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Validation of diagnostic tests to support plant health

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Abstract:
We prepared a list of methods and tests for validation in test performance study (TPS) Round 1, both for laboratory and on-site use, for 6 selected pests: *Erwinia amylovora*, *Pantoea stewartii* subsp. *stewartii*, citrus tristeza virus, plum pox virus, *Fusarium circinatum* and *Bursaphelenchus xylophilus*. 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 prepared in D1.1.

Partners involved Task NIB, NVWA, FERA, ANSES

HISTORY OF CHANGES		
Version	Publication date	Change
1.0	04 March 2019	Initial version
2.0	11 February 2020	Following the review of the project, harmonization of the vocabulary between “in-house”, “prevalidation” and “preliminary study”. Consistency of the use of the term “preliminary study”. Clarification of the definition of “test”.

Contents

1 Purpose

The purpose of this deliverable is to select tests for validation in the frame of WP1 of VALITEST project, in which the aim is to coordinate (prepare and organize) tests validations and running of 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 in EPPO Standard PM 7/76 as the application of a method to a specific pest and a specific matrix. TPS Round 1 (in year 1 and 2) is focused on six preselected pests (*Bursaphelenchus xylophilus*, *Erwinia amylovora*, *Pantoea stewartii* subsp. *stewartii*, citrus tristeza virus, plum pox virus and *Fusarium circinatum*) for which the selected tests are listed in this deliverable. Tests for each of six preselected pests are selected using the criteria from VALITEST Deliverable D1.1.

2 Scope

The tests listed in this deliverable will be validated in TPS in Round 1 (in year 1 and 2). In addition, during the first year of the project, analysis in the frame of WP4 of VALITEST project will conclude with the selection of further pests where test validation is a priority and which will be the focus of TPS in Round 2 (in year 2 and 3) and the process to select the tests for validation, described in this deliverable, will be used as a guidance to select tests for validation in TPS in Round 2. Furthermore, this deliverable is applicable to any TPS organization and could help new EU reference laboratories (in the field of plant health).

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4 Terms, abbreviations and definitions

LAMP - Loop-mediated isothermal amplification

LFD - Lateral flow device

5 Methodology

Tests for validation were chosen based on data from different literature sources and laboratory experience of TPS organizers, who have used some of the tests already and have some validation data from previous preliminary studies. First, we prepared a list of different diagnostic tests, both for laboratory and on-site use, for 6 selected pests: *Erwinia amylovora*, *Pantoea stewartii* subsp. *stewartii*, citrus tristeza virus (CTV), plum pox virus (PPV), *Fusarium circinatum* and *Bursaphelenchus xylophilus*. Then we collected 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 these 6 pests from different sources: from literature (research articles), validation data in EPPO database, EUPHRESCO final reports, EPPO questionnaire for experts, through internet search, email sent to all commercial kits providers known by the partners. We have noticed that in many cases the data are not comparable, because results are presented in different ways (different sample types, units, volumes) and sometimes crucial information (for example about sample preparation and concentration step used) is missing in the research articles or reports. In these cases, the professional experience of TPS organizers proofed invaluable in judging the reported results and other information to be able to select the tests for TPS. It is important also to evaluate the reported information about the number of target and non-target isolates, controls performance, comparison between different available tests and, data of validations. Based on data collected and definition of the scope of diagnosis (see D.1.1.) we prepared a narrow list of pre-selected tests for validation for TPS Round 1, which were subjected to validation in preliminary studies conducted in-house where the TPS organizers checked the performance of the tests to be included in the TPS Round 1. After assessing the results of preliminary studies against weighted criteria (described in Deliverable D1.1) final list of tests was selected for TPS Round 1.

6 Preliminary studies and selection of tests for TPS – Round 1

6.1 Erwinia amylovora

Erwinia amylovora is the causal agent of fire blight. Detection of the pest normally based on plant material with fire blight symptoms. Symptomatic tissue contains relatively high concentration of the causative bacteria, therefore high sensitivity of the detection methods is not a crucial factor for its implementation in the diagnostic procedure. We gained an insight on the diagnostic tests available for *E. amylovora* through thorough literature search. In the first step, we were focused on published laboratory tests with high analytical specificity and at least partial validation data. Majority of the available *E. amylovora* real-time PCR tests include full or partial validation data or TPS data, which form a solid base for suitable assays selection. For the preliminary study we selected 5 different real-time PCR assays (Table 1). All the tests were very sensitive and highly specific, as no cross reactions or false negative identifications were reported. There are available validation data for plant matrixes of interest for all the chosen tests, and no problems regarding testing of plant samples were reported.

We chose two different types of on-site methods, Loop-mediated isothermal amplification (LAMP) and lateral flow devices (LFD) as best suitable representative of molecular and serological methods, respectively. Three different LAMP assays were selected from the literature data for the preliminary study. Assays reported in Moradi *et al.* 2012 and Shin *et al.* 2018 include partial validation on plant matrix of interest and therefore provide valuable information for initial assessment of test performance. Furthermore, the reported analytical sensitivity and analytical specificity of the assays meet the criteria for the tests selection as defined in D1.1 (medium concentration for analytical sensitivity and medium level for analytical specificity). In our preliminary study we included two LFD tests, for which the validation information is available in publications and/or on the EPPO website.

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

Method	Tests for validation:
Laboratory methods	**Molecular (real-time PCR):** Pirc *et al.*, 2009 (Plant Pathology 58, 872–881) (two amplicons, AmsC and ITS) Gottsberger, 2010 (Letters in Applied Microbiology 51, 285–292) Salm and Geider, 2004 (Plant Pathology 53, 602–610)
On-site methods	**Molecular (LAMP and other):** Bühlmann *et al.*, 2013 (Journal of Microbiological Methods, 92:332-339) Moradi *et al.*, 2012 (Eur J Plant Path, 133 (3): 609-620) Shin *et al.*, 2018 (Plant Pathology J, 34 (3): 191-198) **Serological (LFD):** AgriStrip (Bioreba) Pocket Diagnostic

Tests were modified as necessary e.g. LAMP tests were transferred to fluorescent detection and real-time PCR were modified to run with the same enzyme mixes. LFD tests were performed according to manufacturers' recommendations.

Preliminary studies were performed according to EPPO guidelines (PM7/98 (3), EPPO Bulletin 2018, 48 (3): 387-404) and included application of tests on: standard dilutions of DNA isolated from pure bacterial cultures (molecular tests) or dilution of target bacteria (LFD tests), analysis of standard curves of target bacteria in plant material (4 different matrices i.e. plant species), and a set of target and non-target bacteria.

For each test the analysis settings were determined and criteria for positive and negative results identified. Diagnostic parameters of analytical sensitivity (in DNA and in plant material) and analytical specificity were determined.

As expected, the tests exhibited difference in analytical sensitivity however, all molecular tests were able to detect concentrations of *Erwinia amylovora* commonly expected in symptomatic tissues.

The two LFD tests, which were included in the preliminary study, showed similar performance characteristics however, they exhibited lower analytical sensitivity than the molecular tests. While analytical sensitivity is lower than the one observed for molecular tests we assess that the tests are fit for purpose i.e. for detection of *E. amylovora* in symptomatic plants in which bacterial concentrations are expected to be high and gave the added advantage to be easily applicable in the field with little training. However, one test (Agristrip) has significantly more previously obtained and published validation data and has been tested on a range of matrices (e.g. blossom). ELISA method was not selected due to lack of experience for this pest by the TPS organizer.

6.2 Pantoea stewartii subsp. *stewartii*

Pantoea stewartii subsp. *stewartii* (ex *Erwinia stewartii*) is causing serious bacterial disease named Stewart's wilt, mainly on maize plants. The pest is not indigenous to Europe; however, it was introduced to European environment through infected seeds. Infected seeds do not show any characteristic symptoms, therefore testing of the seeds is the only possibility to prevent spread of the pest with planting material. Latently infected maize seeds contain very low concentration of the causative pest, therefore highly sensitive methods are crucial for reliable pest detection. On the other hand, only highly specific tests that are able to distinguish *P. stewartii* subsp. *stewartii* from highly similar and non-pathogenic strains (e.g. *P. stewartii* subsp. *indologenes)* and thus provide accurate diagnosis. Firstly, we performed thorough literature searches to gain an insight on the diagnostic tests available for *P. stewartii* subsp. *stewartii*. Unpublished data and specialist experience were also considered in the test selection process. Synergy was sought with

the Euphresco project 2018-A-275 (Use of new diagnostic tools for detection of *Pantoea stewartii* subsp. *stewartii* from plant and seeds) which is starting in 2019, to collect strains of relevance and exchange experience on different tests. We were focused on sensitive tests with high selectivity; ideally, selectivity of the test would be limited to *P. stewartii* subsp. *stewartii*. For the preliminary studies, we selected 4 different real-time PCR assays and 2 PCR assays (table 2). All the test were sensitive, however, cross-reactions with a closely related subsp. indologenes and some other bacteria have been described for some or the data was missing. The only tests that were reported to be able to specifically detect *P. stewartii* subsp. *stewartii* were Pal *et al.*, 2019 (real-time PCR) and Gehring *et al.*, 2014 (PCR). Loop-mediated isothermal amplification (LAMP) method was chosen for on-site detection. Two different LAMP assays were selected from the literature data for the preliminary study, both reported in Uematsu *et al.*, 2015. The tests were partially validated, however validation did not included testing on maize seed extracts. ELISA method was not selected due to lack of experience for this pest by the TPS organizer.

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 (Journal of Applied Microbiology 104, 1525–1537) Thwaites *et al.* (FERA protocol, EUPH05 *Pantoea stewartii* subsp. *stewartii* Final Report) Wensing *et al.*, 2010 (Applied and Environmental Microbiology, 76:6248-6256) Pal *et al.* (Plant Disease, accepted for publication)*
PCR	AGES, 2016 (EPPO Bulletin 46 (2): 226-236) Gehring *et al.*, 2014 (Journal of Applied Microbiology 116: 1553-1562) (galE locus)
On-site methods	**Molecular (LAMP):** Uematsu *et al.*, 2015 (Journal of General Plant Pathology, 81: 173–179) (cpsD & pstS-glmS loci)

*The test was included subsequently as it was accepted for publication in 2019. The preliminary study is on-going and the test might be rejected later on based on the validation data.

Tests were modified as necessary e.g. LAMP tests were transferred to fluorescent detection and real-time PCR tests were modified to run to run with the same enzyme mixes where possible.

Preliminary studies were performed according to EPPO guidelines (PM7/98 (3), EPPO Bulletin 2018, 48 (3): 387-404) and included application of tests on standard dilutions of DNA isolated from pure bacterial cultures (molecular tests), analysis of standard curves of target bacteria in plant material (3 different matrices i.e. maize seed varieties), and a set of target and non-target bacteria.

For each test analysis, settings were determined and used for analysis of all the generated data. Diagnostic parameters of analytical sensitivity (in DNA and in plant material) and analytical specificity were determined.

Based on the previously published validation data and results from our preliminary studies, the following tests are fit for purpose: (i) real-time PCR tests described by Tambong *et al.* (2008), the test described by Thwaites (previously included in the validations within an Euphresco project) and test described by Wensing *et al.* (2010); (ii) PCR tests described by AGES and Gehring *et al.* (2014; gale locus).

The LAMP tests described by Uematsu *et al.* (2015) were transferred from turbidimetric to fluorescent detection. 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, based on the laboratory results reported for contaminated seeds.

Recently, a new real-time PCR was reported (Pal *et al.*, accepted for publication in Plant Disease, 2019). The test is described for both detection of *P. stewartii* subps. *stewartii* and its ability to distinguish it from isolates of subsp.

indologenes, an important performance characteristic in diagnostics of Stewart's wilt. Therefore, the test was subsequently included in our preliminary study.

6.3 Citrus tristeza virus

Citrus tristeza virus (CTV), genus Closterovirus (Martelli *et al*., 2005), is the causal agent of tristeza, a major disease on Citrus causing decline of trees and impacting fruits production. The virus has a host range restricted to most species of the family Rutaceae (Roistacher, 1991) and can be disseminated long distances by movement of virus-infected plant material and locally by several aphid species in semi-persistent mode (Lee and Bar-Joseph, 2000).

CTV probably originated in Malaysia and other countries of Southeast Asia, the putative area of origin of citrus, and it has been disseminated to almost all citrus-growing countries through the movement of infected plant material (IPPC, 2016).

Types and severity of symptoms induced by CTV are associated with different viral strains. The most virulent isolates (aggressive isolates) cause stem pits in wood of twigs, small and large lateral branches and the main trunk. They also reduce growth of the tree accompanied by a decline in fruit yield, fruit size and quality in severe cases (Saponari *et al*., 2008).

Detection and identification of CTV can be achieved using biological, serological or molecular methods.

Many tests are available on the market, covering these different methods: ELISA, real time PCR, conventional PCR, on site methods (LAMP or LFD) and also direct tissue blot immunoassay.

Available results of interlaboratory studies or intralaboratory validation data were used to make a selection among this large number of tests available.

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 Plantprint Sediag
Real-time RT-PCR	Bertolini *et al.*, 2008 (European Journal of Plant Pathology, 120: 177-188) Saponari *et al.*, 2008 (Journal of Virological Methods, 147: 43–53) Qualiplante/Ipadlab
RT-PCR	Loewe PCR - Olmos *et al.*, 1999 (Nucleic Acids Research, 27: 1564–1565)
On-site methods	**Molecular (LAMP):** Optigene Wang *et al*., 2013 (Scientia Agricultura Sinica, 46: 517-524) **Serological (Immunostrip):** Flashkit (Agdia)
Other methods	**Tissue Print Immunoassay (TPIA)** PlantPrint

Selection of tests was performed based both on a bibliographic review and on experimental investigations conducted by the TPS organiser. Only the most sensitive and specific tests have been selected to be included in the TPS.

Concerning the experimental investigations, for each test included in the above table, a panel of at least 10 target samples (representative of a diversity of CTV isolates and including aggressive isolates) and 10 non target samples (representative of a diversity of citrus species) were analysed to evaluate the inclusivity, the exclusivity and the selectivity of the test.

If the results were constituent with expected results (reference value of the samples), dilutions of target samples were then analysed 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 were selected for the TPS. However, it is worth noting the following exception for the real-time RT-PCR method. The three tests evaluated gave equivalent results but due to the limited number of tests to be included in the TPS, only the tests from Saponari *et al.* (2008) and IPADLAB were retained. The test from Bertolini *et al.* (2008) was excluded, but the primers developed by Bertolini *et al.* (2008) are included in the IPADLAB kit, and consequently will be evaluated.

6.4 Plum pox virus

Plum pox, also known as sharka, is a disease caused by plum pox virus (PPV). PPV may infect a wide variety of *Prunus* species, including almond, apricot, cherry, nectarine, peach, plum as well as wild and ornamental species (e.g *Prunus besseyi, Prunus insititia, Prunus tomentosa, Prunus triloba* and *Prunus spinose*). In fruit trees, infection may eventually result in deformation of fruits and severe yield reduction. At present more than ten different strains and recombinants are distinguished, based on both biological, serological and molecular characteristics. These are; Ancestor Marcus (An), Cherry (C), Cherry Russian (CR), Dideron (D), El Amar (EA), Marcus (M), Recombinant (Rec), Tatarstan (Tat), Turkish (T) and Winona (W). PPV is present in many European countries and is regulated for prunus plants for planting to control the disease (EU II/AII). Therefore, the availability of reliable tests is required to guarantee the absence of PPV in this material. For detection of the virus testing can be performed on symptomatic leaves, flowers, and/or fruits. In plant material without symptoms, both shoots and leaves can be tested. The scope of the TPS is 'detection of PPV in symptomatic and asymptomatic leaves of *Prunus* spp.', thereby focusing on a broad detection of 'all' strains. The TPS will include approx. 15 samples and includes both serological (DAS-ELISA) and molecular methods (real-time RT-PCR and RT-PCR).

Firstly, we performed thorough literature searches, including websites of commercial companies and methods described in the EPPO standard PM7/32 (1) plum pox virus, to gain an insight in the diagnostic tests available for PPV. Unpublished data and specialist experience were also considered in the tests selection process. We were focused on analytical specificity (inclusivity and exclusivity) and analytical sensitivity. For the evaluation 4 RT-PCR tests, 9 real-time RT-PCR tests, 9 DAS-ELISA antibody sets/kits from different commercial companies and 2 commercially available lateral flow tests were selected (table 4). On-site detection method LAMP was not selected due to limited experience by the TPS organizer.

Table 4: Tests selected for preliminary study for plum pox with references. Tests selected for TPS are underlined.

Method	Tests for validation:
RT-PCR	Qualiplante (2X) Wetzel et al., 1991 (Journal of Virological Methods, 33: 355–365) Levy and Hadidi, 1994 (EPPO Bulletin, 24: 595–604)
Real-time RT-PCR	Qualiplante (3X) Schneider et al., 2004 (Journal of Virological Methods, 120: 97–105) Olmos et al., 2005 (Journal of Virological Methods, 128: 151–155) Mavrič Pleško *et al.*, 2011(Acta agriculturae Slovenica, 97:105 – 113) Anonymous, 2018 (Naktuinbouw protocol)
DAS-ELISA	Agdia Agritest Bioreba DSMZ Loewe Neogen Plant Print Diagnostics S.L., Prime Diagnostics SEDIAG
On-site methods	**Serological (LFD):** AgriStrip (Bioreba) ImmunoStrip (Agdia)

In silico analytical specificity of RT-PCR and real-time RT-PCR primers and probes is performed. All primers and probes are made to the most conserved part of the PPV genome: the coding sequence of the C-terminal part of the coat protein and/or the 3' UTR. The primers and probe from the Naktuinbouw real time RT-PCR protocol were the most specific with only one mismatch in the forward primer for one of the tested CR strains. Primer and probes from other test showed 0-3 mismatches depending on the PPV strain. However, these mismatches are most likely not found in a crucial position within the primer or probe (no mismatches in the last 3 nucleotide at the 3' end for primers and 3' and 5' end for probes). The three real-time RT-PCR kits from Qualiplante are all based on the real-time RT-PCR described by Olmos *et al*. (2005) but contain different enzyme mixes. The two RT-PCR kits from Qualiplante are based on the RT-PCR described by Wetzel *et al*. (1991). During the TPS a single reaction-mix for the RT-PCR as well as for the real-time RT-PCR will be prescribed to be able to compare, without biased caused by the use of different enzyme mixes, the results from different participants. Therefore, the tests from Qualiplante will not be included in the TPS.

The analytical specificity of the molecular tests was investigated with RNA purified from PPV infected *Prunus* spp leaves as well as from *Prunus* spp infected with Apple chlorotic leaf spot virus, Cherry virus A, Little cherry virus 1 and Prunus necrotic ringspot virus. All RT-PCR and real-time RT-PCR tests were modified to a one-tube test. For unknown reasons the real-time RT-PCR according to Mavrič Pleško *et al*. (2011) did not give any positive signal and will consequently not be included in the TPS. With the other tests all available PPV strains (An, C, CR, D, EA, M, Rec, T and W) were detected and no cross reaction was found with other viruses. The analytical sensitivity of the RT-PCR and real-time PCR are almost equal. RNA isolates with Cq values of approx. 35 in the real-time RT-PCR were still positive in the RT-PCR. For identification of the virus (strain) the amplicons from the RT-PCR can be sequenced. This is out of the scope of the TPS. The RT-PCR according to Wetzel et al. (1991) and Levy & Hadidi (1994) and the real-time RT-PCR according to Schneider et al. (2004), Olmos et al. (2005) and the Naktuinbouw protocol will be included in the TPS.

For detection detection by ELISA, the method DAS-ELISA was chosen, as this is the standard method performed by the TPS organizer and not all companies can provide antibody sets for DAS-ELISA. Validation information about commercial antibodies for DAS-ELISA vary among companies, going from no information to an extensive list of PVV strains and

other viruses that are tested. It is very difficult to compare the antisera based on these validation data. Also validation data from diagnostics labs are done only with antisera from one company. Therefore the analytical specificity and analytical sensitivity of the antibodies from eight out of the nine companies (DSMZ was out of stock) from table 4 was evaluated for a limited set of PPV strains (An, C, CR, EA, Rec) and for Apple chlorotic leaf spot virus, Cherry virus A, Little cherry virus 1 and Prunus necrotic ringspot virus. PPV Infected *Nicotiana benthamina* plants were used for the evaluation as limited amount of infected *Prunus* spp leaves were available. The DAS-ELISA was performed according to EPPO standard PM7/125 (1) ELISA tests for viruses. This might imply that the used buffers are not the recommended buffers by the companies. All antibodies were able to detect the 5 PPV strains in undiluted samples and did not cross react with the other viruses or *Prunus* sp. leaves. The antisera differ in their analytical sensitivity as tested by serial dilutions of plant extracts. Because of the limited amount of starting material only three antisera sets can be included in the TPS. The most relevant criteria for selection of tests for the PPV TPS, as described in D1.1, are the analytical sensitivity, analytical specificity, and selectivity. As there was no difference in analytical specificity and selectivity among the different antisera, the three most sensitive antisera was chosen to be included in the TPS. These are the antisera of Agdia, Bioreba and SEDIAG.

The TPS organizers do not have experiences with detection by LFD. But the method seemed to be very straight forward. The idea was to use the same sample set for DAS-ELISA also for LFD. However, it turns out that for both selected kits leaf material has to be grinded in supplied buffer-containing bags. As limited sample material is available it is not possible to make an extra set or two extra sets of test samples for detection by LFD. Furthermore, the sample sets would most likely not be homogeneous as virus load can differ between leaves. Therefore, the method LFD will not be included in the TPS.

6.5 Fusarium circinatum

Fusarium circinatum is the causal agent of pitch canker disease which primarily affects *Pinus* sp. Whilst the pest has been reported in some European countries the serious threat to the pine forest industry means this pest is seen as of high importance. There is a wide range of host materials that can be tested for the presence of *Fusarium circinatum*, including infected tree material, seeds and insects.

A review of available published methods, including methods described in the EPPO protocol PM7/91 and previous TPS studies (EUPHRESCO project on pine seed testing) was carried out. The methods described in the EPPO protocol for both plating and molecular methods are widely used within European laboratories with many laboratories deploying multiple methods to confirm positive findings. For this reason, the TPS organisers have decided to include viable cultures as a test material allowing the plating methods to be included in the TPS. DNA extracts will also be included for the molecular methods to provide a standardised DNA concentration for testing methods in case of lab to lab variation during the extraction of culture material. There are currently no described on-site methods for *Fusarium circinatum*.

To be able to harmonise the sample types between methods within the TPS, the TPS organisers have decided to include only pure cultures for the plating method, therefore the isolation of the pest will not be required for this TPS. There is only a single plating method described for pest identification which will be included: Growth on potato dextrose agar (PDA) to study colony morphology and pigmentation along with growth on Spezieller Nahrstoffarmer Agar (SNA) to study formation and type of microconidia and conidiogenous cells. Both mating types will also be included with the samples.

There are no commercial kits presently available for molecular detection of *F. circinatum* (conventional PCR or real-time PCR). Only assays from published literature are available, and two conventional assays (Table 5) and three real-time assays are in use within European laboratories (EPPO survey). The validation data available within the publications is inconsistent, making it hard to compare tests, and no validation data for detection/identification of this pathogen

has been published on the EPPO database. Further preliminary studies have been carried out by the TPS organiser in accordance with EPPO guidelines (PM7/98 (3), EPPO Bulletin 2018, 48 (3): 387-404), to help with selection of tests. These preliminary studies have incorporated the use of different PCR reagents and analytical sensitivity and analytical specificity effects to provide suitable robust reagent(s) for use in the TPS.

RFLP will not be included in the TPS as only one laboratory replying to the EPPO survey reported the use of this method (other individual tests are infrequently used, but the methods are in wider use).

For note, in 2015 two new *Fusarium* spp. were described: *Fusarium marasasianum* and *F. parvisorum* (Herron *et al.* 2015). Both were isolated from diseased roots of *Pinus patula* seedlings in Colombia and have caused disease symptoms in seedlings similar to *F. circinatum*. Existing published assays may have been designed before the new species were described and therefore analytical specificity with the new species must be assessed and where possible this will be reviewed in the validation data.

Table 5: Tests selected for preliminary study for *F. circinatum* with references. Tests selected for TPS are underlined.

Method	**Tests for validation:**
Plating	PDA and SNA for morphological identification of cultures (EPPO 7/91)
PCR	Ramsfield *et al.*, 2008 (Molecular Ecology Resources, 8: 1270-1273) Schweigkofler *et al.*, 2004 (Applied and Environmental Microbiology, 70: 3512-3520)
Real-time PCR	Ioos *et al.*, 2009 (Phytopathology, 99: 582-90) Schweigkofler *et al.*, 2004 (Applied and Environmental Microbiology, 70: 3512-3520) Lamarche *et al*., 2015 (PLoS ONE 10(8): 0134265) Luchi *et al*., 2018 (Applied Micobiology and Biotechnology, 102: 7135-7146)

The PCR assay of Schweigkofler *et al*. is widely used, is included in EPPO protocol PM7/91, and meets analytical sensitivity requirements when used with either agarose gel electrophoresis or real-time fluorescence detection; both versions of this test are selected for inclusion in the TPS. The PCR assay of Ramsfield *et al*. (2008) was found in preliminary study to have a lower analytical sensitivity, and results reported by Rumsfield *et al*. indicate potential cross reactivity with non-target species and lack of inclusivity for isolates of some geographic origins. This test will not be included in the TPS.

The real-time PCR test of Ioos *et al*. is widely used and is included in EPPO protocol PM7/91. The reported analytical sensitivity and specificity of this test justifies its inclusion in the TPS. Our preliminary study has indicated that this assay is very sensitive to procedural changes (e.g. reagent choice, cycling conditions). For this reason, and on the basis of published data and the results of our preliminary study, the TPS will also include the more recently published real-time PCR tests described by Luchi *et al*. (2018) and Lamarche *et al*. (2015).

6.6 Bursaphelenchus xylophilus

Bursaphelenchus xylophilus is the causal agent of the pine wilt disease. Depending of the climatic conditions, the disease can be symptomatic or asymptomatic and it can be spread quickly by different means (wood material, vector…). Additionally, in natural conditions different confusing species of *Bursaphelenchus* are present in pine stands.

Considering all these elements and the quarantine status of the pest, high analytical sensitivity and high analytical specificity are essential for reliable detection.

The literature review conducted so far underlined the numerous molecular tests available, using conventional PCR technology or real-time PCR. 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 the organized TPS.

For PCR (conventional and real time), the tests with consequent validation data available were selected for preliminary studies. A commercial kit based on real time PCR technology is available and wasn't submitted to an interlaboratory study, it also justified to retain this kit in the TPS organization.

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.

Table 5: 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 (ref: RT-N-D-0401) Francois *et al.*, 2007 (Molecular Plant Pathology, 8, 803-809)
PCR	Matsunaga and Togashi, 2004 (Nematology, 6: 273-277) Burgermeister *et al.*, 2009 (PCR RFLP (Nematology, 11, 649–668)
On-site methods	Molecular (LAMP): Kikuchi *et al.*, 2009 (Phytopathology, 99, 1365-1369) Meng, *et al*., 2018 (Forest Pathology, 48, e12404)

Selection of tests was performed based both on tests used in EU laboratories (EPPO survey results) and on the availability of validation data from literature sources and laboratory experience of TPS organizers.

Preliminary studies were performed according to EPPO guidelines (PM7/98 (3), EPPO Bulletin 2018, 48 (3): 387-404) and included analytical sensitivity, analytical specificity, repeatability and reproducibility. Depending on the purpose (detection / identification), analytical sensitivity was evaluated either on isolated nematodes or spiked wood extracts. Analytical specificity was evaluated on populations of *B. xylophilus* of different geographical origins, populations of *Bursaphelenchus* of the group *xylophilus* and other populations of *Bursaphelenchus.* It allowed to evaluate the inclusivity, the exclusivity and the selectivity of the tests.

Only one commercial kit for the detection of *B. xylophilus* is available on the market. It will be therefore evaluated and integrated to the TPS.

Among the two LAMP tests evaluated, the test developed by Meng *et al.* (2018) didn't give any results and will consequently not be included in the TPS.