PepMV real-time RT-PCR (Ling et al, 2007)	
General information	
Type of molecular test :	Real-time RT-PCR TaqMan
Described in EPPO Protocol :	PM 7/113 (1) Pepino mosaic virus
Contact :	RAA van der Vlugt
Link to virus species information :	Pepino mosaic virus (PepMV)
Remarks on molecular tests :	Method based on Ling et al (2007), modified and evaluated in a test performance study (PEPEIRA, 2009); validation data published by Gutierrez-Aguirre et al (2009)

PepMV- Q-bank information

Test specifications

Primer and probe information

Target

Target of amplification :	TGB2: positions 5126–5232 in isolate Ch1 (acc nr DQ000984)
Host sequence accession nr :	EU-tom (AJ438767, AJ606359, AJ606360, AF340024, AF484251); Ch-1 (DQ000984, AY509926); Ch-2 (DQ000985); Peruvian (AJ606361, AM109896); US-2 AY509927)
Forward primer sequence :	KL05-48 forward 1: 5'-ACTCCTAGAGCTGACCTCAC-3' (Ch1, EU-tom, Peruvian) KL05-49 forward 2: 5'-ACTCCTAGAGCTGATCTTAC-3' (Ch2, US2)
Reverse primer sequence :	KL05-51 reverse 1: 5'-TCTCCAGCAACAGGTTGGTA-3' (Ch1, EU-tom, Peruvian) KL05-52 reverse 2: 5'-TCACCTGCAACTGGTTGATA-3' (Ch2, US2)
TaqMan probe sequence :	KL05-50 probe: 5'-FAM-TGTCAGCTTGCATTTACTTCCAAAA-BHQ-3' (All PepMV isolates)
Location of TaqMan probe :	5181-5205 nt in Ch1
Expected amplicon size :	107 nt

Internal control

Target of Internal Control (IC) :	COX (plant endogene cytochrome oxidase)
Forward IC primer sequence :	COX F: 5'-CGTCGCATTCCAGATTATCCA-3'
Reverse IC primer sequence :	COX RW: 5'-CAACTACGGATATATAAGRRCCRRAACTG-3'
TaqMan IC probe sequence :	COXSOL 1511T: 5'-VIC-AGGGCATTCCATCCAGCGTAAGCA-BHQ-3'

PepMV- Q-bank information

(RT-) PCR programme	
PCR Cycling conditions :	30 min 48°C, 10 min 95°C, 40x (15 s 95°C, 1 min 60°C); for ABI machines do NOT run in 9600 emulation mode
Protocol (specification reagents and master mix)	
Link to protocol (pdf) :	PepMV real-time RT-PCR (Ling et al, 2007)
Bibliography	
Bibliography :	1. Gutiérrez-Aguirre et al, 2009 2. Ling et al, 2007

www.q-bank.eu/Virus/BioloMICS.aspx?Link=T&TableKey=2821647000000016&Rec=1&Fields=All

Pepino Mosaic Virus

Tasks:

1. Plan a plate
2. Calculate mix preparation per 1 reaction for each gene
3. Determine number of reactions for each gene*
4. Calculate mix preparation for each gene for all reactions

*Pipetting loss: allow cca 10% more (e.g. if you have 10 reactions, preapare for 11)

Pepino Mosaic Virus-Mastermix preparation

Master mix

Reagent	Working concentration	Volume per reaction (µl)	Final concentration
DNase and RNase free water	-	9.25	-
ABI Taq Gold Buffer A buffer	10x	2.5	1x
$MgCl_2$	25000 µM	5.5	5500 µM
dNTP Mix (each)	6250 µM	2.0	500 µM
PCR forward primers (each)	7.5 µM	1.0	0.3 µM
PCR reverse primers (each)	7.5 µM	1.0	0.3 µM
Probe(s)	5.0 µM	0.5	0.1 µM
MMLV Reverse transcriptase	4 U/µl	0.125	10 U
Taq polymerase	5 U/µl	0.125	0.625 U
Subtotal		24.0	
Total RNA		1.0	
Total		25.0	

Master mix internal positive control

Reagent	Working concentration	Volume per reaction (µl)	Final concentration
DNase and RNase free water	-	11.25	-
ABI Taq Gold Buffer A buffer	10x	2.5	1x
$MgCl_2$	25000 µM	5.5	5500 µM
dNTP Mix (each)	6250 µM	2.0	500 µM
COX-F forward primer	7.5 µM	1.0	0.3 µM
COX-RW reverse primer	7.5 µM	1.0	0.3 µM
COXSOL 1511T probe	5.0 µM	0.5	0.1 µM
MMLV reverse transcriptase	4 U/µl	0.125	10 U
Taq polymerase*	5 U/µl	0.125	0.625 U
Subtotal		24.0	
Total RNA		1.0	
Total		25.0	

*Pipetting loss: allow cca 10% more (e.g. if you have 10 reactions, preapare for 11)

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Pepino Mosaic Virus-Mastermix preparation

MIX				
Components	Final concentration	Volume per reaction (µl)	Volume per .....reactions (µl)	Identification # Cat or lot #
Bufor Gold A (10x)	1x	2,5		
MgCl2 (25 mM)	5,5 mM	5,5		
dNTPs (10 mM)	0,5 mM	1,25		
Primer KL05-48 (7,5 uM)	0,3 uM	0,75		
Primer KL05-49 (7,5 uM)	0,3 uM	0,75		
Primer KL0-51 (7,5 uM)	0,3 uM	0,75		
Primer KL0-52 (7,5 uM)	0,3 uM	0,75		
Probe KL0-50 (5 uM)	0,1 uM	0,5		
MMLV200 U/ul	0.4 U/ul ie 10 U per reaction	0,125 0,125 of predilution (ie 0,5 U per reaction)		
Taq Poly (5U/ul)	0,625 U	0,125		
H2O		11		
		24		
template		1		
Total		25		

*Pipetting loss: allow cca 10% more (e.g. if you have 10 reactions, preapare for 11)

Pepino Mosaic Virus-Mastermix preparation

COX				
Components	Final concentration	Volume per reaction (µl)	Volume per .....reactions (µl)	Identification # Cat or lot #
Buffer Gold A (10x)	1x	2,5		
MgCl2 (25 mM)	5,5 mM	5,5		
dNTPs (10 mM)	0,5 mM	1,25		
Primer COX F (7,5 uM)	0,3 uM	1		
Primer COX R (7,5 uM)	0,3 uM	1		
COX Probe (5 uM)	0,1 uM	0,5		
MMLV200 U/ul (predilution 4U/ul)	0.4 U/ul ie 10 U per reaction	0,125 of predilution (ie. 0,5 U per reaction)		
Taq Poly (5U/ul)	0,625 U	0,125		
H2O		12		
		24		
template		1		
Total		25		

Pepino Mosaic Virus – controls [nucleic acid isolation (sample) controls]

	Description	Control?	When to use?
NIC: Negative Isolation Control	- water/buffer instead of sample - ”healthy sample” of the same type	- contamination of reagents during isolation	**Each run**! (NIC) **Validation!**
PIC: Positive Isolation Control	- conservative gene, present in high copy numbers in the genome (e.g.: COX, 18S rRNA) - naturally infected host tissue or spiked „healthy sample”	- confirmation that the extraction from different plant samples has been successful - to prove that detection of the target from defined sample is possible	**Each run!** For each sample separate! **Validation**!

Pepino Mosaic Virus – controls [PCR controls]

	Description	Control?	When to use?
NTC: **No template control** **(EPPO: NAC)**	- water instead of sample DNA	- contamination of the reaction mix and pipetting	**Each run**! (NTC1) (NTC2)
PC: **Positive DNA target Control** **(EPPO: PAC)**	- Sample containing target sequence	- Errors in PCR reaction	**Each run!** PC

Inhibition, internal control, corrrect Ct, different kits for isolation and amplification , different optical systems, verification in each laboratory when implementing the test for the first time

Diagnostics in practice:

EXPERIMENT

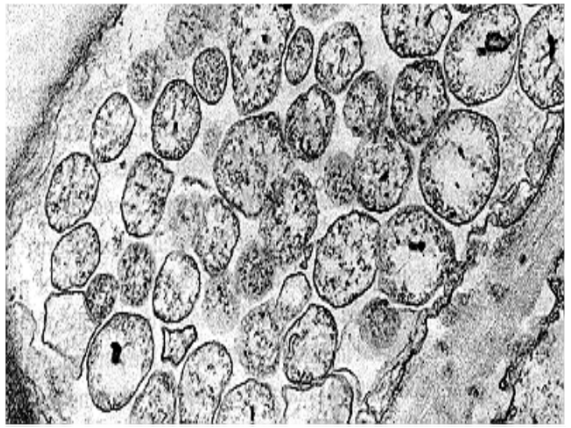
&

REACTION SETUP

Phytoplasmas 16SrX

Phytoplasmas in general

- cell wall-less Gram positive bacteria
- class Mollicutes
- cell and genome size are the smallest among bacteria
- obligate inttracellular parasites, generally restricted to phloem sieve elements
- Transmitted:
  - phloem-feeding leafhoppers, planthoppers and psyllids
  - dodder, micropropagation, grafting and cutting
- propagate within the cytoplasm of both insects and plants
- > 1000 diseases



~~culture phytoplasmas in cell free media~~

~~traditional methods suitable for bacteria~~

Phytoplasmas

- Phytoplasmas circulate inside the plant during the growing season
- The location in the plant restricted to phloem
- Outside the growing season are undetectable in the aerial parts of the plant, but overwinter in the roots
- In the spring, the tree is again re-infected from roots

Apple Prroliferation -symptoms

- Early symptoms observed at terminal buds:

- Leaf rosettes: a rosette of terminal leaves may develop late in the season at the end of shoots in place of normal terminal buds

- „witches brooms”

- late flowers with long petioles

'Candidatus Phytoplasma mali'

Apple Prroliferation -symptoms

„witches brooms” in winter

Leaf rosette in spring

Flower with extralong petiole

Photos:
- E.Seemuller Institut fur pflanzenschutz im Obstbau, Germany
- A Loschi, Universita degali studi di Udine

Apple Proliferation -symptoms

Pear decline-symptoms

There are two types of symptoms, depending on the pads:

- **Slow decline** – tolerant pads/ resist
- **Quick decline** – sensitive pads

- The universal common symptoms include:

- twisting leaves
- premature red coloration of leaves
- drooping leaves
- rare pale green foliage
- poor flowering
- no fruit

Pear decline-symptoms

„Slow decline” in spring

On fruits and leaves

„Slow decline” in autumn

'Candidatus Phytoplasma pyri'

Photos:
- V Vicchi, Plant Protection Service, Italy
- T. Malinowski, Research Institute of Pomology, Poland

Pear decline-symptoms

Apricot chlorotic leafroll mycoplasm- symptoms

- Synonyms
  - **European stone fruit yellows**
  - Apricot chlorotic leafroll
- Wczesne symptomy
  - Przedwczesne wykształcenie liści
  - Liście pojawiają się w czasie i zamiast kwitnienia
- Późne lato
  - Krótsze ogonki liściowe z mniejszymi liśćmi
  - Zakończenie liścia poszarpane i cylindryczne
  - Przedwcześnie pożółkłe lub czerwone liście
  - Liście stają się kruche i szybko opadają

'Candidatus Phytoplasma prunorum'

Apricot chlorotic leafroll mycoplasm- symptoms

Prematured leaves and twisted leaves

Prematured leaves
and reduced flowering

Photos:
- *L Giunchedi, Università degli studi di Bologna Italy*

Phytoplasmas 16 SrX

Draft ISPM 27 Phytoplasmas (general)

EPPO A2 LIST OF PESTS RECOMMENDED FOR REGULATION AS QUARANTINE PESTS

PROKARYOTES

Burkholderia caryophylli A2/55
Clavibacter michiganensis subsp. *insidiosus* A2/49
Clavibacter michiganensis subsp. *michiganensis* A2/50
Clavibacter michiganensis subsp. *sepedonicus* A2/51
Curtobacterium flaccumfaciens pv. *flaccumfaciens* A2/48
Dickeya dianthicola (Erwinia chrysanthemi pv. *dianthicola)* A2/53
Erwinia amylovora A2/52
Pantoea stewartii A2/54
Phytoplasma mali (Apple proliferation) A2/87
Phytoplasma pyri (Pear decline) A2/95
Phytoplasma solani (Stolbur) A2/100
Phytophthora ramorum A2/376
Puccinia horiana A2/80
Stenocarpella macrospora A2/67
Stenocarpella maydis A2/68
Synchytrium endobioticum A2/82
Verticillium albo-atrum & V. dahliae (hop-infecting strains) A2/85

VIRUSES

Beet leaf curl virus A2/90
Beet necrotic yellow vein virus (Benyvirus) A2/160
Blueberry leaf mottle virus (Nepovirus) A2/198
Blueberry scorch virus (Carlavirus) A2/347
Chrysanthemum stunt viroid (Pospiviroid) A2/92

EPPO recommends its member countries to regulate the pests listed below as quarantine pests (A2 pests are locally present in the EPPO region). The EPPO A2 List is reviewed every year by the Working Party on Phytosanitary Regulations and approved by Council.

DRAFT ANNEX TO ISPM 27– PHYTOPLASMAS (2004-018)

Extraction

- Various nucleic acid extraction methods for phytoplasma detection by PCR.
- A lot of use an enrichment step to concentrate the phytoplasmas before nucleic acid extraction (low titre of phytoplasmas woody perennial plants or “difficult” hosts).
- In some simplified methods, plant tissue is ground directly in a commercially available lysis buffer or in CTAB-based buffer. The DNA is then extracted directly from the lysate using commercially available silica spin columns or magnetic beads (KingFisher) or with organic solvents.
- Most extraction methods are well validated for a variety of plant host species.
- The choice of method is dependent on the host being tested and the availability of facilities and equipment.
- For routine diagnostics it is important to validate an extraction method for a particular host to ensure reliability.

European and Mediterranean Plant Protection Organisation
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

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Candidatus *Phytoplasma mali*

DNA extraction for PCR

Shoots, roots, leaves or petioles may be used. Shoots and roots are debarked, and a sample of phloem is removed with a sterile blade. For leaves, only the midrib is taken. Any effective grinding method may be used .The best results are obtained if DNA is extracted from leaf midribs or stems collected from June until the end of September. For PCR, it is advisable to include a phytoplasma enrichment procedure, because low concentrations may escape detection. Electrophoresis is done on a 1% agarose gel in TBE buffer.

DNA extraction

The extraction buffer contains: 2% CTAB (soluble at approximately 50°C), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8. Small rigid plastic bags containing the sample and extraction buffer (5 mL for 1 g of phloem) are triturated using a ball mill. Transfer 1–2 mL extract to 2 mL microtubes. Incubate microtubes, if possible with shaking, for at least 30 min at 65°C, then centrifuge at 2000 *g* for 2 min in a microcentrifuge. Put 1 mL of supernatant in a microtube and add 1 mL of chloroform-octanol solution (24 : 1). Mix the two phases to obtain an emulsion. Centrifuge at 13 000 *g* for 5 min. Transfer the supernatant to a new microtube, and add approximately 800 µL of cold isopropanol. Mix and centrifuge at 15000 *g* for 10 min. Remove the supernatant. Add 500 µL of 70% ethanol and centrifuge at 15 000 *g* for 5 min. Empty microtubes and dry residues. Add 100 µL of distilled water and shake with a vortex to help dissolution.

Alternatively, commercial kits (e.g. DNeasy, Qiagen) or other described methods, e.g. extraction with a silica-suspension as described by Menzel *et al.* (2002), can be used for DNA extraction. The extracts can be stored in a deep-freeze (–80°C) for a year.

Phytoplasmas 16SrX

Tasks:

1. Calculate mix preparation per 1 reaction for each gene
2. Determine number of reactions
3. Calculate mix preparation for all reactions
4. Inhibition, internal control?

Phytoplasmas 16SrX MIX preparation

fO1: 5'-3' CGG AAA CTT TTA GTT TCA GT
rO1: 5-3' AAG TGC CCA ACT AAA TGA T

MIX				
Components	Final concentration	Volume per reaction (µl)	Volume per ..... reactions (µl)	Identification # Cat or lot #
H_2O		26,5 (26,56)		
Buffer (10x) with MgCl2 (15mM)	1 x	4,0		
dNTPs (10 mM)	100 uM	0,4		
Primer fO1 (10 uM)	0,5 uM	2		
Primer rO1 (10 uM)	0,5 uM	2		
TaqPolymerase(5 U/ ul)	0,5 U (0,2 U in standard)	0,1 (0,04)		
		35		
template		5		
Total		40		

Conditions for PCR:
94°C for 2 min; 40 cycles (at 95°C for 20 s, 55°C for 20 s and 72°C for 1 min); 72°C for 4 min; 4°C hold

Prepare 1% agarose gel in TBE buffer
The rO1/fO1 primers produce a 1071 base pair (bp) amplicon

RFLP analysis

If universal primers fU5/rU3 (Lorenz *et al.*, 1995) or R16F2n/R16R2 (Lee *et al.*, 1998) are used, the amplification product may be digested by restriction enzyme *Alu*I to ensure that the phytoplasma belongs to group AP (Seemüller *et al.*, 1998) or to group 16SrX (subgroup A) (Lee *et al.*, 1998).

If AP-or 16SrX-group specific primers fO1/rO1 (Lorenz *et al.*, 1995) are used, the amplification product may be digested by the restriction enzymes *Ssp*I and *Sfe*I (Lorenz *et al.*, 1995) for differentiation of *P. mali* from *P. pyri* and *P. prunorum*.

Trawienie *SspI*

AP MLO	±340 i ±710 bp
PD MLO	Nie trawi

Trawienie *BfmI*

AP MLO	±920 i ±130bp
PD MLO	±540 ±370 i ±130bp

Characterization of the phytoplasma using RFLP

Amplified DNA (10 µL) is added to 10 µL of digestion solution (sterile distilled water, enzyme-specific buffer 1×, 2 units enzyme and amplified DNA). The proportion (10 + 10) may vary depending on the concentration of the amplicons. The mixture is incubated for at least 2 h at 37°C, and then subjected to electrophoresis on 2% agarose gel. Further details on RFLP analysis are given in Appendix 2.

Phytoplasmas [nucleic acid isolation (sample) controls]

	Description	Control?
Negative extraction control	- ”healthy sample” of the same type	- contamination during nucleic acid extraction and/or cross-reaction with the host tissue
Positive extraction control	- naturally infected host tissue or spiked „healthy sample”	- confirmation that the extraction from different plant samples has been successful - to prove that detection of the target from defined sample is possible
Internal control	- A housekeeeping gene	- checks acids extraction and inhibition

PHYTOPLASMAS– controls [PCR controls]

	Description	Control?
Negative amplification control (no template control)	- water instead of sample DNA	- contamination of the reaction mix and pipetting
Positive nucleic acid control	- Extracted DNA containing target sequence	- the efficiency of the test method and specifically the amplification (without extraction)

Development of SOPs (Standard Operating Procedures)

IPPC Standards

EPPO Standards

Your own methode

Standard Operating Procedure in your Lab

Development of SOPs (Standard Operating Procedures)

What should be included in SOP?

- How to make a test
- All information to facilitate identification of all factors affecting a test together with human operators - personel responsible for each step of a test from the beginig to the end
- All information which provides appropriate control over a documentation which is a part of a system

So
have a nice writing of all
your procedures
.....
now and in the future

THANK YOU

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