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DELIVERABLE N° 1.4

Title: TPS reports with description of the method, materials and software used, as well as the data analysis - Round 1

Version 1.0

Validation of diagnostic tests to support plant health

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Abstract:
The aim of the deliverable 1.4. is to present a summary of the results obtained in the Round 1 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, while the list of selected tests for each TPS is available and explained in deliverable D1.2. 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 following studies in the Round 2.

Partners involved: ANSES, FERA, NIB, NVWA

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TERMS, ABBREVIATIONS AND DEFINITIONS

CTV – citrus tristeza virus

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

PDA – Potato dextrose agar

PIC – Positive isolation control

PPV – Plum pox virus

RT-PCR – Reverse transcription polymerase chain reaction

SNA – Synthetic nutrient-poor agar

TPS – Test performance study

1 Purpose of TPS activity

The purpose of this deliverable is to present summary of the results of the six TPSs organized in Round 1 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, and description of the work and results of preliminary studies.

2 Scope of TPS round 1

This document contains the main outcomes supported by the TPS results on six pests, selected based on consortium expertise: *Erwinia amylovora*, *Pantoea stewartii* subsp. *stewartii*, citrus tristeza virus, plum pox virus, *Fusarium circinatum* and *Bursaphelenchus xylophilus*. For each of the six TPSs, the methodology used to perform the tests (scope of TPS, common rules and criteria for the selection of test 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. 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 a supplementary documents to this deliverable and they can be shared upon the explicate request from the interested parties. TPS reports are protected by the confidentiality agreement.

3 Methodology for implementation of TPS

Tests for each TPS were selected 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, while the list of selected tests for each TPS is available and explained in deliverable D1.2. 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. Methodology used in each TPS to perform the tests and to analyse the results from each of six TPSs is described in corresponding TPS reports (supplementary information).

4 Explanation and recommendation for test selection, including selection of commercial kits

This part was prepared in collaboration with WP7. The process of tests selection for TPS was described and explained in more details to make it as transparent as possible. Reasons leading to the decisions on how selection process was done in TPS Round 1 are listed and explained in details below. In the frame of the VALITEST project a list of methods and tests for validation for test performance study (TPS) Round 1 was prepared for 6 selected pests (*Erwinia amylovora*, *Pantoea stewartii* subsp. *stewartii*, citrus tristeza virus, plum pox virus, *Fusarium circinatum* and *Bursaphelenchus xylophilus*), which is described in more details in Deliverable D1.2 (List of tests for validation – Round 1). The view presented in this document is the opinion of TPS Round 1 organizers.

The aim of the project is to evaluate the performances of different tests, including commercial kits in the detection of plant pests. Usually, several tests (encompassing different methods and sometimes commercial kits) are available for the detection of a specific pest. In these cases, the tests are often described with variations, in various literature sources, databases and diagnostic protocols (e.g. EPPO and IPPC) and different commercial kits may also be available on the market. Data on the commercial kits' comparative performance or significance of variations are often limited or not freely available. To alleviate this in the VALITEST project, and in addition to the experience of the partners, companies were contacted with a request for information (performance or additional data) on the relevant tests they offer. One of the important conclusions of this study is that continued discussions between TPS organizers and commercial kit providers will allow better operation of reference laboratories, and especially the EU reference laboratories, both in organizing proficiency testing and TPS studies from one side, and provide better understanding of the specific needs of the diagnostic laboratories for companies, on the other side.

Considering the limited resources and the time constraints of the project, only a limited number of tests could be included. The tests have been compared with all their variations for some pests. It is worth keeping in mind that variations of operating procedures are introduced and consequently described for various reasons. While this is not an exhaustive list some of the most common reasons for variations are: (i) adaptation of sample preparation including the extraction buffer to a particular matrix, (ii) replacement of reagents specified in the original source but no longer available, (iii) use of different instruments, and (iv) optimization of processes (e.g. minimizing

the number of different buffers, polymerases, instruments, DNA extraction kits in a laboratory). An example of a flow of changes for DNA extraction and real-time PCR is schematically represented in Figure 1. Some of these variations are not considered critical and may only require verification (revalidation) of the adapted test while others require a more comprehensive assessment and need to undergo through extensive validation process; this is evaluated case-by-case by the laboratory introducing changes (see e.g. EPPO guidelines PM 7/98). Compared to a simplified representation in Figure 1, each of those steps has many more sources of variations (Figure 2) leading to a potentially, and often practically, very high number of possible combinations requiring to be evaluated to different extent. Taking as example case when sample preparation procedure is followed by extraction of nucleic acids (DNA/RNA) and a molecular test. At each stage several decisions need to be taken e.g. selecting suitable buffers (of which many may be suitable), approach to sample preparation (commuting/macerating), DNA procedure,... Even when testing is considered harmonized, the number of possible combinations increases exponentially with each stage. In this particular case 3 possible selections at three stages, resulting in 27 combinations, which may need to be experimentally tested and compared for one particular matrix. Because in most cases this is not feasible and often does not lead to a conclusive result ('clearly and absolutely the best combination' for each and all samples of the same matrix), limited number of combinations are considered for validations and/or test performance studies based on previous experience and available information.

Parameter	Original publication	User 1	User 2	User 3
DNA extraction	**A (CTAB)**	**A**	**B**	**C**
Real-time PCR instrument	**A (SmartCycler, Cepheid)**	**B**	**A**	**B**
Master mix	**A (own recipe)**	**B**	**C**	**D**
Comments		User 1 decided to use the same DNA extraction but has a different real-time PCR instrument and used a different master mix because the one described in the original publication is no longer available.	User 2 decided to change the DNA extraction to the one typically used in their laboratory (e.g. automatic one to improve standardization and lower hands-on time) but has the same real-time PCR instrument. However, the master mix described in the original publication is no longer available. Therefore, user 2 used a different master mix.	User 3 decided to change the DNA extraction to the one commonly used in their lab, has the same real-time PCR instrument as user 1 but is using another master mix.
		verification	**validation**	**validation**

Figure 1: Example of common modifications to the originally published protocol (test) of DNA extractions and real-time PCR.

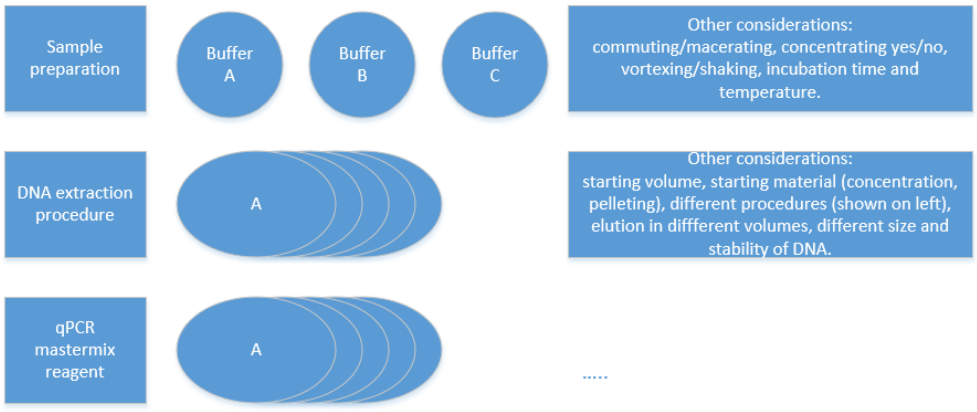

Figure 2: Schematic representation of a typical workflow in testing with molecular tests.

The validation and TPSs within the VALITEST project were organized in accordance with project's budget and time constraints and therefore in many cases it was not possible to include all available methods and tests/kits, in particular where the number of tests available was very high. When limitations existed for the inclusion of commercial kits, the commercial kits from the VALITEST consortium companies have been preferred in case of similar results or validation data. This way more direct communication within the research project for exchange of relevant information regarding the kits was made possible. The approach for the selection of tests is further described below.

4.1 General arguments for selecting /not selecting specific tests for TPS Round 1

The different arguments for not selecting a test for preliminary studies are described below:

1) absence of critical information (e.g. performance) limiting the inclusion of the test into the TPS validation procedure.
2) Kits versus publication: when a commercial kit based on a publication was available on the market, it was not always possible to include it instead/before of the test from publication the kit was based on. The reasoning for the selection was based on the lack of validation data available for commercial test compared to the (same) test from publication. In addition, different quality assurance practices required by the national accreditation bodies applied in different laboratories can ensure the applicability/reliability of the particular test (e.g. *in silico* analysis of amplicons done by sequencing and/or sequence alignment is required to ensure the test is applicable on the range of known isolates/strains of particular pest). Therefore, in some cases TPS organizers preferred to select a test based on a publication, where more information on validation data was provided. For inclusion of the test from commercial kits in the TPS in the framework of this project more transparent information on their validation data and performance is required. This should establish a common policy for test selection in the future.
3) Offer of kits for specific methods and pests too large: In the case of many different kits being available on the market it is extremely difficult to validate them in their original description, e.g. with all the different chemicals and protocols because of limited budget, workload given in the project, limited staff capacity and also the time constraints (dedicated period for TPS was set, which cannot be extended; see also Figure 2). In addition, the project proposal did not include the funding of external laboratories for TPS participation, consequently it was difficult to expect participants, which are not partners in the consortium, to spend additional resources testing all of the different combinations (this is not feasible in routine diagnostics). The extent of the TPS should be reasonable / feasible / affordable for all participants, even those not funded. In TPS Round 1 preselection was based on the expertise of TPS organizers, previous experience, and assessment of feasibility. In TPS Round 2, preselection will preferably also include input from companies providing commercial kits, especially with regards to modifications.
4) Lack of expertise for specific method and specific pest: a TPS organizer, who lacks expertise for one method for a selected pest cannot reliably prepare the material and cannot make the extensive preliminary validation study, which would be needed. Therefore, methods, for which the TPS organizer does not have enough experience for a selected pest, are not included in the TPS. Additionally, in some cases it is difficult to prepare enough homogeneous material for a specific method and pest combination.
5) Lack of offer for kits in specific cases: in some cases, there are no commercial kits available on the European market (e.g. LAMP for *Pantoea stewartii* subsp. *stewartii*) or TPS organizer is not aware of its availability after performing the literature and internet research, while also directly contacting companies producing kits for plant disease detection.

4.2 Suggestions for test selection for TPS Round 2

The different explanations described above (see point 1. General arguments for selecting /not selecting specific tests for TPS Round 1) fully justify having extensive discussions with kits producers / providers to define the extent of the validation and validation conditions for the second round of TPS.

The experience from round 1 of the TPSs highlights the importance of being as transparent as possible with regards to composition of the kits and data and validation data when available. In order to further harmonize the approach to test selection, further discussions between TPSs Round 2 organizers and companies contributing potential tests is expected in order for those test to be included in the second round of the TPS. The outcomes of those discussions could contribute to better understanding between the needs of the diagnostics laboratories and commercial kit providers, and could grant tighter connections between kit producers and end users. In TPS Round 1 some difficulties have been encountered when many kits were available. The companies are encouraged to provide advice on a common protocol (e.g. common buffer for several ELISA tests) to allow for more tests to be validated within the budgetary and time constraints. Based on the feedback from TPS Round 1, the companies strongly suggested to use their kits without any modifications. In practice this means that fewer kits can be evaluated with the resources allocated in the project.

5 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 the diagnostic expertise. The TPS organizer will deposit the validation data in the database for all test included in the particular TPS. Also, TPS organizers will be able to deposit the data obtained in preliminary studies in the EPPO validation database.

5.1 Short reports for test selection for each TPS in Round 1

The selection of the test for the TPS in Round 1 was described in details in the Deliverable D1.2. However, after the deliverable was finalized and during the TPS period some organizers decided to change the selection of test based on the new information they obtained (e.g. new tests were included in the TPS or in-house validation, or some test were excluded). For that reason, in order to provide the final data on the results of test selection process the short reports for the test selection process are given in for all six pest included in TPS R1.

5.1.1 Erwinia amylovora

- For immunofluorescence, there were different commercial kits available at the time of test selection for TPS Round 1: from Loewe, Plant Print, Prime Diagnostics and Sediag. However, immunofluorescence was not selected because priority was given to the on-site serological tests.

- For ELISA there were also a few commercial kits available at the time: from Sediag, Loewe and Plant Print. However, again ELISA was not selected due to lack of experience for this test of the TPS

organizer. In addition, only 4 out of 27 laboratories answering EPPO survey use ELISA test for *Erwinia amylovora* detection in the laboratory.

- For conventional PCR there were two commercial kits available at the time: from AgroDiagnostica and Loewe. However, there were no validation data available, which did not give the TPS organizer a base for test selection.

- For real-time PCR there were also two commercial kits available at the time: from AgroDiagnostica and Qualiplante in collaboration with IpadLab. However, there were no validation data available for the kit from AgroDiagnostica, again not giving the TPS organizer a base for test selection. In case of kit from Qualiplante, which was developed in collaboration with IpadLab, there were no direct validation data available. The kit producers informed the TPS organizer that the kit is based on the test developed and described by Pirc et al. (2009), for which validation data is available. Therefore, the TPS organizer decided to choose the original test from the publication (Pirc et al., 2009) for the TPS to avoid any uncertainties in case of deviations of the commercial kit from the original test. Additionally, real-time PCR test by Gottsberger (2010), which showed similar performance as the tests by Pirc et al. (2009), was also selected for TPS. On the other hand, real-time PCR described by Salm & Geider (2004), which is targeting a plasmid, which is not present in all *Erwinia amylovora* strains, was not included in the TPS.

- For LAMP, there were two commercial kits available at the time: from Enbiotech and Optigene. However, there was no validation data available for the kit from Enbiotech. In the case of the kit from Optigene, which is based on the test by Bühlmann et al. (2013), the TPS organizer decided to choose the original test from publication for preliminary validation study prior the TPS. Additionally, LAMP tests by Moradi et al. (2012) and by Shin et al. (2018) were also included in the validation. Finally, only the test by Shin et al. (2018) was selected for TPS based on the ease of interpretation of the results and other performance characteristics.

- For LFD there were also two commercial kits available at the time: from Bioreba and Pocket Diagnostics. The two LFD tests showed comparable performance in preliminary validation study prior TPS; therefore both were included in the TPS.

For *E. amylovora*, the tests selected for validation based on the scope of TPS and weighted criteria are listed in Table 1.

Table 1: Tests selected for preliminary study for *E. amylovora* with references. Tests selected for TPS are underlined.

Method	**Tests for validation:**
Laboratory methods	**Molecular (real-time PCR):** Pirc *et al.*, 2009 (amplicons ITS and AmsC) Gottsberger, 2010 Salm and Geider, 2004
On-site methods	**Molecular (LAMP and other):** Bühlmann *et al.*, 2013 Moradi *et al.*, 2012 Shin *et al.*, 2018 **Serological (LFD):** AgriStrip (Bioreba) Pocket Diagnostic

5.1.2 Pantoea stewartii subsp. stewartii

- For immunofluorescence, there were some commercial kits available at the time of test selection for TPS Round 1: from Linaris and Loewe. However, immunofluorescence was not selected because it is generally a less suitable method for detection of bacteria in seeds (lots of background in samples, frequent cross-reactions).

- For ELISA there were also some commercial kits available at the time: from Agdia and Loewe. However, again ELISA was not selected due to lack of experience for this pest by the TPS organizer. No laboratory answering the EPPO survey reported use of ELISA for the diagnosis.

- For conventional PCR there were two commercial kits available at the time: from AgroDiagnostica and Loewe. However, there was no validation data available, which did not give the TPS organizer a base for test selection. On the other hand, after performing preliminary validation study prior TPS the TPS organizer found PCR tests by AGES (detecting both *P. stewartii* subsp. *stewartii* and *P. stewartii* subsp. *indologens*) and by Gehring et al. (2014; *galE* locus; detecting *P. stewartii* subsp. stewartii only) to be fit for purpose and included them in the TPS.

- For real-time PCR there was one commercial kit available at the time: from AgroDiagnostica, however without validation data. On the other hand, the TPS organizer found real-time PCR tests described by Tambong et al. (2008), by Thwaites (previously included in the validation within an Euphresco project), by Wensing et al. (2010) and by Pal et al. (2019) to be fit for purpose and included them in the TPS. However, only test by Pal et al. (2019) has the ability to distinguish *P. stewartii* subsp. *stewartii* from *P. stewartii* subsp. *indologenes*, an important performance characteristic in diagnostics of Stewart's wilt.

- For LAMP, there were no known commercial kits at the time. There were only two LAMP tests described by Uematsu et al. (2015) and although both tests showed overall satisfactory performance, they are less sensitive than the tested real-time PCR and PCR tests. Therefore, they are less suitable for detection of *P. stewartii* subsp. *stewartii* in maize seeds in which we can expect low concentrations of the pest and were therefore not selected for the TPS.

For *P. stewartii* subsp. *stewartii,* the tests selected for validation based on the scope of TPS and weighted criteria are listed in Table 2.

Table 2: Tests selected for preliminary study for *P. stewartii* subsp. *stewartii* with references. Tests selected for TPS are underlined.

Method	**Tests for validation:**
Real-time PCR	Tambong *et al.*, 2008 Thwaites *et al.* (FERA protocol, EUPH05 *Pantoea stewartii* subsp. *stewartii* Final Report) Wensing *et al.*, 2010 Pal *et al.*, 2019
Conventional PCR	AGES, 2016 (EPPO Bulletin 46 (2): 226-236) Gehring *et al.*, 2014
On-site methods	**Molecular (LAMP):** Uematsu *et al.*, 2015

5.1.3 Citrus tristeza virus

For conventional RT-PCR, the selection for inclusion in the TPS was based on the best performing kit or method. Currently, Loewe is the only company to offer a ready-to-use kit that seemed promising. Therefore, it was important to include it in the preliminary study to characterize its performance. However, the evaluation of the kit from the preliminary study gave insufficient

results. Therefore, the Loewe kit was not selected. Other conventional RT-PCR were also evaluated and also gave insufficient results: PCR Roy et al. 2005; PCR Nolasco et al. 2002 and PCR Rubio et al. 2001.Alternatively, there is a conventional RT-PCR test developed by Olmos et al. (1999). It has been included in the preliminary study despite the available validation data from different studies shows that this PCR test does not perform as well as existing real-time PCR tests. However, this molecular test is referred to in the IPPC protocol and is used by two EU laboratories answering the preliminary EPPO survey prior to the TPS organization. Moreover, validation data after preliminary study gave more satisfactory results.

- For real-time RT-PCR, the current offer covers kits from Qualiplante and Ipadlab, but these kits are based on the same reagents and protocol (based on Bertolini et al. 2008) as it has been co-developed by the two companies. Consequently, one of the two commercial kits was included in the preliminary study to characterize its performance, which was sufficient for inclusion in the TPS. Additionally two published tests were evaluated. But due to limitation of tests to be included in the TPS and as the tests and kit gave equivalent results, only the test from Saponari et al. (2008) and the kit from Qualiplante/Ipadlab were retained in the TPS. The test from Bertolini et al. (2008) was excluded, but its primers developed are included in the Qualiplante/Ipadlab kit, and consequently are evaluated.

- For LAMP: this technology is promising, especially for on-site testing, and end-users are demanding to get more information and evaluation of such technology for CTV. Consequently, it was decided to select a maximum of 2 LAMP tests for the TPS. The only commercial offer known at that time, was the one from Optigene, but their kit was not ready in time to be included in the TPS. Therefore, the Optigene kit has not been selected. However, the TPS organizer will evaluate it alone (not interlaboratory evaluation) during the TPS period. Finally, the TPS includes the test based on Wang et al. (2013), which gave satisfactory results in preliminary study.

- For Immunostrip, the offer is limited to the Agdia on-site kit. Even if validation data was not available, this kit was included in the TPS, as it offers an on-site approach.

- For ELISA, different kits are available on the market from several companies: Agdia, Agritest, Bioreba, DSMZ, Loewe, Plantprint and Sediag. The kits from these companies were pre-selected for the preliminary study. From the 7 kits evaluated, kits from Agritest, DSMZ and Loewe gave the least satisfactory results during the preliminary study. Nevertheless, the difference in performance for Loewe and DSMZ compared to the kits of other companies (except Agritest) was not significant. The selection of the kits was first based on their performance with the limit of a maximum of 5 ELISA tests (for the benefit of the statistical analysis). The final list of kits includes kits from Agdia, Bioreba, Plantprint, Sediag and Loewe.

- For Tissue Print Immunoassay (TPIA), the PlantPrint test has been selected as it is referred to in numerous publications and is used as a cheap screening test as reported by different EU laboratories in the preliminary EPPO survey.

For citrus tristeza virus, the tests selected for validation based on the scope of TPS and weighted criteria are listed in Table 3.

Table 3: Tests selected for preliminary study for citrus tristeza virus with references. Tests selected for TPS are underlined.

Method	Tests for validation:
ELISA	Agdia Agritest Bioreba DSMZ Loewe Plant Print Diagnostics S. L. Sediag
Real-time RT-PCR	Bertolini *et al.*, 2008 Saponari *et al.*, 2008 Qualiplante/Ipadlab
Conventional RT-PCR	Loewe Olmos *et al.*, 1999 Roy *et al.*, 2005 Nolasco *et al.*, 2002 Rubio *et al.*, 2001
On-site methods	**Molecular (LAMP):** Optigene Wang *et al.*, 2013 **Serological (Immunostrip):** Flashkit (Agdia)
Other methods	**Tissue Print Immunoassay (TPIA)** Plant Print Diagnostics S. L.

5.1.4 Bursaphelenchus xylophilus

The literature review conducted underlined the numerous molecular tests available, using conventional PCR technology or real-time PCR. 23 publications were identified including 14 mentioning a conventional PCR test, 5 real-time PCR methods including a commercial kit and 4 LAMP test.
Initial selection of tests was performed based both on tests reported in EPPO survey results and on the availability of validation data from literature sources and experience from TPS organizers. Available results of interlaboratory studies or intralaboratory validation data were also used to make a selection among the tests available.

- For PCR (conventional and real time) and considering the large offer of tests in literature, only the tests that could fit with the TPS scope defined, were selected for preliminary studies.

The PCR RFLP test described by Burgermeister et al. (2009) is widely used in the EU region and considered in a way as a gold standard. It justifies that this test is included in this TPS.

- For real-time PCR, only one commercial kit (Clear Detections) for the detection of B. xylophilus is available on the market, which implies automatically that the test was included in the TPS. Nevertheless, it has been evaluated in preliminary study to gain experience and if necessary to provide specific advice to TPS participants.

Finally, as on-site testing may be crucial for early detection, tests based on LAMP technology were retained in order to document more completely their performance, as it is not done yet.

For *B. xylophilus*, the tests selected for validation based on the scope of TPS and weighted criteria are listed in Table 4.

Table 4: Tests selected for preliminary study for *B. xylophilus* with references. Tests selected for TPS are underlined.

Method	**Tests for validation:**
Real-time PCR	Real-Time PCR Nematode diagnostic kits Clear®Detections Francois *et al.*, 2007
Conventional PCR	Matsunaga and Togashi, 2004 Burgermeister *et al.*, 2009
On-site methods	**Molecular (LAMP):** Kikuchi *et al.*, 2009 Meng, *et al*., 2018

5.1.5 Plum pox virus

For the diagnostic tests for plum pox virus (PPV), the initial viewpoint was to generate validation data for tests included in the PPV EPPO standard. Also, tests that were considered to be included in the update of the EPPO standard were reviewed. As a result, commercial, molecular based kits were initially out of scope. Following discussions with several project partners, and to be more in line with the overall project aims, a commercial real-time RT PCR kit was included.

- For ELISA, different commercial kits are available on the market. Antisera from Agdia, Agritest, Bioreba, Loewe, PlantPrint, Primediagnostics and SEDIAG were included in the preliminary study. Coating and conjugate antibody dilutions were tested following the manufacturers' instructions. Coating and antigen incubation were performed overnight at 4 °C, whereas the conjugate was incubated for 3 hours at 37 °C following the recommendation in EPPO PM7/125 (1) ELISA tests for viruses. A sample set containing EA, CR, An, C, and M-Rec strains of the virus, non-infected plant material and blanks was tested in several dilutions to determine the sensitivity, specificity and selectivity of the tests included. Under these test conditions, the Agdia, Bioreba and SEDIAG kits outperformed the others. These kits were included in the test performance study, which had to be tested using the manufacturers' protocols.

- For the molecular tests, two conventional RT-PCR tests were tested following EPPO PM7/32, i.e. Wetzel et al. (1991) and Levy and Hadidi (1994). Three real-time RT PCRs for PVV detection were tested, namely Schneider et al. (2004), Olmos et al. (2005), and a test that was considered for inclusion in the PPV EPPO standard: Naktuinbouw et al. (unpublished). Based on the preliminary study, all tests were included in the TPS. In collaboration with relevant project partners, it was decided to include at least a one commercial molecular test which was based on published primers which had already been included in the preliminary study. It was decided that the commercial kit for PPV detection based on the Olmos protocol as provided by Qualiplante was included in the TPS.

For plum pox virus, the tests selected for validation based on the scope of TPS and weighted criteria are listed in Table 5.

Table 5: Tests selected for preliminary study for plum pox virus with references. Tests selected for TPS are underlined.

Method	Tests for validation:
Conventional RT-PCR	Qualiplante (2X) Wetzel *et al.*, 1991 Levy and Hadidi, 1994
Real-time RT-PCR	Qualiplante (3X) Schneider *et al.*, 2004 Olmos *et al.*, 2005, as a commercial kit from Qualiplante cat. no. qPCR-PPV-100Liq Mavrič Pleško *et al.*, 2011 Anonymous, 2018 (Naktuinbouw protocol)
DAS-ELISA	Agdia Agritest Bioreba Loewe Plant Print Diagnostics S. L. Prime Diagnostics SEDIAG

5.1.6 Fusarium circinatum

At the time of test selection for TPS Round 1 there were no commercial kits available for *Fusarium circinatum*.

For *F. circinatum*, the tests selected for validation based on the scope of TPS and weighted criteria are listed in Table 6.

Table 6: Tests selected for preliminary study for *F. circinatum* with references. Tests selected for TPS are underlined.

Method	Tests for validation:
Plating	PDA and SNA media for morphological identification of cultures (EPPO 7/91)
Conventional PCR	Ramsfield *et al.*, 2008 Schweigkofler *et al.*, 2004
Real-time PCR	Ioos *et al.*, 2009 Schweigkofler *et al.*, 2004 Lamarche *et al.*, 2015 Luchi *et al.*, 2018

6 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 will and was already shown to be useful in organization of TPSs in Round 2. The organization would be easier if timelines, rules and criteria which needed to be followed were defined earlier. This requires more preparation work, however, it pays off when the TPS is running. It is worthwhile 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 does not affect the running of the TPS to such an extent. 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 4). 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 4: Number of planned, identified from the literature search/systematic search, included in preliminary studies and finally selected tests for TPS Round 1

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
Erwinia amylovora	2-3	46	51	9	6 (real-time PCR, LFDs and LAMP)	All molecular tests (real-time PCR and LAMP) were able to detect *E. amylovora* high accuracy and good detection, and in concentrations expected in symptomatic plant material with characteristic symptoms. Plant matrix affected performance of all molecular test, but to different extend. Two LFD tests showed equivalent performance in preliminary study, however unexpectedly one of the tests, for currently unknown reason, performed significantly worse in the TPS. Therefore, procedure for test item preparation would need to be reviewed before the results of the TPS for serological panel for both tests can be taken into account.
Pantoea stewartii subsp. *stewartii*	3-5	30	113	8	6 (real-time PCR, conventional PCR)	Molecular tests were able to detect *P. stewartii* with high accuracy and good detection. Real-time PCR tests and the conventional PCR AGES show similar diagnostic sensitivity and specificity and all allow good detection of the target. The conventional PCR based on Gehring *et al.* (2014), as described in TPS, had lower diagnostic sensitivity than other tests, however enables differentiation of subsp. *stewartii* and *indologenes*. The real-time PCR test based on Pal *et al.* (2019) showed good performance with the best diagnostic odds ratio of all tests evaluated and is also able to differentiate between the subsp. *stewartii* and *indologenes*.
Citrus tristeza virus	5-7	22	90	16	11 (ELISA, TPIA, conventional RT-PCR, real-time RT-PCR, RT-LAMP and ImmunoStrip) from which 9 performed by each participant (3 out of 5 ELISA tests performed, different for each participant)	The real-time RT-PCR Ipadlab (Bertolini *et al.* (2008) and the conventional RT-PCR Olmos (as well as the real-time RT-PCR Saponari *et al.* (2008) for laboratories largely experienced with it) present a very high analytical sensitivity, but also a risk of contamination (false positive results). A good confidence can be placed in negative results obtained from these tests. The RT-LAMP PCR Wang *et al.* (2013) and the ELISA tests are less sensitive, but present a high diagnostic specificity, appropriate to detect higher contaminated samples. A good confidence can be placed in positive results obtained from these tests. A strong interlaboratory effect was identified for the real-time RT-PCR Saponari and to a lesser extent, for the RT-LAMP PCR Wang. For the real-time RT-PCR Saponari *et al.* (2008), these differences between laboratories could not be related to specific consumables or equipment used by each group of laboratories. It should rather be linked to the robustness of

						the test and the conditions of implementation of the test. For the RT-LAMP PCR Wang, difference occurred among laboratories at the limit of detection of the test. Concerning the tissue-print tests, quite equivalent results were obtained for TPIA and tissue-print real-time RT-PCR Ipadlab: even if not assessed in the same conditions as other methods, and considering that their results are hardly comparable, these "on-site" protocols can present a good alternative.
Plum pox virus	4-8	22	82	18	8 (conventional RT-PCR, real-time RT-PCR, DAS-ELISA) from which 6 performed by each participant (1 out of 3 ELISA tests performed, different for each participant)	Real-time and conventional PCR tests show similar results and are all fit for purpose. With the exception of one molecular test (Schneider *et al.* 2004; diagnostic sensitivity 98.6%) all molecular tests have a diagnostic sensitivity (SE) of 100%. The diagnostic specificity (SP) varied between 83% and 94%. Serological tests are less sensitive but are still able to detect high virus titers. The SP for DAS-ELISAs varied between 88% and 97%. The SE varied among ELISA tests and was from 48% to 86%.
Fusarium circinatum	3	7	141	7	6 (plating, conventional PCR, real-time PCR)	The plating method had the lowest analytical sensitivity. Lamarche *et al*., (2015), real-time PCR has greatest analytical sensitivity. Luchi *et al*., (2018) real-time PCR and plating had lowest analytical specificity. Lamarche *et al*., (2015), real-time PCR has greatest concordance.
Bursaphelenchus xylophilus	2-5	23	66	6	5 (conventional PCR, real-time PCR, LAMP)	The two conventional PCR tests (Matsunaga and Togashi, 2004 Burgermeister *et al.,* 2009) can be used to identify *Bursaphelenchus xylophilus* (100% of diagnostic specificity), the diagnostic sensitivity of the two tests being substantially equivalent (respectively 92.5% and 97.5%). The real-time PCR tests or the LAMP test were able to detect *Bursaphelenchus xylophilus* with high accuracy. Real-time PCR test François et al. and LAMP test Kikuchi et al. (2009) offer slightly better performance with 100% for diagnostic sensitivity and specificity versus 98.5% for commercial kit. We can note a greater ease of interpretation with the LAMP test.

6.1 Recommendations to companies in test selection process

TPS organizers and also diagnostic laboratories (especially official ones) need as much information as possible to select the test for validation and further use:

- Validation data,
-The information about the target gene or amplicon for molecular kits or relevant information for serological methods,
- Information about the isolates/populations on which the test was evaluated by the kit producer (diagnostic laboratories need to know which isolates are covered by commercial kits),
- Information about the mastermixes, amplification programs (molecular methods) and buffers (DNA/RNA extraction, ELISA) with which the kits were already evaluated → for TPS, if possible, the use of common reagents (for example extraction buffer for ELISA) is recommended when comparing tests (otherwise impossible to prepare the TPS and not feasible in routine diagnostics). This would mean that a maximum number of tests could be compared, according to available resources and testing material.

Sometimes, there are specific conditions (special enzymes included) which cannot be changed for a specific test, while sometimes harmonization is possible. An ideal situation would be that, in the future, harmonization could be achieved between kits producers for the use of different chemicals (e.g. buffers and mastermixes) and protocols (e.g. incubation temperatures and amplification programs) or to give information on all alternative chemicals and protocols which could be used instead of those described in the original protocol (It is not realistic to expect that the TPS organizer or even diagnostic laboratories during validation process will use many different buffers and many different polymerases for testing of one sample). Currently there is no solution and it is important to continue the discussions. This is a very important question to be addressed to allow better operation of the EU reference laboratories (Proficiency testing and TPS studies).

Also another important point is that suppliers should give the rules for the interpretation of the results using their kits (for example cut-off value).

6.2 Reference documents

See under Deliverable D1.2 (List of tests for validation – Round 1).

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