This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement N° 773139

Grant agreement N. 773139

DELIVERABLE N° 3.2

Title: Development of draft Standard Operating Procedures (SOPs) available for the production of the reference materials identified in task 3.1

Validation of diagnostic tests to support plant health

Due date:	Month 10
Actual submission date	04-03-2019 (Month 11)
Start date of the project	01-05-2018
Deliverable lead contractor (organization name)	WR, NIB
Participants (Partners short names)	WR, UNITO, WBF, EPPO, NIB, NVWA, ANSES, IPADLAB, SEDIAG
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Level of dissemination	Public
Type of Deliverable	Report

Abstract:
A general standard operating procedure (SOP) for the production of reference material (RM) for use in plant health diagnostics was developed. The general SOP was designed based on (limited) information on existing SOPs and guidelines available with the consortium partners. The general SOP describes the different steps required in the production process, ranging from the different possible sources of the reference material, tests to confirm its identity, possibly required multiplication steps to the actual production process. For each step in the process, criteria and critical points are identified. The criteria that reference material has to meet, and their minimum required levels as identified and described in Deliverable 3.1 of WP3 form an import basis of the final production process.

Partners involved Task WR, UNITO, WBF, EPPO, NIB, NVWA, ANSES, IPADLAB, SEDIAG

Contents

1 Purpose

As described in the VALITEST project proposal **WP3** aims at developing recommendations for the quality of reference materials for validation purposes. An essential condition for proper validation of methods and execution of test performance studies is the availability of well-defined and characterized reference and control material. The actual production of reference material involves many steps for which currently no common guidelines are shared by the different producing laboratories and companies.

There is clearly a growing market demand for more differentiation and better and more uniformed quality assurance for the reference material used in tests for plant pests. Since most of this reference material will be produced by different suppliers including commercial SMEs (international collections, specific suppliers), there is a clear need for common guidelines and standard operating procedures (SOPs) to ensure sufficient and common quality levels among suppliers. Metrologically sound reference material needs not just to be representative from the biological point of view but it should also have well defined cell count or DNA/RNA copy number (if applicable), homogeneity, stability and purity. This deliverable describes a standard operating procedure (SOP) for the production of different types of reference material (RM) for use in validation studies for diagnostic tests as well as in intralaboratory evaluations such as test performance studies.

2 Scope

The general standard operating procedure (SOP) developed in this deliverable is intended for use in the (future) production of different types of reference material by different plant health laboratories and producers of plant health diagnostics fur use in studies of diagnostic tests either intralaboratory or inter laboratory studies/comparisons.
This SOP will later be evaluated in the production of RM to be used in the Round 2 of the Test Performance Study (TPS) in WP1.

3 Terms, abbreviations and definitions

Generally, reference material used in plant diagnostic testing is used as some sort of positive control. As such is it at some point may be derived from a collection which can either be a working collection, a reference collection or a certified reference collection. It may also be derived from plant material from the field (.e.g for a newly discovered pest for which no collection material is available yet). In addition, when working with reference materials people may have different ideas and expectations on their use and performance. It is therefore important to first define what reference materials are, where they come from and in what context they can and should be used.
A previous FP7 EU-project (Q-collect) focussed on coordination and collaboration between reference collections of plant pests and diseases within the EU. This project defined a number of terms which are also very relevant for this current VALITEST project (see below). These definitions were also implemented in the EPPO Standard PM 7/76 (5); Use of EPPO diagnostic protocols (2018):

Certified reference material (CRM): Reference material derived from a source that certifies the authenticity of the material. Preferably material should come from an internationally recognised source such as a national reference collection. The material should have a unique identification code allowing traceability and the name of the person who certifies its authenticity. Details of how the material was authenticated should also be supplied. If appropriate, information about its activity (e.g. pathogenicity, antigenic properties) under specified

conditions should also be supplied along with any related uncertainty at a stated level of confidence (EPPO PM 7/76 (5)).

Reference material (RM): Material appropriate to the test and diagnosis being performed such as live cultures, infected plant material, DNA/RNA preparations, images of a diagnostic quality or mounted specimens. The reference material used should be documented and appropriate for the test and diagnosis being performed. It should be ensured that it has the features for which it was selected, for example expressing a desired antigen for use in serological diagnosis or display specific physical features (e.g. sporulation) if used for morphological diagnosis (EPPO PM 7/76 (5)).

Reference collection: A collection of individuals maintained for the purpose of study and authentication. Reference collections are generally large undertakings maintained by institutions; instead of having a single representative of each species, they will typically have multiples, so as to illustrate variations and, be able to provide samples externally for comparisons and research. Reference collections are an important source of information about variations of populations within a species. They are also the repository of type strains or holotypes used as the official definition of a particular species.

Working collection: Collections, usually of individuals belonging to a single organism or group of related organisms, maintained for the purpose of scientific investigation by experts. Working collections are usually maintained by individual researchers or research groups with recognized knowledge of the organism(s) in question. They do not usually provide samples externally other than to deposit individuals of interest into one or more reference collections for safe keeping and/or protection of intellectual property.

Quality assurance: part of quality management, focussed on providing confidence that quality requirements will be fulfilled. Dependent on the laboratories own quality management system, samples and materials may be rejected if basic quality requirements are not met. For example, samples clearly contaminated with non-target organisms or samples transported under sub-optimal conditions may be discarded without further testing

Test: the application of a method to a specific pest and a specific matrix.

Multiplication (of RM): if more or a different type of biological material of a certain candidate reference material is needed, the material may be used for multiplication/ amplification. This may for instance mean amplification in or on plants, but also be an amplification of specific target genes.

Pest (IPPC, 2017): any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products.

NAC: negative amplification control.

NC: negative control.

NIC: negative isolation control.

PAC: positive amplification control.

PC: positive control.

PIC: positive isolation control.

Additional definitions and descriptions

The following definitions and descriptions refer specifically to the criteria mentioned for the production of RM. For more background information on these terms please see section 5 (Descriptors for reference material (RM) for validation for tests and TPSs) in the report on Deliverable 3.1 of WP3.

Identity: The reference material should be clearly identified and characterized at least to the extent ensuring its correct identification. Following the recommendation below this should be done by at least two preferably validated and independent tests, based on different physical characteristic of the RM. If the specimen used originates from and is available in several reference collections working according to commonly agreed quality standards this provides additional guarantee into its identity. At minimum, the material should be thoroughly identified to the level of international accepted diagnostic protocols (when available) to ensure it is properly identified. The list of tests used for its identification should be clear from its description.

Traceability: Traceability can be considered as an aspect of both identity and availability in the sense that it may provide some additional guarantees to the correct identity of the materials used to prepare the reference material and its future availability. To ensure traceability it is important to properly identify and describe the matrix as well and ensure its proper identification. Collecting meta-data otherwise used for samples is suitable.

Commutability: a characteristic describing the extent to which they resemble actual samples. A reference material would be considered commutable when a measurement produces the same result for a it as it does for an authentic sample that contained the same analyte concentration.

Homogeneity: The assessment of homogeneity should generally be performed after the samples have been packaged in the final form and before distribution to participants. Homogeneity can be demonstrated prior to packaging where no influence of packaging is reasonably expected (EPPO PM 7/122). This guideline provides further information on the assessment of homogeneity for different types of material also stating: the current available guidelines recommend to test a minimum of 10 randomly chosen samples (for each pest/ matrix/infestation level, including negative samples) in duplicate.

Stability: Samples should be demonstrated to be sufficiently stable to ensure that they will not undergo any significant change, including storage and transport conditions. The number of aliquots to be tested for stability also depends on the quantity of the reference material produced.

Assigned value: Assigned quantity values correspond to the expected result of the test (pest present or absent, concentration of the pest, morphological characteristics of the specimen, etc.) (EPPO Guidelines PM 7/122).

Purity: Purity is defined as a ratio of target pest versus non-targets, particularly non-targets interfering with a test.

4 Methodology

Partners within WP3 as well as commercial laboratory partners within the VALITEST consortium were approached to supply already existing SOPs for use in the production of specific reference material. None of the partners who replied, had such specific SOPs available. Some partners supplied general SOPs on i.e. management and maintenance of (reference) material in collections (ANSES, NVWA, FERA).

Based on these more generic SOPs and personal expertise within WP3 a flow chart was designed to serve as the basis for a SOP for the general production of reference materials (see Figure 1). This flow chart provides an overview of steps to follow for the production of reference material specific for one or more target organism(s) and one or more target test(s). Competence of laboratories and personnel

as well as traceability of materials, methods, test results and personnel involved in the different production steps is imperative. It is therefore assumed that the basic requirements for quality management in plant health diagnosis laboratories as stated in EPPO standard PM 7/84 (2) (2018) are met.

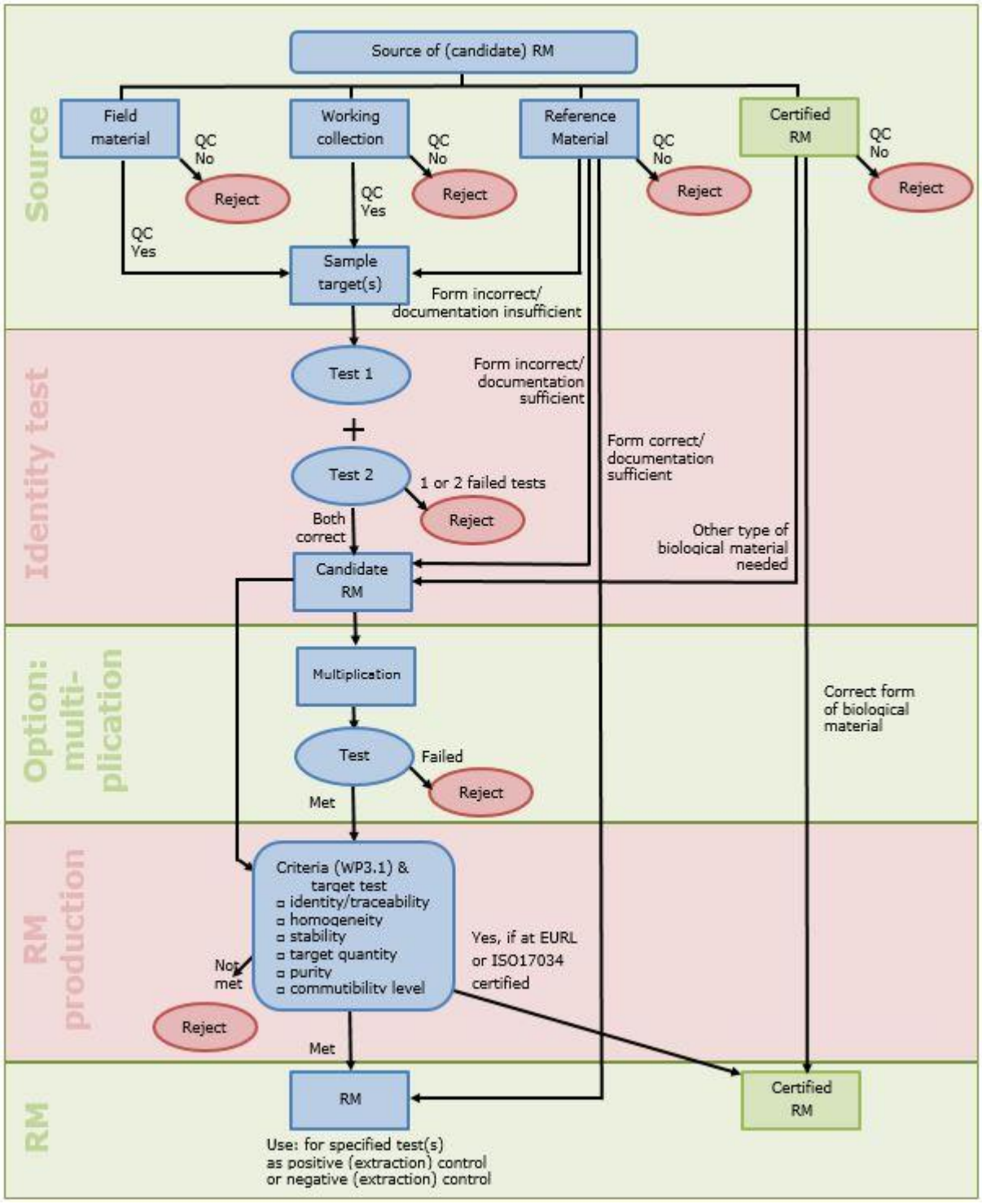

Figure 1: Graphical presentation of the general Standard Operating Procedure (SOP) for the production of reference material to be used in specified tests as either positive or negative samples.

The different steps identified within the SOP are discussed below:

4.1 Sources of (candidate) RM

It is acknowledged that both the physical nature as well as the source of the material intended as positive of negative RM can be quite diverse. Plant health diagnostic deals with a variety of different pests each with their own characteristics and their own specific tests. The majority of pests are not available in pure or cultured form and different (physical) types of samples were defined in Deliverable 3.1 in decreasing order of commutability):

- naturally infested plant material
- artificially infested plant material
- spiked plant material
- purified or isolated organisms
- purified nucleic acids
- synthetic nucleic acids

The (candidate) reference material may come from different sources and in different forms. The source and form do determine the steps necessary in the actual production of the reference material. The first step in the process is therefor to distinguish between the possible sources of the candidate material. The SOP distinguishes four different possible sources:

CRM: material directly obtained from a certified reference collection which is accompanied by adequate documentation to show it meets the criteria defined in D3.1 (i.e. identity, traceability, homogeneity, purity, stability). If for whatever reason delivered CRM is not trusted, reject it. Ideally the institute is ISO 17034 accredited for their collection. When supplied in the correct form as needed for the specified test, it can serve directly as RM. When it is not in the appropriate form, it will need to be converted in its desired form. It should then be regarded and treated as candidate RM. Possibly the candidate RM will need to go through a multiplication step. Following that step (or steps) it needs to be shown that the resulting material meets all the required criteria as identified in D3.1 (see also figure 1, step RM production).

RM: candidate reference material obtained from a recognized collection or supplier. When supplied both in the correct form (suited for the intended test) and with adequate documentation (see criteria D3.1) this material can be regarded as RM and used directly in the specified test. However, when either in the incorrect form or/and without adequate documentation, additional tests need to be performed before the material can be used as reference material.

Working collection: usually this material is maintained by experts and as such may have been already (partly) characterized. However, here we assume it does not meet all requirements for proper form and/or documentation and should therefor go through proper identification steps (and possibly multiplication steps).

Field material: this is material collected directly from the field, usually plant parts. These may or may not show typical symptoms of the pest. The material may (or may not) contain the desired pest and it may contain other organisms which may interfere in one or more of the steps later in the procedure. As working collection material, most likely it will not meet all requirements for proper form and/or

documentation and should therefore go through proper identification steps (and possibly multiplication steps).

Before this material, irrespective of its source, can be processed further an initial check at arrival on its quality by the laboratory receiving the material is mandatory. The criteria of this check depend on the internal quality system requirements of the laboratory as well as documentation supplied, and the conditions set by the supplier (e.g. transport under certain controlled conditions or time restrictions). If these criteria are not met, the material should be rejected (see also Deliverable D3.1 paragraph 5.10). Depending on the source of the material its use may be governed by conditions upon which it was obtained defined by e.g. Nagoya protocol requirements, material transfer agreements and other. These conditions should be met before it can be further processed.

In the actual SOP a description of the target organism should be included as well as a description of the final form and the intended test(s) it is to be used in.

4.2 Identity test

If the material intended for use as RM comes either from field material, a working collection, reference material or even a certified reference collection but either is in the incorrect form or necessary documentation is missing, it should be treated as not sufficiently characterized material and should go through proper identification steps. These should comprise of at least two independent tests (test 1 and 2, both preferably validated), preferably based on different physical characteristics (e.g. serological and genetic) to prove the identity of the pest in the material. If one or both tests fail to confirm its identity the material should be rejected. If both tests confirm the identity of the pest, the material can be regarded as candidate RM. If sufficient material is available, the candidate RM may go in the production phase. If not enough material is available for later use as reference material, a multiplication step may be optional.

4.3 Multiplication (optional)

If not enough material is available for future use, the candidate RM may need to go through one or more multiplication steps. Following this, the identity of the material needs re-confirmation using a preferably validated test, which may be one of the tests used in the identity test. If the identity of the RM cannot be confirmed the material should be rejected.

4.4 Reference material (RM) production

The final step will be the actual production of the reference material. Precise steps and procedures will depend on the actual physical properties and intended end-use (i.e. specific test) of the material. Following production, the material should meet all the general criteria for RM as defined in section 5 (Descriptors for reference material (RM) for validations and TPSs) in the report on Deliverable 3.1 of WP3. Table 1 lists the different criteria to be assessed in the production of RM, their possible values and the minimum criterion that should be reached. As discussed, and described in Deliverable 3.1, the minimum level identified is considered as a required criterion. However, the specific levels of each

criterion required for a specific RM will depend on the scope of its use and purpose. As already stated in Deliverable 3.1 (paragraph 6) it is important to note '*that since criteria are inherently linked to the intended use of the reference materials and may be test-specific, the criteria may be different for different uses and should be defined by the producer*'.
If for the candidate RM the criteria are met, it can be regarded as reference material.

These criteria considered for reference material are:

Identity: The reference material should be clearly identified and characterized by at least two preferably validated and independent tests, based on different physical characteristic of the RM. If the material originates from a reference collection operating under commonly agreed quality standards, this provides additional guarantee into its identity. At minimum, the material should be thoroughly identified to the level of international accepted diagnostic protocols (when available) to ensure it is properly identified. The list of tests used for its identification should be clear from the documentation accompanying the RM.

Traceability: Traceability of the RM covers the origin of the material including prior handling and multiplication (if applicable). If the material is derived from a collection, it should be traceable to a specific specimen in that collection including its history of maintenance and handling in that collection. If it is derived from field material meta data should be documented; i.e. when and where the material was collected, from what plant species and part and whether or not it was showing which symptoms.

Commutability: Commutability in its narrower sense describes the extent to which the reference material is similar to actual samples, i.e. its exchangeability by virtue of being replaceable. The type of test item chosen reflects the intended use of the reference material.
While naturally contaminated samples are often considered as the most appropriate material for reference material, this is not always available. We recognise six classes of commutability, ranging from naturally infested plant material to completely artificial synthetic nucleic acids (see paragraph 4.1; sources of reference material).

Homogeneity: Homogeneity should generally be determined after the reference material has been packaged in its final form. EPPO guideline PM 7/122 provides information on the assessment of homogeneity for different types of material also stating: '*it recommends to test a minimum of 10 randomly chosen samples (for each pest/ matrix/infestation level, including negative samples) in duplicate*'.

Stability: As part of the production process, reference material should be tested to determine it is sufficiently stable for its intended purpose and will not undergo any significant changes for a minimum required period of time, including transport and storage, that may influence the test result.. This minimum period of time and the required conditions should be clearly indicated in the documentation accompanying each individual lot of RM. The number of aliquots to be tested for stability also depends on the quantity of the reference material produced.

Assigned value: Assigned quantity values correspond to the expected result of the test (pest present or absent, concentration of the pest, morphological characteristics of the specimen, etc.) (EPPO Guidelines PM 7/122). Depending on the nature of the RM and its commutability the assigned value may vary:

- Known concentration i.e. absolute quantification of the target pest and/or its components (e.g. DNA copy numbers).
- Level of concentration (high/medium/low) known (as determined through use of at least one semi-quantitative or quantitative test).
- Qualitative status known (positive/negative above the determined limit of detection using at least one test).
- Consensus values from participants in proficiency test. Rules for definition of these values from the participants results should be defined: statistical methods, outliers effect (e.g. a virology interlaboratory comparison may assign the values this way). Uncertainty of assigned values should be defined.
- Originating from plants with known health statues with a recent test result (a given period of time depends on the plant-pest combination and previous experience).

Reports on an assigned value of particular RM should include and reflect the method used to determine them. E.g. if turbidity measurements were used to determine bacterial cells concentrations this should be reported as 'the turbidity of XY which corresponds to xy cells/unit'. Similarly, if the concentration was determined through colony counts, the concentration should be reported as colony forming units/unit also stating the media and growth conditions used.

Purity: Purity is defined as a ratio of target pest versus non-targets, particularly non-targets interfering with a test. Different levels of purity can be defined ranging from an absence of any non-targets to a relative high amount of non-target in your reference material (i.e. a low ratio of target). Assessment of purity may also depend on the level of commutability of the RM (e.g. synthetic nucleic acid vs. plant material with a known health status).
Presence of non-target material cannot always be avoided and does not need to be a problem however, this depends on the exclusivity specificity of the test used i.e. the performance of a test with regard to cross-reaction with a range of non-targets (e.g. closely related organisms, contaminants) (EPPO PM7/76 (5). Determining the presence of non-targets should preferably be done through unbiased methods e.g. high throughput sequencing.

Table 1 lists the different criteria for the production of reference material, the several levels available for each criterion and the minimum level required for each criterion required in the production of reference material.

Table 1: List of descriptors of criteria to be assessed in the production of reference material (RM). Where several levels are available for a descriptor, the lowest corresponds to the minimum criterion for the specific descriptor.

Descriptor	Value	Minimum criterion
Intended use	**should be defined (in this case it equals preparation of RM for the scope of the individual test or TPS)**	**yes**
Identity	**identified to the level of internationally recognized diagnostic protocols**	**yes**
Traceability	traceability to a specimen from a reference culture collection	no
	traceability to a specimen from a working culture collection	no
	traceability provided for the target pest and matrix used (the latter if relevant)	**yes**
Commutability level	naturally infested plant material	no
	artificially infested plant material	no
	spiked plant material	no
	purified organisms	no
	purified nucleic acids	no
	synthetic nucleic acids	**yes**
Homogeneity	**homogenous**	**yes**
Stability	**stable**	**yes**
	stability - short term	no
	stability - long term	no
Assigned value	absolute concentration known	no
	level of concentration known (high/medium/low)	no
	qualitative status known (above LOD level)	no
	originating from plants with known health statues with a recent test result (a given period of time depends on the plant-pest combination and previous experience)	**yes**
Purity	absence of non-targets	no
	absence of interfering non-targets	no
	known ratio of target VS non-target interfering with the test - high	no
	known ratio of target VS non-target interfering with the test - medium	no
	known ratio of target VS non-target interfering with the test - low	**yes**

5 Final notes

The current deliverable describes the requirements and procedures for the production of reference material suitable for intra and interlaboratory studies (e.g. test performance studies, proficiency). These were used to design a general standard operating procedure (SOP). This general SOP is listed in Annex 1. An example of a specific SOP for the production of reference material of *Fusarium circinatum* is listed in Annex 2.

The general SOP will be evaluated with the aim to further improve it in the second round of TPS in VALITEST once the pests and their respective tests for this TSP2 have been identified.

6 Sources of operating procedures

ANSES. Procédure spécifique: Gestion des différents matériaux de référence.

EPPO (2018) PM 7/76 (5) Use of EPPO Diagnostic protocols. *Bulletin OEPP/EPPO Bulletin* 48 (3): 373–377.

EPPO (2018) PM 7/84 (2) Basic requirements for quality management in plant pest diagnosis laboratories. *Bulletin OEPP/EPPO Bulletin* 48 (3): 378–386.

EPPO (2018) PM 7/98 (3) Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity. *Bulletin OEPP/EPPO Bulletin* 48 (3): 387–404.

EPPO (2014) PM7/122 (1) Guidelines for the organization of interlaboratory comparisons by plant pest diagnostic laboratories. *Bulletin OEPP/EPPO Bulletin* 44 (3): 390-399

FERA (2016) The basic procedure of mechanical inoculation, including maintenance of Potato spindle tuber viroid (PSTVd) reference cultures. PLH/195 (5)

FERA (2011) The Restoration of Nematode Specimens Slide Mounted in Anhydrous Glycerol. PLH/849 (1)

NVWA (2016) Opname, beheer en uitgifte collectie- en referentiemateriaal MolBio. I-MOL-077 (3)

VALITEST (2019) Deliverable D3.1

ANNEX 1

General Standard Operating Procedure (SOP) for the production of reference material

title	**General SOP Production of Reference Material**			
code		version 1	date 01-03-2019	Page x of y

Version	date	Relevant changes

1 Scope/intended use

This is a standard operating procedure (SOP) for the production of [specify particular organism] as reference material as [specify particular form of the reference material is to be used in] for use in [specify particular test in which the RM is to be used]. It describes the different steps required in the production process, ranging from the different possible sources of the reference material, tests to confirm its identity, possibly required multiplication steps to the actual production process. For each step in the process, criteria and critical points are identified.

2 Definitions

Assigned value: Assigned quantity values correspond to the expected result of the test (pest present or absent, concentration of the pest, morphological characteristics of the specimen, etc.) (EPPO Guidelines PM 7/122).

Certified reference material (CRM): Reference material derived from a source that certifies the authenticity of the material. Preferably material should come from an internationally recognised source such as a national reference collection. The material should have a unique identification code allowing traceability and the name of the person who certifies its authenticity. Details of how the material was authenticated should also be supplied. If appropriate, information about its activity (e.g. pathogenicity, antigenic properties) under specified conditions should also be supplied along with any related uncertainty at a stated level of confidence (EPPO PM 7/76 (5)).

Commutability: a characteristic describing the extent to which they resemble actual samples. A reference material would be considered commutable when a measurement produces the same result for a it as it does for an authentic sample that contained the same analyte concentration.

Homogeneity: The assessment of homogeneity should generally be performed after the samples have been packaged in the final form and before distribution to participants. Homogeneity can be demonstrated prior to packaging where no influence of packaging is reasonably expected (EPPO PM 7/122). This guideline provides further information on the assessment of homogeneity for different types of material also stating: the current available guidelines recommend to test a minimum of 10 randomly chosen samples (for each pest/ matrix/infestation level, including negative samples) in duplicate.

Identity: The reference material should be clearly identified and characterized at least to the extent ensuring its correct identification. Following the recommendation below this should be done by at least two preferably validated and independent tests, based on different physical characteristic of the RM. If the specimen used originates from and is available in several reference collections working according to commonly agreed quality standards this provides additional guarantee into

its identity. At minimum, the material should be thoroughly identified to the level of international accepted diagnostic protocols (when available) to ensure it is properly identified. The list of tests used for its identification should be clear from its description.

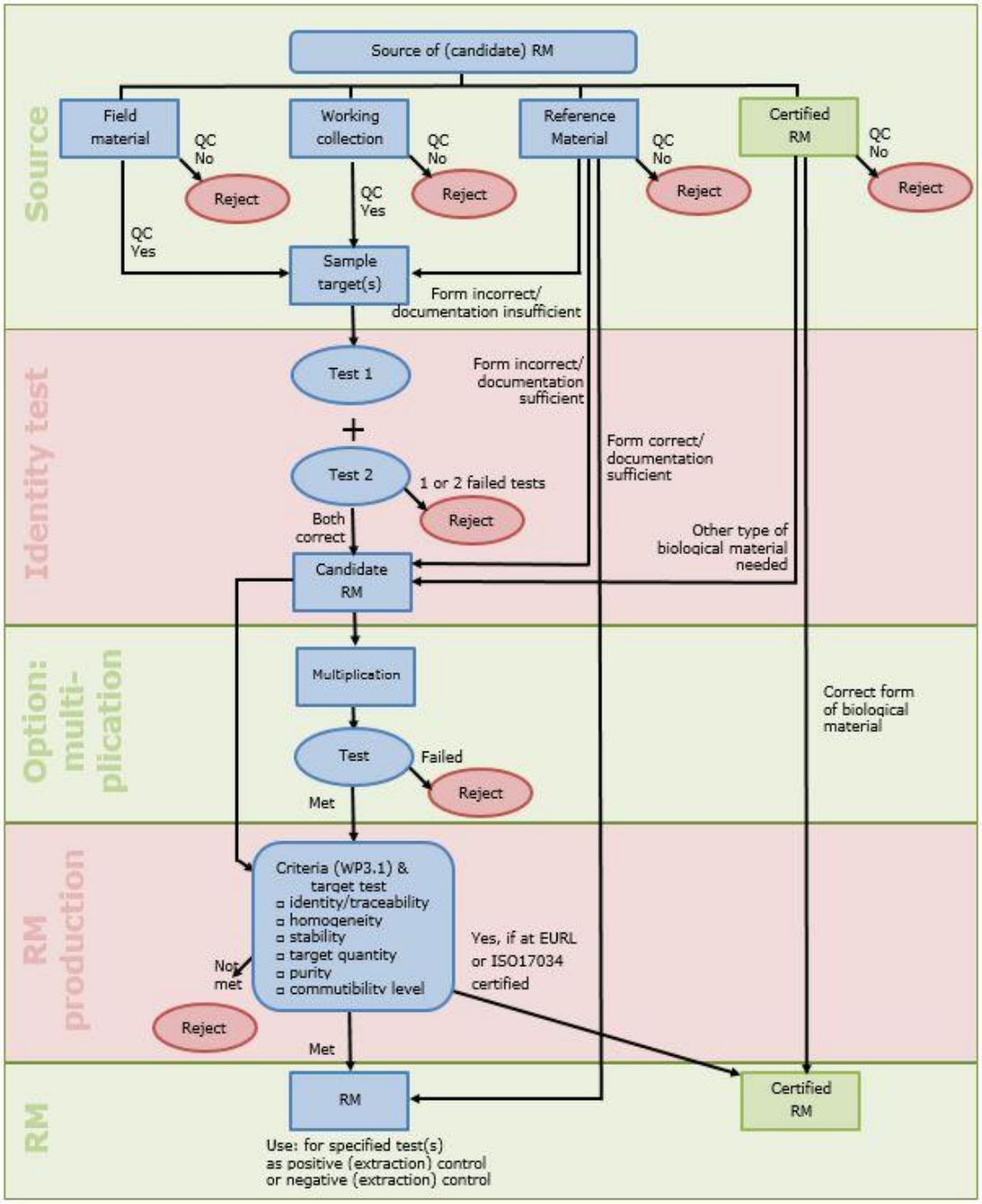

Figure 1: Graphical presentation of the general Standard Operating Procedure (SOP) for the production of reference material to be used in specified tests as either positive or negative samples.

Multiplication (of RM): if more or a different type of biological material of a certain candidate reference material is needed, the material may be used for multiplication/ amplification. This may for instance mean amplification in or on plants, but also be an amplification of specific target genes.

NAC: negative amplification control.

NC: negative control.

NIC: negative isolation control.

PAC: positive amplification control.

PC: positive control.

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Reference material (RM): Material appropriate to the test and diagnosis being performed such as live cultures, infected plant material, DNA/RNA preparations, images of a diagnostic quality or mounted specimens. The reference material used should be documented and appropriate for the test and diagnosis being performed. It should be ensured that it has the features for which it was selected, for example expressing a desired antigen for use in serological diagnosis or display specific physical features (e.g. sporulation) if used for morphological diagnosis (EPPO PM 7/76 (5)).

Reference collection: A collection of individuals maintained for the purpose of study and authentication. Reference collections are generally large undertakings maintained by institutions; instead of having a single representative of each species, they will typically have multiples, so as to illustrate variations and, be able to provide samples externally for comparisons and research. Reference collections are an important source of information about variations of populations within a species. They are also the repository of type strains or holotypes used as the official definition of a particular species.

Stability: Samples should be demonstrated to be sufficiently stable to ensure that they will not undergo any significant change, including storage and transport conditions. The number of aliquots to be tested for stability also depends on the quantity of the reference material produced.

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identify and describe the matrix as well and ensure its proper identification. Collecting meta-data otherwise used for samples is suitable.

Working collection: Collections, usually of individuals belonging to a single organism or group of related organisms, maintained for the purpose of scientific investigation by experts. Working collections are usually maintained by individual researchers or research groups with recognized knowledge of the organism(s) in question. They do not usually provide samples externally other than to deposit individuals of interest into one or more reference collections for safe keeping and/or protection of intellectual property.

3 Source of (candidate) reference material

A description the target organism is required. The source of the material should be identified i.e. field collected material/working collection material/reference material/certified reference material), including all available meta data.

Documentation should be provided here on the handling protocol of target organism and sampling and on sample registration.

Before material, irrespective of its source, can be processed an initial check at arrival on its quality by the laboratory receiving the material is mandatory. The criteria of this check depend on the internal quality system requirements of the laboratory as well as documentation supplied, and the conditions set by the supplier (e.g. transport under certain controlled conditions or time restrictions). If these criteria are not met, the material should be rejected. Depending on the source of the material its use may be governed by conditions upon which it was obtained defined by e.g. Nagoya protocol requirements, material transfer agreements and other. These conditions should be met before it can be further processed.

Both the physical nature as well as the source of the material intended as positive of negative RM can be quite diverse. The majority of pests are not available in pure or cultured form. The physical type of reference material should be defined:

- naturally infested plant material
- artificially infested plant material
- spiked plant material
- purified or isolated organisms
- purified nucleic acids
- synthetic nucleic acids

4 Identification of candidate reference material

If the material intended for use as RM comes either from field material, a working collection, reference material or even a certified reference collection but either is in the incorrect form or necessary documentation is missing, it should be treated as not sufficiently characterized material and should go through proper identification steps.

In principle two independent tests (preferably based on different physical characteristics (e.g. serological and genetic) should be performed for the target organism(s). In the case of destructive sampling in *e.g*. insects or nematodes this may not be possible.

A list of recommended tests for the identification of target species should be given. Standard operation procedures for these test(s) can be given or it should be documented where to find them. The list contains preferably validated tests (accepted diagnostic protocols) but may contain other tests if these are not available *e.g.* in the case of new, emerging species.

If one or both tests fail to confirm its identity the material should be rejected. If both tests confirm the identity of the pest, the material can be regarded as candidate RM. If sufficient material is available, the candidate RM may go in the production phase. If not enough material is available for later use as reference material, a multiplication step may be optional.

5 Multiplication of material

If not enough material is available for future use, the candidate RM may need to go through one or more multiplication steps. Following this, the identity of the material needs re-confirmation using a preferably validated test, which may be one of the tests used in the identity test. If the identity of the RM cannot be confirmed the material should be rejected.

6 Criteria for reference materials

Here the different criteria are listed that are to be assessed during the production of reference material. Table 1 summarizes these and lists possible values of these criteria. It also indicates which minimum value of each criterion is to be met. It is important to note, that since criteria are inherently linked to the intended use of the reference materials and may be test-specific, the criteria may be different for different uses and should be defined by the producer. The **Intended Use** of the reference material should be defined prior to the production of the material.

Identity: The RM should be clearly identified and characterized preferably by two validated and independent tests (section 4). If the material originates from a reference collection operating under commonly agreed quality standards, this provides additional guarantee into its identity. At minimum, the material should be thoroughly identified to the level of international accepted diagnostic protocols (when available) to ensure it is properly identified.

Traceability: Traceability of the RM covers the origin of the material including prior handling and multiplication (if applicable). If the material is derived from a collection, it should be traceable to a specific specimen in that collection including its history of maintenance and handling in that collection. If it is derived from field material meta data should be documented; i.e. when and where the material was collected, from what plant species and part and whether or not it was showing which symptoms. In case RM is mixed with matrix material, the matrix should be properly be identified and described.

Commutability: Describe the extent to which the RM is similar to actual samples.

Homogeneity: Homogeneity should generally be determined after the reference material has been packaged in its final form. EPPO guideline PM 7/122 recommends to test a minimum of 10 randomly chosen samples (for each pest/ matrix/infestation level, including negative samples) in duplicate.

Stability: RM should be tested to determine it is sufficiently stable for its intended purpose and will not undergo any significant changes for a required period of time, including transport and storage, that may influence the test result. Minimum period of time and the required conditions should be clearly indicated in the documentation accompanying each individual lot of RM. The number of aliquots to be tested for stability also depends on the quantity of the reference material produced.

Assigned value: Assigned quantity values correspond to the expected result of the test (pest present or absent, concentration of the pest, morphological characteristics of the specimen, etc.). Include the method used to determine the assigned value.

Purity: defined as a ratio of target pest versus non-targets, particularly non-targets interfering with a test. Different levels of purity can be defined ranging from an absence of any non-targets to a relative high amount of non-target in your reference material. Determining the presence of non-targets should preferably be done through unbiased methods e.g. high throughput sequencing.

7 References

Include any relevant references.

Table 1: List of descriptors of criteria to be assessed in the production of reference material (RM).

Descriptor	Value	Minimum criterion
Intended use	**should be defined (in this case it equals preparation of RM for the scope of the individual test or TPS)**	**yes**
Identity	**identified to the level of internationally recognized diagnostic protocols**	**yes**
Traceability	traceability to a specimen from a reference culture collection	no
	traceability to a specimen from a working culture collection	no
	traceability provided for the target pest and matrix used (the latter if relevant)	**yes**
Commutability level	naturally infested plant material	no
	artificially infested plant material	no
	spiked plant material	no
	purified organisms	no
	purified nucleic acids	no
	synthetic nucleic acids	**yes**
Homogeneity	**Homogenous** *e.g. test 10 aliquots in duplicate for positivity*	**yes**
Stability	**Stable** *Describe storage conditions & period of stability in tests*	**yes**
	stability - short term	no
	stability - long term	no
Assigned value	absolute concentration known	no
	level of concentration known (high/medium/low)	no
	qualitative status known (above LOD level)	no
	originating from plants with known health statues with a recent test result (a given period of time depends on the plant-pest combination and previous experience)	**yes**
Purity	absence of non-targets	no
	absence of interfering non-targets	no
	known ratio of target VS non-target interfering with the test - high	no
	known ratio of target VS non-target interfering with the test - medium	no
	known ratio of target VS non-target interfering with the test - low	**yes**

ANNEX 2

Standard Operating Procedure (SOP) for the production of *Fusarium circinatum*

title	**SOP Production of Reference Material of *Fusarium circinatum***			
code		version 1	date 01-03-2019	page x of y

Version	date	explanation

1 Scope/intended use

This is a standard operating procedure (SOP) for the production of ***Fusarium circinatum*** as reference material as purified organism/purified nucleic acid for use in PCR and Real-time PCR tests. It describes the different steps required in the production process, ranging from the different possible sources of the reference material, tests to confirm its identity, possibly required multiplication steps to the actual production process. For each step in the process, criteria and critical points are identified. For traceability of materials and future adjustments of use, registration of all steps and tests in the production of a given reference material is important and are registered in E-lab under the VALITEST directory.

2 Definitions

Assigned value: Assigned quantity values correspond to the expected result of the test (pest present or absent, concentration of the pest, morphological characteristics of the specimen, etc.) (EPPO Guidelines PM 7/122).

Certified reference material (CRM): Reference material derived from a source that certifies the authenticity of the material. Preferably material should come from an internationally recognised source such as a national reference collection. The material should have a unique identification code allowing traceability and the name of the person who certifies its authenticity. Details of how the material was authenticated should also be supplied. If appropriate, information about its activity (e.g. pathogenicity, antigenic properties) under specified conditions should also be supplied along with any related uncertainty at a stated level of confidence (EPPO PM 7/76 (5)).

Commutability: a characteristic describing the extent to which they resemble actual samples. A reference material would be considered commutable when a measurement produces the same result for a it as it does for an authentic sample that contained the same analyte concentration.

Homogeneity: The assessment of homogeneity should generally be performed after the samples have been packaged in the final form and before distribution to participants. Homogeneity can be demonstrated prior to packaging where no influence of packaging is reasonably expected (EPPO PM 7/122). This guideline provides further information on the assessment of homogeneity for different types of material also stating: the current available guidelines recommend to test a minimum of 10 randomly chosen samples (for each pest/ matrix/infestation level, including negative samples) in duplicate.

Identity: The reference material should be clearly identified and characterized at least to the extent ensuring its correct identification. Following the recommendation below this should be done by at least two preferably validated and independent tests, based on different physical characteristic

of the RM. If the specimen used originates from and is available in several reference collections working according to commonly agreed quality standards this provides additional guarantee into its identity. At minimum, the material should be thoroughly identified to the level of international accepted diagnostic protocols (when available) to ensure it is properly identified. The list of tests used for its identification should be clear from its description.

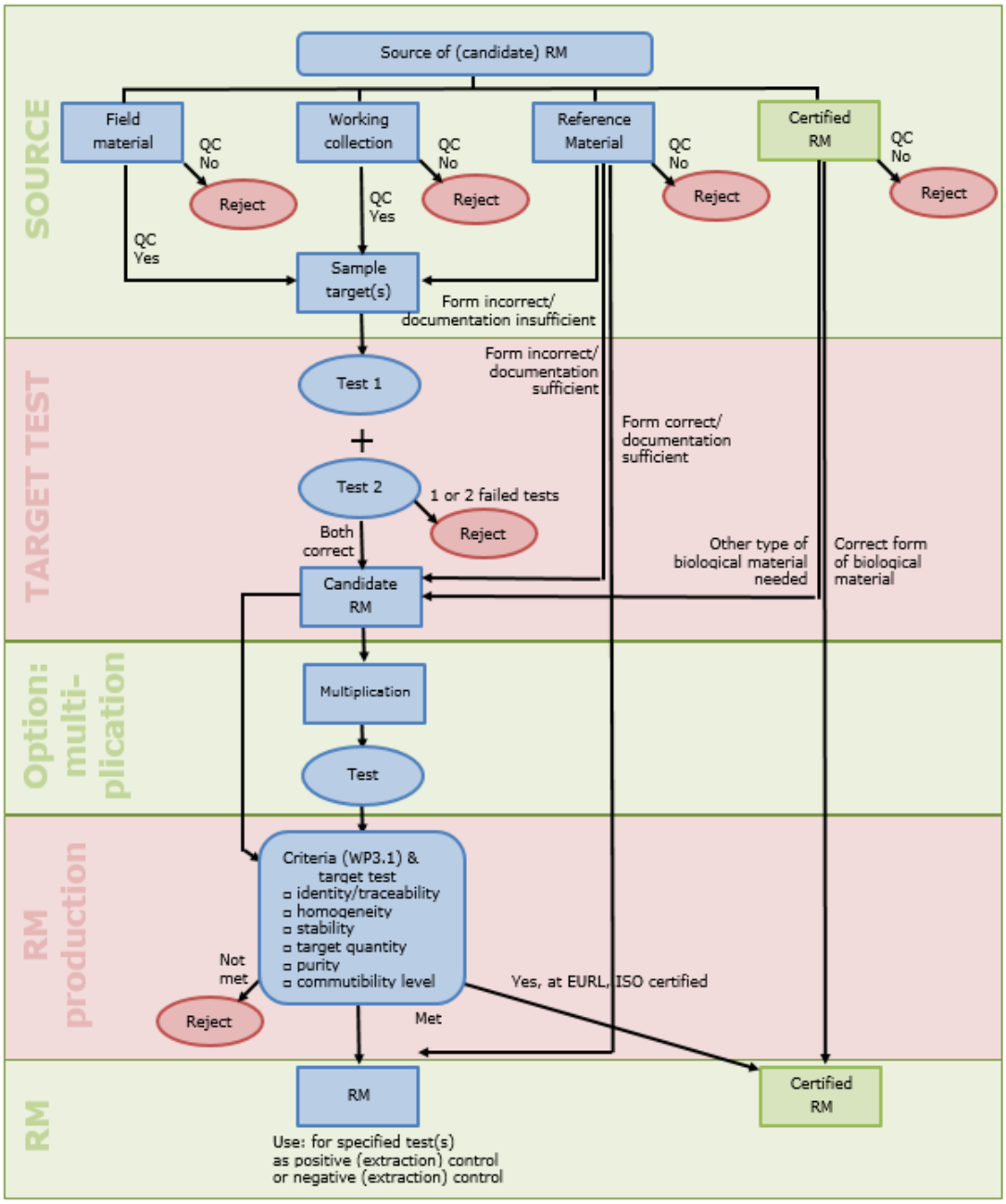

Figure 1: Graphical presentation of the general Standard Operating Procedure (SOP) for the production of reference material to be used in specified tests as either positive or negative samples.

Multiplication (of RM): if more or a different type of biological material of a certain candidate reference material is needed, the material may be used for multiplication/ amplification. This may for instance mean amplification in or on plants, but also be an amplification of specific target genes.

NAC: negative amplification control.

NC: negative control.

NIC: negative isolation control.

PAC: positive amplification control.

PC: positive control.

Pest (IPPC, 2017): any species, strain or biotype of plant, animal or pathogenic agent injurious to plants or plant products.

PIC: positive isolation control.

Purity: Purity is defined as a ratio of target pest versus non-targets, particularly non-targets interfering with a test.

Quality assurance: part of quality management, focussed on providing confidence that quality requirements will be fulfilled. Dependent on the laboratories own quality management system, samples and materials may be rejected if basic quality requirements are not met. For example, samples clearly contaminated with non-target organisms or samples transported under sub-optimal conditions may be discarded without further testing

Reference material (RM): Material appropriate to the test and diagnosis being performed such as live cultures, infected plant material, DNA/RNA preparations, images of a diagnostic quality or mounted specimens. The reference material used should be documented and appropriate for the test and diagnosis being performed. It should be ensured that it has the features for which it was selected, for example expressing a desired antigen for use in serological diagnosis or display specific physical features (e.g. sporulation) if used for morphological diagnosis (EPPO PM 7/76 (5)).

Reference collection: A collection of individuals maintained for the purpose of study and authentication. Reference collections are generally large undertakings maintained by institutions; instead of having a single representative of each species, they will typically have multiples, so as to illustrate variations and, be able to provide samples externally for comparisons and research. Reference collections are an important source of information about variations of populations within a species. They are also the repository of type strains or holotypes used as the official definition of a particular species.

Stability: Samples should be demonstrated to be sufficiently stable to ensure that they will not undergo any significant change, including storage and transport conditions. The number of aliquots to be tested for stability also depends on the quantity of the reference material produced.

Test: the application of a method to a specific pest and a specific matrix.

Traceability: Traceability can be considered as an aspect of both identity and availability in the sense that it may provide some additional guarantees to the correct identity of the materials used to prepare

the reference material and its future availability. To ensure traceability it is important to properly identify and describe the matrix as well and ensure its proper identification. Collecting meta-data otherwise used for samples is suitable.

Working collection: Collections, usually of individuals belonging to a single organism or group of related organisms, maintained for the purpose of scientific investigation by experts. Working collections are usually maintained by individual researchers or research groups with recognized knowledge of the organism(s) in question. They do not usually provide samples externally other than to deposit individuals of interest into one or more reference collections for safe keeping and/or protection of intellectual property.

3 Source of (candidate) reference material

The fungus *F. circinatum* is the causal organisms of pitch canker in pine (pitch because of its often spectacular resin exudates), a destructive disease of *Pinus* spp. and Douglas-fir (*Pseudotsuga menziesii*). The disease results in extensive tree mortality, reduced growth and timber quality. Multiple branch infections may cause severe crown dieback and eventually lead to the death of the tree, while it can also cause root rot. This aggressive fungus may also cryptically infect the Pinus seeds and may cause damping-off in seedlings. Conifer seeds can be colonized by *F. circinatum* internally (where it can remain dormant until seed germination) and externally (Storer et al., 1998).

F. circinatum is predominantly a wound pathogen and enters the host tree through mechanical wounds or feeding holes caused by woodboring insects. The taxon is listed as a quarantine fungus in Europe and several other regional plant protection organizations throughout the world. Whereas long-distance spread of the disease is made possible through the trade of infected pine seeds, local spread is caused by aerial dispersion or insect transportation of the fungal conidia.

The fungus is officially reported in the USA, Mexico, Haiti, South Africa, Japan, Chile (OEPP⁄EPPO, 2005) and has been officially reported in the EPPO region only recently: Spain (Landeras et al., 2005; under eradication), Italy (Carlucci et al., 2007 eradicated), France (OEPP⁄EPPO, 2008 under eradication). In most instances of introduction into new areas the pest was first found in nurseries.

Culture collection = *Reference material*

The type strain of *F. circinatum* (CBS 405.97) was obtained as a lyophilized culture from the Westerdijk Fungal Biodiversity Institute, Utrecht (NL) (PM7/91). Strain material comes under MTA. The material is intended to be used in the form of as purified organism/purified nucleic acid.

4 Identification of candidate reference material

A list of recommended tests for validation of target species is given below. In principle two independent tests (on different characters) should be performed for the target organism(s).

(1) Diagnostic method A: culture and morphology

Cultures are observed on Potato dextrose agar (PDA) and Spezieller-Nährstoffarmer Agar (SNA) plates for morphological identification. The correct morphological identification of *F. circinatum* in pure culture requires experience and a molecular confirmation should be carried out in case of uncertainty (PM7/91).

(2) Diagnostic Method B: Molecular tests

Direct detection in planta using molecular techniques (plant tissue, including seeds) include conventional PCR, SyBr green real-time PCR or dual-labelled probe real-time PCR; Besides IGS amplicon sequencing or WGS can be applied to confirm identity. The molecular tests rely on a previous DNA extraction.

(3) DNA extraction from pure culture

Fungal DNA should be extracted using an appropriate standard method for DNA extraction from fungi e.g. regular commercial plant DNA extraction kits (or other methods reviewed in Irlinger et al., 2008) and analysed following any of the tests.

(4) Molecular methods

There are several molecular methods currently available to confirm the identity of *F. circinatum* isolated in pure culture or to detect and identify directly *F. circinatum* in planta.

- A PCR-RFLP (Restriction Fragment Length Polymorphism) test, with primers and RFLP pattern developed by Steenkamp et al. (1999) and is appropriate for identification of the anamorphic stage of F. circinatum in pure culture only as contaminants or host material may affect the quality and numbers of PCR amplicons.
- SyBr green real-time PCR or conventional PCR tests with primers designed by Schweigkofler et al. (2004) can be useful for identification of the fungus in pure culture, as well as for direct detection of the pathogen in seeds. However, when carried out on plant samples DNA, verification of the nature of the PCR amplicon should be carried out by sequencing for conventional PCR, or by melting analysis for SyBr green real-time PCR PCR cross-reaction might occur with phylogenetically close Fusarium sp., especially with high amounts of Fusarium template DNA.
- Method for real-time PCR with primers and a dual-labelled probe designed by Ioos et al. (2009) can be useful for identification of the fungus in pure culture, as well as for direct detection of the pathogen in plant tissue, including seeds. This method proved to be more sensitive than the conventional PCR (diagnostic sensitivities of 79.1% and 58.6%, respectively; Ioos et al., 2009) and its specificity is strengthened thanks to the combination of specific primers and probe.
- Sequencing: Regions of the IGS rDNA, such as that amplified by the CIRC1A/CIRC4A primers (Schweigkofler et al., 2004), or the region of the translation elongation factor 1-alpha (EF-1alpha) gene amplified by the EF1/EF2 primers (O'Donnell et al., 1998), must be sequenced and used for species identification. The CIRC1A/CIRC4A PCR product may be generated from DNA extracted from a pure fungal culture or from plant tissue or seeds, whereas the EF1/EF2 PCR product may be generated only from DNA extracted from a pure fungal culture. The EF-

1alpha sequence is sufficient to assign the identity of a Fusarium strain to *F. circinatum* (O'Donnell et al., 1998) but other markers may also be useful (e.g. largest RNA polymerase II B-subunit (RPB1), second largest RNA polymerase II B-subunit (RPB2), beta-tubulin, IGS) (Steenkamp et al., 2002; O'Donnell et al., 2010). The universal barcode ITS, while very useful for fungi in general, should not be used for the Fusarium genus as it is not sufficiently polymorphic for several closely related species, including *F. circinatum*.

5 Multiplication of material

Cultures are best grown on potato dextrose agar (PDA), potato dextrose broth (PDB) and Spezieller-Nährstoffarmer Agar (SNA) for growth and spore production. Transfer under aseptic conditions under the required safety conditions. Optimal growth conditions 25-27 °C, in dark or UV light conditions. Isolations of nucleic acids as described above.

6 Criteria for reference materials

Target tests of this reference material are the (real-time) PCR tests described in the section above. All RM criteria and their assessed values are listed in Table 1.

Table 1: List of descriptors of criteria assessed in the production of *Fusarium circinatum* as reference material (RM).

Descriptor	Value	Minimum criterion
Intended use	(real-time) PCR tests described in the section above	**yes**
Identity	**identified to the level of internationally recognized diagnostic protocols:** Culture collection strain CBS 405.97, identified based on morphology and multi-locus sequencing (26S; 18S; ITS and EF1) and in-house sequencing EF-1α as confirmation	**yes**
Traceability	traceability to a specimen from a reference culture collection: Type strain CBS 405.97	**yes**
Commutability level	purified organisms	**yes**
	purified nucleic acids	**yes**
Homogeneity	*Homogeneous (tested: 10 samples checked in duplicate in RT-PCR; all positive)* *Genomic DNA extract in extraction buffer at a concentration of 10 ng/µl (3 ml); in aliquots of 5 µl*	**yes**
Stability	**Stable** (as tested by real-time PCR)	**yes**
	stability - short term; *5 °C/RT 1 week*	
	stability - long term; *-20 °C > 1 years* *-80 °C > several years*	
Assigned value	absolute concentration known: *10 ng/µl*	**yes**
Purity	absence of non-targets	**yes**

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PM7/91 Diagnostics Gibberella circinata EPPO

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