Normes OEPP
EPPO Standards

Diagnostic protocols for regulated pests
Protocoles de diagnostic pour les organismes réglementés

PM 7/29
Approval

EPPO Standards are approved by EPPO Council. The date of approval appears in each individual standard. In the terms of Article II of the IPPC, EPPO Standards are Regional Standards for the members of EPPO.

Review

EPPO Standards are subject to periodic review and amendment. The next review date for this EPPO Standard is decided by the EPPO Working Party on Phytosanitary Regulations.

Amendment record

Amendments will be issued as necessary, numbered and dated. The dates of amendment appear in each individual standard (as appropriate).

Distribution

EPPO Standards are distributed by the EPPO Secretariat to all EPPO member governments. Copies are available to any interested person under particular conditions upon request to the EPPO Secretariat.

Scope

EPPO Diagnostic Protocols for Regulated Pests are intended to be used by National Plant Protection Organizations, in their capacity as bodies responsible for the application of phytosanitary measures to detect and identify the regulated pests of the EPPO and/or European Union lists.

In 1998, EPPO started a new programme to prepare diagnostic protocols for the regulated pests of the EPPO region (including the EU). The work is conducted by the EPPO Panel on Diagnostics and other specialist Panels. The objective of the programme is to develop an internationally agreed diagnostic protocol for each regulated pest. The protocols are based on the many years of experience of EPPO experts. The first drafts are prepared by an assigned expert author(s). They are written according to a ‘common format and content of a diagnostic protocol’ agreed by the Panel on Diagnostics, modified as necessary to fit individual pests. As a general rule, the protocol recommends a particular means of detection or identification which is considered to have advantages (of reliability, ease of use, etc.) over other methods. Other methods may also be mentioned, giving their advantages/disadvantages. If a method not mentioned in the protocol is used, it should be justified.

The following general provisions apply to all diagnostic protocols:

• laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated or that proper positive and negative controls are included.

References


Definitions

Regulated pest: a quarantine pest or regulated non-quarantine pest.

Quarantine pest: a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled.

Outline of requirements

EPPO Diagnostic Protocols for Regulated Pests provide all the information necessary for a named pest to be detected and positively identified by an expert (i.e. a specialist in entomologist, mycology, virology, bacteriology, etc.). Each protocol begins with some short general information on the pest (its appearance, relationship with other organisms, host range, effects on host, geographical distribution and its identity) and then gives details on the detection, identification, comparison with similar species, requirements for a positive diagnosis, list of institutes or individuals where further information on that organism can be obtained, references (on the diagnosis, detection/extraction method, test methods).

Existing EPPO Standards in this series

Nineteen EPPO standards on diagnostic protocols have already been approved and published. Each standard is numbered in the style PM 7/4 (1), meaning an EPPO Standard on Phytosanitary Measures (PM), in series no. 7 (Diagnostic Protocols), in this case standard no. 4, first version. The existing standards are:

PM 7/14 (1) *Ceratocystis fimbriata f. sp. platani*. Bulletin OEPP/EPPO Bulletin 33, 249–256
PM 7/16 (1) *Fusarium oxysporum f. sp. albedinis*. Bulletin OEPP/EPPO Bulletin 33, 265–270

Several of the Standards of the present set result from a different drafting and consultation procedure. They are the output of the DIAGPRO Project of the Commission of the European Union (no. SMT 4-CT98-2252). This project involved four ‘contractor’ diagnostic laboratories (in England, Netherlands, Scotland, Spain) and 50 ‘intercomparison’ laboratories in many European countries (within and outside the European Union), which were involved in ring-testing the draft protocols. The DIAGPRO project was set up in full knowledge of the parallel activity of the EPPO Working Party on Phytosanitary Regulations in drafting diagnostic protocols, and covered regulated pests which were for that reason not included in the EPPO programme. The DIAGPRO protocols have been approved by the Council of EPPO as EPPO Standards in series PM7. They will in future be subject to review by EPPO procedures, on the same terms as other members of the series.
Diagnostic protocols for regulated pests
Protocoles de diagnostic pour les organismes réglementés

Tilletia indica

Specific scope
This standard describes a diagnostic protocol for Tilletia indica.

Introduction
Tilletia indica causes the disease Karnal bunt, or partial bunt, of wheat (Triticum spp.), Triticale (X Triticosecale) is also naturally infected and rye (Secale) is a potential host. T. indica was added to the EC Plant Health Directive 77/93/EEC (now 2000/29/EC) as a I/AI pest in 1996 and phytosanitary requirements applied to seed and grain of Triticum, Secale and X Triticosecale imported from countries where T. indica is known to occur.

Identity
Name: Tilletia indica Mitra.
Synonyms: Neovossia indica (Mitra) Mundkur.
Bayer computer code: NEOVIN.
Phytosanitary categorization: EPPO A1 list, no. 23; EU Annex designation I/AI.

Detection
Symptoms
T. indica is a floret-infecting fungal smut pathogen. Unlike systemic smuts, not all the seeds on an ear are usually infected. Seeds are infected through the germinal end of the grain 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). Seeds are usually only partially colonized, showing various degrees of infection. Point infections are most common, but infection may also spread down the adaxial groove and, in severe cases, the whole grain may appear bunted (Web Fig. 1).

Sampling
Seed lots should be sampled according to current ISTA rules. Grain, e.g. for feed or processing, is typically more difficult to sample because consignments are usually very large, and transported or stored as large, loose bulks. However, for monitoring purposes, grain should be sampled in an appropriate fashion to produce a 1–2 kg thoroughly mixed sample representative of the consignment.

For phytosanitary purposes, detection of T. indica is best achieved by a wash test (CABI/EPPO, 1997); infected parts of the grain typically disintegrate so that the teliospores contaminate other grains in the lot. The most efficient and rapid wash test method for detecting teliospores in a sample is a size-selective sieving and centrifugation technique (Appendix 1; Peterson et al., 2000). This method has, on average, an 82% efficiency of recovery and microscopic examinations typically require only a few slides per 50 g subsample. The number of replicate 50 g subsamples needed to detect differing levels of contamination is given in Table 1.

Direct visual examinations for bunted kernels or teliospores contaminating seed surfaces are not considered reliable methods for phytosanitary purposes. However, Karnal bunt may be detected by visual examination with the naked eye and low power microscopy (× 10–× 70 magnification). To help visualize symptoms, seed can be soaked in 0.2% NaOH for 24 h at 20 °C. This is especially useful for chemically treated seed lots where coloured dyes may obscure symptoms (Mathur & Cunfer, 1993; Agarwal & Mathur, 1992). With severe contamination, teliospores may be seen on the surface of seeds (Mathur & Cunfer, 1993).

¹The Figures in this Standard marked ‘Web Fig.’ are published on the EPPO website www.eppo.org.
Table 1  Number of replicate 50 g subsamples needed to detect differing levels of contamination with specified confidences, assuming an equal distribution of teliospores (Peterson et al., 2000; Inman & Bowyer, EU SMT4-CT98-2252 evaluation, 2000)

<table>
<thead>
<tr>
<th>Contamination level (no. of spores per 50 g sample)</th>
<th>99%</th>
<th>99.9%</th>
<th>99.99%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Identification

Isolation

*T. indica* is a facultative biotroph. To produce cultures, teliospores are soaked in water, quickly surface-sterilized and then germinated on water-agar plates (Appendix 4). After 7–14 days, non-dormant teliospores produce a promycelium bearing 32–128 or more basidiospores (primary sporidia) at its tip. These basidiospores can then be cultured directly on solid or liquid nutrient media.

Morphology

Teliospores globose to subglobose, sometimes with a small hyphal fragment (more common on immature teliospores, but occasionally on mature teliospores), mostly 22–47 µm in diameter, occasionally larger (mean 35–41 µm); pale orange to brown to dark, reddish brown; some teliospores black and opaque (Web Fig. 3); densely ornamented with sharply pointed to truncate spines, occasionally with curved tips, 1.5–5.0 µm high, which in surface view appear as either individual spines (densely echinulate) or as closely spaced, narrow ridges (finely cerebriform) (Web Fig. 3); the spines are covered by a thin hyaline membrane. Sterile cells: globose, subglobose to lacrymiform (tear-shaped), yellowish brown, 10–28 × 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. See also CMI (1983), Table 2, Web Fig. 3, Appendix (3)².

Diagnostic scheme

The diagnostic scheme for *T. indica*, as presented in Fig. 2, describes procedures for detection of teliospores in imported seeds or grain of wheat by a size-selective sieving wash test; morphological identification of teliospores detected in wash tests; isolation and germination of teliospores for molecular confirmation and molecular confirmation of cultures.

Confirmation

Morphological confirmation

If only a few teliospores are present (< 10 teliospores) in a wash test, morphological characters are not considered totally reliable for confident discrimination between *T. indica* and the morphologically similar species that are known contaminants of wheat grain, e.g. *T. walkeri* and *T. horrida* (Table 2; Web Figs 3–5). However, if large numbers of teliospores are present (> 10 spores) then morphological identification may be possible (Appendix 3). The most important discriminatory characters are teliospore size (maximum size and mean), exospore ornamentation and colour (Table 2; Web Figs 3–5; Appendix 3). However, molecular confirmation tests are still recommended.

When suspect teliospores are found in a wash test, the grains in both the washed subsample(s) and the larger submitted sample should be examined for Karnal bunt symptoms. If symptoms are found, these should be confirmed by microscopic examination of the teliospores. Any grass seeds found in the sample should also be examined for signs of bunt infection and, if found, the associated teliospores should be examined microscopically. Molecular confirmation tests are also recommended.

Molecular confirmation

There are three main molecular methods available to confirm presumptive morphological diagnoses: (1) restriction enzyme analysis of the ITS1 region (rDNA Internally Transcribed Spacer Region 1) after PCR application using universal ITS primers; (2) conventional PCR (polymerase chain reaction) assay using species specific primers; (3) PCR assay using species specific primers and a fluorescent probe in a TaqMan system. All these molecular confirmation methods require that teliospores are germinated and cultures produced from the resulting sporidia (Appendix 4) Detailed descriptions of these methods are still in preparation. Although PCR has been used on ungerminated teliospores, this typically requires large numbers of teliospores and even then negative results are considered unreliable (Smith et al., 1996; McDonald et al., 1999).

Possible confusion with similar species

Morphological comparisons

Other tuberculate-spored *Tilletia* species may be confused with *T. indica* (Durán & Fischer, 1961; Durán, 1987). In particularly, the morphologically and genetically similar fungus *Tilletia walkeri* (ryegrass bunt), and also *Tilletia horrida* (rice smut), are known contaminants of wheat seed or grain (Cunfer & Castlebury, 1999; Castlebury & Carris, 1999; Smith et al., 1996). The most important morphological characters that

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²The mounting medium, and heating or warming treatments, can affect teliospore size (Aggarwal et al., 1990; Khanna & Payak, 1968; Castlebury & Carris, 1999). This protocol assumes that spores are mounted in water and not warmed or heated; suspect spores can then be germinated for any subsequent PCR confirmation. However, surface ornamentation can sometimes not be seen clearly in water. In such cases, mounting teliospores in lactoglycerol or Shear’s solution (Mathur & Cunfer, 1993) and gently heating the slides may improve clarity.
discriminate *T. indica*, *T. walkerii* and *T. horrida* are teliospore size (range and mean), exospore ornamentation and colour (Table 2; Web Figs 3–5; Appendix 3). If sufficient numbers of teliospores are present, *T. horrida* teliospores are principally distinguished from *T. indica* by their smaller size, chestnut-brown colour and spines that are frequently curved and that appear as polygonal scales in surface view. *T. walkerii* and *T. indica* have a larger degree of overlap in morphological characters. However, *T. walkerii* teliospores are on average smaller, paler in colour (never black/opaque) and have coarser exospore ornamentation which in surface view gives the appearance of wide, incompletely cerebriform ridges or thick
Table 2  Morphological characteristics of Tilletia indica, Tilletia walkeri and Tilletia horrida

<table>
<thead>
<tr>
<th>Teliospore character</th>
<th>Tilletia indica</th>
<th>Tilletia walkeri</th>
<th>Tilletia horrida</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (range) (\mu m)</td>
<td>22–47 (61)(26–55 (64))</td>
<td>(23–45</td>
<td>17–36 (20–38 (41))</td>
</tr>
<tr>
<td>Size (mean) (\mu m)</td>
<td>35–41</td>
<td>30–31</td>
<td>24–28</td>
</tr>
<tr>
<td>Colour</td>
<td>Pale orange to mainly dark red</td>
<td>Pale yellow to mainly dark red</td>
<td>Pale yellow to mainly light or dark chestnut brown (semiopaque)</td>
</tr>
<tr>
<td>Exospore ornamentation in median view</td>
<td>Spines (occasionally curved), 1.5–5.0 (\mu m) high, covered with a hyaline sheath</td>
<td>Conical to truncate spines, 3–6 (\mu m) high, covered with a hyaline to yellowish-brown sheath</td>
<td>Spines appearing as polygonal scales with maturity, covered with a hyaline to tinted sheath†</td>
</tr>
<tr>
<td>Exospore ornamentation in surface view</td>
<td>Spines densely arranged, either individually (densely echinulate) or in closely spaced, narrow ridges (finely cerebriform)</td>
<td>Spines coarsely arranged, forming wide, incompletely cerebriform (to coralloid) ridges or thick clumps</td>
<td>—</td>
</tr>
</tbody>
</table>

1Authors’ data.
2Based on: Castlebury & Carris (1999); Cunfer & Castlebury (1999); Milbrath et al. (1998); Castlebury (1998).
3As T. barclayana. Castlebury & Carris (1999); CMI Description no. 75 (1965); Durán (1987); Durán & Fischer (1961) Or as T. horrida: Aggarwal et al. (1990); Khanna & Payak (1968); Castlebury (1998).
†Castlebury & Carris (1999) report larger spore sizes (in brackets) for teliospores warmed overnight at 45 °C in Shear’s solution; Castlebury (1998) also reports larger teliospore sizes.
*Milbrath et al. (1998), supported by Author’s data from teliospores ex. Lolium (two isolates ex. Oregon, USA) in water.
‡Author’s data from teliospores ex. Oryza (California, USA; Arkansas, USA) in water; though not reported in the literature, some spores may have ridges in addition to individual spines (see Web Fig. 5).

Molecular comparisons

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) methods, including RAPDs, RFLPs and AFLPs (Pimentel et al., 1998; Laroche 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., 1998). However, within the ITS1 region, T. walkeri has a diagnostically important restriction enzyme site (ScaI) that is not present with T. indica, T. horrida or other closely related species (Pimentel et al., 1998; Levy et al., 1998). With mtDNA, sequence differences have enabled species-specific primers to be designed to T. indica and T. walkери (Frederick et al., 2000). These primers can be used in conventional PCR assays or in a TaqMan system in conjunction with a probe (Frederick et al., 2000). There are currently no species-specific primers for T. horrida, but RFLPs can be used to identify cultures (Pimentel et al., 1998). If species-specific primers for T. walkeri and T. indica do not give positive results on test cultures, RFLPs, RAPDs or AFLPs may be useful tools in identification (Pimentel et al., 1998).

Requirements for a positive diagnosis

The procedures for detection and identification described in this protocol, and the decision scheme in Fig. 2, should have been followed.
If bunted grains are present, a positive diagnosis can be made if the symptoms are confirmed by the presence of teliospores that are morphologically consistent with those described for *T. indica* in this protocol (Table 2; Appendix 3).

If bunted grains are not present, the wash test and procedures for detection described in this protocol should be followed. Since there is considerable overlap in morphological characters between *T. indica* and various other tuberculate-spored species which can potentially contaminate seed or grain, namely *T. walkeri* and *T. horrida*, morphological identification of teliospores is only possible when a large number of teliospores are present (> 10 spores). In such cases, teliospores may be identified morphologically, but only if all key characters conform to one specific species (size range, size mean, colour and exospore ornamentation patterns: (see Appendix 3). Molecular confirmation is still recommended.

If too few spores are present (<10 spores), it may not be possible to discriminate species using morphological characters. In such cases, molecular confirmation by PCR using species – specific primers combined as appropriate with restriction – enzyme analysis is recommended for a positive diagnosis.

**Report on the diagnosis**

A report on the diagnosis should include the following information:

- results obtained by the recommended procedures
- the origin (country; state) and type (host; commodity) of the infected material
- quantity of consignment or lot
- degree of infection/contamination (e.g. the number of positive subsamples and the estimated number of teliospores detected in each positive subsample)
- a description of any disease symptoms (preferably including photographs)
- a description of the teliospores (with colour photographs if possible) compared with descriptions of *T. indica* and morphologically similar species, e.g. *T. walkeri* and *T. horrida*
- if cultures were obtained for molecular confirmation, a description of the colony morphology, especially any pigmentation, and growth rate under defined conditions
- Cultures should be kept (mycelium from broths or mycelial plugs from agar plates can be stored frozen at −80 °C) if molecular confirmation tests were done, a copy of the molecular test result along with positive and negative controls should be provided. For TaqMan assays, the CT-value should be noted
- comments as appropriate on the certainty or uncertainty of the identification.

When cultures have been obtained, these should be cultured onto potato dextrose agar (PDA) and 5-mm diameter mycelial plugs stored at −80 °C in sterile tubes. Similarly, any DNA extractions should be stored at −80 °C.

**Further information**

Further information on this organism can be obtained from: Pest and Disease Identification Team, Central Science Laboratory, Sand Hutton, York YO41 1LZ, United Kingdom.

**Acknowledgements**

This protocol was originally drafted by: A. J. Inman, K. J. D. Hughes and R. J. Bowyer, Central Science Laboratory, York (GB).

This protocol was ring-tested in different European laboratories.

**References**


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; 1.6% active NaOCl); wash water = 0.01% aqueous Tween-20 (detergent); large weigh boats (8 × 8 cm); weighing balance; 250-ml Erlenmeyer glass flask; 100-ml measuring cylinder; Parafilm® or clingfilm; laboratory flask shaker (alternatively, shaking flasks by hand is acceptable); 500-ml Erlenmeyer 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 (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% bleach6. Rinse the bleach thoroughly from the equipment with tap water. Weigh 50 g of grain into a new, disposable, large weigh boat (see Fig. 2, 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 (Web Fig. 8). 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 rev min⁻¹ 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 nylon sieve (11 cm diameter) in a funnel over a clean 500-ml Erlenmeyer flask (Web Fig. 9) then pour the whole contents of the flask (the grain and the wash water) into the sieve (Web Fig. 10). Rinse the 250-ml flask with 20–50 mL of distilled water from an aspirator bottle, then pour evenly over the grain on the 53-µm sieve. Repeat this rinse twice more. Thoroughly rinse the grain on the 53-µm sieve by washing with further distilled water from an aspirator bottle (Web Fig. 10) 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, 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; Web Fig. 9) 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 to an angle of 30–45° (Web Fig. 11) and gently wash the deposit on the

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6 Shear’s solution: 300 mL Mc Ilvaine’s buffer 6 mL Potassium acetate, 120 mL Glycerine 180 mL Ethyl alcohol (95%). Prepare Mc Ilvaine’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 (Na₂HPO₄) 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.
membrane and on the sieve walls on to one side of the membrane using distilled 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 (Web Fig. 12) and place the suspension in a new 15-mL disposable conical centrifuge tube. 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 3–5 mL in the centrifuge tube). If necessary, the 20-µm sieve can be examined under a low power microscope to check for any residual teliospores.

Centrifuge the collected suspension at 1000 g for 3 min. 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.

Pipette a 20-µL aliquot of the suspension onto a microscope slide and place a cover slip (18 × 18 mm) on top. Examine the whole slide immediately (the slide can quickly dry out) for teliospores of T. indica (Web Fig. 13) 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: Web Figs 2 and 3, Tables 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 (Appendix 3) and the general Diagnostic Scheme (Fig. 2), i.e. record morphological characters (e.g. size, colour, ornamentation); examine the sample for bunted seeds; isolate & germinate teliospores for molecular confirmation, if required.

Finally, bleach all equipment used and rinse with water before re-using (see Steps 1 & 2).

### 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

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7 Conical-bottomed tubes are recommended, as are centrifuges with swing-out arms rather than fixed arms, as 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.

8 If warm laboratory conditions cause water preparations to dry out quickly, then Shear’s solution, or just a gycerol 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.

Table 3 Example of a record sheet, with suggested colour and ornamentation codes, that could be used for teliospores detected in wash tests

<table>
<thead>
<tr>
<th>Teliospore number</th>
<th>Size (µm)</th>
<th>Colour</th>
<th>Ornamentation</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
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<td>8</td>
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<td></td>
<td></td>
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<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Examples of colour codes: BO black/opaque, RB 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 cerebriform (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).

(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 ring-tested.

### 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 Web Figs 3, 4, 5 and 13 and Table 2). Follow the Decision Scheme (Web Fig. 15) and examine the submitted grain sample (including the 50 g washed subsamples) for bunted wheat seeds or bunted seeds of other Poaceae, e.g. ryegrass seed (see Fig. 2). If wheat seeds with Karnal bunt symptoms are found, confirm Tilletia indica by microscopic examination of the teliospores in the seed (Fig. 2, Table 2; Table 4). If confirmed, compare the teliospores in the seed (Table 4). If confirmed, compare table 3.

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9 Tuberculate teliospores detected in wash tests of wheat grain are assumed to be either Tilletia 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. 2.
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 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 less than 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
- **samples with mature, dark teliospores less than 25 µm** are most likely to be *T. horrida*, not *T. walkeri* or *T. indica*
- **samples with some black, opaque teliospores** are most probably *T. indica* (*T. walkeri* teliospores are never opaque, black; *T. horrida* teliospores can be dark, semiopaque).

If relatively large numbers of teliospores are present (e.g. over 10 spores), it may be possible to identify the teliospores morphologically if all morphological criteria (size range; mean size; colour; ornamentation) clearly conform to any one species (Table 4). However, molecular confirmation is still recommended if bunted wheat seeds were not found in the grain sample.

If only a few teliospores are detected (e.g. less than 10), or morphological characters are not conclusive, then molecular methods are recommended for confirmation of any presumptive diagnosis (see Appendices IV–VII).

**Appendix 4. Method for isolation and germination of teliospores for molecular confirmation**

**Materials**

Aspirator bottle with distilled water; 20-µm mesh nylon sieve (4 cm diameter); sterile disposable Pasteur pipettes; pipettor (1000-µL capacity) and sterile, disposable pipettor tips (plugged); pipettor (200-µL capacity) and sterile, disposable pipettor tips (plugged); sterile, disposable 3-µL Pasteur pipettes; 15-µL disposable conical bottom centrifuge tubes; centrifuge (to take 15-µL centrifuge tubes); autoclavable disposable waste bottle; autoclave bags; 10% Bleach solution (1 part domestic bleach: 9 parts water; c. 0.3–0.5% active NaOCl); sterile distilled water; sterile disposable spreader; antibiotic

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

<table>
<thead>
<tr>
<th>Sample Size (µm)</th>
<th>Mean Size (diam., µm)</th>
<th>Colour</th>
<th>Spines (ornamentation) in surface view</th>
<th>Median Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 36</td>
<td>36–45</td>
<td>Pale yellow to mostly light or dark chestnut-brown (to semi-opaque)</td>
<td>Echinulate; polygonal scales (to echinulate); occasionally conical to truncate (gaps between spines obvious in profile after bleaching)</td>
<td></td>
</tr>
<tr>
<td>36–41</td>
<td>45–50+</td>
<td>Pale orange, but mostly reddish-brown (never opaque)</td>
<td>Coarse; broad, incompletely cerebriform ridges (to thick clumps)</td>
<td></td>
</tr>
<tr>
<td>30–31</td>
<td>50+</td>
<td>Pale yellow to mostly dark red-brown (to opaque black)</td>
<td>Dense; echinulate or closely spaced narrow ridges (to broad, incompletely cerebriform)</td>
<td></td>
</tr>
<tr>
<td>35–41</td>
<td>45–50+</td>
<td>Pale yellow to mostly dark reddish-brown (to black)</td>
<td>Coarse; broad, incompletely cerebriform ridges (to thick clumps)</td>
<td></td>
</tr>
</tbody>
</table>

**Materials**

Aspirator bottle with distilled water; 20-µm mesh nylon sieve (4 cm diameter); sterile disposable Pasteur pipettes; pipettor (1000-µL capacity) and sterile, disposable pipettor tips (plugged); pipettor (200-µL capacity) and sterile, disposable pipettor tips (plugged); sterile, disposable 3-µL Pasteur pipettes; 15-µL disposable conical bottom centrifuge tubes; centrifuge (to take 15-µL centrifuge tubes); autoclavable disposable waste bottle; autoclave bags; 10% Bleach solution (1 part domestic bleach: 9 parts water; c. 0.3–0.5% active NaOCl); sterile distilled water; sterile disposable spreader; antibiotic

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**Acidified electrolysed water (AEW) with the following properties can be used instead of 10% bleach (Bonde et al., 1999): pH 2.3–2.8; redox potential (ORP) c. 1100 mv; free chlorine (5)–10–15 (ppm). Equipment: Super Oxseed Labo, Advanced H2O Inc., Alameda, US.**
water agar (AWA) plates (2% Technical agar No. 3, Oxoid; 60 mg penicillin-G (Na salt) and 200 mg streptomycin sulphate per L of agar); Parafilm M or electrical tape; scalpel; potato dextrose broth (Difco); dissecting needle; sterile filter paper; 1.5–2.0 mL microcentrifuge tubes

**Method**

Recover suspect teliospores from both the microscope slide and the cover slip by washing them with distilled water over a clean 20-µm sieve. Recover the teliospores from the sieve (Appendix 1), pipetting the suspension into a new 15-mL conical centrifuge tube. Make up the final volume to 3–5 mL with distilled water. Incubate the teliospore suspension overnight at 21 °C to hydrate the teliospores and make fungal and bacterial contaminants more susceptible to subsequent surface sterilization. After incubating overnight, pellet the teliospores by centrifuging at 1200 g for 3 min. Remove the supernatant with a pipettor with a plugged tip, or a disposable Pasteur pipette, taking care not to disturb the pellet; pipette the supernatant into a suitable disposable waste bottle for subsequent quarantine disposal. Resuspend the pellet in 5 mL of 10% domestic bleach11 (0.3–0.5% active NaOCl); replace the centrifuge tube cap and immediately invert the tube three time to ensure that the bleach contacts all inner surfaces. Immediately centrifuge at 1200 g for 1 min, then quickly and aseptically remove the supernatant and resuspend the pellet in 1 mL sterile distilled water (SDW) to wash the pellet. Centrifuge at 1200 g for 5 min, aseptically remove the supernatant and then add another 1 mL SDW to wash the pellet again. Centrifuge at 1200 g for 5 min, aseptically remove the supernatant and resuspend the final pellet in 1 mL SDW.

Transfer 200 µL of the teliospore suspension aseptically onto individual 2% water agar plates12 with antibiotics (AWA) and spread with a sterile spreader. Incubate the AWA plates at 21 °C with a 12-h light cycle (e.g. TLD 18 W/83 Philips white light tubes). Leave for about 5 days before sealing plates with parafilm or electrical tape, or placing the plates inside clear polyethylene bags. After 7–14 days, examine the plates for germinated teliospores bearing a tuft of filiform basidiospores (primary sporidia), or small colonies forming around germinated teliospores (Web Fig. 16). These colonies produce secondary sporidia typically of two types: filiform and allantoid. Cut out small blocks of agar (5 × 5–10 × 10 mm square) bearing germinated teliospores or colonies; invert the agar blocks and stick them onto the inside surface of a Petri dish lid. Place the lids over Petri dish bases containing approximately 5 mL potato dextrose broth so that sporidia can then be released onto the broth surface. Incubate at 21 °C with 12 h light cycle. After 2–3 days, basidiospores deposited onto the broth surface produce small mats of mycelia approximately 0.5–1.0 cm diameter. With a sterile dissecting needle, remove each mycelial mat, touching them onto sterile filter paper to remove excessive broth. Place the mycelia in suitable vials (e.g. 1.5–2.0 mL microcentrifuge tubes) for immediate DNA extraction, or store at −80 °C for subsequent DNA extraction.

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11 Instead of bleach, teliospores can be surface sterilized for 30 min in 5–10 mL of AEW (see above). AEW effectively surface sterilizes teliospores but, compared to a 1–2 min bleach treatment, stimulates rather than reduces teliospore germination (Bonde et al., 1999).

NB. Some teliospores can be killed if the total time in the bleach exceeds 2 min.

12 3-day-old plates are recommended, as these quickly absorb the suspension; excessive surface water can inhibit teliospore germination. Alternatively, prepare the agar plates on the day of use, but pour the liquid agar when cool and do not replace the lids fully until the agar has set.
Fig. 1. Grain infected with *Tilletia indica* (Karnal bunt). Symptoms range from partial bunting (point infections and infections spreading down the adaxial groove) to almost complete bunting. Photograph courtesy of G. L. Peterson, USDA.
Fig. 3. Teliospores of *Tilletia indica* (Karnal bunt of wheat) showing surface ornamentation patterns: spines densely arranged, either individually (densely echinulate) or in closely spaced, narrow ridges (finely cerebriform). (Figures 3–5 are at the same scale; 10 mm = 17 µm.)
Fig. 4. Teliospores of *Tilletia walkeri* (ryegrass bunt) showing surface ornamentation patterns: spines coarsely arranged and forming wide, incompletely cerebriform to coralloid ridges or thick clumps.
(Figures 3-5 are at the same scale; 10 mm = 17 µm.)
Fig. 5. Teliospores of *Tilletia horrida* (rice smut) showing surface ornamentation patterns: polygonal scales or, occasionally, with cerebriform ridges.
(Figures 3–5 are at the same scale; 10 mm = 17 µm.)
Fig. 6. Teliospores of *Tilletia indica* (top) and *T. walker* (bottom) showing teliospore profiles in median view after bleaching and then staining with lactoglycerol-trypsin blue. Note the smoother outline on *T. indica* teliospores compared to the more irregular outline of *T. walker* teliospores with more obvious gaps between spines.

*Tilletia indica*

*Tilletia walker*
Fig 7. Colonies of *Tilletia indica* (right), *T. walkeri* (centre) and *T. horrida* (left) after 7 days (top), 10 days (centre) and 14 days (bottom) on PDA at 19°C and a 12 hour dark/light cycle. Note slower growth, and purple pigmentation after 14 days, for *T. horrida* colonies.
**Fig. 8** 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. 9** 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. 10** 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.

![Image of a 53 µm sieve and an aspirator bottle](image1)

**Fig. 11** 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.

![Image of a Pasteur pipette and a funnel](image2)
**Fig. 12** 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.

**Fig. 13.** *Tilletia indica* teliospores in median view (20–50 µm diam.; mean 35–41 µm).
Fig. 15. Pictorial key to teliospore ornamentation (see also Figs 3–5).

**Tilletia indica**
- Densely echinulate (DE)
- Densely echinulate (DE) to finely cerebriform (FC: closely spaced, narrow ridges)

**Tilletia walkeri**
- Coarsely cerebriform (CO): Coarsely arranged spines forming wide incompletely cerebriform ridges
- Incompletely cerebriform to coralloid ridges (CC)
- Thick clumps (TC)

**Tilletia horrida**
- Spines as polygonal scales (PS), sometimes forming ridges or clumps (left and centre); Sharply pointed, often curved spines (right)
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; allantoid sporidia which are forcibly discharged.