This diagnostic protocol was adopted by the Standards Committee on behalf of the Commission on Phytosanitary Measures in January 2014.

The annex is a prescriptive part of ISPM 27:2006.

ISPM 27
Annex 4

INTERNATIONAL STANDARDS FOR PHYTOSANITARY MEASURES

ISPM 27 DIAGNOSTIC PROTOCOLS

DP 4: *Tilletia indica* Mitra

(2014)

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1. Pest Information

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 (USDA, 2007; Wiese, 1987). It has also been detected in certain areas of the United States and Mexico, and in South Africa (Crous *et al.*, 2001; Fuentes-Davila, 1996).

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). *T. 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).

T. 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 (Figure 1). Seeds are usually only partially colonized, showing varying degrees of infection (Figure 2). 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.

T. 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).

There are other *Tilletia* species that can be confused with *T. indica* and are commonly found in harvested grain or seeds. These include *Tilletia walkeri* (a pathogen of *Lolium perenne* and *Lolium multiflorum*), *T. horrida* (a pathogen of *Oryza* spp.) and *T. ehrhartae* (a pathogen of *Ehrharta calycina*). In Australia, *T. walkeri* and *T. ehrhartae* are found to contaminate harvested seed of *Triticum aestivum*. *T. 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 and Carris, 1999; Pascoe *et al.*, 2005). Because of the morphological similarity of these pathogens, accurate identification is important.

2. Taxonomic Information

Name: *Tilletia indica* Mitra, 1931

Synonyms: *Neovossia indica* (Mitra) Mundkur, 1941

Taxonomic position: Eukaryota, Fungi, Basidiomycota, Ustilaginomycotina, Exobasidiomycetes, Exobasidiomycetidae, Tilletiales, Tilletiaceae

Common name: Karnal bunt or partial bunt

Reference: MycoBank 267835

3. Detection

The diagnostic scheme for *T. indica*, as presented in Figure 3, describes procedures for the detection of teliospores in seeds or grain of host plants. Seeds or grain samples are visually examined for the presence of bunted kernels (section 3.1). If a bunted kernel is detected, teliospores can be removed and *T. indica* can be identified by morphology (section 4.1).

If no bunted kernels are detected in the sample, the sample may be tested for the presence of teliospores by using a size-selective sieve wash test on three subsamples (section 3.2). 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, the number of teliospores 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. If further confirmation is required, the next step is *either* isolation of the teliospores and germination (section 4.2.1) followed by the molecular protocols described in sections 4.3.1–4.3.3 *or* removal of individual teliospores (section 4.2.3) followed by a direct real-time polymerase chain reaction (PCR) on the individual teliospores (section 4.3.4). (Refer to A, B and C in Figure 3.)
- 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. The detection limit may or may not be the same as the regulatory limit.

In this diagnostic protocol, methods (including reference to brand names) are described as published, as these defined the original level of sensitivity, specificity and/or reproducibility achieved.

3.1 Examination of seeds/grain

Direct visual examination either for bunted kernels or for teliospores contaminating seed or grain surfaces is not considered 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). This protocol is based on the examination of a large sample of seeds or grain; the whole sample needs to be examined for bunted kernels (Figure 2) or other Poaceae seeds (for example *Lolium* spp.). The symptoms observed and the presence of the other Poaceae seeds is recorded.

If bunted kernels are present, a positive diagnosis can be made based on the morphology of the teliospores. Microscope slides of the teliospore dust must be made and the morphology of these teliospores described. If the morphology of the teliospores is consistent with that of *T. indica* (refer to section 4.1 and Figures 4–8) a positive diagnosis can be made.

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 infection stand out in stark contrast. This process is especially useful for chemically treated seed lots where coloured dyes may obscure symptoms (Agarwal and Mathur, 1992; Mathur and Cunfer, 1993). With severe infestation and contamination, teliospores may be seen on the surface of seeds (Mathur and Cunfer, 1993).

In the absence of bunted kernels the size-selective sieve wash test (section 3.2) may be used to determine whether *T. indica* is present or not present in the sample. Alternatively, in the absence of bunted kernels *T. indica* may be considered not to be present. If seed of *Lolium* spp. is found contaminating the sample there is a high probability that *T. walkeri* will be detected in the sample.

3.2 Extraction of teliospores from seeds/grain, size-selective sieve wash test

The size-selective sieve wash test 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 is 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. The method is described below and further details are available from Inman *et al.* (2003), Peterson *et al.* (2000) and Wright *et al.* (2003). The detection limit may or may not be the same as the regulatory limit.

It is important that all equipment is soaked before use for 15 min in a bleach solution (1.6% sodium hypochlorite (NaOCl) active ingredient) to eliminate the risk of false positives by cross-contamination from previous samples. Bleach kills teliospores and makes them appear hyaline compared with their normally dark, pigmented appearance. All equipment is rinsed with tap water after soaking.

The 50 g sample of untreated seed is placed in an Erlenmeyer flask (250 ml) with 100 ml 0.01% Tween 20 aqueous solution. The sample is placed on a shaker for 3 min at 200 r.p.m. to release the teliospores, then it is poured onto a 53 µm sieve sitting on top of a 20 µm sieve, which is sitting inside a funnel on top of another flask (500 ml). The flask that contained the sample is then rinsed twice with approximately 50 ml sterile tap water each time: the rinsing water is poured over the sample sitting in the sieve. The sample is further washed with sterile tap water (200–300 ml) using an aspirator bottle to ensure good removal of the teliospores from the seed. The sample and the 53 µm sieve are removed. The 20 µm sieve is tilted to a 45° angle and, using an aspirator bottle filled with sterile tap water, the debris is washed on the sieve from the top to the bottom with a sidewards sweeping motion, going backwards and forwards. This process washes all teliospores recovered from the sample into the lower part of the sieve. The teliospores and debris are then washed into a 15 ml 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. These steps are repeated until the 20 µm sieve appears clean. The final volume in the tube will be approximately 8 ml. If necessary, the 20 µm sieve can be examined under a low power microscope to check for residual teliospores.

The collected suspension is centrifuged at 1000 *g* for 3 min to collect the teliospores, as they are denser than most of the debris collected during the wash test. The equation for calculating the relative centrifugal force (RCF (*g*)) from r.p.m. is RCF = 1.12 r_{max} (r.p.m./100)^2, where r_{max} is the maximum radius (mm) from the centre of rotation to the bottom of the centrifuge tube. The supernatant is carefully removed, without disturbing the pellet, using a new disposable Pasteur pipette. The pellet can then be examined under the microscope. If the pellet is too thick, water can be added to dilute the suspension, and the pellet stirred with a pipette tip to ensure an even suspension is obtained, before examination under the microscope.

The whole pellet suspension is placed in 2 µl lots onto a microscope slide and covered with a coverslip. The slides are examined using bright field microscopy at 20–40× magnification. It is important to examine every square millimetre of the suspension on the slide for the presence of teliospores. If teliospores are found, the morphological characteristics (e.g. size, colour and ornamentation) and the number of teliospores found on each slide are recorded.

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)

	No. replicate samples required for detection according to level of confidence (%)		
Contamination (no. teliospores per 50 g sample)	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 techniques (see Figure 3).

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 could 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 (sections 4.3.1–4.3.4) 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

T. 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 black and opaque (Figures 4 and 5); 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) (Figures 4 and 5); the spines are covered by a thin hyaline membrane (Carris *et al.*, 2006; CMI, 1983).

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 (Carris *et al.*, 2006; CMI, 1983).

If 10 or more teliospores are present in a sieve wash test, then the morphological identification can be confirmed. If fewer than 10 teliospores are detected, morphological characteristics are not considered completely reliable for confident identification (EPPO, 2007). In this case it is recommended that the sample is resampled by preparing new subsamples from the original 1 kg and tested.

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; Figures 4–8). Published reports often vary on spore size. The spore size is affected by the mounting medium and by heating treatments. Ascoe *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 and genetically similar fungus *T. walkeri* and also *T. horrida* are known contaminants of harvested *Triticum aestivum* (Castlebury and Carris, 1999; Cunfer and Castlebury, 1999; Smith *et al.*, 1996). In addition to the *Tilletia* species mentioned in Table 2, other tuberculate-spored *Tilletia* species may be confused with *T. indica* (Durán, 1987; Durán and Fischer, 1961; Pimentel *et al.*, 1998). These species are less likely to be found as contaminants of *Triticum aestivum.* They include *Tilletia barclayana sensu lato* (bunt of various Poaceae, e.g. *Panicum* and *Paspalum*), *Tilletia eragrostidis* (on *Eragrostis*), *Tilletia inolens* (on *Lachnagrostis filiformis*), *Tilletia rugispora* (on *Paspalum*) and *Tilletia bouteloua*e (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 necessary, teliospores can then be rinsed twice in water and stained, for example with trypan blue or cotton blue in lactoglycerol (Figure 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 (section 3.2). There is the standard procedure of recovering the teliospores from the slide and inducing their germination (section 4.2.1) 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 4.2.3).

4.2.1 Germination of teliospores

T. indica is a facultative biotroph. To produce cultures, teliospores are soaked in water, quickly surface-sterilized and then germinated on water agar plates.

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 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 acidified electrolyzed water (AEW). AEW effectively surface-sterilizes teliospores but, compared with a 1–2 min bleach treatment, stimulates rather than reduces teliospore germination (Lonsde *et al.*, 1999). The teliospores are then washed twice by removing the supernatant, resuspending the pellet in 1 ml sterile distilled water (SDW) and centrifuging at 1200 *g* for 5 min.

The pellet is resuspended in 1 ml SDW and 200 µl of the teliospore suspension is placed aseptically onto 2% water agar with antibiotics (WA+A) plates 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. These colonies produce secondary sporidia typically of two types: filiform and allantoid. These can then be cultured directly on solid media (Figure 9) or liquid nutrient media 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 broth. This allows the sporidia to be released onto the broth surface. The dishes are incubated at 21 °C with a 12 h light cycle. After 2–3 days, basidiospores deposited onto the broth 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 fungicides 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 effect germination (Carris *et al.*, 2006). This can be resolved by carrying out direct real-time PCR on individual teliospores (see section 4.3.4).

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[a]	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[b]	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[c]	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[d]	17–25	no data	Very dark chestnut brown when mature. Can be opaque because of masking 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*

Notes: [a]Based on Inman *et al.* (2003). [b]Based on Castlebury, 1998; Milbrath *et al.,* 1998; Castlebury and Carris, 1999; Cunfer and Castlebury, 1999. [c]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. [d]Pascoe *et al.,* 2005.

4.2.2 Germination of similar *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 (Figure 9). In contrast, comparable cultures of *T. horrida* grow significantly more slowly (colonies only 2–3 mm in diameter) because of their higher optimal temperature. *T. horrida* isolates may also produce a reddish purple pigment (Figure 9), both on PDA and potato dextrose broth.

4.2.3 Recovery of single teliospores

After the teliospores are examined and their morphology is 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 a single piece of a coverslip obtained by cutting into tiny pieces (1 × 1 mm^2) is placed that has been sterilized (autoclaved at 121 °C for 15 min or baked at 170 °C for 2 h). A 2 µl drop of Tris-ethylenediaminetetraacetic acid (EDTA) (TE) buffer is placed on 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 as described in section 4.3.4.

4.3 Molecular identification

There are a number of molecular methods available for the identification of *T. indica*. Any of the methods described below may be used, however, it is essential that reference material (positive controls) has been obtained from experts in the area (refer to section 6).

The first three protocols described below work well but rely upon germination of the teliospores so that sufficient DNA can be extracted from the mycelial mat produced. Germination of the teliospores can take up to three weeks. Peterson *et al.* (2000) found the average teliospore germination rate to be 55%, which severely reduces the chances of identifying the teliospores by these three molecular methods. A fourth molecular protocol is then described that does not rely upon germination of the teliospores.

Diagnostically significant differences exist between *T. indica*, *T. walkeri* and *T. horrida* in their nuclear and mitochondrial (mt) DNA. Interspecific polymorphisms have been identified using various PCR methods, including random amplification of polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) (Laroche *et al.*, 1998; Pimentel *et al.*, 1998). In the nuclear ribosomal (r) DNA internal transcribed spacer (ITS) 1 and 2 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*1) that is not present in *T. indica*, *T. horrida* or other closely related species (Levy *et al.*, 2001; Pimentel *et al.*, 1998). mtDNA sequence differences have enabled species-specific primers to be designed for *T. indica* and *T. walkeri* (Frederick *et al.*, 2000). These primers can be used in conventional PCR assays, in a TaqMan® system in conjunction with a probe (Frederick *et al.*, 2000) or real-time multiplex assay with five probes (Tan *et al.* 2009).

4.3.1 Restriction enzyme analysis of the ITS1 region

The target gene region is the ITS region of the nuclear rRNA gene (Pimentel *et al*., 1998). The PCR amplicon produced includes both ITS1 and ITS2 and the conserved fragment 5.8S. This amplicon is approximately 670 base pairs (bp) including primer sequences. Oligonucleotides used for *T. indica*:

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).

DNA is extracted from mycelium. This is done either by grinding up the mycelium using a mortar and pestle or by placing approximately 0.1 g mycelium in a sterile 2 ml microcentrifuge tube one-third full with sterile 0.5 mm glass beads and 1 ml molecular grade water (MGW). The tube is sealed with a screw lid containing an o-ring and oscillated in a beadbeater or in a tissue lyser on quarter power for 5 min. The ground sample is allowed to stand for 30 s, then its DNA is extracted using a proprietary DNA extraction kit for fungi. No DNA cleanup is required. The extracted DNA is used immediately, kept overnight at 4 °C or stored at −20 °C for longer periods.

PCR to produce the restriction amplicon uses the following mastermix (concentration per 20 µl single reaction): 1× PCR buffer (containing 1.5 mM $MgCl_2$ (Applied Biosystems))[1], 0.2 mM of each dNTP, 1.25 µl AmpliTaq (5 U/µl) (Applied Biosystems), 0.5 µM each primer and 1 µl extracted DNA. PCR cycling parameters are: 94 °C denaturation for 2 min; 30 cycles of 94 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min; and a 72 °C extension step for 10 min.

Restriction of the PCR amplicon is done as follows. Restriction mix (concentration per 20 µl single reaction): 7.3 µl MGW, 2.0 µl restriction buffer (Promega)[2], 0.2 µl bovine serum albumin (10 µg/µl), 0.5 µl restriction enzyme (either *Taq*1 or *Sca*1 at 10 U/µl (Promega)) and 10.0 µl neat DNA amplicon solution as produced above (>50 ng/µl DNA). This mix is incubated for 3 h at 37 °C, and the reaction is gently mixed by inversion during incubation. Restricted products are stored at 4 °C before visualizing on a gel. When required, 10 µl reaction product is loaded with a suitable marker and run on a 2% gel.

The assay is positive for *T. indica* if amplified test samples are cut with restriction enzyme *Taq*1 to give five products (occurring at 60, 70, 110, 170 and 260 bp) and there is no cut with *Sca*1. A positive result for *T. walkeri* is obtained if amplified test samples are restricted with *Taq1* to give the same five fragments as with *T. indica*, but *Sca*1 restricts amplified products to give two fragments: at 140 bp and 520 bp. If the amplified product comes from *T. horrida*, *Taq*1 produces four DNA fragments (60, 110, 150 and 335 bp) and *Sca*1 produces no cuts. Other *Tilletia* species give different restriction patterns with these and other enzymes (Pimentel *et al*., 1998).

[1] The use of products of the brand Applied Biosystems in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[2] The use of products of the brand Promega in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

4.3.2 Conventional PCR assay using species-specific primers

This assay was designed by Frederick *et al*. (2000) using mtDNA[3], producing an amplicon of 414 bp. Oligonucleotides used for *T. indica*:

Forward primer Tin 3 (5′-CAA TGT TGG CGT GGC GC-3′)

Reverse primer Tin 4 (5′-CAA CTC CAG TGA TGG CTC CG-3′).

DNA is extracted from mycelium. This is done by grinding 0.5–1.0 g mycelium in a 1.5 ml microcentrifuge tube with 75 µl lysis buffer and then grinding further with a sterile pestle attached to a power drill. An additional 75 µl lysis buffer is added before extracting DNA using a proprietary DNA extraction kit for fungi. No DNA cleanup is required. Extracted DNA is used immediately, kept overnight at 4 °C or stored at −20°C for longer periods.

PCR for this assay uses the following mastermix (concentration per 25 µl single reaction): 1x PCR buffer (containing 10 mM Tris-HCl, 50 mM KCl (pH 8.3), 1.5 mM $MgCl_2$ and 0.001% w/v) gelatin); dATP, dGTP, dCTP and dTTP, each at a concentration of 0.1 µM; each primer at a concentration of 0.1 µM; 0.5 U of *AmpliTaq* DNA polymerase; and 1.0 µl extracted DNA obtained as described above.

PCR cycling parameters are: 94 °C denaturation for 1 min; 25 cycles of 94 °C for 15 s, 65 °C for 15 s and 72 °C for 15 s; and a 72 °C extension step for 6 min.

As required, 10 µl reaction product is loaded with a suitable marker and run on a 2% agarose gel.

When testing for *T. walkeri*, the Tin 3 primer is replaced with 0.1 µl forward primer Tin 11 (5′-TAA TGT TGG CGT GGC AT-3′) (25 µM). This produces an amplicon of 414 bp.

Positive reactions produce a single amplicon of 414 bp for both *T. indica* (primers Tin 3/Tin 4) and *T. walkeri* (primers Tin 11/Tin 4). If the *T. walkeri*- and *T. indica*-specific primers do not produce positive results for the test samples (but 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 (section 4.3.1).

Alternatively, no amplification can result from poor quality DNA. This can be checked by testing extracts with the universal primers (ITS1 and ITS4) described in section 4.3.1. If the samples contain good quality DNA 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 on an agarose gel. If amplification is still not produced, fresh DNA should be extracted and retested.

4.3.3 PCR assay using species-specific primers and a fluorescent probe

This assay was designed by Frederick *et al.* (2000) using genomic DNA, producing an amplicon of 212 bp. Oligonucleotides used for *T. indica*:

Forward primer Tin 3 (5′-CAA TGT TGG CGT GGC GC-3′)

Reverse primer Tin 10 (5′-AGCTCCGCCTCAAGTTCCTC-3′)

RT probe: TaqMan® probe (10 µM) (Applied Biosystems): 5′-(FAM label)-ATT CCC GGC TTC GGC GTC ACT-(TAMRA quencher)-3′.

DNA is extracted from mycelial tissue as described in section 4.3.2.

[3] Ferreira and colleagues submitted the GenBank accession numbers AF218058, AF218059 and AF218060. This mitochondrial sequence shares low homology with a *T. indica* mitochondrial DNA sequence with accession number DQ993184: BLAST results show only approximately 30% homology. The base composition of the AT content in mitochondrial DNA is higher than the GC content, which is generally 30–40% (Kurtzman, 1985), however, the AT content of the three sequences in GenBank submitted by Ferreira and colleagues is 43.5%, which is lower than the GC content (56.55%). (C) The primers TIN3/Tin4 cannot amplify mitochondrial DNA to give the desired amplicon when the primers are derived from the extracted and purified *T. indica* mitochondrial DNA; therefore, the three submitted sequences refer to genomic DNA.

PCR for this assay uses the following mastermix (concentration per 25 µl single reaction): 1× TaqMan® Universal Master Mix, 0.4 µM of either Tin3/Tin10 or Tin11/Tin10 primers and 4 µM of the probe, 12.5 ng genomic DNA for both *T. indica*- and *T. walker*-specific assays (obtained as in section 4.3.2). PCR cycling parameters are: 50 °C for 2 min, 95 °C for 10 min, and 34 cycles of 95 °C for 15 s and 60 °C for 1 min.

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

When testing for *T. walkeri*, Tin 3 is replaced with 1.0 µl forward primer Tin 11 (5′-TAA TGT TGG CGT GGC AT-3′) (25 µM), which produces an amplicon of 212 bp.

T. indica produces amplification with primers Tin 3/Tin 10 and *T. walkeri* with primers Tin 11/Tin 10. 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*. 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*. Restriction enzyme analysis may enable further species identification of these samples if required (section 4.3.1).

No amplification can result from poor quality DNA. This can be checked by testing extracts with the universal primers (ITS1 and ITS4) described in section 4.3.1. If the samples contain good quality DNA 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 on an agarose gel. If amplification is still not produced, fresh DNA should be extracted and retested.

The sensitivity limits of both the *T. indica* and *T. walkeri* assays were found to be 5 pg total DNA. This concentration produced detectable levels of fluorescence (Frederick *et al*., 2000). The species specificity of the assays was tested against DNA extracted from *T. barclayana, Tilletia tritici, Tilletia laevis, Tilletia controversa* and *Tilletia fusca.* None of these isolates amplified in either the *T. indica*- or the *T. walkeri*-specific assays (Frederick *et al*., 2000).

4.3.4 Direct real-time PCR on teliospores

This assay was designed by Tan *et al.* (2010) to use the ITS region that occurs between the nuclear small and large subunit rDNA. It was found that *Tilletia* species have two variable regions (ITS1 and ITS2) separated by the conserved 5.8S rRNA gene (Levy *et al.*, 2001; Tan and Murray, 2006). The protocol is designed to initially amplify *Tilletia*-specific DNA and then use real-time PCR and fluorescent probes to identify the species of *Tilletia*. The ITS1 region in rDNA was targeted in this study for the design of the multiplex assay; a five-plex fluorescent PCR assay to identify closely related *Tilletia* species detected in grain.

An aliquot of the reaction mix is added to the PCR tube (from section 4.2.3) and using the same pipette tip the grass sandwich is crushed into pieces to release the spore material. It is important to ensure the PCR tube is not cut during the crushing.

4.3.4.1 Amplification of *Tilletia* DNA before proceeding to real-time PCR

Amplification of *Tilletia*-specific DNA of various *Tilletia* species is performed 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 and Murray, 2006). Each PCR is performed in 20 µl (single reaction) containing 1.5 mM $MgCl_2$, 200 µM each of the four deoxynucleotides dATP, dTTP, dCTP and dGTP, 0.5 µM each of the primer pair and 0.5 U Taq DNA polymerase (Invitrogen[4]) in 1× buffer (50 mM Tris (pH 9.0), 20 mM NaCl, 1% Triton X-100 and 0.1% gelatin).

[4] The use of products of the brand Invitrogen in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this

The thermal cycling parameters are: an initial cycle of 95 °C for 3 min; 20 cycles of 94 °C for 20 s, 63 °C for 30 s and 72 °C for 30 s, with the annealing temperature decreased by 1 °C per cycle for 5 cycles to 59 °C; and finally a 10 min and 1 min incubation at 72 °C and 4 °C, respectively.

The restricted products may be stored at 4 °C. If visualizing on a gel, 10 µl reaction product is loaded with a suitable marker and run on a 2% agarose gel. The expected fragment size is 260 bp. However, this fragment will not be visible if the PCR is done on a single teliospore, as there will not be enough DNA present.

4.3.4.2 Real-time five-plex fluorescent PCR assay for species identification

Real-time PCR assays with the dual-labelled probes and oligonucleotide primers (Table 3) in 20 µl reactions in 0.1 ml microfuge tubes are performed in the Rotor-Gene 6000 instrument (Qiagen[5]). The five-plex reaction mixture consists of 1× ImmoBuffer (Bioline[6], 5 mM $MgCl_2$, 200 µM of each of the four deoxynucleotides dATP, dTTP, dCTP and dGTP, 1 U Immolase DNA Polymerase (Bioline) and 0.2 µM, 0.4 µM and 0.9 µM of each of the dual-labelled probes, the four forward primers and the four reverse primers, respectively (Table 3). The template DNA is 1 µl PCR product from the PCR amplification of *Tilletia*-specific DNA (section 4.3.4.1).

The thermal cycling parameters are an initial cycle of 95 °C for 10 min followed by 45 cycles of 94 °C for 15 s and 65 °C for 60 s, with the annealing 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. Fluorescence data are recorded to five channels: green, yellow, orange, red and crimson.

The sensitivity of the test for single spores was 10–40% (i.e. out of known positive *T. indica* spores only 10–40% gave positive PCR results) (Tan and Wright, 2009). This sensitivity arises from of 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 ldeterioration of genetic material. The specificity of the probe for *T. indica* was investigated in a DNA mixture of *T. indica*:*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.

Standard curves for each detection of each species should be generated as described in Tan *et al.* (2009) using known concentrations of *Tilletia* spp. DNA. The Ct value (the value of the cycle where the amplification curve crosses the threshold line) obtained is used to set the threshold for that *Tilletia* species being tested. In general, a Ct value greater than that set in this step is considered a negative result.

protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[5] The use of products of the brand Qiagen in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

[6] The use of products of the brand Bioline in this diagnostic protocol implies no approval of them to the exclusion of others that may also be suitable. This information is given for the convenience of users of this protocol and does not constitute an endorsement by the CPM of the chemical, reagent and/or equipment named. Equivalent products may be used if they can be shown to lead to the same results.

Table 3. Sequences and modifications of the primers and probes used in the five-plex fluorescent PCR diagnostic assay for *T. indica* and other related *Tilletia* spp.

Primer pairs (sequence 5′-3′)	Probes (modifications 5′, 3′)	Channel	Target
KB-DL-For: CTTCGGAAGAGTCTCCTT (nt. 64–81[a]) KB-DL- Rev: CCGGACAGGTACTCAG (nt. 127–142)	ACGGAAGGAACGAGGC (nt. 105–120) (6-FAM, BHQ1)	Green	*T. indica*
	ACGGAAGGAACAAGGC (nt. 67–82[b]) (JOE, BHQ1)	Yellow	*T. walkeri*
Hor-DL-For: GGCCAATCTTCTCTACTATC (nt. 40–59[c]) Hor-DL-Rev: CCGGACAGGATCACTA (nt. 87–102)	CAACCCAGACTACGGAGGGTGA (nt. 60–81) (CAL Fluor Red 610, BHQ2)	Orange	*T. horri* (some strains are not detected)
Tri-DL-For: ATTGCCGTACTTCTCTTC (nt. 56–73[d]) Tri-DL-Rev: GTAGTCTTGTGTTTGGATAATAG (nt. 99–112)	AGAGGTCGGCTCTAATCCCATC A (nt. 75–97) (Quasar 670, BHQ2)	ed	road range*
Ehr-DL-For: CGCATTCTTATGCTTCTTG (nt. 72–90[e]) Ehr-DL-Rev: GTTAGGAACCAAAGCCATC (nt. 128–146)	CAGAGTCATTGGTTCTCGGAG C (nt. 104–126) (Quasar 70 BHQ2)	Crimson	*T. ehrhartae*

Notes: GenBank accession numbers are [a]AF39843 AF310180, [c]AF310171, [d]AF398447 and [e]AY770433. The list of the reference material used as place of origin is in Tan *et al.* (2009), and material is held at Elizabeth Macarthur Agricultural Institute (EMAI), NSW D of Primary Industries in Australia (See section 6, contact points. nt., nucleotide.

*Includes *T. caries, T. laevis, T. controversa, T. fusca, T. bromi, T. goloskokovii.*

5. Records

Refer to section 7 of ISPM 27:2006 for the list of information that needs to be recorded and retained.

The report of the diagnosis should include the number of positive subsamples and the estimated number of teliospores detected in each positive subsample. If cultures were obtained for molecular analysis, the colony morphology, especially any pigmentation, and growth rate under defined conditions should be described. Cultures should be kept (mycelium from broths or mycelial plugs from agar plates can be stored frozen at −80 °C).

6. Contact Points for Further Information

Further information on this organism can be obtained from:

Department of Agriculture and Food, Government of Western Australia, South Perth, WA 6151, Australia (Ms Dominie Wright; e-mail: dominie.wright@agric.wa.gov.au; tel: +61 8 9368 3875; fax: + 61 8 474 2658).

Elizabeth Macarthur Agricultural Institute (EMAI), New South Wales Department of Primary Industries, Camden, NSW 2570, Australia (Dr Mui-Keng; email: mui-keng.tan@idpi.nsw.gov.au).

Laboratory of Plant Inspection and Quarantine, Shenzhen Entry-Exit Inspection and Quarantine Bureau, Shenzhen, 518045 Guangdong Province, China (Dr Guiming Zhang; email: zgm2001cn@yahoo.com.cn; tel: +86 755 8211 1148; fax: +86 755 2558 8630).

United States Department of Agriculture (USDA) Agricultural Research Service (ARS), North Atlantic Area (NAA), Fort Detrick, MD 21702, USA (Mr Gary Peterson; email: gary.peterson@ars.usda.gov).

USDA Animal and Plant Health Inspection Service (APHIS), Riverdale, MD, USA (Dr Mary Palm; email: Mary.E.Palm@aphis.usda.gov).

USDA APHIS, Beltsville, MD, USA (Dr John McKemy; email: John.M.McKemy@aphis.usda.gov)

Food and Environment Research Agency, York YO41 1LZ, United Kingdom (Dr Kelvin Hughes; email: Kelvin.Hughes@fera.gsi.gov.uk).

A request for a revision to a diagnostic protocol may be submitted by national plant protection organizations (NPPOs), regional plant protection organizations (RPPOs) or Commission on Phytosanitary Measures (CPM) subsidiary bodies through the IPPC Secretariat (ippc@fao.org), which will in turn forward it to the Technical Panel to develop Diagnostic Protocols (TPDP).

7. Acknowledgements

The basis of this protocol was originally drafted by A.J. Inman, K.J.D. Hughes and R.J. Bowyer, Food and Environment Agency, York, United Kingdom, in 2003. That protocol was ring-tested in European laboratories[7] (Riccioni *et al*., 2002) and has formed the basis of the EPPO protocol PM 7/29(2) (EPPO, 2007).

The protocol has been enhanced by D.G. Wright, Department of Agriculture and Food, Government of Western Australia, Perth, Australia; K.J.D. Hughes, Food and Environment Agency, York, United Kingdom; and G. Zhang, Laboratory of Plant Inspection and Quarantine, Shenzhen, China. V. Cockerell, Science and Advice for Scottish Agriculture, Edinburgh, United Kingdom, reviewed the protocol.

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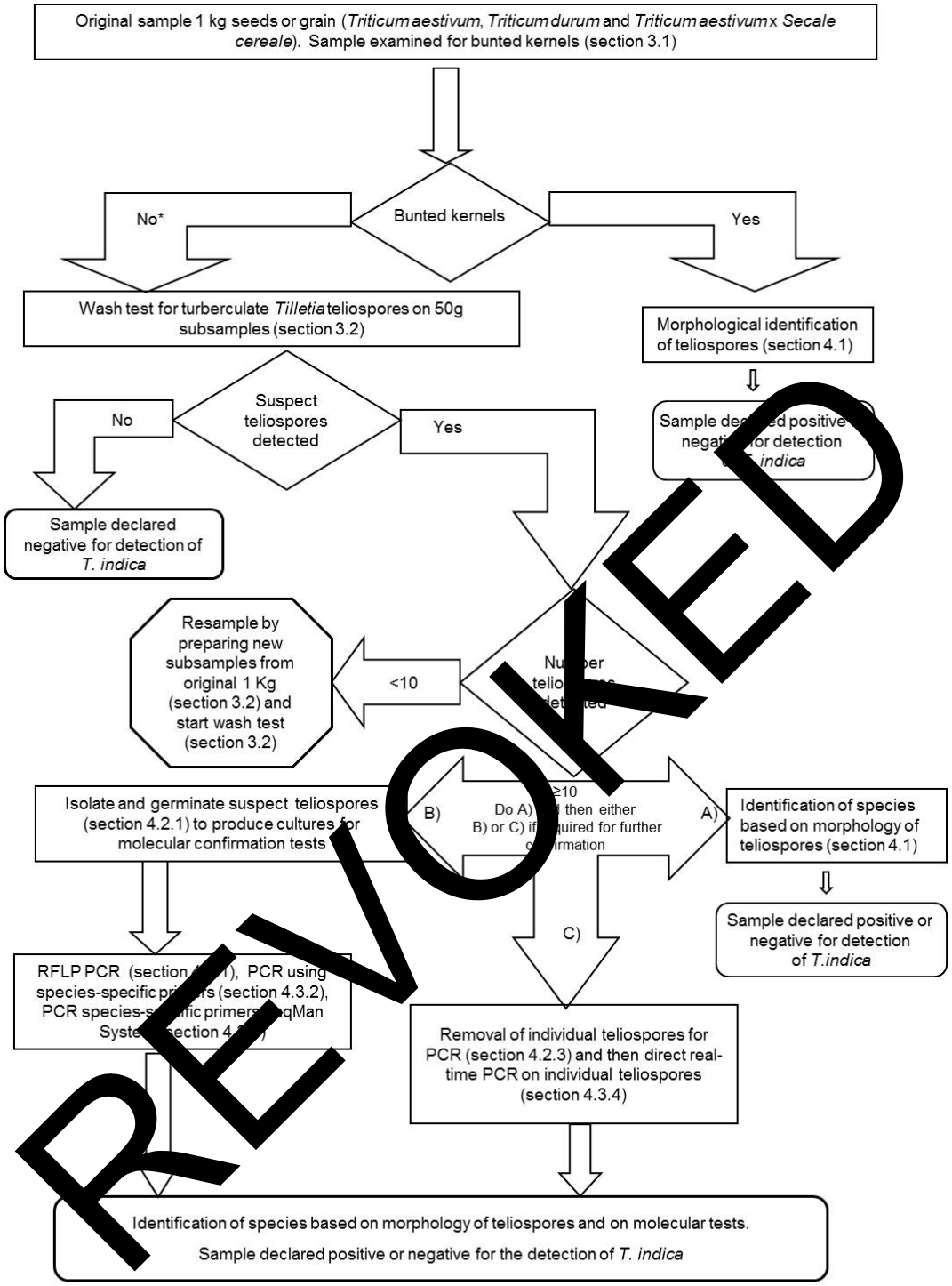
9. Figures

Figure 1. An infected head of wheat showing the symptoms of Karnal bunt.

Photo courtesy Department of Agriculture and Food, Government of Western Australia.

Figure 2. Infected grains of wheat showing the symptoms of Karnal bunt.

Photo courtesy Department of Agriculture and Food, Government of Western Australia.



* In the absence of bunted kernals *T. indica* may be considered not to be present

Figure 3. Flow diagram showing the process to be used for the detection and identification of *Tilletia indica* in seed and grain samples.

PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism.

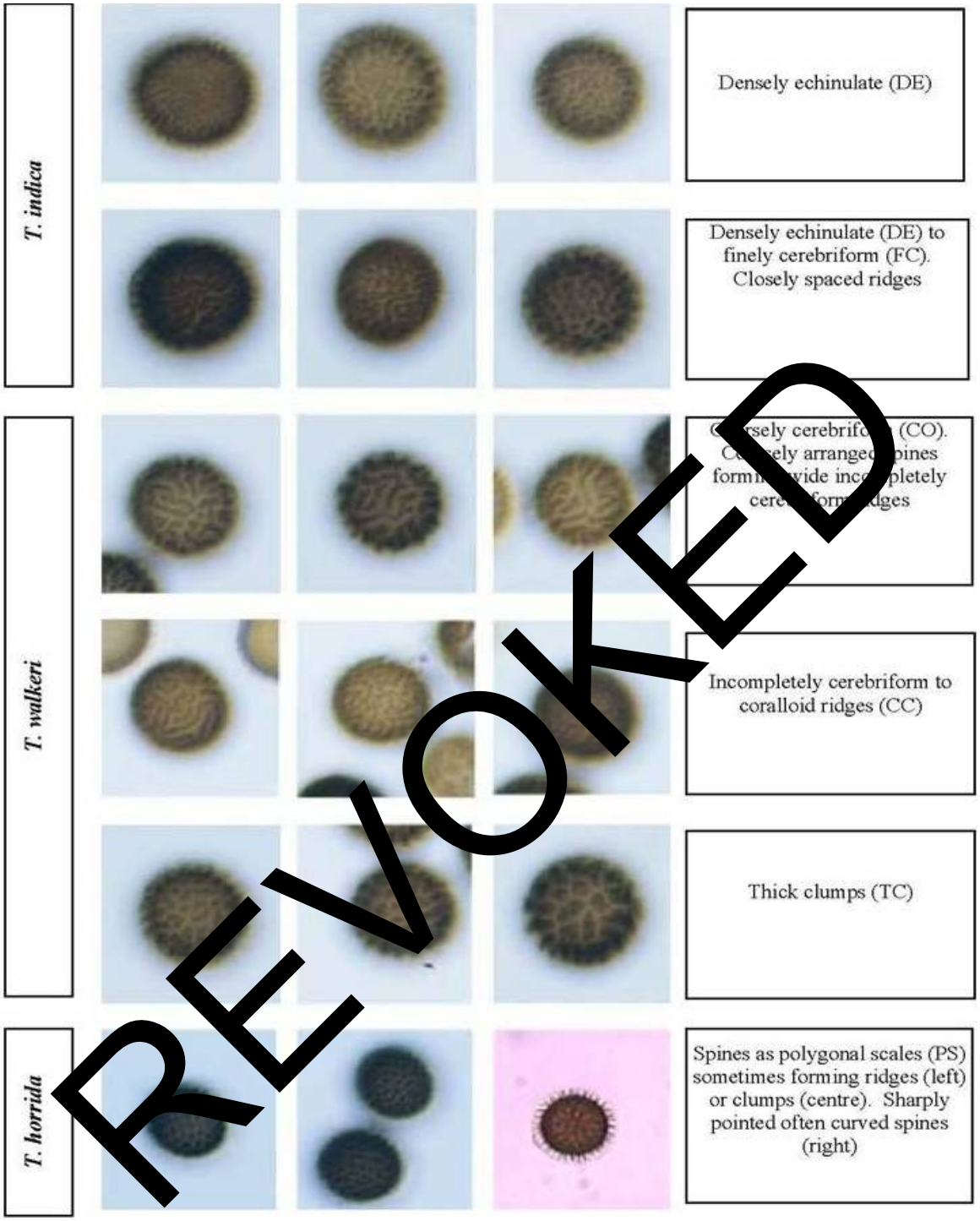


Figure 4. Pictorial key to *Tilletia* teliospore ornamentation. Use in conjunction with Table 2 (section 4.1).

Photos courtesy A. Inman, Central Science Laboratory, York, United Kingdom.

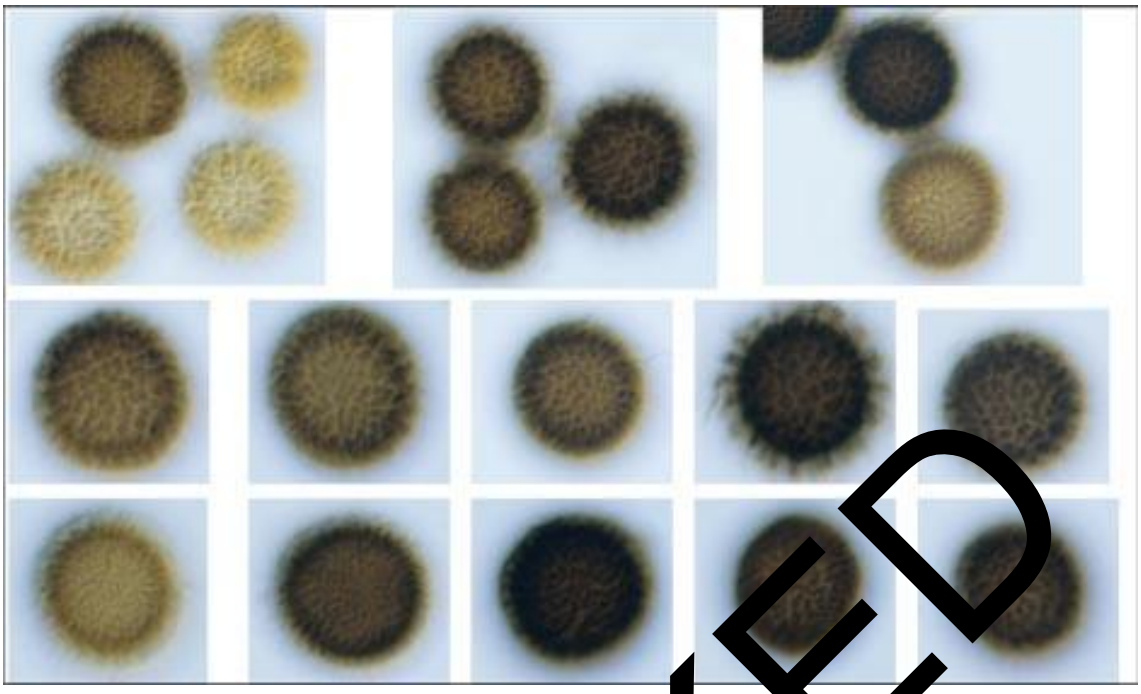


Figure 5. Teliospores of *Tilletia indica* showing surface ornamentation patterns. Spines are densely arranged, either individually (densely echinulate) or in closely spaced narrow ridges (finely cerebriform). Scale: 10 mm = 17 µm.

Photos courtesy A. Inman, Central Science Laboratory, York, United Kingdom.

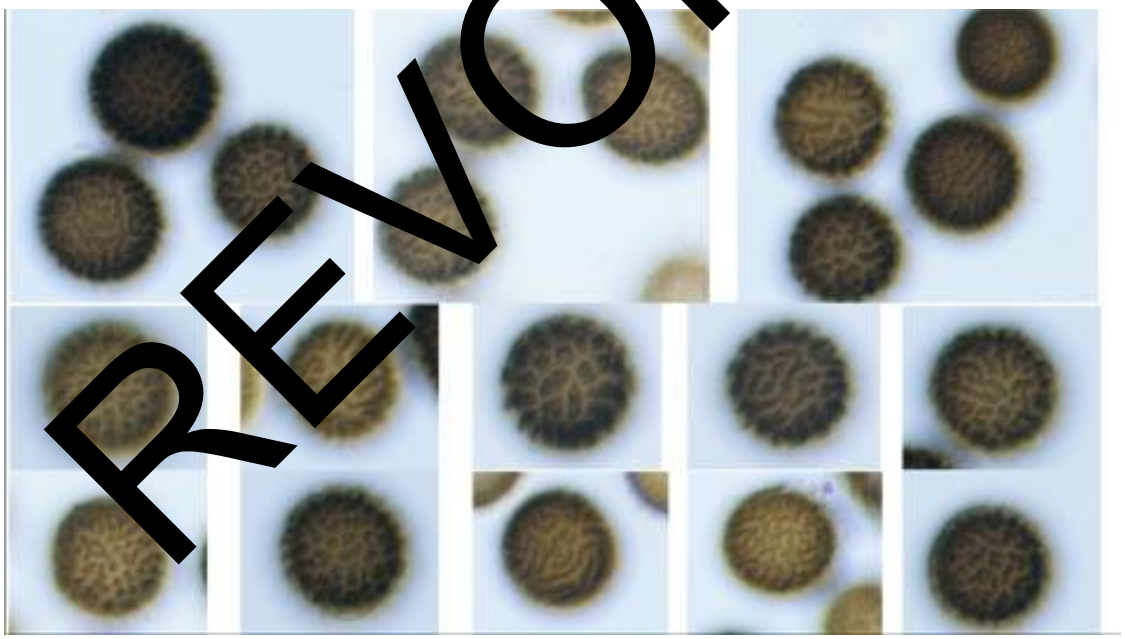


Figure 6. Teliospores of *Tilletia walkeri* showing surface ornamentation patterns. Spines are coarsely arranged and form wide, incompletely cerebriform to coralloid ridges or thick clumps. Scale: 10 mm = 17 µm.

Photos courtesy A. Inman, Central Science Laboratory, York, United Kingdom.

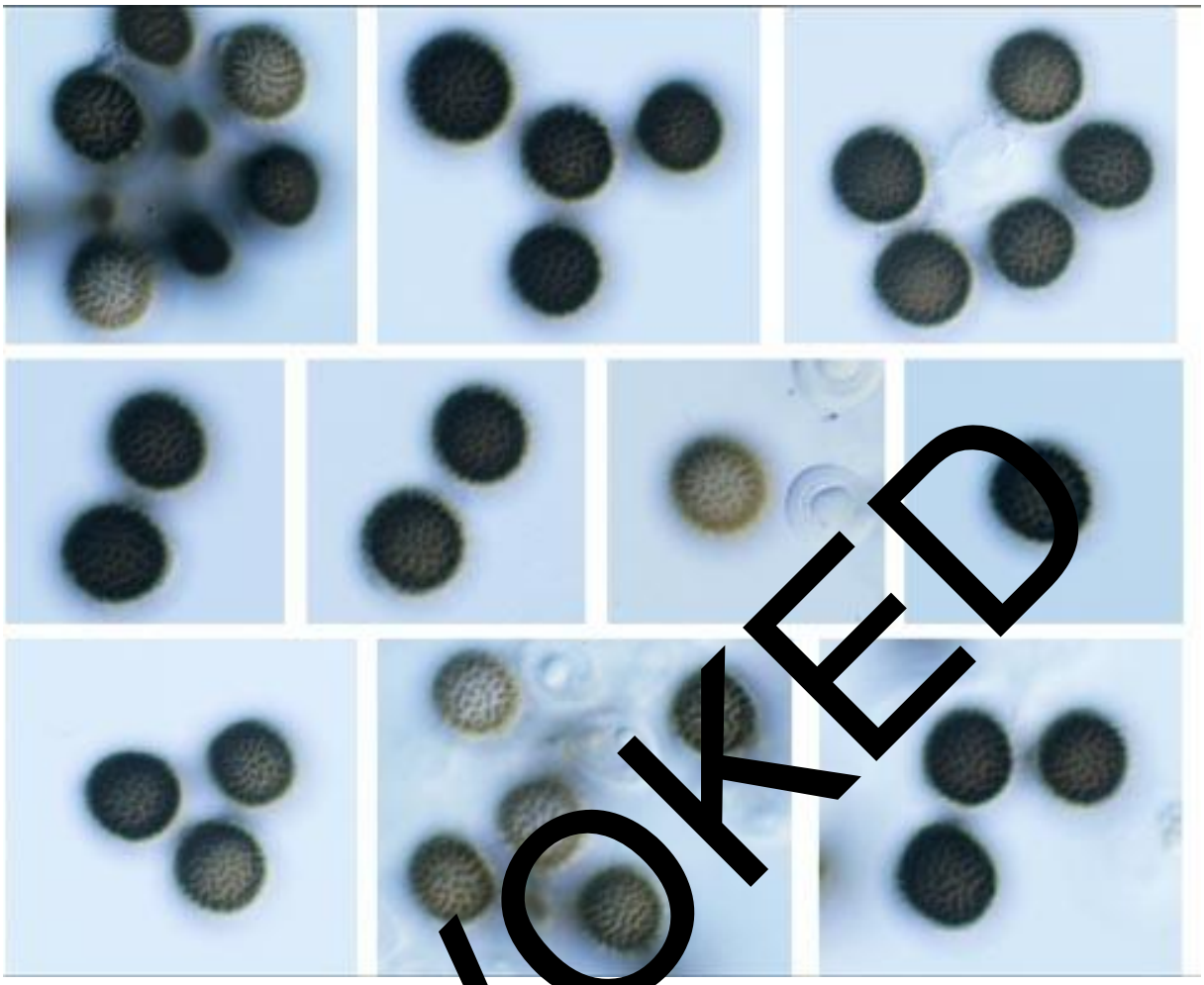
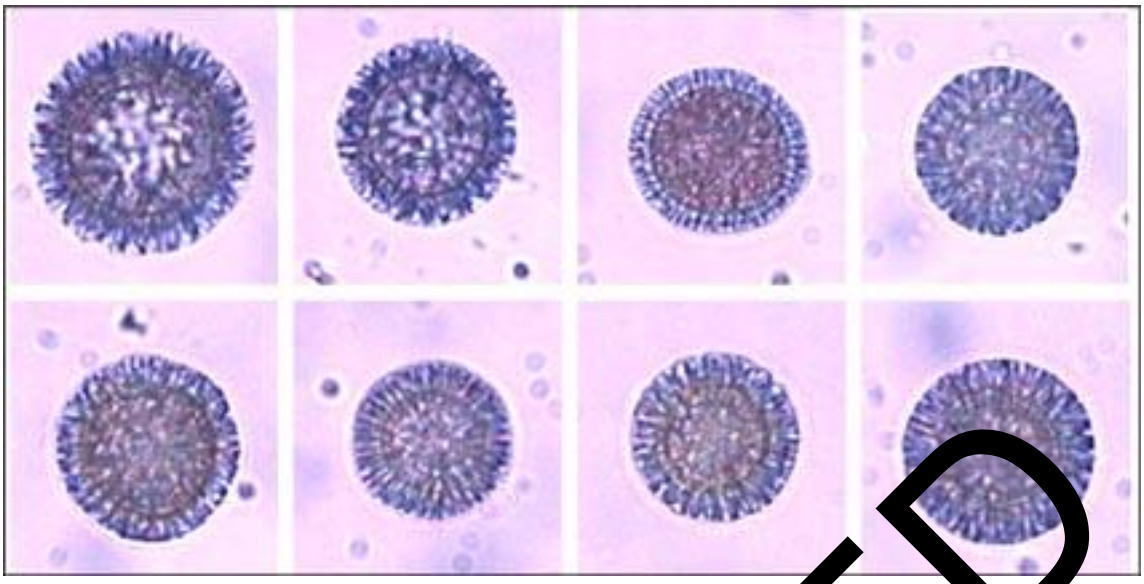
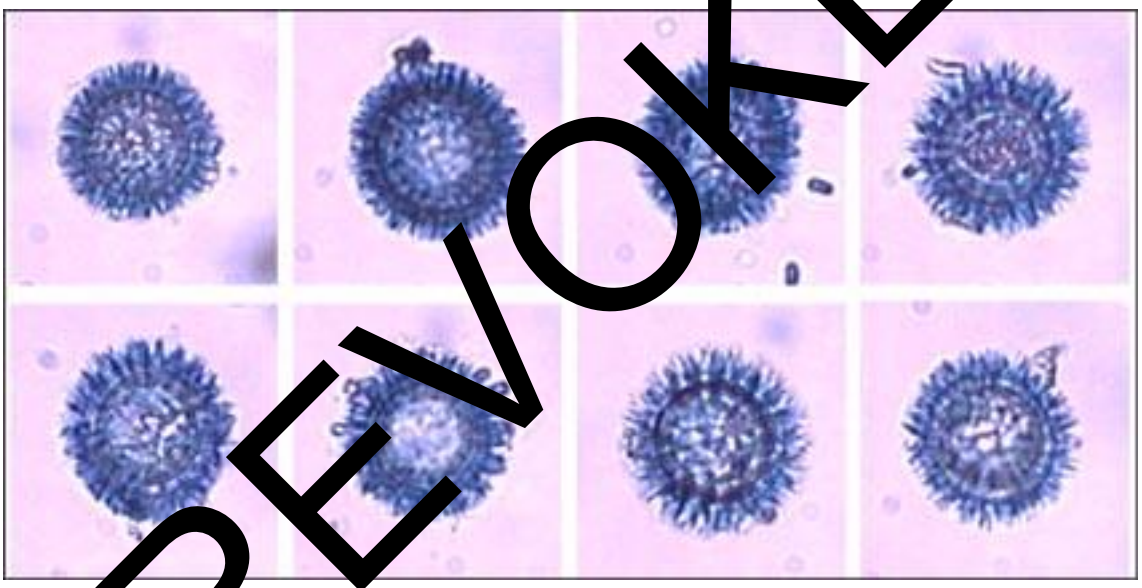


Figure 7. Teliospores of *Tilletia horrida* showing surface ornamentation patterns. Spines are arranged in polygonal scales or, occasionally, cerebriform ridges. Scale: 10 mm = 17 µm.

Photos courtesy A. Inman, Central Science Laboratory, York, United Kingdom.



A



B

Figure 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 A. Inman, Central Science Laboratory, York, United Kingdom.

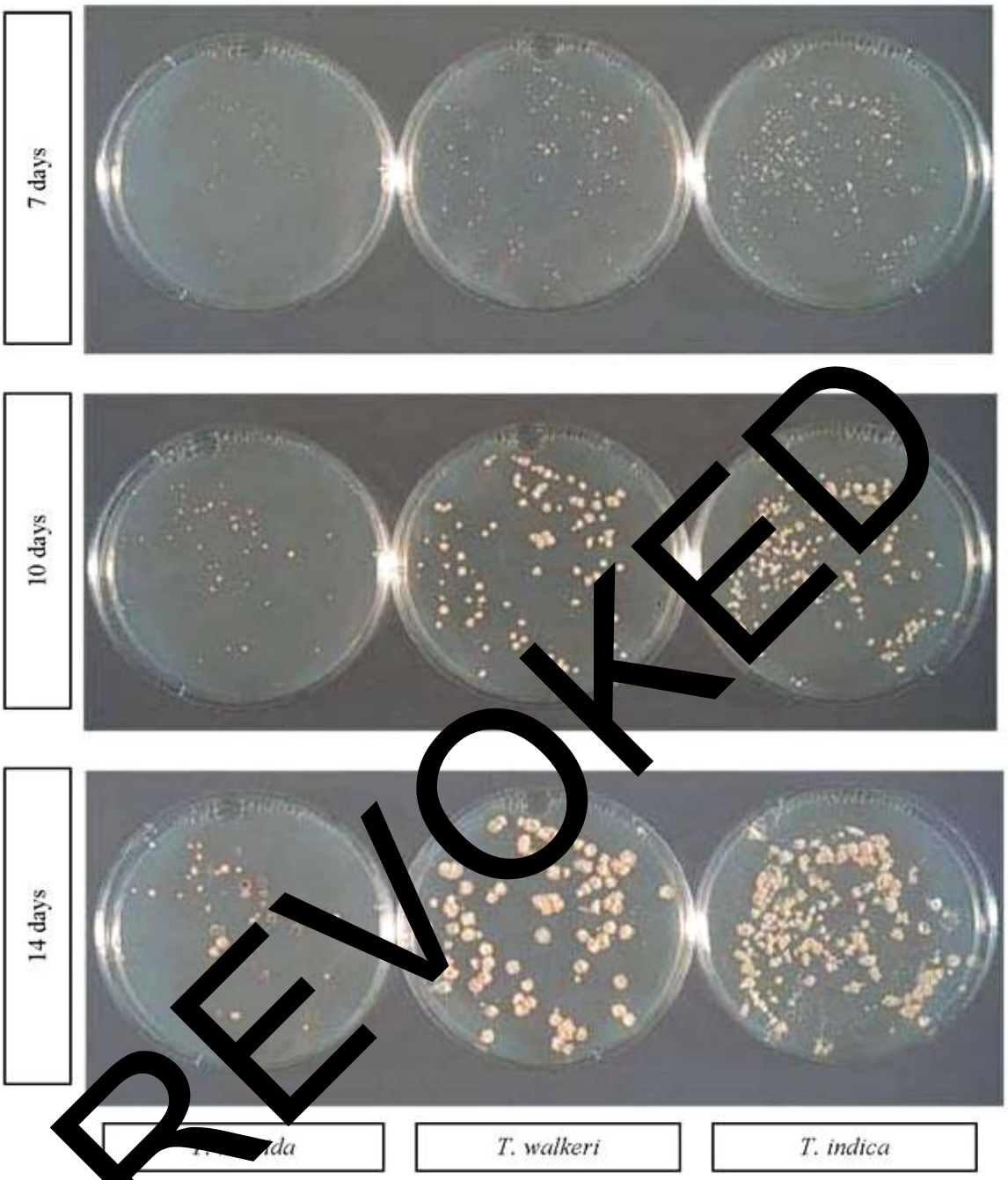


Figure 9. Colonies of *Tilletia indica* (right), *Tilletia walkeri* (centre) and *Tilletia horrida* (left) after 7 days (top), 10 days (centre) and 14 days (bottom) on potato dextrose agar (PDA) at 19 °C and a 12 h dark/light cycle. Note the slower growth and purple pigmentation after 14 days for *T.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

Photos courtesy A. Inman, Central Science Laboratory, York, United Kingdom.

Publication history

This is not an official part of the standard

2006-03 CPM-1 added the subject *Tilletia indica* / *T. controversa* (2004-014) under the topic: Fungi and fungus-like organisms

2012-11 SC approved draft for MC via e-decision

2012-07 member consultation

2013-05 SC approval for adoption via e-decision (returned to TPDP)

2013-06 TPDP revised

2013-10 to SC for approval for adoption via e-decision

2013-10 SC approved the draft for the 45-days notification period via e-decision

2013-12 45 days notification period

2014-01 SC adopted DP on behalf of CPM

2014-08 Secretariat corrected minor formatting issues

ISPM 27. 2006: **Annex 4** *Tilletia indica* Mitra (2014)

Publication history last updated: 2014-09-11