Bulletin OEPP/EPPO Bulletin (2018) 48 (1), 7–31 ISSN 0250-8052. DOI: 10.1111/epp.12452

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes PM 7/29 (3)

Diagnostics
Diagnostic

PM 7/29 (3) *Tilletia indica*

Specific scope

This Standard describes a diagnostic protocol for *Tilletia indica.*[1] It should be used in conjunction with PM 7/76 *Use of EPPO diagnostic protocols*.

Specific approval and amendment

This Standard was originally developed under the EU DIAGPRO Project (SMT 4-CT98-2252) by a partnership of contractor laboratories and interlaboratory comparison in European countries.

First approved as an EPPO Standard in 2003–09.

First revision approved in 2007–09.

Second revision approved on 2017–11.

Although this EPPO Diagnostic Standard differs in terms of format it is in general consistent with the content of the IPPC Standard adopted in 2014 on *Tilletia indica* (Annex 4 to ISPM 27) with the following exceptions. (1) In the EPPO region, as the pest is not present, a higher confidence in the results is required, a sieve wash test should be carried out (optional in the IPPC protocol). (2) When fewer than 10 teliospores are found the options should allow testing the (<10) teliospores with conventional or real-time PCR (this was not an option in the IPPC protocol flow chart, although it was stated that direct real-time PCR could be used on individual teliospores in the text). (3) The method for extracting teliospores from untreated seed or grain by size-selective sieving is slightly different based on the experience in the region (European Union test performance study). The EPPO Diagnostic Standard also includes a test for a direct real-time PCR for use on pellets (developed in 2016). Some additional information on methods for morphological identification, from the former version of the EPPO Standard, which are not in the IPPC protocol are included in this protocol in Appendix 3 as they were considered useful by the members of the Panel on Diagnostics in Mycology.

1. Introduction

Tilletia indica Mitra causes the disease Karnal bunt, also known as partial bunt, of wheat (*Triticum* spp.). Karnal bunt was first described in Karnal, India, in 1931. The pathogen is widespread in parts of South Asia and Southwest Asia (Wiese, 1987; USDA, 2007). It has also been detected in certain areas of the United States and Mexico, and in South Africa (Fuentes-Davila, 1996; Crous *et al.*, 2001).

Hosts include *Triticum aestivum*, *Triticum durum* and *Triticum aestivum* × *Secale cereale*. Records on *Triticum aestivum* × *Secale cereale* are rare; however, *Secale* spp. have been shown to have the potential to be a host (Sansford *et al.*, 2008). *Tilletia indica* has been shown to infect other grass species under glasshouse conditions but has never been detected in the field in these alternative hosts (Inman *et al.*, 2003).

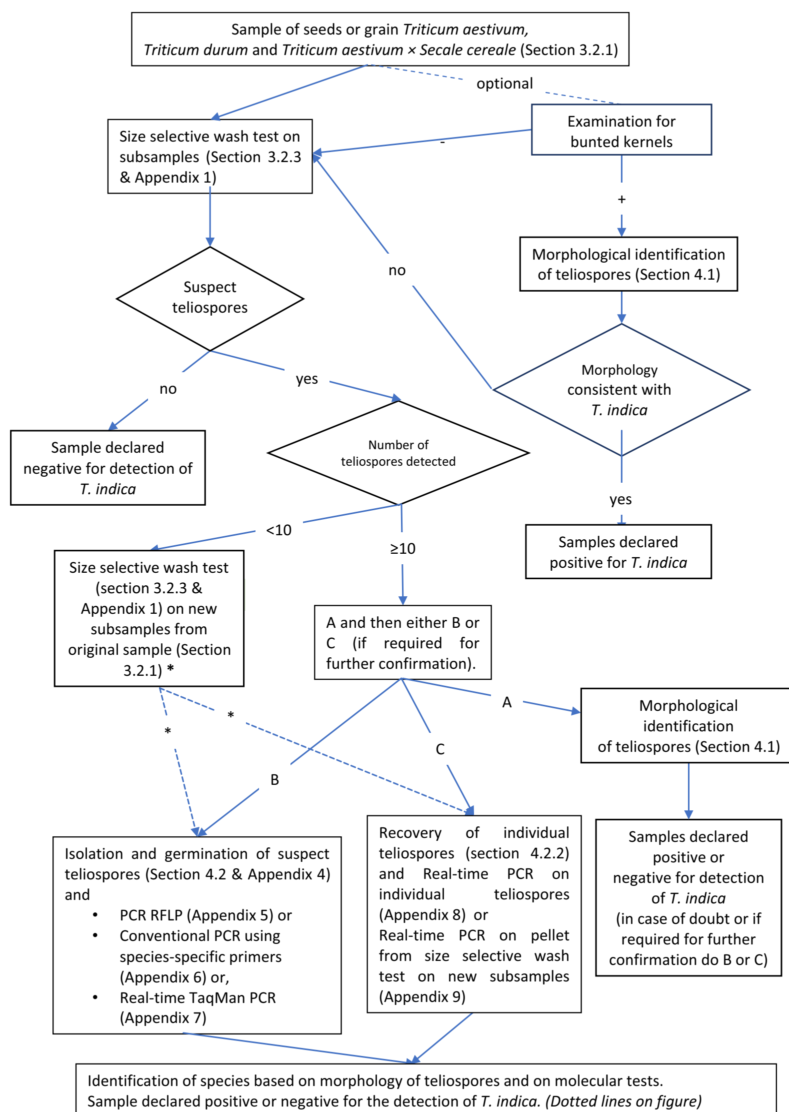
Tilletia indica reduces grain quality by discolouring and imparting an objectionable odour to the grain and products made from it. It also causes a small reduction in yield. Generally, *Triticum aestivum* containing more than 3% bunted kernels is considered unsatisfactory for human consumption (Fuentes-Davila, 1996).

A flow diagram describing the diagnostic procedure for *T. indica* is presented in Fig. 1.

2. Identity

Name: *Tilletia indica* Mitra, 1931
Synonyms: *Neovossia indica* (Mitra) Mundkur, 1941
Taxonomic position: Fungi: Basidiomycota: Ustilaginomycetes: Tilletiales
EPPO Code: NEOVIN
Phytosanitary categorization: EPPO A1 List, no. 23; EU Annex designation I/AI

[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.



*If there are still less than 10 teliospores retrieved, then these teliospores can be used for molecular confirmation by conventional PCR or real-time PCR on cultures or by direct real-time PCR on teliospores or on the pellet from size selective wash test

Fig. 1 Flow diagram showing the process to be used for the detection and identification of *Tilletia indica* in seed and grain samples. [Colour figure can be viewed at wileyonlinelibrary.com]

3. Detection

3.1. Symptoms

Tilletia indica is a floret-infecting smut pathogen. Seeds are infected through the germinal end of the kernel and the fungus develops within the pericarp, where it produces a powdery, brownish-black mass of teliospores. When fresh, the spore masses produce a foetid, decaying fish-like smell (trimethylamine). Unlike systemic smuts, it is not usual for all the seeds on an ear of the host to be infected with *T. indica*, and heads with infected seeds do not differ in appearance from healthy heads (Fig. 2). Seeds are usually only partially colonized, showing varying degrees of infestation (Fig. 3). Therefore it is very difficult to detect the disease in the field. The symptoms are not usually seen until after harvest, unless infestation levels are high.

There are other *Tilletia* species that can be confused with *T. indica* and which are commonly found in harvested grain or seeds. These include *Tilletia walkeri* (a pathogen of *Lolium perenne* and *Lolium multiflorum*), *Tilletia horrida* (a pathogen of *Oryza* spp.) and *Tilletia ehrhartae* (a pathogen of *Ehrharta calycina*). In Australia, *T. walkeri* and *T. ehrhartae* are found to contaminate harvested seed of *Triticum aestivum*. *Tilletia walkeri* and *T. horrida* are present in the United States and are detected in harvested seed of *Triticum aestivum*, especially where *Oryza* spp. and *Lolium* spp. are grown in rotation with *Triticum aestivum* (Castlebury, 1998; Castlebury & Carris, 1999; Pascoe *et al.*, 2005). Because of

Fig. 2 An infected head of wheat showing the symptoms of Karnal bunt. Photo courtesy of the Department of Agriculture and Food, Government of Western Australia.

Fig. 3 Infected grains of wheat showing the symptoms of Karnal bunt. Photo courtesy of the Department of Agriculture and Food, Government of Western Australia.

the morphological similarity of these pathogens, accurate identification is important.

3.2. Detection in seeds and grain

Seeds or grain samples may be visually examined for the presence of bunted kernels (Section 3.2.2). If a bunted kernel is detected, teliospores can be removed and *T. indica* can be identified by morphology (Section 4). In the absence of teliospores of *T. indica*, a wash test should be performed.

If no bunted kernels are detected in the sample, the sample should[2] be tested for the presence of teliospores by using a size-selective sieve wash test on three subsamples (Section 3.2.3). However, such testing may not distinguish between infested grain and grain contaminated with teliospores on the seed surface. If no teliospores are detected after the size-selective sieve wash test, the diagnostic result of the sample is negative. If teliospores are detected see Section 4.

3.2.1. Sampling

This protocol is based on the collection of a 1-kg sample of seeds or grain representative of the consignment; when direct examination of seeds or grain is performed, the whole sample needs to be examined for bunted kernels (Fig. 3) or other Poaceae seeds (e.g. *Lolium* spp.). The symptoms observed and the presence of the other Poaceae seeds is recorded.

3.2.2. Direct examination of seeds/grain

Direct visual examination either for bunted kernels or for teliospores contaminating seed or grain surfaces is not considered to be a reliable method for phytosanitary purposes. However, bunted kernels may be detected by visual examination with the naked eye in conjunction with low-power microscopy (10–40 × magnification). If bunted kernels are present, a positive diagnosis can be made based on the morphology of the teliospores (see Section 4). To help visualize symptoms, kernels can be soaked in 0.2% NaOH for 24 h at 20°C, which mildly bleaches the endosperm and makes the blackened infestation stand out in stark contrast. This process is especially useful for chemically treated seed lots where coloured dyes may obscure symptoms (Agarwal & Mathur, 1992; Mathur & Cunfer, 1993). With severe infestation and contamination, teliospores may be seen on the surface of seeds (Mathur & Cunfer, 1993).

3.2.3. Size-selective sieve wash test

In the absence of bunted kernels the size-selective sieve wash test (Appendix 1) should[2] be used to determine whether *T. indica* is present or not in the sample. If seed of *Lolium* spp. is found contaminating the sample there is a high probability that *T. walkeri* will be detected in the sample.

The size-selective sieve wash test described in Appendix 1 is a reliable method for detecting *T. indica* teliospores in an untreated sample of *Triticum aestivum*, *Triticum durum* or *Triticum aestivum* × *Secale cereale*. It is important that a minimum of three replicate subsamples of 50 g each are tested to ensure detection of teliospores if they are present in the sample (refer to Table 1 for the number of samples required to detect different numbers of teliospores). This method has, on average, an 82% efficiency of recovery, and microscopic examination typically requires only a few slides per 50-g sample. Further details on this test are available from Inman *et al.* (2003), Peterson *et al.* (2000) and Wright *et al.* (2003).

[2]It is noted that in the EPPO region, as the pest is not present, a higher confidence in the results of visual examination is required; therefore the absence of visible bunted kernels should not be taken as evidence of absence of the pest, and a sieve wash test should be carried out in this case (this is optional in the IPPC protocol).

Table 1. Number of replicate 50-g subsamples required to detect different levels of contamination with specified confidences, assuming an equal distribution of teliospores (Peterson *et al.*, 2000)

Contamination level (no. of teliospores per 50-g sample)	No. of replicate samples required for detection according to the level of confidence (%)		
	99%	99.9%	99.99%
1	3	5	6
2	2	3	4
5	1	1	1

4. Identification

Identification of *T. indica* is based on either (a) symptoms on kernels and morphology of teliospores or (b) morphology of teliospores and detection of the unique DNA sequence by one of the PCR tests (see Fig. 1).

When teliospores are detected the number detected will determine which method can be used for identification:

- If 10 or more teliospores are detected, the first step is identification of the species of the teliospores (Section 4.1) by morphology. Microscope slides of the teliospores should be made and the morphology of these teliospores described. If the morphology of the teliospores is consistent with that of *T. indica* (see Section 4.1 and Figs 4–8) a positive diagnosis can be made. In case of doubt on the morphology, the next step is *either* isolation of the teliospores and germination (Section 4.2) followed by the molecular tests described in Section 4.3 *or* direct real-time PCR testing on individual teliospores or on the pellet from the size-selective wash test of a new subsample (Section 4.3) (see A, B and C in Fig. 1).
- If fewer than 10 teliospores are detected, for reliable discrimination between *T. indica* and similar species it is highly recommended that the size-selective sieve wash test is repeated on new subsamples (until 10 teliospores are collected or the full sample has been used). If too few spores are still found (<10 spores), it may not be possible to discriminate species using morphological characters. In such cases, the next step is *either* isolation of the teliospores and germination (Section 4.2) followed by the molecular tests described in Section 4.3 *or* direct real-time PCR testing on individual teliospores or on the pellet from the size-selective wash test (Section 4.3) (see A, B and C in Fig. 1).

4.1. Morphology of teliospores

When suspect teliospores are found in a sieve wash test, the kernels in both the washed subsample(s) and the parent sample can be re-examined for symptoms. If symptoms are found, they should be confirmed by microscopic examination of the teliospores. Any grass seeds found in the sample should also be examined for signs of bunt infestation and, if found, the associated teliospores should be examined microscopically. If the teliospores found in the sieve wash test are the same as those found on bunted kernels a diagnosis can be made. If, however, no bunted kernels are found in the larger sample, testing with one of the molecular tests (Section 4.3) is recommended for identification.

Table 2 lists the morphological characteristics of *T. indica* teliospores as well as teliospores of the common *Tilletia* species that can be found in seeds or grain shipments and confused with *T. indica.*

4.1.1. Morphological identification

Tilletia indica teliospores are globose to subglobose, sometimes with a small hyphal fragment (more common on immature teliospores, but occasionally found on mature teliospores); mostly 22–47 µm in diameter, occasionally larger, up to 64 µm (mean 35–41 µm); pale orange-brown to dark, reddish brown; mature teliospores are black and opaque (Figs 5 and 6); densely ornamented with sharply pointed to truncate spines, occasionally with curved tips, 1.4–5.0 (−7.0) µm high, which in surface view appear as either individual spines (densely echinulate) or closely spaced, narrow ridges (finely cerebriform) (Figs 5 and 6); the spines are covered by a thin hyaline membrane (CMI, 1983; Carris *et al.*, 2006).

Sterile cells of *T. indica* are globose, subglobose to lachrymiform (tear-shaped), yellowish brown, 10–28 µm × 48 µm, with or without an apiculus (short stalk), with smooth walls up to 7 µm thick and laminated. Sterile cells are likely to be uncommon in sieved washings (CMI, 1983; Carris *et al.*, 2006). See also Appendix 3.

4.1.2 Morphological comparison with other Tilletia *species*

The most important morphological characteristics that discriminate *T. indica*, *T. walkeri*, *T. horrida* and *T. ehrhartae* are teliospore size (range and mean), ornamentation and colour (Table 2, Figs 4–8, Appendix 3). Published reports often vary on spore size. The spore size is affected by the mounting medium and by heat treatments. Pascoe *et al.* (2005) showed that in Australia *T. walkeri* and *T. ehrhartae* are common contaminants of harvested *Triticum aestivum.* In the United States, the morphologically similar and genetically closely related fungus *T. walkeri* and also *T. horrida* are known contaminants of harvested *Triticum aestivum* (Smith *et al.*, 1996; Castlebury & Carris, 1999; Cunfer & Castlebury, 1999). In addition to the *Tilletia* species mentioned in Table 2, other tuberculate-spored *Tilletia* species may be confused with *T. indica* (Durán & Fischer, 1961; Durán, 1987; Pimentel *et al.*, 1998). These species are less likely to be found as contaminants of *Triticum aestivum.* They include *Tilletia barclayana* (smut of various Poaceae, e.g. *Panicum* and *Paspalum*), *Tilletia eragrostidis* (on *Eragrostis*), *Tilletia inolens* (on *Lachnagrostis filiformis*), *Tilletia rugispora* (on *Paspalum*) and *Tilletia boutelouae* (on *Bouteloua gracilis*). None of these morphologically similar species has been found to naturally infest *Triticum aestivum.*

The median teliospore spin profiles can be enhanced by bleaching the teliospores in 10% NaOCl for 15–20 min. If

Table 2. Morphological characteristics of teliospores of *Tilletia indica*, *Tilletia walkeri*, *Tilletia horrida* and *Tilletia ehrhartae*, and hosts associated with these four species

Species	Teliospore size (µm)	Teliospore size (mean) (µm)	Teliospore colour	Teliospore shape	Teliospore sheath	Teliospore spines	Host
T. indica[*]	22–64	35–41	Pale orange-brown to dark reddish brown, mature spores black to opaque	Globose to subglobose	Present	1.4–5(–7) µm. In surface view, densely echinulate or as closely spaced, narrow ridges (finely cerebriform). In median view, smoother more complete outline due to spines being densely arranged occasionally with curved tips	*Triticum* spp.
T. walkeri[†]	28–35	30–31	Pale yellow to dark reddish brown (never black or opaque)	Globose	Present, extending to tips of projections, hyaline to yellowish brown	3–6 µm. Coarse ± cerebriform. Wide incompletely cerebriform ridges in surface view. In median view, profile is irregular with gaps between spines	*Lolium perenne* and *Lolium multiflorum*
T. horrida[‡]	14–36 (mature <25)	24–28	Light to dark chestnut brown, can be semi-opaque	Globose to subglobose	Present, extending to the ends of the spines, hyaline to tinted	1.5–4 µm. Frequently curved, and appear as polygonal scales in surface view.	*Oryza* spp.
T. ehrhartae[§]	17–25	No data	Very dark olivaceous brown when mature. Can be opaque because of melanization of the scales	Globose to subglobose	Present, extending to the apex of the spines or slightly beyond	1–2.5 µm Cylindrical or slightly tapered spines. In surface view, rarely cerebriform. Larger, acute polygonal scales. In median view, broadly truncated to slightly rounded at apex	*Ehrharta calycina*

*Based on Inman *et al.* (2003).
[†]Based on Castlebury (1998), Milbrath *et al.* (1998), Castlebury and Carris (1999), Cunfer and Castlebury (1999).
[‡]As *T. barclayana*: Durán and Fischer (1961), CMI (1965), Durán (1987), Castlebury and Carris (1999). As *T. horrida*: Khanna and Payak (1968), Aggarwal *et al.* (1990) , Castlebury (1998).
[§]Pascoe *et al.* (2005).

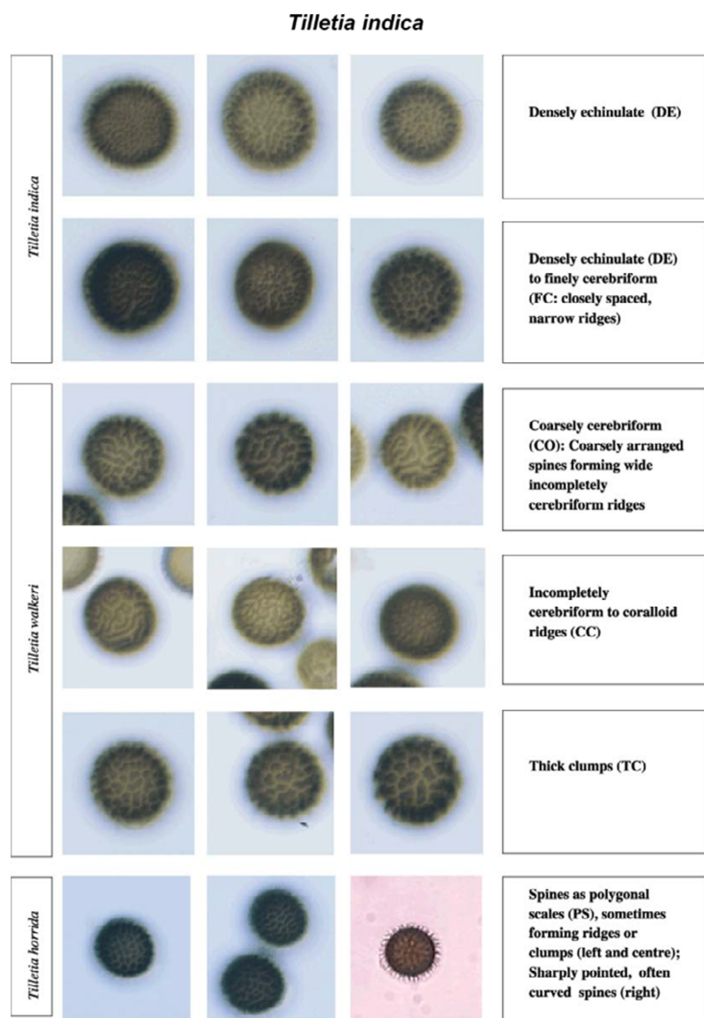


Fig. 4 Pictorial key to *Tilletia* teliospore ornamentation to be used in conjunction with Table 2 (Section 4.1). Photos courtesy of A. Inman, Fera, York, United Kingdom.

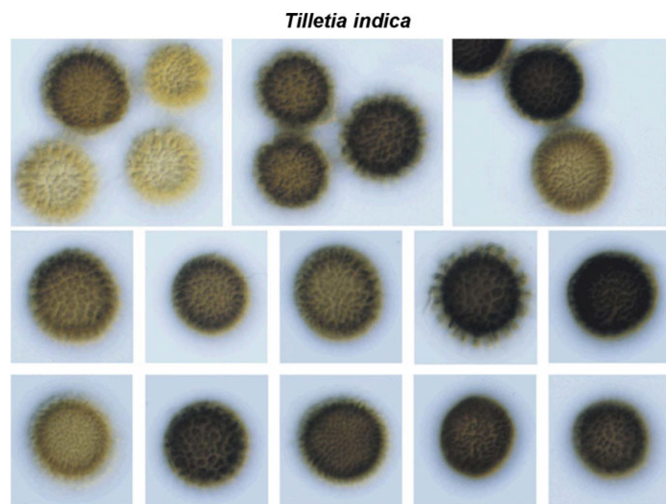


Fig. 5 Teliospores of *Tilletia indica* showing surface ornamentation patterns. Spines are densely arranged, either individually (densely echinulate) or in closely spaced, narrow ridges (finely cerebriform). Photos courtesy of A. Inman, Fera, York, United Kingdom.

necessary, teliospores can then be rinsed twice in water and stained, for example with trypan blue or cotton blue in lactoglycerol (Fig. 8).

4.2 Isolation and germination of teliospores

There are now two methods available to confirm the identification of teliospores detected in the sieve wash test

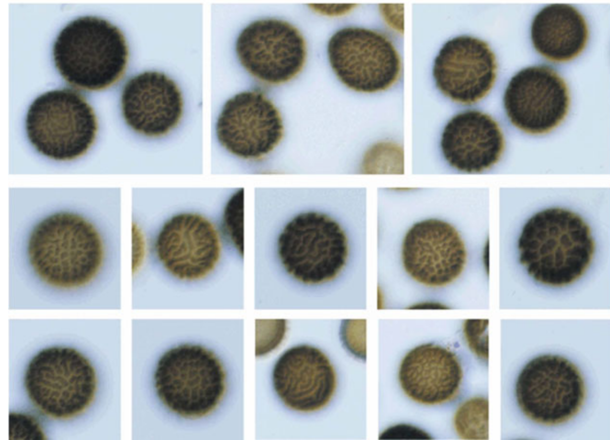


Fig. 6 Teliospores of *Tilletia walkeri* showing surface ornamentation patterns. Spines are coarsely arranged and form wide, incompletely cerebriform to coralloid ridges or thick clumps. Photos courtesy of A. Inman, Fera, York, United Kingdom.

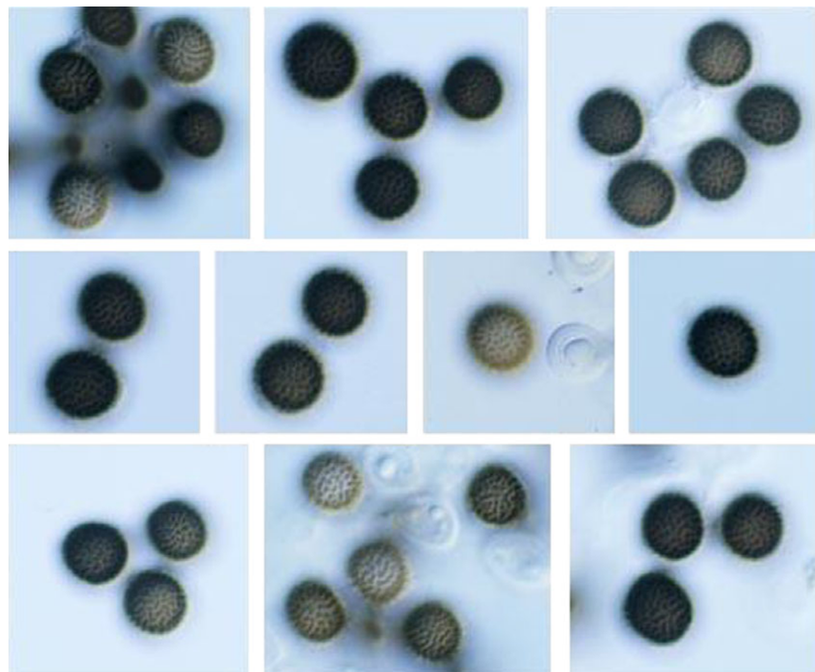


Fig. 7 Teliospores of *Tilletia horrida* showing surface ornamentation patterns. Spines are arranged in polygonal scales or, occasionally, cerebriform ridges. Photos courtesy of A. Inman, Fera, York, United Kingdom.

(Section 3.2.3). There is the standard procedure of recovering the teliospores from the slide and inducing their germination (see Appendix 4) and a new procedure developed by Tan *et al.* (2009) that enables PCR to be done directly on a single teliospore recovered from the slide (Section 4.2.2).

4.2.1. Confusion with other Tilletia species

In culture, *T. walkeri* and *T. indica* produce very similar colonies. On potato dextrose agar (PDA) after 14 days at 19°C with a 12-h light cycle, both species typically produce white to cream-coloured slow-growing irregular crustose colonies approximately 4–6 mm in diameter (Fig. 9). In contrast, comparable cultures of *T. horrida* grow significantly more slowly (colonies are only 2–3 mm in diameter) because of their higher optimal temperature. *Tilletia horrida* isolates may also produce a reddish purple pigment (Fig. 9), on both PDA and potato dextrose broth.

4.2.2. Recovery of single teliospores

After the teliospores have been examined and their morphology recorded, the slide is allowed to dry out, either with or without the coverslip on. When the coverslip is removed, it is placed on the slide upside down so it can be checked for teliospores adhering to it.

On another slide is placed a single piece of a coverslip (obtained by cutting it into tiny pieces of 1 × 1 mm^2) that has been sterilized (autoclaved at 121°C for 15 min or baked at 170°C for 2 h). A 1 μL drop of Tris-

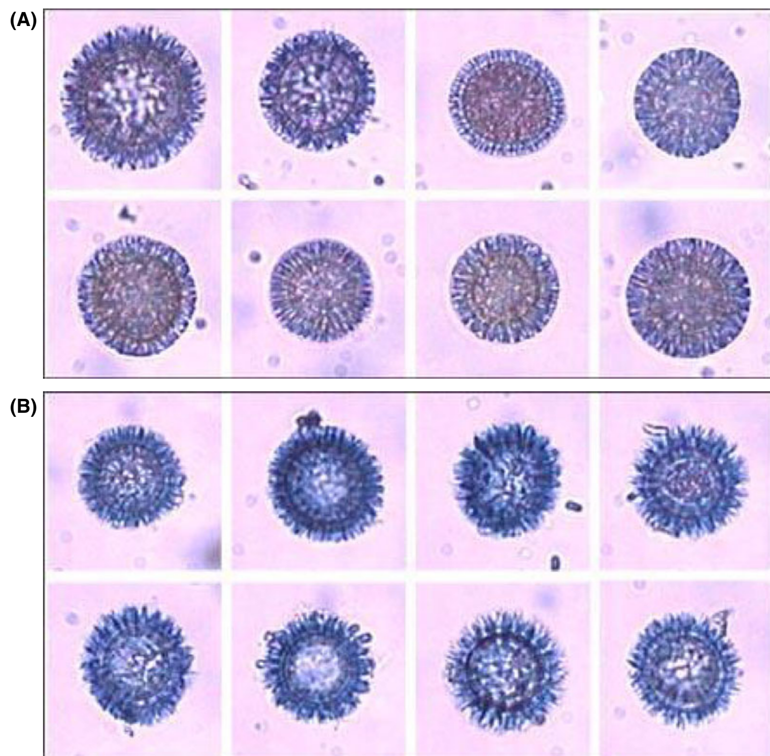


Fig. 8 Teliospores of *Tilletia indica* (A) and *Tilletia walkeri* (B) showing teliospore profiles in median view after bleaching and then staining with lactoglycerol-trypan blue. Note the smoother outline of *T. indica* teliospores compared with the more irregular outline of *T. walkeri* teliospores, which have more obvious gaps between spines. Photos courtesy of A. Inman, Fera, York, United Kingdom.

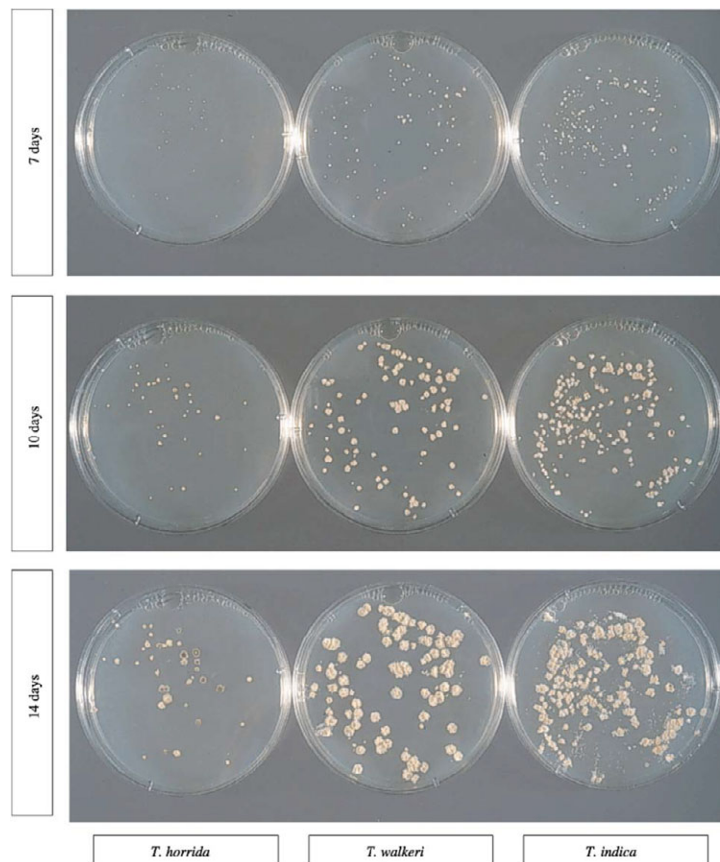


Fig. 9 Colonies of *Tilletia indica* (right), *Tilletia walkeri* (centre) and *Tilletia horrida* (left) after 7 days (top), 10 days (centre) and 14 days (bottom) on potato dextrose agar at 19°C and a 12-h dark/light cycle. Note the slower growth and purple pigmentation after 14 days for *T. horrida* colonies. Photos courtesy of A. Inman, Fera, York, United Kingdom.

ethylenediaminetetraacetic acid (EDTA) (TE) buffer is placed onto this piece of coverslip. Under either a compound or a dissecting microscope, a single teliospore is picked off with a very fine needle and placed into the droplet of TE buffer. The teliospore will transfer to the droplet. Using forceps, another sterilized small piece of coverslip is placed on top to make a sandwich. The teliospore is crushed by using the forceps to press down on the coverslip, and then the glass sandwich is transferred into a 0.2-mL PCR tube. The coverslip is crushed further with a pipette tip (Tan *et al.*, 2009).

The procedure then followed is described in Appendix 8.

4.3 Molecular tests

There are four molecular tests available to confirm presumptive morphological diagnoses.

The first three molecular tests above require that teliospores are germinated and cultures produced from the resulting sporidia, which may take several weeks (Appendix 1). Detailed descriptions of these techniques are found in Appendices 1, 2 and 4. Germination of teliospores for molecular confirmation may not always be possible, for example if grain is treated with NaOH (as in the case of examining fungicide-treated grain; Appendix 2) or teliospores are mounted in Shear's solution (Appendix 1). Increasing the number of sieved replicates may increase the number of teliospores recovered and hence the number of teliospore which can be germinated.

The fourth molecular test can be used directly on ungerminated teliospores recovered from microscope slides (Appendix 8) or mixed in the pellet from the size-selective sieve wash test Appendix 9)

- PCR restriction fragment length polymorphism (RFLP) adapted from Pimentel *et al.* (1998) (Appendix 5)
- conventional PCR (Frederick *et al.*, 2000) (Appendix 6)
- real-time TaqMan PCR (Frederick *et al.*, 2000) (Appendix 7)
- multiplex real-time-PCR (Tan *et al.*, 2009) on teliospores and adapted for pellets (Appendices 8 and 9).

Diagnostically significant differences exist between *T. indica*, *T. walkeri* and *T. horrida* in their nuclear and mitochondrial DNA. Interspecific polymorphisms have been identified using various polymerase chain reaction (PCR) tests, including random amplified polymorphic DNA (RAPD), RFLPs and amplified fragment length polymorphism (AFLP) (Laroche *et al.*, 1998; Pimentel *et al.*, 1998). In the nuclear ribosomal (rDNA) ITS1 and ITS2 regions, there is a >98% similarity between *T. walkeri* and *T. indica* sequences (Levy *et al.*, 2001). However, within the ITS1 region, *T. walkeri* has a diagnostically important restriction enzyme site (*Sca*I) that is not present with *T. indica*, *T. horrida* or other closely related species (Levy *et al.*, 1998; Pimentel *et al.*, 1998). A multiplex real-time-PCR test has also been set up that use species-specific primers and probes designed on the ITS1 region for *T. indica*, *T. walkeri*, *T. horrida* and other *Tilletia* species (Tan *et al.*, 2009). With mitochondrial DNA (mtDNA), sequence differences have enabled species-specific primers to be designed to *T. indica* and *T. walkeri* (Frederick *et al.*, 2000). These primers can be used in conventional PCR tests or in a TaqMan system in conjunction with a probe (Frederick *et al.*, 2000).

5. Reference material

Reference material can be provided on request from Fera, Sand Hutton, York YO41 1LZ, York, GB.

6. Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis*.

7. Performance criteria

When performance criteria are available, these are provided with the description of the test. Validation data is 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: Fera, Sand Hutton, York YO41 1LZ, GB.

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: A. J. Inman, K. J. D. Hughes and R. J. Bowyer, Fera, Sand Hutton, York

YO41 1LZ, GB. This revision has been prepared by K. Hughes, L. Riccioni and M. T. Valente. It was reviewed by the European Mycological Network and the Panel on Diagnostics in Mycology.

The size-selective sieve wash test was evaluated during a test performance study in different European laboratories.[3] The multiplex real-time PCR was evaluated with an international test performance study[4] The direct real-time PCR on the pellet was evaluated with an Italian test performance study with 9 laboratories.[5]

References

Agarwal R, Joshi LM & Singh DV (1990) Morphological differences between teliospores of Neovossia indica and N. horrida. *Indian Phytopathology* **43**, 439–442.

Agarwal VK & Mathur SB (1992) Detection of Karnal bunt in wheat seed samples treated with fungicides. *FAO Plant Protection Bulletin* **40**, 148–153.

Bonde MR, Nester SE, Smilanick JL, Frederick RD & Schaad NW (1999) Comparison of effects of acidic electrolysed water and NaOCl on *Tilletia indica* teliospore germination. *Plant Disease* **83**, 627–632.

Carris LM, Castlebury LA & Goates BJ (2006) Nonsystematic bunt fungi-*Tilletia indica* and *T. horrida*: a review of history, systematics and biology. *Annual Review of Phytopathology* **44**, 113–133.

Castlebury LA (1998) Morphological characterisation of *Tilletia indica* and similar fungi. In *Bunts and Smuts of Wheat: An International Symposium* (eds. Malik VS & Mathur DE), pp. 97–105. NAPPO, Ottawa (CA).

Castlebury LA & Carris LM (1999) *Tilletia walkeri*, a new species on *Lolium multiflorum* and *L. perenne*. *Mycologia* **91**, 121–131.

CMI (1965) *Description of Pathogenic Fungi and Bacteria, no. 75. Tilletia barclayana*. CAB International, Wallingford (GB).

CMI (1983) *Description of Pathogenic Fungi and Bacteria, no. 748. Tilletia indica*. CAB International, Wallingford (GB).

Crous PW, van Jaarsveld AB, Castlebury LA, Carris LM, Frederick RD & Pretorius ZA (2001) Karnal bunt of wheat newly reported from the African continent. *Plant Disease* **85**, 561.

[3]A. Radova, State Phytosanitary Administration, Olomouc (CZ); I. Vloutoglou, Benaki Phytopathological Institute, Athens (GR); A. Porta-Puglia, Istituto Sperimentale per la Patologia Vegetale, Rome (IT); C. Montuschi, Servizio Fitosanitario Regionale, Bologna (IT); I. Heurneman-van Brouwershaven, Plantenziektenkundige Dienst, Wageningen (NL); M. de Jesus Gomes, E. Diogo and M. R. Malheiros, Direcção-Geral de Protecção das Culturas, Lisboa (PT); V. Cockerell, Scottish Agricultural Science Agency, East Craigs, Edinburgh (GB); A. Barnes, Fera, Sand Hutton, York (GB).

[4]Laboratories in FERA: CRA-PAV Plant Pathology Research Centre, Rome (IT); SASA; Shanghai Inspection and Quarantine Bureau (CN); Shenzhen Entry–Exit Inspection and Quarantine Bureau (CN); DAFWA; DPI VIC and NSW DPI (Tan M.-K., Wright D. (2009) Final Report CRC20004-Karnal Bunt Detection. Cooperative Research Centre for National Plant Biosecurity, Canberra, AU).

[5]Servizio Fitosanitario Regione (SFR) Lombardia, Fondazione Edmund Mach, Agenzia Regionale per lo Sviluppo Rurale (ERSA) Friuli Venezia Giulia, SFR Emilia-Romagna, Centro di Ricerca Sperimentazione e Formazione in Agricoltura (CRSFA) 'Basile Caramia', SFR Toscana, SFR Liguria, SFR Piemonte, Food Safety Lab S.r.l (Bari).

Cunfer BM & Castlebury LA (1999) *Tilletia walkeri* on annual ryegrass in wheat fields in the southeastern United States. *Plant Disease* **83**, 685–689.

Durán R (1987) *Ustilaginales of Mexico: Taxonomy, Symptomatology, Spore Germination and Basidial Cytology*. Washington State University, Seattle (US).

Durán R & Fischer GW (1961) *The Genus Tilletia*. Washington State University, Seattle (US).

Frederick RD, Snyder KE, Tooley PW, Berthier-Schaad Y, Peterson GL, Bonde MR *et al.* (2000) Identification and differentiation of *Tilletia indica* and *T. walkeri* using the polymerase chain reaction. *Phytopathology* **90**, 951–960.

Fuentes-Davila G (1996) Karnal bunt. In *Bunt and Smut Diseases of Wheat: Concepts and Methods of Disease Management* (eds. Wilcoxson RD & Saari EE), pp. 26–32. International Maize and Wheat Improvement Center (CIMMYT), Mexico (US). 74 pp.

Hughes KJD, Inman AJ & Cooke DEL (2000) Comparative testing of nested PCR-based methods with bait-plant tests for detecting *Phytophthora fragariae* var. *fragariae* in infected strawberry roots from fruit crops in the UK. *Bulletin OEPP/EPPO Bulletin* **30**, 533–538.

Inman AJ, Hughes KJD & Bowyer R (2003) Protocol for extracting teliospores from untreated seed or grain by size-selective sieving. In *EU Recommended Protocol for the Diagnosis of a Quarantine Organism – Tilletia indica*, pp. 21–26. http://www.csl.gov.uk/special/Interest/tipro.pdf [accessed on 29 October 2007]

ISPM 27 (2006) *Diagnostic Protocols for Regulated Pests*. IPPC, FAO, Rome (IT).

Khanna A & Payak MM (1968) Teliospore morphology of some smut fungi. II. Light microscopy. *Mycologia* **60**, 655–662.

Laroche A, Gaudet DA, Despins T, Lee A & Kristjansson G (1998) Distinction between strains of Karnal bunt and grass bunt using amplified fragment length polymorphism (AFLP). In *Bunts and Smuts of Wheat: an International Symposium* (eds. Malik VS & Mathur DE), p. 127. NAPPO, Ottawa (CA).

Levy L, Castlebury LA, Carris LM, Meyer RJ & Pimentel G (2001) Internal transcribed spacer sequence-based phylogeny and polymerase chain reaction-restriction fragment length polymorphism differentiation of *Tilletia walkeri* and *T. indica*. *Phytopathology* **91**, 935–940.

Levy L, Meyer RJ, Carris L, Peterson G & Tschanz AT (1998) Differentiation of *Tilletia indica* from the undescribed *Tilletia* species on ryegrass by its sequence differences. *Proceedings of the 12th Biennial Workshop on Smut Fungi*, p. 29.

Mathur SB & Cunfer BM (1993) *Seed-Borne Diseases and Seed Health Testing of Wheat*, pp. 31–43. Government Institute of Seed Pathology for Developing Countries, Copenhagen (DK).

Milbrath GM, Pakdel R & Hilburn D (1998) Karnal bunt spores in ryegrass (*Lolium* spp.). In *Bunts and Smuts of Wheat: an International Symposium*(eds. Malik VS & Mathur DE), pp. 113–116. NAPPO, Ottawa (CA).

NAPPO (1999) *NAPPO Standards for Phytosanitary Measures: a harmonized procedure for morphologically distinguishing teliospores of Karnal bunt, ryegrass bunt and rice bunt*. http://www.nappo.org [accessed on 01 June 2017].

Pascoe IG, Priest MJ, Shivas RG & Cunnington JH (2005) Ustilospores of *Tilletia ehrhartae*, a smut of *Ehrharta calycina*, are common contaminants of Australian wheat grain, and a potential source of confusion with *Tilletia indica*, the cause of Karnal bunt of wheat. *Plant Pathology* **54**, 161–168.

Peterson GL, Bonde MR & Phillips JG (2000) Size-selective sieving for detecting teliospores of *Tilletia indica* in wheat seed samples. *Plant Disease* **84**, 999–1007.

Pimentel G, Carris LM, Levy L & Meyer R (1998) Genetic variability among isolates of *Tilletia barclayana, T. indica* and allied species. *Mycologia* **90**, 1017–1027.

Sansford CE, Baker RHA, Brennan JP, Ewert F, Gioli B, Inman AJ *et al.* (2008) The new pest risk analysis for *Tilletia indica,* the cause of Karnal bunt of wheat, continues to support the quarantine status of the pathogen in Europe. *Plant Pathology* **57**, 603–611.

Smith OP, Peterson GL, Beck RJ, Schaad NW & Bonde MR (1996) Development of a PCR-based method for identification of *Tilletia indica*. *Phytopathology* **86**, 115–122.

Tan MK, Ghalayini A, Sharma I, Yi JP, Shivas R, Priest M *et al.* (2009) A one-tube fluorescent assay for the quarantine detection and identification of *Tilletia indica* and other grass bunts in wheat. *Australasian Plant Pathology* **38**(2), 101–109.

Tan MK & Murray GM (2006) A molecular protocol using quenched FRET probes for the quarantine surveillance of *Tilletia indica*, the causal agent of Karnal bunt of wheat. *Mycological Research* **110**, 203–210.

Tan MK, Timmer LW, Broadbent P, Priest M & Cain P (1996) Differentiation by Molecular Analysis of Elsinoe spp. Causing Scab Diseases of Citrus and Its Epidemiological Implications. *Phytopathology* **86**, 1039–1044.

USDA (United States Department of Agriculture) (2007) *Karnal Bunt Manual*. USDA, Frederick (US). 160 pp. http://www.aphis.usda.gov/import_export/plants/manuals/online_manuals.shtml [accessed on February 2012].

Valente MT & Riccioni L (2016) Optimization and validation of a diagnostic real-time PCR for *Tilletia indica* Karnal bunt. In XXII National Congress Italian Society for Plant Pathology (SIPaV), 19–22 September 2016, Rome, Italy.

White TJ, Bruns T, Lee S & Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols, A Guide to Methods and Applications* (eds. Innis MA, Gelfand DH, Sninsky JJ & White TJ), pp. 315–322. Academic Press, London (GB).

Wiese MV (ed.) (1987) *Compendium of Wheat Diseases*, 2nd edn., p. 112. APS Press, Saint Paul (US).

Wright D, Murray G & Tan M-K (2003) *National Diagnostic Protocol for the Identification of Tilletia indica, the Cause of Karnal Bunt*. Department of Agriculture and Food, Government of Western Australia, Perth (AU).

Appendix 1 – Method for extracting teliospores from untreated seed or grain by size-selective sieving (based on Peterson *et al.*, 2000)

Materials

30% bleach solution (3 parts household bleach, 7 parts water; approximately 1.6% active NaOCl); wash water = 0.01% aqueous Tween-20 (detergent); large weigh boats (8 cm × 8 cm); weighing balance; 250-mL Erlenmeyer glass flask; 100-mL measuring cylinder; Parafilm 'M' or clingfilm; laboratory flask shaker (alternatively, shaking flasks by hand is acceptable); 500-mL Erlenmeyer glass flask × 2; funnel (approx. 13 cm diameter); 53 (50 or 70) μm mesh nylon sieve (11 cm external diameter), mesh from Spectrum Laboratories; 20-μm mesh nylon sieve (4 cm external diameter), mesh from BDH or Spectrum Laboratories; aspirator bottle with distilled water; sterile disposable 3-mL Pasteur pipettes; pipettor (100-μL capacity) plus disposable pipettor tips (plugged); pipettor (1000-μL capacity) plus disposable pipettor tips (plugged); 15-mL sterile, disposable conical-bottom centrifuge tubes; centrifuge (to take 15-mL centrifuge tubes above); autoclavable, disposable waste bottle; autoclave bags; glass microscope slides (76 × 21 mm); microscope cover slips (18 × 18 mm); compound microscope (×100–×400 magnification); dissecting microscope (×10–×70 magnification); Shear's solution[6] (As an alternative mounting medium to water if slides are prone to drying; however, Shear's starts to kill teliospores after a few minutes' exposure and little germination can be expected after exposure of 1 h.)

Method

Bleach the sieves, funnels and flasks by immersion for 15 min in 30% bleach[7] Rinse the bleach thoroughly from the equipment with tap water. Weigh 50 g of grain into a new, disposable, large weigh boat (see Table 1, for the number of 50-g subsamples required to detect different levels of contamination; 3 replicates detects a level of 1 spore per 50 g sample with a 99% confidence). Pour the 50-g subsample of grains into a 250-mL Erlenmeyer flask (Fig. 10). Add 100 mL of 0.01% Tween-20 aqueous solution to the flask. Seal the top of the flask (e.g. with Parafilm or clingfilm).

Place the flask on a flask shaker set at an appropriate speed (e.g. 350 oscillations min^{-1}, or 200 rpm for an orbital shaker) to ensure good agitation for 3 min to release any teliospores from the grain. Alternatively the flask can be shaken or swirled by hand. Place a 53-μm mesh nylon sieve (11 cm diameter) in a funnel over a clean 500-mL Erlenmeyer flask[8] (Fig. 11) then pour the whole contents of the flask (the grain and the wash water) into the sieve (Fig. 12). Rinse the 250-mL flask with 20–50 mL of distilled water/sterile tap water from an aspirator bottle, then pour evenly over the grain on the 53-μm sieve. Repeat this

[6]Shear's solution: 300 mL McIlvaine's buffer, 6 mL potassium acetate, 120 mL glycerine 180 mL, ethyl alcohol (95%). Prepare McIlvaine's buffer (Mathur & Cunfer, 1993) as follows: dissolve 19.212 g citric acid in 1000 mL distilled water and mix thoroughly; dissolve 28.392 g of disodium phosphate (Na_2HPO_4) in 1000 mL of distilled water and mix thoroughly; mix 8.25 mL of citric acid solution with 291.75 mL of disodium phosphate solution and mix thoroughly.

[7]Bleach eliminates the risk of false positives by cross-contamination from previous samples; bleach kills teliospores and makes them appear hyaline compared with the normally dark, pigmented spores. The bleach solution should be changed regularly, as appropriate.

[8]This is a slight deviation from the technique described in the IPPC protocol. The test as described here has been used in an EU test performance study and experts in the region are more confident with this technique.

Fig. 10 Size-selective sieving wash test: 20 µm mesh nylon sieve (mounted between 4-cm diameter cylinders, left), 53-µm sieve (mounted between 11-cm diameter cylinders, right) and a 50-g grain sample in a 250-mL Erlenmeyer flask with 100 mL of 0.01% Tween-20 aqueous solution.

Fig. 12 Size-selective sieving wash test: grain and washings poured onto a 53-µm sieve over a 500-mL Erlenmeyer flask, together with an aspirator bottle for subsequent rinsing of the grain on the sieve.

Fig. 11 Size-selective sieving wash test: arrangement of sieves (20-µm sieve, left; 53-µm sieve, right) mounted in funnels over 500-mL Erlenmeyer flasks in preparation for size-selective sieving of wash water from a 50-g grain sample.

Fig. 13 Size-selective sieving wash test: the final sieve fraction being washed to one side of the 20-µm sieve with water from a disposable Pasteur pipette in preparation for recovery.

rinse once or twice more until the flask appears clean. Thoroughly rinse the grain on the 53-µm sieve by washing with further distilled water/sterile tap water from an aspirator bottle (Fig. 12) to give a final collected volume of 300–400 mL.

Remove the 53-µm sieve from the funnel and rinse the funnel with two aliquots of 10–20 mL of distilled water/sterile tap water, collecting the water in the same 500 mL flask. (NB: Keep the washed grain sample(s) and also the remainder of the submitted sample that has not been tested in case there is a need to examine grain directly for disease symptoms – see Appendix 3). Place a 20-µm mesh nylon sieve (4 cm diameter; Fig. 11) in a funnel over a second 500-mL Erlenmeyer flask. Pour the collected washings from the earlier washings through the 20-µm nylon sieve. (NB: Wet the sieve membrane prior to use and gently tap the outside of the PVC sieve-holder repeatedly to facilitate a good rate of sieving; otherwise the membrane can quickly become blocked.)

Rinse the first 500-mL flask twice with 20 mL of water and pour through the 20-µm sieve. Tilt the 20-µm sieve to an angle of 30–45° (Fig. 13) and gently wash the deposit on the membrane and on the sieve walls on to one side of the membrane using distilled water/sterile tap water or 0.01% Tween-20 detergent from an aspirator bottle or a disposable Pasteur pipette. Recover the suspension that collects at the edge of the 20-µm sieve using a clean, disposable Pasteur pipette (Fig. 14) and place the suspension in a new 15-mL disposable conical centrifuge tube. It is important that polypropylene tubes are used as the teliospores will stick to the sides of polycarbonate tubes, giving false results. Repeat these steps until the 20-µm sieve appears clean (this may require 5–10 repeats, and typically results in a final collected volume of about 8 mL in the centrifuge tube). If necessary, the 20-µm sieve can be examined under

Fig. 14 Size-selective sieving wash test: the final sieve fraction being recovered from the 20-μm sieve with water from a disposable Pasteur pipette for subsequent centrifugation in a conical centrifuge tube.

a low-power microscope to check for any residual teliospores.

Centrifuge the collected suspension at 1000*g* for 3 min.[9] The equation for calculating the relative centrifugal force (RCF (*g*)) from rpm is RCF = $1.12r_{\max}$ (rpm/100)^2, where $r_{\max}$ is the maximum radius (mm) from the centre of rotation to the bottom of the centrifuge tube. Carefully remove the supernatant using a 1-mL pipettor with a plugged, disposable pipette tip, or a new disposable Pasteur pipette. Take care not to disturb the pellet (discard the removed supernatant into a disposable waste vessel for quarantine disposal). Re-suspend the pellet using distilled water to give a final volume of 50–100 μL, or more if the pellet volume requires.[10]

Pipette a 20-μL aliquot of the suspension onto a microscope slide and place a cover slip (18 mm × 18 mm) on top. Examine the whole slide immediately (the slide can quickly dry out) for teliospores of *T. indica* (Fig. 15) using a compound microscope at ×100–×400 magnification. Assess the characteristics of any teliospores at ×100 magnification. Teliospores of *T. indica* are mainly 25–45 μm in diameter, pale orange but mostly reddish-brown to opaque-black, and densely echinulate (for reference see Fig. 4, Table 2 and Appendix 3). Repeat with further 20-μL aliquots until the whole suspension has been examined.

If suspect teliospores are found, refer to, and follow, the morphological diagnostic method (see Appendix 3) and the flow diagram (Fig. 1), i.e. record morphological characters (e.g. size, colour, ornamentation), examine the sample for bunted seeds and isolate and germinate teliospores for molecular confirmation, if required.

Finally, bleach all equipment used and rinse with water before re-using (as described at the beginning of this section).

Appendix 2 – Method for extracting teliospores from fungicide-treated seed by size-selective sieving (adapted from Agarwal & Mathur, 1992 and based on Peterson *et al.*, 2000)

Follow the method in Appendix 1, but after weighing the 50-g sample add to it 100 mL of 0.2% (or 1%) sodium hydroxide (NaOH) and incubate for 24 h before adding 100 mL of 0.01% Tween-20 aqueous solution to the flask. NaOH can help to remove most of the fungicide, allowing subsequent sieving. Without the NaOH treatment, the 20-μm sieve may become blocked by fungicide. The NaOH treatment does not affect teliospore size or colour characteristics, but does kill the teliospores (Bowyer & Inman, unpublished). An alternative to using NaOH is to use just the 53-μm sieve and not the 20 μm sieve if this becomes easily blocked. This method has not been tested in an inter-laboratory comparison.

Appendix 3 – Method for morphological identification

If tuberculate teliospores are found in a wash test, record the morphological characteristics of the teliospores using Table 3 (refer also to Figs 4–6 and 13 and Table 2).[11] Follow the pictorial key (Fig. 4) and examine the submitted grain sample (including the 50-g washed subsamples) for bunted wheat seeds (see Fig. 3) or bunted seeds of other Poaceae (e.g. ryegrass seed). If wheat seeds with Karnal bunt symptoms are found, confirm *T. indica* by microscopic examination of the teliospores in the seed (Fig. 4, Tables 2 and 4). If bunted ryegrass seeds are found, but no bunted wheat seeds, confirm *T. walkeri* by microscopic examination of the teliospores in the seed (Table 4). If confirmed, compare teliospores from the seed with those found in the wash test. If the teliospores are identical make a diagnosis. Molecular confirmation of the teliospores from the wash

[9]Conical-bottomed tubes are recommended, as are centrifuges with swing-out arms rather than fixed arms because these give better pellets. If debris is seen to adhere to the inside walls of the centrifuge tubes, re-suspend in 0.01% Tween-20 and repeat the centrifugation.

[10]If warm laboratory conditions cause water preparations to dry out quickly, then Shear's solution, or just a glycerol solution, can be used as an alternative to water. However, teliospores start to be killed after a few minutes' exposure in Shear's and little germination can be expected after exposure of 1 h. Slides should be assessed immediately (within 10–20 min) and any spores recovered immediately from the slide (see Appendix 4) and washed in water to allow germination and the recommended molecular confirmations.

[11]Tuberculate teliospores detected in wash tests of wheat grain are assumed to be either *T. indica*, *T. walkeri* or *T. horrida*. Other tuberculate-spored *Tilletia* species that infect various grasses cannot be excluded as contaminants but have not previously been found contaminating wheat (see Fig. 1).

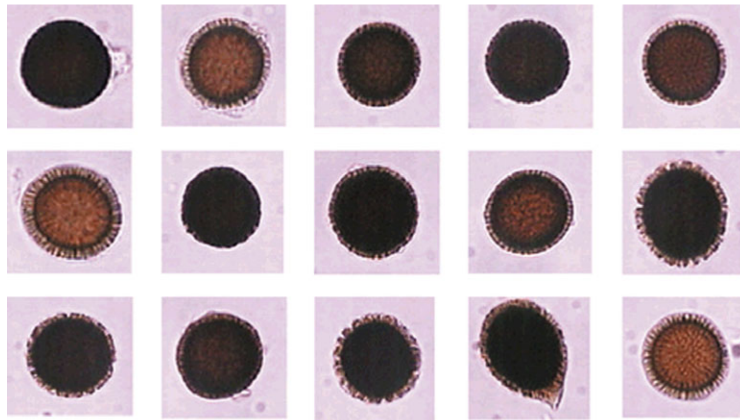


Fig. 15 *Tilletia indica* teliospores in median view (20–50 μm diameter, mean 35–41 μm).

Table 3. Example record sheet, with suggested colour and ornamentation codes

Teliospore number	Size (μm) (Diameter)	Colour	Ornamentation	Notes
1				
2				
etc.				
Size range	Mean ± SD		Provisional identification	

Examples of colour codes: BO, black/opaque; RBm reddish brown; CB, chestnut brown; P (PY, PO, PB), pale (yellow/orange/brown). Examples of ornamentation codes: DE, densely echinulate (spines densely and individually arranged); FC, finely cerebriforn (spines forming closely spaced narrow ridges); CC, coralloid (ridges much branched); CO, coarsely cerebriform (spines coarsely arranged forming wide, incompletely cerebriform ridges); TC, thick clumps (spines forming thick clumps); PS, polygonal scales (curved in profile).

test is still recommended. If wheat seeds infected with *T. indica* or ryegrass seeds infected with *T. walkeri* are not found, make a presumptive identification of teliospores found in the wash test: use Table 4 in conjunction with the following guiding diagnostic principles (adapted from NAPPO, 1999):

- samples with teliospores all <36 μm, with curved spines, are most likely to be *T. horrida*
- samples with teliospores >36 μm are most likely to be *T. indica*
- samples with teliospores mostly 28–35 μm, translucent brown, never black/opaque, very spherical, with blunt spines with distinct gaps between (made more obvious in profile after bleaching) are most likely to be *T. walkeri*, especially if grain is from areas where ryegrass is grown alongside wheat or where ryegrass seeds are present in the sample

Table 4. Scheme for morphologically distinguishing teliospores of *Tilletia indica*, *Tilletia walkeri, Tilletia horrida* and *Tilletia ehrhartae* detected in size-selective sieving wash tests that use 20-lm and 53-lm sieves. character conforms to species

√ character conforms to species

	Max Size (diam., μm)[*]				Mean size (diam., μm)[*]				Colour[†]				Spines (ornamentation) in surface view[†] and median profile[†‡§]			
	< 25	< 36	36–45	45–50+	18-20	24–28	30–31	35–41	Pale yellow to mostly light or dark chestnut-brown (to semi-opaque)	Pale yellow to mostly reddish-brown (never opaque)	Pale orange but mostly dark reddish-brown to opaque black	Very dark olivaceous brown when mature (can be opaque due to melanization of the scales)	Echinulate; polygonal scales in surface view; occasionally cerebriform ridges or rarely clumps. Sharply pointed, becoming truncate, occasionally curved	Coarse; broad, incompletely cerebriform ridges (to coralloid), or thick clumps. Conical to truncate (gaps between spines obvious in profile after bleaching)[‡]	Dense; echinulate or closely spaced narrow ridges (finely cerebriform) Sharply pointed to truncate, occasionally curved (few or no gaps between spines after bleaching)[‡]	Cylindrical or slightly tapered. Rarely cerebriform. Larger, acute polygonal scales. Broadly truncated to slightly rounded at apex.
Sample[¶] (√ boxes)																
T. horrida		√			√	√			√				√			
T. walkeri			√				√			√				√		
T. indica				√				√			√				√	
T. ehrhartae	25				√							√				√

[*] Table 2; [†] Table 2 and Figs 3–5; [‡] Fig. 6; [§] Fig. 14; [¶] Refer to Fig. 2 (Flow-diagram)

- samples with mature, dark teliospores <25 µm are most likely to be *T. horrida*, not *T. walkeri* or *T. indica*.

Appendix 4 – Method for isolation and germination of teliospores

The teliospores can be recovered from the slides and coverslips by washing them with distilled water over the 20-µm sieve and then into a clean sterile conical centrifuge tube (as in Section 3.2). The volume should be approximately 3–5 mL. The tubes are incubated overnight at 21°C to hydrate the teliospores and make fungal and bacterial contaminants more susceptible to subsequent surface sterilization. After overnight incubation, the teliospores are pelleted by centrifugation at 1200*g* for 3 min.

The supernatant is removed and the teliospores are sterilized by suspending the pellet in 5 mL of bleach (0.3–0.5% NaOCl active ingredient), inverting the tube quickly three times and centrifuging at 1200*g* for 1 min. Some teliospores can be killed if the total time in the bleach exceeds 2 min. As an alternative to bleach treatment, teliospores can be surface-sterilized for 30 min in 5–10 mL of acidified electrolyzed water (AEW). AEW effectively surface-sterilizes teliospores but, compared with a 1–2 min bleach treatment, stimulates rather than reduces teliospore germination (Bonde *et al.*, 1999). The teliospores are then washed twice by removing the supernatant, resuspending the pellet in 1 mL of sterile distilled water (SDW) and centrifuging at 1200*g* for 5 min.

The pellet is resuspended in 1 mL of SDW and 200 µL of the teliospore suspension is placed aseptically onto 5 plates of 2% water agar with antibiotics (WA+A) and spread with a sterile spreader. The antibiotics used are 60 mg penicillin-G (Na salt) and 200 mg streptomycin sulphate per litre of agar (EPPO, 2007). The WA+A plates are incubated at 21°C with a 12-h light cycle. The plates are left for about 5 days before being sealed or placed inside clear polyethylene bags.

After 7–14 days, non-dormant teliospores produce a promycelium bearing 32–128 or more basidiospores (primary sporidia) at its tip (Fig. 16). These colonies produce secondary sporidia typically of two types: filiform and allantoid. These can then be cultured directly on solid medium (Fig. 9) or liquid nutrient medium such as potato dextrose broth. Small blocks of agar (1 cm × 1 cm) bearing germinated teliospores or colonies are cut out and then stuck to the underside of a Petri dish lid so that the germinated teliospore is facing the surface of the medium. This allows the sporidia to be released onto the media surface. The dishes are incubated at 21°C with a 12-h light cycle. After 2–3 days, basidiospores deposited onto the medium surface produce small mats of mycelia of approximately 0.5–1.0 cm diameter. Each mycelial mat is removed with a sterile dissecting needle, and touched onto sterile filter paper to remove excessive broth. The mycelium is placed in suitable vials (e.g. 1.5–2.0 mL microcentrifuge tubes) for immediate DNA extraction, or stored at −80°C for later DNA extraction.

Germination of teliospores for molecular analysis may not always be possible; for example, if seeds are treated with NaOH, as in the case of fungicide-treated grain. Increasing the number of sieved replicates may increase the number of teliospores recovered and hence the number of teliospores that can be germinated. Teliospores can have a period of dormancy, which can affect germination (Carris *et al.*, 2006). This can be resolved by carrying out direct real-time PCR on individual teliospores (see Appendix 8) or directly on the pellet obtained from the wash test (Appendix 9).

Appendix 5 – PCR restriction analysis of *T. indica* to distinguish from *T. walkeri* and *T. horrida*

1. General information

1.1 Protocol developed by Pimentel *et al.* (1998) and adapted for use at CREA, Italy.

Fig. 16 *Tilletia indica* teliospores germinating on water agar after 10–14 days, producing a tuft of primary sporidia (basidiospores) at the apex of the promycelium. Primary sporidia germinate *in situ* to produce small colonies which produce secondary sporidia of two types: further filiform sporidia and allantoid sporidia, which are forcibly discharged.

1.2 Test mycelium is grown as described in Appendix 4.
1.3 The target gene region is the internal transcribed spacers (ITS) of the nuclear ribosomal RNA gene.
1.4 The PCR amplicon produced includes both ITS1 and ITS2 and the conserved fragment 5.8S. This amplicon is approximately 670 bp including primer sequences.
1.5 Oligonucleotides used: forward primer ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′); reverse primer ITS4 (5′-TCC TCC GCT TAT TGA TAT GC 3′) (White *et al.*, 1990).
1.6 AmpliTaq® polymerase 5 U μL^{-1} (Applied Biosystems) used for PCR amplification and enzymes *Taq*I and *Sca*I (Promega) used for amplicon restriction.
1.7 Buffers: 10× PCR buffer containing 15 mM $MgCl_2$ (Applied Biosystems), 10× restriction enzyme buffer (Promega).
1.8 Bovine serum albumin 0.2 μL [10 μg μL^{-1}] is added to the restriction mix as a reaction additive.
1.9 Amplification is performed using a standard thermal cycler. 3 thermocyclers were used in this publication: Coy TempCycler II, models 110s and Multicycler (Coy Laboratory Products Inc., Grass Lake, Michigan, US), and a GeneAmp PCR 9700 system (PE Applied Biosystems, Roche Molecular Systems inc., Branchburg, New Jersey, US).

2. Methods

2.1 Nucleic acid extraction and purification
2.1.1 DNA is extracted from mycelium produced as described in Appendix 4. Approximately 0.1 g is placed in a sterile 2 mL microcentrifuge tube 1/3 full with sterile 0.5-mm glass beads (Stratech) and 1 mL of molecular-grade water. Seal the tube with a screw-lid containing an O-ring and oscillate the tube in a BeadBeater (Stratech) on 1/4 power for 5 min or grind-up the mycelium in a mortar.
2.1.2 Allow the ground sample to stand for 30 s then extract its DNA using a proprietary DNA extraction kit for fungi, for example Nucleospin (Machery Nagel), or a more traditional method such as described by Hughes *et al.* (2000).
2.1.3 No DNA cleanup is required. Either use the extracted DNA immediately or store overnight at 4°C or at −20°C for longer periods
2.2 Polymerase chain reaction to produce restriction amplicon
2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water*	n.a.	31.75	n.a.
Taq DNA polymerase buffer containing 15 mM $MgCl_2$ (Applied Biosystems)	10×	5.0	1 x
dNTPs	10 mM	1	0.2 mM
Forward primer	5 μM	5	0.5 μM
Reverse primer	5 μM	5	0.5 μM
AmpliTaq® polymerase (Applied Biosystems)	5 U μL^{-1}	1.25	0.125 U μL^{-1}
Subtotal		49	
Template DNA (obtained as described above)		1.0	
Total		50	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-μm filtered) and nuclease-free water.

2.2.2 PCR conditions: Initial denaturation at 94°C for 2 min; 30 cycles at 94°C for 1 min, 54°C for 1 min and 72 for 1 min; final extension at 72°C for 10 min.
2.3 Restriction of PCR amplicon
2.3.1 Restriction mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water*	n.a.	7.3	n.a.
Restriction buffer (Promega)	10×	2.0	1×
Bovine serum albumin	10 μg μL^{-1}	0.2	0.1 μg μL^{-1}
Restriction enzyme *Taq*I or *Sca*I (Promega)	10 U μL^{-1}	0.5	0.25 U μL^{-1}
Subtotal		10	
PCR product	>50 ng μL^{-1}	10	>25 ng μL^{-1}
Total		20	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-μm filtered) and nuclease-free water.

2.3.2 Incubation temperature, time for restriction. Three hours at 65°C for *Taq*I and 37°C for restriction enzyme *Sca*I; gently mix the reaction by inversion during incubation
2.3.3 If required store restricted products at 4°C, before visualizing as described below.

3. Essential procedural information

As required mix 10 µL of reaction products with a running marker and run on a 2% agarose gel.

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.
- 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 PCR tests not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative to (or in addition to) the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. IPC also ensure that inhibitory compounds from the DNA extract do not prevent the amplification reaction. Positive internal controls can either be 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. 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 IAC) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2 Interpretation of results

In order to assign results from PCR-based tests the following criteria should be adopted:

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of the expected size.

When these conditions are met:

- A test will be considered positive for *T. indica* if amplicons of 670 bp are produced and cut with restriction enzyme *Taq*I to give five products (Fig. 17) and there is no cut with *Sca*I (Fig. 18).
- A test will be considered positive for *T. walkeri* if amplicons of 670 bp are produced and cut with *Taq*I to give the same 5 fragments as with *T. indica* (Fig. 17), but *Sca*I restricts amplified products to give two fragments (Fig. 18).
- A test will be considered positive for *T. horrida* if amplicons of 670 bp are produced and cut with *Taq*I to give four DNA fragments (Fig. 17) and *Sca*I produces no cuts (Fig. 18).

	T. indica	*T. walkeri*	*T. horrida*
			355
	260	260	
	170	170	150
	110	110	110
	70	70	
	60	60	60
Total bp.	670	670	675

Fig. 17 Schematic of PCR-RFLP patterns from the ITS region using restriction enzyme *Taq*I. Individual products are shown as thick lines against product size (base pairs, bp).

	T. indica	*T. walkeri*	*T. horrida*
	670 (NC)	520	670 (NC)
		140	
Total bp.	670	660	670

Fig. 18 Schematic of PCR-RFLP patterns from the its region using restriction enzyme *Sca*I. Individual products are shown as thick lines against product size (base pairs, bp). nc = not cut.

- Other *Tilletia* species give different restriction patterns with these or other enzymes (see Pimentel *et al.*, 1998).
- 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

No performance criteria are available.

Appendix 6 – Identification of *Tilletia indica* by conventional PCR.

1. General Information

1.1 Protocol developed by Frederick *et al.* (2000).
1.2 Test mycelium is grown as described in Appendix 4.
1.3 The target region is mitochondrial DNA producing an amplicon of 414 bp. Oligonucleotides used: forward primer, Tin 3 (5′-CAA TGT TGG CGT GGC GGC GC-3′); reverse primer, Tin 4 (5′-CAA CTC CAG TGA TGG CTC CG-3′). To test for *T. walkeri* replace Tin 3 with the forward primer Tin 11 (5′-TAA TGT TGG CGT GGC GGC AT-3′, this also produces an amplicon of 414 bp.
1.4 AmpliTaq® polymerase 5 U μL^{-1} (Applied Biosystems) used for PCR amplification.
1.5 Buffers: 10× PCR buffer containing 15 mM $MgCl_2$ (Applied Biosystems).
1.6 Amplification is performed using a standard thermal cycler.

2. Methods

2.1 Nucleic acid extraction and purification
2.1.1 DNA is extracted from mycelium produced as described in Appendix 4. Approximately 0.1 g is placed in a sterile 2 mL microcentrifuge tube 1/3 full with sterile 0.5-mm glass beads (Stratech) and 1 mL of molecular-grade water. Seal the tube with a screw-lid containing an O-ring and oscillate the tube in a BeadBeater (Stratech) on 1/4 power for 5 min or grind the mycelium in a mortar.
2.1.2 Allow the ground sample to stand for 30 s then extract its DNA using a proprietary DNA extraction kit for fungi, for example Nucleospin (Machery Nagel), or a more traditional method such as described by Hughes *et al.* (2000).
2.1.3 No DNA cleanup is required. Either use the extracted DNA immediately or store overnight at 4°C or at −20°C for longer periods
2.2 Conventional PCR
2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	n.a.	20.95	n.a.
Taq DNA polymerase buffer containing 15 mM $MgCl_2$ (Applied Biosystems)	10×	2.5	1×
dNTPs	10 µM	0.25	0.1 µM
Forward primer	25 µM	0.1	0.1 µM
Reverse primer	25 µM	0.1	0.1 µM
AmpliTaq® polymerase (Applied Biosystems)	5 U μL^{-1}	0.1	0.02 U μL^{-1}
Subtotal		24	
Template DNA (obtained as described above)		1.0	
Total		25	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free water.

2.2.2 PCR conditions*:* initial denaturation at 94°C for 1 min; 25 cycles at 94°C for 15 s, 65°C for 15 s and 72°C for 15 s; final extension at 72°C for 6 min.

3. Essential procedural information

As required mix 10 µL of reaction products with a running marker and run on a 2% agarose gel

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.
- 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 PCR tests not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As alternative to (or in addition to) the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. IPC also ensure that inhibitory compounds from the DNA extract do not prevent the amplification reaction. 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 a conserved non-pest target nucleic acid that is also present in the sample (e.g. 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 IAC) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2 Interpretation of results

In order to assign results from PCR-based tests the following criteria should be adopted:

Verification of the controls

- NIC and NAC should produce no amplicons.
- PIC and PAC should produce amplicons of the expected size.

When these conditions are met:

- A test will be considered positive for both for *T. indica* (primers Tin 3/Tin 4) and *T. walkeri* (primers Tin 11/Tin 4) if amplicons of 414 bp are produced.
- A test will be considered negative both for *T. indica* (primers Tin 3/Tin 4) and *T. walkeri* (primers Tin 11/Tin 4) if no band or a band of a different size is produced. If positive control DNA samples are positive, then the sample extractions belong to another *Tilletia* species such as *T. horrida*. Restriction enzyme analysis may enable further species identification of these samples if required (Appendix 5). Alternatively no amplification can result from poor-quality DNA or the presence of inhibitory compounds, which can be checked by testing extracts with the universal primers (ITS1 and ITS4) described in Appendix 5. If the samples contain good-quality DNA and not inhibitory compounds, and hence test samples are not *T. indica* or *T. walkeri* but another *Tilletia* species, then a single band (approximately 670 bp) will be produced when PCR amplicons are run out on an agarose gel. However, if amplification is still not produced, fresh DNA should then be extracted and retested.
- Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical specificity

Thirty-four *T. indica* isolates were tested and positively detected with primers Tin 3/Tin 4. Ten isolates of *T. walkeri* were tested and did not give any cross-reaction.

Appendix 7 – Identification of *Tilletia indica* by real-time PCR

1. General Information

1.1 Protocol developed by Frederick *et al.* (2000).

1.2 Test mycelium and control cultures are grown as described in Appendix 4.

1.3 The target region is mitochondrial DNA producing an amplicon of 212 bp. Oligonucleotides used: the forward primer is the same as for conventional PCR (Tin 3) while the reverse primer is Tin 10 (5′-AGCTCCGCCTCAAGTTCCTC-3′). To test for *T. walkeri*, replace Tin 3 with the forward primer Tin 11 (5′-TAA TGT TGG CGT GGC GGC AT-3′), which also produces an amplicon of 212 bp.

1.4 Real-time probe: TaqMan probe [10 μM] (Applied Biosystems) 5′ (FAM label)-ATT CCC GGC TTC GGC GTC ACT- (TAMRA quencher) 3′.

1.5 2 X universal TaqMan master mix is used containing Taq polymerase, reaction buffer containing $MgCl_2$ and nucleotides (Applied Biosystems).

1.6 Amplification is performed using a real-time PCR thermal cycler.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 DNA is extracted from mycelium produced as described in Appendix 4, approximately 0.1 g is placed in a sterile 2-mL microcentrifuge tube 1/3 full with sterile 0.5-mm glass beads (Stratech) and 1 mL of molecular-grade water. Seal the tube with a screw-lid containing an O-ring and oscillate the tube in a BeadBeater (Stratech) on ¼ power for 5 min or grind up the mycelium in a mortar.

2.1.2 Allow the ground sample to stand for 30 s then extract its DNA using a proprietary DNA extraction kit for fungi, for example Nucleospin (Machery Nagel), or a more traditional method such as that described by Hughes *et al.* (2000).

2.1.3 No DNA cleanup is required. Either use the extracted DNA immediately or store overnight at 4°C or at −20°C for longer periods.

2.1.4 Optical reaction tubes and caps should be used to allow real-time amplification to be monitored.

2.2 Real-time PCR

2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water*	n.a.	8.5	n.a.
Universal TaqMan master mix (Applied Biosystems)	2×	12.5	1×
Forward primer	10 μM	1	0.4 μM
Reverse primer	10 μM	1	0.4 μM
TaqMan probe (Applied Biosystems)	10 μM	1	0.4 μM
Subtotal		24	
Template DNA (obtained as described above)	12.5 ng μL^{-1}	1.0	0.5 ng μL^{-1}
Total		25	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-μm filtered) and nuclease-free water.

2.2.2 PCR conditions*:* 50°C for 2 min; initial denaturation at 95°C for 10 min; 34 cycles at 95°C for 15 s and 60°C for 1 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 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.
- 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 PCR tests not performed on isolated organisms, the PAC should preferably be near to the limit of detection.

As an alternative to (or in addition to) the external positive controls (PIC and PAC), internal positive controls (IPC) can be used to monitor each individual sample separately. IPC also ensure that inhibitory compounds from the DNA extract do not prevent the amplification reaction. Positive internal controls can be 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. 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 IAC) or amplification of a duplicate sample spiked with the target nucleic acid.

3.2 Interpretation of results

In order to assign results from PCR-based tests the following criteria should be adopted:

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 for *T. indica* if it produces an exponential amplification curve with primers Tin 3/Tin 10 while *T. walkeri* needs primers Tin 11/Tin 10.
- A test will be considered negative both for *T. indica* (primers Tin 3/Tin 10) and *T. walkeri* (primers Tin 11/Tin 10) if it does not produce an amplification curve or if it produces a curve which is not exponential when testing for *T. indica* and the threshold cycle (Ct) of the sample is >33 the result indicates that it is negative for *T. indica* and is highly likely to be another species of *Tilletia*. Likewise, when testing for *T. walkeri* and the Ct is >33, the result indicates that it is negative for *T. walkeri* and is highly likely to be another species of *Tilletia*. If neither primer set produces amplification but control samples react as expected then the sample extractions belong to another *Tilletia* species, such as *T. horrida*. Restriction enzyme analysis (Appendix 5) or real-time-PCR (Appendix 8) may enable further species identification of these samples if required. Alternatively no amplification can result from poor-quality DNA or the presence of inhibitory compounds, which can be checked by testing extracts with the universal primers (ITS1 and ITS4) described in Appendix 5. If the samples contain good-quality DNA/no inhibitory compounds, and hence test samples are not *T. indica* or *T. walkeri* but another *Tilletia* species, then a single band (approximately 670 bp) will be produced when PCR amplicons are run out on an agarose gel. However, if amplification is still not produced, fresh DNA should then be extracted and retested.
- Tests should be repeated if any contradictory or unclear results are obtained.

Note: As a Ct cut-off value is dependent on equipment, material and chemistry it needs to be verified in each laboratory when implementing the test.

4. Performance criteria available

4.1 Analytical sensitivity

The sensitivity limit for both *T. indica* and *T. walkeri* was found to be 5 pg total DNA.

This concentration produced detectable levels of fluorescence.

4.2 Analytical specificity

The specificity of the test was tested against DNA extracted from *Tilletia barclayana sensu lato*, *Tilletia tritici*, *Tilletia laevis*, *Tilletia controversa* and *Tilletia fusca.* None of these isolates was amplified in either the *T. indica*- or the *T. walkeri*-specific tests

Appendix 8 – Direct real-time PCR on teliospores

1. General Information

1.1 This protocol was developed by Tan *et al.* (2009) and is based on a multiplex real-time PCR.

1.2 The nucleic acid source is a single teliospore.

1.3 The target region is the internal transcribed spacer ITS1 region in rDNA.

1.4 The real-time PCR test is set up with a multiplex format, enabling the simultaneous detection of *T. indica*, *T. walkeri*, *T. ehrhartae*, *T. horrida* and one group of species with very similar morphology to the type species *Tilletia caries.*

1.5 Before proceeding to the real-time PCR, an amplification step to enrich *Tilletia*-specific DNA is required with primers MK56 (5′-GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) (Tan *et al.*, 1996) and Tilletia-R (5′-CAA GAG ATC CGT TGT CAA AAG TTG-3′) (Tan & Murray, 2006).

1.6 The multiplex real-time PCR is carried out with 5 dual-labelled probes and 4 oligonucleotide primers pairs.

1.7 The GenBank ITS sequences of various *Tilletia* species were aligned for the design of four pairs of primers and five dual-labelled probes.

1.8 Oligonucleotides and probes:

1.11.1 *T. indica* and *T. walkeri*

Forward primer KB-DL-For: 5′-CTTCGGAAGAGT CTCCTT-3′

Reverse primer KB-DL-Rev: 5′-CCGGACAGGTAC TCAG-3′

Hydrolysis probe *T. indica*: 5′-FAM-ACGGAAGGA ACGAGGC-BHQ1-3′

Hydrolysis probe *T. walkeri*: 5′-JOE-ACGGAAGG AACAAGGC-BHQ1-3′

1.11.2 *T. horrida*

Forward primer Hor-DL-For: 5′-GGCCAATCTTCT CTACTATC-3′

Reverse primer Hor-DL-Rev: 5′-CCGGACAGGATC ACTA-3′

Hydrolysis probe *T. horrida*: 5′-CALFluorRed610-CAACCCAGACTACGGAGGGTGA-BHQ2-3′

1.11.3 *T. ehrhartae*

Forward primer Ehr-DL-For: 5′-CGCATTCTTATG CTTCTTG-3′

Reverse primer Ehr-DL-Rev: 5′-GTTAGGAACCAA AGCCATC-3′

Hydrolysis probe *T. ehrhartae*: 5′-Quasar 705-CAG AGTCATTGGTTCTTCGGAGC-BHQ2-3′

1.11.4 Broad range (*T. caries*, *T. laevis*, *T. controversa*, *T. fusca*, *T. bromi*, *T. goloskokovii*)

Forward primer Tri-DL-For: 5′-ATTGCCGTACTT CTCTTC-3′

Reverse primer Tri-DL-Rev: 5′-GTAGTCTTGTGTT TGGATAATAG-3′

Hydrolysis probe broad range: 5′-Quasar 670-AGAG GTCGGCTCTAATCCCATCA-BHQ2-3′

1.9 Taq DNA polymerase (Invitrogen) in 1× buffer (50 mM Tris, pH 9.0, 20 mM NaCl, 1% Trition X-100, 0.1% gelatin) used for PCR amplification of *Tilletia*-specific DNA.

1.10 Immolase DNA polymerase (Bioline) used for real-time PCR tests with the dual-labelled probes and oligonucleotide primers.

2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Teliospores are produced as described in Appendix 4.

2.1.2 After the teliospores have been examined and their morphology recorded, the slide is allowed to dry out, either with or without the coverslip on. When the coverslip is removed, it is placed on the slide upside down so it can be checked for teliospores adhering to it. On another slide is placed a single piece of a coverslip (obtained by cutting a coverslip into tiny pieces of 1 mm × 1 mm) that has been sterilized (autoclaved at 121°C for 15 min or baked at 170°C for 2 h). A 1-µL drop of Tris-ethylenediaminetetraacetic acid (TE) buffer is placed onto this piece of coverslip. Under either a compound or a dissecting microscope, a single teliospore is picked off with a very fine needle and placed into the droplet of TE buffer. The teliospore will transfer to the droplet. Using forceps another sterilized small piece of coverslip is placed on top to make a sandwich. The teliospore is crushed by using

the forceps to press down on the coverslip, and then the glass sandwich is transferred into a 0.2-mL PCR tube. After dispensing into the tube an appropriate volume of PCR mix (see Section 2.2) for the amplification of *Tilletia*-specific DNA, the same pipette tip was used to further crush the spore in the PCR mix (Tan *et al.*, 2009) releasing the spore material.

2.2 PCR amplification of *Tilletia*-specific DNA

2.2.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	n.a.	14.9	n.a.
PCR buffer:	10×	2	1×
$MgCl_2$	50 mM	0.6	1.5 mM
dNTPs	10 mM	0.4	0.2 mM
Forward primer MK56	10 µM	1	0.5 µM
Reverse primer Tilletia-R	10 µM	1	0.5 µM
Taq DNA polymerase (Invitrogen)	5 U μL^{-1}	0.1	0.025 U μL^{-1}
Total		20	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free water.

2.2.2 PCR conditions*:* initial denaturation at 95°C for 3 min; 20 cycles of 20 s denaturation at 94°C, 30 sec annealing at 63°C, 30 s extension at 72°C with the annealing temperature decreased by 1°C per cycle for 5 cycles to 59 °C; 10 min final extension at 72°C; and 1 min incubation at 4°C.

2.3 Real-time PCR

2.3.1 Master mix

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water*	N.A.	2	N.A.
PCR buffer (ImmoBuffer, Bioline):	10×	2	1×
$MgCl_2$	50 mM	2	5 mM
dNTPs	10 mM	0.4	0.2 mM
Forward primer KB-DL-For	10 µM	0.8	0.4 µM
Reverse primer KB-DL-Rev	10 µM	1.8	0.9 µM
Forward primer Hor-DL-For	10 µM	0.8	0.4 µM
Reverse primer Hor-DL-Rev	10 µM	1.8	0.9 µM
Forward primer Ehr-DL-For	10 µM	0.8	0.4 µM
Reverse primer Ehr-DL-Rev	10 µM	1.8	0.9 µM
Forward primer Tri-DL-For	10 µM	0.8	0.4 µM
Reverse primer Tri-DL-Rev	10 µM	1.8	0.9 µM
Hydrolysis probe *T. indica*	10 µM	0.4	0.2 µM
Hydrolysis probe *T. walkeri*	10 µM	0.4	0.2 µM
Hydrolysis probe *T. horrida*	10 µM	0.4	0.2 µM
Hydrolysis probe *T. ehrhartae*	10 µM	0.4	0.2 µM
Hydrolysis probe broad range	10 µM	0.4	0.2 µM
Immolase DNA Polymerase (Bioline)	5 U μL^{-1}	0.2	0.05 U μL^{-1}
Subtotal		19	
DNA†	n.a.	1	n.a.
Total		20	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-µm filtered) and nuclease-free water.

†The template DNA is the PCR product from the PCR amplification *of Tilletia*-specific DNA (Section 2.2).

2.3.2 PCR conditions*:* initial denaturation at 95°C for 10 min; 40 cycles of 15 s denaturation at 94°C, 60 s annealing/extension at 65°C, with the annealing/extension temperature decreased by 1°C per cycle for 6 cycles to 60°C; the dynamic tube normalization option was used to determine the average background of each individual sample before amplification commenced.

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 (in real-time PCR): nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated (in real-time PCR): nucleic acid extraction and subsequent amplification of the target organism or a sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).

- Two negative amplification controls (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 for enrichment PCR and for real-time PCR.
- Two positive amplification controls (PAC) to monitor the efficiency of the amplification of the target organism for the enrichment PCR and for the real-time PCR. 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). The PAC should preferably be near to the limit of detection.

3.2 Interpretation of results

Verification of the controls

Conventional PCR

- The PAC in the enrichment PCR should produce an amplicon of 270 bp.
- The NAC in the enrichment PCR should give no amplification.

Real-time PCR

- PIC and PAC in real-time PCR should produce exponential curves relative to the fluorophore for each target organism analysed.
- NIC and NAC curves 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 data

The sensitivity of the test is a single teliospore of *T. indica*.

4.2 Analytical specificity data

The specificity of the probe for *T. indica* was investigated in a DNA mixture of *T. indica* and *T. walkeri* or *T. ehrhartae* or *T. caries*, in ratios of 1:0.1 pg and 0.1:1 pg (appropriate concentration range indicated from single-spore analysis). The specificity of the primers was tested and they were found not to react with other *Tilletia* species.

4.3 Diagnostic sensitivity

Diagnostic sensitivity ranges from 10 to 40% (i.e. out of known positive *T. indica* spores only 10–40% gave positive PCR results) (Tan *et al*., 2009). This sensitivity is due to a number of reasons, including the fact that all *T. indica* spores and bunted grain had to be autoclaved twice so there may have been a deterioration of genetic material.

Appendix 9 – Direct Real-time PCR on pellet

1. General Information

1.1 This protocol is based on the protocol developed by Tan *et al.*, 2009 (Appendix 8), with some changes for DNA extraction and enrichment PCR steps (Valente & Riccioni, 2016).

1.2 The nucleic acid source is the pellet obtained from 'washing test' of 50 g of a wheat seed sample as described in Appendix 1.

1.3 The target region is the internal transcribed spacer ITS1 region in rDNA

1.4 The real-time PCR test is set up with a triplex format, enabling the simultaneous detection of *T. indica*, *T. walkeri* and *T. horrida*

1.5 Before proceeding to the real-time PCR, an amplification step to enrich *Tilletia*-specific DNA is required with primers MK56 (5′-GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) (Tan *et al.*, 1996) and Tilletia-R (5′-CAA GAG ATC CGT TGT CAA AAG TTG-3′) (Tan & Murray, 2006).

1.6 The multiplex real-time PCR is carried out with 3 dual-labelled probes and 2 oligonucleotide primers pairs.

1.7 The GenBank ITS sequences of various *Tilletia* species were aligned for the design of two pairs of primers and three dual-labelled probes

1.8 Oligonucleotides and probes

1.8.1 *T. indica* and *T. walkeri*

Forward primer KB-DL-For: 5′-CTTCGGAAG AGTCTCCTT-3′

Reverse primer KB-DL-Rev: 5′-CCGGACAG GTACTCAG-3′

Hydrolysis probe *T. indica*: 5′-FAM-ACGGA AGGAACGAGGC-BHQ1-3′

Hydrolysis probe *T. walkeri*: 5′-JOE-ACGGA AGGAACAAGGC-BHQ1-3′

1.8.2 *T. horrida*

Forward primer Hor-DL-For: 5′-GGCCAATC TTCTCTACTATC-3′

Reverse primer Hor-DL-Rev: 5′-CCGGACAG GATCACTA-3′

Hydrolysis probe *T. horrida*: 5′-CALFluor Red610-CAACCCAGACTACGGAGGGTGA-BHQ2-3′

1.9 BioTaq DNA polymerase (Bioline) is used for PCR amplification of *Tilletia*-specific DNA.

1.10 Immolase DNA polymerase (Bioline) used for real-time PCR.

2. Methods

2.1 Nucleic acid extraction

2.1.1 The pellet is produced from the 'washing test' described in Appendix 1.

2.1.2 The pellet is centrifuged for 5 min at 7000*g* and the supernatant is gently removed with a P100 micropipette. The pellet is resuspended in 60 μL of 20× Amplification Buffer (1.34 M Tris-HCl pH 8.8; 320 mM $(NH_4)_2SO_4$; 0.2% Tween 20) and is vigorously homogenized with a sterile micropestle for about 1 min. The sample is incubated at 94°C for 3 min in a block thermostat or pre-heated water bath; 180 μL of sterile MilliQ water is added and the sample is mixed by vortexing and centrifuged for 3 min at 10 000–13 000*g*. The supernatant (containing the template DNA) is recovered into a new 1.5-mL microtubes and should be directly amplified by the enrichment PCR as soon as possible.

2.2 PCR amplification of *Tilletia*-specific DNA

2.2.1 The enrichment amplification protocol has undergone some changes compared to the original described by Tan *et al.* (2009) in order to maximize its effectiveness. The amplification reaction is prepared in ice.

2.2.2 Master mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water*	n.a.	10.4	n.a.
$MgCl_2$ (Bioline)	50 mM	2	5 mM
dNTPs (Bioline)	10 mM	0.4	0.2 mM
Ultrapure BSA (Ambion)	10 mg mL^{-1}	1	0.5 mg mL^{-1}
Forward primer MK56	10 μM	1	0.5 μM
Reverse primer Tilletia-R	10 μM	1	0.5 μM
BioTaq DNA polymerase (Bioline)	5 U μL^{-1}	0.2	0.05 U μL^{-1}
Subtotal		16	
DNA	n.a.	4	n.a.
Total		20	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-μm filtered) and nuclease-free water.

2.2.3 PCR conditions: initial denaturation at 95°C for 3 min; 20 cycles of 20 s denaturation at 94°C, 30 s annealing at 63°C, 30 s extension at 72°C with the annealing temperature decreased by 1°C per cycle for 5 cycles to 59 °C; 10 min final extension at 72°C; and 1 min incubation at 4°C.

2.3 Real-time PCR

2.3.1 Master mix

Reagent	Working concentration	Volume per reaction (μL)	Final concentration
Molecular-grade water*	n.a.	7	n.a.
PCR buffer (Bioline):	10×	2	1×
$MgCl_2$	50 mM	2	5 mM
dNTPs	10 mM	0.4	0.2 mM
Forward primer KB-DL-For	10 μM	0.8	0.4 μM
Reverse primer KB-DL-Rev	10 μM	1.8	0.9 μM
Forward primer Hor-DL-For	10 μM	0.8	0.4 μM
Reverse primer Hor-DL-Rev	10 μM	1.8	0.9 μM
Hydrolysis probe *T. indica*	10 μM	0.4	0.2 μM
Hydrolysis probe *T. walkeri*	10 μM	0.4	0.2 μM
Hydrolysis probe *T. horrida*	10 μM	0.4	0.2 μM
Immolase DNA polymerase (Bioline)	5 U μL^{-1}	0.2	0.05 U μL^{-1}
Subtotal		18	
DNA†	N.A.	2	N.A.
Total		20	

*Molecular-grade water should be used preferably, or prepared purified (deionized or distilled), sterile (autoclaved or 0.45-μm filtered) and nuclease-free water.

†The template DNA is the PCR product from the PCR amplification of *Tilletia*-specific DNA (Section 2.2).

2.3.2 PCR conditions: initial denaturation at 95°C for 10 min; 40 cycles of 15 s denaturation at 94°C, 60 s annealing/extension at 65°C, with the annealing/extension temperature decreased by 1°C per cycle for 6 cycles to 60°C; the dynamic tube normalization option is used to determine the average background of each individual sample before amplification commences; allelic discrimination analysis has to be set to unequivocally identify each target species.

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 (in real-time PCR): nucleic acid extraction and subsequent amplification of a sample of uninfected host tissue or clean extraction buffer.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated (in real-time PCR): nucleic acid extraction and subsequent amplification of the target organism or a sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).

- Two negative amplification controls (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 for enrichment PCR and for real-time PCR.
- Two positive amplification controls (PAC) to monitor the efficiency of the amplification of the target organism for the enrichment PCR and for the real-time PCR. 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). The PAC should preferably be near to the limit of detection.

3.2 Interpretation of results

Verification of the controls

- The PAC in the enrichment PCR should produce an amplicon of 270 bp.
- The NAC in the enrichment PCR should give no amplification.
- PIC and PAC in real-time PCR should produce exponential curves relative to the fluorophore for each target organism analysed.
- NIC and NAC curves should give no amplification.

When these conditions are met:

- A test will be considered positive if it produces an exponential amplification curve with a Ct equal to or less than the Ct of the PAC and allelic discrimination analysis will give the corresponding identification results.
- 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 data

The sensitivity of the test is five teliospores of *T. indica*.

4.2 Analytical specificity data

The specificity of the probe for *T. indica* was investigated in a DNA, *T. walkeri* and *T. horrida*. The specificity of the primers was tested and they were found not to react with other *Tilletia* species.

4.3 Diagnostic sensitivity

Diagnostic sensitivity was investigated in a test performance study (TPS) organized with 9 laboratories and was found to be 83%. This value has been obtained using autoclaved teliospores and there may have been a deterioration of genetic material.

4.4 Diagnostic specificity

The diagnostic specificity was investigated in a TPS organized with 9 laboratories and it was found to be 85%; in this case it was observed that all the samples mistakenly identified as *T. indica* (false positives) contained *T. walkeri* spores. The *T. indica* probe showed a slight cross-reaction with the amplified *T. walkeri* DNA, due to the different thermocyclers used. Performance of an allelic discrimination analysis on the data from 4 laboratories increased the specificity from 79% to 100%.

4.5 Data on repeatability

Repeatability was assessed in a TPS organized with 9 laboratories and was found to be 76%. Performance of an allelic discrimination analysis on the data from 4 laboratories increased the specificity from 71% to 86%.

4.6 Data on reproducibility

Reproducibility was assessed in a TPS organized with 9 laboratories and was found to be 50%. Performance of an allelic discrimination analysis on the data from 4 laboratories increased the specificity from 50% to 59%.