Diagnostic protocol for
Tilletia indica, the cause of
karnal bunt

**The International Diagnostic Protocol for Tilletia indica
(ISPM-27 DP04) was released March 2014**

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This version of the National Diagnostic Protocol (NDP) for karnal bunt is current as at the date contained in the version control box on the front of this document.

NDPs are updated every 5 years or before this time if required (i.e. when new techniques become available).

The most current version of this document is available from the SPHDS website:
http://plantbiosecuritydiagnostics.net.au/resource-hub/priority-pest-diagnostic-resources/

Where an IPPC diagnostic protocol exists it should be used in preference to the NDP. NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

https://www.ippc.int/core-activities/standards-setting/ispms

Contents

1 Introduction

1.1 General introduction

Karnal bunt is one of five bunt and smut diseases that affect wheat throughout the world (Wilcoxson and Saari, 1996). None of these are toxic to humans or livestock, but some can affect the appearance and smell of grain products. Three occur in Australia and in most other wheat growing countries: common bunt (caused by *Tilletia caries* and *T. laevis*); loose smut (*Ustilago tritici*) and flag smut (*Urocystis tritici*). karnal bunt (*T. indica*) and dwarf bunt (*T. contraversa*) have more restricted distributions world-wide and are subject to quarantine regulations in many countries.

Karnal bunt is a serious disease for international trade because it reduces grain quality and has a restricted distribution, being limited to areas within the Indian subcontinent, neighbouring Middle East, Mexico, the south-western United States of America and South Africa (Fuentes-Davila, 1996; Crous *et al.,* 2001). Flour made from infected wheat is unfit for human consumption because it has an objectionable smell and taste.

The disease is caused by the fungal pathogen *Tilletia indica* Mitra, also known as *Neovossia indica* (Mitra) Mundkur, which is the name preferred by most Indian researchers.   The pathogen affects wheat, durum and triticale. It was first found in wheat being sold in Karnal in northern India in 1930, with the town giving its name to the new bunt (Mitra, 1931).

Karnal bunt replaces part of the wheat seed with a black powder consisting of millions of teliospores.   Bunted grain smells foul like rotting fish due to the presence of the volatile chemical trimethylamine.   Thus the disease 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.

The disease cycle of karnal bunt differs from that of common bunt, loose smut and flag smut, hence seed treatments that are highly effective for controlling these latter diseases are ineffective for controlling karnal bunt. The introduction of *T. indica* to Australia would impose costs through disruption of export markets and the use of specific control measures to maintain the high quality of Australian wheat grain.

T. indica is regarded as a high threat because:

• It reduces grain quality, producing masses of dark powdery spores that discolour the grain and grain products, and having an objectionable 'dead fish' smell;

• It has a restricted world distribution, leading to many countries imposing stringent quarantine regulations that can prevent sale of wheat grain from infested areas even if the grain is otherwise of sound quality.

Australia imposes strict quarantine regulations to prevent the entry of *T. indica*. To be effective, the country requires an internationally recognised means of testing imports for presence of the fungus, providing surveillance to demonstrate that the country is free of the pathogen, and to enable an incursion to be identified quickly and accurately.

1.2   Host range

Hosts include *Triticum aesativum* (wheat), *Triticum durum* (durum wheat), and *Triticum aestivum* x *Secale cereale* (triticale). Records on triticale are rare, and rye (*Secale*) has 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).

1.3   Symptoms

Karnal bunt affects some of the seeds in the wheat head, but it is extremely difficult to find infected kernals. Heads with infected seeds do not differ in appearance from healthy heads and so the symptoms are not usually seen until after harvest (Figure 1). Symptoms on seed range from a pinpoint sized spot to a black sorus that runs the length of the groove, and occasionally most of the seed can be replaced (Figures 2 and 3). The sorus is composed of a mass of dark brown to black powdery teliospores which are evident when the seed coat is broken (Figure 4).   When fresh, the affected grain has an unpleasant foetid smell varying from rotten fish to mouse-like.   This smell is due to the presence of the volatile chemical trimethylamine.   Flour milled from such seed will be grey and may have the odour.

1.3.1   Confusion with other symptoms

Symptoms of common bunt generally replaces all seeds in the head completely.   The bunted seeds are greyish and readily broken at harvest or crushed between the fingers to show a black, slightly greasy mass of teliospores. Trimethylamine is also present so grain affected by common bunt has the same smell as karnal bunt.

Dwarf bunt causes identical seed symptoms to common bunt. Loose smut replaces the floral parts with a mass of black teliospores and is readily seen after the crop comes into head. These spores generally disperse before harvest leaving a bare rachis. Sometimes some spores remain in a hard mass on the rachis and these masses can contaminate the harvested grain. They differ from bunt in being hard and present on the broken rachis rather than on seed, and lack the unpleasant odour. Flag smut affects the leaves, producing stripes of black powdery teliospores in the leaves. This material is not usually present as large pieces in harvested grain, although flag smut spores can adhere to seed.

Figure 1. An infected head of wheat showing the symptoms of Karnal bunt in USA (Photograph: D. Wright, Department of Agriculture and Food, Western Australia).

Figure 2. Infected grains of wheat with Karnal bunt in USA (Photograph: D. Wright, Department of Agriculture and Food, Western Australia).

Figure 3. Range of Karnal bunt symptoms seen on individuals grains of wheat in USA (a) The embryo end of the seed is infected and (b) will continue along the crease. (c) The grain coat has a holey appearance and (d) collapse when infection is severe (Photographs: D. Wright, Department of Agriculture and Food, Western Australia).
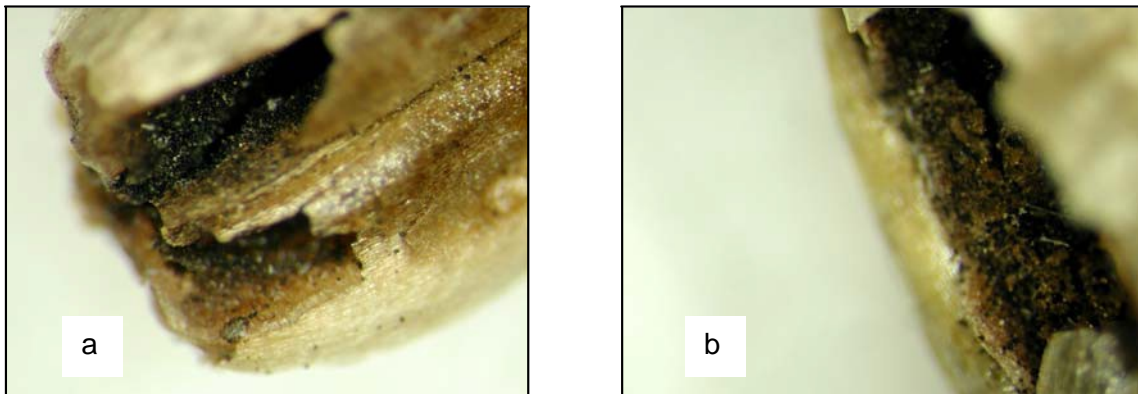

Figure 4. Teliospores of *T. indica* will be evident where the grain coat has broken (Photographs: D. Wright, Department of Agriculture and Food, Western Australia).

2 Taxonomic Information

Kingdom: Fungi

Division: Basidiomycotina

Class: Ustilaginomycetes

Order: Tilletiales

Family: Tilletiaceae

Species   *Tilletia indica*

Scientific Name: *Tilletia indica* Mitra

Synomyms: *Neovossia indica* (Mitra) Mundkur

Common Names: Karnal bunt, partial bunt, stinking bunt

3 Detection

The diagnostic scheme for *T. indica* (Figure 5) 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/or germination of teliospores for molecular confirmation and molecular confirmation of cultures or teliospores.

3.1 Examination of bunted grain

Direct visual examinations for bunted kernels or teliospores contaminating seed surfaces are not considered reliable methods for phytosanitary purposes. However, bunted grain may be detected by visual examination with the naked eye and low power microscopy (10 – 40 times magnification). The whole submitted sample (minimum 1 kg per 30 000 tonnes) needs to be examined for bunted wheat seeds or other Poaceae seeds (for example ryegrass). The sample is assessed for symptoms of Karnal bunt (Figures 3 and 4), and for presence of other poaceae seeds. Record the symptoms observed and the presence of the other poaceae seeds.

If bunted grains are present, a semi - positive diagnosis can be made based on the morphology of the teliospores. Microscope slides of the teliospores must be made and the morphology of these spores described. If the morphology of the teliospores is consistent with those described for *T. indica* (refer to morphology section 4.1 and Figures 7-11) a positive diagnosis can be made.

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 and Cunfer, 1993; Agarwal and Mathur, 1992). With severe contamination, teliospores may be seen on the surface of seeds (Mathur and Cunfer, 1993).

NB: The absence of bunted grain does not indicate that *T. indica* is not present. It is important to proceed to the selective sieve wash method for determining whether *T. indica* is present or not present on the grain. If ryegrass grain is found contaminating the sample there is a high probability that *T. walkeri* will be detected in the sample as well.
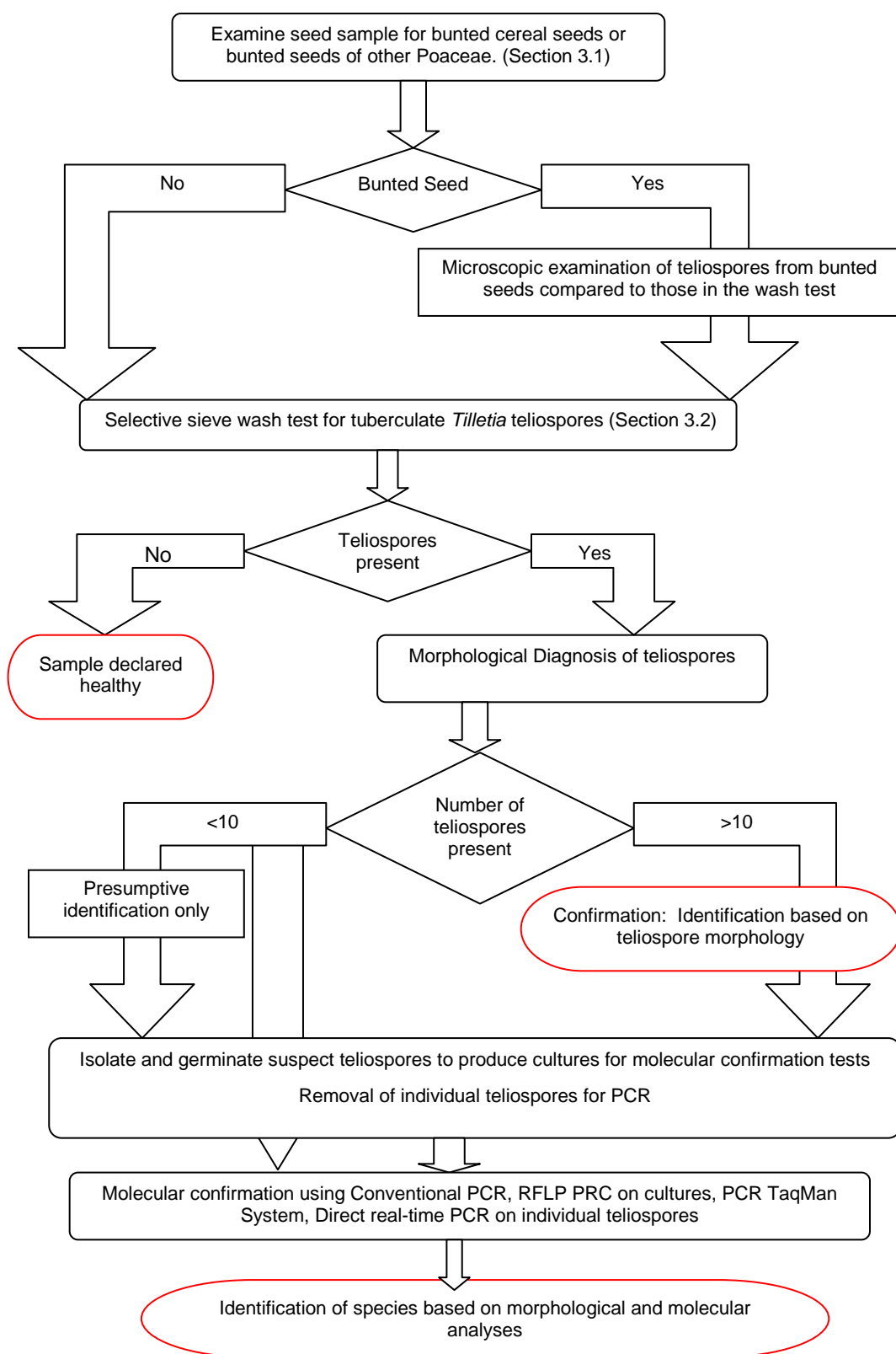
Figure 5. Flow diagram of protocols to be used for the analysis of suspect grain sample.

3.2 Extraction of spores from grain (selective sieve wash method)

This method describes the most reliable method for detection of *T. indica* spores in a sample of wheat (Peterson *et al* 2000, Inman *et. al.,* 2003, and Wright *et.al.,* 2003). It is important that a minimum of 3 replicates of 50g is done to ensure detection of teliospores if they are present in the sample (refer to Table 1 for number of samples required to detect different numbers of teliospores). This method has, on average, an 82% efficiency of recovery and microscopic examinations typically require only a few slides per 50 g sample.

Before beginning it is important that all equipment is soaked for 15 minutes in a bleach solution of (1.6%a.i of NaOCl), to eliminate the risk of false positives by cross contamination from previous samples. The bleach kills teliospores and makes them appear hyaline compared with the normally dark, pigmented spores. All equipment is rinsed with tap water after soaking.

The 50g sample of wheat is placed in an Erlenmyer flask (250 ml) with a 100 ml of 0.01% Tween 20 aqueous solution. The samples are then placed on a shaker for three minutes at 200 RPM to release the spores from the grain. The solution and grain are then poured onto a 53µm sieve, which is sitting on top of a 20 µm sieve, which is sitting inside a funnel on top of another flask (500 ml) (Figure 6a). The flasks that contained the grain are then rinsed twice (approx 100 ml) with sterile tap water and this is then poured over the grain sitting in the sieve. The grain is further washed with sterile tap water (approx 200 – 300 ml) using an aspirator bottle to ensure a good removal of the spores from the grain. The grain and the 53µm sieve are removed. The 20 µm sieve is tilted to a 45 degree angle (Figure 6b), 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 is to wash all spores recovered from the grain into the lower side of the sieve. The spores and debris are then washed into to a 15 ml conical centrifuge tube (Figure 6c). NOTE: 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 20µm sieve appears clean. The final volume in the tube will be approximately 8 ml. If necessary, the 20µm sieve can be examined under a low power microscope to check for any residual teliospores.

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).

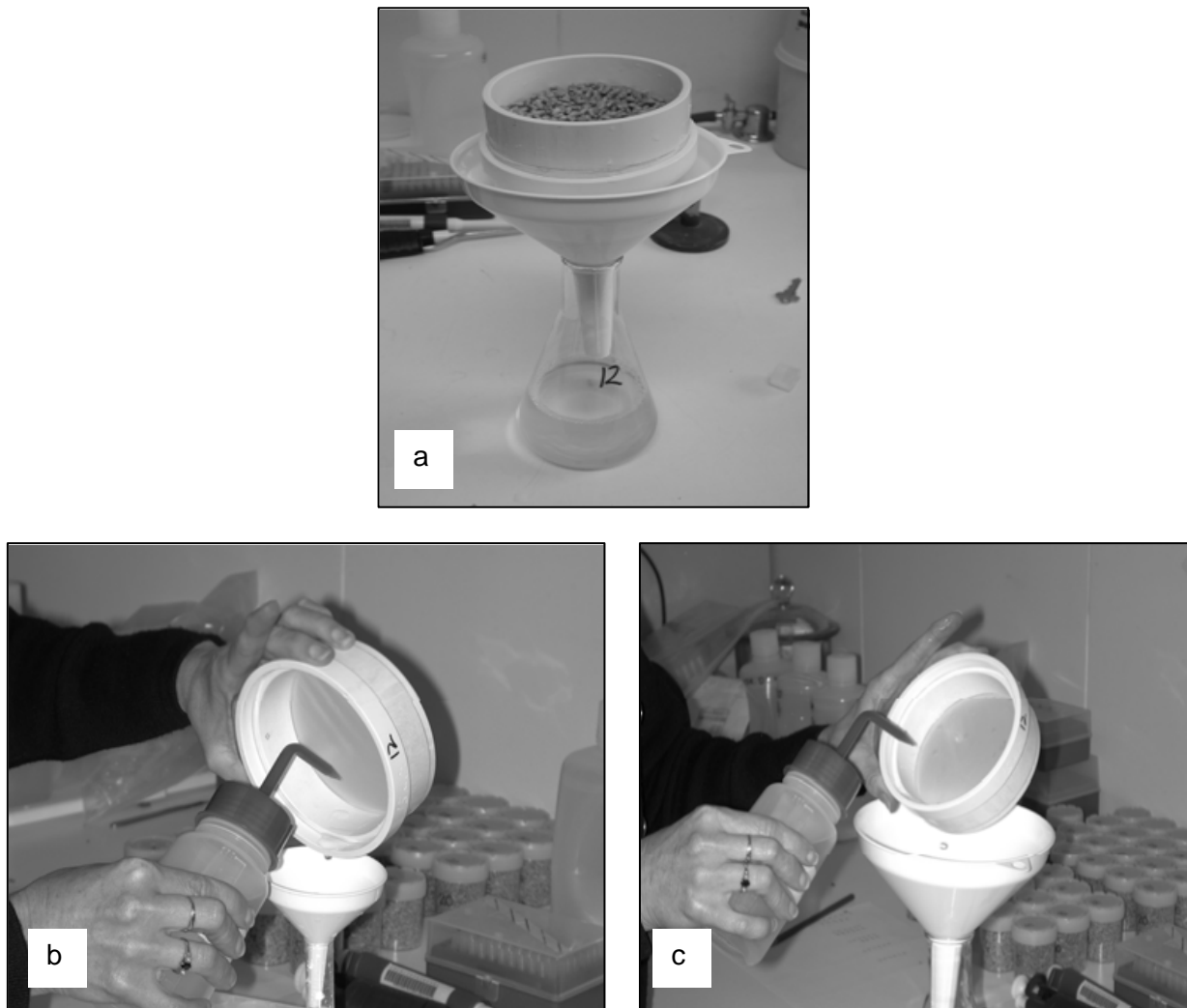| No. of replicate samples required for detection according to level of confidence (%)			
Contamination level (no. of spores per 50 g sample)	99%	99.9%	99.99%
1	3	5	6
2	2	3	4
5	1	1	1

Figure 6. Extraction of spores from grain (a) Set up for the selective sieve wash test. Two sieves (50 µm and 15 µm) are placed within funnel on top of the 500 ml Erlenmyer flask. (b) Washing the deposit on the membrane to one side of the 15 µm sieve (c) Washing the deposit from the 15 µm sieve into the centrifuge tube.

The collected suspension is centrifuged at 1000 g for 3 min[1] (this is to collect the spores as they are denser than most of the debris detected during the wash test). The supernatant is carefully removed using a new disposable Pasteur pipette, without disturbing the pellet. The pellet can now be examined under the microscope. If the pellet appears to be quite thick, extra water can be added to dilute the suspension before examination under the microscope. The pellet is stirred with a pipette tip before examining to ensure an even suspension is obtained.

The whole pellet suspension is examined by placing 20 µL lots onto a microscope slide, covered with a cover slip. It is important to examine every square millimetre of the suspension on the slide for presence of teliospores. The slides are examined using bright field microscopy, at 20 - 40 x magnification. If suspect teliospores are found, it is important to record the morphological characteristics (e.g. size, colour and ornamentation) and record the number of spores found on each slide.

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[1] Equation for calculating the Relative Centrifugal Force (RCF(x g)) from RPM:
RCF = 1.2 r_{max}(RPM/100)^2, where r_{max} is the radius (mm) from the centre of rotation to the bottom of the centrifuge tube.

4 Identification

Formal identification of *Tilletia indica* is based on symptoms on seed, morphology of the teliospores, and detection of the unique DNA sequence by PCR techniques.

4.1 Morphology

When suspect teliospores are found in a wash test, the grains in both the washed sub-sample(s) and the larger submitted sample should be re-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. If the teliospores found in the wash test are the same as those found on bunted grain a diagnosis can be made. If however, there are no bunted grains found in the larger sample, molecular tests are recommended for confirmation.

Table 2 lists the common *Tilletia* species that can be found in grain shipments and confused with *Tilletia indica.*

4.1.1 Morphological confirmation

Tilletia indica teliospores are globose to sub-globose, 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 / brown to dark, reddish brown; mature teliospores are black and opaque (Figure 7); 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) (Figure 8); the spines are covered by a thin hyaline membrane (CMI 1983; Carris *et al.,* 2006).

Sterile cells of *T. indica* are globose, sub-globose 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 (CMI 1983, Carris *et al.,* 2006).

If ≥ 10 teliospores are present in a wash test, then the morphological identification can be confirmed. If ≤10 teliospores are detected, morphological characters are not considered totally reliable for confident discrimination between *T. indica* and the morphologically similar species *T. walkeri T. horrida,* and *T. ehrhartae.* The most important discriminatory characters are teliospore size (maximum size and mean), exospore ornamentation and colour (Table 2; Figs 7-11). However, regardless of the number of teliospores detected molecular confirmation tests are still recommended on either the cultures or on the teliospores.

4.1.2 Morphological comparisons with other Tilletia species

Other tuberculate-spored *Tilletia* species may be confused with *T. indica* (Durán & Fischer, 1961; Durán, 1987, Pimentel *et al.*, 1998). These species are less likely to be found as contaminants of wheat, but they include: *Tilletia barclayana* (smut of various *Poaceae*, e.g. *Panicum* and *Paspalum*), *Tilletia eragrostidis* (on *Eragrostis*), *Tilletia ehrhartae (on Ehrharta calycina), Tilletia inolens* (on *Lachnagrostis filiformis*), *Tilletia rugispora* (on *Paspalum*), *Tilletia boutelouae* (on *Bouteloua gracilis*). However, Pascoe *et al.* (2005) showed that *T. walkeri* and *T. ehrhartae* are common contaminants of Australian wheat. None of these morphologically similar species have been found to naturally infect wheat. In the USA, 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 and Castlebury, 1999; Castlebury and Carris, 1999; Smith *et al.*, 1996). The most important morphological

characters that discriminate *T. indica*, *T. walkeri, T. horrida* and *T. ehrhartae* are teliospore size (range and mean), exospore ornamentation and colour [2](Table 2; Figures 7-11).

The median profiles can be enhanced by bleaching the teliospores in 10% sodium hypochlorite for 15–20 min. If necessary, spores can then be rinsed twice in water and stained, e.g. with trypan blue or cotton blue in lactoglycerol (Figure 11).

Table 3 is a scheme for morphologically distinguishing similar *Tilletia* species. The table can be photocopied and filled in by using ticks in the relevant boxes.

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[2] The literature on spore sizes is often variable. Spore size is affected by the mounting medium and by heating treatments. For rice smut (*T. horrida*, synonym *T. barclayana*), data from rice is potentially more reliable than data based on *T. barclayana sensu lato* from various *Poaceae* as the latter is considered a species complex. The rice pathogen is considered distinct from those on *Paspalum* and *Panicum* but it is not known whether it is distinct from *T. barclayana* s.s. on *Pennisetum* (Pimentel, 1998; Castlebury, 1998)

Table 2. Morphological characteristics of *Tilletia indica*, *T.walkeri*, *T. horrida,* and *T. ehrhartae.*

Species	Spore size range	Spore size mean	Spore colour	Spore shape	Spore sheath	Spore spines	Host
T. indica[a]	28-54	35-41	Brown to dark reddish brown, opaque	Globose to sub globose	Present	1.4-5 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* sp.
T. walkeri[b]	28-35	30-31	Pale yellow to dark reddish brown (never black / opaque)	Globose	Present. Extending to tips of projections, hyaline to yellowish brown	36 µm Coarse +/- cerebriform. Wide incompletely cerebriform ridges in surface view In median view, profile is irregular with gaps between spines	*Lolium* sp.
T. horrida[c]	14-36 (mature <25)		Light to dark chestnut brown, Can be semi opaque	Globose to sub globose	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* sp.
T. ehrhartae[d]	17-25		Very dark olivaceous brown when mature. Can be opaque due to melanisation of the scales.	Globose to sub globose	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, Paspalum*

[a]Authors' data. [b]Based on: Castlebury & Carris (1999); Cunfer & Castlebury (1999); Milbrath *et al.,* (1998); Castlebury (1998). [c]As *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). [d]Pascoe *et al.,* (2005).

Table 3. 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.

	Max Size (diameter, µm)[a]				Mean size (diameter, µm)[a]			Colour [b]				Spines (ornamentation) in surface view [b] and median profile [b, c]			
	<28	<36	36–45	45–50+	24–28	30–31	35–41	Olivaceous brown (very dark). Opaque	Pale yellow to mostly light or dark chestnut-brown (to semi-opaque)	Pale yellow to mostly reddish-brown (never opaque)	Pale orange but mostly dark reddish-brown to opaque black	Polygonal scales in surface view. Median view, cylindrical or slightly tapered spines, broadly truncate to slightly rounded at the apex.	Echinulate; polygonal scales in surface view; occasionally cerebriform ridges or rarely clumps. Sharply pointed, becoming truncate, occasionally curved	Coarse; broad, incompletely cerebriform ridges (to coralloid), or thick clumps. Conical to truncate (gaps between spines obvious in profile after bleaching)[c]	Dense; echinulate or closely spaced narrow ridges (finely cerebriform) Sharply pointed to truncate, occasionally curved (few or no gaps between spines after bleaching)[c]
Sample Tick boxes															
T. ehrhartae	▓			▓	▓			▓				▓			
T. horrida		▓		▓	▓				▓				▓		
T. walkeri			▓		▓					▓				▓	
T. indica				▓			▓				▓				▓

[a] Table 2; [b, c] Table 2 and Figs 6–12; ▓ Character conforms to *Tilletia* species
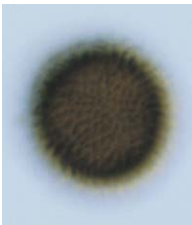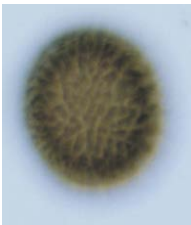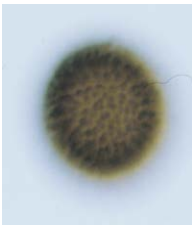
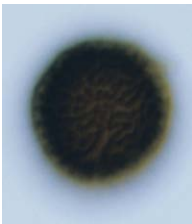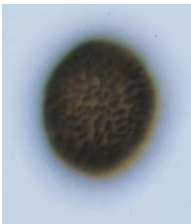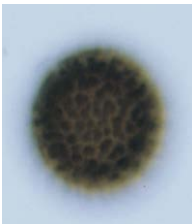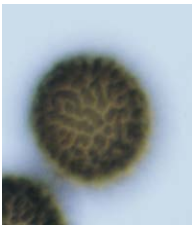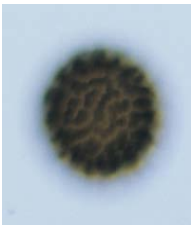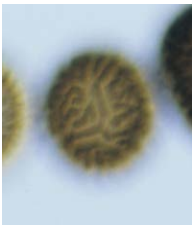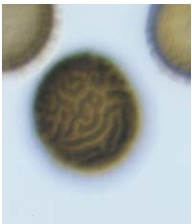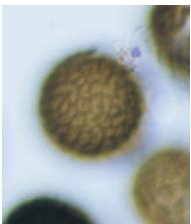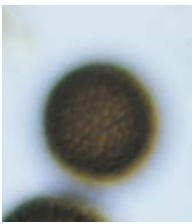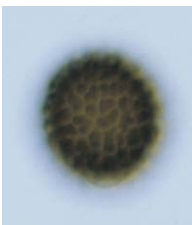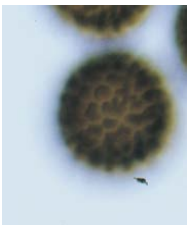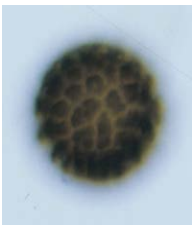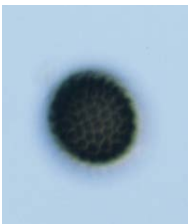**Figure 7**. Pictorial key to teliospore ornamentation. Use in conjunction with Tables 2 and 3. Photographs courtesy of Dr Alan Inman, Central Science Laboratory, York.
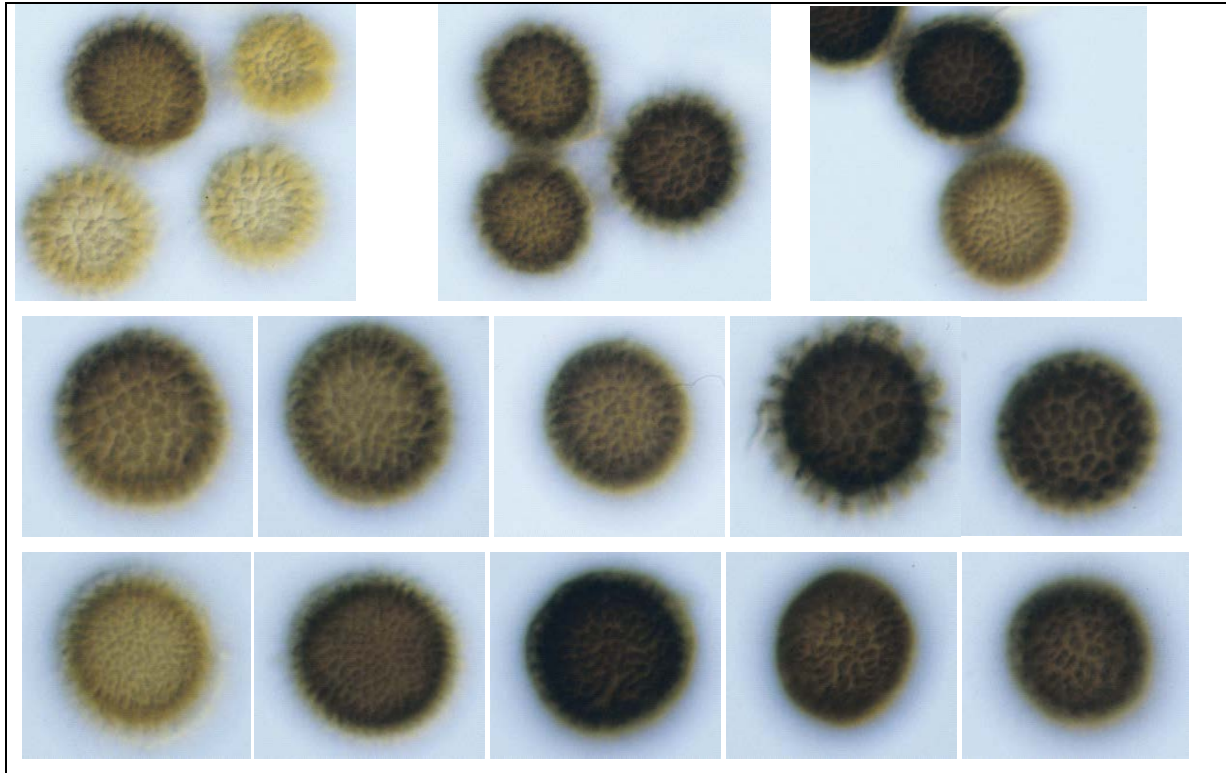
Figure 8. Teliospores of *Tilletia indica* showing surface ornamentation patterns. Spines are densely arranged, either individually (densely echinulate) or in closely spaced, narrow ridges (finely cerebriform). Scale: 10mm = 17 µm. Photographs courtesy of Dr Alan Inman, Central Sciences Laboratory, York.
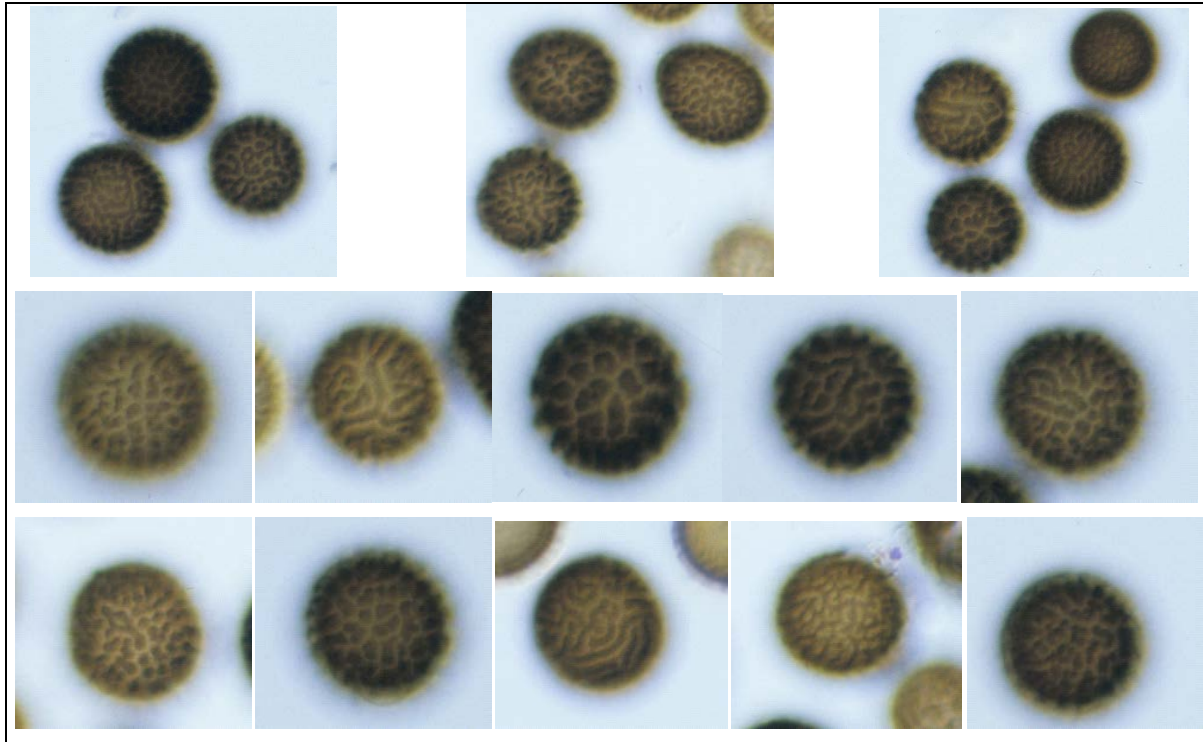
Figure 9. Teliospores of *Tilletia walkeri* showing surface ornamentation patterns. Spines are coarsely arranged and forming wide, incompletely cerebriform to coralloid ridges or thick clumps. Scale: 10mm = 17 µm. Photographs courtesy of Dr Alan Inman, Central Sciences Laboratory, York.
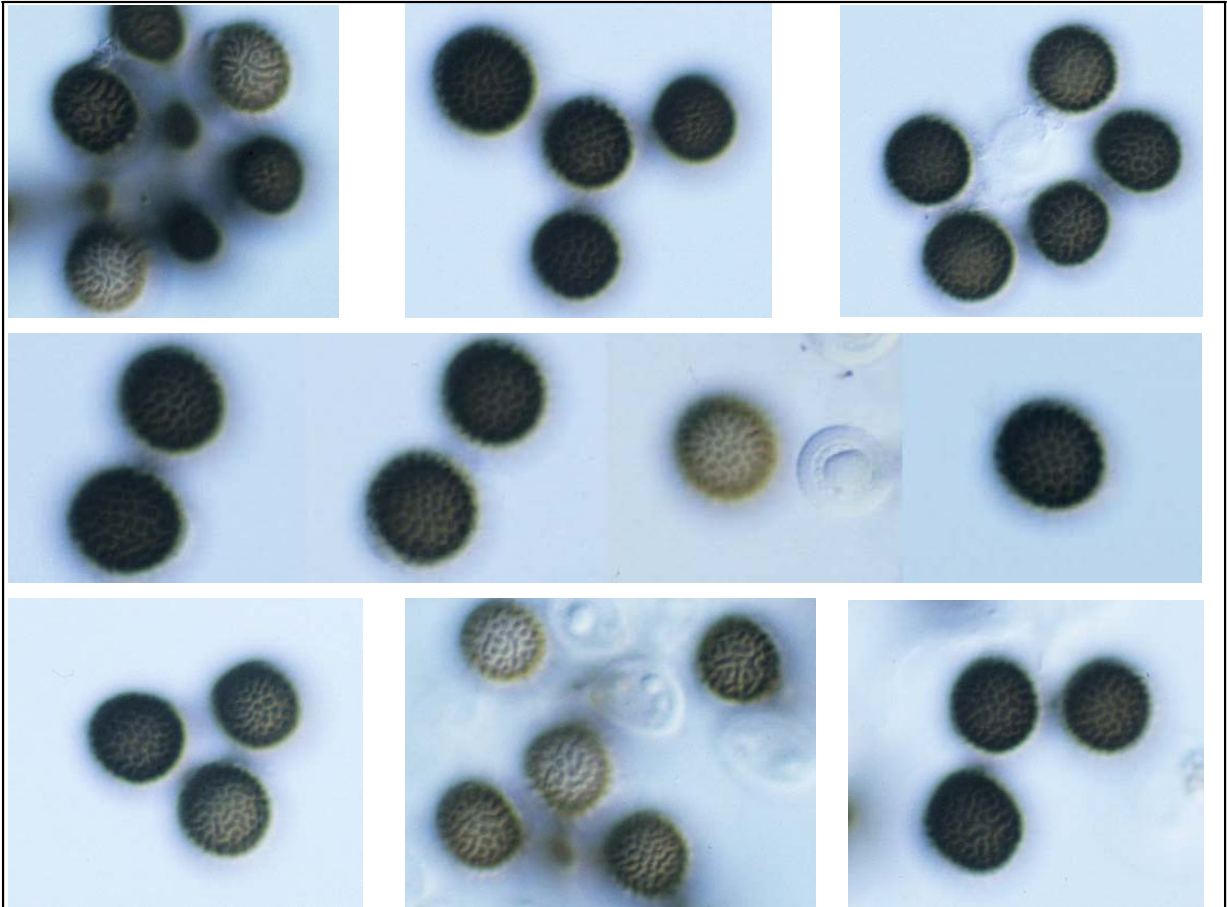
Figure 10. Teliospores of *Tilletia horrida* showing surface ornamentation patterns. Polygonal scales or, occasionally, with cerebriform ridges. Scale: 10mm = 17 µm. Photographs are the courtesy of Dr Alan Inman, Central Sciences Laboratory, York.
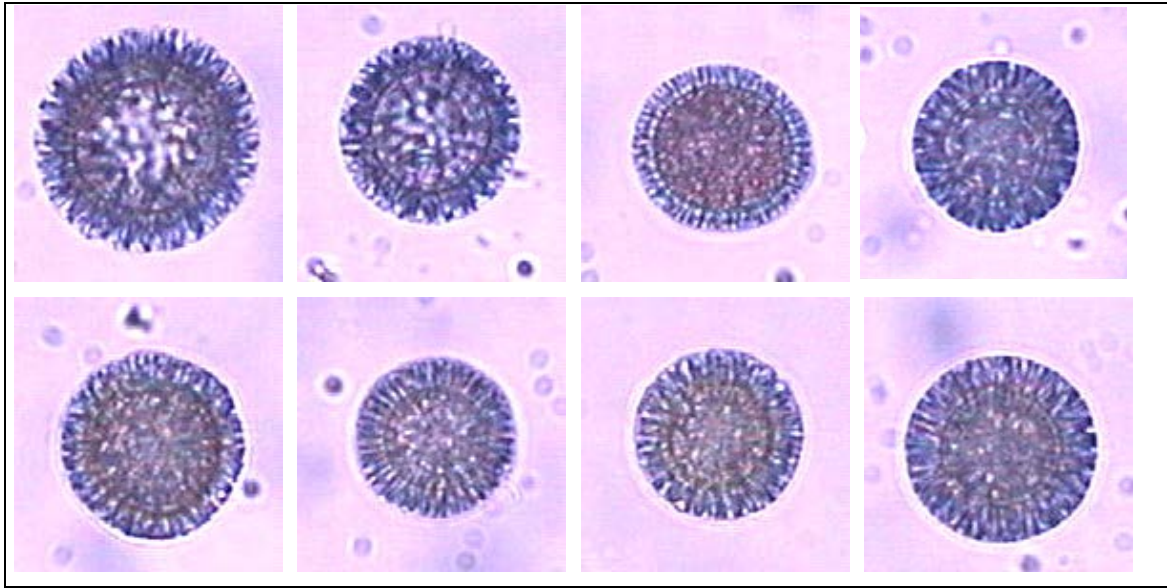
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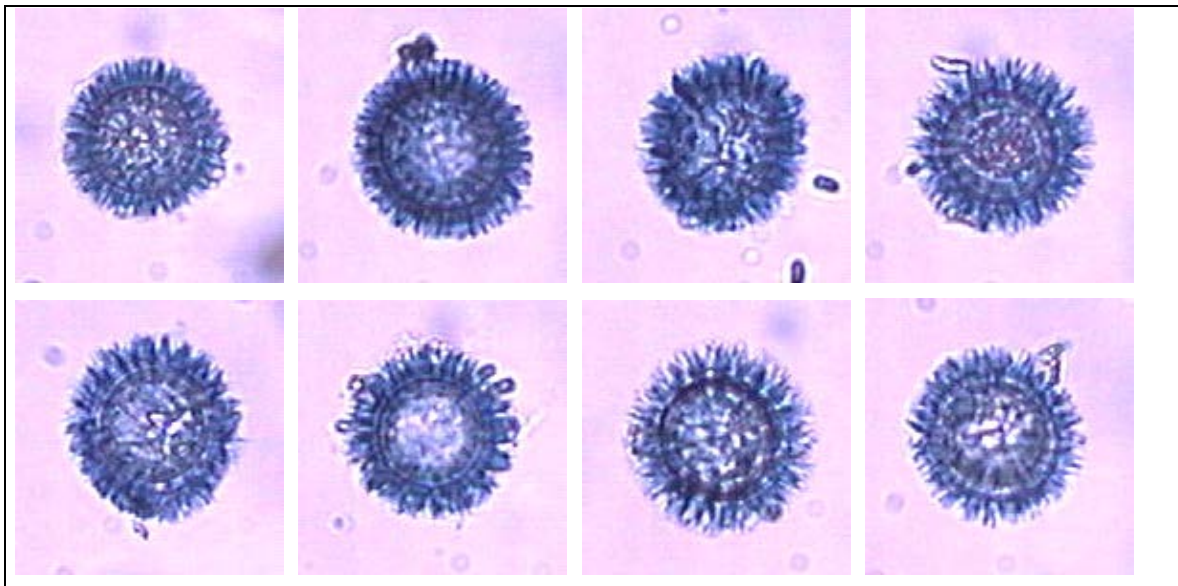
B: *Tilletia walkeri*

Figure 11. Teliospores of *Tilletia indica* (top) and *T. walkeri* (bottom) showing teliospore profiles in median view after bleaching and then staining with lactoglycerol-trypan blue. Note: The smoother outline on *T. indica* teliospores compared to the more irregular outline of *T. walkeri* teliospores with more obvious gaps between spines. Photos courtesy of Dr Alan Inman, Central Sciences Laboratory, York.
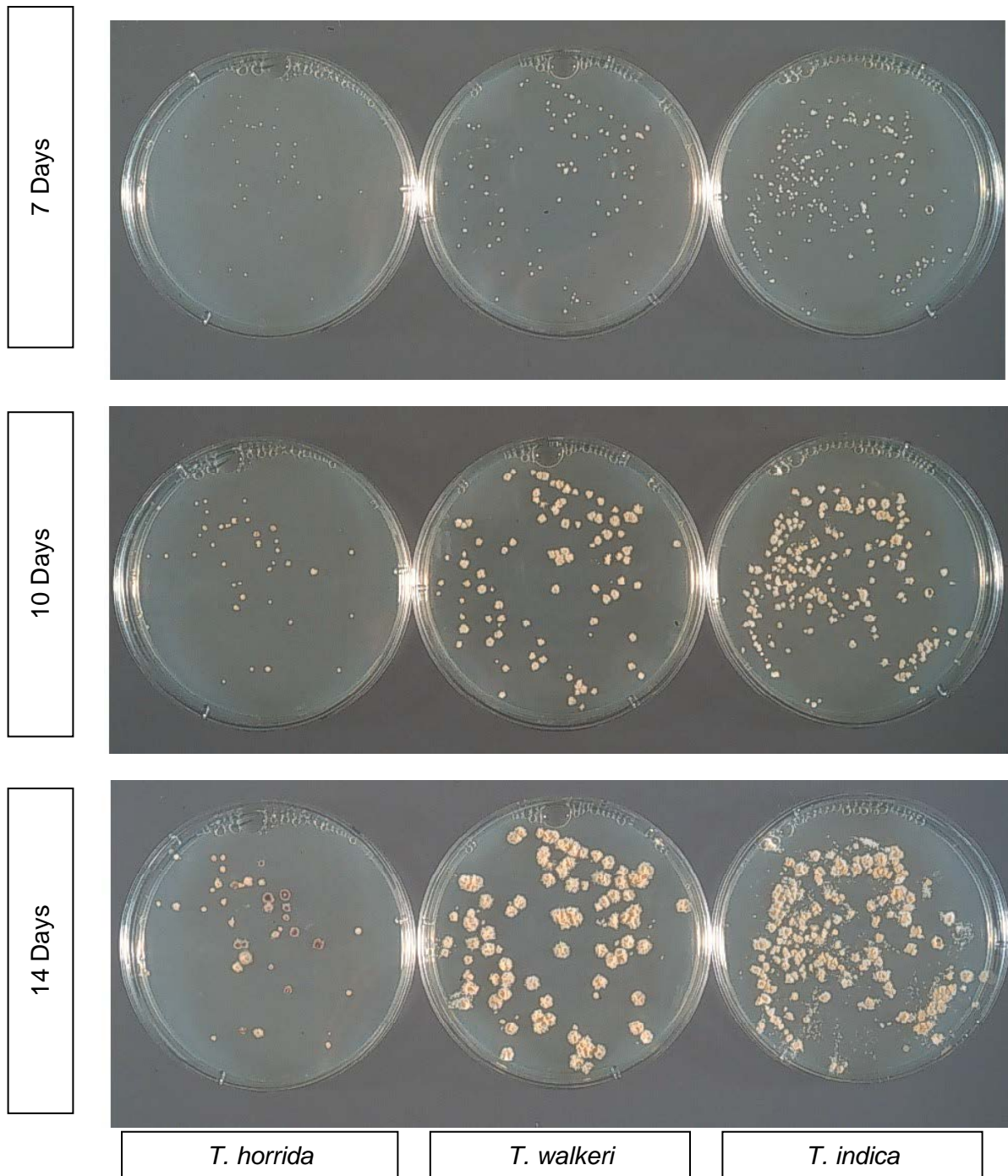
Figure 12. Colonies of *Tilletia indica* (right), *T. walkeri* (centre) and *T. horrida* (left) after 7 days (top), 10 days (centre) and 14 days (bottom) on PDA at 19°C and a 12 hour dark/light cycle. Note: slower growth, and purple pigmentation after 14 days, for *T horrida* colonies. Photos courtesy of Dr. Alan Inman, Central Sciences Laboratory.

4.2   Isolation and Germination of teliospores

There are now two methods available to confirm the identification of spores detected in the sieve wash test. There is the standard procedure of recovering the spores from the slide and a new procedure developed by Tan *et al.*, (2009) which enables PCR to be done directly on a single spore.

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 cover slips by washing them with distilled water over the 20 μm sieve. The spores are recovered from the sieve as in step 3.2 into a clean sterile conical centrifuge tube. The volume should be approx 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 incubating overnight, pellet the teliospores by centrifuging at 1200 x *g* for 3 min. The spores are then sterilised by removing the supernatant and suspending the pellet in 5 ml of bleach[3] (0.3–0.5% active NaOCl) and inverting the tube quickly three times and centrifuging at 1200 x *g* for 1 minute. The spores are then washed twice by removing the supernatant and resuspend the pellet in 1 ml sterile distilled water (SDW) and centrifuging at 1200 x *g* for 5 min.

The pellet is then plated onto 2% water agar plated with antibiotics (AWA) by resuspending in1 ml of SDW and placing 200 μL of the teliospore suspension aseptically onto the plates and spreading 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 or placing the plates 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 or liquid nutrient media, such as potato dextrose broth. Cut out small blocks of agar (1 x 1 cm) bearing germinated teliospores or colonies. Stick the agar blocks 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. 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. Remove each mycelial mat, with a sterile dissecting needle, 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.

Note: Germination of teliospores for molecular confirmation may not always be possible, for example if grain is treated with NaOH as in the case of examining fungicide treated grain. Increasing the number of sieved replicates may increase the number of teliospores recovered and hence the number of teliospores which can be germinated. Teliospores can have a period of dormancy which can effect germination (Carris *et al.*, 2006) and it should not be assumed that teliospores, which do not germinate, are not viable.

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[3] Instead of bleach, teliospores can be surface sterilized for 30 min in 5–10 ml of acidified electrolysed water (AEW). 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.

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, about 4–6 mm in diameter (Figure 12). In comparison, comparable cultures of *T. horrida* grow significantly more slowly (colonies only 2–3 mm in diameter) due to their higher temperature optima. *T. horrida* isolates may also produce a reddish-purple pigment (Figure 12), both on PDA and potato dextrose broth.

4.2.3 Recovery of a single teliospores

After examination of the teliospores and recording of the morphology, allow the slide to dry out, either with or without the cover slip on. When removing the cover slip place it on the slide upside down so it can be checked for teliospores adhering to it.

On another slide place a single piece of a cover slip (1 x 1 mm^2) that has been sterilised (autoclave at 121°C for 15 minutes). Place a 1 μL drop of TE buffer onto this piece of cover slip. Using either a compound or dissecting microscope, pick off a single teliospores using a very fine needle, and place this into the TE buffer droplet. The teliospores will transfer to the droplet. Make a sandwich by placing another small piece of a cover slip using forceps onto this. Using the forceps press down on the cover slip to crush the teliospores, and then transfer the glass sandwich into a 0.2 ml PCR tube.

4.3 Molecular confirmation

There are a number of molecular methods available to confirm the presumptive morphological diagnoses of *T. indica*. The three main protocols described rely upon the germination of the teliospores, so that DNA can be extracted from the mycelial mat produced. The germination of the teliospores can take up to three weeks. It is acceptable to use any one of the methods described below for confirmation of species. It is essential that reference material has been obtained from either Dr. Kelvin Hughes at the Central Science Laboratory, York, UK, or from Dr Mui-Keng Tan, NSW DPI. Please note: these methods work well, but are dependent upon the germination of the teliospores so that enough DNA can be extracted for the protocols to work. Peterson *et al.,* (2000) found that the average teliospore germination rate was 55%, which severely reduces the chances of identifying the teliospores by molecular methods.

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.*,2001). However, within the ITS1 region, *T. walkeri* has a diagnostically important restriction enzyme site (Sca 1) 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. walkeri* (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).

4.3.1 Restriction enzyme analysis of the ITS1 region

The target gene region is the internal transcribed spacers (ITS) of the nuclear ribosomal RNA gene. The PCR amplicon produced includes both ITS 1 and 2 and the conserved fragment 5.8 S. This amplicon is approximately 670 bp including primer sequences (Pimental et al, 1998). Oligonucleotides used: Forward primer ITS1 (5' TCC GTA GGT GAA CCT GCG G 3'); Reverse primer ITS4 (5'-TCC TCC GCT TAT TGA TAT GC 3') (White *et al.*, 1990).

DNA is extracted from mycelium.

1. Grind up the mycelium using a mortar and pestle or by placing approx. 0.1 g of mycelium in a sterile 2 ml microcentrifuge tube $^1/_3$ full with sterile 0.5 mm glass beads (Stratech) and 1 ml molecular grade water (MGW).
2. Seal tube with screw-lid containing an o-ring and oscillate the tube in a BeadBeater (Stratech) on ¼ power for 5 min.
3. Allow ground sample to stand for 30s then extract its DNA using a proprietary DNA extraction kit for fungi e.g. Nucleospin (Machery Nagel). No DNA cleanup is required.
4. Either use extracted DNA immediately, store overnight at 4°C or at - 20°C for longer periods.

Polymerase Chain Reaction (PCR) to produce restriction amplicon uses the following master mix (concentration per 50µl single reaction) and PCR cycling parameters (Table 4). Restriction of PCR amplicon is shown in Table 5.

Table 4. Quantitative PCR reaction master mix for detection of *T. indica.*

Reagent	Volume per reaction
Molecular Grade Water (MGW)	28.75 µl
10 X PCR buffer containing 15mM MgCl$_2$ (Applied Biosystems)	5.0 µl
dNTPs [10 mM] (dATP, dTTP, dCTP, dTTP) [final concentration of these are 0.2 mM each]	1.0 µl each
AmpliTaq [5U/µl] (Applied Biosystems)	1.25 µl
ITS1 Primer [5 µM] (5' TCC GTA GGT GAA CCT GCG G 3');	5.0 µl
ITS4 Primer [5 µM] (5'-TCC TCC GCT TAT TGA TAT GC 3')	5.0 µl
Extracted DNA	1 µl
Total reaction volume	**50 µl**

Conditions: 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 72°C extension step for 10 min.

Table 5. Restriction of PCR amplicon

Reagent	Volume per reaction
Molecular Grade Water (MGW)	7.3 µl
Restriction buffer (Promega)	2.0 µl
Bovine serum albumin [10µg/µl]	0.2 µl
Restriction enzyme, either Taq 1 or Sca 1 at 10 units /µl (Promega)	0.5 µl
Neat DNA amplicon solution [> 50 ng/µl DNA]	10.0 µl
Total reaction volume	**20.0 µl**

This is then incubated for three hours at 37°C, gently mixing the reaction by inversion during incubation. If required store restricted products at 4°C, before visualising as described below. As required mix 10 µl of reaction products with a running marker and run on a 2 % agarose gel.

4.3.1.1 Identification of species

A positive confirmation of *T. indica* is obtained if amplified test samples are cut with restriction enzyme Taq 1 to give five products (Figure 13) and there is no cut with Sca 1 (Figure 14). A positive confirmation of *T. walkeri* is obtained if amplified test samples are restricted with Taq 1 to give the same 5 fragments as with *T. indica* (Figure 14), but Sca 1 restricts amplified products to give two fragments (Figure 14). If the amplified product comes from *T. horrida*, Taq1 produces four DNA fragments (Figure 13) and Sca 1 produces no cuts (Figure 13). Other *Tilletia* species give different restriction patterns with these or other enzymes (see Pimentel *et al.*, 1998).

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

Figure 13. Schematic of PCR-RFLP patterns from the ITS region using restriction enzyme Taq1. Individual products shown as thick lines against product size (base pairs, bp.).

	T. indica	*T. walkeri*	*T. horrida*
	▬ 670 (NC)	520 ▬	▬ 670 (NC)
		140 ▬	
Total b.p.	670	660	670

Figure 14. Schematic of PCR-RFLP patterns from the its region using restriction enzyme Sca1. Individual products shown as thick lines against product size (base pairs, bp.); nc = not cut.

4.3.2 Conventional PCR assay using species specific primers

The assay is designed to using the mitrochondrial DNA producing an amplicon of 163 bp. Oligonucleotides used for *T. indica* are: Forward primer Tin 3 (5' CAA TGT TGG CGT GGC GGC GC 3') and reverse primer Tin 4 (5' CAA CTC CAG TGA TGG CTC CG 3') (Frederick *et al.*, 2000).

The Polymerase Chain Reaction (PCR) to produce restriction amplicon is shown in Table 6. PCR cycling parameters: 94°C denaturation for 1 min, 25 cycles of 94°C for 15s, 65°C for 15s and 72°C for 15s, 72°C extension step for 6 min. As required mix 10 µl of reaction products with a running marker and run on a 2 % gel.

When testing for *T. walkeri* the following primer is used: Replace Tin 3 with 0.1 µl of forward primer Tin 11 (5' TAA TGT TGG CGT GGC GGC AT 3') [25 µM]. This produces an amplicon of 163 bp.

Table 6. Restriction of PCR amplicon

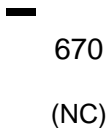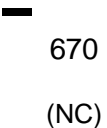| Reagent	Volume per reaction
Molecular Grade Water (MGW)	20.2 µl
10 X PCR buffer containing 15mM MgCl$_2$ (Applied Biosystems)	2.5 µl
dNTPs [10 mM] (dATP, dTTP, dCTP, dTTP) (final concentration of 0.1 mM each)	0.25 µl each
AmpliTaq [5U/µl] (Applied Biosystems),	0.1 µl
Tin 3 Primer [25 µM] (5' TCC GTA GGT GAA CCT GCG G 3');	0.1 µl
Tin 4 Primer [25 µM] (5'-TCC TCC GCT TAT TGA TAT GC 3')	0.1 µl
Neat extracted DNA	1.0 µl
Total reaction volume	**25.0 µl**

4.3.2.1 Identification of species

Positive reactions produce a single amplicon of 414 bp for both for *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 (c. 670 bp) will be produced when PCR amplicons are run out on an agarose gel. However, if amplification is still not produced, fresh DNA should then be extracted and retested.

4.3.3 PCR assay using species specific primers and fluorescent probe TaqMan system

The assay is designed to use the mitochondrial DNA producing an amplicon of 163 bp. Oligonucleotides used: The forward and reverse primers are the same as for conventional PCR (Tin 3 and Tin 4). Real-time probe: TaqMan probe [10 μM] (Applied Biosystems) [5'] (FAM label)- ATT CCC GGC TTC GGC GTC ACT- (TAMRA quencher) [3'].

Polymerase Chain Reaction to produce restriction amplicon uses the following mastermix (concentration per 25μl single reaction); 8.5 μL of MGW, 12.5 μL of 2 X universal TaqMan master mix, 1.0 μL of each primer [10 μM], 1.0 μL of TaqMan probe [10 μM], 1.0 μL neat extracted DNA obtained as described above. PCR cycling parameters: 50°C for 2 min, 95°C for 10 min, 34 cycles of 95°C for 15s 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*. Replace Tin 3 with 1.0 μl of forward primer Tin 11 (5' TAA TGT TGG CGT GGC GGC AT 3') [25 μM] to test for *T. walkeri*, which produces an amplicon of 163 bp.

4.3.3.1 Identification of species

T. indica produces amplification with primers Tin 3/Tin 4 while *T. walkeri* needs primers Tin11/Tin 4. If neither primer sets produce amplification, but control samples react as expected then the sample extractions belong to another *Tilletia* species, such as *T. horrida*. For example, when testing for *T. indica* and the Cts of the sample is >40, 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 Cts is >40, 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).

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 (c. 670 bp) will be produced when PCR amplicons are run out on an agarose gel. However, if amplification is still not produced, fresh DNA should then be extracted and retested.

4.3.4 Direct real-time PCR on teliospores

This assay is designed to use the internal transcribed spacer (ITS) that occurs between the nuclear small and large subunit ribosomal DNA (rDNA). It was found that the *Tilletia* species has 2 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 using real-time PCR and fluorescent probes identify the species of *Tilletia*. The ITS1 region in rDNA was targeted in this study for the design of the multiplex assay, which enables a five-plex fluorescent PCR assay to identify closely related *Tilletia* species detected in grain (Tan *et al.*, 2009).

4.3.4.1 Tilletia specific DNA amplification

An aliquot of the reaction mix is added to the PCR tube and using the same pipette tip crush the glass sandwich into pieces to release the spore material. <u>Precaution</u>: Ensure the PCR tube is not cut during the crushing.

Amplification of *Tilletia* specific DNA of various *Tilletia* species was performed with primers, MK56 and Tilletia-R (Tan and Murray 2006). Each PCR reaction was performed in 20 μL (single reaction). 20 μL of 1.5 mM MgCl$_2$, 200 μM of each of the four deoxynucleotides dATP, dTTP,

dCTP and dTTP; 0.5 μM of each of the primer pair and 0.5 U *Taq* DNA Polymerase (Invitrogen USA) in 1X buffer L (50 mM Tris, pH 9.0, 20 mM NaCl, 1% Trition X-100, 0.1% gelatin).

The thermal cycling parameters included an initial cycle of 95°C for 3 min, followed by 20 cycles of 94°C for 20 s, 63°C for 30 s, 72°C 30 s, with the annealing temperature decreased by1°C / cycle for 5 cycles to 59°C; and finally followed by a 10 minute and 1 minute incubation at72°C and 4°C respectively.

Expected fragment size is 260 bp. This fragment will not be visible in samples with a very low count of *Tilletia* spores (as low as one).

4.3.4.2 *Five-plex fluorescent PCR assay for identification of species*

Real-time PCR assays with the dual-labelled probes and oligonucleotide primers (Table 7) in 20 μL reactions in 0.1 ml microfuge tubes were performed in the Rotor Gene 6000 instrument (Corbett Research, Australia). The five-plex reaction mixture consisted of 1x ImmoBuffer (Bioline, UK), 5 mM MgCl$_2$, 200 μM of each of the four deoxynucleotides, dATP, dTTP, dCTP and dTTP, 1U Immolase DNA Polymerase (Bioline, UK) 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 2). The template DNA was one μL of PCR product from the PCR amplification of *Tilletia*-specific DNA or a known DNA concentration of a *Tilletia* sp.

The thermal cycling parameters included an initial cycle of 95^{0}C for 10 min, followed by 40 cycles of 94°C for 15 s, 65°C for 60 s with the annealing temperature decreased by 1°C / cycle for 6 cycles to 60°C. Dynamic tube normalisation option was used to determine the average background of each individual sample before amplification commences.   Fluorescence data was recorded to 5 channels (green, yellow, orange, red and crimson; Figure 15).

Table 7. Sequences and modifications of the primers and probes used in the multiple diagnostic assay for *T. indica* and other related *Tilletia* spp.

Primer Pairs (Sequence 5'-3')	Probes	Channel	Target
KB-DL-For: CTTCGGAAGAGTCTCCTT (nt.64-81[a])	ACGGAAGGAACGAGGC (nt. 105-120) (6-FAM, BHQ1)	Green	*T. indica*
KB-DL- Rev: CCGGACAGGTACTCAG (nt.127-142)	ACGGAAGGAACAAGGC (nt. 67-82) (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. horrida*
Tri-DL-For: ATTGCCGTACTTCTCTTC (nt.56-73[d]) Tri-DL-Rev: GTAGTCTTGTGTTTGGATAATAG (nt. 99-112)	AGAGGTCGGCTCTAATCCCATCA (nt. 75-97) (Quasar 670, BHQ2)	Red	Broad range*
Ehr-DL-For: CGCATTCTTATGCTTCTTG (nt.72-90[e]) Ehr-DL-Rev: GTTAGGAACCAAAGCCATC (nt.128-146)	CAGAGTCATTGGTTCTTCGGAGC (nt. 104-126) (Quasar 705, BHQ2)	Crimson	*T. ehrhartae*

*Includes *T. caries*, *T. laevis*, *T. contraversa*, *T. fusca*, *T. bromi, T. goloskokovii* GenBank No.
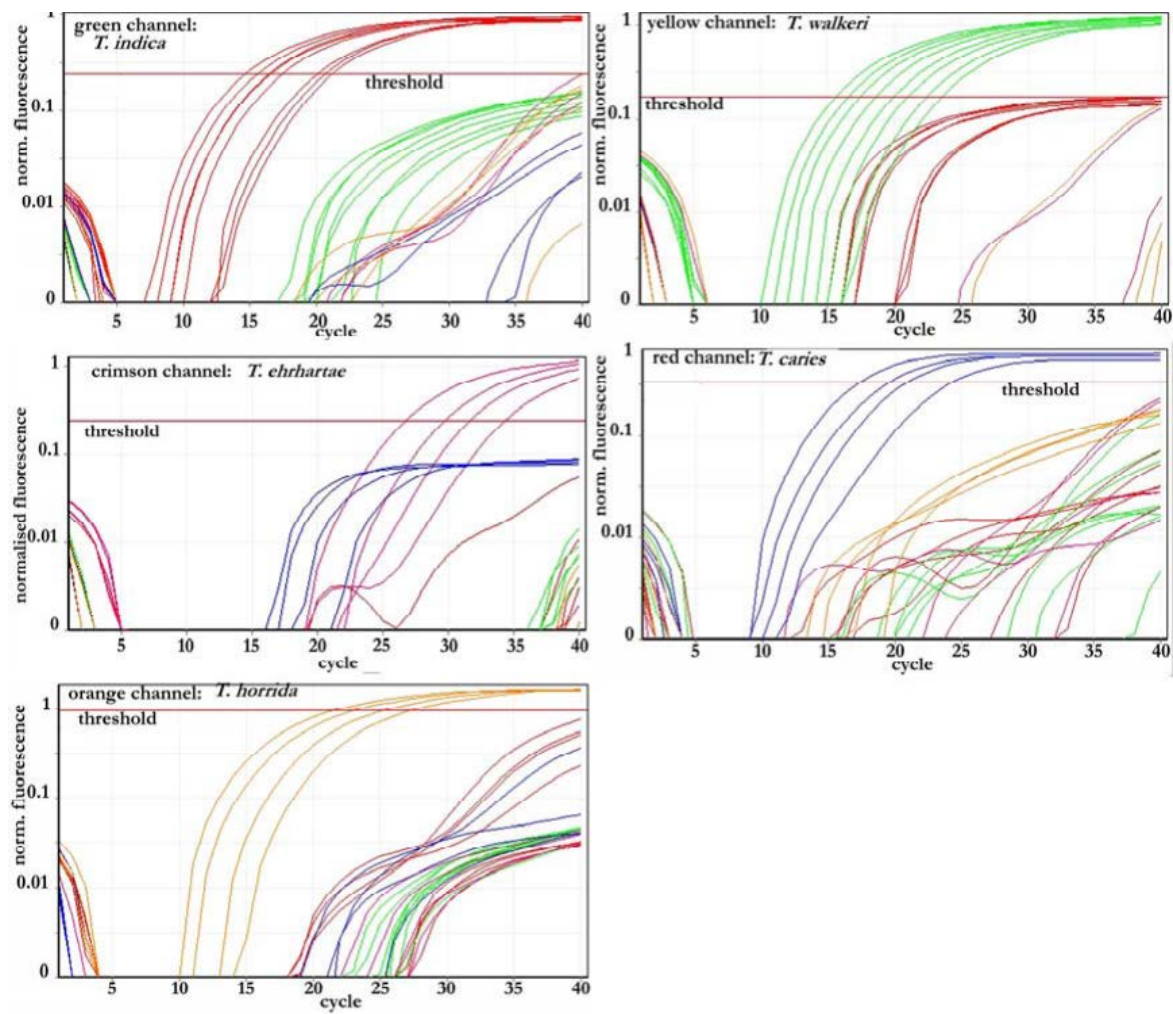[a]AF398434, [b]AF310180, [c]AF310171, [d]AF398447, [e]AY770433

Figure 15. Fluorescence data acquired simultaneously by five channels in Rotor Gene 6000 and normalised to a log scale for *T. indica* (P2), *T. walkeri* (Tw4), *T. ehrhartae* (BRIP45363), *T. horrida* (Th2) and *T. caries* (S6) in a five-plex real-time PCR assay.

5 Records

The report on the diagnosis should include the following information:

- Quantity of the consignment or lot including consignment number etc for trace back.

- The number of positive subsamples and the estimated number of teliospores detected in each positive subsample)

- 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 real-time PCR and TaqMan assays, the CT-value should be noted and DNA extractions should be stored at −80 °C.

6 Contact points for further information

Further information on this organism can be obtained from:

Dr Kelvin Hughes, Pest and Disease Identification Team, Central Science Laboratory, Sand Hutton, York YO41 1LZ, United Kingdom. Email: k.hughes@csl.gov.uk

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Mr Gary Peterson, Research Biologist, USDA ARS NAA, Fort Detrick MD 21702 USA. Email: gary.peterson@ars.usda.gov

7 Acknowledgements

This basis of this protocol was originally drafted by: Alan Inman, Kelvin Hughes and Richard Bowyer (2003) Central Science Laboratory, York (GB). This protocol was ring-tested in different European laboratories[4]. Dominie Wright, Gordon Murray and Mui-Keng Tan (2003) then developed the first national diagnostic protocol for Australia with their help.

This current National Diagnostic Protocol (NDP) is based on the International Diagnostic Protocol that is currently being completed for IPPC by D.G Wright, Department of Agriculture and Food, Western Australia, Australia; K.J.D Hughes, Central Science Laboratory, Sand Hutton, York, United Kingdom and Guiming Zhang, Laboratory of Plant Inspection and Quarantine, Shenzhen City, 518045 Guangdong Province, China.

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