**Diagnostics**
**Diagnostic**

# *Gibberella circinata*

## Specific scope

This standard describes a diagnostic protocol for *Gibberella circinata*[1].

## Specific approval and amendment

Approved in 2009–09.

## Introduction

*Gibberella circinata* is the causal agent of pitch canker disease. The disease almost exclusively affects *Pinus* sp., but was also described on Douglas-fir (*Pseudotsuga menziesii*). This disease is a serious threat to the pine forests wherever it occurs (especially on plantations of *Pinus radiata*), due to extensive tree mortality, reduced growth and timber quality. Multiple branch infection may cause severe crown dieback and eventually lead to the death of the tree. This aggressive fungus may also cryptically infect the *Pinus* seeds and may cause damping-off in seedlings. Conifer seeds can be colonized by *G. circinata* internally (where it can remain dormant until seed germination) and externally (Storer *et al.*, 1998).

*Fusarium circinatum,* anamorph of *G. circinata,* 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 areas to disease-free areas may be driven by infected seed movement and movement of infected plant material (Storer *et al.*, 1998).

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

[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.

The fungus has gone through a number of anamorph name changes, with the same teleomorph *Gibberella fujikuroi*. Originally described as *Fusarium lateritium* f. sp. *pini* (Snyder *et al.*, 1949), it was successively renamed *Fusarium moniliforme* f. sp. *subglutinans* (Kuhlman *et al.*, 1978), *F. subglutinans* f. sp. *pini*, 'H' mating population (Correll *et al.*, 1991, 1992) and was finally ranked at the species level as *F. circinatum,* with a new teleomorph recognition: *G. circinata* (Nirenberg & O'Donnell, 1998). The *G. fujikuroi* sp. complex encompasses at least 36 anamorphic *Fusarium* sp. (O'Donnell *et al.*, 1998), from which the new species *G. circinata* is now excluded. Numerous *Fusarium* sp. residing in this complex, already fully described or still poorly documented, are morphologically similar and identification may require several techniques to be undertaken. In particular, Steenkamp *et al.* (1999) report that some of the distinguishing morphological characters may be inadequate or insufficient to make a definite identification of *F. circinatum* (Nirenberg & O'Donnell, 1998).

Although an official ISTA method was published in 2002 to detect *F. moniliforme* f. sp. *subglutinans* in seeds of *Pinus taeda* and *Pinus elliotii* (ISTA, 2002), the latter is not recommended as the morphological features indicated as typical for *F. moniliforme* f. sp. *subglutinans* in this method are based on a substrate not showing the characteristic sterile hyphae of this pathogen and are not sufficient to ensure a reliable identification of *F. circinatum* Nirenberg & O'Donnell (anamorphic stage of *G. circinata*).

## Identity

**Name**: *Gibberella circinata* Nirenberg & O'Donnell
**Anamorph**: *Fusarium circinatum* Nirenberg & O'Donnell
**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*

Notes on taxonomy and nomenclature: For many years, the pitch canker pathogen was known only as an anamorph (form or pathotype of *F. subglutinans*; Correll *et al.*, 1991). Since its teleomorph was discovered following *in vitro* crosses, it is known as *G. circinata* (Nirenberg & O'Donnell, 1998). However, since only the anamorphic form of *G. circinata* will be observed in pure culture after isolation, *F. circinatum* will consistently be used in the morphological descriptions throughout this protocol.

**EPPO code**: GIBBCI

**Phytosanitary categorization**: EPPO A1 list no. 306.

## Detection

### Symptoms

The fungus causes cankers that girdle branches, aerial roots and even trunks of *Pinus* sp., often associated with conspicuous and sometimes spectacular resin exudates ('pitch') in response to the fungal infection (Fig. 1A,B). *Gibberella circinata* may also be soil-borne, and can infect seeds cryptically (Storer *et al.*, 1998). The fungus can also cause root rot (Coutinho *et al.*, 1997). Symptoms may be observed at any time of the year.

Infected seedlings show usual damping off symptoms, but are not distinctive to a *G. circinata* infection: needles turn red, brown or chlorotic and die from the base upwards or the seedling dies (Fig. 2).

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 and 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 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 a 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. 3), on the main stems and even on the trunk, associated with the typical resin bleeding (Fig. 1A,B). However, the symptoms in older trees can be mistaken with those caused by *Sphaeropsis sapinea* (Fr.) Dyco & Sutton (synonym *Diplodia pinea*), 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 cortical and subcortical tissue of the trunk. Removal of the bark shows subcortical lesions with brown and resin-impregnated tissues.

### Sampling procedure

Despite the fact that they may exhibit different susceptibility levels to *G. circinata*, all the *Pinus* sp. along with douglas-fir (*Pseudotsuga menziesii),* may be potentially attacked by this fungus. There are two methods to be used, depending of the type of material to be sampled (plant tissue/seeds).

*Plant tissue (except seeds)*

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 the most active. The pieces of tissue should be wrapped with sheets of sponge towels or newspapers and placed in a sealed plastic bag.


**Fig. 1** (A,B) Copious resin exudates (pitch) beneath cankers caused by *Gibberella circinata* (courtesy of J Armengol Instituto, Agroforestal Mediterraneo, Universidad politeenica de Valencia/CNRS and R Ioos, Station de Mycologie, Malzéville, FR).

**Fig. 2** Seedling damping-off caused by *Gibberella circinata* in nursery (courtesy of E Landeras, Laboratorio de Sanidad Vegetal, Oviedo, ES.).

**Fig. 3** Shoot cankers caused by *Gibberella circinata* (courtesy of J Armengol, Institute Agroforestal Mediterraneo. Universidal Politeenica de CNRS.).

Sections of shoots, twigs or aerial roots should be collected by visual inspection upon observation of the symptoms indicated above. The sample should include the lesion edge and a few centimeters of healthy-looking tissue ahead of the lesion. The pieces of tissue are wrapped with sheets of sponge towels or newspapers and placed in a sealed plastic bag.

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.

*Seeds*

Depending on the method chosen for the identification, the number of seeds to be analyzed per lot may be different. The total number of seeds is 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.). Sample size recommended by ISTA is 400 seeds for plating (ISTA, 2009). However, larger samples (e.g. 1000 seeds) can easily be processed by biological enrichment before DNA analyses (Ioos *et al.*, 2009). As no symptoms can be seen on seeds, the lot should be sampled randomly. As counting of seeds may be laborious in some cases, the sampled seeds may be weighed instead of being counted. 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* sp. The seeds will be subsequently analyzed without any surface disinfection, as *G. circinata* may be present on the seed husk, as well as inside the seed.

### Isolation on semi-selective medium

*Plant tissue except seeds*

Isolations from symptomatic conifer trees or seedlings are made onto media including Komada, Dichloran Chloramphenicol

**Table 1** Mean thousand seed weight for the major *Pinus* and *Pseudotsuga* sp. (source: French Forestry Board 'Office National des Forêts'). TSW, thousand seed weight

| Species | Indicative TSW (g) | Species | Indicative TSW (g) |
|---|---|---|---|
| *Pinus aristata* | 22 | *Pinus mugo* subsp *pumilio* | 6 |
| *Pinus armandi* | 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 griffithi* | 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 leucodermis* | 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 |

**Table 2** Sequence and target of the PCR primers and probes combinations

| Primer | Sequence (5′-3′) | Size (bp) | Target | Reference |
|---|---|---|---|---|
| H3-1a | ACT AAG CAG ACC GCC CGC AGG | ca 520 | Histone H3 gene | Steenkamp *et al.* (1999) |
| H3-1b* | GCG GGC GAG CTG GAT GTC CTT | | | |
| CIRC1A | CTT GGC TCG AGA AGG G | 360 | IGS rDNA region | Schweigkofler *et al.* (2004) |
| CIRC4A* | ACC TAC CCT ACA CCT CTC ACT | | | |
| FCIR-F | TCG ATG TGT CGT CTC TGG AC | 146 | IGS rDNA region | Ioos *et al.* (2009) |
| FCIR-R* | CGA TCC TCA AAT CGA CCA AGA | | | |
| FCIR-P | FAM-CGA GTC TGG CGG GAC TTT GTG C-BHQ1 | | | |
| ITS1 | TCC GTA GGT GAA CCT GCG G | ca 580 | ITS rDNA region | White *et al.* (1990) |
| ITS4* | TCC TCC GCT TAT TGA TAT GC | | | |
| 18S uni-F | GCA AGG CTG AAA CTT AAA GGA A | 150 | 18S rDNA | Ioos *et al.* (2009) |
| 18S uni-R* | CCA CCA CCC ATA GAA TCA AGA | | | |
| 18S uni-P | JOE-ACG GAA GGG CAC CAC CAG GAG T-BHQ1 | | | |

*reverse primers.

Peptone Agar (DCPA) or onto Potato dextrose agar 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*. On seedlings the pathogen is isolated from the lower part of the stem and from the roots. 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 (Fig. 4). During incubation, the plates are observed periodically and all the *Fusarium* spp. colonies are transferred to Potato dextrose agar (PDA) and to Spezieller-Nährstoffarmer Agar (SNA) (Appendix 1) for morphological identification. This method is very efficient and reliable to isolate 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.

*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). During incubation, the plates are observed periodically and all the *Fusarium* spp. colonies are transferred to Potato dextrose agar (PDA) and to Spezieller-Nährstoffarmer Agar (SNA) (Appendix 1) for morphological identification. This method is efficient and reliable to isolate any *Fusarium* spp. from the seeds and does not require expensive equipment, though 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) have demonstrated that agar plating of pine seeds may not be able to detect dormant (quiescent) propagules of *F. circinatum*, thus leading to an unknown risk of false negative results.

### Direct detection *in planta* using molecular techniques (plant tissue, including seeds)

See *Identification* section for description. These methods are fast, efficient and reliable in detecting *G. circinata* 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 conventional PCR requires confirmation as cross-reaction with phylogenetically closely related species might occur and lead to false positive results.

**Fig. 4** Sub cortical necrosis caused by *Gibberella circinata* beneath a canker observed on a stem (courtesy of E Landeras, Laboratorio de Sanidad Vegetal, Oviedo, ES).

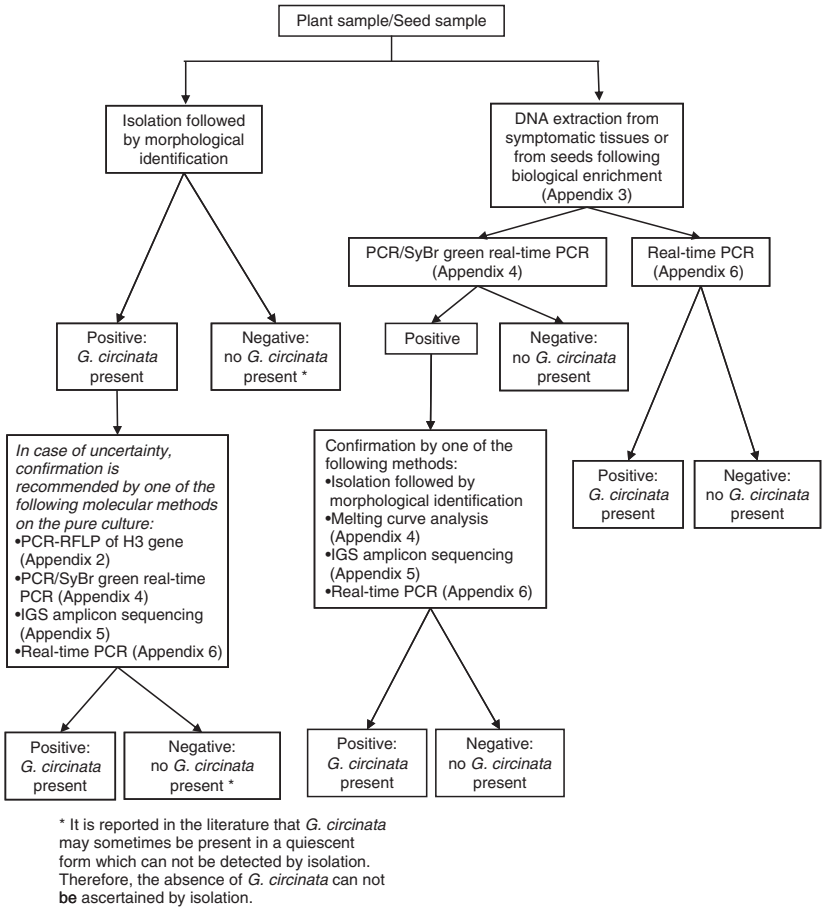


**Fig. 5** Flow diagram for diagnosis of *Gibberella circinata* on plant tissue and on seeds.

## Identification

The procedures for the identification of *G. circinata* on *Pinus* spp. and *Pseudotsuga menziesii* will consist of (i) isolating the fungus from the plant tissue on semi-selective culture media followed by morphological and, in the case of uncertainty, molecular identification, or (ii) directly detecting the fungus *in planta* by conventional PCR, SyBr green real-time PCR or dual-labelled probe real-time PCR. A flow diagram indicating the different combinations of methods is shown in Fig. 5.

### Morphological characteristics in pure culture

For morphological identification, the isolates are grown on PDA to study colony morphology and pigmentation, and on SNA (Appendix 1) to study formation and type of microconidia and conidiogenous cells. SNA and PDA plates are incubated at room temperature. 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.* (2002). On PDA, *F. circinatum* grows relatively rapidly (average growth of 4.7 mm/day at 20°C; Nirenberg & O'Donnell, 1998). After 10 days, the colony should have an entire margin, white

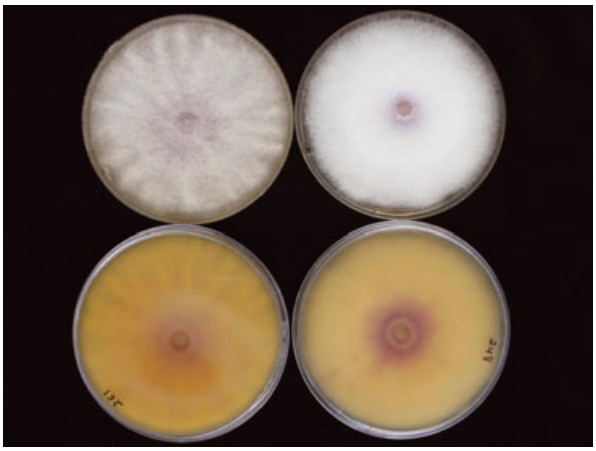

**Fig. 6** Cultural aspect of the anamorphic stage of *Gibberella. circinata* (*F. circinatum*) on potato dextrose agar (left: *Fusarium circinatum* MAT-1; right: *Fusarium circinatum* MAT-2). MAT-1 mating type produces typical coiled sterile hyphae on Spezieller-Nährstoffarmer Agar (SNA), whereas MAT-2 mating type produces not distinctively coiled or even uncoiled sterile hyphae (courtesy of A. Pérez-Sierra, Instituto Agroforestal Mediterraneo, Universidad Politecnica de Valencia (ES)) (see also Fig. 9A,B).

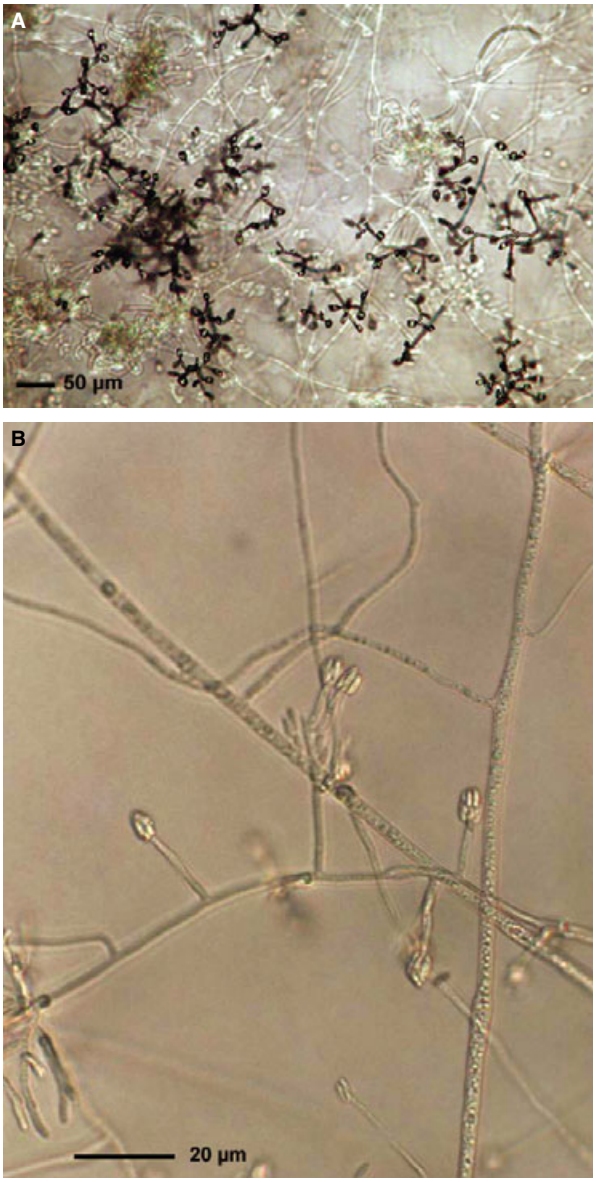


**Fig. 7** (A) Erect conidiophores bearing microconidia arranged in false heads of *Fusarium circinatum*, observed directly on Spezieller-Nährstoffarmer Agar (SNA) medium (×200 magnification) (courtesy of R. Ioos, Station de Mycologie, Malzéville (FR)) and (B) on a microscopic slide (×400 magnification)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

cottony or off-white aerial mycelium with a salmon tinge in the middle or with a purple or dark violet pigment in the agar (Fig. 6). On SNA, microconidia are aggregated in false heads (Fig. 7A,B), with branched conidiophores, mono and polyphialidic- conidiophores (Fig. 8A,B), obovoid microconidia in aerial mycelium, mostly nonseptate or with occasionally 1-septum. Chlamydospores are absent. The sterile hyphae (coiled/not

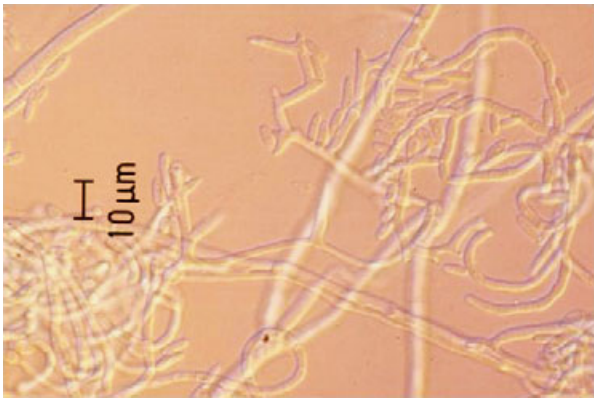


**Fig. 8** Mono- and polyphialidic conidiophores of *Fusarium circinatum* observed on Spezieller-Nährstoffarmer Agar (SNA) medium (courtesy of J. Armengol).

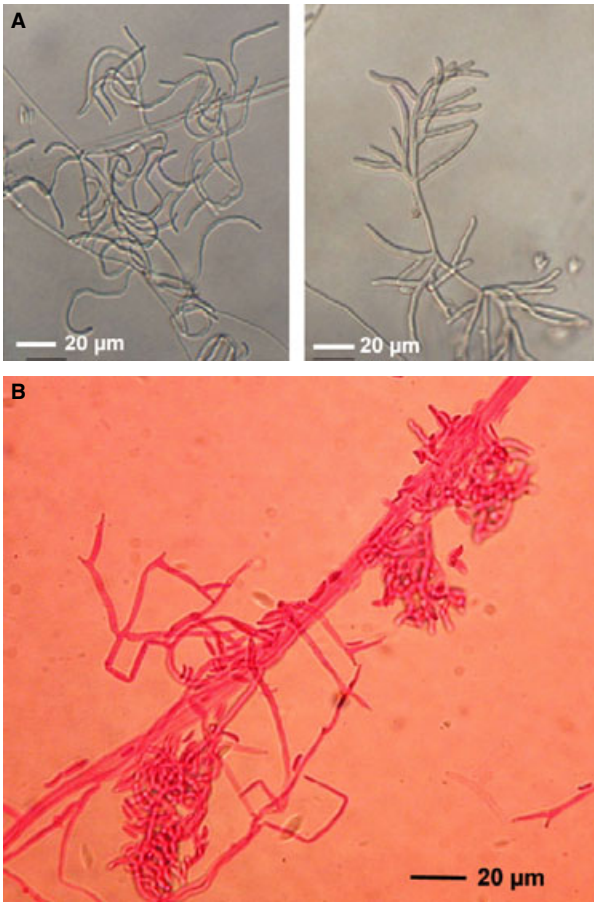


**Fig. 9** (A) Coiled and not distinctively coiled sterile hyphae produced on Spezieller-Nährstoffarmer Agar (SNA) medium by MAT-1 (left) and MAT-2 (right) mating type isolates of *Fusarium circinatum*, respectively (courtesy of A. Pérez-Sierra, Instituto Agroforestal Mediterraneo, Universidad Politecnica de Valencia (ES)). (B) Groups of coiled sterile hyphaes and polyphialidic conidiophores produced on Spezieller-Nährstoffarmer Agar (SNA) (courtesy of R. Ioos, Station de Mycologie, Malzéville (FR)).

distinctively coiled) are characteristic of *F. circinatum* and are observed clearly on this medium (Fig. 9A,B). The epithet '*circinatum*' refers to these typical coiled hyphae, also called 'circinate' hyphae.

### Molecular methods

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

- A PCR-RFLP (Restriction Fragment Length Polymorphism) test, with primers and RFLP pattern developed by Steenkamp *et al.* (1999) is presented in Appendix 2 and is appropriate for identification of the anamorphic stage of *G. circinata* in pure culture only as contaminants or host material may affect the quality and numbers of PCR amplicons.
- SyBr green real-time PCR or conventional PCR tests with primers designed by Schweigkofler *et al.* (2004) can be useful for identification of the fungus in pure culture, as well as for direct detection of the pathogen in seeds, and is presented in Appendix 4. However, when carried out on plant samples DNA, verification of the nature of the PCR amplicon should be carried out by sequencing for conventional PCR, or by melting analysis for SyBr green real-time PCR (Appendix 5). Indeed, infection by other *Fusarium* spp. is frequent and cryptic speciation was reported in the *Gibberella fujikuroi* sp. complex (Steenkamp *et al.*, 2002). PCR cross-reaction might occur with phylogenetically close *Fusarium* sp., especially with high amounts of *Fusarium* template DNA
- Method for real-time PCR with primers and a dual-labelled probe designed by Ioos *et al.* (2009) can be useful for identification of the fungus in pure culture, as well as for direct detection of the pathogen in plant tissue, including seeds, and is presented in Appendix 6. This method proved to be more sensitive than the conventional PCR (diagnostic sensitivities of 79.1% and 58.6%, respectively; Ioos *et al.*, 2009) described in Appendix 4 and its specificity is strengthened thanks to the combination of specific primers and probe.

Another conventional PCR test has been developed by Ramsfield *et al.* (2008) but there is no experience with this test in the EPPO region, therefore it is not described in the protocol.

*DNA extraction from pure culture*

Fungal DNA should be extracted using an appropriate standard method for DNA extraction from fungi e.g. regular commercial plant DNA extraction kits (or other methods reviewed in Irlinger *et al.*, 2008) and analyzed following any of the tests presented in Appendices 2, 4 or 6.

*DNA extraction from plant tissue (except seeds)*

Total DNA from potentially infected plant tissue should be extracted as described in Appendix 3 and analyzed following any of the tests presented in Appendices 4 or 6.

*DNA extraction from seeds*

Total DNA from potentially infected seeds should be extracted as described in Appendix 3 and analysed following any of the tests presented in Appendices 4 or 6.

## Reference cultures

The type strain of *G. circinata* (CBS 405.97) and other strains (CBS 117843, Spain; CBS 119864, South Africa; CBS 100197, USA) are available from CBS, Utrecht (NL).

## Reporting and documentation

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

## Further information

Further information on this organism can be obtained from:

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## Appendix 1 – Composition of the different culture media

**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 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 (PNCB, 75% w/w): 1.0 g
- Oxgall: 0.5 g
- $Na_2B_4O_7$ $10H_2O$: 1.0 g
- Streptomycin: 6 mL/L of stock solution (5 g of streptomycin in 100 mL distilled water).

**PDAS**

Potato dextrose agar supplemented with 0.5 mg/mL of streptomycin sulphate salt (775 units/mg solid).

**Dichloran Chloramphenicol Peptone Agar (DCPA)**

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

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

- Bacteriological peptone, 15.0 g
- $KH_2PO_4$, 1.0 g
- $MgSO_4(7H_2O)$: 0.5 g
- Chloramphenicol: 0.2 g
- 2.6-dichloro-4-nitroanilin (dichloran) (0.2% W/V in ethanol, 1.0 mL): 2 mg
- Violet crystal (0.05% W/V in water, 1.0 mL): 0.0005 g
- Technical agar: 20.0 g
- Distilled water: to 1.0 L

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

This medium should be mandatory used for 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 1.0 L

Optionally, two 1-$cm^2$ square pieces of sterile filter paper may be laid on the surface of the agar since *Fusarium sporodochia* are sometimes more likely to be produced at the edge of the paper.

## Appendix 2 – Identification at species level by PCR-RFLP (Steenkamp *et al.*, 1999)

### (1) General information

Steenkamp *et al.* (1999) described a technique based on a PCR-RFLP carried out on the histone H3 gene to identify the anamorphic stage for *G. circinata* from pure culture. Total DNA extracted from a pure *Fusarium circinatum* (anamorphic stage of *G. circinata*) culture is the nucleic acid source.

The PCR test targets the histone H3 gene and produces a 515 bp amplicon for *G. circinata* (sequences of partial *G. circinata* H3 gene may be retrieved from Genbank, accessions AF150847 to AF150853). The histone H3 gene is first amplified in *Gibberella* spp. using the primer pair H3-1a (forward) and H3-1b (reverse) (Table 2).

### (2) Methods

*Nucleic acid extraction and purification*

Fungal DNA should be extracted using an appropriated standard method for DNA extraction from fungi (Irlinger *et al.*, 2008). Purified DNA should be frozen until analysis.

*PCR reaction*

The PCR reaction mixture includes:

- 1× PCR buffer (supplied with the DNA polymerase),
- 0.25 mM each dNTP,
- 2.5 mM $MgCl_2$,
- 0.2 µM of each H3-1a and H3-1b primers,
- 0.05 U/µL DNA polymerase
- 25–50 ng of template DNA,
- Molecular grade water (MGW) is added to reach the final reaction volume (20 µL).

The PCR reaction conditions are carried out on a thermocycler equipped with a heated lid and include an initial denaturation at 92°C for 1 min, followed by 30 cycles of denaturation at 92°C for 1 min, annealing at 63°C for 1 min and elongation at 72°C for 1 min. A final elongation step is done at 72°C for 5 min. The PCR product is kept at 5°C or less, until restriction analysis.

*Enzymatic digestion*

Histone H3 PCR products obtained are thereafter consecutively digested with restriction enzymes *Cfo*I and *Dde*I, respectively. Consecutive enzymatic digestion is performed by addition of 5 U *Cfo*I to 15 µL of unpurified PCR product followed by incubation at 37°C for 3 h. Subsequently, the sodium chloride concentration is adjusted to 100 mM and 5 U of *Dde*I is added to the reaction mixture. This is followed by further 5-h incubation at 37°C. However, Pérez-Sierra *et al.* (2007)showed that it was possible to add both enzymes simultaneously without adjustment of the sodium chloride concentration, and to simply incubate the mixture for 5 h at 37°C.

The digested amplicons are separated by electrophoresis in 2–3% agarose gels followed by ethidium bromide staining.

The PCR-RFLP pattern of *G. circinata* (refered to as the anamorph *F. subglutinans* f. sp. *pini*, mating population 'H', by Steenkamp *et al.* (1999) consists of bands of 250, 232, and 33 (not observed on the gel) bp.

### (3) Essential procedural information

Total DNA obtained from a reference culture of *G. circinata* should be processed in parallel with the DNA samples to be analyzed in order (i) to have a reference control pattern on the final electrophoresis gel and (ii) to ensure that the PCR/enzymatic process was correctly achieved.

## Appendix 3 – DNA extraction for *in planta* detection of *Gibberella circinata*

### (1) 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 first collected then subsequently cut into smaller pieces (<2–3 mm, each side) into a sterile plastic Petri dish.

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, 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 (Macherey-Nagel, Hoerdt, France), 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 4 and 6).

### (2) Seeds

*Biological enrichment*

This procedure was initially described by Ioos *et al.* (2009) and should be followed when the presence of *G. circinata* is checked by a conventional or real-time PCR test carried out directly on a seed DNA extract (Appendix 4 and 6). The purpose of this preliminary biological enrichment step is to increase the biomass of viable *G. circinata* propagules, prior to DNA extraction and molecular testing.

As recommended by ISTA for agar plating technique (ISTA, 2002), at least 400 seeds per seed lot are incubated at 22 ± 3°C for 72 hrs 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 in a way 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 is subsequently ground with a mixer mill till a homogenous solution is obtained. Sterile water or sterile PDB may be added at this step in case 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® mini-prep (Macherey-Nagel, Hoerdt, France), which proved to be efficient, but other DNA extraction protocols may be used providing that they proved 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.

## Appendix 4 – Identification at species level by conventional or SyBr green real-time PCR (Schweigkofler *et al.*, 2004)

### (1) General information

Schweigkofler *et al.* (2004) described a technique based on a conventional or a SyBrgreen real-time PCR designed from the rDNA IGS (Inter Genic Spacer) region to identify the anamorphic stage of *G. circinata* in pure culture or in trapped airborne spores, but may be adapted to the analysis of seeds following the biological enrichment step (See *Identification* section, Ioos R., pers. comm.).

The PCR test targets a region of the IGS and produces a 360 bp amplicon for *G. circinata* (sequences of the IGS region for *G. circinata* may be retrieved from Genbank, accessions AFAY249397 to AY249403). A specific region of the IGS is amplified with *G. circinata* DNA using the primer pair CIRC1A (forward) and CIRC4A (reverse) (Table 2).

### (2) Methods

*Nucleic acid extraction and purification*

See Appendix 3.

*Conventional PCR reaction*

A *G. circinata*-specific IGS portion is amplified by PCR as follows.

The PCR reaction mixture includes:

- 1× PCR buffer supplied with the DNA polymerase,
- 0.25 mM each dNTP,
- 2 mM $MgCl_2$,
- 0.5 µM of each CIRC1A and CIRC4A primers,
- 0.05 U/µL DNA polymerase
- 6.25 µL of template DNA,
- Molecular grade water is (MGW) added to reach the final reaction volume (25 µL).

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

The PCR reaction conditions 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 ethidium bromide staining. A DNA template containing amplifiable *G. circinata* DNA will yield a 360-bp fragment after a CIRC1A/CIRC4A PCR.

*SyBr green real-time PCR reaction*

A *G. circinata*-specific IGS portion is amplified by PCR as follows.

The PCR reaction mixture includes:

- 1× PCR buffer supplied with the DNA polymerase,

- 0.25 mM each dNTP,
- 5 mM $MgCl_2$,
- 0.5 µM of each CIRC1A and CIRC4A primers,
- SyBrgreen dye (concentration to be adjusted following the manufacturer's recommendation)
- 0.05 U/µL DNA polymerase
- 6.25 µL of template DNA,
- Molecular grade water is (MGW) added to reach the final reaction volume (25 µL).

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

The real-time PCR reactions are carried out in a suitable PCR instrument equipped with a system capable of fluorescence monitoring.

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 *G. circinata* 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 *G. circinata* 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

A **DNA extraction negative control** (blank tube) should be included for each DNA extraction series in order to ensure the absence of contamination during this step.

A **PCR negative control** containing no target DNA should be included in every test in order to ensure the absence of contamination during PCR.

A **PCR positive control** should be used (genomic DNA from a reference strain of *G. circinata*, or subcloned *G. circinata* CIRC1A/CIRC4A PCR product). When testing plant and seed samples, the positive control should correspond to the limit of detection of the test (LOD). This **LOD positive control**[2] should be included in order to assess the performance of the PCR run and to ensure that the negative results are caused by an absence or a too low level of the PCR target in the DNA sample, rather than by an insufficient PCR efficiency.

The quality of the DNA extract should be assessed by a relevant means e.g. by spectrophotometry, by using an *ad hoc* **internal amplification control** or by testing the extract in PCR with the fungal ribosomal genes primers ITS1 and ITS4 (White *et al.*, 1990). In the latter case, the PCR conditions are those described above, simply replacing the FCIRC1A/-CIRC4A primers with ITS1 and ITS4 primers (Table 2), and decreasing the annealing temperature to 50°C. A positive signal (approximately 600 bp) following this test would mean that the DNA extract was amplifiable: DNA was successfully extracted and the level of co-extracted inhibiting compounds was sufficiently low.

Interpretation of results:

- A sample will be considered positive if it produces amplicons of 360 bp and provided that the contamination controls are negative.
- A sample will be considered negative if (i) it produces no band of 360 bp; (ii) provided that the sample DNA extract proved to be amplifiable and that no significant inhibition occurred; and (iii) if used, that the LOD positive control tested in the PCR run yielded a 360 bp amplicon.
- Plant samples whose DNA extract yields a Ct inferior or equal to $Ct_{LOD}$ should be considered as infected by *G. circinata,* provided that the negative controls (PCR and DNA extraction) do not yield Ct.
- Plant samples whose DNA extract does not yield a Ct inferior or equal to $Ct_{LOD}$ should be considered as non-infected by *G. circinata,* or infected below the detection threshold of the technique, provided that the sample DNA extract proved to be amplifiable and that no significant inhibition occurred.
- Tests should be repeated if any contradictory or unclear results are obtained.

## Appendix 5 – Confirmation of *Gibberella circinata* by sequencing

As the CIRC1A/CIRC4A conventional PCR test might cross react with phylogenetically close species (including non- or ill-described species of the *G. fujikuroi* complex), especially when testing 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 primer 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* sp. (http://www.ncbi.nlm.nih.gov). The sequence lying between CIRC1A and CIRC4A on the IGS region is sufficiently discriminant to identify *G. circinata*.

[2]LOD positive control is made of diluted genomic DNA from a reference strain of *G. circinata*, or diluted subcloned *G. circinata* CIRC1A/CIRC4A PCR product. It can be defined as the lowest target amount giving positive result in at least 95% of the times, thus ensuring a ≤5% false negative rate.

## Appendix 6 – Identification at species level by dual-labelled probe real-time PCR (Ioos *et al.*, 2009)

### (1) General information

Ioos *et al.* (2009) described a technique based on a real-time PCR designed from the rDNA IGS (Intergenic spacer region) to identify the anamorphic stage of *G. circinata* (*F. circinatum)* in pure culture or directly in plant samples.

The PCR test targets a region of the IGS and produces a 149 bp amplicon for *G. circinata* (sequences of the IGS region for *G. circinata* may be retrieved from Genbank, accessions AFAY249397 to AY249403). A specific region of the IGS is first amplified with *G. circinata* DNA using the primer pair FCIR-F (forward) and FCIR-R (reverse) and detected by a fluorescent probe FCIR-P (Table 2).

### (2) Methods

*Nucleic acid extraction and purification*

See Appendix 3.

*Real-time PCR reaction*

A *G. circinata*-specific IGS portion is amplified by real-time PCR as follows.

The real-time PCR reaction mixture includes:

- 1 × PCR buffer supplied with the DNA polymerase,
- 0.20 mM each dNTP,
- 5 mM $MgCl_2$,
- 0.2 µM of each FCIR-F and FCIR-R primers,
- 0.1 µM of FCIR-P probe,
- 0.025 U/µL Hotstart DNA polymerase,
- 25–50 ng of template DNA,
- Molecular grade water (MGW) is added to reach the final reaction volume (20 µL).

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

The real-time PCR reactions are carried out in a suitable PCR instrument equipped with a system capable of fluorescence monitoring.

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, 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 *G. circinata* 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 *G. circinata* 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

A **DNA extraction negative control** should be included for each DNA extraction series in order to ensure the absence of contamination during this step (blank tube containing sterile MGW, or 500 µL of PD Broth for seed samples).

A **PCR negative control** (no template control, containing for instance MGW) should be included in every experiment to check the absence of contamination during PCR.

A **PCR limit of detection (LOD) positive control**[3] should be used in order to assess the performance of the PCR run and to ensure that the negative results are caused by an absence or a too low level of the PCR target in the DNA sample, rather than by an insufficient PCR efficiency.

The quality of the DNA extract should be assessed by a relevant means, e.g. by spectrophotometry, by testing the extract in conventional PCR, with the universal fungal ribosomal genes primers ITS1 and ITS4 (See Appendix 4) or in real-time PCR, with other universal plant and fungal primers and probe such as 18S uni-F/-R/-P (Ioos *et al.*, 2009) or other universal tests described in the scientific literature. A positive signal (approximately 600 bp) following ITS1/ITS4 PCR or a Ct yielded with 18S uni-F/-R/-P real-time PCR test would mean that the DNA extract was amplifiable: DNA was successfully extracted and the level of co-extracted inhibiting compounds was sufficiently low.

Interpretation of results:

- Plant samples whose DNA extract yields a Ct inferior or equal to $Ct_{LOD}$ should be considered as infected by *G. circinata,* provided that the negative controls (PCR and DNA extraction) do not yield Ct.
- Plant samples whose DNA extract does not yield a Ct inferior or equal to $Ct_{LOD}$ should be considered as non-infected by *G. circinata,* or infected below the detection threshold of the technique, provided that the sample DNA extract proved to be amplifiable and that no significant inhibition occurred.
- Tests should be repeated if any contradictory or unclear results are obtained. Doubtful or borderline results should be re-analyzed using the same or another technique (e.g. sequencing).

[3]LOD positive control is made of diluted genomic DNA from a reference strain of *G. circinata*, or diluted subcloned *G. circinata* FCIR-F/FCIR-R PCR product. It can be defined as the lowest target amount giving positive result in at least 95% of the times, thus ensuring a ≤5% false negative rate.