International Rules for Seed Testing 2020

Validated Seed Health Testing Methods

7-009: Detection of *Fusarium circinatum* in *Pinus* spp. (pine) and *Pseudotsuga menziesii* (Douglas fir) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2019, Hyderabad, India

Effective from 1 January 2020

Validation reports

See References. Copies are available by e-mail from the ISTA Secretariat at ista.office@ista.ch.

Please send comments, suggestions or reports of problems relating to this method to the ISTA Seed Health Committee, c/o ISTA Secretariat.

Disclaimer

Whilst ISTA has taken care to ensure the accuracy of the methods and information described in this method description, ISTA shall not be liable for any loss or damage, etc. resulting from the use of this method.

Safety precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during weighing out of ingredients. It is assumed that persons carrying out this test are in a laboratory suitable for carrying out microbiological procedures and familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic techniques. Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Note on the use of the translations

The electronic version of the International Rules for Seed Testing includes the English, French, German and Spanish versions. If there are any questions on interpretation of the ISTA Rules, the English version is the definitive version.

Published by
The International Seed Testing Association (ISTA)
Zürichstr. 50, CH-8303 Bassersdorf, Switzerland

Online ISSN 2310-3655

7-009: Detection of *Fusarium circinatum* in *Pinus* spp. (pine) and *Pseudotsuga menziesii* (Douglas fir) seed

Host: *Pinus* spp.; *Pseudotsuga menziesii* (Mirb.) Franco
Pathogen(s): *Fusarium circinatum* Nirenberg & O'Donnell (syn. *Fusarium subglutinans* f. sp. *pini* Hepting, syn. *Fusarium lateritium* f. sp. *pini* Hepting, syn. *Gibberella circinata*)

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Revision history

Version 1.0, 2001-11-20: Revised J. Sheppard, V. Cockerell
Reprinted 2003
Version 1.1, 2008-01-01: Treated seed revised; Reporting results revised
Version 1.2, 2011-01-01: *Fusarium moniliforme* var. *subglutinans* changed to *Fusarium circinatum*
Version 1.3, 2012-01-01: Missing reference details added (Cockerell & Koenraadt, 2007)
Version 1.4, 2014-01-01: Addition of positive control; scientific name of pathogen changed from *Fusarium circinatum*
Version 2.0, 2015-01-01: Method revised and replaced previous method
Version 2.1, 2017-01-01: Reporting results revised
Version 2.2, 2018-01-01: Changes to the taxonomic names of fungi

Background

Fusarium circinatum is the causal agent of pitch canker disease. The disease almost exclusively affects *Pinus* spp., but was also described on Douglas fir (*Pseudotsuga menziesii*). This disease is a serious threat to the pine forests wherever it occurs (especially on plantations of *Pinus radiata*), due to extensive tree mortality, reduced growth and timber quality. Conifer seeds can be colonised by *F. circinatum* internally (where it can remain dormant until seed germination) and externally (Storer *et al.*, 1998).

Although an official ISTA method was published in 2002 to detect *F. moniliforme* f. sp. *subglutinans* in seeds of *Pinus taeda* and *Pinus elliotii* (ISTA, 2002), the morphological features indicated as typical for *F. moniliforme* f. sp. *subglutinans* in this method were based on a substrate not showing the characteristic sterile hyphae of this pathogen and were not sufficient to ensure a reliable identification of *F. circinatum* Nirenberg & O'Donnell (anamorphic stage of *Gibberella circinata*). This method and the accompanying figures are taken from the EPPO diagnostic protocol PM 7/91 (EPPO, 2009).This method has been evaluated through a European ring test (Ioos *et al.*, 2013). This method is very efficient and reliable to isolate any *Fusarium* spp. from infected seeds and does not require expensive equipment. However, the correct morphological identification of *F. circinatum* in pure culture requires experience and a molecular confirmation should be carried out in case of uncertainty, such as those described in EPPO (2009) and in Ioos *et al.* (2013). In addition, Storer *et al.* (1998) have demonstrated that agar plating of pine seeds may not be able to detect dormant (quiescent) propagules of *F. circinatum*, thus leading to an unknown risk of false negative results.

This protocol replaces the former ISTA procotol '7-009: Detection of *Fusarium moniliforme* var. *subglutinans* on *Pinus taeda* and *P. elliotii* (pine)', which did not take into consideration the more recent taxonomic re-assignation of *Fusarium moniliforme* var. *subglutinans* to *F. circinatum* (Nirenberg & O'Donnell, 1998), and in particular the production of typical sterile hyphae by this species.

This method and the accompanying figures are taken with permission from the EPPO diagnostic protocol PM 7/91 (EPPO, 2009).

Treated seed

This method has not been validated for treated seeds. Seed treatments may affect the performance of the method. (Definition of treatment: any process, physical, biological or chemical, to which a seed lot is subjected, including seed coatings. See 7.2.3.)

Sample size

The sample (total number of seeds tested) and subsample size to be tested depends on the desired tolerance standard (maximum acceptable percentage of seeds infected) and detection limit (theoretical minimum number of pathogen propagules per seed which can be detected). The minimum recommended sample size is 400 seeds.

Materials

Reference material: reference cultures or other appropriate material

Media: PDA, DCPA and SNA

Petri dishes: When sowing density is given by a number of seeds per Petri dish, a diameter of 90 mm is assumed

Incubator: Incubator set at 22 ±6 °C, with a day/night alternation for light, or incubate at room temperature (22 ±6 °C) on the bench with natural lighting

Sample preparation

It is vital to exclude any possibility of cross-contamination between seed samples. It is therefore essential to disinfect all surfaces, containers, hands etc. both before and after handling each sample. This can be achieved by swabbing/spraying equipment and gloved hands with 70 % ethanol.

The seeds must be analysed without any surface disinfection, as *F. circinatum* may be present on the seed husk, as well as inside the seed.

Methods

Critical control points are indicated by CCP.

1. Plating: Seeds are directly plated onto *Fusarium* DCPA semi-selective media. Depending on the size of the seeds, from three to five seeds can be plated per Petri dish.
2. Incubation: Plates are incubated at room temperature or in an incubator (22 ±6 °C), with a day/night alternation of light, or incubated at room temperature on the bench with natural lighting.
3. Reference material: Subculture a reference culture to a DCPA plate at the same time the seeds are plated and incubate with the test plates.
4. Examination: During incubation, the plates are observed periodically at a magnification of ×100 without removing the lid. All the *Fusarium* spp. colonies for which microconidia are aggregated in false heads (Fig. 2a, b), with branched conidiophores and mono- and polyphialidic conidiophores, are transferred in aseptic conditions to potato dextrose agar (PDA) and to spezieller nährstoffarmer agar (SNA) for species assignation, based on morphology. For morphological identification, the isolates are grown on PDA to study colony morphology and pigmentation, and on SNA to study formation and type of microconidia and conidiogenous cells. All isolates grown on PDA and SNA are examined after 10 days and confirmed as *F. circinatum* based on the morphological features described by Nirenberg & O'Donnell (1998) and Britz *et al.* (2002).
   On PDA, *F. circinatum* grows relatively rapidly (average growth of 4.7 mm/day at 20 °C; Nirenberg & O'Donnell, 1998). After 10 days, the colony should have an entire margin, white cottony or off-white aerial mycelium with a salmon tinge in the middle or with a purple or dark violet pigment in the agar (Fig. 1).
   On SNA, microconidia are aggregated in false heads (Fig. 2a, b), with branched conidiophores and mono- and polyphialidic conidiophores (Fig. 3), and obovoid microconidia in aerial mycelium, mostly nonseptate or with occasionally one septum. Chlamydospores are absent. The sterile hyphae (coiled/not distinctively coiled) are characteristic of *F. circinatum* and are observed clearly on this medium (Fig. 4a, b). The epithet '*circinatum*' refers to these typical coiled hyphae, also called 'circinate' hyphae. In case of doubt about the presence of typical sterile hyphae, a molecular confirmation should be carried out, such as those described in EPPO (2009) and in Ioos *et al.* (2013).
5. Record the number of infected seeds in each plate.

Note 1: *Fusarium circinatum* is classified as a quarantine fungus for numerous national plant protection organisations. All the isolates of *F. circinatum* or culture putatively identified as *F. circinatum* should be incubated and handled with an appropriate level of biosafety containment.

Note 2: This method is based on the isolation of the target fungus in culture from the seeds, and thus requires that viable and non-quiescent propagules of the fungus are present. It was, however, demonstrated that agar plating of pine seeds may not be able to detect dormant (quiescent) propagules of *F. circinatum*, thus leading to an unknown risk of false negative results (Storer *et al.*, 1998)

General methods

Reporting results: The result of a seed health test should indicate the scientific name of the pathogen detected and the test method used. When reported on an ISTA Certificate, results are entered under 'Other Determinations'.

The report must indicate the number of seeds tested.

In the case of a negative result (pathogen not detected), the results must be reported as 'not detected'.

In the case of a positive result, the report must indicate the number of positive subsamples out of the total number tested.

Quality assurance

Specific training

This test should only be performed by persons who have been trained in fungal identification or under the direct supervision of someone who has.

Critical control points (CCP)

Preparation of plates: The source of agar may influence the results. The level of available nutrients may vary from manufacturer to manufacturer. Whenever a new batch of agar is used, a check on the quality should be made, using a reference lot with a known infection level, or a reference isolate and sustainability of isolate measured. Pay particular attention to the growth characteristics of reference isolates.

Media and solutions

Potato dextrose agar (PDA)

Potato dextrose agar: 39.0 g
Distilled/deionised water: to 1000 ml

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add distilled/deionised water to 1000 ml.
3. Dissolve and dispense into final containers.
4. Autoclave at 121 °C and 15 psi for 15 min.

Dichloran chloramphenicol peptone agar (DCPA)

(slightly modified by Ioos *et al.,* 2004; after Andrews & Pitt, 1986)

This medium is suitable for isolation of *Fusarium circinatum* from seeds, but not for identification.

Bacteriological peptone: 15.0 g
KH_2PO_4: 1.0 g
$MgSO_4$ ($7H_2O$): 0.5 g
Chloramphenicol: 0.2 g
2.6-Dichloro-4-nitroanilin (dichloran)(0.2 % W/V in ethanol): 2 mg
Violet crystal (0.05 % W/V in water, 1.0 ml): 0.5 mg
Technical agar: 20.0 g
Distilled/deionised water: to 1000 ml

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add distilled/deionised water to 1000 ml.
3. Dissolve and dispense into final containers.
4. Autoclave at 121 °C and 15 psi for 15 min.

Storage

Provided containers are tightly closed, the media may be stored in a fridge for several months before use, but not exceeding 3 months for DCPA.

Spezieller nährstoffarmer agar (SNA)

(Gerlach & Nirenberg, 1982)

This medium should be mandatory used for identification of *F. circinatum*, based on morphological features.

KH_2PO_4: 1.0 g
KNO_3: 1.0 g
$MgSO_4$ $7H_2O$: 0.5 g
KCl: 0.5 g
Glucose: 0.2 g
Sucrose: 0.2 g
Technical agar: 20.0 g
Distilled/deionised water: to 1000 ml

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add distilled/deionised water to 1000 ml.
3. Dissolve and dispense into final containers.
4. Autoclave at 121 °C and 15 psi for 15 min.

Optionally, two 1 cm square pieces of sterile filter paper may be laid on the surface of the agar, since *Fusarium* sporodochia are sometimes more likely to be produced at the edge of the paper.

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Validation references

Test performance study of diagnostic procedures for identification and detection of *Gibberella circinata* in pine seeds was carried out in the framework of a EUPHRESCO project (Ioos *et al.* 2013)

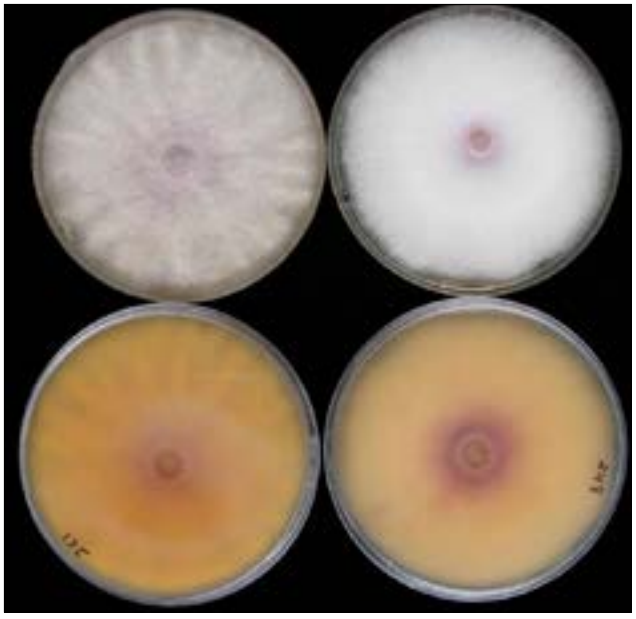

Figure 1. Cultural aspect of the anamorphic stage of *Fusarium circinatum* on potato dextrose agar (left: *Fusarium circinatum* MAT-1; right: *Fusarium circinatum* MAT-2). The MAT-1 mating type produces typical coiled sterile hyphae on spezieller nährstoffarmer Agar (SNA), whereas the MAT-2 mating type produces not distinctively coiled or even uncoiled sterile hyphae (see also Figs. 4a and b). (Courtesy of A. Pérez-Sierra)

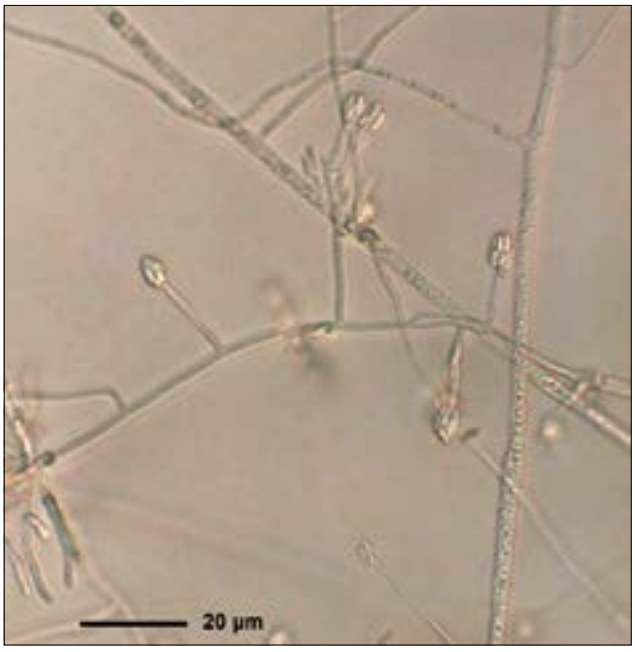

Figure 2a. Erect conidiophores bearing microconidia arranged in false heads of *Fusarium circinatum*, observed on a microscope slide. ×400. (Courtesy of A. Pérez-Sierra)

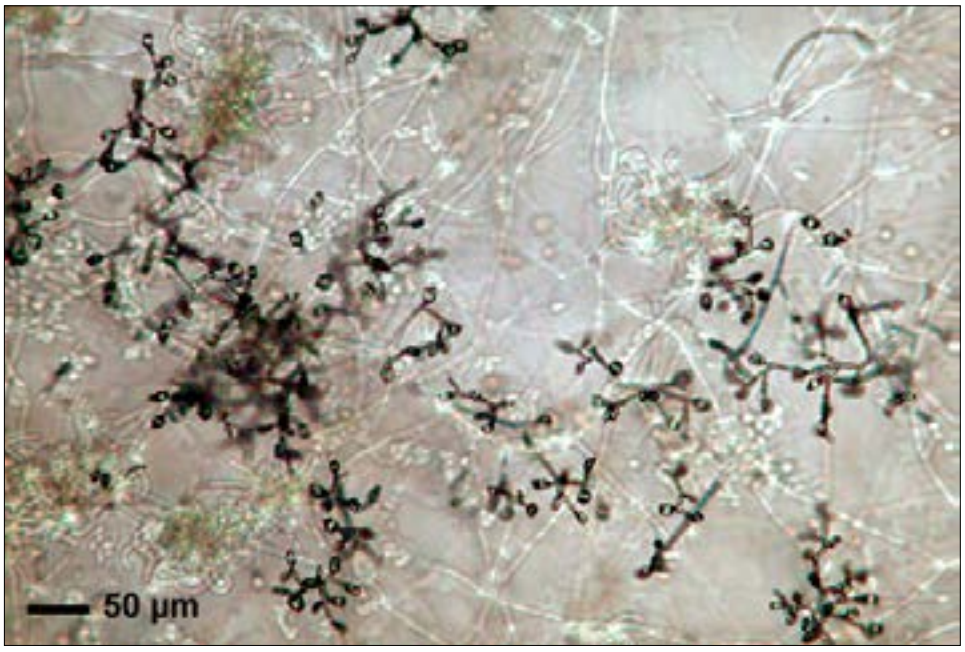

Figure 2b. Erect conidiophores bearing microconidia arranged in false heads of *Fusarium circinatum*, observed directly on spezieller nährstoffarmer agar (SNA) medium. ×200. (Courtesy of R. Ioos)

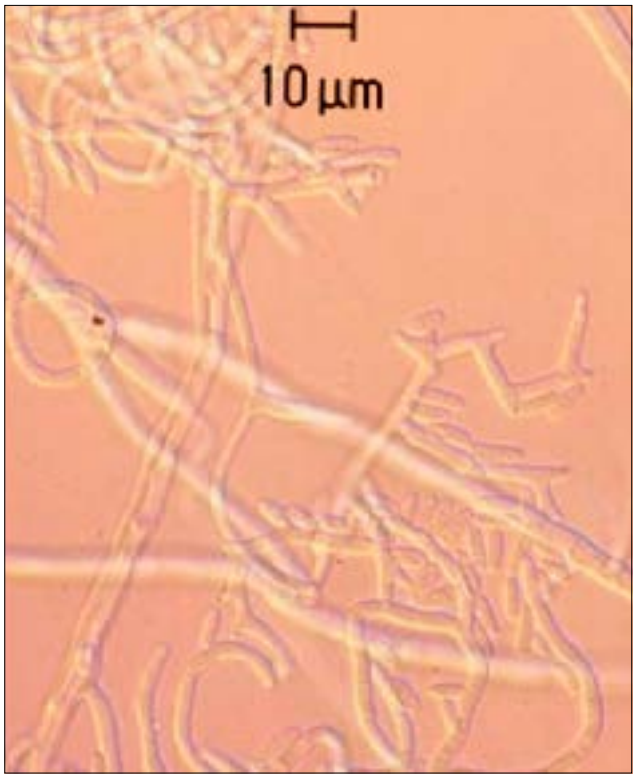

Figure 3. Mono- and polyphialidic conidiophores of *Fusarium circinatum* observed on spezieller nährstoffarmer agar (SNA) medium. (Courtesy of J. Armengol)

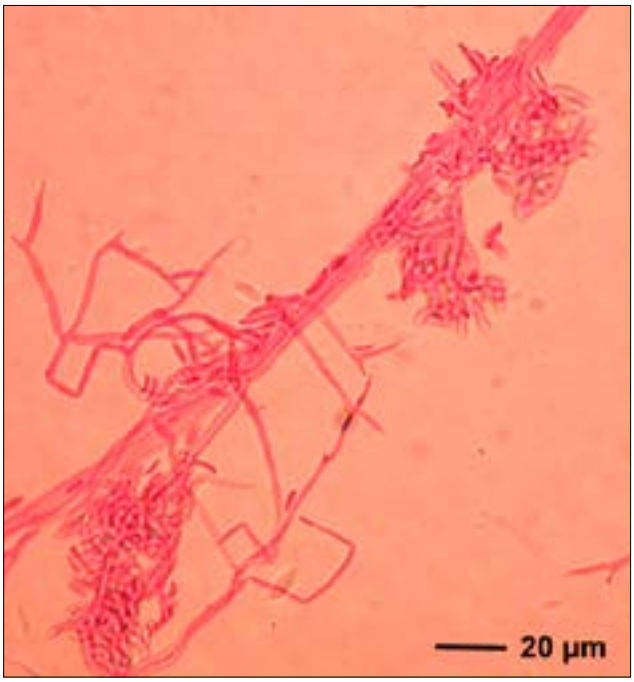

Figure 4a. Groups of coiled sterile hyphae and polyphialidic conidiophores produced on spezieller nährstoffarmer agar (SNA). (Courtesy of R. Ioos)

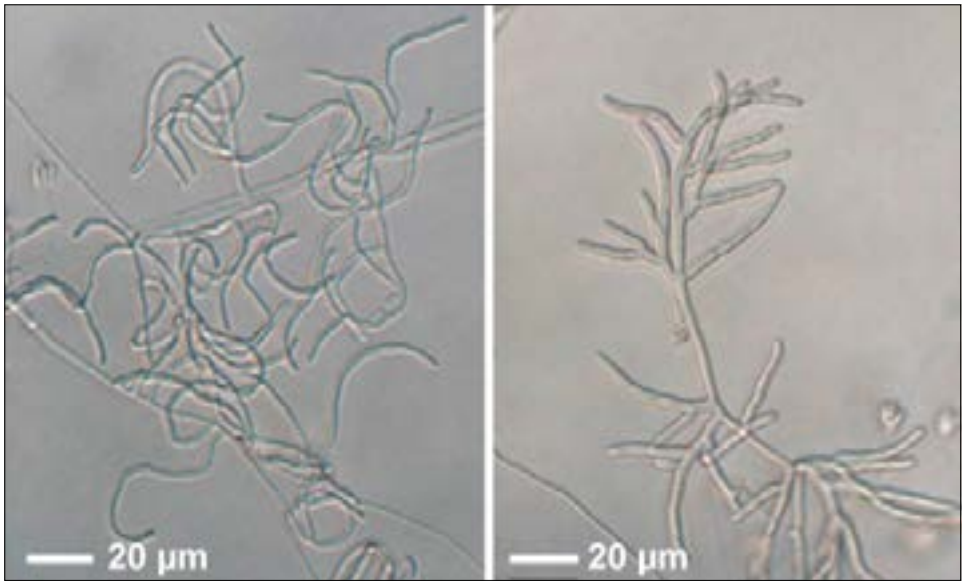

Figure 4b. Coiled and not distinctively coiled sterile hyphae produced on spezieller nährstoffarmer agar (SNA) medium by MAT-1 (left) and MAT-2 (right) mating type isolates of *Fusarium circinatum*, respectively. (Courtesy of A. Pérez-Sierra)