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Valitest

Validation of diagnostic tests to support plant health

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Abstract:
A list of methods and tests for validation for test performance study (TPS) Round 2 (for laboratory and/or on-site use) was prepared for six selected pests: *Xanthomonas citri* pv. *citri*, *Xylophilus ampelinus*, *Cryphonectria parasitica*, tomato spotted wilt tospovirus, tomato brown rugose fruit virus and plum pox virus. The listed tests were first validated in preliminary studies by TPS organizers in order to select the final tests for TPS, based on the scope and criteria also described in this deliverable.

Partners involved: ANSES, CREA, FERA, NIB, UNITO

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TERMS, ABBREVIATIONS AND DEFINITIONS

BPeMV – Bell pepper mottle virus

EPPO – European and Mediterranean Plant Protection Organization

ISF - International Seed Federation

LAMP - Loop-mediated isothermal amplification

LFD - Lateral flow device

NAC – Negative amplification control

NC – Negative control

NIC – Negative isolation control

PAC – Positive amplification control

PC – Positive control

PIC – Positive isolation control

PPV – Plum pox virus

TMGMV – Tobacco mild green mosaic virus

TMV – Tobacco mosaic virus

ToBRFV - Tomato brown rugose fruit virus

ToMV – Tomato mosaic virus

TPS – Test performance study

TSWV - Tomato spotted wilt tospovirus

Xcc - *Xanthomonas citri* pv. *citri*

1 Purpose

This document describes the process of selection of tests for validation in Round 2 in the frame of WP1 of the VALITEST project. The aim of WP1 is to coordinate (prepare and organize) tests validations and run TPSs for prioritized pests in a range of matrices and for a range of diagnostic technology related platforms (both laboratory and on site-based). Test is defined as the application of a method to a specific pest and a specific matrix. TPS Round 2 is focused on six pests, selected based on the results of the survey organized in the framework of WP4 and also taking into account the list of pests categorized by the EU (WP1 in collaboration with WP4 and EPPO), allowing the priorities to be better aligned to the needs of stakeholders and to the market: *Xanthomonas citri* pv. *citri*, *Xylophilus ampelinus*, *Cryphonectria parasitica*, tomato spotted wilt tospovirus (TSWV), tomato brown rugose fruit virus (ToBRFV) and plum pox virus (PPV; only on-site testing). Selected tests for each of the six pests are listed in this deliverable. Tests were selected using the modified criteria from deliverable D1.1.

2 Scope

The tests listed in this deliverable will be validated in TPS Round 2 (in year 2 and 3). The rules and the process to select the tests for validation, described in deliverables D1.1 and D1.2, were used as a guidance to select tests for validation in TPS Round 2. Similarly to the deliverable D1.2, this deliverable is applicable to any TPS organization and could help new EU reference laboratories (in the field of plant health).

3 Methodology

Tests for validation were chosen based on available validation data collected from different sources and laboratory experience of TPS organizers. First, the scope of testing was defined for each of the six pests (*X. citri* pv. *citri*, *X. ampelinus*, *C. parasitica*, TSWV, ToBRFV and PPV) to be able to set the strategy for method/test selection. As explained in the deliverable D1.1, to be able to set weighted criteria for tests selection it is necessary to precisely define the scope of testing for a specific pest included in the TPS. Then a list of different diagnostic tests (for laboratory and/or on-site use) was prepared. Validation data (e.g. about the type of matrix, extraction method, sample type, analytical sensitivity, analytical specificity, diagnostic sensitivity, diagnostic specificity, repeatability, accuracy and reproducibility) for diagnostic tests for the six pests were collected from different sources: extensive literature searches, internet searches, EPPO database on diagnostic expertise (section validation data), experience of TPS organizers and correspondence with commercial kits providers. Organizers of TPS Round 2 discussed with commercial kit providers regarding the possible modifications of kit components/protocols to be able to compare as many tests as possible and include them in the TPS Round 2. As during the process of selection of tests for TPS Round 1, the professional experience of TPS organizers proved invaluable in judging the reported results and other information to be able to select the tests for TPS (in some cases data are not comparable, sometimes crucial information is missing in the research articles or reports). Weighted criteria were set for selection of tests from the large list of identified tests according to the TPS scope which helped to define a narrow list of pre-selected tests for validation, which were subjected to validation in preliminary studies where the TPS organizers checked the performance of the tests to be included in the TPS. After assessing the results of preliminary studies against weighted criteria final list of tests was selected for TPS Round 2.

3.1 Setting the weighted criteria for selection of tests for TPS – Round 2

As in the TPS Round 1, also in the TPS Round 2 weighted criteria had to be set to be able to objectively select tests from a list of tests for a specific pest, each having advantages and disadvantages under specific circumstances or needs, depending on the scope of the TPS. Criteria for selection of tests, defined in preparation for TPS Round 1 (deliverable D1.1) were modified according to experience gained from TPS Round 1 (see the tables with criteria further below prepared for each pest). Some criteria needed to be added, to better justify the selection of a test, e.g. the criterion about the appropriately selected target (gene/protein) of the test. Also, some criteria were made more specific, or removed, as they were not important for selection of tests or the information was not always possible to obtain. Further on, the weights, which are assigned to the criteria, sometimes differ between the pests (depending on the scope of testing for each pest) and between tests for on-site use and laboratory use. Further explanation regarding the criteria and their use for test selection is given in the deliverable D1.1.

4 *Xanthomonas citri* pv. *citri*

4.1 General information on the pest

Xanthomonas citri pv. *citri (Xcc)* is the causal agent of Asiatic citrus canker (ACC). It causes damages to many cultivated species of Rutaceae (EPPO, 1979) – primarily *Citrus* spp., *Fortunella* spp. and *Poncirus* spp. – grown under tropical and subtropical conditions in many countries of Asia, South America, Oceania and Africa as well as in Florida, United States (CABI, 2006; EPPO, 2006).

Pathotype strains A have the greatest economic impact on citrus industry. These strains are distributed worldwide and induce canker symptoms on a broad range of citrus hosts. Typical strains of *Xcc* with a restricted host range have been identified and are designated as strains A* and Aw (Sun *et al.*, 2004; Vernière *et al*., 1998). Strain A* affects *Citrus aurantiifolia* (Mexican lime) under natural conditions in Asia. Strain Aw causes canker in *Citrus aurantiifolia* (Mexican lime) and *Citrus macrophylla* (Alemow) in Florida, United States under natural conditions (Cubero and Graham, 2002, 2004).

Other Xanthomonas cause also citrus diseases but have a lower economic impact compared to *Xcc* strains. *Xanthomonas citri* pv. *aurantifolii* (strains B and C) causes a minor canker of diminishing importance on a narrow host range (lime, lemon) in South America. *Xanthomonas alfalfae* pv. *citrumelo* is responsible of a leaf spot of the rootstock Swingle citrumelo (*Poncirus trifoliata* × *Citrus paradisi*) and to a lesser extent, to grapefruit (*C. paradise)* in citrus nurseries in Florida.

It is therefore particularly relevant in this study to evalute precisely the analytical specificty of the different tests used for the detection of *Xcc*.

4.2 Scope of the TPS

Table 1: Definition of scope for *X. citri* pv. *citri*

	Methods		
	PCR	**Real-time PCR**	**LAMP**
sample type (DNA, plant material with deactiv. pests, etc.)	DNA	DNA (+ ImmunoStrips® (Agdia) and Whatman™ FTA cards for direct real-time PCR)	DNA (+ ImmunoStrips® (Agdia) and Whatman™ FTA cards for direct LAMP PCR)
matrix (type of plant material: seed, leaves, etc.)	Leaves/ fruits	Leaves/ fruits	Leaves/ fruits
suitable for: symptomatic / asymptomatic sample	symptomatic/ asymptomatic	symptomatic/ asymptomatic	symptomatic/ asymptomatic
purpose: detection / identification	detection	detection	Detection
type of controls needed (NIC, NAC, PAC, PIC, IC, etc)	PAC NAC	PAC NAC	PAC NAC
max no. of samples (including controls)	26	26 (14 ImmunoStrips® and 14 Whatman™ FTA cards for laboratories implementing direct real-time PCR)	26 (14 ImmunoStrips® and 14 Whatman™ FTA cards for laboratories implementing direct real-time PCR)
max no. of participants	20	20 (10 for laboratories implementing direct real-time PCR)	20 (10 for laboratories implementing direct real-time PCR)

4.3 Setting the weighted criteria for selection of tests for TPS

Selection of tests included in preliminary studies and in TPS has been done based on pre-defined criteria listed in the table below.

Table 2: Criteria for selection of tests for TPS for *X. citri* pv. *citri*

Criteria	Descriptor (%, number, text)	Target	Relative Weight (lab)	Relative Weight (on-site)
Validation data (prior preliminary studies)				
Is the target (gene/protein) appropriately selected?	Yes/No	Yes	high	high
Available validation data	Yes/No	Yes	medium	medium
Validation data available for selected matrix	Yes/No	Yes	low	low
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)	Lowest level	medium	low
Analytical sensitivity in plant material (selected matrix)	Conc. (absolute value if possible or relative conc. or low/medium/high)	Highest level	high	medium
Diagnostic sensitivity (comparison of different tests)	%	100%	high	high
Analytical specificity	Level	100%	high	high
a) Exclusivity (Non-target organism): False positives	Level	0%	high	high
b) Inclusivity (Target organisms): False negatives	Level	0%	high	high
Selectivity	Presence of cross reactions with matrix	No	high	high
Repeatability (near LOD)	Level	100% at LOD	medium	medium
Reproducibility/ robustness	%	100% at LOD	medium	medium
Results of interlaboratory comparisons available	Yes/No	Yes	low	low
Additional information (not a criterion!)				
Type of matrix				
Extraction method				
Use on sympt./asympt.				
Other				
Validation data (after preliminary studies)				
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)	Lowest level	high	low
Analytical sensitivity in plant material (selected matrix)	Conc. (absolute value if possible or relative conc. or low/medium/high)	Highest level	high	medium
Diagnostic sensitivity (comparison of different tests)	%	100%	high	high
Analytical specificity	Level	100%	high	high
a) Exclusivity (Non-target organism): False positives	Level	0%	high	high
b) Inclusivity (Target organisms): False negatives	Level	0%	high	high
Selectivity	Presence of cross reactions with matrix	No	high	high
Repeatability (near LOD)	Level	100% at LOD	medium	medium

Reproducibility/ robustness	%	100% at LOD	medium	medium
APPLICABILITY				
Applicability in different matrixes	Level	Fruits and leaves	high	high
Amount of material which is included in one sample	Amount of plant units tested	NA	low	low
Standardized preparation of the reaction (e.g., ready to use reagents)	Yes/No	Yes	low	high
Availability and relevance of controls (in the case of kits)	Yes/No	Yes	very low	high
PROTOCOLS				
Available detailed protocols	Yes/No	Yes	medium	medium
Simple test procedure	Yes/No	Yes	medium	high
Simplicity of data analysis	Yes/No	Yes	medium	medium
User friendly test	Yes/No	Yes	medium	high
Time needed to complete analysis (less than one hour/ one day/ several days)	Duration in time unit	the fastest	medium	high
Easy to multiplex?	Yes/No	NA	NA	NA
Database/library dependent (yes/ no) (for example fatty acids profiling, sequencing,...)	NA	NA	NA	NA
CHEMICALS				
Stability of chemicals at ambient temperature	Yes/No	Yes	low	high
EQUIPMENT				
No equipment/ instrument needed (relevant only for on-site tests)	Yes/No	Yes	NA	NA
Test not exclusively developed for a specific instrument	Yes/No	Yes	medium	medium
Cost of obligatory equipment/ instruments (up to 10.000 EUR/ 10.000-50.000 EUR/ more than 50.000 EUR?)	Cost in euro	NA	medium	medium

4.4 Preliminary study and selection of tests for TPS

Detection and identification of *Xcc* can be achieved by using serological or molecular methods. The TPS aims to compare molecular methods. The methods selected for TPS are those described in IPPC and EPPO Diagnostic protocols, methods found in the literature and available commercial kits. All selected methods are well established in the laboratory of TPS organizer. The most important criteria for test selection are analytical specificity (exclusivity and inclusivity) and analytical sensitivity.

Table 3 : Tests selected for preliminary study for *X. citri* pv. *citri* with references. Tests selected for TPS are underlined.

Method	Tests for validation
Conventional PCR	Hartung *et al.*, 1993 (2/3) Hartung *et al.*, 1996 (4/7) Cubero *et al.*, 2002 (J-pth1/2) Cubero *et al.*, 2002 (J-Rxg/J-RXc2) Kingsley *et al.*, 2000 (King F/R) Mavrodieva *et al.*, 2004 (VM3/4) Coletta-Filho *et al.*, 2006 (Xac01/Xac02) Park *et al.*, 2006 (XACF/XACR) Miyoshi *et al.*, 1998 (XCF/XCR) Robène *et al.*, 2020 (to be published) (XcciF/R) Commercial kit Loewe® based on Hartung *et al.*, 1993 (2/3)

Real-time PCR	Kingsley *et al.*, 2000 (King F/R) Cubero *et al*., 2005 (J-pth3/4 and probe J-Taqpth2) PlantPrint Mavrodieva *et al*., 2004 (VM3/4) Robène *et al*., 2020 (to be published) (Xcci1051F/R and probe P-Xcci-1051)
LAMP-PCR	Rigano *et al*., 2010 (with OptiGene amplification kit)
Direct molecular tests performed from ImmunoStrips® (Agdia) and Whatman™ FTA cards (only for a limited number of participants)	Real-time PCR test adapted from Cubero *et al*., 2005 (Jpth3/4 and probe J-Taqpth2) Real-time PCR test Robène *et al*., 2020 (to be published) (Xcci1051F/R and probe P-Xcci-1051) LAMP PCR test adapted from Rigano *et al*., 2010 (with OptiGene amplification kit)

Selection of tests was performed based both on a bibliographic review and on experimental investigations conducted by the TPS organizer.

Concerning the experimental investigations, for each test included in the above table, a panel of target samples (representative of a diversity of Xcc strains) and of non target samples (including non-target organisms and a diversity of citrus species) was analyzed to evaluate the analytical specificity, the diagnostic specificity and the diagnostic sensitivity.

If the results were as expected, dilutions of target samples were then analyzed with repetitions for each selected test to evaluate its repeatability and its analytical sensitivity.

For each method, only the tests giving the best results in terms of performance (considering the scope) were selected for the TPS. Although, the conventional PCR (Cubero et al., 2002; J-pth1/2) was not one of the best performing tests, it is considered as a reference test for *Xcc* detection and was consequently included in the TPS.

In addition, direct molecular tests performed from ImmunoStrip® (Agdia) and Whatman™ FTA cards were included in the TPS to evaluate simplified protocols which present practical advantages (simplified extraction and ease of conservation/transport of samples).

5 *Xylophilus ampelinus*

5.1 General information on the pest

Xylophilius ampelinus is a bacterial pathogen causing 'bacterial blight' of grapevine which has the potential to severely affect vineyards; the highest valued fruit crop across the globe. All *Vitis vinifera* subspecies are at risk of infection, with most of the geographic distribution restricted to South Africa, Greece, Turkey, and France. Control of *X. ampelinus* using chemical agents has so far proved ineffective however copper-containing agents have shown to have a protective effect and inhibit the spread of the disease (Komatsu & Kondo, 2015). *X. ampelinus* prefers humid and wet conditions which favor its spread from vine to vine within the vineyard. The bacteria can survive temperatures down to freezing for short periods of time and does not exhibit any natural heat restrictions to growth. Symptoms can appear on all aerial parts of the plant. Distribution of the bacterial in the plant is irregular.

Distribution of the bacteria in the plant is irregular and can vary within and between years. Symptoms presented can be mistaken for other diseases such as *Phomopsis viticola* which can make diagnosis and treatment more complicated (EPPO PM 7/96).

5.2 Scope of the TPS

The scope of the TPS is the detection and identificiation of *X. ampelinus* in grape vine material.

Table 4: Definition of scope for *X. ampelinus*

	Methods			
	IF	**DAS-ELISA**	**PCR**	**Real-time PCR**
Sample type (DNA, plant material with pests, etc.)	Spiked plant material	Spiked plant material	Spiked plant material	Spiked plant material
Matrix (type of plant material: seed, leaves, etc.)	Stem material	Stem material	Stem material	Stem material
Suitable for: symptomatic / asymptomatic sample	symptomatic / asymptomatic	symptomatic / asymptomatic	symptomatic / asymptomatic	symptomatic / asymptomatic
Purpose: detection / identification	detection	detection	detection	detection
Type of controls needed (NIC, NAC, PAC, PIC, IC, etc)	PC; NC (buffer)	PC; NC (plant); NC (buffer)	NIC; NAC; PIC; PAC	NIC; NAC; PIC; PAC
No. of samples	24	24	24	24
Max no. of participants	20	20	20	20

5.3 Setting the weighted criteria for selection of tests for TPS

Selection of tests included in preliminary studies and in TPS has been done based on pre-defined criteria listed in the table below.

Table 5: Criteria for selection of tests for TPS for *X. ampelinus*

Criteria	**Descriptor (%, number, text)**	**Target**	**Relative Weight (lab)**
Validation data (prior preliminary studies)			
Is the target (gene/protein) appropriately selected?	Yes/No	Yes	high
Available validation data	Yes/No	Yes	low
Validation data available for selected matrix	Yes/No	yes	medium
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)	low	high
Analytical sensitivity in plant material (selected matrix)	Conc. (absolute value if possible or relative conc. or low/medium/high)	low	high
Diagnostic sensitivity (comparison of different tests)	%	low	high
Analytical specificity	Level	medium	high
a) Exclusivity (Non-target organism): False positives	Level	low	high
b) Inclusivity (Target organisms): False negatives	Level	low	high
Selectivity	Presence of cross reactions with matrix		
Repeatability (near LOD)	Level	high	high
Reproducibility/ robustness	%	high	high
Results of interlaboratory comparisons available	Yes/No		
Additional information (not a criterion!)			
Type of matrix			
Extraction method			
Use on sympt./asympt.			

Other			
Validation data (after preliminary studies)			
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)	low	high
Analytical sensitivity in plant material (selected matrix)	Conc. (absolute value if possible or relative conc. or low/medium/high)	low	high
Diagnostic sensitivity (comparison of different tests)	%	low	high
Analytical specificity	Level	low	high
a) Exclusivity (Non-target organism): False positives	Level	low	high
b) Inclusivity (Target organisms): False negatives	Level	low	high
Selectivity	Presence of cross reactions with matrix	no	high
Repeatability (near LOD)	Level	high	high
Reproducibility/ robustness	%	high	high
APPLICABILITY			
Applicability in different matrixes	Level	high	low
Amount of material which is included in one sample	Amount of plant units tested	low	low
Standardized preparation of the reaction (e.g., ready to use reagents)	Yes/No	yes	low
Availability and relevance of controls (in the case of kits)	Yes/No	yes	low
PROTOCOLS			
Available detailed protocols	Yes/No	yes	medium
Simple test procedure	Yes/No	yes	low
Simplicity of data analysis	Yes/No	yes	low
User friendly test	Yes/No	yes	low
Time needed to complete analysis (less than one hour/ one day/ several days)	Duration in time unit	fastest (for each method)	low
Easy to multiplex?	Yes/No	yes	low
Database/library dependent (yes/ no) (for example fatty acids profiling, sequencing,...)	NA	NA	NA
CHEMICALS			
Stability of chemicals at ambient temperature	Yes/No	yes	low
EQUIPMENT			
No equipment/ instrument needed (relevant only for on-site tests)	Yes/No	NA	NA
Test not exclusively developed for a specific instrument	Yes/No	Yes	medium
Cost of obligatory equipment/ instruments (up to 10.000 EUR/ 10.000-50.000 EUR/ more than 50.000 EUR?)	Cost in euro	as low as possible	low

5.4 Preliminary study and selection of tests for TPS

Methods selected for TPS for *X. ampelinus* are those described in the EPPO Diagnostic Protocol, from literature search and available commercial kits. All selected methods are well established in the laboratory of TPS organizer. Analytical sensitivity and specificity will be evaluated on DNA extracted from pure cultures or pure cultures of *X. ampelinus* from different geographical regions and other relevant species. Repeatability and reproducibility will also be evaluated.

A review of available commercial diagnostic kits, published methods, including methods described in the EPPO protocol PM7/96 was carried out. In total, 1 LFD, 2 IF, 2 ELISA, 1 conventional PCR kit, 3 conventional PCR published assays and 1 Real-time PCR assay were identified and selected for evaluation.

Table 6 : Tests selected for preliminary study for *X. ampelinus* with references. Tests selected for TPS are underlined.

Method	**Tests for validation:**
LFD	Loewe
IF	Loewe Plantprint diagnostics
ELISA	Loewe Plantprint diagnostics
Conventional PCR	Loewe Botha *et al.*, 2001 PCR (Plant Pathology 50: 515-526) Manceau *et al.*, 2005 (OEPP/EPPO Bulleting 35: 55-60) Manceau *et al.*, 2000 (European Journal of Plant Pathology 106: 243-253)
Real-time PCR	Dreo *et al.*, 2007 real-time PCR (Plant Pathology 56; 9-16) adapted from EPPO PM7/96 appendix 4

Results from our preliminary studies indicate that all the methods underlined above provided satisfactory validation results for inclusion in the TPS. The PCR assay of Manceau *et al.* (2005) is specifically mentioned in the EPPO protocol PM7/96. Our findings show all PCR assays have a similar level of analytical sensitivity and analytical specificity and have therefore all been included in the TPS.

The only method which is not being taken forward to the TPS is the LFD test. A low level of analytical sensitivity was found when testing pure suspension of bacterial cells with only a faint band on the LDF strip being identified at 10^6 cells. ELISA and IF kits proved to be less sensitive compared to the molecular methods. Although there is variation in the level of analytical sensitivity between methods, the EPPO protocol outlines an isolation method which would enable high concentrations of the pathogen to be produced increasing the suitability of these methods when screening for *Xylophilius ampelinus* from infected vine material.

6 *Cryphonectria parasitica*

6.1 General information on the pest

The fungus *C. parasitica* is the causal agent of chestnut blight causing serious damage in orchards and in forests since its introduction in Europe during the 30's. The main host of this pathogen in the EPPO region is *Castanea* spp. (especially *Castanea sativa*). *C. parasitica* infection process starts with the penetration of the pathogen in the susceptible host trees through wounds. The fungus then grows and spreads in and beneath the bark. Once the pathogen has colonized the wood, it causes perennial necrotic lesions called cankers on the bark of trunks and branches of the host, eventually leading to wilting of the plant part distal to the infection (Rigling & Prospero, 2018). Distinctive symptoms on tree bark are represented by the formation of small orange-brown areas and the presence of yellow to red asexual and sexual fungal reproductive structures. Below the cankers, the host tree may react by producing epicormic shoots (Rigling & Prospero, 2018). In addition to its important ecological impacts, *C. parasitica* causes significant economic losses especially for European regions that depend on the

production of chestnut trees. The pathogen is currently included in the EPPO A2 List of pests recommended for regulation as quarantine pests (version 2019-09).

The type strain of *C. parasitica* was obtained as a cryopreserved culture from the Mycotheca Universitatis Taurinenesis (MUT) collection. Strain material comes under MTA. The material is intended to be used in the form of as purified organism/purified nucleic acid.

Three molecular methods that were found in the literature were selected and tested for inclusion in the TPS for *C. parasitica*. All selected methods are well established in the laboratory of TPS organizer. Morphological methods were not included in the TPS. The EPPO Standard PM 7/45(1) only includes morphological methods. No commercial kits are available.

6.2 Scope of the TPS

Table 7: Definition of scope for *C. parasitica*

	Methods	
	PCR	**Real-time PCR**
Sample type (DNA, plant material with deactiv. pests, etc.)	Plant material with deactivated pathogen/ DNA extracts	Plant material with deactivated pathogen/ DNA extracts
Matrix (type of plant material: seed, leaves, etc.)	Spiked wood material	Spiked wood material
Suitable for: symptomatic / asymptomatic sample	Symptomatic / asymptomatic	Symptomatic / asymptomatic
Purpose: detection / identification	Detection	Detection
Type of controls needed (NIC, NAC, PAC, PIC, IC, etc)	NAC NIC PAC PIC	NAC NIC PAC PIC
No. of samples	8 Spiked wood material samples/ 8 DNA sample extracts	8 Spiked wood material samples/ 8 DNA sample extracts
Max no. of participants	15	15

6.3 Setting the weighted criteria for selection of tests for TPS

Selection of tests included in preliminary studies and in TPS has been done based on pre-defined criteria listed in the table below.

Table 8: Criteria for selection of tests for TPS for *C. parasitica*

Criteria	**Descriptor (%, number, text)**	**Target**	**Relative Weight (lab)**
Validation data (prior preliminary studies)			
Is the target (gene/protein) appropriately selected?	Yes/No	Yes	high
Available validation data	Yes/No	Yes	medium
Validation data available for selected matrix	Yes/No	Yes	medium
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)	Low	high
Analytical sensitivity in plant material (selected matrix)	Conc. (absolute value if possible or relative conc. or low/medium/high)	Medium	high
Diagnostic sensitivity (comparison of different tests)	%	100%	high
Analytical specificity	Level	100%	high

a) Exclusivity (Non-target organism): False positives	Level	0%	high
b) Inclusivity (Target organisms): False negatives	Level	0%	high
Selectivity	Presence of cross reactions with matrix	No	high
Repeatability (near LOD)	Level	100% at LOD	medium
Reproducibility/ robustness	%	100% at LOD	medium
Results of interlaboratory comparisons available	Yes/No	No	medium
Additional information (not a criterion!)			
Type of matrix			
Extraction method			
Use on sympt./asympt.			
Other			
Validation data (after preliminary studies)			
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)		high
Analytical sensitivity in plant material (selected matrix)	Conc. (absolute value if possible or relative conc. or low/medium/high)	Yes	high
Diagnostic sensitivity (comparison of different tests)	%	100%	high
Analytical specificity	Level	100%	high
a) Exclusivity (Non-target organism): False positives	Level	0%	high
b) Inclusivity (Target organisms): False negatives	Level	0%	high
Selectivity	Presence of cross reactions with matrix	No	high
Repeatability (near LOD)	Level	100% at LOD	high
Reproducibility/ robustness	%	100% at LOD	high
APPLICABILITY			
Applicability in different matrixes	Level	NA	low
Amount of material which is included in one sample	Amount of plant units tested	1	low
Standardized preparation of the reaction (e.g., ready to use reagents)	Yes/No	Yes	low
Availability and relevance of controls (in the case of kits)	Yes/No	Yes	low
PROTOCOLS			
Available detailed protocols	Yes/No	Yes	high
Simple test procedure	Yes/No	Yes	low
Simplicity of data analysis	Yes/No	Yes	low
User friendly test	Yes/No	Yes	low
Time needed to complete analysis (less than one hour/ one day/ several days)	Duration in time unit	one day	medium
Easy to multiplex?	Yes/No	NA	NA
Database/library dependent (yes/ no) (for example fatty acids profiling, sequencing,...)	NA	NA	NA
CHEMICALS			
Stability of chemicals at ambient temperature	Yes/No	No	low
EQUIPMENT			
No equipment/ instrument needed (relevant only for on-site tests)	Yes/No	Yes	NA
Test not exclusively developed for a specific instrument	Yes/No	Yes	low
Cost of obligatory equipment/ instruments (up to 10.000 EUR/ 10.000-50.000 EUR/ more than 50.000 EUR?)	10000-50000	NA	medium

6.4 Preliminary study and selection of tests for TPS

A list and the description of recommended tests for validation of the target species are given below.

The tests selected, one conventional PCR and two real-time PCR, can be used for direct detection in planta (plant tissue, including wood) and from purified cultures (Table 9). The molecular tests rely on a previous DNA extraction.

- Conventional PCR with primers designed by Popov et al. (2010) on gene Mf2/1, encoding the mating type 2 pheromones precursor of *C. parasitica.* This method has proved to be specific. Eight representative isolates were screened and confirmed suitable for use as pure cultures on plant materials such as ground wood samples of *Castanea sativa*.
- Real-time PCR with primers and a dual-labelled minor groove binder probe designed by Rubio et al. (2017) on *C. parasitica* translation elongation factor (EF1-α) sequence can be useful for identification of the fungus in pure culture and for direct detection of the pathogen in spiked ground wood samples. This method showed a high analytical sensitivity (limit of detection at 2 pg of pathogen genomic DNA) and high specificity.
- Real-time PCR with primers and a Taqman®probe designed by Chandelier et al. (2019) on *C. parasitica* ITS sequence can be also useful for identification of the fungus in pure culture. This method has proved to be more sensitive (limit of detection at 2 fg of pathogen genomic DNA or 1 spore) than the test developed by Rubio et al. (2017) and its specificity is strengthened thanks to the combination of specific primers and probe. However, in preliminary studies the method was not found suitable for detection of the pathogen in spiked wood material, because it produced false positive results in healthy ground wood, therefore not confirming the results obtained by Chandelier et al (2019).

In case a follow up on species identification of the pathogen is required, the regions of the universal barcode ITS (White et al. 1990) and of the ß-tubulin (Glass & Donaldson, 1995) can be sequenced. The ITS or the ß-tubulin sequences are sufficient to correctly identify *C. parasitica* within the genus *Cryphonectria* (Bragança et al., 2011).

Table 9 : Tests selected for preliminary study for *C. parasitica* with references. Tests selected for TPS are underlined.

Method	**Tests for validation:**
Conventional PCR	Popov et al., 2010
Real-time PCR	Rubio et al., 2017 Chandelier et al., 2019

7 Tomato spotted wilt tospovirus

7.1 General information on the pest

Tomato spotted wilt tospovirus (TSWV) takes second place on the list of top ten economically most important plant viruses (Scholthof et al., 2011; Rybicki, 2015). Host range of TSWV counts more than 1000 plant species including some very important vegetables, such as tomato and pepper, and a variety of ornamentals. TSWV is one of the most common viruses in temperate, tropical and sub-tropical climate worldwide causing frequent devastating epidemics in different crop species. For tomato, TSWV is considered as one of the major constraints to successful production. TSWV is causing a variety of symptoms on plants depending on cultivar, growth stage, time of infection and

environmental conditions. On tomato, symptoms caused by TSWV are visible on leaves and petioles, fruits and stems. Sometimes whole plant is affected. Typical symptoms are leaf bronzing, ringspots and line patterns on leaves and fruits, uneven fruit ripening, necrotic lines on leaf petioles and stems, and tip necrosis that could result in plant dwarfing. Infections in earlies stages of plant growth usually resulting in devastating yield losses.

Besides with TSWV, tomato can be affected by a number of other tospoviruses, e.g., alstroemeria necrotic streak virus (ANSV), groundnut bud necrosis virus (GBNV), groundnut ringspot tospovirus (GRSV), tomato chlorotic spot tospovirus (TCSV), tomato yellow (fruit) ring virus (TYRV), tomato zonate spot virus (TZSV), tomato necrotic ringspot virus (TNRV), watermelon silver mottle tospovirus (WSMoV) and capsicum chlorosis orthotospovirus (CaCV) (EFSA, 2012). Symptoms caused by those tospoviruses, and also in case of infection with some other viruses, can be similar therefore laboratory testing is needed to identify the causing virus species.

In EU, TSWV is no longer on the list of quarantine pests (Regulation (EU) 2019/2072, 28 November 2019), however it is still very important pathogen and has a status of regulated non-quarantine pest. The importance of TSWV for the production of agricultural plants is highlighted by the significant loses that could occur as a consequence of TSWV infection and its extensive host range. An increasing problem is the emergence of TSWV resistance-breaking) isolates (Turina et al., 2012). The resistance-breaking isolates are able to overcome the Sw-5 resistance gene in tomato and to cause significant losses, and since that is the only commercially available resistance to TSWV in tomato, it is very important to limit or prevent further spread of those isolates (Aramburu and Martı´, 2003; Ciuffo et al., 2005; Turina et al., 2012). Therefore, accurate detection and identification of TSWV is very important step in establishing effective control strategies.

7.2 Scope of the TPS

The scope of the test performance study is: detection and identification of tomato spotted wilt tospovirus in symptomatic leaves of tomato (*Solanum lycopersicum* L.).

Table 10: Definition of scope for TSWV

	Methods			
	ELISA	**conventional RT-PCR**	**real-time RT-PCR**	**methods applicable for on-site: LFD**
sample type (DNA, plant material with deactiv. pests, etc.)	infected/ non-infected plant material	infected/ non-infected plant material	infected/ non-infected plant material	infected/ non-infected plant material
matrix (type of plant material: seed, leaves, etc.)	leaves of tomato	leaves of tomato	leaves of tomato	leaves of tomato
suitable for: symptomatic / asymptomatic sample	symptomatic	symptomatic	symptomatic	symptomatic
purpose: detection / identification	detection and identification	detection and identification	detection and identification	detection and identification
type of controls needed (NIC, NAC, PAC, PIC, IC, etc)	PC NC	PAC PIC NAC NIC	PAC PIC NAC NIC IC	PC NC
no. of samples	22	22	22	22
max no. of participants	20	20	20	20

TPS starting material will include extracts of tomato leaves with or without target virus. Different virus isolates (i.e., leaf extracts of infected *Nicotiana* sp.), in various concentration, will be spiked into tomato leaf extracts. Worldwide population of TSWV isolates is very diverse, concerning host plant origin, geographical origin, and molecular and serological properties. To ensure that selected test covers majority of known TSWV isolates we will include TSWV isolates of different origin (geographical and biological). Additionally, to test the selectivity of chosen tests the TPS will include isolates of different tospovirus species, which are very similar to TSWV based on serological and molecular properties. The TPS will include around 22 samples. The number of participants per method will be approximately 20 laboratories. The purpose of the TPS will be detection and identification of the TSWV using both serological (ELISA) and molecular methods (real-time RT-PCR and RT-PCR). In addition, some tests applicable for on-site use will be evaluated. The process how methods and tests were selected, and the criteria used for selection of tests are described below. All selected methods are well established in the laboratory of TPS organizer.

7.3 Setting the weighted criteria for selection of tests for TPS

There are numerous tests available for detection and/or identification of TSWV. Tests are based on biological, serological and molecular properties of the pathogen. Biological tests, such as mechanical inoculation of test plants, do not allow identification, and serological detection, though widely used is often hampered by cross-reactions with other similar tospovirus species (Hassani-Mehraban et al., 2016). Molecular tests, e.g. RT-PCR and real-time RT-PCR were developed based on amplification of different genomic parts. Molecular tests are usually more sensitive compared to biological and serological tests, but they can also cross-react with other tospovirus species or they don't detect all TSWV isolates. Consequently, the main challenge for the detection and identification of TSWV is the selection of appropriate method and test.

In order to find the most appropriate test for detection and identification of TSWV in symptomatic leaves of tomato, we performed thorough literature search. Then we selected the most promising methods and tests that have been included in the preliminary studies. Based on the results of preliminary studies, tests that will be included in TPS have been selected. Selection of tests included in preliminary studies and in TPS has been done based on pre-defined criteria listed in the Table 11. The most important criteria for test selection are analytical specificity (exclusivity and inclusivity) and analytical sensitivity.

Table 11: Criteria for selection of tests for TPS for TSWV

Criteria	Descriptor (%, number, text)	Target	Relative Weight (lab)	Relative Weight (on-site)
Validation data (prior preliminary studies)				
Is the target (gene/protein) appropriately selected?	Yes/No	yes	high	high
Available validation data	Yes/No	yes	medium	medium
Validation data available for selected matrix	Yes/No	yes	medium	medium
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)	low dilution of RNA	medium	low
Analytical sensitivity in plant material (selected matrix)	Conc. (absolute value if possible or relative conc. or low/medium/high)	low dilution of plant material	medium	low

Diagnostic sensitivity (comparison of different tests)	%	within the same method: more sensitive test	medium	low
Analytical specificity	Level	specificity: TSWV only	high	high
a) Exclusivity (Non-target organism): False positives	Level	0%	high	high
b) Inclusivity (Target organisms): False negatives	Level	0%	high	high
Selectivity	Presence of cross reactions with matrix	no	high	high
Repeatability (near LOD)	Level	100% at LOD	medium	medium
Reproducibility/ robustness	%	100% at LOD	medium	medium
Results of interlaboratory comparisons available	Yes/No	yes	low	low
Additional information (not a criterion!)				
Type of matrix				
Extraction method				
Use on sympt./asympt.				
Other - Used successfully in different labs (according to literature) - part of the target genome				
Validation data (after preliminary studies)				
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)	low dilution of RNA	medium	low
Analytical sensitivity in plant material (selected matrix)	Conc. (absolute value if possible or relative conc. or low/medium/high)	low dilution of plant material	high	medium
Diagnostic sensitivity (comparison of different tests)	%	within the same method: more sensitive test	high	medium
Analytical specificity	Level	specificity: TSWV only	high	high
a) Exclusivity (Non-target organism): False positives	Level	0%	high	high
b) Inclusivity (Target organisms): False negatives	Level	0%	high	high
Selectivity	Presence of cross reactions with matrix	no	high	high
Repeatability (near LOD)	Level	100% at LOD	high	medium
Reproducibility/ robustness	%	100% at LOD	high	medium
APPLICABILITY				
Applicability in different matrixes	Level		medium	medium
Amount of material which is included in one sample	Amount of plant units tested		medium	medium
Standardized preparation of the reaction (e.g., ready to use reagents)	Yes/No		low	high
Availability and relevance of controls (in the case of kits)	Yes/No		medium	high
PROTOCOLS				
Available detailed protocols	Yes/No	Yes	high	high
Simple test procedure	Yes/No	Yes	low	high

Simplicity of data analysis	Yes/No	Yes	low	high
User friendly test	Yes/No	Yes	low	high
Time needed to complete analysis (less than one hour/ one day/ several days)	Duration in time unit	fastest	low	high
Easy to multiplex?	Yes/No	NA	low	low
Database/library dependent (yes/ no) (for example fatty acids profiling, sequencing,...)	NA	NA	NA	NA
CHEMICALS				
Stability of chemicals at ambient temperature	Yes/No	Yes	low	high
EQUIPMENT				
No equipment/ instrument needed (relevant only for on-site tests)	Yes/No	Yes	NA	NA
Test not exclusively developed for a specific instrument	Yes/No	yes	high	high
Cost of obligatory equipment/ instruments (up to 10.000 EUR/ 10.000-50.000 EUR/ more than 50.000 EUR?)	Cost in euro	NA	low	high

7.4 Preliminary study and selection of tests for TPS

7.4.1 Literature search for available methods and tests for detection and identification of TSWV

Since TSWV is one the most important plant viruses worldwide, methods and tests for its detection and identification are numerous. After extensive search of scientific papers and commercial providers of tests for plant pathogen detection (information was collected from their websites and through direct contact) in total 76 different tests were evaluated for being included in the preliminary study:

- 13x ELISA (DAS-, TAS-, B-fast, ELISA with specific single chain antibodies)
- 2x luminex
- 2x tissue-blot immunoassay (TBIA)
- 2x dot-blot immunoassay (DBIA)
- 4x on-site detection (lateral flow devices (LFD), Rapid immune gold)
- 2x dot-blot hybridization
- 36x reverse transcription (RT)-PCR or immunocapture (IC) RT-PCR
- 8x real-time RT-PCR (SYBR green, TaqMan)
- 4x RT- loop-mediated isothermal amplification (LAMP) or IC-RT-LAMP
- 1x RT- thermostable Helicase-Dependent DNA Amplification (RT-HAD)
- 1x hyperspectral Imaging and Outlier Removal Auxiliary Classifier Generative Adversarial Nets (OR-AC-GAN)
- 1x microarray

Validation data of tests varied among companies and publications. For some tests, extensive validation data are available, for some not. Therefore, comparison of different tests based on available validation data is very difficult. For molecular tests, in addition to available validation data, analytical specificity has been checked with *In silico* analysis. The aim of this literature search and *In silico* analysis was to find tests which will detect all TSWV known strains and will not cross-react with other tospoviruses.

Regarding ELISA method, which was taken into consideration because widely used, 13 tests were evaluated. Some tests were excluded because the commercial provider stopped production of the test or was in the process of changing the antisera or because TPS organizer did not get the required information on validation data not even through direct communication with the company. ELISA with

Specific Single-Chain Antibodies which is described in one scientific publication was excluded because antibodies are not commercially available. In total, five ELISA test were selected for preliminary study (Table 12).

Tests based on Luminex technology were not selected for preliminary study because it requires specific equipment which is not available in many diagnostic laboratories, and the TPS organizer do not have experience with the method. TBIA and DBIA tests were not selected for preliminary study because of to the lack of validation data and because the interpretation of the results in some cases is difficult and the result depends on the experience of the person reading them. In addition, TPS organizer don't have experience with these methods.

Concerning methods for on-site detection, four tests were taken into consideration and two were selected for preliminary study (Table 12). These tests were selected because of their practicality for on-site use. Two tests were excluded because there is no commercial kit available.

Altogether 52 molecular tests were considered. LAMP method was not selected because the protocols are in Chinese and Japanese only. IC-RT-LAMP tests were not selected because the performance of those tests requires several steps and in addition, IC-RT-LAMP is not widely used in diagnostic laboratories in EU (no EU research publications and not included in EPPO and IPPC diagnostic protocols for TSWV detection). SYBR green real-time RT-PCR, dot blot hybridization, RT-HAD, OR-AC-GAN and microarray were not selected because they are not frequently used in diagnostic laboratories and the lack of validation data. In addition, OR-AC-GAN and microarray requires usage of specific equipment which is not considered as standard laboratory equipment. Among 34 conventional RT-PCRs, eight were selected for preliminary study based on the availability of validation data and based on the results of *In silico* analysis (Table 12). Among eight selected tests, seven are considered to be TSWV specific and one is generic test for tospoviruses. The generic test (Hassani-Merhaban et al. 2016) allows the detection of American clade 1 of tospoviruses, which includes TSWV. This generic test was selected because it allows identification of tospovirus species by Sanger sequencing of the RT-PCR product. IC-RT-PCR tests were not selected because the performance of those tests requires several more steps compared to conventional RT-PCR. In addition, IC-RT-PCR is not widely used in diagnostic laboratories in EU (no EU research publications and not included in EPPO and IPPC diagnostic protocols for TSWV detection). From five available TaqMan real-time RT-PCRs, four were selected for preliminary study based on the availability of validation data and based on the results of *In silico* analysis (Table 12). One commercial TaqMan real-time RT-PCR was not selected for preliminary study, because the protocol is available in Russian language only.

Table 12 : Tests selected for preliminary study for TSWV with references. Tests selected for TPS are underlined.

Method	**Tests for validation:**
ELISA	TAS-ELISA DSMZ TAS-ELISA Sediag DAS-ELISA Bioreba DAS-ELISA Agdia B-FAST DSMZ
On-site: LFD	Immunostrip Agdia AgriStpip Bioreba
Conventional RT-PCR	RNA PCR reaction kit Loewe RT-PCR generic for tospoviruses Hassani-Mehraban *et al.* 2016 (Journal of Virological Methods, 233, 89-96)

	Hassani-Mehraban *et al.* 2016 (Journal of Virological Methods, 233, 89-96) Mumford *et al.* 1994 (Journal of Virological Methods, 46(3), 303-311) Zarzyńska-Nowak *et al.* 2018 (Canadian Journal of Plant Pathology, 40, 580-586) Fineti Sialer *et al.* 2002 (Journal of Plant Pathology, 84(3), 145-152) Vučurović *et al.* 2012 (European Journal of Plant Pathology, 133(4), 935-947) Panno *et al.* 2012 (Journal of Virological Methods, 186, 152– 156)
Real-time RT-PCR	Boonham *et al.* 2002 (Journal of Virological Methods, 101(1-2), 37-48) Roberts *et al.* 2000 (Journal of Virological Methods, 88(1), 1-8) Debreczeni *et al.* 2011 (Journal of Virological Methods, 176(1-2), 32-37) Mortimer-Jones et al. 2009 (Journal of Virological Methods, 161, 289-296)

7.4.2 Evaluation of the possibility to use the same buffers for all ELISA test

For the commercially available ELISA tests, validation data vary among companies. Therefore, it was very difficult to compare and select tests based on that information. The only option was, that the TPS organizer performed preliminary study on all available tests. In order to do the preliminary study, the TPS organizer suggested the modification of the protocol in a way that the same panel of samples can be tested with all different ELISA tests. With this modification the TPS organizer would be able to prepare enough homogeneous fresh plant material for all tests, and this would also lower the costs.

Commercial providers of 5 antisera (4 companies) selected for preliminary study were asked for the permission to use their protocol with modified buffers. The TPS organizer asked commercial providers if extraction buffer PBST-Tween + 2% PVP + 0.2% BSA, and coating buffer, wash buffer, substrate buffer and alkaline phosphatase as recommended in Appendix 3 of EPPO PM 7/125 could be used with their antisera. The TPS organizer specified that all other conditions (e.g., ELISA plate, time and temperature for incubation) specified in the manufacturer's instruction would be followed.

One company (DSMZ) granted the permission to the TPS organizer to use suggested buffers, because these are not essentially different as those recommended in their instructions. Other three companies were concerned that suggested modification will affect the performance of their test. However, they agreed to reconsider the suggested changes of the protocol after a study in which modified buffers and buffers recommended in manufacturer's protocol would be tested in parallel on a small-scale study.

The small-scale study was performed by the TPS organizer and results were communicated to the respective company for them to decide if the result of the modified protocol could be considered comparable with their standard protocol. This assessment included evaluation of analytical specificity and analytical sensitivity. Analytical specificity was tested on a seven different tospoviruses. Analytical sensitivity was tested on a series of 10-fold dilutions of two different TSWV isolates. Dilutions were obtained by mixing the extract of TSWV isolate with the extract of healthy tomato. Comparison of the obtained results was presented to the respective companies.

TheTPS organizer was given permission to perform the test with modified protocol from two companies (Bioreba and Sediag) while one company (Agdia) considered that the results with modified protocol could not be comparable with their protocol. Since one of the companies did not agree to use modified protocol, after considering all available options and following the communication with companies the TPS organizer decided that the ELISA tests would be performed without suggested protocol modifications.

7.4.3 Stability testing of the plant extracts

The plan of TPS organizer is to send samples of a plant extract in order to provide enough and homogenous testing material for all participants. Plant extract will be prepared by homogenizing the plant material in the extraction bags with corresponding extraction buffer for each test: for commercial kits their suggested buffers and dilution ratios will be used, for molecular methods PBST-Tween + 2% PVP + 0.2% BSA extraction buffer will be used. To confirm the stability of such extracts, the preliminary stability testing has been already carried out. The extracts were stored at < -15 °C and then tested in different time after storage to mimic the time, transport and storage conditions of samples that will be used in TPS.

7.4.4 Tests selected for TPS: based on preliminary studies data

Final selection of the best performing tests which will be included in the TPS was done after the preliminary studies, which was conducted according to the EPPO guidelines (PM7/98 (3)). Preliminary studies included testing on a panel of different isolates of TSWV, other tospoviruses, and on healthy samples. These was done to evaluate the inclusivity, exclusivity and selectivity of each test. To evaluate the analytical sensitivity of each test, serial dilutions of TSWV samples were analyzed. Among 19 tests that were included in preliminary studies (Table 12), eight tests were selected for TPS: two DAS-ELISA, two test for on-site detection, one conventional RT-PCR and three real-time RT-PCRs.

During the process of preliminary studies, all ELISA tests and tests for on-site detection were performed according to manufacturers' instructions. All molecular tests were performed on the same panel of RNA samples. Samples for molecular test were firstly homogenized in the PBST-Tween + 2% PVP + 0.2% BSA extraction buffer, the extract obtained was used as a starting material for the extraction of total RNAs with the RNeasy Plant Mini kit (Qiagen). Commercial RT-PCR test was performed according to manufacturers' instruction. The other 11 molecular tests were modified to be performed with the same enzyme mixes: One-Step RT-PCR kit (Qiagen) for conventional RT-PCR, AgPath-IDTM One-step RT-PCR kit (Ambion, Thermo Scientific™) for real-time RT-PCRs.

Results of preliminary studies showed that all five ELISA tests cross-react with some non-target tospoviruses. Although the aim was to find test specific to TSWV, ELISA was not excluded because of its robustness and because it is widely used in many diagnostic laboratories. The final selection among ELISA tests was done based on the results of the evaluation of analytical sensitivity. Selected ELISA tests (Agdia and Bioreba) had 10- up to 100-times higher analytical sensitivity compared to non-selected ELISA tests.

Both tests for on-site detection of TSWV also cross-reacted with some non-target tospoviruses, and sensitivity of both is lower compared to ELISA and RT-PCR based methods. The reason to include on-site tests in spite of these drawbacks is because of their practicality for use in the field. For TPS, both on-site tests were selected because showed comparable results.

Among the conventional RT-PCRs included in preliminary studies, seven were for specific detection of TSWV and one was for generic detection of the tospoviruses of American clade 1 (in this case Sanger sequencing is needed for the identification of species). Among seven specific tests, only one based on the publication of Hassani-Merhaban et al. (2016) did not cross-react with other tested non-target tospoviruses included in the preliminary studies. This specific test was also 10x more sensitive than generic RT-PCR test and provides final identification much faster compared to the generic test, therefore only this specific RT-PCR test was selected for inclusion in TPS. The analytical sensitivity of commercial RT-PCR test was 1.000x lower compared to the selected RT-PCR.

Regarding the real-time RT-PCR, three tests were comparable in the terms of sensitivity and selectivity: Boonham *et al.* (2002), Roberts et al. (2000) and Mortimer-Jones et al. (2009). Therefore all these three tests were selected for the inclusion in the TPS. The test from publication of Debreczeni *et al.* (2011)

strongly cross-reacted with some non-target tospovirus isolates and based on that it will not be included in the TPS.

8 Tomato brown rugose fruit virus

8.1 General information on the pest

Tomato brown rugose fruit virus (ToBRFV, tobamovirus genus) is an emerging virus first identified in tomato in Israel (2014) and Jordan (2015) and now spread in different Mediterranean and European countries (https://gd.eppo.int/taxon/tobrfv). Since November 2019 emergence measures were adopted to prevent the spread of the virus in the EU territory (Commission implementing decision (EU) 2019/1615). The contingency plan is restricted only to ToBRFV and does not include any other Tobamoviruses. ToBRFV has a single-stranded positive sense RNA genome located in rigid elongated particles. The virus is transmitted via seed (teguments contamination), and by contact during crop cultivation. Symptoms are not confined in the characteristic brown rugose patches developed on fruit, but they can include mosaic and discoloring on the leaves. Due to these symptoms, fruits from infected plants become unmarketable. Like other tobamoviruses, ToBRFV can survive for long time in infected plant residues, in contaminated soil, on cultivation tools and worker clothes, in irrigation systems and greenhouse structures (as poles, nets, pallets, etc.). ToBRFV has been found in *Solanum lycopersicum* (tomato) in plant cultivated in greenhouse and in *Capsicum* spp.

8.2 Scope of the TPS

Table 13: Definition of scope for ToBRFV

	Methods			
	DAS-ELISA	**RT-PCR**	**Real-time PCR**	**Other methods applicable for on-site: Immunostrip for tobamovirus**
Sample type (DNA, plant material with deactiv. pests, etc.)	Freeze dried leaves and fruits extract	Freeze dried leaves and fruits extract	Freeze dried leaves and fruits extract	Freeze dried leaves extract
Matrix (type of plant material: seed, leaves, etc.)	Leaves/ fruits of tomato and pepper	Leaves/ fruits of tomato and pepper	Leaves/ fruits of tomato and pepper	Leaves of tomato and pepper
Suitable for: symptomatic / asymptomatic sample	symptomatic / asymptomatic	symptomatic / asymptomatic	symptomatic / asymptomatic	symptomatic / asymptomatic
Purpose: detection / identification	detection	detection	detection	Early warning
Type of controls needed (NIC, NAC, PAC, PIC, IC, etc)	PC; NC (plant); NC (buffer)	NIC; NAC; PIC; PAC	NIC; NAC; PIC; PAC	PC; NC (plant);
No. of samples	22	22	22	22
Max no. of participants	25	25	25	25

8.3 Setting the weighted criteria for selection of tests for TPS

Selection of tests included in preliminary studies and in TPS has been done based on pre-defined criteria listed in the table below.

Table 14: Criteria for selection of tests for TPS for ToBRFV

Criteria	Descriptor (%, number, text)	Target	Relative Weight (lab)	Relative Weight (on-site)
Validation data (prior preliminary studies)				
Is the target (gene/protein) appropriately selected?	Yes/No	Yes	High	High
Available validation data	Yes/No	No	Low	Low
Validation data available for selected matrix	Yes/No	No	Low	Low
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)	NA	NA	NA
Analytical sensitivity in plant material (selected matrix)	Conc. (absolute value if possible or relative conc. or low/medium/high)	high	High	Medium
Diagnostic sensitivity (comparison of different tests)	%	100%	High	Medium
Analytical specificity	Level	High	High	High
a) Exclusivity (Non-target organism): False positives	Level	0%	High	High
b) Inclusivity (Target organisms): False negatives	Level	0%	High	High
Selectivity	Presence of cross reactions with matrix	No	High	High
Repeatability (near LOD)	Level	High	High	Medium
Reproducibility/ robustness	%	100%	High	Medium
Results of interlaboratory comparisons available	Yes/No	No	low	Low
Additional information (not a criterion!)				
Type of matrix		leaves/fruits		
Extraction method		kits		
Use on sympt./asympt.		sympt/asymp		
Other				
Validation data (after preliminary studies)				
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)	NA		
Analytical sensitivity in plant material (selected matrix)	Conc. (absolute value if possible or relative conc. or low/medium/high)	high	High	High
Diagnostic sensitivity (comparison of different tests)	%	100%	High	High
Analytical specificity	Level	High	High	High
a) Exclusivity (Non-target organism): False positives	Level	0%	High	High
b) Inclusivity (Target organisms): False negatives	Level	0%	High	High
Selectivity	Presence of cross reactions with matrix	No	High	High
Repeatability (near LOD)	Level	high	High	Medium

Reproducibility/ robustness	%	100%	High	Medium
APPLICABILITY				
Applicability in different matrixes	Level	Medium	Medium	Medium
Amount of material which is included in one sample	Amount of plant units tested	25 plants/sample	Medium	Medium
Standardized preparation of the reaction (e.g., ready to use reagents)	Yes/No	Yes	Medium	High
Availability and relevance of controls (in the case of kits)	Yes/No	Yes	high	High
PROTOCOLS				
Available detailed protocols	Yes/No	Yes	Medium	Medium
Simple test procedure	Yes/No	Yes	Medium	Medium
Simplicity of data analysis	Yes/No	Yes	High	High
User friendly test	Yes/No	Yes	Medium	High
Time needed to complete analysis (less than one hour/ one day/ several days)	Duration in time unit	one day/less than one hour	Medium	High
Easy to multiplex?	Yes/No	Yes	Medium	NA
Database/library dependent (yes/ no) (for example fatty acids profiling, sequencing,...)	NA	NA	NA	NA
CHEMICALS				
Stability of chemicals at ambient temperature	Yes/No	Yes	Low	High
EQUIPMENT				
No equipment/ instrument needed (relevant only for on-site tests)	Yes/No	Yes	NA	High
Test not exclusively developed for a specific instrument	Yes/No	Yes	High	High
Cost of obligatory equipment/ instruments (up to 10.000 EUR/ 10.000-50.000 EUR/ more than 50.000 EUR?)	Cost in euro	10000 to 50000	Low	Low

8.4 Preliminary study and selection of tests for TPS

Up today, no validated diagnostic protocols nor EPPO standard are available for the detection of ToBRFV. For these reasons, the methods selected for TPS are those described in literature, from the International seed Federation (ISF) protocol and available commercial kits. All selected methods are well established in the laboratory of TPS organizer. The most important criteria for test selection have been specificity (exclusivity and inclusivity) and sensitivity.

Indeed, the selected tests should unequivocally detect and identify ToBRFV without cross reaction with other viruses.

Accordingly, the scope of the TPS is the specific detection of ToBRFV in tomato and pepper leaves and fruits. The TPS will include 22 blind samples and NIC, PIC and PAC.

As no EPPO standard nor validated protocols are available for ToBRFV, we performed thorough literature searches, including websites of commercial companies and methods. It has been found: 3 DAS-ELISA antibody sets/kits from different commercial companies; 4 already design primer pairs and a commercial kit for RT-PCR; 3 real-time RT-PCR tests and 1 commercially available lateral flow test.

Table 15 : Tests selected for preliminary study for ToBRFV with references. Tests selected for TPS are underlined.

Method	Tests for validation:
DAS-ELISA	Agdia DSMZ Loewe
Conventional RT-PCR	Alkowani et al., 2019 Ling et al., 2019 Panno et al., 2019a Rodriguez-Mendoza et al., 2019 Loewe
Real-time RT-PCR	ISHI Veg, 2019 Menzel and Winter 2019 Panno et al., 2019b
On-site: LFD	ImmunoStrip (Agdia)

According to the information obtained from the different producers, all three DAS-ELISA kits resulted specific in ToBRFV detection (good inclusivity) but cross reacted with other Tobamoviruses (poor exclusivity), so we decided to not include DAS-ELISA in the ToBRFV TPS. The same situation was obtained for the lateral flow that was not considered in the TPS.

Analytical specificity of RT-PCR and Real Time RT-PCR tests has been analysed with RNA extracted from isolates of ToBRFV, tomato mosaic virus (ToMV), tobacco mosaic virus (TMV), tobacco mild green mosaic virus (TMGMV) and bell pepper mosaic virus (BPeMV). All the tests were adapted to be a one-step-one tube and to work with a commercial amplification kit. In these conditions, the RT-PCR published by Ling et al., 2019 and Panno et al., 2019a, cross reacted with other tobamoviruses, so they were excluded by the TPS. On the contrary, the couple of primer published by Rodriguez-Mendoza et al., 2019, also incorporate in the Loewe RT-PCR kit, confirmed a high analytical specificity so they have been selected and the complete commercial kit has been included in TPS.

The remaining tests have been evaluated for their analytical sensitivity. All the three real time RT-PCR protocols showed the same LOD value both for tomato and pepper infected plants. The two end point RT-PCR protocols showed a different LOD value, both acceptable.

9 Plum pox virus (on-site testing)

9.1 General information on the pest

Plum pox, also known as sharka, is caused by plum pox virus (PPV). PPV may infect a wide variety of *Prunus* species used as commercial varieties or rootstocks, including almond, apricot, cherry, nectarine, peach, plum, as well as wild and ornamental species. In fruit trees, infection may eventually result in deformation of fruits and severe yield reduction. Plum pox virus isolates can currently be classified into ten monophyletic strains: D (Dideron), M (Marcus), Rec (Recombinant), C (Cherry), EA (El Amar), W (Winona), T (Turkish), CR (Cherry Russian), CV (Cherry Volga) and An (Ancestor Marcus). The strains have specific genome sequences and may vary in their symptomatology, pathogenicity, host range, epidemiology and aphid transmissibility (EPPO PM 7/32 (2) in preparation). PPV is present in many European countries. To control the disease, PPV is a regulated non-quarantine pest for EU: plants of a list of *Prunus* species intended for planting shall not be introduced into, or moved within the European Union in the presence of PPV, or of symptoms caused by PPV above a detection threshold (Commission implementing regulation (EU) 2019/2072, appendix IV). Therefore, the availability of reliable detection tests is required. To detect the virus, testing can be performed on symptomatic

leaves, shoots, flowers, and fruits. On asymptomatic plant material, both shoots and leaves can be tested.

9.2 Scope of the TPS

Table 16: Definition of scope for PPV

PPV on-site testing	**Methods**	
	On-site LFD (serological)	**On-site LFD RPA (molecular)**
sample type (DNA, plant material with deactiv. pests, etc.)	Infected/ non-infected plant material	Infected/ non-infected plant material
matrix (type of plant material: seed, leaves, etc.)	Freeze-dried ground leaves	Freeze-dried ground leaves
suitable for: symptomatic / asymptomatic sample	Symptomatic / Asymptomatic	Symptomatic / Asymptomatic
purpose: detection / identification	Detection	Detection
type of controls needed (NIC, NAC, PAC, PIC, IC, etc)	PC, NC	PC, NC
no. of samples	22	22
max no. of participants	20	20

9.3 Setting the weighted criteria for selection of tests for TPS

Selection of tests included in preliminary studies and in TPS has been done based on pre-defined criteria listed in the table below.

Table 17: Criteria for selection of tests for TPS for PPV

Criteria	**Descriptor (%, number, text)**	**Target**	**Relative Weight (on-site)**
Validation data (prior preliminary studies)			
Is the target (gene/protein) appropriately selected?	Yes/No	yes	High
Available validation data	Yes/No	yes	Low
Validation data available for selected matrix	Yes/No	yes	Low
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)	NA	NA
Analytical sensitivity in plant material (selected matrix)	Conc. (absolute value if possible or relative conc. or low/medium/high)	Lowest level	Low
Diagnostic sensitivity (comparison of different tests)	%	100%	Medium
Analytical specificity	Level	100%	High
a) Exclusivity (Non-target organism): False positives	Level	0%	High
b) Inclusivity (Target organisms): False negatives	Level	0%	High
Selectivity	Presence of cross reactions with matrix	no	High
Repeatability (near LOD)	Level	100 % near LOD	Medium
Reproducibility/ robustness	%	100 % near LOD	Medium
Results of interlaboratory comparisons available	Yes/No	yes	Low

Additional information (not a criterion!)			
Type of matrix		Leaves of *Prunus* spp	
Extraction method		On-site extraction for LAMP	
Use on sympt./asympt.		Symptomatic	
Other		Low number of assays available for kits	
Validation data (after preliminary studies)			
Analytical sensitivity (LOD) (pure culture or DNA diluted in water)	Conc. (absolute value if possible or relative conc. or low/medium/high)	NA	NA
Analytical sensitivity in plant material (selected matrix)	Relative concentrations from dilutions of target	Lowest level	Medium
Diagnostic sensitivity (comparison of different tests)	%	100%	Medium
Analytical specificity	% of true positive detected and true negative not detected	100%	High
a) Exclusivity (Non-target organism): False positives	% of non target strains detected	0%	High
b) Inclusivity (Target organisms): False negatives	% of target strains / samples not detected	0%	High
Selectivity	presence of cross reactions with matrix (% of non target samples detected)	0%	High
Repeatability (near LOD)	% of agreement between repetitions	100% (not specifically near LOD)	Medium
Reproducibility/ robustness	% of agreement between repetitions in different conditions	100% (not specifically near LOD)	Medium
APPLICABILITY			
Applicability in different matrixes	Level	In all Prunus spp fresh leaves	High
Amount of material which is included in one sample	Amount of plant units tested	Highest	Low
Standardized preparation of the reaction (e.g., ready to use reagents)	Yes/No	Yes	High
Availability and relevance of controls (in the case of kits)	Yes/No	Yes	High
PROTOCOLS			
Available detailed protocols	Yes/No	Yes	High
Simple test procedure	Yes/No	Yes	High
Simplicity of data analysis	Yes/No	Yes	High
User friendly test	Yes/No	Yes	High
Time needed to complete analysis (less than one hour/ one day/ several days)	Duration in time unit	Hour	Medium
Easy to multiplex?	Yes/No	NA	NA
Database/library dependent (yes/ no) (for example fatty acids profiling, sequencing,...)	NA	NA	NA
CHEMICALS			
Stability of chemicals at ambient temperature	Yes/No	Yes	High
EQUIPMENT			
No equipment/ instrument needed (relevant only for on-site tests)	Yes/No	no	Medium

Test not exclusively developed for a specific instrument	Yes/No	yes	High
Cost of obligatory equipment/ instruments (up to 10.000 EUR/ 10.000-50.000 EUR/ more than 50.000 EUR?)	Cost in euro	Up to 10 000	High

9.4 Preliminary study and selection of tests for TPS

Table 18 : Tests selected for preliminary study for PPV with references. Tests selected for TPS are underlined.

Method	**Tests for validation:**
On-site: LFD RPA (molecular)	AmplifyRP® Acceler8™ (Agdia)
On-site: LFD (serological)	ImmunoStrip® (Agdia) Agristrip® (Bioreba)
On-site: LAMP	bKit-PPV (Qualiplante) with EXT-001 (Optigene) extraction kit

Firstly, we performed an in-depth literature search, including websites of commercial companies. Tests selected for the TPS are available commercial kits and described on internet sites or pending. They can be performed on-site and may include only a few steps in a room or laboratory (tests were excluded from the selection). Two LFD (serological), one LFD RPA (molecular) and one on-site extraction / LAMP test (molecular) were selected. Before preliminary studies, provider's protocols needed to be adapted to the freeze-dried plant leaf material. For each test included in the above table, a panel of plant reference materials was analyzed. The selection of tests was based on the consistency of the obtained results with the expected results (assigned value of the plant reference materials), focusing on diagnostic specificity and inclusivity.

Preliminary studies could not be performed for the on-site detection LAMP and this test could not be selected for the TPS. Finally, the TPS panels will include a maximum of 22 samples to be analyzed using two on-site LFD serologic tests and one on-site LFD RPA test (molecular). Further assessment will be performed for all the tests by the TPS organizer (including the on-site extraction / LAMP), using fresh plant material and following the provider's instructions.

10 Conclusions

Number of tests selected for the TPS for six selected pests is the same or higher as in the plan described in the Annex 1 of the Grant Agreement (Table 19): 13 tests for *X. citri* pv. *citri*, 9 tests for *X. ampelinus*, 3 tests for *C. parasitica*, 8 tests for TSWV, 5 tests for ToBRFV and 3 tests for PPV. In some cases the decision to choose only a few from many available commercial and non-commercial tests required a great deal of effort from the TPS organizers to do the research and preliminary studies to select the most promising tests according to the criteria and scope of TPS.

Table 19: Number of planned, identified from the literature search/systematic search, included in preliminary studies and finally selected tests for TPS Round 2

Pest	No. of planned tests	No. of tests identified from the literature search/systematic search	No. of tests included in preliminary studies	Tests selected for TPS (methods)
Xanthomonas citri* pv. *citri	5 to 8	24	21	16 (PCR, real-time PCR, LAMP-PCR, direct molecular tests performed from ImmunoStrips® (Agdia) and Whatman™ FTA cards)
Xylophilus ampelinus	5 to 8	10	10	9 (IF, ELISA, PCR, real-time PCR)
Cryphonectria parasitica	1 to 4	3	3	3 (PCR, real-time PCR)
Tomato spotted wilt tospovirus	5 to 8	76	19	8 (ELISA, LFD, RT-PCR, real-time RT-PCR)
Tomato brown rugose fruit virus	5 to 8	12	12	5 (RT-PCR, real time RT-PCR)
Plum pox virus (on-site testing)	1 to 4	4	4	3 (LFD RPA, LFD)

This deliverable can be seen as an example how learning from one task (TPS Round 1) can be used to improve future similar projects or tasks (TPS Round 2). A lot of material for the TPS was prepared already in Round 1 (criteria, rules, TPS documents), which was used as starting point for TPS Round 2. Furthermore, challenges and experiences gathered from organization of TPS Round 1 have helped to improve the whole process of preparation and organization of TPS Round 2. Most importantly, description and recommendations for TPS organization in the scope of VALITEST project are applicable to any TPS organization and could help new EU reference laboratories (in the field of plant health).

One of the most important lessons learned by TPS organizers was that intensive communication with commercial kit providers is needed to achieve harmonization between both sides and much more constructive discussions were achieved in the preparation for TPS Round 2. Discussions regarding the possible modifications of kit components/protocols have proved to be very valuable in planning of the TPS as this was essential especially to improve the process of preliminary study, which is very time-consuming and cost-intensive, however necessary for the organization of a TPS on such level as in VALITEST project.

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