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**Diagnostics**
**Diagnostic**

# PM 7/91 (2) *Fusarium circinatum* (formerly *Gibberella circinata*)

## Specific scope

This Standard describes a diagnostic protocol for *Fusarium circinatum*.[1]

It should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

When revising this protocol, the expert working group carefully considered the IPPC Standard (IPPC, 2017) adopted in 2017 on *Fusarium circinatum* ISPM 27 (Annex 22 to ISPM 27). However, this revision also took into account more recent information on the increased diversity and new *Fusarium* species reported in the literature (e.g. Mullett *et al*., 2017) and the performance characteristics of the various tests assessed during the Pinestrength COST Action project (FP1406; Ioos *et al.,* 2019). This new information led to the recommendation that positive results (from isolation or real-time PCR) should be confirmed as indicated in the flow diagram. Additional information, such as sampling from seedlings (both symptomatic and asymptomatic) and vectors was also added.

## Specific approval and amendment

First version approved in 2009-09.
Revision approved in 2019-06.

## 1. Introduction

*Fusarium circinatum* Nirenberg & O'Donnell, 1998 (formerly *Gibberella circinata*) is the causal agent of pitch canker disease. The disease almost exclusively affects *Pinus* species, but was also described on Douglas-fir (*Pseudotsuga menziesii*; Gordon *et al*., 1996). Moreover, it has been demonstrated that *Picea abies* seedlings at an early stage are susceptible to *F. circinatum* (Martínez-Alvarez *et al*., 2014; Martín-García *et al*., 2017). This disease is a serious threat to pine forests wherever it occurs (especially on plantations of *P. radiata*) due to extensive tree mortality, reduced growth and timber quality. Multiple branch infection may cause severe crown dieback and eventually lead to the death of the tree. This aggressive fungus may also cryptically infect *Pinus* seeds and may cause damping-off in seedlings. Seeds can be colonized by *F. circinatum* internally (where it can remain dormant until seed germination) and externally (Storer *et al*., 1998).

*Fusarium circinatum* is predominantly a wound pathogen and enters the host tree through mechanical wounds or feeding holes caused by woodboring insects. The fungus may move from tree to tree by aerial dispersion of the conidiospores or through vectoring by feeding insects (Gordon *et al*., 2001; Schweigkofler *et al*., 2004). However, long-range dispersal of the pathogen from affected to disease-free areas may be driven by infected seed movement and movement of infected plant material (Storer *et al*., 1998).

Outside the EPPO region, the fungus is present in the USA, Mexico, Haiti, Uruguay, Chile, South Africa, Japan and South Korea (EPPO, 2018). In the EPPO region, the first official reports were in Spain (Landeras *et al*., 2005), where it has restricted distribution, and subsequently in Portugal in 2007, where it has few occurrences (EPPO, 2018). In Italy (Carlucci *et al*., 2007) and France (EPPO, 2008) outbreaks have been eradicated. In most instances of introduction into new areas, the pest was first found in nurseries.

The fungus has gone through a number of name changes. According to the 'one species/one name' rule in mycology, the preferred name is now *Fusarium circinatum* Nirenberg & O'Donnell, 1998. *F. circinatum* is now a well-defined species within the *F. fujikuroi* species complex.

The numerous *Fusarium* species residing in this complex, some fully described and others poorly documented, are morphologically similar. Therefore, identification may require a combination of multiple techniques. In particular, Steenkamp *et al.* (1999)

[1]Use of brand names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.


 

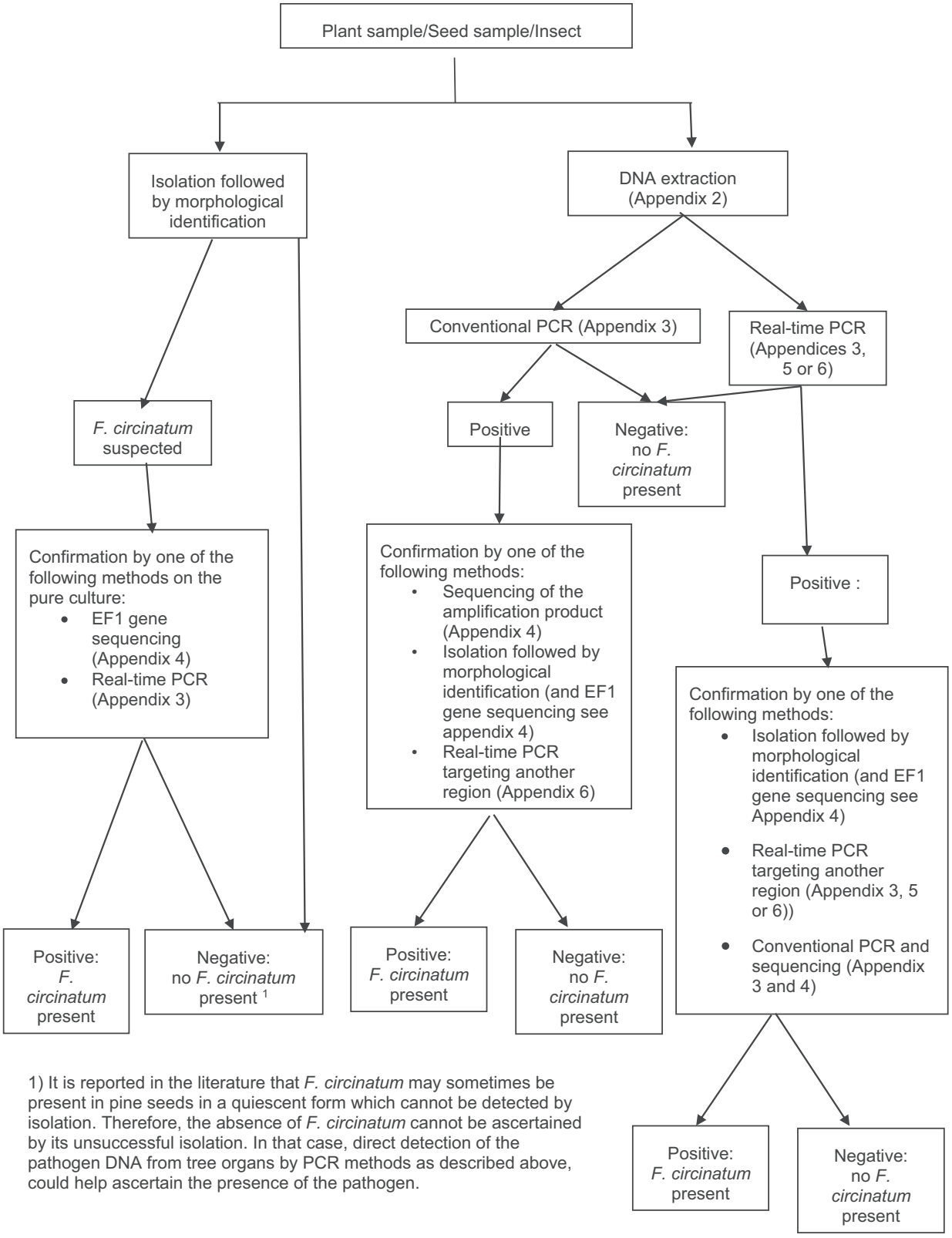


**Fig. 1** Flow diagram of the diagnostic procedure for *Fusarium circinatum* from plant tissue, seeds and insects.

report that some of the distinguishing morphological characteristics may be inadequate or insufficient to make a definite identification of *F. circinatum* (Nirenberg & O'Donnell, 1998).

A flow diagram describing the diagnostic procedure for *Fusarium circinatum* is presented in Fig. 1.

## 2. Identity

**Name**: *Fusarium circinatum* Nirenberg & O'Donnell, 1998
**Synonyms**: *Gibberella circinata* Nirenberg & O'Donnell (former synonyms *Fusarium subglutinans* f. sp. *pini* Hepting; *F. moniliforme* Sheldon var. *subglutinans* Wollenweber *F. lateritium* f. sp. *pini* Hepting; *Fusarium subglutinans* (Wollenweber & Reinking) Nelson *et al*. f. sp. *pini* Correll *et al*.)
**Taxonomic position**: *Fungi*: *Ascomycota*: *Hypocreales*: *Nectriaceae*
**EPPO Code**: GIBBCI
**Phytosanitary categorization**: EPPO A2 list no. 306.

## 3. Detection

### 3.1. Disease symptoms

The fungus causes cankers that girdle branches, aerial roots and even trunks of *Pinus* species, and is often associated with conspicuous and sometimes copious resin exudates ('pitch') in response to the fungal infection (Fig. 2A,B). Infected seedlings show damping-off symptoms which are not distinctive for *F. circinatum* infection: either needles turn red, brown or chlorotic and die from the base upwards or the seedling dies (Fig. 3). However, seedlings may not display any symptoms of the disease, but still be latently infected by the fungus (Storer *et al*., 1998; Swett & Gordon, 2011). Symptoms of the disease may be observed at any time of year.

**Fig. 3** Seedling damping-off caused by *Fusarium circinatum* in a nursery. Courtesy of E Landeras, Laboratorio de Sanidad Vegetal, Oviedo (ES).

*F. circinatum* can also be seed-borne and can infect seeds asymptomatically (Storer *et al*., 1998). The fungus can also cause root rot (Coutinho *et al*., 1997). *Fusarium circinatum* can be spread from tree to tree by insects. Various Coleoptera, such as *Ips* species and *Pityophthorus* species, have been reported to be potential vectors of the pathogen (Romón *et al*., 2007; Erbilgin *et al*., 2008).

Root infections are most often observed on seedlings in nurseries but can also occur on exposed roots of larger trees in landscape plantings. Symptoms on roots are brown discoloration and disintegration of the cortex, which are similar to symptoms caused by other root rot pathogens. Consecutive above-ground symptoms are generally not apparent until the pathogen reaches the crown after it


**Fig. 2** Copious resin exudates (pitch) beneath cankers caused by *Fusarium circinatum*. Courtesy of J Armengol Instituto, Agroforestal Mediterraneo, Universidad politeenica de Valencia/CNRS and R Ioos, Station de Mycologie, Malzéville (FR).

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, due to repeated infections, may lead to significant crown dieback. The female cones on infected branches may also become affected and abort before reaching full size. Cankers may thereafter appear on the shoots (Fig. 4), on the main stems and even on the trunk, associated with typical resin bleeding (Fig. 2A,B). However, the symptoms in older trees can be mistaken for those caused by *Diplodia sapinea* (Fr.) Fuckel. In addition, Bosnian pine (*P. heldreichii* syn. *P. leucodermis*) occasionally shows resinosis of stem and branches associated with bark necroses, but not caused by *F. circinatum*. The phenomenon is regarded as likely to be of physiological origin, therefore the diagnosis should be based on testing. Sometimes this resin bleeding may coat the trunk and lower branches for several metres below the infection level. The stem cankers are flat or slightly sunken and may sometimes affect large surfaces of the cortical and subcortical tissue of the trunk. Removal of the bark shows subcortical lesions with brown and resin-impregnated tissues (Fig. 5).

**Fig. 4** Shoot cankers caused by *Fusarium circinatum*. Courtesy of J Armengol, Institute Agroforestal Mediterraneo, Universidal Politeenica de CNRS.

**Fig. 5** Sub-cortical necrosis caused by *Fusarium circinatum* beneath a canker observed on a stem. Courtesy of E Landeras, Laboratorio de Sanidad Vegetal, Oviedo (ES).

### 3.2. Test sample requirements

Despite the fact that they may exhibit different susceptibility levels to *F. circinatum*, all *Pinus* species and the Douglas-fir (*Pseudotsuga menziesii*) can potentially be attacked by this fungus. There are four methods that can be used for sampling depending of the type of material to be sampled (plant tissue, seedlings, seeds or insects).

#### *3.2.1. Plant tissue showing symptoms (except seedlings)*

For trunk or branch cankers, the inner bark in 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 to try to collect portions of the lesion edge, where the fungus is most active. The pieces of tissue should be wrapped with sheets of dry sponge towels or newspapers and placed in a sealed plastic bag.

Sections of shoots, twigs or aerial roots should be collected by visual inspection upon observation of the symptoms indicated above. As for trunk or branch cankers, the sample should include the lesion edge and a few centimetres of healthy looking tissue ahead of the lesion and be stored/transported in the same manner.

All samples of plant material should be sent to the laboratory as soon as possible after sampling or refrigerated until transfer. When received in the laboratory, the samples should be kept in a refrigerator until analysis. The sample should be analysed within 8 days.

#### *3.2.2. Seedlings*

Infected seedlings may be symptomatic or asymptomatic; it is preferable to collect symptomatic seedlings. Sampling asymptomatic seedlings for analysis may be required in some circumstances (e.g. monitoring plan set by the National Plant Protection Organization, surveillance zone around an outbreak in a nursery). In these cases, sampling should be as per an appropriate plan. For both symptomatic and asymptomatic seedlings, the culture substrate should be removed gently by hand, and the seedling should be wrapped with sheets of sponge towels or newspapers and placed in a sealed plastic bag and handled until analysis as described for plant tissue in section 3.2.1.

#### *3.2.3. Seeds*

Depending on the method chosen for identification, the number of seeds to be analysed may differ per lot. The

**Table 1.** Mean thousand seed weight (TSW) for the major *Pinus* and *Pseudotsuga* species (French Forestry Board – Office National des Forêts)

| Species | Indicative TSW (g) | Species | Indicative TSW (g) |
|---|---|---|---|
| *Pinus aristata* | 22 | *Pinus mugo* subsp. *pumilio* | 6 |
| *Pinus armandii* | 245 | *Pinus nigra* subsp. *koekelare* | 21 |
| *Pinus banksiana* | 4 | *Pinus nigra* var. *austriaca* | 20 |
| *Pinus bungeana* | 130 | *Pinus nigra* var. *calabrica* | 18 |
| *Pinus brutia* | 53 | *Pinus nigra* var. *corsicana* | 15 |
| *Pinus canariensis* | 120 | *Pinus nigra* subsp. *salzmannii* | 16 |
| *Pinus cembra* | 350 | *Pinus palustris* | 75 |
| *Pinus contorta* var. *latifolia* | 5 | *Pinus parviflora* | 125 |
| *Pinus coulteri* | 330 | *Pinus pinaster* | 55 |
| *Pinus eldarica* | 62 | *Pinus pinea* | 895 |
| *Pinus densiflora* | 18 | *Pinus ponderosa* | 42 |
| *Pinus gerardiana* | 295 | *Pinus pumila* | 105 |
| *Pinus griffithii* | 58 | *Pinus radiata* | 29 |
| *Pinus halepensis* | 18 | *Pinus rigida* | 7 |
| *Pinus jeffreyi* | 110 | *Pinus strobus* | 14 |
| *Pinus koraiensis* | 460 | *Pinus sylvestris* | 7 |
| *Pinus lambertiana* | 300 | *Pinus tabuliformis* | 32 |
| *Pinus heldreichii* | 25 | *Pinus taeda* | 27 |
| *Pinus montana uncinata* | 9 | *Pinus thunbergii* | 14 |
| *Pinus uncinata* | 19 | *Pinus wallichiana* | 50 |
| *Pinus mugo* subsp. *mugo* | 7 | *Pseudotsuga menziesii* | 13 |

total number of seeds to be tested in order to detect the pest at different infection levels in a lot needs to be determined statistically (useful guidance is given in Tables 1 and 2 of ISPM no. 31 *Methodologies for sampling of consignments*; IPPC, 2008). Levels of infection in seeds can be very low (AM Pérez-Sierra, pers. comm.). The sample size for plating recommended by ISTA is 400 seeds (ISTA, 2015). However, larger samples (e.g. 1000 seeds) can easily be processed by biological enrichment before DNA analysis (Ioos *et al*., 2009). Since symptoms cannot be observed on seeds, the lot should be sampled randomly. Counting of seeds may be laborious when large sample sizes are used, and the sampled seeds may then be weighed instead. One thousand seeds may be collected in accordance with Table 1, which gives examples of mean thousand-seed-weight for the major *Pinus* or *Pseudotsuga* species. The seeds will subsequently be analysed without any surface disinfection, as *F. circinatum* may be present on the seed husk, as well as inside the seed.

#### *3.2.4. Potential vector insects*

Monitoring the presence of insects carrying propagules of *F. circinatum* by trapping may be a good means to study and anticipate the natural spread of the disease on a local scale.

*Ips* spp. can be trapped using appropriate trapping systems such as flight-intercept slit traps baited with ipsdienol pheromone, or 'crosstraps' baited with 2-(undecyloxy)-ethanol,[2] mainly attracting *Monochamus* spp. but also Scolytidae (Fourrier *et al*., 2015). After capture, the insects should be stored in plastic bottles and sent to the laboratory as soon as possible or kept refrigerated until transfer. When received in the laboratory, the samples should be kept in a refrigerator until analysis. The sample should be analysed within 8 days.

A similar method could probably be used for vectors other than *Ips* spp., such as *Tomicus piniperda, Pityophthorus pubescens* and *Hylurgops palliates*, but these methods have not been validated (Romón *et al*., 2007; Bezos *et al*., 2015).

### 3.3. Isolation on semi-selective medium

#### *3.3.1. Insects and plant tissue except seeds*

Isolations from insects, symptomatic conifer trees or symptomatic or asymptomatic seedlings are made onto one of the following medium: Komada, Dichloran Chloramphenicol Peptone Agar (DCPA) or potato dextrose agar (PDA) supplemented with streptomycin sulphate (PDAS; see Appendix 1). Plant material should be surface-sterilized for up to 1 min in a 1.5% solution of sodium hypochlorite and rinsed in sterile distilled water to eliminate saprophytic organisms from the plant material, which would otherwise overgrow any *Fusarium*. For symptomatic or asymptomatic 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. For symptomatic seedlings, the pathogen is isolated from the leading edge of the lesions. For asymptomatic seedlings, isolation is carried out from 1-cm sections taken just above and just

[2]The trapping method using ethanol solution is suitable for samples for PCR but not for samples for isolation

below the collar. For insects, the samples should have been appropriately handled prior to isolation (see section 3.2.4).

In the case of 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 (Fig. 4). Plates are incubated at room temperature (22 ± 6°C) with day/night light alternance. During incubation, the plates are observed periodically, and all the *Fusarium* spp. colonies are transferred to PDA and to Spezieller-Nährstoffarmer Agar (SNA; Appendix 1) for morphological identification. As *F. circinatum* does not produce chlamydospores, *Fusarium* colonies showing chlamydospore should not be transferred for further identification. This method is very efficient and reliable for isolating any *Fusarium* spp. from infected tissue 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.

*3.3.2. Seeds*

Seeds are directly plated onto *Fusarium* semi-selective media (e.g. Komada's medium, DCPA medium; see Appendix 1) without previous surface disinfection. Plates are incubated at room temperature (22 ± 6°C) with day/night light alternance. During incubation, the plates are observed periodically, and all the *Fusarium* spp. colonies are transferred to PDA and SNA (Appendix 1) for morphological identification. As *F. circinatum* does not produce chlamydospores, *Fusarium* colonies showing chlamydospores should not be transferred for further identification. This method is efficient and reliable to isolate any *Fusarium* spp. from the seeds and does not require expensive equipment, though it is time- and space-consuming when serial analyses are conducted. However, the correct morphological identification of *F. circinatum* in pure culture requires experience and in case of uncertainty a molecular confirmation should be carried out. In addition, Storer *et al*. (1998) demonstrated that agar plating of pine seeds may sometimes fail to detect dormant (quiescent) propagules of *F. circinatum*, thus leading to an unknown risk of false-negative results.

### 3.4. Direct detection in planta (plant tissue, including seeds) and on insects using molecular techniques

See *Identification* section for description. These methods are fast, efficient and reliable in detecting *F. circinatum* specifically, without previous agar plating, thus saving a lot of space and time, but require molecular biology facilities and instruments. In addition, as these techniques target the DNA of the fungus, active and quiescent forms of the pathogen should be equally detected. However, positive results with PCR (conventional or real-time) require confirmation as cross-reaction with phylogenetically closely related species might occur and lead to false-positive results.

## 4. Identification

The procedures for the identification of *F. circinatum* on *Pinus* spp. and *Pseudotsuga menziesii* will initially consist of (a) isolating the fungus from the plant tissue on semi-selective culture media followed by morphological identification or (b) directly detecting the fungus *in planta* by conventional or real-time PCR. Based on the literature reporting increased diversity and new *Fusarium* species (Herron *et al*., 2015; Mullett *et al*., 2017) and the performance characteristics of the various tests assessed during the Pinestrength COST Action project (FP1406; Ioos *et al.,* 2019) positive results should be confirmed as indicated in Fig. 1, which indicates the different combinations of methods.

### 4.1. Morphological characteristics in pure culture

It is reported in the literature that *F. circinatum* may sometimes be present in a quiescent form which cannot be detected by isolation (Storer *et al*., 1998), therefore absence of *F. circinatum* cannot be ascertained by unsuccessful isolation. A negative result with isolation will therefore not be as reliable as a negative result using a molecular test because of the possible quiescent state of the pathogen. In that case, direct detection of the pathogen DNA from tree organs by PCR methods as described above could help ascertain the presence of the pathogen.

For morphological identification, the isolates are grown on PDA to study colony morphology and pigmentation, and on SNA (Appendix 1) to study features of microconidia, macroconidia and conidiogenous cells. SNA and PDA plates are incubated at room temperature (22 ± 6°C). All isolates 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*. (2002a,b). The morphological features are presented in Table 2 and allow discrimination from similar species (in terms of morphology or host range) including those described by Herron *et al*. (2015), which are also found on pine.

On PDA, *F. circinatum* grows relatively quickly (average growth of 4.7 mm $day^{-1}$ at 20°C; Nirenberg & O'Donnell, 1998). After 10 days, the colony should have an entire margin (occasionally lobate or undulate), 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, alternatively colonies can be white, whitish purple, white with a purple centre, whitish yellow or purple (Mullett *et al*., 2017; Fig. 6).

On SNA, microconidia are aggregated in false heads (Fig. 7A,B), with branched conidiophores, mono and polyphialidic-conidiophores (Fig. 8) obovoid microconidia in aerial mycelium, mostly nonseptate or with occasionally 1-septum.

Macroconidia should preferably be sampled from sporodochia (Fig. 9) and not from aerial mycelium (if sampled from aerial mycelium the size can be different).

**Table 2.** Morphological features that allow discrimination of *Fusarium circinatum* from similar species (in terms of morphology or host range)

| Species and strain(s) studied. | Macroconidia* size (µm) | Number of septa | Macroconidia amount | Microconidia size (µm) | Microconidia amount | Conidiogenous cells | False heads/ chains | Sterile hyphae | Host | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|
| *Fusarium circinatum* (Nirenberg & O'Donnell, 1998; Leslie & Summerell, 2007) PD 5319336 CBS 117843 CBS 405.97 KSU H-10850 KSU H-10847 FC-76 FC 100 | 33.7–42.7 × 3.4–3.7 | 3 | Not abundant | 8.5–10.9 × 2.8–3.6 | Abundant | Mono and poly | False heads | Present** (In most cases MAT1 circinate, MAT2 not circinate) | *Pinus* spp., *Pseudotsuga menziesii* | **In rare cases sterile hyphae not present. |
| *Fusarium fracticaudum* (Herron *et al*., 2015) CMW 25237 | 38–63.5 × 2.5–4.5 | **3–5** | Abundant | 8–13 × 1.5-3 | Abundant | Mono and poly | False heads | **Not present** | *Pinus* sp. | |
| *Fusarium marasasianum* (Herron *et al*., 2015) CMW 25261 | 23.5–44.5 × 2.5–4 | 0–3 | **Abundant** | 7.5–18 × 2-3.5 | **Scarce** | Mono and poly | False heads | Present (circinate hyphae) | *Pinus* sp. | |
| *Fusarium parvisorum* (Herron *et al*., 2015) CMW 60897 | **12.5–29.5 × 1.5–3** | 1–3 | Not abundant | 7–13 × 1.5–3 | Not abundant | Mono and poly | False heads | Present (circinate hyphae) | *Pinus* sp. | |
| *Fusarium pininemorale* (Herron *et al*., 2015) CMW 25243 | 35–52 × 2–3.5 | 3–4 | Abundant | 5–16.5 × 1.5–3 | Scarce | Mono and poly | False heads | **Not present** | *Pinus* sp. | |
| *Fusarium sororula* (Herron *et al*., 2015) CMW 40578 | 20–42.5 × 2–4* | 1–3 | Scarce | 5.5–15.5 × 1.5–3 | Abundant | Mono and poly | False heads | **Not present** | *Pinus* sp. | *Sporodochia are sparse, so macroconidia are rare. Size of some aerial macroconidia is smaller |
| *Fusarium pseudocircinatum* (Nirenberg & O'Donnell, 1998; Leslie & Summerell, 2007) KSU 10761 | 22.4–33.9 × 2.6–3.3* | 3 | Scarce/not abundant | 6.0–8.3 × 2.1–2.9 | Abundant | Mono and poly | False heads and **short chains** | Present (circinate hyphae) | Variety of substrates from tropical regions | *Sporodochia are sparse, so macroconidia are rare. Size of some aerial macroconidia is smaller |

(continued)

Table 2 *(continued)*

| Species and strain(s) studied. | Macroconidia* size (µm) | Number of septa | Macroconidia amount | Microconidia size (µm) | Microconidia amount | Conidiogenous cells | False heads/ chains | Sterile hyphae | Host | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|
| *Fusarium sterilihyphosum* (Britz *et al.*, 2002b; Leslie & Summerell, 2007) KSU 11783 | 28.4–47.1 × 2.4–4.1* | **3–5** | Scarce/not abundant | 4.5–14.2 × 1.6–3.5 | Abundant | Mono and poly | False heads | Present (circinate hyphae) | ***Mangifera indica*** | *Sporodochia are sparse so macroconidia are rare. Size of some macroconidia is smaller |

Bold and underlined: most clearly different characteristic compared with *F. circinatum*. Examined on SNA and in accordance with literature.
Bold: Different characteristic compared with *F. circinatum*. Examined on SNA and in accordance with literature. The value of this characteristic might be doubtful because only one isolate per species was checked for the morphological characteristics, except for *Fusarium circinatum*.
Table based on table developed at National Plant Protection Organization (NL).
*From sporodochia (and not from aerial mycelium).




**Fig. 6** Examples of colony morphologies observed with *Fusarium circinatum* on PDA (22°C ± 3°C, 12 h NUV light, 12 h dark. Photo taken after 14 days incubation). Courtesy of P. van Rijswick National Plant Protection Organization (NL).




**Fig. 7** (A) Erect conidiophores bearing microconidia arranged in false heads of *Fusarium circinatum*, observed directly on SNA medium (×200 magnification). Courtesy of R. Ioos, ANSES, Malzéville (FR). (B) Erect conidiophores bearing microconidia arranged in false heads of *Fusarium circinatum*, observed directly on a microscopic slide (×400 magnification). Courtesy of A. Pérez-Sierra, Instituto Agroforestal Mediterraneo, Universidad Politecnica de Valencia (ES).

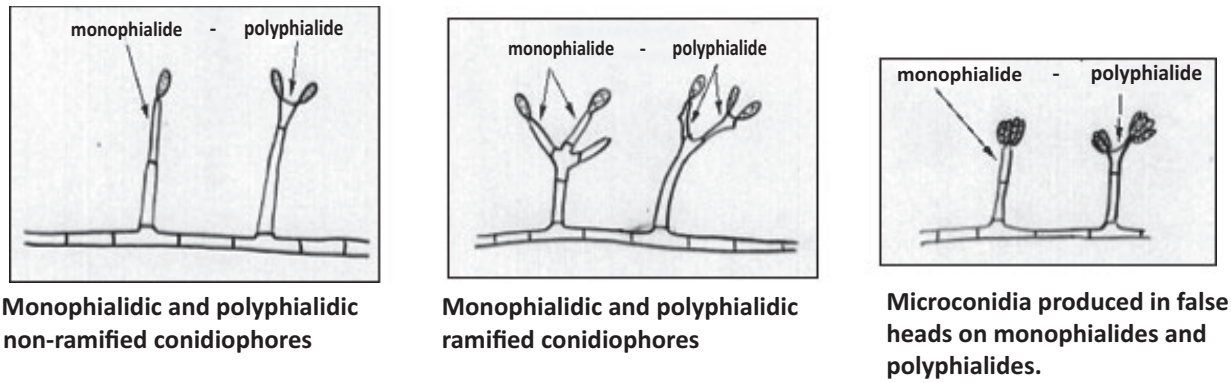


**Fig. 8** Mono and polyphialidic- conidiophores observed for *F. circinatum*. Courtesy of R. Ioos, ANSES, Malzéville (FR).

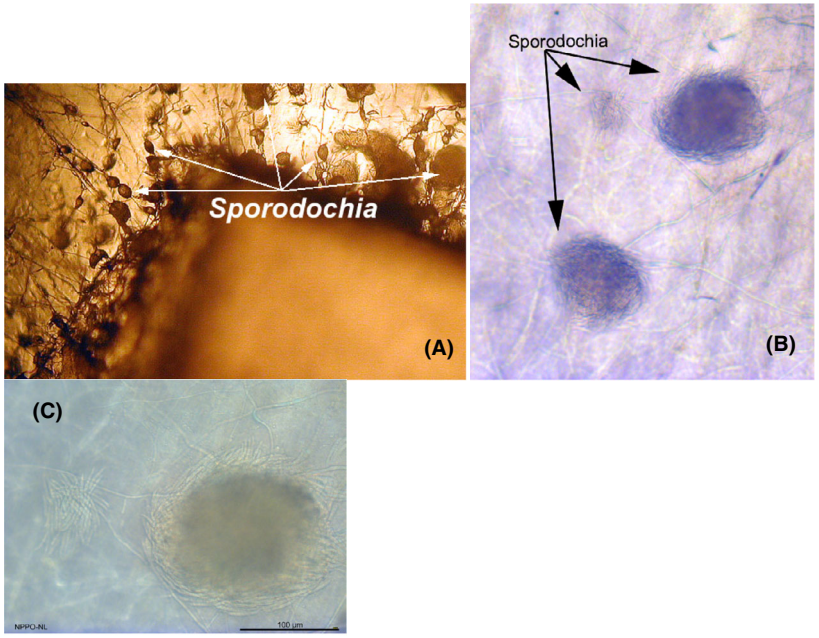


**Fig. 9** (A) *Fusarium* sporodochia produced around an agar plug on SNA. Courtesy of R. Ioos, ANSES, Malzéville (FR). (B) and (C) *Fusarium* sp. sporodochia on agar at different magnifications. Courtesy of P. van Rijswick, National Plant Protection Organization (NL).

Chlamydospores are absent. The epithet '*circinatum*' refers to the typical sterile coiled hyphae, also called 'circinate' hyphae produced by this *Fusarium* species. The sterile hyphae (coiled/not distinctively coiled) are observed clearly on SNA (Fig. 10A,B). However, this feature can also be lost over time (R. Ioos, personal observation) and a small number of isolates from different geographical origins have been reported that are either not distinctly coiled or where coils are absent (Mullett *et al*., 2017).

### 4.2. 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* or from insects.

- A 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 all plant tissue and insects, and are presented in Appendix 3. Verification of the nature of the PCR amplicon can be carried out by sequencing as described in Appendix 4.
- EF1 gene sequencing is described in Appendix 4 and can be used for confirmation of the identification of the fungus in pure culture,
- A real-time PCR test with primers and a dual-labelled probe designed by Ioos *et al*. (2009) for direct detection of the pathogen in plant tissue, including seeds, is presented in Appendix 5. This test was adapted by Fourrier *et al*. (2015) for the direct detection of the pathogen on

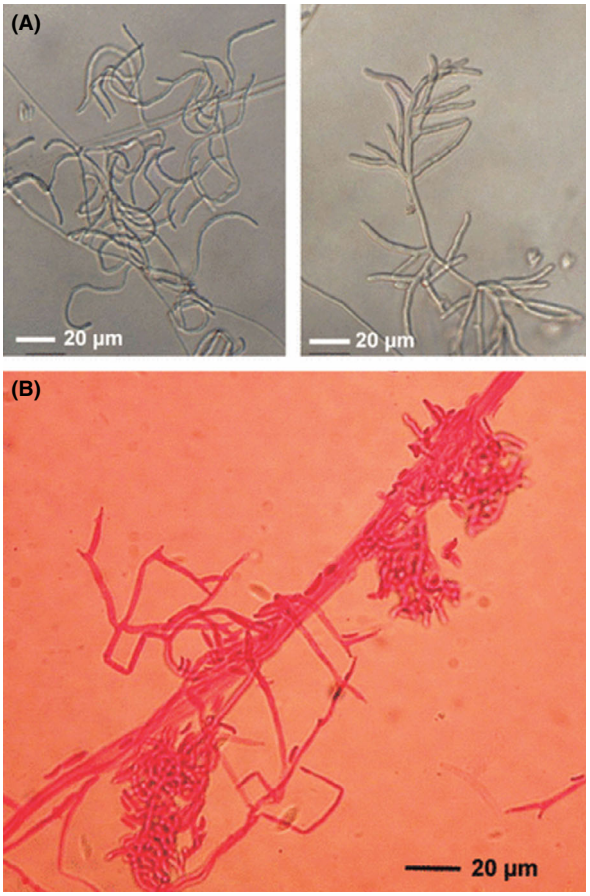


**Fig. 10** (A) Coiled and not distinctively coiled sterile hyphae produced on SNA medium by MAT-1 (left) and MAT-2 (right) mating type isolates of *Fusarium circinatum*, respectively. Courtesy of A. Pérez-Sierra, Instituto Agroforestal Mediterraneo, Universidad Politecnica de Valencia (ES). (B) Groups of coiled sterile hyphaes and polyphialidic conidiophores produced on SNA. Courtesy of R. Ioos, ANSES, Malzéville (FR).

insects. This method has been developed on *Ips* spp., but may be used for other potential vectors.

- A real-time PCR test with primers and a dual-labelled probe designed by Luchi *et al*. (2018) can be used for direct detection of the pathogen in insects or in plant tissue, including seeds and for confirmation of the identification of the fungus in pure culture (Appendix 6). Based on the results of the Pinestrength project, this test targeting a single copy gene (EF1) had high analytical specificity (no cross-reactions observed with species found on pine) but lower analytical sensitivity than tests based on multiple copy genes such as IGS.

According to the experience gained during the Pinestrength COST Action project (FP1406; Ioos *et al.,* 2019), the above PCR tests were selected predominantly based on their inclusivity and limited cross-reactions with other *Fusarium* species. Other tests are described in the literature (Ramsfield *et al*., 2008; Dreaden *et al*., 2012; Fourie *et al*., 2014; Lamarche *et al*., 2015), but there is limited/no experience with them in the EPPO region, therefore they are not described in the protocol.

*4.2.1. DNA extraction*

DNA extraction from the different matrices is described in Appendix 2.

## 5. Reference cultures

The type strain of *F. circinatum* (CBS 405.97) and other strains (CBS 117843, Spain; CBS 119864, South Africa; CBS 100197, USA) are available from the Westerdijk Institute, Utrecht (NL).

## 6. Reporting and documentation

Guidelines on reporting and documentation are given in EPPO standard PM 7/77 *Documentation and reporting of a diagnosis*.

## 7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data are also available in the EPPO Database on Diagnostic Expertise ( http://dc.eppo.int), and it is recommended to consult this database as additional information may be available there (e.g. more detailed information on analytical specificity, full validation reports, etc.).

## 8. Further information

Further information on this organism can be obtained from:

Ioos R, ANSES Laboratoire de la Santé des Végétaux (ANSES LSV), Unité de Mycologie, Domaine de Pixérécourt, Bât. E., 54220 Malzéville (FR). Tel: +33 (0) 383290002, Fax: +33 (0) 383290022, e-mail: renaud.ioos@anses.fr.

Pérez-Sierra AM, Tree Health Diagnostic & Advisory Service, Forest Research, Alice Holt Lodge, Wrecclesham, Farnham, Surrey GU10 4LH (GB).

e-mail: ana.perez-sierra@forestry.gov.uk.

## 9. Feedback on this Diagnostic Protocol

If you have any feedback concerning this diagnostic protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.int.

## 10. Protocol revision

An annual review process is in place to identify the need for revision of diagnostic protocols. Protocols identified as needing revision are marked as such on the EPPO website.

When errata and corrigenda are in press, this will also be marked on the website.

## Acknowledgements

This protocol was originally drafted by:

Ioos R, ANSES Laboratoire de la Santé des Végétaux, Unité de Mycologie, Malzéville (FR) and A. M. Pérez-Sierra, Tree Health Diagnostic & Advisory Service, Forest Research, Alice Holt Lodge, Wrecclesham, Farnham, Surrey GU10 4LH (GB).

The revision was prepared by an Expert Working Group comprising Ioos R, A. Chandelier, K. Heungens, C. Douanla-Meli. P. van Rijswick provided useful guidance and the basis for the table on morphology. The protocol was reviewed by the EPPO Panel on Diagnostics in Mycology.

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## Appendix 1 – Composition of the different culture media

**All media are sterilized by autoclaving at 121°C for 15 min, except when stated otherwise.**

**Komada medium** (Komada, 1975)

This medium is suitable for isolation of *Fusarium circinatum* from plant tissue, including seeds, but not for identification. The basal medium contains:

| | |
|---|---|
| $K_2HPO_4$ | 1.0 g |
| KCl | 0.5 g |
| $MgSO_4$ $7H_2O$ | 0.5 g |
| Fe-Na-EDTA | 10 mg |
| L-Asparagine | 2.0 g |
| D-Galactose | 20.0 g |
| Technical agar | 15.0 g |
| Distilled water | To make up to 1.0 L |
| The pH is adjusted to 3.8 ± 0.2 with 10% phosphoric acid. The basal medium is autoclaved and slightly cooled before adding the following filter-sterilized supplemental solutions: | |
| Pentachloronitrobenzene | 1.0 g |
| Oxgall | 0.5 g |
| $Na_2B_4O_7$ $10H_2O$ | 1.0 g |
| Streptomycin | 6 mL $L^{-1}$ of stock solution (5 g of streptomycin in 100 mL distilled water) |

**PDAS**

Potato dextrose agar supplemented with 0.5 mg $mL^{-1}$ of streptomycin sulphate salt (775 units/mg solid). This medium is suitable for isolation of *F. circinatum* from plant tissue, including seeds, but not for identification.

**Dichloran Chloramphenicol Peptone Agar (DCPA**; slightly modified by Ioos *et al*., 2004; after Andrews & Pitt, 1986)

This medium is suitable for isolation of *F. circinatum* from plant tissue, including seeds, but not for identification. It can be noted that chloramphenicol and dichloran withstand autoclaving. The medium contains:

| | |
|---|---|
| Bacteriological peptone | 15.0 g |
| $KH_2PO_4$ | 1.0 g |
| $MgSO_4$ | 0.5 g |
| Chloramphenicol | 0.2 g |
| 2.6-dichloro-4-nitroanilin (dichloran) | 2 mg (prepared as a 0.2% W/V solution in ethanol, add 1.0 mL) |
| Violet crystal | 0.0005 g (prepared as a 0.05% W/V solution in water, add 1.0 mL) |
| Technical agar | 20.0 g |
| Distilled water | To make up to 1.0 L |

**Spezieller-Nährstoffarmer Agar (SNA**; Gerlach & Nirenberg, 1982)

This medium should be used in identification of *F. circinatum* based on morphological features. The medium contains:

| | |
|---|---|
| $KH_2PO_4$ | 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 water | To make up to 1.0 L |

Two 1-$cm^2$ square pieces of sterile filter paper may be laid on the surface of the agar as production of *Fusarium* sporodochia may be enhanced at the edge of the paper.

## Appendix 2 – DNA extraction for detection of *F. circinatum* from plant or insect material

### Plant tissue (except seeds)

#### Grinding

Potentially infected plant tissues are collected from the sample and first roughly cut using a sterile scalpel blade, without prior surface disinfection step. Small pieces of approximately 0.5–1 $cm^2$ should be collected then subsequently cut into smaller pieces (<2–3 mm, each side) on a sterile solid surface.

The sample is then transferred into a 2 mL microcentrifuge tube corresponding to approximately 200 µL and ground for 2 min with two 3-mm steel or tungsten carbide beads and 400 µL of the lysis buffer provided by the DNA extraction kit (Nucleospin Plant II® miniprep (Macherey-Nagel, Hoerdt, France), at a frequency of 30 Hz with a bead beater (Tissuelyser®, Qiagen or equivalent). The samples may also be ground in a mortar by a pestle with liquid nitrogen, or using other efficient grinding techniques.

#### DNA extraction and purification

Total DNA should be extracted preferably following the extraction protocol described by Ioos *et al*. (2009) using the commercial DNA extraction kit Nucleospin Plant II® miniprep, which proved to be efficient. However, other DNA extraction protocols may be used providing that they proved to yield total DNA at least equivalent with at least similar quality and quantity.

Total DNA is extracted following the manufacturer's instructions with slight modifications. First, the chemical lysis incubation step is extended to 20 min, using the PL1 lysis buffer. After this incubation step, the sample is centrifuged 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 by the manufacturer and stored frozen until analysis. Total DNA is directly used as a template for conventional or real-time PCR (Appendices 3, 5 and 6).

## Seeds

#### Biological enrichment

This procedure was initially described by Ioos *et al*. (2009) and should be followed when the presence of *F. circinatum* is checked by a conventional or real-time PCR test (Appendices 3 and 6). The purpose of this preliminary biological enrichment step is to increase the biomass of viable *F. circinatum* propagules prior to DNA extraction and molecular testing.

As recommended by ISTA for agar plating technique (ISTA, 2015), at least 400 seeds per seed lot are incubated at 22 ± 3°C for 72 h in a cell culture flask with potato dextrose broth (PDB, Difco, Beckton, Dickinson and Co, Sparks, MD, USA). However, larger sample sizes (e.g. 1000 seeds in Ioos *et al*., 2009) can easily be processed by this test and may increase the chance to detect the fungus when present at low infection levels. The flask's size should be chosen so that the entire seed sample can be spread more or less as a 'single seed'-thick layer. 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.

#### Grinding

After incubation, the whole content of the flask (seeds and PDB) is transferred aseptically into a decontaminated mixer bowl of appropriate volume and subsequently ground with a mixer mill (e.g. Microtron MB 550 mixermill, Kinematica, Lucern, Switzerland) till a homogenous suspension is obtained. Sterile water or sterile PDB may be added at this step if the ground sample remains too thick. Two sub-samples of approximately 500 µL are then collected and transferred aseptically into individual 2-mL microcentrifuge tubes for DNA extraction.

#### DNA extraction and purification

Total DNA should be extracted preferably following the extraction protocol described by Ioos *et al*. (2009) using the commercial DNA extraction kit Nucleospin Plant II® miniprep (Macherey-Nagel, Hoerdt, France), which proved to be efficient, but other DNA extraction protocols may be used providing that they prove equivalent in yield and quality of DNA.

Total DNA is extracted individually from the two 500 µL sub-samples following the manufacturer's instructions with slight modifications. Proceed as described above for plant tissue.

## Insects

#### Grinding

Up to ten potentially contaminated individual *Ips* are transferred into a 2-mL Lysing Matrix A tube (MP Biomedicals, Santa Ana, CA, USA) containing a garnet matrix and one 1/4-inch (0.64 cm) ceramic sphere and 400 µL of DNeasy plant mini kit AP1 lysis buffer (Qiagen, Hilden, Germany) are added. The tube is immediately shaken for 1 min at a frequency of 6 units using the FastPrep-24 system (MP Biomedicals). The samples may also be ground in a mortar by a pestle with liquid nitrogen or using other efficient grinding techniques.

#### DNA extraction and purification

Total DNA should be extracted preferably following the extraction protocol described by Fourrier *et al*. (2015) using the commercial DNA extraction kit DNeasy plant mini (Qiagen), which proved to be efficient. However, other DNA extraction protocols may be used providing that they proved to yield at least a similar quality and quantity of total DNA.

Total DNA is extracted following the manufacturer's instructions with slight modifications. First, the chemical lysis incubation step with AP1 buffer starts after grinding and is extended to 15 min. After this incubation step, the homogenate is centrifuged 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 by the manufacturer, diluted 10-fold in 1× Tris-EDTA buffer (Sigma-Aldrich, Lyon, France) and stored frozen until analysis. Total DNA diluted 10-fold is used as a template for conventional or real-time PCR (Appendices 3, 5 and 6).

## Appendix 3 – Identification at species level by conventional or SYBR® Green real-time PCR (Schweigkofler *et al.*, 2004)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

### 1. General Information

1.1 The test was originally developed for identification of *F. circinatum* from pure culture or trapped airborne spores; it may be used for plant tissue or adapted to the analysis of seeds following the biological enrichment step (see Identification section, Ioos R., personal communication) using conventional or SYBR® Green real-time PCR.

1.2 The protocol was established by Schweigkofler *et al.* (2004).

1.3 The targeted gene is the rDNA IGS (Inter Genic Spacer) region (sequences of the IGS region for *F. circinatum* may be retrieved from GenBank, accessions AY249397 to AY249403).

1.4 A specific region of the IGS is amplified using the primer pair CIRC1A (forward) and CIRC4A (reverse).

1.5 Oligonucleotides:
Forward primer (CIRC1A): 5′ CTT GGC TCG AGA AGG G 3′
Reverse primer (CIRC4A): 5′ ACC TAC CCT ACA CCT CTC ACT 3′

1.6 The amplicon size is 360 bp.

1.7 Real-time PCR reactions were carried out using the iCycler from Bio-Rad (Hercules, CA, USA).

### 2. Methods

2.1 Nucleic Acid Extraction and Purification
See Appendix 2.

2.2 Conventional PCR

2.2.1 Master Mix

| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
|---|---|---|---|
| Molecular grade water* | NA | 12 | NA |
| PCR buffer (supplied with the DNA polymerase) | 10× | 2.5 | 1× |
| $MgCl_2$ | 50 mM | 1 | 2 mM |
| dNTPs | 2.5 mM | 2.5 | 0.25 mM (each dNTP) |
| Forward primer (CIRC1A) | 50 µM | 0.25 | 0.5 µM |
| Reverse primer (CIRC4A) | 50 µM | 0.25 | 0.5 µM |
| DNA polymerase (Platinum *Taq* Polymerase) | 5 U $\mu L^{-1}$ | 0.25 | 0.05 U $\mu L^{-1}$ |
| Subtotal | | 18.75 | |
| Template DNA | | 6.25 µL | |
| Total | | 25 | |

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

Each DNA extract should be tested by at least two replicate reactions.

2.2.2 PCR conditions

The PCR reaction should be carried out in a thermocycler equipped with a heated lid and include an initial denaturation at 94°C for 3 min, followed by 45 cycles for denaturation at 94°C for 35 s, annealing at 66°C for 55 s and elongation at 72°C for 50 s. A final elongation step is done at 72°C for 12 min.

The PCR products are separated by electrophoresis in a 1% agarose gel followed by staining. A DNA template containing amplifiable *F. circinatum* DNA will yield a 360-bp fragment after a CIRC1A/CIRC4A PCR.

2.3 SYBR Green real-time PCR reaction

A *F. circinatum*-specific IGS portion is amplified by PCR as follows.

2.3.1 Master mix

| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
|---|---|---|---|
| Molecular grade water† | NA | 8 | NA |
| PCR buffer (supplied with the DNA polymerase) | 10× | 2.5 | 1× |
| $MgCl_2$ | 50 mM | 2.5 | 5 mM |
| dNTPs | 2.5 mM | 2.5 | 0.25 mM (each dNTP) |
| SYBR® Green dye (Applied-Biosystems; concentration to be adjusted following the manufacturer's recommendation) | Data not available | 1.25 | Data not available |

Table *(continued)*

| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
|---|---|---|---|
| Fluorescein (Bio-Rad*) | Data not available | 1.25 | Data not available |
| Forward primer (CIRC1A) | 50 µM | 0.25 | 0.5 µM |
| Reverse primer (CIRC4A) | 50 µM | 0.25 | 0.5 µM |
| DNA polymerase | 5 U $\mu L^{-1}$ | 0.25 | 0.05 U $\mu L^{-1}$ |
| Subtotal | | 18.75 µL | |
| Template DNA | | 6.25 µL | |
| Total | | 25 | |

*Fluorescein can be omitted for platforms for which it is not relevant (volume of molecular water to be adjusted).
†Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

Each DNA extract should be tested by at least two replicate reactions.

2.3.2 Real-time PCR conditions

The PCR reaction conditions include an initial denaturation at 95°C for 3–10 min (according to the type of DNA polymerase), followed by 45 cycles for denaturation at 94°C for 35 s, annealing at 66°C for 55 s and extension at 72°C for 50 s. The fluorescence of the reporter dye is monitored at the end of each extension step.

The accumulation of *F. circinatum* PCR amplicons is monitored in real-time by the measurement of the specific fluorescence of the SyBr Green dye incorporated into the PCR product. A DNA template containing amplifiable *F. circinatum* DNA will yield a cycle threshold (Ct) value. The Ct value represents the estimated cycle number from which the level of fluorescence becomes significantly superior to the background fluorescence level.

The nature of the amplicons should be checked by yielding melting curves at the end of the amplification process and by comparison to the melting curves yielded with the PCR positive control.

## 3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification, preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the limit of detection (LOD).

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can be genes either present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

*Other possible controls*

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results

Conventional PCR

*Verification of the controls*

- NIC and NAC should produce no amplicons.
- PIC, PAC (and if relevant IC) should produce amplicons of the expected size.

*When these conditions are met*

- A test will be considered positive if it produces amplicons of 360 bp.
- A sample will be considered negative if it produces no band of 360 bp or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

The results should be confirmed by another method (see Fig. 1) which includes the sequencing of the amplicon as described in Appendix 4.

Real-time PCR

*Verification of the controls*

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

- The melting temperature value should be as expected (88.0 ± 0.5°C).

*When these conditions are met*

- A test will be considered positive if it produces an exponential amplification curve and if the amplicon shows the expected melting temperature value (88.0 ± 0.5°C).
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential or if the amplicon shows an unexpected melting temperature value.

Tests should be repeated if any contradictory or unclear results are obtained.

### 4. Performance criteria available

4.1 Analytical sensitivity

The analytical sensitivity of the test has been published in Schweigkofler *et al*. (2004) and the LOD is 10 pg of the target per PCR tube for real-time PCR. Analytical sensitivity for conventional PCR is not known.

4.2 Analytical specificity

The specificity of the primers was shown by PCR analyses of seven *F. circinatum* strains, 12 strains of *Fusarium* species belonging to clade 2 of the *F. fujikuroi* complex and 11 other *Fusarium* species (Schweigkofler *et al*., 2004). Only DNA from *F. circinatum* was amplified.

4.3 Additional performance values

The Pinestrength COST Action project (FP1406; Ioos *et al.,* 2019) calculated additional performance criteria based on the results of an international collaborative study.For real-time PCR, the diagnostic specificity was 94.4%, the diagnostic sensitivity was 83.6% and the concordance level was 96.8%.For conventional PCR, the diagnostic specificity was 92.4%, the diagnostic sensitivity was 84.3% and the concordance level was 93.0%.

## Appendix 4 – Confirmation of *F. circinatum* by sequencing

### 1. CIRC1A/CIRC4A sequencing

As the CIRC1A/CIRC4A conventional PCR test (see Appendix 3) might cross-react with phylogenetically close species (including non-described or ill-described species of the *F. fujikuroi* complex), especially when testing a high amount of *Fusarium* template DNA, the nature of the CIRC1A/CIRC4A amplicon can be verified by sequencing. Send an appropriate CIRC1A/CIRC4A PCR product for two-way sequencing with CIRC1A and CIRC4A as forward and reverse primer, respectively. The consensus sequence, from which the primers' sequences are trimmed prior to this, is compared by BLAST with those deposited in GenBank for numerous phylogenetically close *Fusarium* species (www.ncbi.nlm.nih.gov; see Appendix 3, section 1.3. for reliable sequences from the target organism). The sequence lying between CIRC1A and CIRC4A on the IGS region is sufficiently discriminant to identify *F. circinatum*.

### 2. EF1 gene partial sequencing (for identification of pure culture)

2.1 General Information

2.1.1 The protocol was established by O'Donnell *et al*. (1998), but the master mix composition and the PCR parameters are as used by ANSES (FR) laboratory (as not all details were given in the original publication).

2.1.2 The targeted region is a portion of the Translation elongation Factor 1 alpha (EF1).

2.1.3 A specific region of the EF1 is amplified using the primer pair EF1 (forward) and EF2 (reverse).

2.1.4 OligonucleotidesForward primer (EF1): 5′ ATG GGT AAG GAR GAC AAG AC 3′Reverse primer (EF2): 5′ GGA RGT ACC AGT SAT CAT GTT 3′

2.1.5 The amplicon size is around 640 bp.

2.2 Master Mix

| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
|---|---|---|---|
| Molecular grade water* | NA | 12.7 | NA |
| PCR buffer (supplied with the DNA polymerase) | 10× | 2 | 1× |
| $MgCl_2$ | 25 mM | 1.2 | 1.5 mM |
| dNTPs | 25 mM | 0.2 | 0.25 mM (each dNTP) |
| Forward primer (EF1) | 10 µM | 0.9 | 0.45 µM |
| Reverse primer (EF2) | 10 µM | 0.9 | 0.45 µM |
| DNA polymerase (platinum *Taq* polymerase) | 5 U $\mu L^{-1}$ | 0.1 | 0.025 U $\mu L^{-1}$ |
| Subtotal | | 18 | |
| Template DNA | | 2 | |
| Total | | 20 | |

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

Each DNA extract should be amplified by at least two replicate reactions that are subsequently pooled for sequencing.

2.3 PCR conditions

The PCR reaction should be carried out in a thermocycler equipped with a heated lid and include an initial denaturation at 94°C for 5 min, followed by 45 cycles for denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 60 s. A final elongation step is done at 72°C for 6 min.

The PCR products are separated by electrophoresis in a 1% agarose gel followed by ethidium bromide staining.

2.4 Interpretation

Starting with DNA extracted from a pure culture, a portion of the EF1 gene may be amplified and sequenced to verify the identity of the isolate. Send an appropriate EF1/EF2 PCR product for two-way sequencing with primer EF1 and EF2 as forward and reverse primer, respectively. The consensus sequence, from which the primers' sequences are eliminated prior to this, is compared by BLAST with those deposited in GenBank for numerous phylogenetically close *Fusarium* species ( www.ncbi.nlm.nih.gov). The sequence lying between EF1 and EF2 on the EF1 region is sufficiently discriminant to identify *F. circinatum*. The level of identity between the EF1 sequence of a reference strain of *F. circinatum* (e.g. GenBank accession number AF160295) should be between 99 and 100%.

## Appendix 5 – Identification at species level by dual-labelled probe real-time PCR (Ioos *et al.*, 2009)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

### 1. General Information

1.1 The test was recommended for use on seeds and has now been adapted to include other plant tissues and insects.

1.2 The test was developed by Ioos *et al*. (2009).

1.3 The PCR test targets a region of the intergenic spacer region (IGR; sequences of the IGS region for *F. circinatum* may be retrieved from GenBank, accessions AY249397 to AY249403).

1.4 A specific region of the IGS is amplified.

1.5 An amplicon of 149 bp is produced (including primer sequences).

1.6 OligonucleotidesForward primer (FCIR-F): 5′ TCG ATG TGT CGT CTC TGG AC 3′Reverse primer (FCIR-R): 5′ CGA TCC TCA AAT CGA CCA AGA 3′Probe (FCIR-P): 5′ FAM-CGA GTC TGG CGG GAC TTT GTG C-BHQ1 3′

1.7 Real-time PCR reactions were performed with a Rotor-Gene 6500 (Corbett Research, Mortlake, Australia).

1.8 Analyses were carried using the Rotor-gene with an autogain optimization for each channel performed before the first fluorescence acquisition.

### 2. Methods

2.1 Nucleic Acid Extraction and Purification

See Appendix 2.

2.2 Real-time PCR reaction

A *F. circinatum*-specific IGS portion is amplified by real-time PCR as follows.

2.3 Real-time Polymerase Chain Reaction – real-time PCR

2.3.1 Master Mix

| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
|---|---|---|---|
| Molecular grade water† | NA | 12.5 | NA |
| Real-time (RT) PCR buffer (supplied with the DNA polymerase) | 10× | 2 | 1× |
| $MgCl_2$ | 50 mM | 2 | 5 mM |
| dNTPs | 5 mM each | 0.8 | 0.20 mM (each dNTP) |
| Forward primer (FCIR-F) | 30 µM | 0.2 | 0.3 µM |
| Reverse primer (FCIR-R) | 30 µM | 0.2 | 0.3 µM |
| Probe 1 (FCIR-P) | 10 µM | 0.2 | 0.1 µM |
| Hotstart DNA polymerase | 5 U $\mu L^{-1}$ | 0.1 | 0.025 U $\mu L^{-1}$ |
| Subtotal | | 18 | |
| Template DNA | | 2 µL | |
| Total | | 20 | |

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

†Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

Each DNA extract should be tested by at least two replicate reactions.

2.4 PCR conditions

The PCR reaction conditions include an initial denaturation at 95°C for 10 min, followed by 40 cycles for denaturation at 95°C for 15 s, and annealing/extension at 70°C for 55 s. The fluorescence of the reporter dye is monitored at the end of each annealing/extension 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. The Ct value represents the estimated cycle number from which the level of fluorescence becomes significantly superior to the background fluorescence level.

### 3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively:

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target

organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the LOD.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can be either genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:
- specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

*Other possible controls*
- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 Interpretation of results: in order to assign results from a PCR-based test the following criteria should be followed

Real-time PCR

*Verification of the controls*
- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.
- NIC and NAC should give no amplification.

*When these conditions are met*
- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

### 4. Performance criteria available

4.1 Analytical sensitivity
The analytical sensitivity was determined by Ioos *et al*. (2009) using serial dilutions of *F. circinatum* DNA as templates. Assay results showed a linear relationship between Ct values and log (initial concentration of the target) down to a concentration of 80 fg per PCR tube and the correlation coefficient ($r^2$) was 0.99. The LOD was 8 fg per PCR tube but fell outside the linear response of the assay.

4.2 Analytical specificity
The analytical specificity was determined by Ioos *et al*. (2009), with DNA from nine *F. circinatum* isolates originating from the USA, Japan, South Africa, Spain and France, and with DNA from 37 strains of *Fusarium* species closely related to *F. circinatum* or commonly isolated from pine. Only DNA from the strains of *F. circinatum* yield Ct values with the real-time test. The results of an international collaborative study carried out during the Pinestrength COST Action project (FP1406; Ioos *et al.,* 2019) provide additional data regarding analytical specificity. In certain conditions (other laboratories, reagent brands, etc.), unexpected cross-reactions were sometimes obtained with DNA from *F. subglutinans* and *F. begoniae*.

4.3 Other performance criteria
The Pinestrength COST Action project (FP1406; Ioos *et al.,* 2019) calculated additional performance criteria based on the results of an international collaborative study. The diagnostic specificity was 86.4%, the diagnostic sensitivity was 83.3% and the concordance level was 88.5%.

## Appendix 6 – Identification at species level by dual-labelled probe real-time PCR (Luchi *et al.*, 2018)

The test below is described as it was carried out to generate the validation data provided in section 4. Other equipment, kits or reagents may be used provided that a verification (see PM 7/98) is carried out.

### 1. General Information

1.1 The test was originally developed for identification of *F. circinatum* from pine tissue and can be combined in a duplex format to also detect *Caliciopsis pinea,* another pine pathogen.

1.2 The test was developed by Luchi *et al*. (2018)

1.3 The PCR test targets a region of the translation Elongation Factor 1-alpha (EF1) gene (the sequence of the EF1 region for *F. circinatum* may be retrieved from GenBank, accession JN092335)

1.4 A specific region of the EF1 is amplified.

1.5 An amplicon of 54 bp is produced (including primer sequences).

1.6 OligonucleotidesForward primer (Fcrc-F): 5′ CGA GCG ATG CGC GTT T 3′Reverse primer (Fcrc-R): 5′ ACG TGA CGA TGC GCT CAG T

3′Probe (Fcrc-Pr): 5′ FAM-CCC TCC CAT TGC CAC –MGBNFQ[3] 3′

1.7 Real-time PCR reactions were performed with a StepOnePlus real-time PCR system (Applied Biosystems, Life Science, Foster City, CA, USA).

1.8 Analyses were carried using StepOne software (Applied Biosystems) after manual adjustment of the baseline and fluorescent threshold.

## 2. Methods

2.1 Nucleic Acid Extraction and Purification

See Appendix 2.

2.2 Real-time PCR reaction.

A *F. circinatum*-specific EF1 portion is amplified by real-time PCR as follows.

2.3 Real-time Polymerase Chain Reaction – real-time PCR

2.3.1 Master Mix

| Reagent | Working concentration | Volume per reaction (µL) | Final concentration |
|---|---|---|---|
| Molecular grade water* | NA | 6.5 | NA |
| TaqMan universal PCR Master mix (Applied Biosystems) | 2× | 12.5 | 1× |
| Forward primer (Fcrc-F) | 30 µM | 0.25 | 0.3 µM |
| Reverse primer (Fcrc-R) | 30 µM | 0.25 | 0.3 µM |
| Probe (Fcrc-Pr) | 10 µM | 0.5 | 0.2 µM |
| Subtotal | | 20 | |
| Template DNA | | 5 µL | |
| Total | | 25 | |

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.22 µm filtered) and nuclease-free.

Each DNA extract should be tested by at least two replicate reactions.

2.4 PCR conditions

The PCR reaction conditions include an initial uracil-DNA glycosylase incubation at 50°C for 2 min, followed a DNA denaturation/DNA polymerase activation at 95°C for 10 min, followed by 50 cycles for denaturation at 95°C for 30 s and annealing/extension at 60°C for 60 s. The fluorescence of the reporter dye is monitored at the end of each annealing/extension 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 Fcrc-Pr probe. A DNA template containing amplifiable *F. circinatum* DNA will yield a cycle threshold (Ct) value. The Ct value represents the estimated cycle number from which the level of fluorescence becomes significantly superior to the background fluorescence level.

[3]Minor Groove Binder Nonfluorescent Quencher. DNA probes with conjugated minor groove binder (MGB) groups form extremely stable duplexes with single-stranded DNA targets, allowing shorter probes to be used.

## 3. Essential procedural information

3.1 Controls

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid extraction and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: nucleic acid extraction and subsequent amplification preferably of a sample of uninfected matrix or if not available clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a matrix sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of molecular grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product). For PCRs not performed on isolated organisms, the PAC should preferably be near to the LOD.

As an alternative (or in addition) to the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. Positive internal controls can be either genes present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls can include:

- specific amplification or co-amplification of endogenous nucleic acid, using conserved primers that amplify conserved non-pest target nucleic acid that is also present in the sample (e.g. plant cytochrome oxidase gene or eukaryotic 18S rDNA)
- amplification of samples spiked with exogenous nucleic (control sequence) acid that has no relation with the target nucleic acid (e.g. synthetic internal amplification controls) or amplification of a duplicate sample spiked with the target nucleic acid.

*Other possible controls*

- Inhibition control (IC) to monitor inhibitory effects introduced by the nucleic acid extract. Same matrix spiked with nucleic acid from the target organism.

3.2 **Interpretation of results: in order to assign results from PCR-based test the following criteria should be followed:**

Real-time PCR

*Verification of the controls*

- The PIC and PAC (as well as IC and IPC as applicable) amplification curves should be exponential.

- NIC and NAC should give no amplification.

*When these conditions are met*

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

## 4. Performance criteria available

4.1 Analytical sensitivity

The analytical sensitivity was determined by Luchi *et al*. (2018) using serial dilutions of *F. circinatum* genomic DNA as templates. The LOD of the assay was 0.32 pg per tube. A standard curve was built with *F. circinatum* gDNA extracts ranging from 25 ng per tube to 40 pg per tube. The slope was −3.59 and the linear correlation $r^2$ was 0.99.

4.2 Analytical specificity

The analytical specificity was determined by Luchi *et al*. (2018), with DNA from 41 *F. circinatum* isolates originating from the USA, Japan, South Africa, Spain, Chile and France, and with DNA from 37 strains of *Fusarium* species closely related to *F. circinatum* or commonly isolated from pine. Only DNA from the strains of *F. circinatum* and DNA from *F. temperatum* yielded Ct values with the real-time test.

4.3 Other performance criteria

Luchi *et al*. (2018) determined the inter and intra assay variabilities using standard DNAs used to build the standard curve, run in three separate assays (inter assay variability) and three replicates run in one assay (intra assay variability). The inter assay coefficient of variation (CV) was 0.50% and the intra assay CV was 0.91%.The Pinestrength COST Action project (FP1406; Ioos *et al.,* 2019) calculated additional performance criteria based on the results of an international collaborative study. The diagnostic was specificity 97.0%, the diagnostic sensitivity was 84.8% and the concordance level was 97.7%.