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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/111 (1)

Diagnostics
Diagnostic

PM 7/111 (1) *Fusarium foetens*

Specific scope

This standard describes a diagnostic protocol for *Fusarium foetens*[1].

Specific approval and amendment

Approved as an EPPO Standard in 2012–09.

Introduction

Fusarium foetens causes a destructive wilt of *Begonia* × *hiemalis* (known as *B. elatior* hybrid) (Schroers *et al.*, 2004; Brand & Wienberg, 2005; Elmer, 2008). It has been responsible for severe damage in the begonia flower industry. It is highly pathogenic to various *Begonia* × *hiemalis* cultivars. Other hybrids were described as either susceptible (e.g. *Begonia* × *tuberhybrida*, *Begonia* × *cheimantha*) (Brand & Wienberg, 2005) or unsusceptible (e.g. *Begonia* × *semperflorens-cultorum*, *Begonia* × *tuberhybrida*) (Elmer, 2008). Some *B. rex* cultivars can show stunted growth on infection with *F. foetens*, while other species (e.g. *B. boliviensis*, *B. cinnabarina*, *B. coccinea*, *B. partita* and *B. schmidtiana*) are not recorded to develop typical symptoms (Brand & Wienberg, 2005; Elmer, 2008). Further studies are necessary to determine the specific pathogenicity of *F. foetens* to *Begonia* species. Pathogenicity testing by the National Plant Protection Organisation of Netherlands further revealed that the fungus is not a pathogen on *Saintpaulia ionantha*, *Impatiens walleriana*, *Pelargonium zonale* and *Euphorbia pulcherrima*, which are commonly grown in nurseries along with *Begonia* × *hiemalis*. *Fusarium foetens* caused vascular discolorations in *Cyclamen persicum* but its significance is not yet determined.

A pest risk assessment for *F. foetens* was published in 2002 (Baayen *et al.*, 2002) and updated recently (van der Gaag & van Raak, 2010). In a study by Huvenne *et al.* (2011), 16 potential host species were inoculated, including *Cyclamen persicum*, *Rosa* mini, *Exacum affine*, *Saintpaulia ionantha*, *Euphorbia pulcherrima*, *Calathea* spp., *Spathiphyllum* 'Alfa', *Delphinium*, *Campanula isophylla*, *Rudbeckia* and *Valeriana officinalis.* They found that although *F. foetens* can sustain itself on all plant species tested, and even accumulate to high levels in the root system of some of these species, it produced symptoms only on *Begonia*.

Experimental data suggest that *F. foetens* probably infects its hosts via the hypocotyls or roots, and that the infections develop systemically. Irrigation systems and fungus gnats may distribute *F. foetens* within *Begonia* growing facilities. The data available so far suggest that *F. foetens* is soilborne. No data currently suggest that wind is important for its distribution.

Morphological and phylogenetic studies revealed that this *Begonia* pathogen differs from *Fusarium beganiae* and other taxa of the *Fusarium oxysporum* species complex (Schroers *et al.*, 2004). The data currently available suggest that *F. foetens* does not recombine sexually. Morphologically, *F. foetens* resembles *F. oxysporum* although they are phylogenetically distinct.

Fusarium foetens was not isolated in Europe until 2000. The fungus has been detected on *Begonia* in Canada, USA, Japan, Netherlands, Germany and Norway (Neubauer & Nirenberg, 2002; Elmer *et al.*, 2004; Schroers *et al.*, 2004; Sekine *et al.*, 2008; Tian *et al.*, 2010), and intercepted in the UK (Jones, 2002). It has been eradicated in France (EPPO, 2011). In Europe, until now symptoms of *F. foetens* have been seen only on its susceptible *Begonia* hosts. Its ability to survive in soil and infect certain plant species asymptomatically may allow its further spread and constitutes a potential threat to other ornamental crops.

The diagnostic procedure for *F. foetens* is presented in Fig. 1.

[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.

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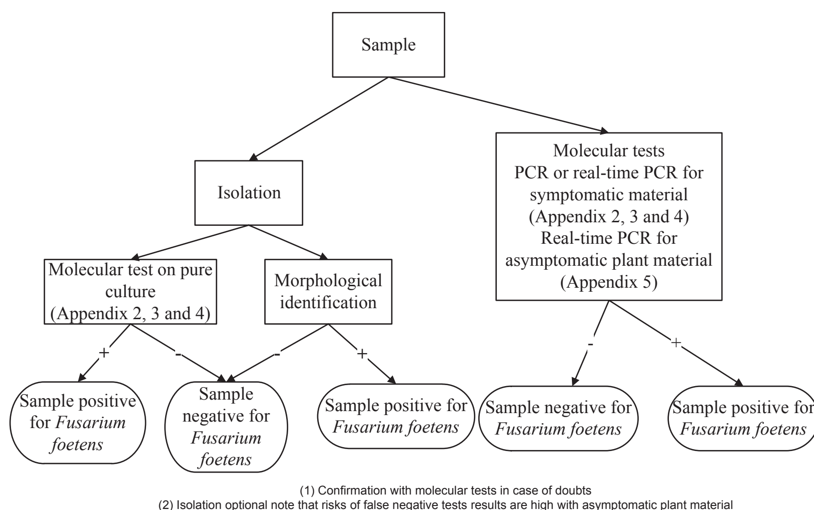

Fig. 1 Flow diagram for detection and identification of *Fusarium foetens*

Identity

Name: *Fusarium foetens* Schroers, O'Donnell, Baayen & Hooftman
Teleomorph: Not known.
Synonyms: No synonyms known. Potentially misidentified as *Fusarium oxysporum* Schltdl.
Taxonomic position: Fungi: Ascomycota: Hypocreales: Nectriaceae
EPPO code: FUSAFO
Phytosanitary categorization: EPPO A2 List. No. 345

Detection

Symptoms

Fusarium foetens causes a destructive wilt that leads to entire collapse of susceptible *Begonia* hosts within a few weeks. Early symptoms include decreased growth and development and dwarfing of young plants, development of a shiny appearance of leaves and vein yellowing. The developing disease is characterized by wilting leaves that become brownish first distally, then entirely; vascular bundles of roots, stems and infected leaves develop brown discoloration; a quickly developing rot of the main stem is followed by the entire collapse of the plant. Pale to light orange-coloured sporodochia normally develop on necrotic stem tissue. The sporodochia, developing directly on the plant surface or from a mat of white mycelium, can cluster as small speckles or aggregates of such (Fig. 2).

Fig. 2 Sporodochia of *Fusarium foetens* on stems of *Begonia* after incubation (courtesy of M Heupel, DE)

Sampling procedure

Sampling symptomatic plants
Tools used for sampling or dissecting plants should be disinfected. Individual potted plants can be sampled, and it is recommended to protect the upper part of the plant from contact with the growing medium during transport. Samples should be packed in dry packing material.

Packaging should be sealed and samples sent to the laboratory as soon as possible, as testing should be conducted within 5 days after sampling. Upon arrival at the laboratory, samples should be processed immediately or refrigerated.

Sampling of asymptomatic plants
Entire cuttings should be sampled when asymptomatic batches of plants are to be screened. When detection is based on isolation, the lower stem base should be analysed, whereas when detection is based on molecular tests, roots should be analysed For maximum sensitivity in detection, it is desirable to plant the cuttings in a sterilized potting substrate and maintain them in a guaranteed *F. foetens*-free greenhouse for up to 4 weeks under conditions used in

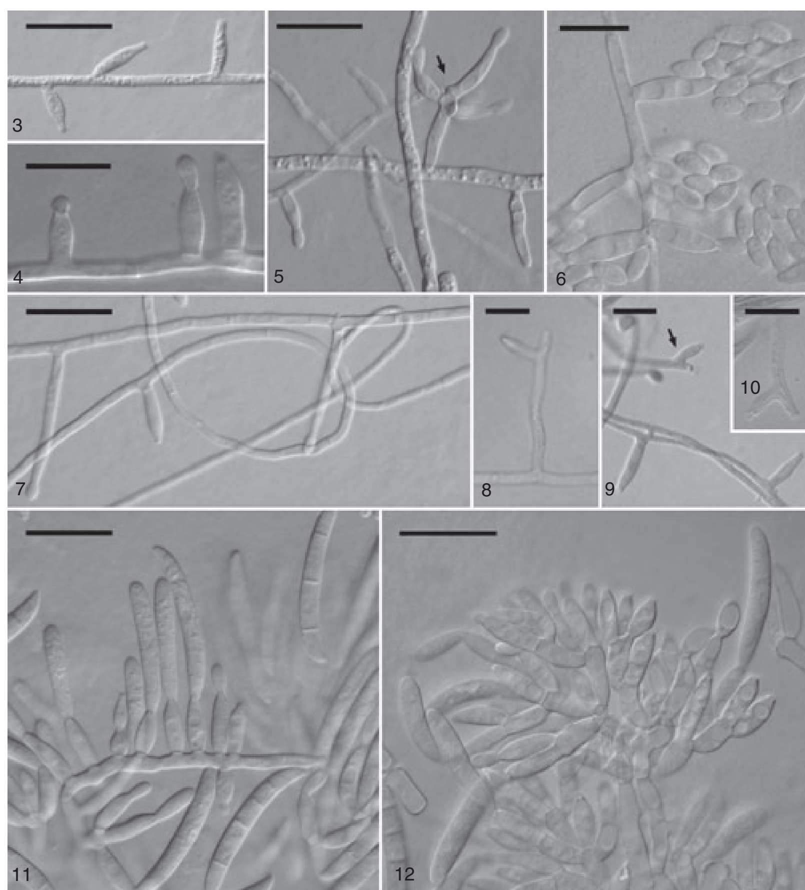

Fig. 3–12 *Fusarium foetens* morphological characters (photos courtesy of HJ Schroers)

pathogenicity tests, such as 25–28°C, 16 h light and 65% relative humidity (Huvenne *et al.*, 2011). This will increase the pathogen concentration in the plants and avoid false negatives (Huvenne *et al.*, 2011). For guidance on sampling, refer to ISPM 31 *Methodologies for sampling of consignments* (FAO, 2008).

Isolation

Isolation from symptomatic material

For details of media, see Appendix 1.

Pieces or blocks of symptomatic plant material are washed in tap water and surface disinfected (e.g. 5 s in 70% ethanol). Small pieces of approximately 5 mm^2 are excised and aseptically placed on the surface of synthetic nutrient-poor agar (SNA) or diluted potato dextrose agar (dPDA) amended with antibiotics for the suppression of bacterial growth. The plates can be kept at room temperature and under general laboratory conditions. Single hyphal tips are removed from the emerging colonies and transferred to Petri dishes containing SNA. Semi-selective media have been used successfully in laboratories in the EPPO region (Dichloran Chloramphenicol Peptone Agar (DCPA), Komada medium).

Fusarium foetens may also be isolated by removing a small mass of conidia from one sporodochium and suspending the conidia in sterile water. To obtain single-spore isolates, suspensions are diluted (e.g. in series) and plated on media. Alternatively, single-spore isolates are obtained by other methods such as using a micromanipulator (Gams *et al.*, 1998).

For isolation from asymptomatic plants

Cross-sections of the surface-disinfected stem base should be plated. Aseptic dissection of the vascular system from the cross-sections and plating only the parts of the vascular system can enhance the selectiveness of the isolation. Then proceed as above.

Identification

Identification of *F. foetens* is possible based on morphological characters. In case of doubt, molecular tests should also be performed. For symptomatic plants, the presence of

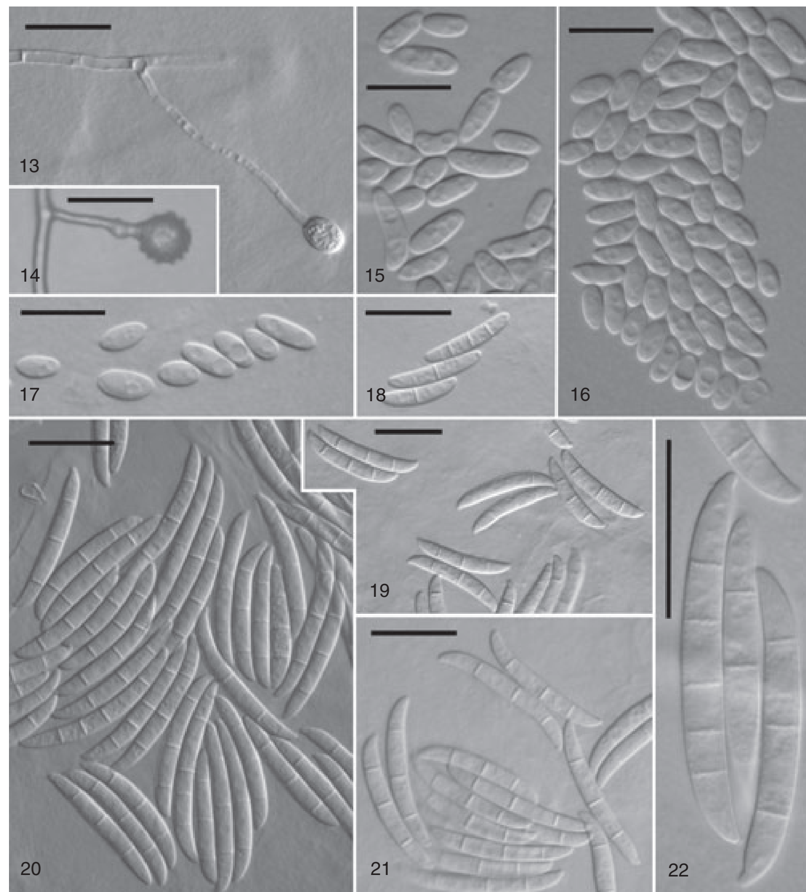

Fig. 13–22 *Fusarium foetens* conidia (photos courtesy of HJ Schroers)

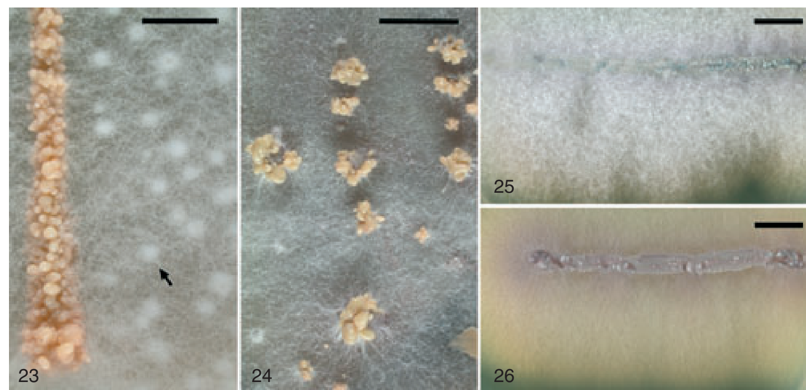

Fig. 23–26 *Fusarium foetens* in culture (photos courtesy of HJ Schroers)

symptoms and a positive PCR test is sufficient to consider the sample as positive.

Morphological identification

Until now, little phenotypic variation has been documented in *F. foetens* although Tschöpe *et al.* (2007) differentiated white and pink strains. Morphological identifications follow Schroers *et al.* (2004). They segregate *F. foetens* morphologically from the following.

- *Fusarium begoniae* Nirenberg & O'Donnell, the common agent of flower, leaf and stem blight (de Gruyter *et al.*, 1994; as *Fusarium sacchari* (Butler) W. Gams var. *elongatum* Nirenberg), which does not produce chlamydospores and forms polyphialides abundantly (Nirenberg & O'Donnell, 1998).

- Members of the *F. oxysporum* species complex not producing polyphialides and without, or with a less pungent, colony odour (Schroers *et al.*, 2004).

For identification according to the morphology of conidiophores and macro- and microconidia, a pure culture grown on SNA for 10–14 days at 20–25°C should be used.

Several samples of the sparsely developing aerial mycelium should be studied to evaluate the abundantly formed monophialides and rare polyphialides scattered and arising laterally on the aerial mycelium. Several cultures on potato dextrose agar (PDA) incubated for 7–10 days together in a cellophane bag allow the detection of the pungent odour once the bag is opened without requiring opening of the Petri dishes.

Morphological description adapted from Schroers *et al.* (2004)

Size of colonies after 4 days	SNA 32–36–43 mm diameter PDA 30–34–37 mm diameter
Size of colonies after 7 days	SNA 57–68–79 mm diameter PDA 51–62.5–68 mm diameter
Description of colonies	Aerial mycelium white, on SNA sparsely developed, faint, sometimes almost absent but abundantly formed near or on filter paper, consisting of single hyphae or ropes of few hyphae; on PDA abundantly produced, forming thick white tufts evenly covering the whole (Fig. 25) or only irregular portions of a colony. Colony reverse on PDA when incubated in the dark unpigmented in most areas or with spots, sectors, or the central half appearing brownish (6D5), reddish brown (8E6), greyish ruby (12E5), or dark green (29F4); on PDA when incubated under near-UV light with brownish orange to brownish hues (5C5–5D5, 6D5, 6E5), or reddish brown (9D4) to greyish red (10D4). For colour codes see Kornerup & Wanscher (1978).Tschöpe *et al.* (2007) distinguished between strains forming either red-coloured or white to pink-coloured colonies on PDA. Odour of colonies on SNA indistinct, of colonies on PDA pungent, irritating. Teleomorph unknown.
Conidiophores on aerial mycelium	Formed laterally from hyphae of the aerial mycelium producing microconidia or in sporodochia producing macroconidia. Bearing solitary monophialides (Figs 3–7), occasionally short supporting cells with whorls of up to 3 monophialides (Fig. 5), or more rarely, solitary polyphialides (Figs 8–10); phialides either cylindrical and slightly tapering toward the tip (long phialides in Fig. 5) or narrowly flask-shaped (Figs 3 and 5), with widest point in the middle, (3.5–) 12.5 (–33.5) µm long, (1.8–)2.4 (–3.3) µm wide at base, (2.1–)3.3 (–4.4) µm at widest point, and (1.1–)1.5 (–1.9) µm near the aperture (*n* = 82).
Microconidia	Predominantly 0-septate (Figs 15–17) to rarely 3-septate (Fig. 18), held in hyaline heads; 0-septate conidia ovoidal to ellipsoidal, sometimes allantoidal with hilum mostly visible and apex rounded (Figs 15–17), (4.5–)6.5(–13.5) x (2.1–)2.8(–4.3) µm (*n* = 438); 1–3-septate conidia fusiform to slightly curved, generally without recognizable foot-cell (Fig. 18); 1-septate conidia (12–)16 (–24) x (2.6–)3.4(–4.2) µm (*n* = 31).
Sporodochia	Formed on agar surface (Figs 23 and 24), submerged, or on a prosenchymatous stroma, bearing monophialides that arise laterally in dense aggregations (Fig. 11) or terminally in adpressed whorls from verticillately branched supporting cells (Fig. 11).
Macroconidia	Predominantly 3(–5)- septate, slightly curved; the central cell(s) frequently almost straight, typically widest in the middle; basal cell indistinctly pedicellate (Figs 9–22); 3-septate conidia (22.5–) 34(– 47.5) x (3.4–) 4.4(–5.3) µm (*n* = 387); 4- and 5-septate conidia not conspicuously longer than 3-septate conidia; masses of conidia on SNA flat hemispherical, up to 200 µm diameter, pale to light orange (5A3–5A4); masses of conidia on PDA light orange, brownish orange or light brown (5A4, 5C4, 5D4) when incubated under near-UV light or hyaline, cream-coloured, or greyish blue (23C4) when incubated in the dark.
Chlamydospores	Globose to subglobose, rare or abundant, mostly terminal, smooth or warted, 7–13 3 7–11 µm (Figs 13 and 15).

Molecular tests

Total DNA from infected plant tissue or from mycelium developing in pure culture systems can be obtained by using commercial kits according to the manufacturer's instructions (see Appendices 2–4). In general, disrupting and homogenizing the material with a bead beater or similar machines with a stainless steel bead or similar beads is essential. The molecular tests described below for the identification of *F. foetens* are based on direct sequencing (Appendix 2), species-specific endpoint PCR and electrophoresis (Appendix 3), and real-time PCR (Appendix 4).

Fungal DNA may be present only in small quantities in asymptomatic plants such as rooted cuttings. In order to increase sensitivity, DNA should be extracted from washed root systems (e.g. after tissue disruption with a Homex tissue macerator) and an appropriate DNA extraction technique (Appendix 5) should be used, followed by real-time PCR (Appendix 4).

Reference material

Strains can be obtained from CBS or NRRL. CBS, CBS-KNAW Fungal Biodiversity Center, Uppsalalaan 8, 3584 CT Utrecht, Netherlands; NRRL, Bacterial Foodborne Pathogens & Mycology Research Unit, National Center for Agricultural Utilization Research, 1815 N. University Street, Peoria, IL 61604, USA.

Reporting and documentation

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

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).

Further information

Further information on this organism can be obtained from Schroers H-J, Agricultural Institute of Slovenia, Hacquetova 17, SI-1000 Ljubljana, Slovenia, Tel: +386 (0) 1 2805276, e-mail: hans.schroers@kis.si or van Raak M, National Reference Laboratory, Geertjesweg 15, 6700 HC Wageningen, Tel +31 317 496834, e-mail marcel.van.raak@minlnv.nl

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

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

The protocol was initially drafted by Schroers H-J, Agricultural Institute of Slovenia, Hacquetova 17, SI-1000 Ljubljana, Slovenia, e-mail: hans.schroers@kis.si.

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Appendix 1 – Preparation of agar media

Antibacterial stock solution according to Kirk *et al.* (2008)

For isolation of *F. foetens*, a solution of the antibacterial penicillin G and streptomycin sulfate should supplement agar media such as SNA or dPDA (see below) for the suppression of bacteria.

Ingredients for antibacterial stock solution	
Penicillin G	1.2 g
Streptomycin sulfate	1.0 g
Distilled water	40 mL

Autoclave the sterile water for 15 min. Cool down the water in the refrigerator. Add and dissolve penicillin G and streptomycin sulfate. Remove an aliquot of 8 mL by using a 10 or 20 mL syringe. Add the penicillin G and streptomycin sulfate solution (8 mL) to 1000 mL agar medium (approximately 50°C) through a sterile 0.45 μm pore membrane. Mix gently and immediately pour the medium into plastic Petri dishes.

Synthetic nutrient-poor agar (SNA) (Nirenberg, 1976)

Suitable for the isolation and identification of *F. foetens* based on conidiophores and micro- and macroconidia. If used for isolation, antibacterial penicillin G and streptomycin sulfate should be added (see above). If used for identification, SNA without antibiotics must be used but autoclaved pieces of filter paper (approximately 1.0–1.5 cm^2) can be added on top of the solidified SNA to enhance sporulation.

Ingredients for SNA	
KH_2PO_4	1 g
KNO_3	1 g
$MgSO_4 \cdot 7H_2O$	0.5 g
KCl	0.5 g
Glucose	0.2 g
Sucrose	0.2 g
Technical agar	15 g (or according to the manufacturer's instructions)
Distilled water	1 L

Combine all ingredients and suspend in water. Heat with frequent agitation and boil for 1 min to dissolve the ingredients completely. Sterilize by autoclaving at 121°C for 15 min. Cool medium to approximately 50°C. Pour into sterilized plastic Petri dishes. If SNA is used for isolation, add an appropriate amount of antibiotic stock solution through a sterile filter (see above) into approximately 50°C-warm SNA before pouring the medium into Petri dishes.

Diluted potato dextrose agar (dPDA)

A diluted PDA supplemented with antimicrobial penicillin G and streptomycin sulfate (see above) can be used for isolating *F. foetens*. Full PDA should not be used.

Ingredients for dPDA	
PDA (Difco)	13 g (or one-third of the amount recommended by another manufacturer for full PDA)
Technical agar	10 g (or according to the manufacturer's instructions)
Distilled water	1 L

Combine all ingredients and heat gently to dissolve. Sterilize by autoclaving at 121°C for 15 min. Cool medium to approximately 50°C. Add an appropriate amount of antibiotic stock solution through a sterile filter (see above) into approximately 50°C-warm dPDA before pouring the medium into Petri dishes.

Potato dextrose agar (PDA)

Suitable for studying macromorphological characters of *F. foetens* such as pigmentation and surface structure.

Ingredients for PDA	
PDA (Difco)	39 g (or according to manufacturer's instructions)
Distilled water	1 L

Combine all ingredients and heat gently to dissolve. Sterilize by autoclaving at 121°C for 15 min. Cool medium to approximately 50°C. Pour the medium into Petri dishes immediately.

Dichloran chloramphenicol peptone agar (DCPA)

Ingredients for DCPA	
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	1.0 L

Komada medium

Ingredients for the basal medium	
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	1 L

The pH is adjusted to 3.8 ± 0.2 with 10% phosphoric acid. The basal medium is autoclaved and cooled slightly before adding the following filter-sterilized supplementary solution.

Ingredients for supplementary solution	
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^{-1} stock solution (5 g streptomycin in 100 mL distilled water)

Appendix 2 – Identification of *F. foetens* by conventional PCR and direct sequencing

1. General information

The following protocol allows amplification of a fragment of the partial *TEF1* gene region from *Fusarium*. Sequencing the fragment allows identification of *Fusarium* species using BLAST searches against databases established at FUSARIUM-ID (http://isolate.fusariumdb.org), GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) or the Fungal Biodiversity Centre CBS (http://www.cbs.knaw.nl/fusarium).

1.1 The described marker was used repeatedly for the phylogenetic characterization of *Fusarium* species (O'Donnell *et al.*, 1998; also providing the sequences of the primers). PCR cycle parameters are from Geiser *et al.* (2005).

1.2 Nucleic acid source: mycelium or conidia from pure culture.

1.3 Name of targeted gene: partial *TEF1*. GenBank accession number of ex type strain CBS 110286 (= NRRL 31852): AY320087.

1.4 Amplicon location in *Fusarium graminearum* (http://mips.helmholtz-muenchen.de/genre/proj/FGDB/singleGeneReport.html?entry=FGSG_08811): supercontig 3.5, starting coordinate 532189. Protein name: Elongation factor 1-alpha (EF-1-alpha).

1.5 Amplicon size in base pairs (including primer sequences and introns): 714 bp.

1.6 Forward primer for PCR amplification (5′–3′): EF-1, ATGGGTAAGGARGACAAGAC. Reverse primer for PCR amplification (5′–3′): EF-2, GGARGTACCAGTSATCATGTT. Purification grade for oligonucleotide primers: salt-free or higher.

1.7 Enzyme used in PCR reaction: native *Taq* DNA polymerase. Producer: Fermentas (5 units per µL) (or others).

1.8 Deoxynucleotide triphosphates (dNTPs). Producer: Promega (or others), 100 mM.

1.9 PCR buffer. Producer: Fermentas (or others), provided together with Taq DNA polymerase. Specifically: Taq buffer 10× with $(NH_4)_2SO_4$, pH 8.8 at 25°C, but other PCR buffers can also be used.

1.10 Reaction additives: $MgCl_2$, 25 mM. Producer: Fermentas (or others), provided together with Taq DNA polymerase.

1.11 Source/quality of water: molecular-grade water (MGW), nuclease-free (Promega or others).

1.12 Thermal cycler: Applied Biosystems GeneAmp PCR System 9700 (or others).

2. Methods

2.1 Extraction of nucleic acid (DNA)

2.1.1 DNA is extracted using commercial kits [e.g. DNeasy Plant (Qiagen), NucleoSpin Plant II (Macherey-Nagel GmbH & Co), Gentra Puregene Cell Kit (Qiagen)] following the manufacturer's recommendations.

2.1.2 An amount of biomass as recommended by the producer of these kits is collected in a 2.0 mL safe-lock microcentrifuge tube (e.g. Eppendorf). A stainless steel grinding ball (approx. 4 mm diameter) is added to disrupt the biomass using a tissuelyser system (e.g. Qiagen Retsch) for 60 s at the highest speed.

2.1.3 If applicable, the specifically supplied support protocols for the extraction of genomic DNA from fungi should be followed. DNA can be stored at 4°C overnight or at −20°C for longer periods.

2.2 Polymerase chain reaction (PCR)

2.2.1 Master mix

The final volume used for PCR should be 30 to 50 µL. The specifications provided in the table refer to a final volume of 50 µL per PCR reaction.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
PCR-grade water		To make up to 50	
PCR buffer	10×	5	1×
$MgCl_2$	25 mM	3	1.5 mM
dNTPs	2.5 mM	4	0.2 mM (of each of the dNTPs)

(continued)

Table (continued)

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Forward primer (EF-1)	100 µM	0.3	0.6 µM
Reverse primer (EF-2)	100 µM	0.3	0.6 µM
Taq polymerase (e.g. Fermentas)	5 U μL^{-1}	0.2	1 U
Genomic DNA extract		1	
Total		50	

2.2.2 PCR cycling parameters

94°C for 5 min, 35 cycles of 94°C for 60 s, 53°C for 60 s, 72°C for 1 min (60 s), 72°C for 7 min followed by cooling to 4°C.

3. Essential procedural information for conventional PCR

3.1 Controls:

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

- Negative isolation control (NIC) to monitor contamination during nucleic acid extraction: clean extraction buffer.
- Positive isolation control (PIC) to ensure nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organisms (e.g. mycelium or spores from a reference culture).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of PCR-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, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

3.2. Interpretation of the conventional PCR

Verification of controls:

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of 714 bp.

When these conditions are met:

- A test will be considered positive if amplicons of 714 bp are produced. A test will be considered negative if it produces no band or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

When the conventional PCR is positive, sequencing should be performed.

4. Performance criteria available

No information available on performance criteria.

5. Purification of PCR product

The PCR product is purified using a commercial kit, for example QIAquick PCR Purification Kit (Qiagen) or others.

6. Sequencing and evaluation

Primers for cycle sequencing reactions:

Forward EF-1 (see 1.6) or EF-11(5′–3′), GTGGGGCA TTTACCCCGCC

Reverse EF-2 (see 1.6) or EF-21(5′–3′), GAGTGGCGG GGTAAATGCC or EF-22(5′–3′), AGGAACCCTTACCG AGCTC.

Sequencing can be done in-house or may be subcontracted to another laboratory.

Sequencing chromatograms are viewed, edited and trimmed as far as necessary using appropriate software (Sequencher from Applied Biosystems, or others) and the obtained sequence is used for a BLAST query (http://isolate.fusariumdb.org, http://blast.ncbi.nlm.nih.gov/Blast.cgi, http://www.cbs.knaw.nl/fusarium).

The required identity of the full-length product is 100% (excluding the primers if EF-1/EF-2 have been used, as not all base pairs are specific in those primers).

Appendix 3 – Identification of *Fusarium foetens* using species-specific primers in conventional PCR

1. General information

1.1 The protocol was developed by de Weerdt *et al.* (2006) and is based on a primer annealing to a species-specific site of the partial *TEF1* gene region.

1.2 Nucleic acid source: mycelium or conidia from pure culture, fungal structures (e.g. sporodochia) with or without dissected symptomatic plant tissue or dissected symptomatic plant tissue.

1.3 Name of targeted gene: partial *TEF1*. GenBank accession number of ex type strain CBS 110286 (= NRRL 31852): AY320087.

1.4 Protein name: Elongation factor 1-alpha (EF-1-alpha).

1.5 Amplicon size in base pairs (including primer sequences): 276 bp.

1.6 Forward primer (orientation 5′–3′): Ff_EF49f, CTCTCCTCGACAATGAGCTT. Reverse primer (orientation 5′–3′): Ff_EF324r, CAAAAAAAA TTACGGTCACATC.

1.7 Enzyme used in PCR reaction: HotStarTaq polymerase (Qiagen Multiplex PCR kit, ready-made pre-mix).

1.8 Deoxynucleotide Triphosphates (dNTPs) (Qiagen Multiplex PCR kit, ready-made pre-mix).

1.9 Multiplex PCR buffer (Qiagen Multiplex PCR kit, ready-made pre-mix).

1.10 Reaction additives: $MgCl_2$ (Qiagen Multiplex PCR kit, ready-made pre-mix).

1.11 Source/quality of water: RNase-Free Water Ultrapure quality (MGW) (Qiagen Multiplex PCR kit).

1.12 Thermal cycler: MJ Research PTC 200 Peltier thermal cycler.

2. Methods

2.1 Extraction of nucleic acid (DNA)

2.1.1 For DNA extraction, see Appendix 2, 2.1.1.

2.2 Polymerase chain reaction (PCR)

2.2.1 Master mix

The specifications provided in the table refer to a final volume of 25 μL per PCR reaction.

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
PCR-grade water		To make up to 25	
Multiplex PCR Master Mix, including, Multiplex PCR Buffer, $MgCl_2$, dNTPs, HotStarTaq DNA polymerase	2×	12.5	1×
Forward primer (Ff_EF49f)	10 μM	0.5	0.2 μM
Reverse primer (Ff_EF324r)	10 μM	0.5	0.2 μM
Total DNA extract		2	
Total		25	

2.2.2. PCR cycling parameters

95°C for 10 min (activation of hot start DNA polymerase), 35 cycles of 95°C for 30 s, 55°C for 60 s, 72°C for 60 s, 72°C for 5 min

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 isolation 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 of a sample of uninfected host tissue (when working with plant samples) or clean extraction buffer (when working with pure cultures).
- Positive isolation control (PIC) to ensure 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 PCR-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 pure cultures, the PAC should preferably be near to the limit of detection.

As 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 may be genes either present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls may include the following.

- 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.

3.2 Interpretation of results

Verification of controls:

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of 275 bp.
- When relevant, the IPC should produce the expected amplicon.

When these conditions are met:

- A test will be considered positive if amplicons of 275 bp are produced.
- A test will be considered negative if it produces no band, or a band of a different size.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data: the 275-bp fragment was amplified in all samples from plant parts of plants showing symptoms.

4.2 Analytical specificity data (de Weerdt *et al.*, 2006)

The specificity was tested on 16 isolates of *F. foetens* and isolates of *F. asparagi*, *F. oxysporum* f. sp. *cubense*, *F. oxysporum* f. sp. *dianthi*, *F. oxysporum* f. sp. *gladioli*, *F. oxysporum* f. sp. *lilii*, *F. oxysporum* f. sp. *lini*, *F. oxysporum* f. sp. *lycopersici*, *F. commune*, *F. begoniae*, *Phytophthora cryptogea*, *P. drechsleri* and *Botrytis cinerea*.

Appendix 4 – Identification of *Fusarium foetens* by real-time PCR targeting mtSSU rDNA

1. General information

1.1 The protocol was developed by de Weerdt *et al.* (2006) and is based on an *F. foetens*-specific

TaqMan probe annealing to the mitochondrial small subunit of the ribosomal DNA.

1.2 Nucleic acid source: mycelium or conidia from pure culture, fungal structures (e.g. sporodochia) with or without dissected symptomatic plant tissue, dissected symptomatic plant tissue or asymptomatic plant tissue.

1.3 Name of targeted gene: partial mitochondrial small subunit ribosomal DNA (mtSSU rDNA). GenBank accession number of ex type strain CBS 110286 (= NRRL 31852): AY320105.

1.4 Amplicon location: not determined.

1.5 Amplicon size in base pairs (including primer sequences and introns): 127 bp.

1.6 Forward primer (orientation 5′–3′): Ff_MT_495f, GTAAATGATGAATGCCATAGGTTAGATTA (Sigma-Genosys). Reverse primer (orientation 5′–3′): Ff_MT_621r, CTCTTGAGGTGAAATGCTTA-CACTTT (Sigma-Genosys). *Fusarium foetens*-specific Taqman probe (fluorophore 6-carboxyfluorescein, FAM) and quencher (dihydrocyclopyrroloindole tripeptide minor groove binder, MGB) (orientation 5′–3′): P_Ff_MT, FAM-TGACAGACTATAATTAAAAATATCTG-MGB (Applied Biosystems).

1.7 Enzyme used in PCR reaction: HotGoldStar DNA polymerase (5 units per μL) (part of the qPCR core kit, Eurogentec), uracil-*N*-glycosylase (AmpErase UNG).

1.8 Deoxynucleotide triphosphates (dNTPs with dUTP) (5 mM) (part of the qPCR core kit, Eurogentec).

1.9 PCR buffer (10×) containing ROX passive reference (part of the qPCR core kit, Eurogentec).

1.10 Reaction additives: $MgCl_2$ (50 mM) (part of the qPCR core kit, Eurogentec).

1.11 Source/quality of water: molecular-grade water nuclease-free (MGW) (Promega, or others).

1.12 Thermal cycler: GeneAmp PCR System 9700 equipped with ABI PRISM sequence detection system software (Applied Biosystems) in 0.2 mL optical grade PCR tubes (Biozym).

2. Methods

2.1 Extraction of nucleic acid (DNA)

2.1.1 DNA extraction from pure culture, fungal structures (e.g. sporodochia) with or without dissected symptomatic plant tissue or dissected symptomatic plant tissue.

DNA is extracted using commercial kits [e.g. DNeasy Plant (Qiagen), NucleoSpin Plant II (Macherey-Nagel GmbH & Co), Puregene kit (Gentra)] following the manufacturer's recommendations.

An amount of biomass as recommended by the producer of these kits is collected in a 2.0 mL safe-lock microcentrifuge tube (e.g. Eppendorf). A stainless steel grinding ball (approx. 4 mm diameter) is added to disrupt the biomass using a tissuelyser system (e.g. Qiagen Retsch) for 60 s at the highest speed.

If applicable, the specifically supplied support protocols for the extraction of genomic DNA from fungi should be followed. DNA can be stored at 4°C overnight or at −20°C for longer periods.

2.1.2 DNA extraction from asymptomatic plant tissue

The procedure for tissue selection and maceration is described in Appendix 5. DNA can be extracted from the pellets of macerated root tissue using commercial DNA extraction kits, following the manufacturer's recommendations. In Huvenne *et al.* (2011), the Invisorb Spin Plant Mini Kit (Stratec, Germany) performed significantly better for recovery of *F. foetens* DNA from infected plant tissue than the other methods tested, but other commercial kits might result in adequate DNA recovery. The DNA extract is eluted in 100 μL and used in real-time PCR as described below (2.2), except that 5 μL DNA extract and a different brand of polymerase (TaqMan Universal Master Mix from Applied Biosystems) are used.

2.2 Polymerase chain reaction (PCR)

2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
PCR-grade water		To make up to 30	
PCR buffer	10×	3	1×
$MgCl_2$	25 mM	6	5 mM
dNTPs	10 mM	0.6	0.2 mM (of each of the dNTPs)
Forward primer (Ff_MT_495f)	10 μM	0.9	0.3 μM
Reverse primer (Ff_MT_621r)	10 μM	0.9	0.3 μM
Probe (P_Ff_MT)	10 μM	0.9	0.3 μM
HotGoldStar DNA polymerase	5 U μL^{-1}	0.15	0.75 U
AmpErase UNG	1 U μL^{-1}	0.15	0.15 U
Genomic DNA extract		2	
Total		30	

2.2.2 Real-time PCR cycling parameters

50°C for 2 min (degradation of uracil-containing DNA with AmpErase), 95°C for 10 min (inactivation of AmpErase), 40 cycles of 95°C for 15 s, 60°C for 60 s (amplification and FAM signal emission)

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 isolation 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 of a sample of uninfected host tissue (when working with plant samples) or clean extraction buffer (when working with pure cultures).
- Positive isolation control (PIC) to ensure 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 PCR-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 pure cultures, the PAC should preferably be near to the limit of detection.

As 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 may be genes either present in the matrix DNA or added to the DNA solutions.

Alternative internal positive controls may include the following.

- 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.

3.2. Interpretation of results

According to de Weerdt *et al.* (2006) Ct-positive test samples show a Ct value ranging from 20 to 29 for symptomatic material. For asymptomatic material a Ct value of 35 or less is considered a very reliable positive test result (Huvenne *et al.*, 2011). The cycle cut-off values needs to be verified in each laboratory when implementing the test for the first time.

Verification of controls:

- The PIC and PAC amplification curves should be exponential.
- NIC and NAC should be negative.
- PIC, PAC and IPC should have a Ct value below the cut-off value.

When these conditions are met:

- A test on asymptomatic material will be considered positive if it produces an exponential amplification curve, a Ct value below the cut-off value.
- A test on symptomatic material will be considered positive if it produces a Ct value below the cut-off value.
- A test will be considered negative if it produces no exponential amplification curve and a Ct value equal to or above the cut-off value.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Pure culture, fungal structures (e.g. sporodochia) with or without dissected symptomatic plant tissue or dissected symptomatic plant tissue.

- Analytical sensitivity data
  The fungus could be detected in all samples that included *F. foetens* fungal structures and/or *F. foetens* symptomatic plant tissue.
- Analytical specificity data (de Weerdt *et al.*, 2006)
  The specificity was tested on 28 isolates of *Fusarium oxysporum* species, *F. begoniae* or *F. commune* from different geographical origins and on *P. cryptogea*, *P. drechsleri* or *B. cinerea*.

4.2 Asymptomatic plant material

- Analytical sensitivity

Using the methods described in this appendix and Appendix 5, the number of spore equivalents detectable per root system was 2310 or less at a Ct value of 35 (Huvenne *et al.*, 2011). The theoretical detection limit, at a Ct of 40, was as low as 84 spore equivalents.

- Analytical specificity (see above)

Appendix 5 – Tissue maceration for asymptomatic plants

1. General information

This procedure relates only to the analysis of asymptomatic plants using molecular techniques, and is based on Huvenne *et al.* (2011). For symptomatic plant material or cultures, methods described in Appendices 2–4 should be used.

The protocol was developed to optimize the analytical sensitivity and to avoid inter-sample contamination. It is based on selecting the plant part containing the highest amount of *F. foetens* DNA, performing good quality tissue maceration with a minimal risk of inter-sample contamination, and using a DNA extraction technique with optimal recovery of *F. foetens* DNA.

2. Methods

Root systems should be selected, as they are the plant part with the highest concentration of *F. foetens* DNA. Roots are rinsed free of potting substrate as far as possi-

ble, dissected from the above-ground parts and weighed. Samples are placed in plastic maceration bags (Bioreba, Switzerland) together with half their weight in 100 mM Tris–HCl pH 8 buffer (e.g. 5 mL sterile buffer added to a plant sample of 10 g) and crushed with a Homex macerator (Bioreba). These Bioreba bags contain a fine-meshed gauze that allows easy separation of the liquefied and the fibrous plant parts. The roots are macerated until the root structure is no longer visible. This takes approximately 30–180 s, depending on the size of the root system. The maceration liquid is collected, and a 2 mL subsample is centrifuged for 7 min at 14 000 rpm. The entire pellet is used for DNA extraction and subsequent real-time PCR-based detection of *F. foetens* DNA. Alternatively, the root systems can be macerated by blending them in buffer, for example using a Grindomix GM200 (Retsch, Germany), after which an amount of blended material resulting in a pellet of approximately 200 mg can be processed for DNA extraction. This alternative method results in a similar final sensitivity but requires rigorous inter-sample decontamination of the blending equipment.