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DELIVERABLE N° 1.5

Title: TPS reports with description of the method, materials and software used, as well as the data analysis - Round 2

Version 2.0

Validation of diagnostic tests to support plant health

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Abstract:

The aim of the deliverable 1.5. is to present a summary of the results obtained in the Round 2 of the test performance studies (TPS) organized by WP1 on six prioritized pests. Tests selection for each TPS was conducted following the "Common rules for selection of tests for TPS" and based on the "Weighted criteria for selection of tests for TPS", both described in deliverable D1.1 (Minimum performance parameters to select tests for validation and selection of laboratories for TPS), while the list of selected tests for each TPS is available and explained in deliverable D1.3 (List of tests for validation - Round 2). TPS participants were selected following the "Common rules for selection of participants for TPS" and based on the "Criteria for selection of participants of TPS", also both described in deliverable D1.1. For each of the six TPSs, the methodology used to perform the tests, the results of preliminary studies to select the tests, the results of the TPS and their thorough analysis and interpretation are described in corresponding TPS reports (supplementary information available upon request under confidentiality agreement). The validation data obtained during the six TPSs will be available in the validation section of the EPPO database on the diagnostic expertise. Main outcomes for each of the TPSs are highlighted as well as difficulties noticed during the organization process, which will improve organization of the future studies in the field of plant pests.

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

HISTORY OF CHANGES		
Version	Publication date	Change
1.0	06 May 2021	Initial version
2.0	28 October 2021	Correction of the name of the company BIOREBA Correction of the name of the commercial kit AgriStrip BIOREBA

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TERMS, ABBREVIATIONS AND DEFINITIONS

DBIA – Dot-blot immunoassay

XCC – *Xanthomonas citri* pv. *citri*

XCA – *Xanthomonas citri* pv. *aurantifolii*

XCB – *Xanthomonas citri* pv *bilvae*

ELISA – Enzyme linked immunosorbent assay

EPPO – European and Mediterranean Plant Protection Organization

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

PCR – Polymerase chain reaction

PIC – Positive isolation control

PPV – plum pox virus

RT-PCR – Reverse transcription polymerase chain reaction

RT-qPCR – Reverse transcription quantitative polymerase chain reaction

TBIA – Tissue-blot immunoassay

ToBRFV - tomato brown rugose fruit virus

TPS – Test performance study

TSWV - tomato spotted wilt tospovirus

1 Purpose of TPS activity

The purpose of this deliverable is to present summary of the results of the six TPSs organized in Round 2 in the frame of WP1 of VALITEST project with the objective to obtain validation data on different tests using different methods. Test is defined in EPPO Standard PM 7/76 as the application of a method to a specific pest and a specific matrix. The aim of WP1 is to coordinate the preparation and organization of test validations and the performance of TPSs for prioritized pests in a range of matrices and for a variety of diagnostic technology platforms used both for laboratory and on site-based testing. TPS participants received TPS reports with the results of the study they have participated in. Each report contains information on selecting participants, planning the TPS, selecting tests for TPS, description of the work, results of preliminary studies, results of the TPS and their thorough analysis and interpretation.

2 Common rules and criteria for selection of TPS organizers

The programme of the second round of TPS includes six pests selected based on the results of the survey organized in the framework of WP4 and on the list of pests categorized by the EU (Table 1). Details on the selection of pests are given in D4.1 (Report on stakeholder priorities for tests and general prioritisation framework).

Selection of competent laboratories which will organize the TPS is one of the critical steps in obtaining relevant results in TPS. For the Round 2 of TPSs, organizers were selected through an online poll where each VALITEST consortium partner expressed their interest in organizing the TPS on the selected prioritized pests. In order to ensure a transparent process for the selection of TPS organizers, selected TPS organizers need to fulfil the criteria defined in D1.1 (Minimum performance parameters to select tests for validation and selection of laboratories for TPS). In addition, other requirements were asked (sections 2.1 to 2.5). The final list of TPS organizers for Round 2 was approved by the VALITEST consortium (Table 1).

Table 1: List of pests and associated TPS organizers selected for the second validation round

Pest	**Pest group**	**EU pest status**	**Comments for prioritization for validation**	**Tests commerciall y available**	**TPS Organiser**
tomato brown rugose fruit virus	Virus	Alert list	Major concerns for growers of tomato and peppers	Yes	CREA
tomato spotted wilt virus	Virus	Quarantine	Harmful for ornamental plants, vegetables and industrial crops	Yes	NIB
plum pox virus (on site testing)	Virus	Quarantine	Important for fruit trees certification. New strains emerging.	Yes, field testing	ANSES
Xanthomonas citri pv. citri	Bacteria	Quarantine	Major concerns for Citrus leaves, stems and fruits Not present in the EU	Yes	ANSES
Xylophilus ampelinus	Bacteria	Quarantine	Major pest on grapevine. Impact on trade and EU exports	Yes	FERA
Cryphonectria parasitica	Fungi	Quarantine	Major concerns for chestnut and other susceptible tree genera and species	Yes	UNITO

2.1 Involvement in diagnostic activities (performing routine analyses) with different methods

A potential TPS organizer needs to have significant experience in performing diagnostic activities and performing routine analyses using the range of different methods and matrices for the pest in question.

2.2 Experience with the development of diagnostic tests

Personnel in the laboratory organizing TPS needs to demonstrate a continued experience in the development of diagnostic test in their field of work and to have adequate knowledge, competency and experience. Records of their qualifications must be available.

2.3 Experience with the obtaining documentation needed for the shipment of material

Since the time limitations have significant impact to the course of the TPS, the TPS organizer is required to have sufficient experience in requesting and obtaining the letter of authorization (LoA) from the authorities. Additionally, TPS material frequently requires specific shipment conditions, therefore it is essential for TPS organizer to have previous experience with shipment of material for TPS/PT.

2.4 Collaboration in national and international bodies in the field of plant pest detection

For TPS organizer it is preferable to be a part of EPPO panels or similar organizations, like International Plant Protection Convention (IPPC).

2.5 Technical requirements

TPS organizer must use the correct terminology and processes, have established quality assurance (QA) system, be able to prepare reference material, ensure the validation processes are properly documented, etc. In addition, TPS organizers should have successful participated in at least one previous interlaboratory comparison.

3 Scope of TPS round 2

This document describes the main outcomes of the TPS organised on six pests selected based on consortium expertise: tomato spotted wilt tospovirus, tomato brown rugose fruit virus, plum pox virus, *Cryphonectria parasitica*, *Xylophilus ampelinus* and *Xanthomonas citri* pv. *citri*. For each of the six TPSs, the methodology used to organize the TPS (scope of TPS, common rules and criteria for the selection of tests and participants for TPS), the results of preliminary studies to select the tests, the results of the TPS and their thorough analysis and interpretation are described in corresponding TPS reports. For each TPS, conclusions on the performance of the tests are summarized and in some cases also the main challenges are discussed and recommendations are given for future similar studies. The complete TPS reports for each study are available as supplementary documents to this deliverable and they can be shared upon request. TPS reports are protected by a confidentiality agreement.

4 Methodology for the implementation of TPS

Tests for each TPS were selected following the "Common rules for selection of tests for TPS" described in deliverable D1.1 (Minimum performance parameters to select tests for validation and selection of laboratories for TPS), while the list of selected tests for each TPS is available and explained in deliverable D1.3 (List of tests for validation - Round 2). TPS participants were selected based on the criteria for the selection of TPS participants described in deliverable D1.1. It has to be noted that the criteria have been slightly modified between TPS Round 1 and Round 2. The updated table "Criteria for selection of participants of TPS" is available in Appendix.

For each of six TPSs descriptions how to perform the tests and analyse the results is described in the corresponding TPS reports (supplementary information).

5 Explanation and recommendation for the selection of tests (including commercial kits)

This part was prepared in collaboration with WP7. The list of methods and tests selected for validation for TPS Round 2 is described in more details in Deliverable D1.3 (List of tests for validation – Round 2).

The process of tests selection for TPS was described and explained in more details in the points 4 and 4.1 in D1.4 (TPS reports with description of the method, materials and software used, as well as the data analysis - Round 1) to make it as transparent as possible.

In this document, reasons leading to the decisions on the selection process in TPS Round 2 are listed and explained in details. The view presented in this document is the opinion of TPS Round 2 organizers.

In addition to the suggestions given after the Round 1 of the TPS, the experience gained during the test selection process for the TPSs organized in Round 2 proves that extensive discussions with kits producers / providers enables better understanding between laboratories and kit providers on the specific needs of laboratories and on the consequential need to slightly deviate from the recommendations/manufacturer instructions (e.g. the use of a different matrix). The tight time schedules and financial constraints can be limiting factors for the selection of the tests when many commercial kits are available, especially for the same method (e.g. ELISA, LFD). Therefore, for Round 2, one TPS organizer, after discussion with ELISA kit providers, evaluated the possibility to use common buffers for several ELISA tests before the selection of the tests. This allowed commercial ELISA kit providers to gain the knowledge how their test is performing in different conditions, while fulfilling the aim of the TPS organizer to include more tests in the comparison and at the same time respect time and material constraints. After careful consideration of the results obtained, some ELISA kits providers approved the use of their kit with changed buffers, while others considered that the different buffers significantly affected the performance of their test. Nevertheless, transparent and fruitful discussion among TPS organizers and commercial kits providers during the organization of TPS Round 2 was mutually beneficial and helped a better understanding of the needs of diagnostic laboratories or other potential users of the commercial kits and kits providers.

6 TPS reports

The complete technical report of each TPS is available as supplementary documents upon request and under confidentiality agreement because the results of the TPS are still unpublished. Confidentiality of the data in the technical reports is in line with the Data Management Plan (DMP) of the VALITEST

project: "*Open access data may compromise the quality trademark of partners. The question of, whether or not the performance of the various tests evaluated will be disclosed, will be discussed on a case-by-case basis by WP1 leaders and kits providers.*"
The validation data obtained during the six TPSs will be available in the validation section of the EPPO database on diagnostic expertise. TPS organizers will deposit in the database the validation data of all the tests included in a particular TPS and if relevant, the data obtained in preliminary studies.

The selection of the tests for TPS Round 2 is described in details in the Deliverable D1.3 (List of tests for validation - Round 2). However, after the finalisation of this deliverable some organizers decided to change the tests selected based on new information they obtained (e.g. new tests were evaluated in in-house validation and included in the TPS, or some tests were excluded). For that reason, in order to provide the final data on the results of test selection process, short reports on the test selection process are given for the six pests included in the TPS Round 2

6.1 Short report for test selection for the TPS on tomato spotted wilt tospovirus

In total 76 different tests were evaluated to be included in the preliminary study and TPS:

- 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.

Thirteen ELISA tests were evaluated as such tests are widely used. Some tests were excluded because the commercial provider stopped the production of the test or was in the process of changing the antisera or because the TPS organizer did not get the required information on validation data including 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 tests were selected for preliminary study (Table 2).

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 because 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 can be difficult and depends on the experience of the person reading them. In addition, TPS organizer don't have experience with these methods.

Concerning on-site detection methods, four tests were taken into consideration and two were selected for preliminary study (Table 2). 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 performing those tests requires additional steps compared to conventional LAMP assays and because 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 use 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 2). Among the eight selected tests, seven are considered to be TSWV specific and one is a 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 performing those tests requires 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). Out of 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. One commercial TaqMan real-time RT-PCR was not selected for preliminary study, because the protocol is only available in Russian.

In total, 19 tests were selected for preliminary studies (Table 2): five DAS-ELISA test, two lateral flow devices applicable for on-site detection, eight RT-PCR tests and four RT-qPCR tests.

Table 2: Methods and tests selected for preliminary study. Tests selected for TPS are written in boldface.

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** **AgriStrip 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 (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)**

6.2 Short report for test selection for the TPS on tomato brown rugose fruit virus

In total 14 different tests were evaluated to be included in the preliminary study and TPS:

- 3x DAS-ELISA
- 7x reverse transcription (RT)-PCR
- 3x real-time RT-PCR (TaqMan)
- 1x on-site test: LFD

The final selection of the tests to be included in the TPS was made based on some minimum performance characteristics that were established by the TPS organizer in advance. Briefly, laboratory-tests with high analytical specificity were selected. Regarding the on-site tests, the weight of this performance criteria was less stringent because those tests can be used for an early detection of the virus that results must be confirmed later with a laboratory test.

Validation data already available for the selected tests varied among companies and publications. For none of the tests complete validation data was not available.

Regarding DAS-ELISA method, which was taken into consideration because it is widely used, 3 tests commercially available at that time were evaluated. The validation data provided by companies highlight the occurrence of cross reactions with other tobamoviruses. Consequently, none of them was selected for inclusion in preliminary study (Table 3).

For the 10 molecular tests (7 conventional RT-PCR and 3 real-time), in addition to available validation data, analytical specificity has been checked with *in silico* analysis. Moreover, the tests were evaluated for their suitability for diagnostic purposes. One test was excluded due to the lack in specificity and another one was excluded due to the length of the amplification product which is more suitable for virus characterization. Eight molecular tests (5 conventional RT-PCR and 3 real-time) were selected and included in the preliminary study (Table 3).

Concerning the only available test for on-site detection method, the organizers included it in the preliminary study though the information provided by the commercial company highlight a cross-reaction with another tobamovirus, due to the utility of this diagnostic method in early diagnosis of the virus (Table 3).

In total, 9 tests were selected for preliminary studies (Table 3): 5 RT-PCR tests, 3 RT-qPCR tests and one lateral flow device applicable for on-site detection.

Table 3: Methods and tests selected for preliminary study. Tests selected for TPS are written in boldface.

Method	Tests for validation:
On-site: LFD	Immunostrip Agdia
Conventional RT-PCR	**Alkowni et al. 2019 (Journal of Plant Pathology, 101, 719–723)** Ling et al. 2019 (Plant Disease, 103(6), 1439) Panno et al. 2019a (Plant Disease, 103(6), 1443) **Rodriguez-Mendoza et al. 2019 (Mexican Journal of Phytopathology, 37(2), 345-356)** **Tomato brown rugose fruit virus – Complete One-Step Reverse transcriptase PCR Reaction Kit (Loewe)**
Real-time RT-PCR	**ISHI-Veg 2019** **Menzel and Winter 2019** **Panno et al. 2019b (PeerJ, 7, e7928)**

6.3 Short report for test selection for the TPS on plum pox virus

An in-depth literature search has been performed, including websites of commercial companies. Tests selected for the TPS were available commercial kits described on internet sites or waiting to be described. They can be performed on-site although they may include a few steps in a room or laboratory (laboratory tests were excluded from the selection).

In total, 4 tests were selected for preliminary studies (Table 4): two LFD (serological), one LFD RPA (molecular) and one on-site extraction / LAMP test (molecular).

Before preliminary studies, provider's protocols needed to be adapted to the freeze-dried plant leaf material. For each test included in the Table 4, 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 included 22 samples to be analyzed using two on-site LFD serologic tests and one on-site LFD RPA test (molecular).

Table 4: Methods and tests selected for preliminary study. Tests selected for TPS are written in boldface.

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

6.4 Short report for test selection for the TPS on *Cryphonectria parasitica*

A review of available published methods was carried out. The tests described in the EPPO protocol (2005) only comprise plating on culture media which is widely used within European laboratories. For this reason, molecular tests were selected based on published protocols. There are currently no described on-site tests for *C. parasitica*. There are no commercial kits presently available for molecular detection (conventional PCR or real-time PCR). Three tests described in published literature are available: one conventional PCR test and two real-time PCR tests (Table 5) and were selected based on their performance as described by the respective authors. The performance of those tests was further investigated during preliminary studies in accordance with EPPO guidelines (PM7/98 (4), EPPO Bulletin

2019), to support the selection of the tests. These validation studies incorporated the use of different fungal isolates, PCR reagents and to provide suitable robust reagent(s) for use in the TPS.

Table 5: Methods and tests selected for preliminary study. Tests selected for TPS are written in boldface.

Method	Tests for validation to be selected
PCR	**Popov et al. 2010 (Microbiology, 79, 223-228)**
Real-time PCR	**Chandelier et al. 2019 (European Journal of Plant Pathology, 153, 29-46)** **Rubio et al. 2017 (Annals of Applied Biology, 171, 52-61)**

6.5 Short report for test selection for the TPS on *Xylophilus ampelinus*

A review of commercially available diagnostic kits and published tests, including tests described in EPPO protocol PM7/96, was carried out. One LFD, 2 immunofluorescence (IF) tests, 2 ELISAs, 1 conventional PCR kit, 3 published conventional PCR tests and 1 published real-time PCR test were identified, and all tests were included in preliminary studies (Table 6).

Table 6: Methods and tests selected for preliminary study. Tests selected for TPS are written in boldface.

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

6.6 Short report for test selection for the TPS on *Xanthomonas citri* pv. *citri*

Detection and identification of *Xcc* can be achieved by using serological or molecular methods. The TPS aims to compare molecular methods only. The tests selected for the TPS are those described in IPPC and EPPO Diagnostic protocols, tests found in the literature and available commercial kits. All selected tests 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.

The selection of the tests was based on a bibliographic review and on preliminary studies conducted by the TPS organizer.

In total 20 tests were selected for preliminary studies (Table 7): eleven conventional PCR tests, five real-time PCR tests, one LAMP test and three direct molecular tests performed from ImmunoStrips® (Agdia) and Whatman™ FTA cards (only for a limited number of participants).

For each test included in Table 7, 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 the 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).

Table 7: Methods and tests selected for preliminary study. Tests selected for TPS are written in boldface.

Method	Tests for validation
Conventional PCR	Hartung et al. 1993 (2/3) (Applied and Environmental Microbiology 59(4), 1143-1148) Hartung et al. 1996 (4/7) (Phytopathology 86(1), 95-101) **Cubero et al. 2002 (J-pth1/2) (Environmental Microbiology, 68(3), 1257–1264)** Cubero et al. 2002 (J-Rxg/J-RXc2) (Environmental Microbiology, 68(3), 1257-1264) Kingsley et al. 2000 (King F/R) (Phytopathology 90, S42) **Mavrodieva et al. 2004 (VM3/4) (Phytopathology,37(1), 61-68)** Coletta-Filho et al. 2006 (Xac01/Xac02) (Journal of Applied Microbiology 100(2), 279-85) **Park et al. 2006 (XACF/XACR) (Microbiological Research 161, 145-149)** **Miyoshi et al. 1998 (XCF/XCR) (Japanese Journal of Phytopathology, 64(4), 249-254)** **Robène et al. 2020 (XcciF/R) (BMC Microbiology, 20, 296)** **Commercial kit Loewe® based on Hartung et al. 1993 (2/3)**
Real-time PCR	Kingsley et al. 2000 (King F/R) (Phytopathology 90, S42) **Cubero et al. 2005 (J-pth3/4 and probe J-Taqpth2) (Phytopathology, 95(11), 1333-1340)** PlantPrint **Mavrodieva et al. 2004 (VM3/4) (VM3/4) (Phytopathology,37(1), 61-68)** **Robène et al. 2020 (Xcci1051F/R and probe P-Xcci-1051) (BMC Microbiology, 20, 296)**
LAMP-PCR	**Rigano et al. 2010 (with OptiGene amplification kit) (BMC Microbiology, 10, 176)**
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) (Phytopathology, 95(11), 1333-1340)** **Real-time PCR test Robène et al. 2020 (Xcci1051F/R and probe P-Xcci-1051) (BMC Microbiology, 20, 296)** **LAMP PCR test adapted from Rigano et al. 2010 (with OptiGene amplification kit) (BMC Microbiology, 10, 176)**

7 General conclusions

Organization of a TPS is a very complex and demanding process. With the organization of the six TPSs in Round 1, a substantial knowledge was gained which was shown to be useful in organization of TPSs in Round 2. The organization is easier if timelines, rules and criteria which needed to be followed are defined in advance. This requires more preparation work, however, it is more efficient when the TPS is running. It is valuable to foresee some possible scenarios and difficulties, even if exhaustiveness

cannot be reached (e.g. the Covid-19 pandemic in 2020 was not anticipated). This way reaction time to act is shorter and limits the impact on the running of the TPS. It was learned in Round 1 that possible delays need to be taken into account. For example delays of samples dispatch due to delays in obtaining the Letter of authorization or import permit can lead to less time available for the TPS participants to perform the tests. Therefore, the TPS participants need to be informed in advance if they will need to provide the Letter of authorization and in any case they need to be given enough time to prepare for the TPS, order specific chemicals, perform the tests and still have time to repeat some tests if needed. TPS organizers also learned that even though communication with TPS participants is sometimes time consuming, it is crucial to avoid later misunderstandings and exclusion of the results from the analysis.

The main outcomes (on the performance of the tests) of each of the six TPSs are listed below (Table 8). It is necessary to point out that in most cases more tests were selected for TPS than initially planned in the project's proposal. Even more tests were included in preliminary studies as in some cases there were many tests identified by systematic search (literature, internet, discussions with commercial kit providers, survey,…) which needed to be considered for the TPS.

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).

Table 8: 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	Number of data sets collected	No. of tests included in preliminary studies	Tests selected for TPS (methods)	Main outcomes*
tomato spotted wilt virus	5-8	76	115	19	8 (2 DAS-ELISA tests, 2 lateral flow devices applicable for on-site detection, 1 RT-PCR test and 3 RT-qPCR tests)	All test included in this TPS are fit for purpose. Serological tests are sensitive enough for the detection in symptomatic material, and on-site serological tests (LFD) are practical to be used in the fields to obtain quick result. For final identification molecular tests are required due to cross-reactions of all serological tests with some other tospoviruses. Between two ELISA tests, ELISA Agdia showed higher specificity, while both ELISA, Agdia and BIOREBA, were similar in terms of diagnostic sensitivity. Regarding test applicable for on-site detection, ImmunoStrip Agdia was more sensitive, but less specific in comparison with AgriStrip BIOREBA. Molecular tests are more sensitive and specific compared to serological tests. Real time RT-PCRs showed the highest diagnostic sensitivity, while RT-PCR had the highest diagnostic specificity. All three RT-qPCR showed similar performances. Repeatability for all tests: > 92% Reproducibility for all tests: > 92%
tomato brown rugose fruit virus	5-8	14	151	9	5 (2 RT-PCR, 3 RT-qPCR)	All test included in this TPS are fit for purpose. Real time RT-PCRs showed the highest diagnostic sensitivity, while RT-PCR had the highest diagnostic specificity. All three RT real timePCR showed similar performances. Repeatability for all tests: ranging from 79 to 88% Reproducibility for all tests: ranging from 73 to 87%
plum pox virus (on-site testing)	1-4	5	35	4	3 (1 LFD RPA, 2 LFD)	Due to the influence of freeze-dried matrices, some tests needed to be adapted. The TPS results are the results of AgriStrip adapted test, ImmunoStrip® and AmplifyRP® Acceler8™ adapted test. In the tested conditions (use of freeze dried plant material and specific adaptations for some tests), all three tests showed good results of overall performance (concordant results rate and accuracy). All the three tests also showed a good ability to detect positive (non-diluted) or negative samples (diagnostic sensitivity and diagnostic specificity). Analytical sensitivity results showed a limit of detection which can be interpreted as rather high for both AgriStrip and ImmunoStrip®. It was lower for AmplifyRP® and this test may have detected even more diluted samples than the TPS could show.

						The TPS demonstrated the ability of the three tests to: - detect PPV in symptomatic leaves with high/medium viral loads - detect different isolates and strains of PPV - provide negative results for healthy Prunus - provide highly repeatable results (high confidence in one result per sample) - provide highly reproducible results (high confidence in results given by different operators) The likelihood positive ratios were very high for the three tests, leading to the conclusion of a high reliability of a positive test result. The likelihood negative ratios were also very high for the three tests, leading to the conclusion of a high reliability of a negative test result. Through all the results obtained for the three tests, it can be concluded that they are suitable for on-site testing.
Cryphonectria parasitica	3	3	44	3	3 (1 conventional PCR, 2 real time PCR)	The preliminary studies showed the suitability of the three molecular methods for the purpose of detection and identification. The highest specificity was obtained with the conventional PCR test for DNA samples, and the lowest with the real-time test by Rubio et al. (2017). On the other hand, higher specificity was obtained with real-time PCR test by Rubio et al. (2017) than with conventional PCR test for spiked wood samples. Real-time PCR test by Chandelier et al. (2019) showed higher sensitivity compared to real-time PCR by Rubio et al. (2017) when testing DNA samples resulting in less false negative results and earlier Ct ranges in the same samples.
Xylophilus ampelinus	5-8	10	68	10	9 (2 IF, 2 ELISA, 4 PCR, 1 real-time PCR)	The PCR-based tests (conventional and real-time) were observed to be more sensitive (analytical sensitivity) than the serological tests. All of the assessed performance characteristics were similar across the five PCR-based methods included in the TPS. Estimated diagnostic specificity was high but <1 for all PCR-based tests. However, the observed false positive results were sporadic and not suggestive of cross reactivity with either the non-target organism included in the sample panel (*X. campestris* pv. *viticola*) or healthy host plant (vine). The IF tests had lower analytical sensitivity than PCR and more false positive results were observed for IF than ELISA. All performance characteristics were similar for the two IF tests included in the TPS. ELISA had the lowest sensitivity (analytical sensitivity and diagnostic sensitivity) of the methods included in the TPS. The Plant Print Diagnostics ELISA was also observed to be less reproducible (lower concordance) than the Loewe ELISA, which is consistent with the lower analytical sensitivity observed for this test.

						It should be noted that fewer laboratories submitted results for the serological tests (4 laboratories) than the minimum number recommended in EPPO Standard 7/122 (10 laboratories). The results are therefore associated with a relatively high level of uncertainty and should be interpreted with caution.
Xanthomonas citri pv. *citri*	5-8	20	187	20	13 (6 conventional PCR, 3 real-time PCR, 1 LAMP and 3 direct molecular tests performed from Immunostrips or Whatman™ FTA cards)	The real-time PCR Robène *et al.* (2020) test showed a very high analytical sensitivity and a very good exclusivity (no detection of *Xca* (*X. citri* pv. *aurantifolii*) and *Xcb* (*X citri* pv *bilvae*) but also a risk of contamination (false positive results). A very good confidence can be placed in negative results obtained from this test. NB: The real-time PCR Cubero *et al*. (2005) test also showed a very high analytical sensitivity but there were some problems of exclusivity (detection of *Xcb* and non-systematic detection of *Xca*). The conventional PCR Robène *et al.* (2020) test was a bit less sensitive but showed a very high diagnostic specificity. A very good confidence can be placed in positive results obtained from this test. The conventional PCR Park *et al*. (2006) and Miyoshi *et al*. (1998) tests showed problems of exclusivity (detection of *Xcb*). The conventional PCR Loewe test based on Hartung *et al*. (1993) showed problems of inclusivity (strain Aw not detected) and problems of exclusivity (detection of *Xcb*). The conventional PCR Cubero *et al*. (2002) and Mavrodieva *et al*. (2004) tests, the real-time PCR Cubero *et al*. (2005) and Mavrodieva *et al*. (2004) tests and the LAMP PCR Rigano *et al*. (2010) showed problems of exclusivity (detection of *Xca* and *Xcb*). NB: in addition, for the conventional PCR Cubero *et al*. (2002) test, many laboratories reported the presence of many aspecific bands which could interfere with the interpretation of the results. NB: One laboratory reported difficulties in interpreting the LAMP PCR results (need of optimization for an easier interpretation). An interlaboratory effect was identified for quite all the tests around the detection level (most important for the real-time PCR Mavrodieva *et al*. (2004)). Considering the detection of both *Xcc* and *Xca* (target= both *Xcc* & *Xca*), the conventional PCR Mavrodieva *et al*. (2004) test showed the best performance, however, it also detected *Xcb*. For the direct molecular tests, the best results were obtained from Whatman™ FTA cards (vs. Immunostrips®). From Whatman samples, quite equivalent results were obtained between the tests: good diagnostic specificity, but low

						diagnostic sensitivity, due to the analytical sensitivity and difficulties in detecting the dilution 10^3 CFU/mL (1 to 2 decimal log lower than for DNA extract of the same sample analysed with the same molecular tools). However, field protocols can be a good alternative in particular for the combination Whatman™ FTA cards and the LAMP PCR Rigano *et al*. (2010) test. Considering the detection of *Xcc*, very good molecular tools are available with very high analytical specificity (inclusivity and exclusivity) and analytical sensitivity. The conventional PCR Robène *et al.* (2020) test is the best one concerning the analytical specificity, but a bit less sensitive than the real-time PCR Robène *et al.* (2020) test. The real-time PCR Robène *et al.* (2020) test is the best one concerning the analytical sensitivity, but with some risk of contaminations on non-target samples inducing a lower specificity than the conventional PCR Robène *et al.* (2020). Considering the detection of *Xca*, it can be noted a lack of performing molecular tools specific to *Xca.* Considering the direct molecular tests, Whatman™ FTA cards should be preferred (vs. Immunostrips®) in combination with the LAMP PCR Rigano *et al*. (2010) test for a tool 100% field.

*Disclaimer: the results only reflect the specific study case and reflects only the results on reagents at the time when they were included in the study

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APPENDIX

Criteria for selection of participants in TPS Round 2

General information	Descriptor	Target	Relative weight
Time schedule described in the invitation letter compatible with participant's availability and participant is committed to perform analysis and report on the results in the time frame defined	Yes/No	Yes	High
Authorized by the national competent authority to work with the specific pest (viable pest/ inactivated pest/ DNA/ RNA will be shipped)	Yes/No	Yes	High
Traceability in place / QA in place	Yes/No	Yes	High
Possibility to obtain an import document or Letter of Authority (EU countries) (only necessary when viable pests are sent)	Yes/No	Yes	High
Possibility to obtain an import document or Letter of Authority (EU countries) within 4 weeks to receive samples containing the specific pest (only necessary when viable pests are sent)	Yes/No	Yes	High
Technical expertise for the pests group (routine analyses, method developments, publications, participation in congress, etc.)	nb of years or validation data submitted to EPPO database or other publications	>1 year; advantage if validation data submitted/published	Medium (validation data: advantage)*
Previous participation in TPS or PT	Yes /No	Yes	Medium
Constraints for delivery?	Yes/No (if yes explanations)	No	Medium
Any problems or limitations with delivery on dry ice?	Yes/No (if yes explanations)	Preferably No	Medium
Ability/willing to perform all the methods described in the invitation letter (Note: it is necessary to perform ALL tests for the selected method)	Yes/No	Yes	Medium

critical criteria indicated in blue
important points indicated in green

Expertise	Descriptor	Target	Relative weight*
- ELISA	nb of years or nb of samples or validation data submitted to EPPO database or other publications	>1 year or > 30 samples; advantage if validation data submitted/published	Medium (validation data: advantage)
- (RT-)PCR	nb of years or nb of samples or validation data submitted to EPPO database or other publications	>1 year or > 30 samples; advantage if validation data submitted/published	Medium (validation data: advantage)
- Real-time (RT-)PCR	nb of years or nb of samples or validation data submitted to EPPO database or other publications	>1 year or > 30 samples; advantage if validation data submitted/published	Medium (validation data: advantage)

*Criteria can be differently weighted to allow the selection of the participants for the defined scope

Equipment	Descriptor	Target	Relative weight
UV-microscope	Yes/No	Yes with appropriate characteristics	High
Plate reader (company/model of instrument, wavelength of filters)	Yes/No	Yes with appropriate characteristics	High
Thermal cycler / gel electrophoresis system / gel imaging system (company/model of instrument)	Yes/No	Yes with appropriate characteristics	High
Thermal cycler (company/model of instrument)	Yes/No	Yes with appropriate characteristics	High
a) Channels available (FAM, VIC,...)	Wavelength filter	Appropriate wavelength filter	
b) For multiplexing (instrument with at least two channels)	Yes/No	Yes	