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DIAGNOSTIC PROTOCOLS

DP 22:
Fusarium circinatum

Produced by the Secretariat of the
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ISPM 27
Diagnostic protocols for regulated pests

DP 22: *Fusarium circinatum*

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1. Pest Information

Fusarium circinatum is an ascomycete fungus formerly described as the anamorph of *Gibberella circinata* (Geiser *et al.*, 2013) and it is the causal agent of pitch canker disease. The disease almost exclusively affects *Pinus* spp., but has also been described on *Pseudotsuga menziesii* (Douglas fir). *F. circinatum* has been found in asymptomatic grasses (Poaceae) near native stands of pine trees with symptoms of the disease (Swett and Gordon, 2012; Swett *et al.*, 2014). The disease affects plantations and nurseries in several countries worldwide and is a serious threat to pine forests wherever it occurs (especially on *Pinus radiata*) as it results in extensive tree mortality, reduced tree growth and reduced timber quality. *F. circinatum* causes cankers that girdle branches, aerial roots and even trunks of *Pinus* spp. Cankers are often associated with conspicuous resin exudates ("pitch"). Multiple-branch infections may cause severe crown dieback and eventually lead to the death of the tree. This fungus may also infect *Pinus* spp. seeds and may cause damping off in seedlings in nurseries. It has been found in regions of North, Central and South America, Asia and South Africa and has been officially reported in parts of southern Europe. Information on its distribution, updated regularly, is available in the European and Mediterranean Plant Protection Organization (EPPO) Global Database (https://gd.eppo.int/) and the CABI Invasive Species Compendium (http://www.cabi.org/isc/datasheet/25153).

F. circinatum is predominantly a wound pathogen that enters the host tree through mechanical wounds or the feeding holes of wood-boring insects. If a wound is not deep enough for the pathogen to reach water within host tissues, ambient moisture or very high relative humidity is required for spore germination. Conidia of *F. circinatum* germinate over a wide range of temperatures; slowly at 10 °C and progressively faster with increasing temperature, up to an optimum around 20 °C (Inman *et al.*, 2008). In nature *F. circinatum* is known to propagate only asexually, through production of microconidia and macroconidia. Both spore types are borne in a viscous liquid and appear better suited to dispersal by splashing water or attachment to motile organisms than to aerial dispersal. However, microconidia and macroconidia can become airborne and they are presumably the primary propagules recovered by air sampling in areas where pitch canker is found (Correll *et al.*, 1991). The fungus may be spread from tree to tree by aerial dispersal of the conidia or through vectors (Gordon *et al.*, 2001; Schweigkofler *et al.*, 2004). However, long-range dispersal of the pathogen from affected areas to disease-free areas may be driven by the movement of infected seeds or infected plant material (Storer *et al.*, 1998) or via vectors associated with logs and other unmanufactured wood articles (Tkacz *et al.*, 1998). Conifer seeds can be colonized by *F. circinatum* internally (where it can remain dormant until seed germination) and externally on the seed coat (Storer *et al.*, 1998). In many pine species, seed contamination may be restricted to the seed coat (Dwinell, 1999).

F. circinatum may also produce perithecia, which contain meiotically derived spores (ascospores). However, perithecia are rarely produced on culture media under laboratory conditions and they have not been observed in nature.

2. Taxonomic Information

Name: *Fusarium circinatum* Nirenberg & O'Donnell, 1998

Synonyms: *Fusarium subglutinans* f.sp. *pini* J.C. Correll, T.R. Gordon, A.H. McCain, J.W. Fox, C.S. Koehler, D.L. Wood & M.E. Schultz, 1991; *Gibberella circinata* Nirenberg & O'Donnell ex Britz, T.A. Cout., M.J. Wingf. & Marasas, 2002

Taxonomic position: Eukaryota, Fungi, Dikarya, Ascomycota, Pezizomycotina, Sordariomycetes, Hypocreomycetidae, Hypocreales, Nectriaceae

Common name: Pine pitch canker (in English only)

MycoBank: MB#444883

3. Detection

Although they may exhibit different levels of susceptibility to *F. circinatum*, all *Pinus* spp., along with *P. menziesii*, may be affected by the fungus, and the symptoms can be observed at any time of year. In addition, *F. circinatum* can affect plants of different ages, ranging from seedlings to mature trees, and it can be detected on all plant parts (roots, trunk, branches, shoots, cones and seeds). *F. circinatum* may also be soil-borne. There are no published methods for the isolation of *F. circinatum* from soil. This protocol describes the identification of *F. circinatum* on asymptomatic and symptomatic plant tissue and on seeds. The requirement for detection of *F. circinatum* is outlined in the flow chart in Figure 1. Plants and trees should be inspected for any typical symptoms of pine pitch canker (section 3.1.1) whereas seeds may be analysed by random sampling (section 3.2.2). Diagnostic method A, isolation and culture (section 3.3) and diagnostic method B, molecular tests (section 3.4), may both be used for plant tissue and seeds.

Because of the high diversity and complexity of the *Fusarium* genus, and challenges in using morphological characters to distinguish *F. circinatum* from other members of the *Fusarium fujikuroi* species complex, it is recommended that diagnosis by both method A and method B is confirmed by DNA sequence analysis of the isolated fungus, particularly if the outcome is critical (e.g. post-entry quarantine sample, new host record, new country record). PCR cross-reaction might occur with phylogenetically close *Fusarium* spp., such as the *Fusarium* species recently described from Colombia (Herron *et al.*, 2015).

3.1 Symptoms

3.1.1 Trees

Root infection. Symptoms – brown discoloration and disintegration of the cortex – are similar to symptoms caused by other root rot pathogens. Root symptoms may lead to above-ground symptoms, which are generally not apparent until the pathogen reaches the crown after it girdles the stem, causing yellowing of the foliage. Resin-soaked tissue may then be observed after removal of the bark on the lower part of the stem.

Aerial infection. Symptoms include yellowing of the needles, which turn red in time and finally drop, and dieback of the shoots. Multiple branch tip dieback, a result of repeated infections, may lead to a significant crown dieback. Cankers might appear on the shoots, on the main branches and even on the trunk, associated with conspicuous resin exudates (pitch) in response to the fungal infection (Figure 2). The cankers can girdle branches and even trunks.

Symptoms in older trees can be mistaken for those caused by *Sphaeropsis sapinea* (Fr.) Dyco & B. Sutton (synonym *Diplodia pinea*) (Sutton, 1980) or feeding damage caused by wood-boring insects. Therefore, the diagnosis should be based on laboratory testing. The resin bleeding sometimes coats the trunk and lower branches for several metres below the level of the infection. Stem cankers are flat or slightly sunken and sometimes affect large areas of cortical and subcortical tissue of the trunk. Removal of the bark reveals subcortical lesions with brown and resin-impregnated tissues (Figure 3).

Female cones. On infected branches female cones may also become affected and abort before reaching full size. However, depending on the timing and severity of infection, an infected cone may remain symptomless.

3.1.2 Seedlings and seed contamination

Seeds can be infected (Storer *et al.*, 1998). Infected seedlings usually show damping off symptoms: the needles turn red, brown or chlorotic and die from the base up, or the seedling dies (Figure 4). In some cases affected seedlings may show brown discoloration on roots and the lower part of stems. However, *F. circinatum* may infect seedlings without apparent symptoms.

It is reported in the literature that *F. circinatum* may sometimes be present in a quiescent form that cannot be detected in seeds by isolation (Storer *et al.*, 1998). Therefore, the absence of *F. circinatum*

cannot be ascertained by isolation from seeds. In contrast, non-viable propagules of *F. circinatum* may generate positive results using the molecular tests.

3.2 Sampling and sample preparation

3.2.1 Plant tissue (except seeds)

Whole seedlings should be placed in plastic bags that are then sealed and kept under cool conditions (4 °C) until they are sent to the laboratory. In the laboratory, the samples should be kept in a refrigerator at 4 °C until analysis, which should be preferably within two days of arrival.

For trunk or branch cankers, the inner bark of the area directly around the visible lesion should be cut repeatedly with a sterile blade until a canker margin is observed. Pieces of tissue, including phloem and xylem, should be removed in order to collect portions of the lesion edge, where the fungus is most active. The pieces of tissue should be wrapped in sheets of paper and placed in a plastic bag that is then sealed. All samples of plant material should be sent to the laboratory as soon as possible after sampling, and kept under cool conditions (4 °C) until transfer. In the laboratory, the samples must be kept in a refrigerator at 4 °C, to be analysed within two days of arrival.

3.2.2 Seeds

As no symptoms can be observed on seeds, the lot should be sampled randomly. As counting of seeds may be laborious, the sampled seeds may be weighed instead of counted. Depending on the method chosen for the identification, the total number of seeds to be tested per lot in order to detect the pathogen at different levels of infection in the lot may be different and needs to be determined statistically (useful guidance is given in tables 1 and 2 of ISPM 31 (*Methodologies for sampling of consignments*)). Sample size recommended by the International Seed Testing Association (ISTA) is 400 seeds for plating (ISTA, 2016). However, larger samples (e.g. 1 000 seeds) can easily be processed by biological enrichment before DNA analysis (Ioos *et al.*, 2009).

Seeds may be analysed by isolation and culture (section 3.3.2) or by conventional or real-time polymerase chain reaction (PCR) after a biological enrichment step (section 3.4.1.2). These methods have been compared in the framework of a European collaborative study, and performance values have been calculated for each of the methods (Ioos *et al.*, 2013).

3.3 Diagnostic method A: Isolation and culture

3.3.1 Plant tissue (except seeds)

For symptomatic seedlings the pathogen is isolated from the lower part of the stem or from the roots. The roots and the lower part of the stem are washed thoroughly with water and isolations are made from the leading edge of the lesions.

On mature trees, isolations are made from cankers. The cankers are washed thoroughly with water, and isolations are made from wood chips taken from the edge of the lesion found beneath the affected bark.

Plant material should be surface-sterilized for up to 1 min in a 1.5% solution of active sodium hypochlorite or 50% alcohol, and rinsed twice in sterile distilled water (Pérez-Sierra *et al.*, 2007). Selective media, such as dichloran chloramphenicol peptone agar (DCPA) or Komada's medium, are recommended for isolations. Potato dextrose agar supplemented with 0.5 mg/ml streptomycin sulphate salt (775 units/mg solid) (PDAS) can also be used (EPPO, 2005) (section 3.3.3).

Plates are incubated at 22 °C ± 6 °C under near ultraviolet (UV) light or in daylight. During incubation, the plates are observed daily and all the *Fusarium* spp. colonies are transferred to potato dextrose agar (PDA) and to Spezieller Nährstoffarmer agar (SNA) and incubated at 22 °C ± 6 °C under near UV light or in daylight for ten days.

3.3.2 Seeds

Seeds are analysed without any surface disinfection as *F. circinatum* may be present on the seed husk as well as inside the seed. Seeds are plated directly onto DCPA, Komada's medium or PDAS.

Plates are incubated at 22 °C ± 6 °C under near UV light or in daylight. During incubation, the plates are observed daily and all the *Fusarium* spp. colonies are transferred to PDA and to SNA (section 3.3.3) for morphological identification (section 4.1).

Although this method is time- and space-consuming when serial analyses are conducted, it does not require expensive equipment and it is efficient and reliable for isolating any *Fusarium* spp. from seeds. However, Storer *et al.* (1998) demonstrated that agar plating of pine seeds may not be able to detect dormant (quiescent) propagules of *F. circinatum.*

3.3.3 Culture media

Dichloran chloramphenicol peptone agar. DCPA is suitable for isolation of *Fusarium* spp. from plant tissue, including seeds, but not for identification. The medium, slightly modified by Ioos *et al.* (2004) after Andrews and Pitt (1986), contains 15.0 g bacteriological peptone, 1.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g chloramphenicol, 2 mg 2,6-dichloro-4-nitroaniline (dichloran) (0.2% (w/v) in ethanol, 1.0 ml), 0.0005 g crystal violet (0.05% (w/v) in water, 1.0 ml) and 20.0 g technical agar made up to 1 litre with distilled water.

Komada's medium. This medium is suitable for isolation of *Fusarium* spp. from plant tissue, including seeds, but not for identification. The base medium contains 1.0 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 10 mg Fe-Na-ethylenediaminetetraacetic acid (EDTA), 2.0 g L-asparagine, 20.0 g D-galactose and 15.0 g technical agar, made up to 1 litre with distilled water. The pH is adjusted to 3.8 ± 0.2 with 10% phosphoric acid. The medium is autoclaved at 121 °C for 15 min and slightly cooled before adding the following filter-sterilized supplements: 1.0 g pentachloronitrobenzene (PNCB) (75% (w/w)), 0.5 g ox-gall, 1.0 g $Na_2B_4O_7 \cdot 10H_2O$ and 6 ml/litre stock solution streptomycin (5 g streptomycin in 100 ml distilled water) (Komada, 1975).

Potato dextrose agar. PDA is used to study *Fusarium* spp. colony morphology and pigmentation. The medium contains 15 g dextrose, 20 g agar and the broth from 200 g white potatoes made up to 1 litre with distilled water (Hawksworth *et al.*, 1995). Commercially available preparations of PDA are as suitable as those made in the laboratory. PDAS can be used for isolation.

Spezieller Nährstoffarmer agar. SNA should be used to study the formation and type of microconidia, macroconidia and conidiogenous cells, but it is not recommended for isolation. The medium contains 1.0 g KH_2PO_4, 1.0 g KNO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose and 20.0 g technical agar made up to 1 litre with distilled water. Optionally, one or two 1 cm^2 pieces of sterile filter paper may be laid on the surface of the agar – *Fusarium* sporodochia are sometimes more likely to be produced at the edge of the paper (Gerlach and Nirenberg, 1982).

3.4 Diagnostic method B: Molecular tests

There are several molecular methods currently available for confirming the identity of *F. circinatum* isolates (identification by sequence analysis) or to detect and/or identify it directly *in planta* (conventional PCR, SYBR Green real-time PCR or real-time PCR using a hydrolysis probe). These methods are fast, efficient and reliable in detecting *F. circinatum* specifically, without agar plating, thus saving a lot of space and time, but they require facilities equipped for molecular testing. In addition, as these techniques target the DNA of the fungus, viable and non-viable cells of the pathogen are equally detected.

The real-time PCR using a hydrolysis probe offers enhanced specificity over the conventional PCR and the SYBR Green real-time PCR. Positive results obtained following real-time PCR using a hydrolysis probe are conclusive, whereas positive results obtained following conventional PCR or SYBR Green real-time PCR should be confirmed by sequence analysis.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

3.4.1 Preparation of material

3.4.1.1 Plant tissue (except seeds)

Potentially infected plant tissues (symptomatic and asymptomatic) are picked from the sample and first cut roughly using a sterile scalpel blade, without a prior surface disinfection step. Small pieces of approximately 0.5–1.0 cm^2 should be first collected then subsequently cut into smaller pieces (<2–3 mm^2, each side) into a sterile plastic Petri dish. The amount of tissue required for each reaction is recommended in the manufacturer's instructions for the DNA extraction kit being used. The sample, corresponding to approximately 200 µl, is transferred to a 2 ml microcentrifuge tube and ground for 2 min with two 3 mm steel or tungsten carbide beads and the quantity of lysis buffer recommended by the manufacturer and provided in the DNA extraction kit, at a frequency of 30 Hz with a bead beater (TissueLyser from Qiagen[1], or equivalent). The samples may also be ground in a mortar by a pestle with liquid nitrogen, or by using other efficient grinding techniques, such as a FastPrep homogenizer (MP Biomedicals[1]).

3.4.1.2 Seeds

A preliminary biological enrichment procedure should be followed when the presence of *F. circinatum* is tested by conventional or real-time PCR carried out directly on a seed DNA extract. The purpose of this preliminary biological enrichment step is to increase the biomass of viable *F. circinatum* propagules before DNA extraction and molecular testing. At least 400 seeds per seed lot are incubated at 22 °C ± 3 °C for 72 h in a cell culture flask with Difco Potato Dextrose Broth (PDB) (Becton, Dickinson and Company[1]) (ISTA, 2016). Depending on the species of *Pinus*, the average size of the seed may vary greatly and the quantity of PDB per flask should be manually adjusted so that the seed layer is almost completely overlaid by the liquid medium. After incubation, the entire contents of the flask (seeds and PDB) are transferred aseptically to a decontaminated mixer bowl of appropriate volume and are ground with a mixer mill until a homogenous solution is obtained. Sterile water or sterile PDB may be added at this step if the ground sample is too dense for pipetting. Two subsamples of approximately 500 µl are collected and transferred aseptically to individual 2 ml microcentrifuge tubes for DNA extraction.

3.4.1.3 Fungal culture

Fungal material is harvested from a pure culture grown for seven days on PDA by scraping the aerial mycelium using a sterile scalpel blade or a sterile needle. A pellet of approximately 2–3 mm diameter may be used directly for DNA extraction. It is recommended that before extraction the fungal material is ground in a mortar by a pestle with extraction buffer, or by using other efficient grinding techniques, such as a FastPrep homogenizer (MP Biomedicals[1]).

3.4.2 Nucleic acid extraction

Total DNA from plant tissue, seeds or fungal culture should be extracted preferably following the extraction protocol described by Ioos *et al.* (2009) using a commercial plant DNA extraction kit such as the NucleoSpin Plant II kit (Macherey-Nagel[1]), which has been proved to be efficient. Total DNA is extracted following the manufacturer's instructions with slight modifications. First, the chemical lysis incubation step (with lysis buffer) is extended to 20 min. After this incubation, the sample is centrifuged

[1]In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved. The use of names of reagents, chemicals or equipment in these diagnostic protocols implies no approval of them to the exclusion of others that may also be suitable. Laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated.

for 5 min at approximately 11 000 *g* to compact the debris and only the supernatant is recovered to be further processed following the manufacturer's instructions. Total DNA is finally eluted with 100 µl of the elution buffer provided in the kit and stored frozen until analysis. Total DNA or a 1:10 dilution, depending on the presence of inhibiting compounds, is used as a template for conventional or real-time PCR.

A quick DNA extraction method modified from Truett *et al.* (2000) can be used for fungal material. In this method, a small amount of aerial mycelium is disrupted in 40 µl of 25 mM NaOH, pH 12, in a 1.5 ml tube and 20 µl of the resulting solution is incubated in a 0.2 ml microcentrifuge tube for 15 min at 100 ºC and 5 min at 5 ºC in a thermocycler, then 20 µl of 40 mM Tris-HCl, pH 5, is added. The resulting lysate can be used directly as a template for PCR.

3.4.3 Detection of *Fusarium circinatum* by conventional PCR

A conventional PCR test with CIRC1A/CIRC4A primers, from the ribosomal (r)DNA intergenic spacer (IGS) region, designed by Schweigkofler *et al.* (2004), can be used for direct detection of the pathogen in plant tissue or seeds as well for identification of the fungus in pure culture. In all cases, the nature of the PCR amplicon should be verified by sequencing. Infection by other *Fusarium* spp. is frequent and cryptic speciation has been reported in the *Fusarium fujikuroi* species complex (Steenkamp *et al.*, 2002). In addition, PCR cross-reaction might occur with phylogenetically close *Fusarium* spp., especially when a large amount of *Fusarium* template DNA is used.

The primers are:

CIRC1A (forward): 5′-CTT GGC TCG AGA AGG G-3′

CIRC4A (reverse): 5′-ACC TAC CCT ACA CCT CTC ACT-3′

Using these primers and the PCR detailed in Table 1, a region of *F. circinatum*-specific IGS is amplified.

Table 1. CIRC1A/CIRC4A conventional PCR master mix composition, cycling parameters and amplicons

Reagent	Final concentration
PCR-grade water	–[†]
PCR buffer	1×
MgCl2	2 mM
dNTPs	250 µM
Primer CIRC1A	0.5 µM
Primer CIRC4A	0.5 µM
DNA polymerase	1 U
DNA (volume)	6.25 µl
Cycling parameters	
Initial denaturation[‡]	94 °C for 3 min
Number of cycles	45
Denaturation	94 °C for 35 s
Annealing	66 °C for 55 s
Elongation	72 °C for 50 s
Final elongation	72 °C for 12 min
Expected amplicons	
Size	360 bp

[†] For a final reaction volume of 25 µl.

[‡] According to the DNA polymerase manufacturer's instruction.

bp, base pairs; PCR, polymerase chain reaction.

The PCR products are separated by electrophoresis in a 1–2% agarose gel and visualized under UV light after staining.

3.4.3.1 Interpretation of results from conventional PCR

A sample will be considered positive if it produces a 360 base pair (bp) PCR product whose sequence shows 99–100% identity with a *F. circinatum* reference sequence (section 4.2), provided that the negative amplification control and negative extraction control are negative.

A sample will be considered negative if it does not produce a 360 bp PCR product, provided that the positive nucleic acid control and internal control are positive, or if it produces a 360 bp PCR product whose sequence does not show 99–100% identity with a *F. circinatum* reference sequence.

3.4.4 Detection of *Fusarium circinatum* by SYBR Green real-time PCR

A SYBR Green real-time PCR test with CIRC1A/CIRC4A primers designed by Schweigkofler *et al.* (2004) (see section 3.4.3 for their sequence) can be used for direct detection of the pathogen in plant tissue or seeds as well as for identification of the fungus in pure culture. In all cases, the nature of the PCR amplicon should be verified by sequencing for the same reasons as those presented in section 3.4.3.

Using these primers and the PCR detailed in Table 2, a region of *F. circinatum*-specific IGS is amplified.

Table 2. CIRC1A/CIRC4A SYBR Green real-time PCR master mix composition, cycling parameters and amplicons

Reagent	Final concentration
PCR-grade water	–†
PCR buffer	1×
MgCl2	2 mM
dNTPs	250 µM
Primer CIRC1A	0.5 µM
Primer CIRC4A	0.5 µM
SYBR Green	X‡
DNA polymerase	1 U
DNA (volume)	6.25 µl
Cycling parameters	
Initial denaturation*	94 °C for 3 min
Number of cycles	45
Denaturation	94 °C for 35 s
Annealing	66 °C for 55 s
Elongation	72 °C for 50 s
Expected amplicons	
Size	360 bp

† For a final reaction volume of 25 µl.

‡ Following the manufacturer's recommendation. May be directly included in a ready-to-use SYBR Green master mix.

* According to the DNA polymerase manufacturer's instruction.

bp, base pairs; PCR, polymerase chain reaction.

3.4.4.1 Interpretation of results from SYBR Green real-time PCR

The nature of the amplicons should be checked by the melting curves yielded at the end of the amplification and by comparison with the melting curves yielded with the positive nucleic acid control.

A sample will be considered positive if it produces a PCR product with a melting peak temperature identical to that of the positive nucleic acid control and whose sequence shows 99–100% identity with a *F. circinatum* reference sequence (section 4.2), provided that the amplification curve is exponential and that the negative amplification control and negative extraction control are negative.

A sample will be considered negative if it does not produce a PCR product with a melting peak temperature identical to that of the positive nucleic acid control, provided that the positive nucleic acid control and internal control are positive, or if it produces a PCR product whose sequence does not show 99–100% identity with a *F. circinatum* reference sequence.

3.4.5 Detection and identification of *Fusarium circinatum* by real-time PCR using a hydrolysis probe

Ioos *et al.* (2009) described a technique based on a real-time PCR using a hydrolysis probe designed from the rDNA IGS region to identify the anamorphic stage of *F. circinatum* in pure culture or directly in plant samples. This PCR test produces a 149 bp amplicon for *F. circinatum* (sequences of the IGS region for *F. circinatum* may be retrieved from GenBank, accession numbers AY249397 to AY249403). A *F. circinatum*-specific region of IGS is amplified using the primer pair FCIR-F/FCIR-R and is detected by a fluorescent hydrolysis probe, FCIR-P. This method has proved to be more sensitive than the conventional CIRC1A/CIRC4A PCR by detecting as little as 8 fg target DNA per reaction, and its specificity is higher (Ioos *et al.*, 2009).

The primers and probe are:

FCIR-F (forward primer): 5′-TCG ATG TGT CGT CTC TGG AC-3′

FCIR-R (reverse primer): 5′-CGA TCC TCA AAT CGA CCA AGA-3′

FCIR-P (probe): 5′-FAM-CGA GTC TGG CGG GAC TTT GTG C-BHQ1-3′

Using these primers and the PCR detailed in Table 3, a region of *F. circinatum* IGS is amplified.

Table 3. FCIR-F/-R/-P real-time PCR using a hydrolysis probe master mix composition and cycling parameters

Reagent	Final concentration
PCR-grade water	–[†]
PCR buffer	1×
MgCl2	5 mM
dNTPs	200 µM
Primer FCIR-F	0.3 µM
Primer FCIR-R	0.3 µM
Probe FCIR-P	0.1 µM
DNA polymerase	0.5 U
DNA (volume)	2 µl
Cycling parameters	
Initial denaturation[‡]	95 °C for 10 min
Number of cycles	40
Denaturation	95 °C for 15 s
Annealing-elongation[‡]	70 °C for 55 s
Expected amplicons	
Size	149 bp

[†] For a final reaction volume of 20 µl.

[‡] According to the DNA polymerase manufacturer's instruction.

3.4.5.1 Interpretation of results from real-time PCR using a hydrolysis probe

The fluorescence of the reporter dye is monitored at the end of each annealing-elongation step. The accumulation of *F. circinatum* PCR amplicons is monitored in real time by the measurement of the specific fluorescence of the reporter dye cleaved from the FCIR-P probe. A DNA template containing amplifiable *F. circinatum* DNA will yield a cycle threshold (Ct) value.

A sample will be considered positive if it produces a Ct value of <40, provided that the amplification curve is exponential and that the negative amplification control and negative extraction control are negative.

A sample will be considered negative if it produces a Ct value of ≥40, provided that the positive nucleic acid control and internal control are positive.

3.4.6 Controls for molecular tests

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – should be considered for each series of nucleic acid isolation and amplification of the target nucleic acid. For PCR a positive nucleic acid control, an internal control and a negative amplification control (no template control) are the minimum controls that should be used.

For the PCR tests described in this diagnostic protocol, it is also recommended that a negative extraction control be included.

Positive nucleic acid control. This control is used to monitor the efficiency of the test method (apart from the extraction). Pre-prepared (stored) genomic DNA from a reference strain of *F. circinatum* or subcloned *F. circinatum* PCR product (CIRC1A/CIRC4A for conventional PCR and SYBR Green real-time PCR; FCIR-F/FCIR-R for real-time PCR with a hydrolysis probe) may be used.

Internal control. For conventional and real-time PCR, internal controls should be incorporated into the protocol to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure or degradation or the presence of PCR inhibitors.

The quality of the DNA extract should be assessed by a relevant method; for example, by spectrophotometry, by using an ad hoc internal amplification control, or by testing the extract in a PCR with universal plant or fungal primers described in the scientific literature.

For conventional PCR and SYBR Green real-time PCR, the ITS1/ITS4 primers targeting the internal transcribed spacers in fungal and plant rDNA (White *et al.*, 1990) may be used in place of the CIRC1A/CIRC4A primers, under the same PCR conditions except for an annealing temperature of 50 °C. The primers are:

ITS1 (forward): 5′-TCC GTA GGT GAA CCT GCG G-3′

ITS4 (reverse): 5′-TCC TCC GCT TAT TGA TAT GC-3′

For real-time PCR using a hydrolysis probe, the 18S uni-F/-R/-P primers and probe targeting plant 18S rDNA (Ioos *et al.*, 2009) may be used in place of the FCIR-F/-R/-P primers and probe, decreasing the annealing-elongation temperature to 65 °C and reading the fluorescence in the appropriate wavelength range for the JOE reporter dye. The primers and probe are:

18S uni-F (forward primer): 5′-GCA AGG CTG AAA CTT AAA GGA A-3′

18S uni-R (reverse primer): 5′-CCA CCA CCC ATA GAA TCA AGA-3′

18S uni-P (probe): 5′-JOE-ACG GAA GGG CAC CAC CAG GAG T-BHQ1-3′

A positive signal with ITS1/ITS4 PCR or a positive Ct value (to be determined by the diagnostic laboratory) with 18S uni-F/-R/-P real-time PCR would indicate that the DNA was successfully extracted, the level of co-extracted inhibiting compounds was sufficiently low, and the DNA was able to be amplified.

Negative amplification control (no template control). This control is necessary for conventional and real-time PCR to rule out false positives due to contamination during preparation of the reaction mixture. PCR-grade water that was used to prepare the reaction mixture is added at the amplification stage.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and/or cross-reaction with the host tissue. The control comprises nucleic acid that is extracted from uninfected host tissue and subsequently amplified, or alternatively PCR-grade water. It is recommended that multiple controls be included when large numbers of positive samples are expected.

4. Identification

The requirement for identification of *F. circinatum* is outlined in the flow chart in Figure 1. However, morphological characters of species in the *Fusarium fujikuroi* species complex might be very similar and PCR cross-reaction might occur with phylogenetically close *Fusarium* spp. Therefore, it is recommended that the fungus is isolated and confirmed by sequence analysis.

4.1 Identification of *Fusarium circinatum* by cultural and morphological characteristics

To study colony morphology and pigmentation the isolates are grown on PDA; plates are incubated at 22 °C ± 6 °C under near UV light or in daylight for ten days. On PDA, *F. circinatum* grows relatively rapidly (average 4.7 mm/day at 20 °C) (Nirenberg and O'Donnell, 1998). After ten days, the colony should have an entire margin and white cottony or off-white aerial mycelium with sometimes a salmon-coloured tinge in the middle and/or with a purple to dark violet or yellow pigment in the agar (Figure 5).

To study the formation and type of microconidia, macroconidia and conidiogenous cells the isolates are grown on SNA; plates are incubated at 22 °C ± 6 °C under near UV light or in daylight. Some strains form sterile hyphae only under dark conditions (Aoki *et al.*, 2001); therefore, incubation in the dark may be needed for some strains to form sterile hyphae. Isolates are examined after ten days and confirmed as *F. circinatum* based on the morphological features described by Nirenberg and O'Donnell (1998) and Britz *et al.* (2002). On SNA, microconidia are aggregated in false heads, with branched conidiophores, monophialidic and polyphialidic conidiophores, and obovoid microconidia in aerial mycelium, mostly non-septate or occasionally one-septate (Figure 6(A)). Macroconidia are typically three-septate, with walls that are slightly curved, an apical cell that narrows to an inwardly (i.e. toward the ventral side) curved tip, and a foot-shaped basal cell (Figure 6(B)). Chlamydospores are absent. The above-mentioned characters are typical of several species within the *Fusarium fujikuroi* species complex, particularly *Fusarium subglutinans*. The production of distinctive flexuous/sinuous sterile hyphae, referred to as "coiled" or "circinate" hyphae, distinguishes *F. circinatum* and some other species in the complex, including some recently described species from pine, from *F. subglutinans* (Figure 7). These sinuous hyphae should not be confused with the commonly observed truly coiled hyphae (likely perithecial initials) at the surface of the agar (Figure 8), which may be produced by several species of *Fusarium*, including *Fusarium pseudocircinatum*.

The isolate observed in pure culture can reliably and confidently be assigned to the species *F. circinatum* if all the morphological features described above are observed. Table 4 presents a comparison of *F. circinatum* with other *Fusarium* species that have similar characteristics and that *F. circinatum* may therefore be confused with. In case of doubt, or if at least one characteristic cannot be clearly observed, then a DNA sequence analysis should be conducted (section 4.2).

Table 4. Main cultural and morphological characteristics of commonly encountered *Fusarium* species on pine producing microconidia

Fusarium species	Arrangement of microconidia	Monophialide/polyphialide	Presence of sterile sinuous hyphae
F. circinatum	False head only, on short conidiophores	Monophialides and polyphialides	Yes, more or less clearly sinuous, depending on the isolate
F. subglutinans	False head only, on short conidiophores	Monophialides and polyphialides	No
F. verticillioides	Chains and false head on short conidiophores	Monophialides only	No
F. oxysporum	False head only, on very short (sometimes inconspicuous) conidiophores	Short monophialides only	No
F. solani	False head only, on long conidiophores	Monophialides only, often quite long	No
F. pseudocircinatum	False heads and short chains	Monophialides and occasionally polyphialides	Yes, but distinctively spiral-shaped and unlike those of *F. circinatum*

Source: After Leslie and Summerell (2006).

4.2 Identification of *Fusarium circinatum* by sequence analysis

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.

4.2.1 Identification of *Fusarium circinatum* in pure culture by sequence analysis

Identification of doubtful isolates in pure culture may be ascertained by analysis of the sequence of a barcode or of another relevant phylogenetic marker. In the case of *Fusarium*, several genes may be used for identification with a high level of certainty. The *EF-1alpha* sequence is sufficient to assign the identity of a *Fusarium* strain to *F. circinatum* (O'Donnell *et al.*, 1998; Geiser, 2004) 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*. Moreover, species within the *Fusarium fujikuroi* species complex possess non-orthologous copies of the ITS2 region, which can lead to incorrect phylogenetic inferences (O'Donnell and Cigelnik, 1997). It is recommended that positive results from all PCR tests be verified by sequence analysis.

4.2.1.1 EF-1alpha *sequencing*

The primers are:

EF1 (forward): 5′-ATG GGT AAG GAR GAC AAG AC-3′

EF2 (reverse): 5′-GGA RGT ACC AGT SAT CAT GTT-3′

Using these primers and the PCR detailed in Table 5, a portion of the *EF-1alpha* gene is amplified.

Table 5. EF1/EF2 conventional PCR master mix composition, cycling parameters and amplicons

Reagent	Final concentration
PCR-grade water	–†
PCR buffer	1×
MgCl2	1.5 mM
dNTPs	250 µM
Primer EF1	0.45 µM
Primer EF2	0.45 µM
DNA polymerase	0.5 U
DNA (volume)	2 µl
Cycling parameters	
Initial denaturation‡	94 °C for 5 min
Number of cycles	45
Denaturation	95 °C for 30 s
Annealing	55 °C for 30 s
Elongation	72 °C for 60 s
Final elongation	72 °C for 6 min
Expected amplicons	
Size	Approximately 640 bp

† For a final reaction volume of 20 µl.

‡ According to the DNA polymerase manufacturer's instruction.

bp, base pairs; PCR, polymerase chain reaction.

The *EF-1alpha* PCR product is sequenced; a two-way sequencing with primers EF1 and EF2 as forward and reverse primer, respectively. The consensus sequence, from which the primers' sequences are trimmed, is compared by the Basic Local Alignment Search Tool (BLAST) with those deposited in GenBank (https://www.ncbi.nlm.nih.gov) for numerous phylogenetically close *Fusarium* spp. or with those deposited in the Fusarium-ID database (http://isolate.fusariumdb.org/), selecting the *EF-1alpha* data set. The sequence lying between EF1 and EF2 is sufficiently discriminant to identify *F. circinatum*. The level of identity with the *EF-1alpha* sequence of a reference strain of *F. circinatum* (e.g. GenBank accession number AF160295) should be between 99% and 100%.

4.2.1.2 CIRC1A/CIRC4A sequencing

The CIRC1A/CIRC4A PCR product is sequenced; a two-way sequencing with primers CIRC1A and CIRC4A as forward and reverse primer, respectively. The consensus sequence, from which the primers' sequences are trimmed, is compared by BLAST with those deposited in GenBank (https://www.ncbi.nlm.nih.gov) for numerous phylogenetically close *Fusarium* spp. The sequence lying between CIRC1A and CIRC4A is sufficiently discriminant to identify *F. circinatum*. The level of identity with the IGS sequence of a reference strain of *F. circinatum* (e.g. GenBank accession number AY249397) should be between 99% and 100%.

5. Records

Records and evidence should be retained as described in section 2.5 of ISPM 27 (*Diagnostic protocols for regulated pests*).

6. Contact Points for Further Information

Further information on this protocol can be obtained from:

Grupo de Investigación en Hongos Fitopatógenos, Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Camino de Vera s/n, 46022 Valencia, Spain (Mónica Berbegal Martínez; e-mail: mobermar@etsia.upv.es; tel.: +34 963 879 254; fax: +34 963 879 269).

Laboratoire de la Santé des Végétaux – Unité de Mycologie, Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES), Domaine de Pixérécourt – Bât. E, CS 40009, F54220 Malzéville, France (Renaud Ioos; e-mail: renaud.ioos@anses.fr; tel.: +33 383 290 080; fax: +33 383 290 022).

Forest Research, Alice Holt Lodge, Farnham, Surrey GU10 4LH, England, United Kingdom (Ana Pérez-Sierra; e-mail: ana.perez-sierra@forestry.gsi.gov.uk; tel.: +44 0300 067 5716; fax: +44 142023653).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel on Diagnostic Protocols (TPDP).

7. Acknowledgements

The first draft of this protocol was written by Mónica Berbegal Martínez (Instituto Agroforestal Mediterráneo, Universidad Politécnica de Valencia, Spain (see preceding section)), Renaud Ioos (Laboratoire de la Santé des Végétaux, ANSES, France (see preceding section)) and Ana Pérez-Sierra (Forest Research, United Kingdom (see preceding section)).

8. References

The present annex may refer to International Standards for Phytosanitary Measures (ISPMs). ISPMs are available on the International Phytosanitary Portal (IPP) at https://www.ippc.int/core-activities/standards-setting/ispms.

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9. Figures

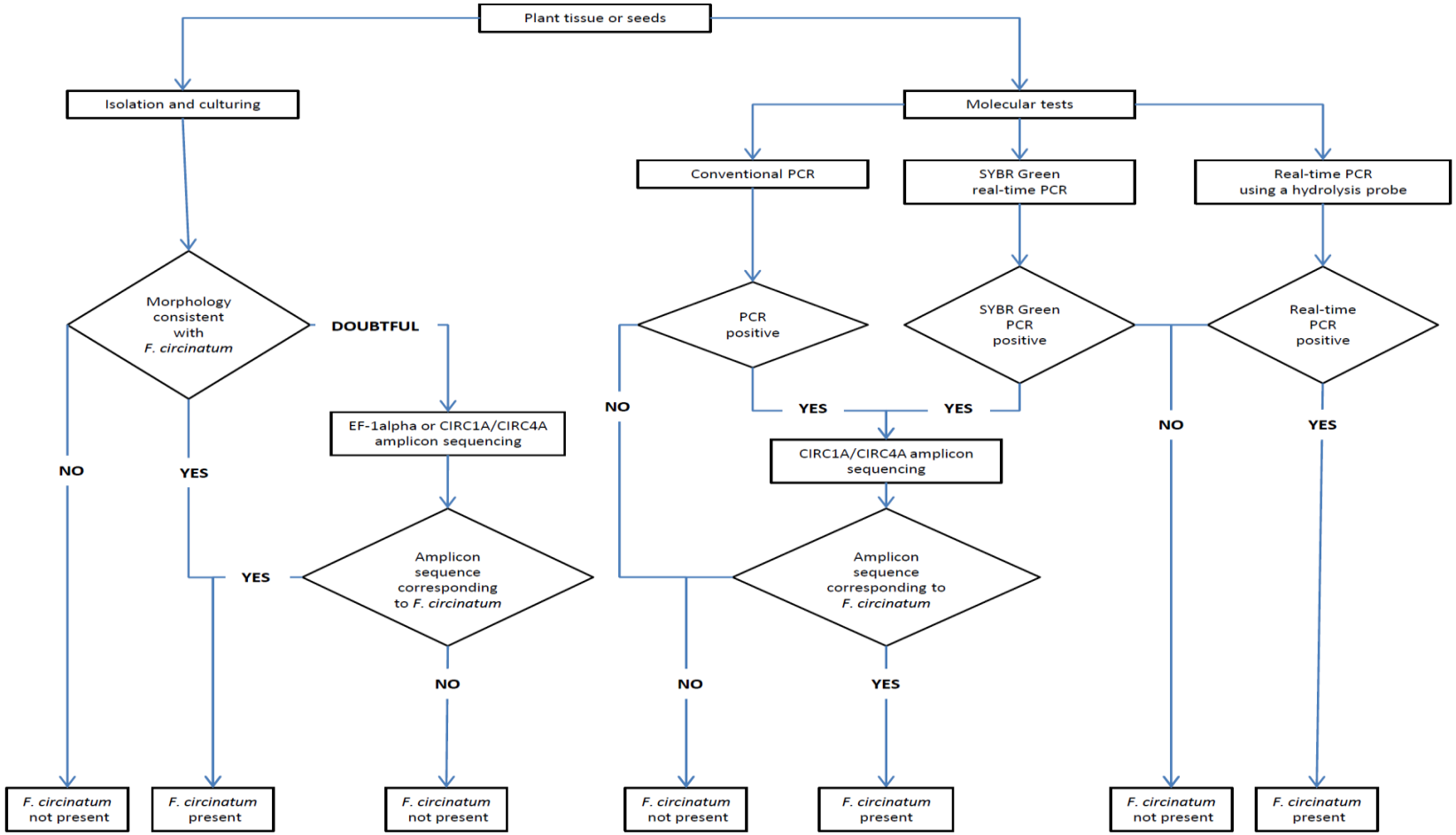

Figure 1. Flow chart for the identification of *Fusarium circinatum* in a sample of plant tissue or seeds.

* It is recommended that the fungus be isolated and confirmed by sequence analysis.

PCR, polymerase chain reaction.

Figure 2. Canker on a *Pinus radiata* trunk caused by *Fusarium circinatum* associated with conspicuous and sometimes resinous exudates.
Photo courtesy A. Pérez-Sierra, Forest Research, United Kingdom.

Figure 3. Removal of the bark on a *P. radiata* trunk shows subcortical lesions with brown and resin-impregnated tissues caused by *F. circinatum*.
Photo courtesy A. Pérez-Sierra, Forest Research, United Kingdom.

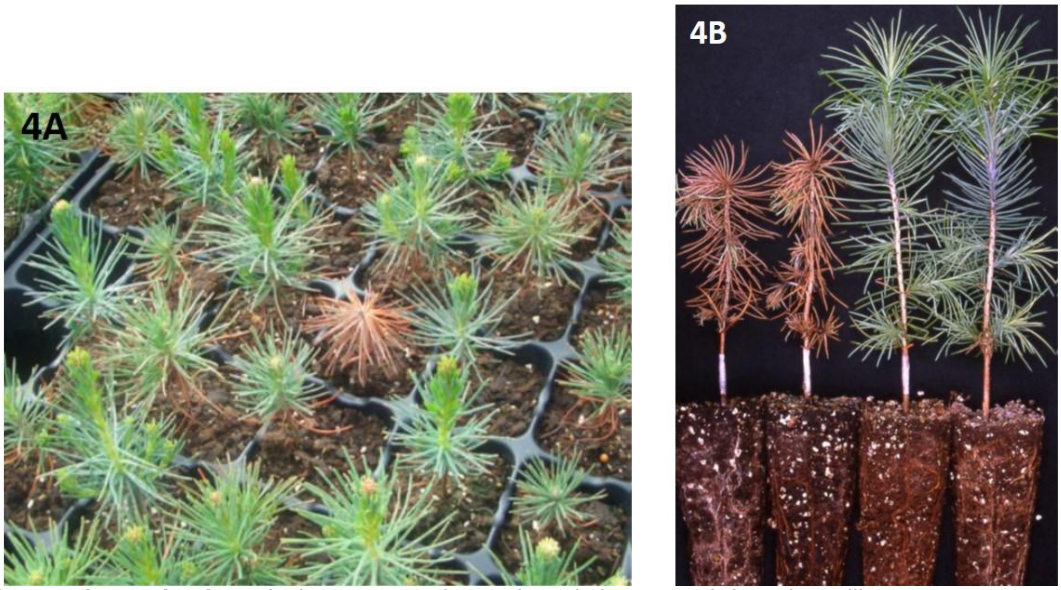

Figure 4 (4A and 4B). Typical symptoms of *Fusarium circinatum* on infected seedlings.
Photos courtesy E. Landeras, Laboratorio de Sanidad Vegetal, Oviedo, Spain.

Figure 5. Colony morphology of *Fusarium circinatum* after 14 days on potato dextrose agar, and the reversed plate. Photos courtesy M. Berbegal Martínez, Universidad Politécnica de Valencia, Spain.

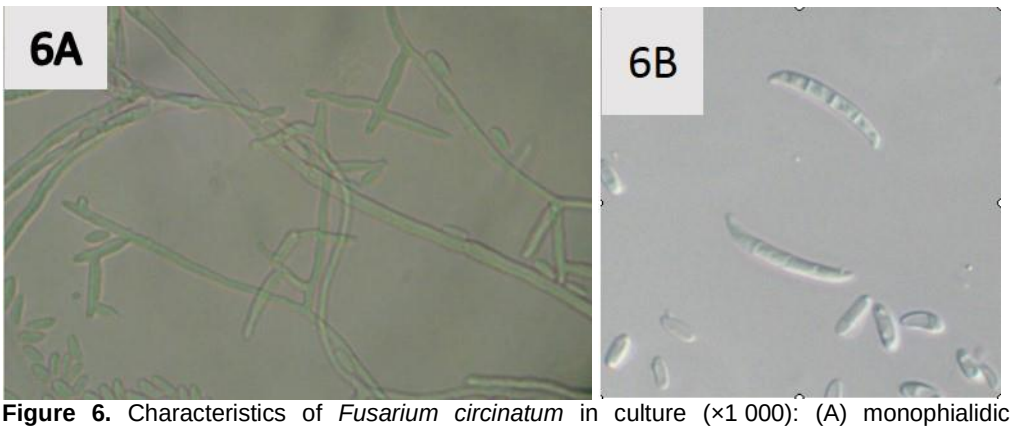

Figure 6. Characteristics of *Fusarium circinatum* in culture (×1 000): (A) monophialidic and polyphialidic conidiophores and microconidia; and (B) macroconidia and microconidia.
Photos courtesy A. Pérez-Sierra, Forest Research, United Kingdom.

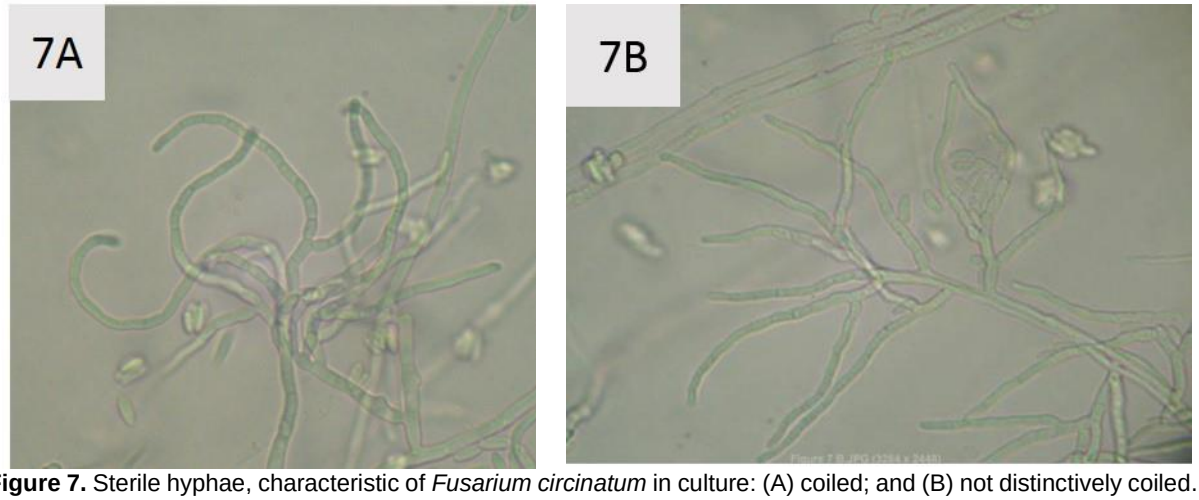

Figure 7. Sterile hyphae, characteristic of *Fusarium circinatum* in culture: (A) coiled; and (B) not distinctively coiled.
Photos courtesy A. Pérez-Sierra, Forest Research, United Kingdom.

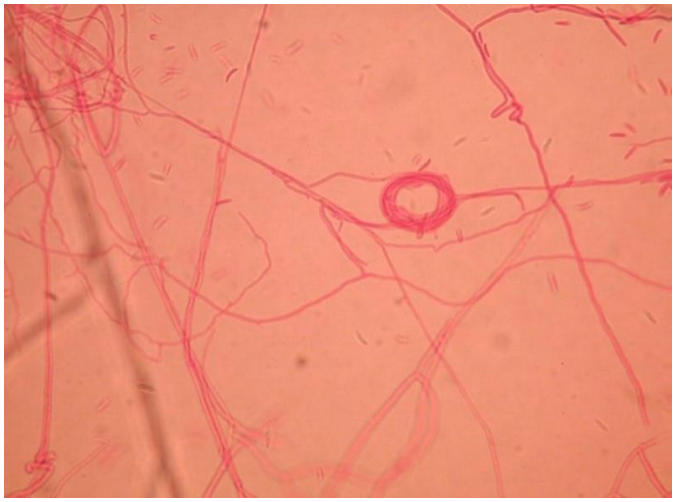

Figure 8. Commonly observed “spiral-wrapped” hyphae at the surface of agar, which may be produced by several species of *Fusarium*.
Photo courtesy R. Ioos, ANSES, Malzéville, France.

Publication history

This is not an official part of the standard

2006-05 SC added original subject: *Gibberella circinata* (syn. of *Fusarium circinatum*) (2006-021)

2015-03 Expert consultation on draft DPs

2015-06 TPDP revised draft

2015-11 SC noted title change from “*Fusarium moniliformis / moniliforme* syn. *F. circinatum*” to “*Fusarium circinatum*”

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2016-07 First consultation

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IPPC

The International Plant Protection Convention (IPPC) is an international plant health agreement that aims to protect cultivated and wild plants by preventing the introduction and spread of pests. International travel and trade are greater than ever before. As people and commodities move around the world, organisms that present risks to plants travel with them.

Organization

- There are over 180 contracting parties to the IPPC.
- Each contracting party has a national plant protection organization (NPPO) and an Official IPPC contact point.
- Nine regional plant protection organizations (RPPOs) work to facilitate the implementation of the IPPC in countries.
- IPPC liaises with relevant international organizations to help build regional and national capacities.
- The Secretariat is provided by the Food and Agriculture Organization of the United Nations (FAO).

International Plant Protection Convention (IPPC)

Viale delle Terme di Caracalla, 00153 Rome, Italy
Tel: +39 06 5705 4812 - Fax: +39 06 5705 4819
Email: ippc@fao.org - Web: www.ippc.int