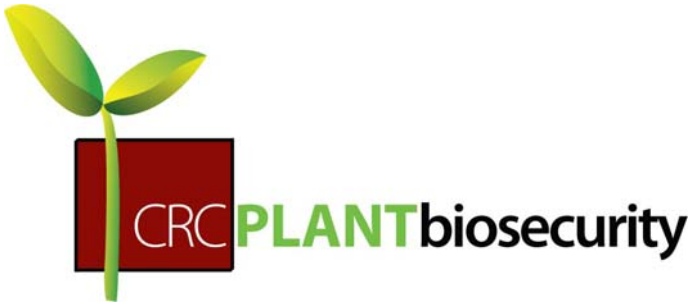


Cooperative Research Centre
for National Plant Biosecurity

Final Report

CRC20004

Enhancing the detection of *Tilletia indica*, the cause of Karnal bunt

Dr. Mui-Keng Tan and Ms Domine Wright

30 June 2009

Project Leader contact details:

Name: Dr. Mui-Keng Tan
Address: Industy and Investment NSW, EMAI, Private Bag 4008, NARELLAN 2567, NSW.
61 2 46406445 (Ph)
61 2 46406300 (Fax)
mui-keng.tan@industry.nsw.gov.au (email)

CRCNPB contact details:

Cooperative Research Centre for National Plant Biosecurity
LPO Box 5012
Bruce ACT 5012

Phone: +61 (0)2 6201 2882
Fax: +61 (0)2 6201 5067
Email: info@crcplantbiosecurity.com.au
Web: www.crcplantbiosecurity.com.au

Table of contents

1. Executive Summary

Tilletia indica, the cause of Karnal bunt of wheat, is the target of strict quarantine regulations by most wheat growing countries and its presence raises trade barriers to wheat exports. The international diagnostic protocol currently used in Australia involves the tentative identification of the spores based on morphology followed by germination of the spores and a molecular protocol to confirm the identity. Quarantine is extremely important to Australia as we are free of Karnal Bunt. This project reports on the development of an enhanced protocol to address the limitations in the current protocol. These limitations include a) the identification of morphologically similar species of *T. indica* and *T. walkeri* when there are less than 10 spores present in the sample and b) the germination of spores for molecular confirmation have found to be inconsistent and adds another 14 days to the test before confirmation of identification can occur.

A molecular assay has been developed which possesses the sensitivity and specificity to detect a small number (<5) of spores from grain washings as well as on genetic material directly released from a single spore. The one tube molecular assay is able to detect and identify *T. indica* and other grass bunts including T*. walkeri, T. ehrhartae, T. horrida* and a group of species with very similar morphology to the type species, *T. caries*. The ability of the protocol to distinguish *T. indica* and *T. walkeri* which differ by only one nucleotide difference at the target genomic region would make less related (i.e. possess more nucleotide differences) fungal species, e.g. rust spores that may be found in wheat grains unlikely to have a cross-reaction in the protocol.

Microscopy is currently used to detect and identify *Tilletia* spores in grain samples. The incorporation of the sensitive molecular assay to substitute the microscopy step in the detection of suspect samples would relieve mycologists of the tedious, repetitive task of the microscopic examination of grain samples. The capability of the assay for direct molecular diagnosis of a single spore eliminates the germination step required for confirmation in the current protocol. Confirmation in the enhanced protocol also includes a microscopy step to provide physical evidence of teliospores with morphological criteria of *T. indica*. This will ensure a near zero-risk of a false positive that may be generated in a highly sensitive molecular technique.

An economic analysis has been performed to compare the economic costs of the current and the enhanced protocol and their relative performance in different scenarios. The enhanced protocol is significantly more economical and sensitive in surveillance situations where the pathogen is not known to be present. The enhanced protocol reduces the elapsed time before a definitive identification can be achieved when international trade is interrupted which would result in savings to the industry. The results indicated that the current protocol would be more economical in the monitoring and identification of *T. indica* in current infested areas.

A national and an international ring test involving only *T. indica* and *T. walkeri*, the two closely related *Tilletia* species have attested to the reliability and accuracy of the protocol.

The protocol is intended for use by scientific technical officers or scientists with a high proficiency in the use and interpretation of real-time PCR methodology in routine molecular diagnosis, in addition to expertise in the morphological identification and differentiation of *T. indica* and closely related *Tilletia*species. Ideally, diagnosticians should undertake training with the authors of this report before using the protocol for routine diagnosis.

2. Aims and objectives

Karnal bunt (KB) of wheat is caused by the smut fungus, *Tilletia indica*. It replaces part of the seed with a black powdery mass containing millions of spores and produces a strong unpleasant odour like rotten fish. This is due to the production of trimethylamine that makes it unpalatable. The fishy smell has drastic consequences for the marketability of wheat. The fungus is thus subjected to very strict quarantine regulations in Australia and other countries not known to have the pathogen.

T. indica was added to the European Commission Plant Health Directive 77/93/EEC (now 2000/29/EC) as a I /AI pest in 1996 and phytosanitary requirements applied to seed and grain of *Triticum*, *Secale* and *X Triticosecale* imported from countries where *T. indica* is known to occur.

The pathogen is difficult to detect in the field as the heads with infected seeds do not differ in appearance from healthy heads and not all of the heads in a crop are infected, and not all of the grains in a head are infected. Thus infected grain is not detected until after harvest and in some situations, the partially bunted grain is broken up and can be mistaken for common bunt caused by *T. caries.* Because the partially bunted grain is broken up during harvest, the spores of *T. indica* are released and caused contamination of clean grain during the harvest. Cross contamination of clean grain also occurs during transport of the grain.

Its overseas history shows that an incursion can cause the pathogen to spread across thousands of kilometres. KB was first described in Karnal, India in 1931. It was reported in Mexico in 1972, Arizona, California and Texas in 1996 and in South Africa in 2000. The threat of KB to Australia from the dispersal of spores is low but clearly possible given Australia imports grain commodities and grain handling machinery and has a large number of travellers between Australia and countries where KB has been known to occur such as USA and India.

Research in India had shown that relative humidity and temperature from heading to anthesis are the two most important factors that correlate with the disease level. Modelling studies have shown that conditions from heading to anthesis are favourable for the establishment of KB in large parts of Europe and Australia once spores gain entry into the countries.

The economic impact of a KB incursion is extreme. It has the potential to reduce grain quality and, through quarantine regulations, restrict the sale of Australian wheat to overseas buyers. The combined cost of quality loss and sale restrictions was estimated to be 18% of the value of Australian production ($490,900,000 per year) in a study by Murray and Brennan (1998). A study by Stansbury and McKirdy (2002) estimated the potential loss in Western Australia to range from 8 to 25% of the value of production.

There are thus enormous economic benefits to implement strategies that will reduce risk of the disease to a zero-tolerance level in Australia where there is currently no record of the disease. Strategies would include surveillance at entry and within the country in conjunction with the availability of very sensitive and accurate diagnostic tools.

A good detection protocol for a quarantine pathogen must meet two important criteria: the sensitivity criterion to detect very small or low quantities of the pathogen and the specificity criterion to identify the true pathogen and differentiate from morphologically similar and closely related pathogens. *Tilletia* spp. reported as contaminants in Australian wheat grain included *T. caries*, *T. laevis*, *T. ehrhartae* and *T. walkeri*. Conventional taxonomic separation based on colour, spore sizes and ornamentation is labour intensive and requires considerable expertise and a significant quantity of ~50 spores of each species for a statistically valid result.

T. walkeri is morphologically similar to *T. indica* and infects ryegrass but does not infect wheat and so unequivocal differentiation from *T. indica* is critical from a quarantine perspective. *T. ehrhartae* infects *Ehrharta calycina* (perennial veldt grass) and has smaller spores but has similar surface ornamentations. The frequent presence of spores of both *T. ehrhartae* and *T. walkeri* in Australian wheat grain increases the risk for possible misidentifications if only a very few spores are detected.

The current national diagnostic protocol (Wright et al. 2003) involves the tentative identification of the spores based on morphology followed by germination and a molecular protocol to confirm the identity. Molecular methods in Wright et al. 2003 require the germination of spores to provide the template DNA for molecular confirmation. Germination of *Tilletia* spores takes at least two weeks and in some cases the spores do not germinate. The reason for this is not understood. The existing molecular protocols have not included the simultaneous identification of other common contaminating grass bunts like *T. caries* (common bunt), *T. horrida* and *T. ehrhartae* in the same assay for *T. indica* and *T. walkeri*.

The objectives of the project were to:

- develop a procedure for the extraction of spores from other commodities that may be contaminated with wheat and from grain dust
- develop a cost-effective, reliable and highly sensitive molecular assay to identify and differentiate *T. indica* and other common grass bunts found in wheat grain that does not require spore germination, and
- develop an enhanced protocol for the quarantine detection of *T. indica* and d) to compare the economic costs of the current and enhanced protocol in an incursion scenario.

3. Key findings

3.1 Improved extraction of spores from grain and other commodities (Wright D.)

Seed lots should be sampled according to current International Seed Testing Association (ISTA) rules (ISTA 2006). Grain, for feed or processing, is typically more difficult to sample than grain for planting because consignments are usually very large, and transported or stored as large, loose bulks. However, for monitoring purposes, grain should be sampled in an appropriate fashion to produce a 1–2 kg thoroughly mixed sample representative of the consignment.

For phytosanitary purposes, detection of *T. indica* is best achieved by a wash test (CABI/EPPO, 1997); because infected parts of the grain typically disintegrate so that the teliospores contaminate other grains in the lot. The most efficient and rapid wash test method for detecting teliospores in a sample is a size-selective sieving and centrifugation technique (Inman *et al.* 2003, Wright *et al.* 2003). This method has, on average, an 82% efficiency of recovery and microscopic examinations typically require only a few slides per 50 g subsample. The number of replicate 50 g subsamples needed to detect one spore to 99% confidence level is three (Peterson *et al*. 2000).

A: The development of new sieves

Currently the sieves used in the national diagnostic protocol (Wright *et al* 2003) are made by hand, and after a number of uses the mesh in the sieves disintegrates due to the use of bleach to sterilise them. We investigated the manufacture of plastic moulded sieves in the correct size as well as development of new sieves in which the mesh could be replaced easily.

The cost of developing and manufacturing plastic moulded sieves was prohibitive and this course was not pursued further. For example, one company quoted $50 000 to develop the moulds and then there were additional costs for the manufacture of the sieves. Many of the companies wanted a standing order, which of course we could not supply.

The making of the sieves by hand, as before, using local plumbing supplies was investigated to incorporate a sleeve that would hold the mesh in place and allow the mesh to be replaced when required. After many attempts some sieves were made. However, when compared with the existing sieves, it was found that the extraction efficiency of the prototype sieves was significantly decreased.

Extraction efficiencies were tested using spiked grain samples with a known number of spores. The total number of spores detected in the prototype sieves was consistently lower than in the existing sieves. This suggested that the spores were being lost either along the rim of the prototype sieves or through the mesh of the sieves. The mesh is regularly checked for spores and it was found with the prototype sieves that the mesh was not tight enough and spores were being left behind on the mesh.

We concluded that the most cost effective, efficient and robust extraction process is to regularly replace the existing hand made sieves. Material costs are relatively small and they can be manufactured without the need for expensive moulds or other machinery.

B: Increasing the efficiency of examining pellets

In the national diagnostic protocol (Wright *et al* 2003), examination of the pellet after washing the grain, is very time consuming due to the presence of other *Tilletia* species, fungal spores and other contaminants. Therefore, methods to reduce the presence of other fungal spores and contaminants in the pellet were investigated

A number of options were examined to decrease the time taken to examine the pellet under the microscope for spores, and to separate the teliospores more selectively, thus making it easier for identification. This included the use of different sieves (based on micron sizes), density gradients, and stains.

Selective sieve sizes

In the national diagnostic protocol (Wright *et al* 2003), 15 and 50 µm sieves are used to catch fungal spores and pollen that is washed from the grain sample. The 15 µm sieve is used so that *Tilletia caries* and *T. laevis* can be detected in the sample. If a 20 µm sieve was used these pathogens would not be detected. The two sieves are stacked on top of each other in the protocol, so the use of more mesh sizes between these two sieves was investigated.

The following sieve sizes were tested; 44 µm, 38 µm, 30 µm, 25 µm, and 20 µm. These sizes were selected based upon the spore size of *Tilletia* species most likely to be detected within the sample.

The national diagnostic protocol was followed with this minor amendment of the extra sieves. Two stacks were required due to stability problems when trying to stack seven sieves on top of each other. One stack was made up with the 50, 44, 38 and 30 µm sieves and the other stack contained the 25, 20 and 15 µm sieves (Figure 3.1A). The grain washings were passed through the first stack of sieves. The water was collected from this washing and then passed through the second stack of sieves. The flask was rinsed to ensure no spores were left behind in the process. Each sieve was then rinsed into a centrifuge tube and completed the steps within the protocol. Pellets obtained at the end of the process were then examined under the microscope.

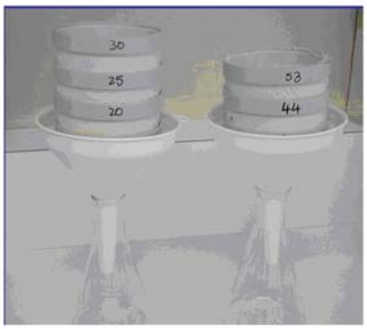


Figure 3.1A: New stack sieves for extraction of spores from grain washings

A time trial was then set up to examine the feasibility of using the new method. The following was noted during the time trial:

a) Washing the grain samples using the standard sieves was faster than using the two stack system. This was because the water drained faster through the 50 and 15 µm stack than the other stacks with more sieves.
b) Less water was required for washing the grain with the standard system compared to the new stacking system.
c) Having to use two stacks of the new sieves meant that the amount of equipment used was doubled and more bench space was required.
d) The number of samples that could be processed concurrently was reduced due to the space and equipment issue. For example, it is normally possible to wash out two grain samples with three reps each at the same time. Using the new stacked sieves, one sample with three reps was difficult to manage.
e) The time taken to examine the pellet from each sieve layer was faster and easier, than examining the single pellet from the standard sieve set up. However, the total time taken to examine the pellet from each layer was greater than from the standard sieve set up (Table 3.1A).

Table 3.1A. The average time in minutes to look at one slide of each pellet from the different sieve sizes tested. The trial was repeated three times with the same operator each time.

	Time (minutes)			
Layer (µm)	64A (Seven sieves set up)	68B (standard sieves set up)	34A (Seven sieves set up)	34B (standard set up)
44	4.29		3.10	
38	3.03		4.16	
30	3.29		4.36	
25	4.19		5.38	
20	3.59		6.00	
15	5.05	11.28	6.40	7.34
Total time	**23.44**	**11.28**	**29.40**	**7.34**

Sucrose gradients

Sucrose gradients are used in plant pathology as a tool to extract and separate particles of different sizes from soils and other biological material. For example, nematodes can be extracted from soils using sucrose gradient combined with centrifugation (Southey 1986) and the same with vesicular arbuscular mycorrhizas (Schenk 1982), and for protein and cellular organelle isolation (Murray 2007). The particles, in this case the spores, move to a layer in the gradient at which the density of the spore matches that of the surrounding sucrose. As we could not predetermine the density of the spore, experiments were set with a range of gradients to see if the different fungal species could be separated by density.

Sucrose gradients were made with layers at the following concentrations; 5, 13, 22, 31 and 44%, (top to bottom) in 15 mL centrifuge tubes (Figure 3.1B). These densities were the most widely found to be used in various applications. Tubes were inoculated with 50 spores of *T. caries*, *T. ehrhartae*, and *T. laevis*. The tubes were then left in the cold room overnight (12-16hrs) to allow the spores to settle and a linear gradient to form with the sucrose. Samples were then centrifuged at 1100 rpm at 4°C for 30 minutes. Each layer was then examined for presence of spores and other contaminants.

Upon analysis of these tubes, it was found that there was no stratification achieved and that the spores were throughout the 13, 22 and 31% layers, hence there was no observable difference in the composition of each layer. The other difficulty with this system is that the tubes need to be kept cold for the sucrose to remain in their density gradients, and the layering was not easy to achieve. This work was repeated a number of times and it was possible to get the layering to happen with practice. After analysing the layers for presence of spores we found that the densities chosen for the sucrose layers were not great enough for separation of the spores. Thus, we concluded that the chosen sucrose gradients cannot be readily applied for the separation of the spores of *Tilletia* species.

Figure 3.1B. Sucrose gradients in 50 mL centrifuge tubes

Use of mycological stains and other water soluble ink dyes for staining

The selective sieve wash protocol in the national diagnostic protocol (Wright *et al* 2003) produces a pellet which , contains plant pollen of many plant species in addition to fungal spores. For example, pollen of *Acacia* and *Pinus* species are often seen. These are often difficult to distinguish from fungal spores because they may have similar shape and ornamentation and consequently increase the time taken to examine the pellet. The use of a pollen stain [Calberla's solution(5 ml glycerol, 10 ml 95% ethanol, 15 ml distilled water, two drops saturated, aqueous solution of basic fuchsin)], (Fiorella 2007) removed some of this confusion. Pollen in the pellet stained pink and could be readily ignored when examining the

slides (Figure 3.1C). We concluded that two drops of the pollen stain were best added to the pellet in the centrifuge tube before examination.

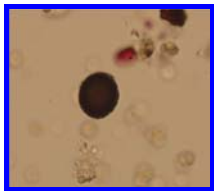


Figure 3.1C. The use of pollen stain when examining pellet

Further use of staining was explored, to determine whether *Tilletia* spores could be selectively stained. Iodine is a starch stain and was tested to determine whether starch grains released from seed during the washing process could be ignored. Starch is released during the washing process as the wheat examined in Australia tends to be a soft wheat rather than a hard wheat. Cotton blue was also tested, it highlighted the ornamentation on fungal spores. Neither of these stains was very successful, as the iodine made it difficult to detect the darker coloured spores such as *T. walkeri* and the cotton blue only helped with highlighting the lighter coloured spores such as rust, or *T. caries.*

Various water soluble inks were then tried to see if having a coloured background would help with observing the ornamentation of the *Tilletia* spores. The colours chosen were blue, green and red. The most successful colour out of these three was green ink (Figure 3.1D) which provided a good contrast background, did not interfere with use of the pollen stain and both dark and light coloured spores were easily detected. The ornamentation of spores was also easy to define. Both the red and blue ink reduced the clarity of the spore ornamentation and colour, thus making detection of the spores more difficult. The red background was also found to be quite hard on the eyes when examining a number of slides.

In conclusion, the examination of pellets from washed grain is enhanced by the use of pollen stain and green water soluble ink. All grain sample pellets that are examined at Department of Agriculture and Food, Western Australia, have pollen stain added to the pellet and a droplet of green ink added to the slide before examining under the microscope.

The above work was presented as a poster at the CRCNPB Science Exchange in November 2007 (Appendix 1).

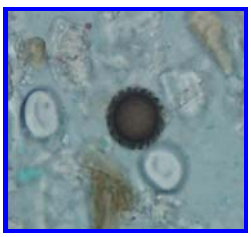


Figure 3.1D. Green ink background highlighting spores found in a pellet.

C: Methods examined for the removal of dust and other contaminants in the pellet

Methods to test other bulk commodities where wheat grain could be a contaminant were also required in case of an incursion. In the case of an incursion, being able to test a large area rapidly was thought to be prudent. The ability to examine screenings from seed cleaners was investigated along with dust removed from filters in silos. To be able to do this modifications and enhancements to the national diagnostic protocol would be required. The ability to examine grain dust for the presence of spores would also be useful for testing shipping containers that had not been cleaned correctly. The other application that this methodology could be used for is to be able to carry out large scale survey work.

The ability to examine grain dust for spores was thought to be an important modification required as it allowed filters from silos, harvesters, and dust at the bottom of sea containers to be tested. Testing of the sea containers was important as it is a possible pathway for *T. indica* to enter Australia as these are often used for international grain shipments. Quite often the containers are not cleaned properly before the next shipment occurs. The dust used in the following experiments was collected from a filter at a train loading point from a Co-operative Bulk Handling silo. The dust was spiked with a known number of *Tilletia* spores.

The dust contained all sort of particles that ranged in size, and could not be identified. Quite a lot of the dust particles were collected on the 15 µm sieve and hence made it difficult to look at the spores. These unidentifiable particles will be referred to as debris. We conducted experiments with density gradient solutions and differential centrifugation with the aim of separating debris from fungal spores and to produce a smaller and less complex pellet to examine.

Sucrose gradients

The sucrose gradient protocol described above was used. Spores of *T. ehrhartae* were added to the dust sample. Tween 20 was required to make the dust hydrophilic rather than hydrophobic. The dust sample was mixed into a Tween 20 solution (5 mL) and then washed through a 50µm sieve before centrifuging and removing the supernatant. A further 5 mL of Tween 20 was added to the pellet, before adding the pellet to the sucrose gradient. Analysis showed that debris from the dust and pollen was found in all layers except for the 40% layer. A pellet was formed below the 40% layer where over 75% of the spores were detected. However, there was still debris present in the pellet making identification of the spores difficult. Both the spores and debris had greater density than those present in the sucrose gradient, therefore we concluded that a more saturated solution was required.

Supersaturated sucrose gradients

Supersaturated sucrose gradients (40%, 50%, 60%, 70% and 80% concentration) and the effect of adding Tween 20 to the sucrose solution was examined. The above protocol used with the sucrose gradient work was repeated using supersaturated sucrose gradients. The dust sample was mixed into a Tween 20 solution (5 mL) and then washed through a 50µm

sieve before centrifuging and removing the supernatant. A further 5 mL of Tween 20 was added to the pellet, before adding the pellet to the sucrose gradient. All density layers were examined for the presence of spores.

The results found that a pellet still did form below the 80% layer. The majority of the spores were detected in this layer, however, spores had settled throughout all layers. This was not satisfactory as it created more work as all layers had to be examined for spores and the spores did not settle out according to species. In the bottom pellet, dust and debris could still be detected in the sample making spore identification difficult.

To determine if there was an effect from adding Tween 20 to the sucrose gradients, Tween 20 was added either to the bottom of the sucrose layers or on top of the layers. The tubes were then centrifuged at 500 RCF for five minutes at 4°C for 10 minutes. In each case the Tween layer rose above the sucrose gradient layers. So we concluded that the Tween 20 was not effecting the density gradients made.

In conclusion, the use of sucrose gradients and supersaturated gradients did not provide a suitable method for separating spores based on species. It also did not provide a suitable means separating the spores from the dust.

Salt concentration gradients

The above experiment for the sucrose gradient was repeated using salt gradients. The concentrations of the salt gradients were 5%, 8.75%, 12.5%, 16.25% and 20% based on what had been tried for vesicular-arbuscular mycorrhizal work (Schenk 1982). A gradient of 40% was unobtainable as the solution became supersaturated.

The results from this work were not very satisfactory. The majority of the spores ended up in the bottom of the solution and some of the spores that were added to the solution were not detected. The spores may have broken up due to the osmotic potential of the solutions. The reason for the loss of the spores could not be determined.

Glycerol centrifugation

The above methods (sucrose and salt gradients) were not suitable for the removal of spores from grain dust. The extraction of VAM propagules from soil relies upon various methods such as differential sieving, and glycerol centrifugation (Schenk, 1982). The glycerol centrifugation technique was found to remove the lighter organic components of the soil.

As above with the VAM work, we found that by centrifuging the dust in 50% glycerol solution caused the lighter particles to float to the surface of the tube. The spores and heavier particles settled to the bottom of the tube. No spores were detected in the lighter particles on the surface of the tubes. This step removed the problems with the dust clogging up the sieves and the loss of spores consequently. However, analysis of the pellet showed that there was still a large amount of debris present, and this made it difficult to identify spores.

Thus, a second suspension in 50% glycerol was tried after collecting the contents found on the 15 µm sieve. A slow spin speed was selected to try and remove some of the heavier particles from the supernatant and keep the spores in suspension. The pellet is kept just in case it

needs to be examined for spores that have become attached to the heavier particles. The third spin at a higher speed gave a pellet that contained the spores. Results found that a greater recovery of the spores was achieved by resuspending the particles and spores collected after the standard wash test. Very few spores were found in the supernatant and >90% of the spores were detected in the final pellet. Very little of the dust debris was found in the pellet. Although, this method required extra washing and further centrifugation steps it was found to be reliable and removed most of the debris associated with examining dust for spores.

The recommended protocol for grain dust samples

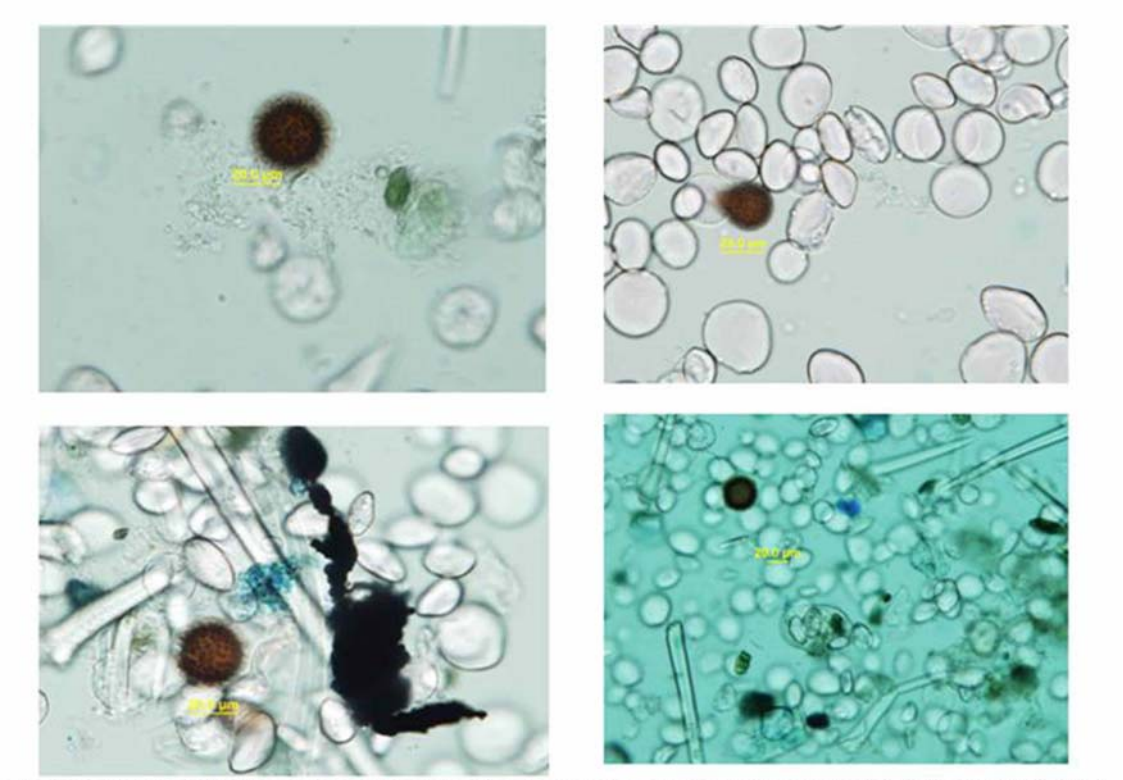
The dust for analysis was weighed out (0.5g) into a 50 mL centrifuge tube and 50 mL of glycerol (50%) added to it. This was then centrifuged for three mins at 1000 x *g*. After spinning, the debris floating on the surface of the tube was removed and discarded. The supernatant and pellet remaining in the tube are then processed according to the standard diagnostic protocol using a selective sieve wash method (Wright *et al* 2003). However, the following steps are required after washing the debris and spores from the 15 µm sieve into a 50 mL centrifuge tube. Remove the supernatant after spinning at 1000 x *g* for three minutes, and add 50 mL glycerol solution (50%) to the tube. Centrifuge the tubes at <500 x *g* for three minutes. The pellet in this step is kept for further analysis if required while the supernatant undergoes the extraction process again. The supernatant is transferred to another 50 mL centrifuge tube and re-spun at 1000 x *g* for three minutes. The supernatant is discarded and the pellet is examined for identification and detection of *Tilletia* spores.

Glycerol centrifugation is the preferred method has been written up and was presented at the enhanced protocol workshops both nationally and internationally (Section 3.5).

3.2 The development of a sensitive molecular assay that does not require germination (Tan M-K)

This project has targeted a highly repeated element in the genome to develop a molecular assay for the detection and identification of *T. indica* and other *Tilletia* species in wheat grains. The repetitive element is located in long tandem arrays at one or a few chromosomal locations representing the nucleolar organiser regions in eukaryotes. This study has found that the very high copy numbers facilitated the amplification of a sufficient concentration of target template DNA from one un-germinated spore for downstream diagnosis by real time PCR.

DNA templates for molecular analysis include total DNA extracts from materials in grain washings and DNA released from a single spore. Grain washings contain a large amount of starch granules and biological particles including pollen grains, rust spores and various grass bunt spores (Fig. 3.2A). A single spore assay is performed by picking a suspect spore and placed between two pieces of ~3-4 mm^2 coverslip glass with the aid of a microscope. The glass sandwich is pressed tight with a pair of forceps to crush the spore to release DNA. The sandwich containing the crushed spore is added into reaction mix and the glass sandwich is then crushed with a pipette tip to release the DNA for amplification in the PCR. Full details are in Tan et al. 2009.



Grain washings contain spores, numerous starch granules, pollen grains and other biological particles

Fig. 3.2A

The molecular protocol consists of firstly an amplification step to enrich *Tilletia*-specific DNA from a low concentration of *Tilletia*-specific DNA in a total DNA extract or a single spore (Fig. 3.2B). The pair of primers was designed to amplify the DNA fragment from *Tilletia* species only. *Tilletia* species amplified in this work included *T. indica*, *T. walkeri*, *T. horrida*, *T. caries*, *T. laevis*, *T. ehrhartae*, *T. bromi* and *T. contraversa*. Only twenty amplification cycles (as opposed to >35 cycles in a standard PCR reaction) are used in the enrichment step to avoid the over-representation of a species in a DNA mixture of *Tilletia* sp.

The second step is the multiplex fluorescent assay of the amplicon from the enrichment step to identify and differentiate four species, *T. indica*, *T. walkeri*, *T. horrida* and *T. ehrhartae* and one group of species with very similar morphology to the type species, *T. caries*. Species-specific regions on the *Tilletia*-specific fragment were targeted to design probes for real-time PCR analysis (Fig. 3.2B). Five target regions were used as probes for five species. The primer pair and probe designed for *T. caries* targets a range of closely-related *Tilletia* species including *T. laevis*, *T. contraversa*, *T. fusca*, *T. cerebrina*, *T. bromi* and *T. goloskokovii* as predicted from the alignment of the sequence data.

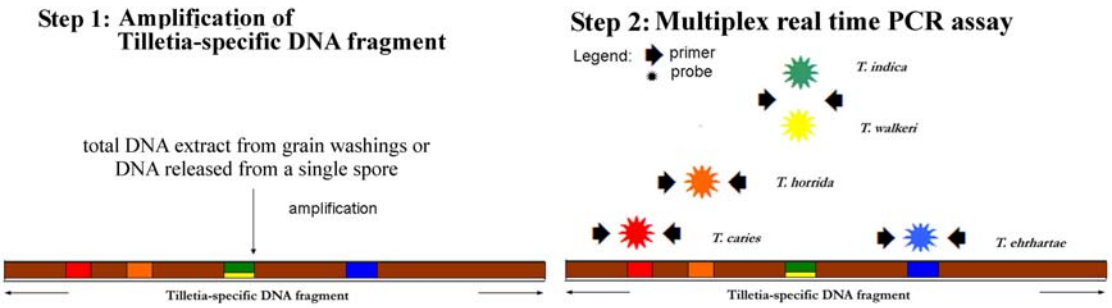


Fig. 3.2B. The two-step molecular assay of T. indica and related grass bunts.

The different probe colours enabled the five species to be analysed simultaneously in one tube containing the specific probes and primers. Fluorescence data from the real time PCR were captured simultaneously to five different fluorescence spectra in a real-time PCR machine (Fig. 3.2C).

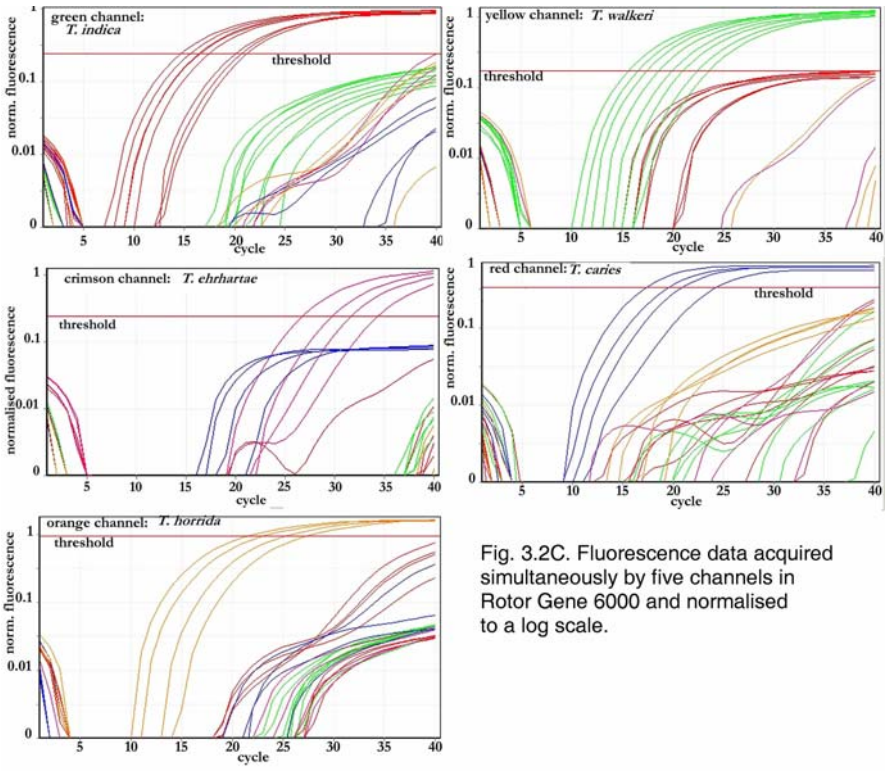


Fig. 3.2C. Fluorescence data acquired simultaneously by five channels in Rotor Gene 6000 and normalised to a log scale.

The molecular assay has the sensitivity to detect five or fewer spores in grain washings from 50g grain samples as well as on genetic material directly released from a single spore.

This work has been published in *Australasian Plant Pathology* **38**, 101-109 and the technical details for the performance of the assay are in Appendix 1.

3.3 *The development of an enhanced protocol for the quarantine detection of* T. indica (Tan M-K)

The current national diagnostic protocol (Wright *et al.* 2003) involves the visual examination of the grain sample for bunted grains, selective sieve-washing of the grain samples to extract the teliospores, microscopic examination to identify the spores by morphology followed by germination of the spores and then a molecular protocol to confirm the identity (Fig. 3.3A).

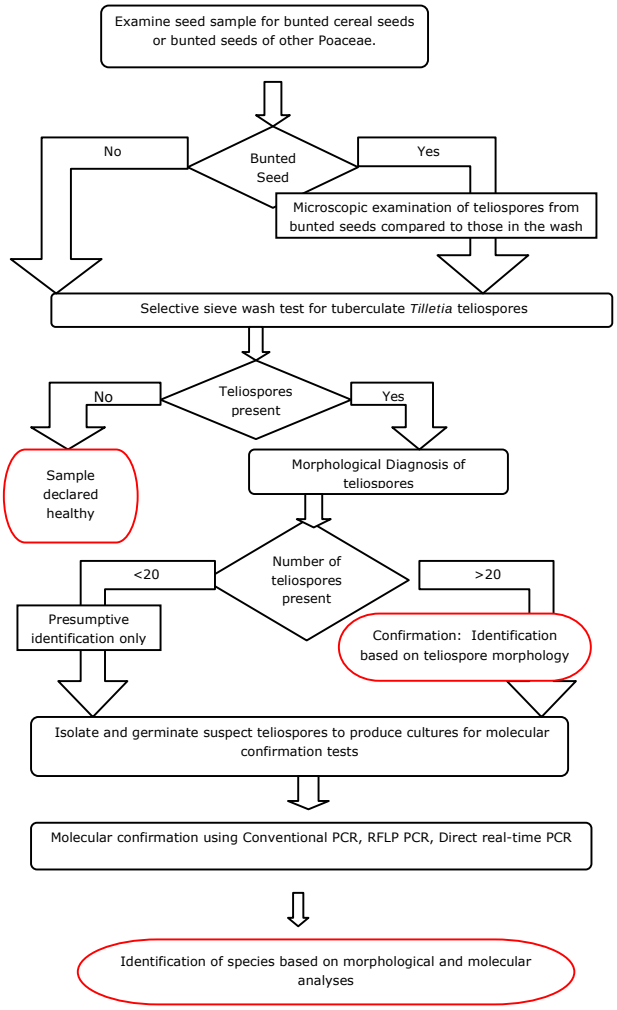


Fig. 3.3A Flow diagram of the national diagnostic protocol for *T. indica* (Wright et al. 2003).

The selective sieve-wash test is an efficient and rapid method for detecting teliospores in a given sample and is indispensable in the enhanced protocol. The minimum number of 50 g samples required to detect one teliospore to 99% level of confidence was reported to be three (Inman *et al*. 2003). Hence each sample comprises three replicate 50 g sub-samples. Microscopic examination and morphological diagnosis of teliospores in the pellet are slow and tedious and the use of a faster and less laborious method to detect and identify the teliospores in the pellet is preferable in the event of a quarantine incursion.

The real time PCR assay is highly sensitive and is suitable to substitute the tedious, repetitive task of microscopic examination of the pellet. In the development of the enhanced protocol (Fig. 3.3B), the pellet from the selective-sieve wash test was suspended to a homogenous suspension and divided into two equal aliquots. One half was used for analysis and the other half was kept for the subsequent confirmation of putative positive samples.

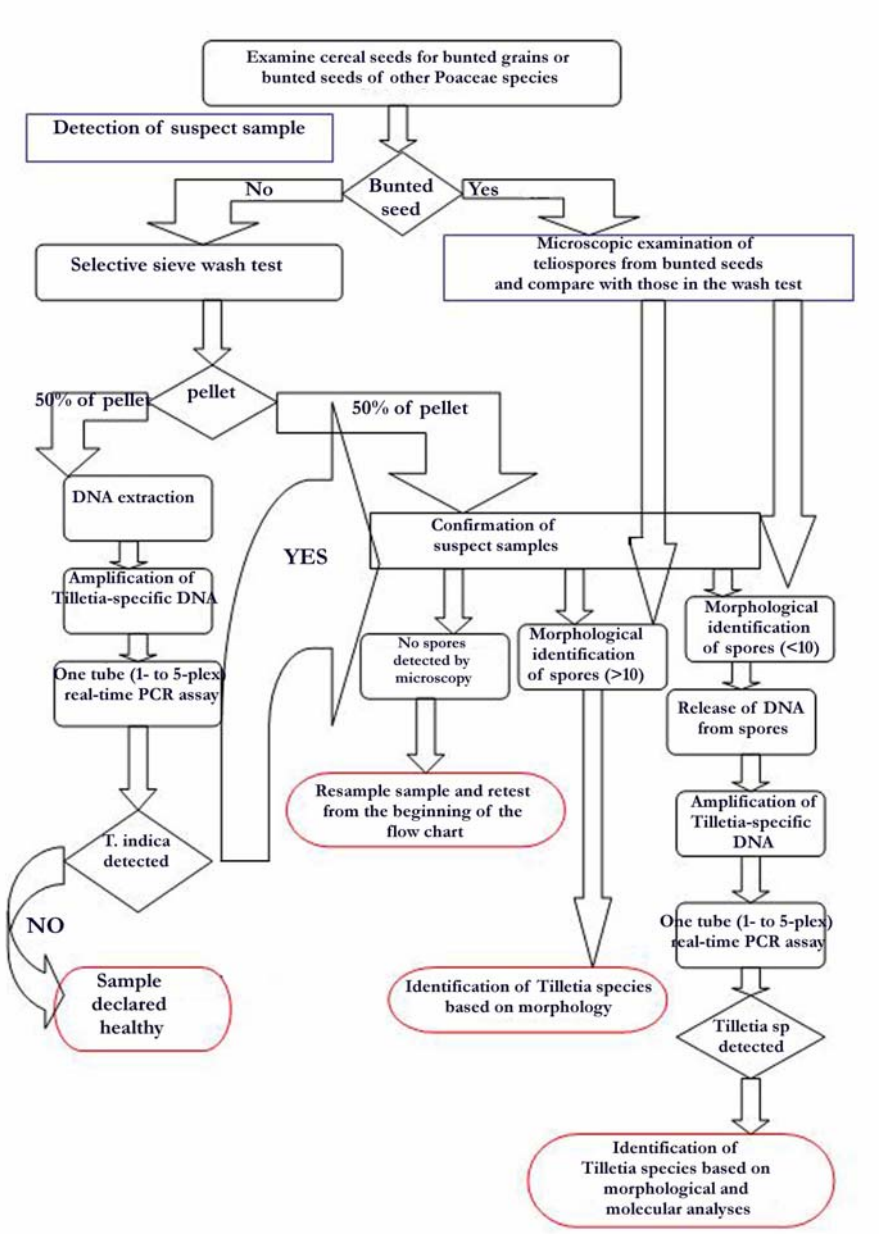


Fig. 3.3B Flow diagram of the enhanced protocol for the detection of T. indica and other related Tilletia spp.

Molecular analysis of the first half of the pellet in the enhanced protocol involves total DNA extraction from half of the sieve-wash pellet, enrichment of *Tilletia*-specific DNA (step 1 of Section 3.2) and finally a one-tube assay to identify and distinguish *T. indica* and other *Tilletia* spp. contaminants (step 2 of Section 3.2). Any samples that have been tested positive for *T. indica* or *Tilletia spp.* will be confirmed using the other half of the pellet.

Confirmation involved both microscopy and a molecular assay on the other half of the pellet. Microscopy of putative positive samples was required to provide physical evidence of teliospores with morphological criteria of *T. indica*. In a quarantine incursion, the percentage of putative positive samples is likely to be very low and hence this microscopy step for confirmation will involve a small number of samples. Assuming the presence of 10% positive *T. indica* samples and a small percentage (5%) of false positives in a batch of 1500 samples, the number of samples required for microscopic examination and identification in the enhanced protocol is 158 putative positives. In contrast, the microscopy step in the current protocol employed in the detection for suspect samples would involve a larger number (1,500 in this instance) of samples making this a considerably more labour-intensive and time-consuming step.

In the confirmation stage, the other half pellet was examined microscopically for morphological identification of spores of *T. indica* or other *Tilletia* species present. The national diagnostic protocol (Wright *et al.* 2003) specifies that if the number of teliospores with morphological criteria of *T. indica* is greater than 10, identification of sample is based on teliospore morphology. Hence, if the number of suspect spores in the half pellet with morphological criteria of *T. indica* is greater than 10, identification is based on teliospore morphology and molecular diagnosis of the first half of the pellet. Confirmation using molecular diagnosis is not necessary in these 'clearly positive' samples (Fig. 3.3B).

If the number of spores is less than 10 in the half pellet, individual spores are isolated with the aid of the microscope and analysed directly in the molecular assay and thus bypassing the germination step. Thus the combination of a microscopy step and molecular methods in the confirmation stage will give a near zero risk of a false positive in the diagnostic process.

3.4 Economic analysis of the enhanced protocol (Tan M-K)

The objective of the analysis was to compare two laboratory protocols for the detection and identification of *T. indica* by analysing the differences between the protocols on two criteria: (a) the variable costs of the direct operations, which determine the extra funds required under each protocol following the detection of suspect sample(s) of KB and (b) the elapsed time to confirmation of the presence or absence of KB, during which the wheat industry would be totally disrupted.

The two protocols assessed were:

1) Existing national diagnostic protocol based on germination of suspect teliospores to produce material for PCR analysis as confirmation for the detection based on detailed microscope examination.
2) Enhanced protocol based on molecular assessment of suspect samples using a multiplex real-time PCR assay and confirmed by microscope examination and molecular analysis.

The variable costs included in the analysis were:

a) Costs of labour involved in the analyses, including salary and 30% on-costs;
b) Direct operating costs, including consumables such as chemicals and materials.

Costs not included in the analysis were:

(a) Capital costs of buildings and equipment used, since these were considered 'sunk costs' for this analysis. Sunk costs relate to pre-existing facilities already accounted for by past activities or other on-going activities not associated with a suspected outbreak of the exotic disease KB.
(b) Sample registration and data management, since these are common to both protocols.
(c) Overhead costs such as power, heating and cooling, etc, that are likely to be common to both protocols.

Costs of carrying out the two protocols were calculated using a spreadsheet-based budgeting approach in an incursion scenario needing to assess 500 samples of grain, from each of which three 50g sub-samples are taken for analysis, giving a total of 1,500 sub-samples for analysis.

All laboratory operations involved in both protocols were identified. Next, information was collected about the time needed to complete each operation or procedure and the physical quantities of specific labour inputs (plant pathologist, technical officer, technical assistant and molecular biologist) required for each operation or procedure. Thus for each operation, information was collected on (a) Who performs the operation? (b) How many can be done in a day? and (c) How long does it take for the whole operation? From those estimates, the time involved for each person was estimated for the operation, and the labour costs for each operation were then determined by relating that figure to their salary and associated costs. The cost effects of changes in the labour input can be readily estimated from the spreadsheet, and the change in labour cost can be estimated for any changes in the number of samples evaluated in an incursion.

The estimated costs of chemicals and consumables (which included laboratory consumables, chemicals, enzymes, glass wares, plastic disposables) required for the different analyses and procedures were included as direct operating costs. For the economic analysis, cost estimates for each step in the molecular diagnosis component were derived from operations on a 96-sample plate format in the DNA extraction and PCR (both conventional and real-time) steps.

Labour and time requirements were provided by laboratory supervisors and technicians. Personnel costs (salary and benefits) were obtained from the Project Costing Module of NSW DPI (now integrated into I&I NSW). Prices of consumable inputs were obtained from current purchasing supplies at Elizabeth Macarthur Agricultural Institute. The information collected provided the input data for the economic analysis.

Cost estimates were generated using typical values for input quantities (and their prices), based on the current testing protocol. For comparison, the typical values under alternative scenarios with the enhanced protocol were then also estimated.

Microscopic examination for morphological identification of *T. indica* and other related bunt species in the current protocol is very rate-limiting. In the national diagnostic protocol, the wash pellet is suspended in about 200 μl of water and about five slides are prepared for examination. Assuming the presence of *T. indica* in 10% of sub-samples, all five slides need to be examined for 90% of sub-samples to confirm the absence of the pathogen. It was estimated that eight plant pathologists were required to perform microscopic examinations of 92 sub-samples per day.

At least two replicate slides need to be microscopically examined for morphological identification of the positive sub-samples, which was assumed to be 10% in this study.

Morphological identification requires the assessment and the documentation of microscopic characteristics and the estimated time taken for at least two slides of each sub-sample was 30 minutes. The total labour time required for microscope examination and identification in an incursion scenario involving 500 samples was estimated to be 989 hours with labour input entirely from plant pathologists or mycologists.

The current protocol requires the microscopic identification of more than 10 teliospores for a confirmed diagnosis of *T. indica* in the sample (Wright *et al*. 2003). If the number of teliospores is less than 10, the suspect teliospores will be isolated for germination for molecular confirmation tests. Teliospores of *T. indica* take two weeks to germinate and this may not occur. This is the other rate-limiting step in the current protocol, incurring two weeks before the molecular confirmation step can be performed.

Detection for suspect samples in the enhanced protocol replaced the microscopy step with the use of a highly sensitive fluorescent molecular assay on DNA extracted from one-half of the pellet. The time estimation for the analysis of the 1,500 sub-samples for each of the molecular steps involving DNA extraction and PCR analysis was done using a 96-sample scale format on which most current molecular analysis operates.

Confirmation in the enhanced protocol involves both morphological and molecular identification of the suspect sample and it bypasses the germination step. Single teliospores from the other half of the pellet were identified under the microscope and documented. If the number of teliospores with morphological criteria of *T. indica* is greater than 10, the sample would be confirmed as a 'clear' positive based on molecular detection from the first half of the pellet and the subsequent microscopic identification.

On the basis of 10% positive *T. indica* samples and a small percentage (5%) of false positives, the total labour time for microscopy in the enhanced protocol is 119 hours as opposed to 989 hours in the current protocol. Repetitive microscopic examination of a very large number of samples from grain washings is tedious and extremely strenuous for the eyes and the very significant reduction in microscopy in the enhanced protocol will spare plant pathologists from potential occupational health hazards associated with sore eyes and back pain, besides resulting in a significant time savings.

Given the expected scheduling of the operations in the current protocol, it was estimated that it requires 35 days to complete the diagnosis of 500 samples. The bottleneck in the current protocol occurs in the 'germination step' where no work can be performed on a particular positive sample for a time period of nine days.

In the enhanced protocol, molecular technology replaces the microscopy step in the detection for suspect samples. The three sequential steps involving DNA extraction from the half-pellet, amplification of *Tilletia*-species specific DNA and the multiplex real time PCR assay (Tan *et al*. 2008) can be streamlined for high throughput processing in batches of 96 samples. This work flow will enable the completion of the detection for 1,500 suspect sub-samples in day 13 of the incursion scenario. The entire three-step operations could be performed by trained technical officers with minimal supervision from the molecular biologist.

The total variable cost for an incursion scenario involving 500 grain samples (1,500 sub-samples) was estimated to be 33.7% lower for the enhanced protocol with a significant difference in the elapsed time. It will take 35 days for the national diagnostic protocol and only 13 days for the enhanced protocol.

Sensitivity of results to changes in key parameters

The relative performance of the two protocols was assessed under different inputs of labour resources, different number of quarantine samples and different percentages of negative samples.

Analysis has shown that the total variable costs incurred for both protocols are directly proportional in a linear scale with the number of samples (Fig. 3.4Aa). The numbers of elapsed days for both protocols have been estimated to also increase with the number of samples (Fig. 3.4Ab).

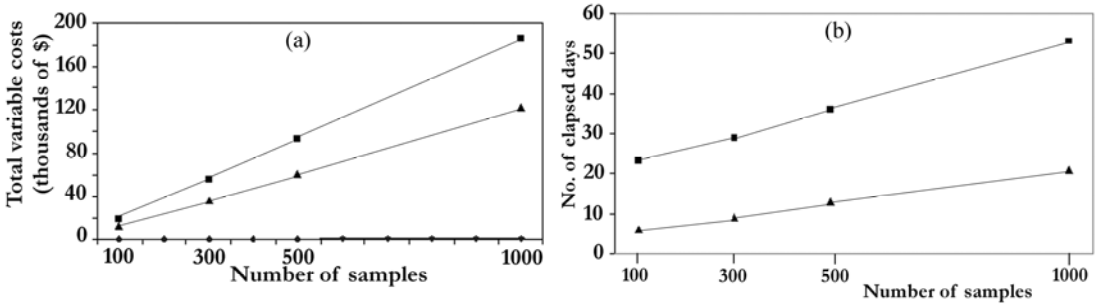


Fig. 3.4A. Comparative analysis of the current (▪) and the enhanced (▲) protocol in (a) total variable costs incurred and (b) the number of elapsed days with different number of samples.

It was found that varying the input of different labour categories for the incursion scenario of 500 samples with 10% of positive samples has little effect on the total variable costs but a huge impact on the number of elapsed days to the completion of the diagnosis. For instance, increasing the number of technicians in the sieve wash from three to nine does not reduce the elapsed time for the national diagnostic protocol but will reduce the number of elapsed days from 18 to 12 in the enhanced protocol (Fig. 3.4B).

Microscopy is the rate limiting step in the current protocol and variation in the number of plant pathologists have been found to effect the number of elapsed days in the current protocol but no impact on the enhanced protocol. For instance, increasing the number of pathologists from four to 6=six will reduce the number of elapsed days by 11 (Fig. 3.4B) but will not effect the number of elapsed days for the enhanced protocol. However, the rate of reduction in the number of elapsed days decreases with further similar input in the number of pathologists. The data have shown that a gradual step-wise increase of two pathologists from six to 12 reduced the number of elapsed days by five, three and three respectively.

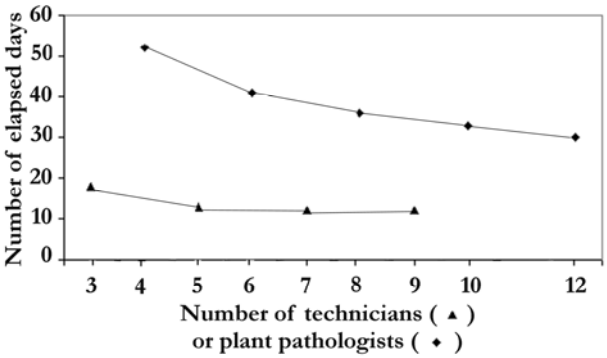


Fig. 3.4B. Comparative analysis of variation in the number of technicians and the number of plant pathologists on the number of elapsed days to completion of diagnosis in the enhanced and the current protocol respectively.

This analysis indicates that an incursion scenario with a specified number of samples and an estimate of a percentage of negative samples will have an optimum requirement for certain labour inputs (technicians in the enhanced protocol and plant pathologists in the current protocol) without any effect on the operating costs. Any additional labour input beyond the optimum will not reduce the number of elapsed days.

A comparison of the total variable costs of the two protocols was made for incursion scenarios of 500 samples with different percentages of negative samples and assuming a labour input of five technicians and eight plant pathologists (Fig. 3.4C). Total variable costs decrease with increasing percentages of negative samples for both protocols, with the rate of decrease greater in the enhanced protocol. The two graphs intersect with 51% of negative samples, making total variable cost lower for the enhanced protocol when the percentage of negative samples is greater than 51%.

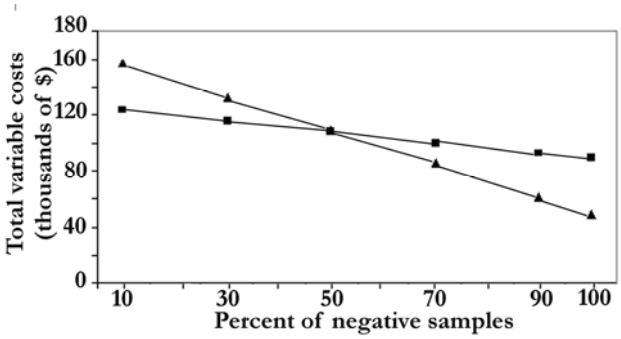


Fig. 3.4C. Comparison of total variable costs (with a labour input of 5 technicians and 8 plant pathologists) incurred in the current (■) and the enhanced (▲) protocol with variation in the percentages of negative samples in an incursion scenario involving 500 samples.

Brennan and Warham (1990) estimated that at an average infection level of 0.4%, the average percent of samples with infected grain is 37%. These figures suggest that a KB incursion would involve an extremely low level of infection. In situations where the number of

negative samples is high to very high (as in a quarantine incursion or a survey to establish area of freedom), the enhanced protocol would be significantly more economical and sensitive than the current protocol. However, in infested areas where more than 50% of the samples collected contain one or more teliospores (e.g. Allen *et al*. 2008), the current protocol would be more economical in the detection and identification of the pathogen, although the number of days elapsing before confirmation would still be considerably higher.

3.5 Ring testing of protocol at national and international level (Tan M-K)

A workshop on the enhanced protocol was conducted at EMAI, NSW DPI on 5 and 6 March 2008. There were 16 participants with representatives from Grains Research and Development Cooperation (GRDC), OCPPO, Plant Health Australia (PHA), Department of Agriculture and Food, Western Australia (DAFWA), Queensland Primary Industries and Fisheries (QDPI), South Australian Research and Development Institute (SARDI), (NSWDPI) and Department of Primary Industries, Victoria (DPI VIC). One nominated researcher from each state agency (NSW DPI, DAFWA, QDPI and DPI VIC) undertook the full training for the enhanced protocol. A workshop manual for the enhanced protocol was produced which included an improved protocol for the extraction of *Tilletia* spores from grain dust.

A national ring test of the new detection protocol was conducted after the March 2008 workshop. Assay kits for *T. indica* and *T. walkeri* were sent out in April 2008 to participants (trained in the workshop) from laboratories in New South Wales, Western Australia, Victoria and South Australia.

Dr. Mui-Keng Tan and Ms Dominie Wright undertook an overseas trip to conduct two workshops on the enhanced protocol at the Phytosanitary Export Testing Laboratory, CSL (now changed to Food and Environment Research Agency, UK (FERA), Sand Hutton, York, UK and at the CRA-PAV Plant Pathology Research Centre, Rome, Italy. Each workshop had six-nine attendees. The workshop in UK included participants from FERA, York and Science and Advice for Scottish Agriculture, UK (SASA), Edinburgh. Attendees at the workshop in Rome travelled from other laboratories within Italy and also Malta.

An international ring test of the protocol was conducted in April 2009. Participants included laboratories in FERA; CRA-PAV Plant Pathology Research Centre, Rome, Italy; SASA; Shanghai Inspection and Quarantine Bureau, People's Republic of China (PRC); Shenzhen Entry-exit Inspection and Quarantine Bureau, PRC; DAFWA; DPI VIC and NSW DPI.

The grain samples were set up to test for T. indica and/or its closely related species, T. walkeri only. There were five treatments of:

No spores:

1) T. indica (4 spores),
2) T. walkeri (4 spores)
3) T. indica and T. walkeri (4+4), and
4) T. indica and T. walkeri (10+10).

Each treatment is a 50g grain sample spiked with the appropriate number and combination of spores. Bunted grains containing *T. indica* spores used in the ring-test had been autoclaved twice (once in India and once on arrival in Australia) under high temperature (121^0C) and pressure (15psi) for 30 minutes. Each ring test comprised three replicates of each treatment. A randomised block design (Appendix 2) was used to produce the different sets of samples for the ring test.

Results received from different laboratories (Appendix 2) were collated (Table 1). Two participants did not differentiate the spores observed in the second half pellet and proceeded to identify by molecular diagnosis. Two participants did not perform the 'single spore' molecular confirmation due to lack of training.

Sterilisation using high temperature and pressure of bunted wheat grains containing *T. indica* spores used in the ring tests (and in the project) had caused a significant portion of the spores to lose their genetic material. Most of the spores observed under the microscope appeared broken in one or more points of the episporial surface. Those with intact spore walls would not be viable but would possess dead genetic material for analysis. This had been confirmed by the analysis of multiple 10 replicates of single spore molecular assays of autoclaved *T. indica* spores which have produced only 10-40% positive identifications.

The requirement to work with autoclaved spores was predicted to hamper the correlation of results with the treatments. However, the option of working with viable spores was never considered due to its status as a quarantine pathogen. The spores of *T. walkeri* had been in long term storage (>5 years) in Shear's solution and some would have similarly lost their genetic material.

Table 1: Results of the International ring test collated from six participating laboratories.

	Diagnosis of 1st half pellet		Diagnosis of 2nd half pellet			
	Molecular Diagnosis of DNA extracts from pellet %*		Microscopy %*		'single spore' molecular confirmation %*	
Treatment (Ti, Tw)	*T. indica*	*T. walkeri*	*T. indica*	*T. walkeri*	*T. indica*	*T. walkeri*
1 (0,0)	20.0	11.1	0	0	NA	NA
2(4,0)	33.3	33.3	33.3	33.3	8.3	0
3 (0,4)	16.7	27.8	8.3	16.7	0	8.3
4(4,4)	16.7	16.7	66.7	58.3	33.3	8.3
5(10,10)	27.8	27.8	50	66.7	8.3	8.3

Ti : *T. indica*; Tw: *T. walkeri*, *: This gives the % of identification of the indicated species.

The results of the international ring test (Table 1) cannot reflect the proficiency of the tests, as a portion of the spores lost their DNA. Consequently, results from some test samples could not be correlated to their treatments and this explains the low percentage of correlation obtained

(Table 1). For instance, Lab I (Appendix 2) had obtained only four positive identifications from 30 single spore analyses. This figure (13.3%) corroborates with our analysis results (10-40%) using multiple sets of 10 single spores.

Both the national and international ring tests have shown that it is feasible to extract sufficient DNA from the washings of 50 g grain samples containing five or fewer number of spores to give a putative positive result which can be confirmed by morphological identification of spores and/or PCR diagnosis from single spores. Note that a half pellet was analysed in the detection of suspect samples (Fig. 3.3B) and so the number of spores present may be ≤ 5. The collated results showed that successful putative positive detections were obtained for 16.7 to 33.3 % of treatments (Treatments 2 to 5). The use of sterilised spores was the main factor for the cause of poor correlation. Other factors included inefficient DNA extraction and/or absence/loss of spores in the half pellet analysed.

Results from Treatment 1 suggested that the probability of a false positive in the analysis of the first half pellet is 11 to 20 %. Each ring test consisted of only 15 assays. Analysis of a significantly large number of samples will help to refine and approximate the true threshold level in a real-time molecular test and reduce the level of false positives. These false positives were all eliminated in the confirmation steps on the second half of pellet.

The percent of mis-identifications of morphologically very similar spores of *T. indica* and *T. walkeri* ranged from 8.3 to 33.3% (Table 1). This is evident in Treatment two and three where the number of spores is low (4). This reiterates the unreliability of identification and differentiation of closely related species with overlapping morphological criteria by microscopy when only a few spores were examined. The number of spores found in half pellets from spiked samples varied from zero to more than half the expected number of spiked spores (e.g. 10.3, 10.4, in Appendix 2). This suggests the possible loss of spores during the grain washings and/or the asymmetrical distribution of spores in the two halves of the pellet. The mis-identifications in the microscopy step are resolved by single spore molecular analysis to give a correct diagnosis. Participants who have been trained in the molecular diagnosis from single spores have indicated that the use of a single spore sandwiched between two pieces (~2-4 mm^2) of cover-slip glass for direct PCR analysis gets easier with practice.

The final diagnosis from the collated results of the diagnosis of the first half pellet followed by confirmation using microscopy and single spore analysis (Table 1) has shown that each of the five different treatments has been correctly identified attesting to the sensitivity, accuracy and reliability of the protocol. The constraints of using sterilised spores and the low number (3) of replicates for each treatment make the statistical assessment of the protocol proficiency for individual laboratories not feasible.

The techniques involved in the protocol include differential size sieving, DNA extraction, PCR, real-time PCR, obtaining a single spore sandwich and microscopy. The range of techniques is broad and different laboratories have different strengths. Operator variability is thus a huge contributing factor to the variation obtained in the results of the ring-test (Appendix 2). The results showed that most laboratories did not have a complete mastery of the different facets of the protocol. Hence specific training is an essential pre-requisite for personnel involved in quarantine diagnosis.

The protocol has been developed using a target region with known nucleotide differences between *T. indica* and the other common grass bunts found in wheat samples. Sequence alignment data of *Tilletia* and other fungal species currently show no basis for cross reaction with other fungal species. This protocol is currently able to distinguish *T. indica* and *T. walkeri* which differs by only one nucleotide difference at this genomic region. Other fungal species, e.g. rust spores that may be found in wheat grains will be less related (i.e. possess more nucleotide differences) to *T. indica* and are thus unlikely to have a cross-reaction in the protocol.

The protocol uses real-time fluorescent technique which is highly sensitive and is thus highly susceptible to contamination resulting in false positive as with any highly sensitive technique involving PCR. Thus the protocol has to be conducted with strict controls in an accredited quality management system. Parameters to be considered include a 'clean' area free from the possible contamination from other DNA or biological materials to perform the PCR set-ups. This area must be dedicated for PCR only.

Operator variability in pipetting is a significant cause for variation in PCR outcomes. Technical personnel differ in their dexterity with pipetting for quantitative assays. There are various controls to assess the accuracy of pipetting of the operator e.g. the reproducibility of replicates or triplicates in a dilution series. The Error values (which are the mean squared error of the single data points fit to the regression line) for each of the replicate assays should be below 0.2 to be acceptable criteria for the accuracy of the pipetting. The correlation coefficient ($R^{\wedge}2$ values) of a standard curve from the dilution series of a DNA control is another indicator of accuracy and reproducibility of the PCR set-ups. A good $R^{\wedge}2$ value is around 0.99. The acceptable critera for $R^{\wedge}2$ values is above 0.95. **Thus the reliability of the diagnosis could be assessed by the $R^{\wedge}2$ values generated from the standard curve that was performed in the same PCR run.** Only some of the laboratories (10.2, 10.4, 10.5, 10.7 in Appendix 2) provided these values in the ring test. Most diagnostic laboratories thus are moving in the direction to use automated liquid handling systems with accurate sensor systems to overcome the problems associated with operator variability.

Setting the correct noiseband level is another very critical factor in a real-time PCR assay. If the noiseband is too low, there will be a significant percentage of false positives. If it is too high, some low positives will be missed. **The standard curves for each of the five *Tilletia* species over the expected concentration range in the samples must be taken into consideration to determine the noiseband in the multiplex real time PCR assay.** The true noiseband will emerge with diagnosis of a sufficiently large number of samples.

4. Implications for stakeholders

The multiplex real-time molecular protocol for the detection of *T. indica* and other related grass bunts is able to provide unequivocal identification and differentiation of *T. indica* and other related grass bunts found in wheat grains when only a few spores have been observed. In the current protocol, detection for suspect samples is based on morphological differentiation using microscopic examination by mycologists. In a quarantine situation, most of the samples examined will be expected to be negative and thus this mindless, tedious laborious task can be substituted by the sensitive fluorescent real-time PCR molecular assay.

Presence of the pathogen has huge economic implications on the wheat trade from the area and a suspect sample must be confirmed both by morphology criteria of the spores and by molecular diagnosis to give a near zero risk of a wrong diagnosis. Development of the methodology in this project for the direct release of DNA from single spores for PCR amplification eliminates the germination step required for confirmation. This has greatly reduced the number of elapsed days to a definitive identification and will enable diagnostic laboratories to mount a much more rapid outcome in a quarantine situation. This is critical if shipments of wheat grain are being held at ports pending an outcome of diagnosis.

Currently, harvested wheat grain is not tested on a national scale due to the cost and time involved in diagnosis. Molecular biology operations have the potential for being structured into an automatic set-up for high throughput analysis with cost savings from economies of scale (see Section 3.4). In addition, elimination of the spore germination step has resulted in significant savings to the time required for the confirmation of a suspect sample (see Section 3.4). The savings in cost and time make the enhanced protocol an economically sustainable tool to be used in a grass bunts surveillance program. Such programs are an important part of Australia's ongoing efforts to provide evidence to markets that the country is free of the pathogen, which will help increase market access for Australian grain.

Automated liquid handling systems for high throughput nucleic acid extraction, PCR set-ups and analysis are an integral part of life sciences research. Thus the enhanced protocol can be integrated in a diagnostic section of a centralised national or state facility for animal and plant biosecurity research. Such a facility must have an industry recognised accredited quality management systems (e.g. ISO 17025) to ensure the reliability and reproducibility of the sensitive protocol. Robotic systems will also reduce variability of diagnosis outcomes associated with operator variability in pipetting micro volumes of liquid. Technical personnel involved must have the training in molecular diagnostics as well as the experience and flair in working at the threshold limits of DNA extraction and molecular detection.

The risk of an incursion is low but real in today's global economy that allows affordable international travel between countries with and without the pathogen. Genetic variability studies of isolates of *T. indica* present in different geographical regions like India, Mexico, USA and South Africa should be conducted to provide crucial information on the geographical movement of the pathogen. It will also provide governments with pivotal information needed to avoid an introduction of the pathogen.

Pre-emptive breeding to incorporate Karnal bunt resistance will be a useful strategy to breed resistant germplasm suited to the Australian climate.

5. Recommendations

- The enhanced molecular protocol should be implemented as a 'beta' version in a national or state facility for animal and plant biosecurity with an industry recognised accredited quality management systems (e.g. ISO 17025). This will allow the fine-tuning of the protocol to ensure its reliability and reproducibility.

- The enhanced protocol should be used in a nation wide survey to establish area of freedom with rigorous scientific data. This will increase market access for Australian grain. The large number of samples in the survey will be valuable to test drive and enable the optimisation of the 'beta' version of the protocol.
- The enhanced protocol could be used for subsequent regular (e.g. annual) surveillance to ensure continuous area of freedom.

6. Abbreviations / glossary

ABBREVIATION	FULL TITLE
CRCNPB	Cooperative Research Centre for National Plant Biosecurity
GRDC	Grains Research and Development Corporation
OCPPO	Office of the Chief Plant Protection Officer
PHA	Plant Health Australia
DAFWA	Department of Agriculture and Food, Western Australia
QDPI	Primary Industries and Fisheries, Department of Employment, Economic Development and Innovation, Queensland
SARDI	South Australian Research and Development Institute, SA
I&I NSW	Industry and Investment NSW
NSW DPI	NSW Department of Primary Industries
DPI VIC	Department of Primary Industries, Victoria
CSL	Central Science laboratory
FERA	Food and Environment Research Agency, UK
SASA	Science and Advice for Scottish Agriculture, UK
EMAI	The Elizabeth Macarthur Agricultural Institute
PRC	People's Republic of China
KB	Karnal bunt
PCR	Polymerase chain reaction

7. Plain English website summary

Please complete table using plain English. This information will be published on CRCNPB's website for a public audience.

CRC project no:	CRC20004
Project title:	Enhancing the detection of *Tilletia indica*, the cause of Karnal bunt
Project leader:	Dr. Mui-Keng Tan
Project team:	Ms Aida Ghalayini Ms Dominie Wright Dr. John Brennan Dr. Gordon Murray
Research outcomes:	• The development of a one tube, fluorescent PCR assay for the simultaneous detection and unequivocal identification of *T. indica* and closely related *Tilletia* spp. when only a few spores have been observed. The key features of the assay are: o Elimination of spore germination step o Direct molecular analysis of a single spore. o Simultaneous detection of *T. indica* and related grass bunts resulting in reduced labour and reagent costs. • The development of an enhanced protocol for the quarantine detection of *T. indica* and other grass bunts in wheat grains. The combination of a microscopy step and direct molecular analysis of single spores in the confirmation stage will give a near zero risk of a false positive in the diagnostic process. • An economic comparison of current and enhanced protocol for quarantine detection and surveillance of *T. indica*, the cause of Karnal bunt in wheat.
Research implications:	• The enhanced protocol should be integrated as a beta version in a diagnostic section of a centralised national or state facility for animal and plant biosecurity. This will enable the refinement of the protocol for high throughput diagnosis and the development of a capacity for an emergency response to Karnal bunt. • The enhanced protocol bypasses the germination step in identification and so the number of days elapsing before definitive confirmation is considerably lower than in the

	current protocol. This is critical in an incursion when huge quantities of wheat grain are being held at ports for export pending an outcome of diagnosis. Use of the enhanced protocol will result in massive savings to the industry. • The savings in cost and time in the enhanced protocol relative to the current protocol make it an economically sustainable tool to be used in a grass bunts surveillance program to ensure continuous freedom of the disease and increase market access.
Research publications:	1. M-K Tan and A. Ghalayini (2008) A 5-plex real-time qPCR assay for quarantine detection and identification of *Tilletia indica*. Journal of Plant Pathology 90 (2, Supplement) 150. 2. Mui-Keng Tan, Aida Ghalayini, Indu Sharma, Jianping Yi, Roger Shivas, Michael Priest, Dominie Wright (2009) A one tube fluorescent assay for the quarantine detection and identification of *Tilletia indica* and other grass bunts in wheat. Australasian Plant Pathology 38:101-109 3. Three workshops on the enhanced protocol have been conducted. They were held at EMAI, NSW DPI; Phytosanitary Export Testing Laboratory, FERA, Sand Hutton, York, UK and CRA-Centro di Ricerca per la Patologia Vegetale, Rome, Italy. 4. M-K Tan, JP Brennan, D Wright, and GM Murray. An enhanced protocol for the surveillance and quarantine detection of Karnal bunt in wheat. Australasian Plant pathology. Submitted Manuscript 33(09). 5. Wright D, Goldsmith P, Jordan M and Tan MK (2007) Comparison of methods for the improvement of detecting Tilletia like ustilospores in wheat grain samples. CRC National Plant Biosecurity Science Exchange.
Acknowledgements:	GRDC NSW DPI CRCNPB DAFWA

8. References

Allen TW, Maples HW, Workneh F, Stein JM, Rush CM (2008) Distribution and recovery of *Tilletia indica* teliospores from regulated wheat fields in Texas. *Plant Disease* **92**(3):344-350.

Brennan JP, Warham EJ (1990) *Economic losses from Karnal bunt of wheat in Mexico.* CIMMYT Economics Working Paper 90/02, International Maize and Wheat Improvement Center,

Mexico, D.F.

Inman AJ, Hughes KJD, Bowyer RJ (2003) EU Recommended protocol for the diagnosis of a quarantine organism '*Tilletia indica*'. Central Sciences Laboratory, York, UK.

Jon Fiorella Fox Lane School Bedford, NY (2007) http://www.accessexcellence.org/MTC/96PT/Share/fiorella.php

Murray A (2007) http://www.koko.gov.my/CocoaBioTech/Purification%20Protein35.html. Accessed on the 25th August 2009.

Murray GM, Brennan JP (1998) The risk to Australia from *Tilletia indica*, the cause of Karnal bunt of wheat. *Australasian Plant Pathology* **27**:212–225.

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

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

Schenk NC (1982) Methods and principles of mycorrhizal research, pg 29-35. APS St Paul, Minnesota.

Southey JF (1986) Laboratory methods for work with Plant and Soil nematodes. Ministry of Agriculture, Fisheries and Food, London.

Stansbury CD, McKirdy SJ, Diggle AJ, Riley IT (2002) Modeling the risk of entry, establishment, spread, containment, and economic impact of *Tilletia indica*, the cause of Karnal bunt of wheat, using an Australian context. *Phytopathology* **92**:321-331.

Tan M-K and A. Ghalayini (2008) A 5-plex real time qPCR assay for quarantine detection and identification of *Tilletia indica*. ICPP 9th International Congress of Plant Pathology, 24-29th Aug 2008, Torino, Italy.

Tan M-K, Ghalayini A, Sharma I, Yi J, Shivas R, Priest M, Wright D (2009) A one-tube fluorescent assay for the quarantine detection and identification of *Tilletia indica* and other grass bunts in wheat. *Australasian Plant Pathology* **38**:101-109.

Tan M-K, Murray GM (2006) A molecular protocol using quenched FRET probes for the quarantine surveillance *of Tilletia indica*, the causal agent of Karnal bunt of wheat. *Mycological Research* **110**:203-10.

Wright D, Goldsmith P, Jordan M and Tan MK (2007) Comparison of methods for the improvement of detecting Tilletia like ustilospores in wheat grain samples. CRC National Plant Biosecurity Science Exchange.

Wright D, Murray GM, Tan M-K (2003) national diagnostic protocol for the identification of

Tilletia indica, the cause of Karnal Bunt, Department of Agriculture Western Australia, Australia.

Wright D. Goldsmith P. Caper D. and McKirdy SJ (2008) Survey of West Australian wheat for Tilletia indica-like ustilospores. ICPP 9th International Congress of Plant Pathology, 24-29th Aug 2008, Torino, Italy.

Wright DG, Murray G and Tan MK (2003). A national diagnostic protocol for the identification of *Tilletia indica* – the cause of Karnal bunt.

9. Appendix 1

Multiplex real time PCR Detection Protocol for T. indica and related grass bunts

9.1 Step 1: Enrichment of *Tilletia*-specific DNA in the total DNA extract or DNA released from a single spore

Principle

This new method is based on that published by Tan and Murray (2006).

Reagents

Unless otherwise stated all water used is sterile MilliQ water.

1. Ethyl alcohol (95%) or Industrial Methylated Spirit.
2. Sterile MilliQ Water.
3. 5x PCR Master Mix (See table below).
4. 10X Primer Mix (See table below)
5. Extracted DNA.
6. Agarose gel.
7. TBE buffer.
8. Loading Buffer.
9. Molecular weight marker.
10. Ethidium bromide stain. **NB:** Ethidium bromide is a carcinogen and appropriate gloves, safety glasses and laboratory coat should be worn at all times.

The individual components of the Master Mix and the Primer mix are given below.

5x PCR Master Mix: for 50 reactions of 20μl/Rx

Component	*Stock*	*Vol μl*
Amp. Buffer (Buffer L*), 5X	10X	100
MgCL2 (Invitrogen); 7.5 mM	50 mM	30
dNTP mix; 1 mM	10 mM	20
BioTaq DNA Polymerase (Invitrogen); 50 U	5 U/μl	10
Water		40
TOTAL		200

* 1X Buffer L: [50mM Tris (pH 9), 20mM NaCL, 1% Trition X-100, 0.1% gelatin]

10X Primer Mix: for 50 reactions of 20μl/Rx

Component	*Stock*	*Vol μl*
MK56[a] ; 5 μM	100 μM	5
Tilletia-R[b] ; 5 μM	100 μM	5
water		90
TOTAL		100

[a]MK56 (5'-gta ggt gaa cct gcg gaa gga tca tt) Tan et al. 1996
[b]Tilletia-R (5'-caa gag atc cgt tgt caa aag ttg) Tan & Murray 2006

Equipment

Refer to operating manuals for general usage.

1. Thermocycler and loading rack
2. Micropipettes (10 μl, 40 μl, 200 μl) and associated sterile tips
3. Microcentrifuge tubes (0.2 ml, 1.5 ml)
4. 96-well PCR plates for high throughput
5. Small esky containing ice as all reagents and extracted DNA needs to be kept cold
6. Electrophoresis tank and power pack
7. Ethidium bromide staining dish
8. Gel Documentation System

Procedure

(1) Thaw the reagents on ice.

(2) Prepare a reaction mix according to the table below for the appropriate number of reactions.

Components required for reaction mix.

Component	*Volume/reaction μl*
Reaction mix	
5 X PCR Master Mix	4
10x primer mix	2
water	13
Template DNA	1
Added at step 4	
TOTAL	20 μl

(3) Mix the reaction mix thoroughly and dispense 19 μl into 0.2 ml PCR tubes or plates.

(4) Add template DNA to the individual PCR tubes or plates containing the reaction mix.

(5) Perform PCR on a thermal cycler. The temperature profile as used on an Eppendorf Mastercycler

Note: In the amplification using a crushed spore, the reaction mix is added directly into tube with the crushed spore.

Temp. Profile for Eppendorf Mastercycler

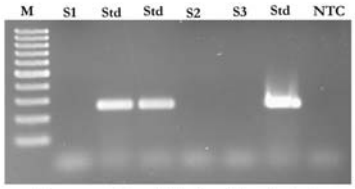
Perform PCR using the following program:

95°C	3 min,	
63 °C	1 min	
72 °C	1 min	
94 °C	20 sec	
TD 63-59 °C	30 sec	20 cycles (start touch down (TD) at cycle 2 at 1°C/cycle)
72 °C	30 sec	
72 °C	10 min	
4 °C	1 min	

(6) Run the PCR products on 2 % Agarose gel.

Results

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). A positive control reaction is thus required to ensure the reagents and the PCR reactions are working.



Fluorescence of an ethidium bromide stained gel from electrophoresis of PCR products from an enrichment step. M is a marker lane. Std are DNA standards of Tilletia species and SI-S3 are test DNA samples.

9.2 Step 2: Detection and Identification of *T. indica* and contaminating grass bunts

Principle

This method is based on the publication of Tan *et. al* (2009).

Reagent

Unless otherwise stated all water used is sterile MilliQ water.

1. MilliQ water.
2. 4 X PCR Master Mix (Refer to Table below)
3. 10 X Primer-probe Mix (Refer to Table below)
4. DNA extract or teliospores from pellet.

The individual components of the Master Mix and the Primer mix are given:

4x PCR Master Mix (multiplex): for 50 reactions of 20µl/Rx

Component	*Stock*	*Vol µl*
Amp. Buffer (Bioline), 4X	10X	100
MgCL2 (Bioline), 20 mM	50mM	100
dNTP mix, 0.8 mM	10mM	20
Immolase DNA Polymerase (Bioline), 50 U	5U/µl	10
water		20
TOTAL		250

10X Primer and Probe Mix: for 50 reactions of 20µl/Rx

Component	*Stock*	*Vol (µl)*
KB-DL-For1, 4 µM	100 µM	4
KB-DL-Rev1, 9 µM	100 µM	9
Ehr-DL-For, 4 µM	100 µM	4
Ehr-DL-Rev, 9 µM	100 µM	9
Tri-DL-For , 4 µM	100 µM	4
Tri-DL-Rev, 9 µM	100 µM	9
Hor-DL-For, 4 µM	100 µM	4
Hor-DL-Rev, 9 µM	100 µM	9
T. indica probe, 5' FAM, 3' BHQ1, 2 µM	100 µM	2
T. walkeri probe, 5' JOE, 3' BHQ1, 2.5 µM	100 µM	2.5
T. ehrhartae probe, 5' Q705, 3' BHQ2, 2 µM	100 µM	2
T. tritici probe, 5' Q670, 3' BHQ2, 2 µM	100 µM	2
T. horrida probe, 5' CFR, 3' BHQ2, 2 µM	100 µM	2
Water		37.5
TOTAL		100

Table Sequences and modifications of the primers and probes used in the multiplex 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. 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

Equipment

Refer to operating manuals for general usage.

(1) LightCycler® 480 or Rotogene 6000.

(2) Micropipettes

(3) Pipette tips

(4) Microcentrifuge tubes (0.2 ml).

(5) Small esky containing ice as all reagents and extracted DNA needs to be kept cold.

(6) Multiwell Plate 96 (04 729 692 001) or

(7) 0.2 ml PCR tubes (PN#3001-001) or 0.1 ml PCR tubes (PN# 3001-002)

Procedure

1. Thaw the components of the kit.
2. For multiplex PCR, make a 2- fold dilution series of each of the 5 *Tilletia* species standards from 10 ng/μl to 0.0625 ng/μl (5 dilutions/ *Tilletia* sp. standard).

3. Prepare a reaction mix according to Table below for the appropriate number of reactions.

Components required for reaction mix.

Component	*Volume/reaction µl*
Reaction mix	
4 X PCR Master Mix	5
10x primer-probe mix	2
MilliQ water	12
Template DNA	1
Added at step 5	
TOTAL	20 µl

4. Mix the reaction mix thoroughly and dispense appropriate volumes into either:
    - LightCycler® 480 Multiwell Plate 96 (04 729 692 001) for use on LightCycler® 480 (Roche), **or**
    - 0.2 ml PCR tubes (PN#3001-001) or 0.1 ml PCR tubes (PN# 3001-002) for use on RotorGene 6000 (Qiagen)
    - Appropropriate tubes/plates for use on other real-time PCR machines
5. Add template DNA to the individual PCR wells/tubes containing the reaction mix.
6. Run the reactions on the real-time machine in accordance to machine specifications

The temperature profile used:

- **LightCycler® 480**:

  Activation: 95 °C 10 min x 1 cycle

  Amplification: 94 °C 15 s, 65°C 60 s x 40 cycles, TD to 60 °C, rate= 1 °C /cycle,

  [data acquiring to 5 channels: Cyan500(450-500), FAM (483-533), HEX (523-568), Red 610 (558-610), Cy5(615-670)].

  Cool: 50 °C 1 min x1 cycle

- **RotorGene 6000:**

Activation: 95 °C 10 min x 1 cycle

Amplification: 94 °C 15 s, 65 °C 60 s x 40 cycles, TD to 60 °C, rate= 1 °C /cycle,

[data acquiring to 5 channels: green, yellow, orange, red and crimson] dynamic tube normalisation option.

Cool: 50 °C 1 min x 1 cycle

Results

If *T. indica* or another *Tilletia* species is detected, the confirmation step of the flow diagram has to be followed. This means: The other part of the pellet needs to be examined using

the microscopy technique described in the national diagnostic protocol (Wright *et al* 2003), and then individual spores selected and retested using the new enhanced procedure.

10. Appendix 2: Results of the International Ring Test

Table 10.1 Treatments used in the international ring test

Lab	Sample #	Random #	Infected/ uninfected	# T. indica spores	# T. walkeri spores	Treatment label
A	1	0.158479742	1	4	0	2
A	2	0.218256296	1	4	0	2
A	3	0.226156672	1	4	4	4
A	4	0.251148682	1	0	4	3
A	5	0.295342241	1	10	10	5
A	6	0.302460392	0	0	0	1
A	7	0.445703235	1	4	0	2
A	8	0.452946785	0	0	0	1
A	9	0.453685492	0	0	0	1
A	10	0.608591668	1	4	4	4
A	11	0.839962878	1	0	4	3
A	12	0.84725221	1	10	10	5
A	13	0.856557013	1	4	4	4
A	14	0.922108239	1	10	10	5
A	15	0.926525385	1	0	4	3
B	1	0.000108103	1	4	0	2
B	2	0.005755727	1	10	10	5
B	3	0.051901698	0	0	0	1
B	4	0.099264938	1	4	0	2
B	5	0.449181979	1	4	4	4
B	6	0.463349256	0	0	0	1
B	7	0.500344523	1	4	4	4
B	8	0.521049735	1	4	4	4
B	9	0.717615514	1	10	10	5
B	10	0.718771634	1	10	10	5
B	11	0.762325953	1	0	4	3
B	12	0.770413757	1	0	4	3
B	13	0.862692666	1	4	0	2
B	14	0.93854999	0	0	0	1
B	15	0.942515139	1	0	4	3
C	1	0.024641715	1	4	0	2
C	2	0.226830687	1	0	4	3
C	3	0.233472771	1	0	4	3
C	4	0.272186485	1	4	0	2
C	5	0.275722573	0	0	0	1
C	6	0.316843272	1	10	10	5
C	7	0.496256983	0	0	0	1
C	8	0.50432296	1	4	0	2
C	9	0.584490112	1	4	4	4
C	10	0.601087535	0	0	0	1
C	11	0.618696917	1	10	10	5
C	12	0.733809722	1	4	4	4
C	13	0.910454839	1	0	4	3
C	14	0.931347059	1	10	10	5

C	15	0.945544847	1	4	4	4
D	1	0.005770506	1	0	4	3
D	2	0.036310487	1	4	4	4
D	3	0.232757666	1	4	0	2
D	4	0.243514685	1	10	10	5
D	5	0.290442832	1	4	0	2
D	6	0.369980618	1	10	10	5
D	7	0.385346697	1	10	10	5
D	8	0.484972983	0	0	0	1
D	9	0.635651839	1	4	0	2
D	10	0.741542307	1	0	4	3
D	11	0.81458421	1	4	4	4
D	12	0.860164561	0	0	0	1
D	13	0.878814035	0	0	0	1
D	14	0.956014923	1	0	4	3
D	15	0.985039411	1	4	4	4
E	1	0.047708227	1	0	4	3
E	2	0.123738458	1	4	0	2
E	3	0.198679303	0	0	0	1
E	4	0.206175832	1	4	0	2
E	5	0.218995535	1	4	4	4
E	6	0.327261345	0	0	0	1
E	7	0.507377159	1	4	0	2
E	8	0.628833086	1	10	10	5
E	9	0.654312235	1	4	4	4
E	10	0.733025492	1	10	10	5
E	11	0.734076182	1	4	4	4
E	12	0.774190195	0	0	0	1
E	13	0.83955061	1	0	4	3
E	14	0.878866519	1	10	10	5
E	15	0.93906556	1	0	4	3
F	1	0.064633224	1	0	4	3
F	2	0.199410275	1	10	10	5
F	3	0.230107355	1	0	4	3
F	4	0.282467022	1	4	0	2
F	5	0.287669755	1	4	4	4
F	6	0.409500684	0	0	0	1
F	7	0.469373964	0	0	0	1
F	8	0.501707297	1	4	0	2
F	9	0.52698168	1	4	4	4
F	10	0.673477301	1	0	4	3
F	11	0.713145206	1	10	10	5
F	12	0.733832448	1	10	10	5
F	13	0.863585212	1	4	0	2
F	14	0.872966064	1	4	4	4
F	15	0.925484552	0	0	0	1
G	1	0.005340627	1	4	0	2
G	2	0.006679531	1	4	0	2
G	3	0.016656372	1	10	10	5
G	4	0.201563822	1	0	4	3
G	5	0.301560735	1	0	4	3
G	6	0.374154394	0	0	0	1

G	7	0.474674841	0	0	0	1
G	8	0.552065178	1	0	4	3
G	9	0.583666306	1	10	10	5
G	10	0.619143216	1	4	4	4
G	11	0.658621764	1	10	10	5
G	12	0.761797359	1	4	4	4
G	13	0.815172885	0	0	0	1
G	14	0.944062936	1	4	4	4
G	15	0.995855592	1	4	0	2
H	1	0.104837017	0	0	0	1
H	2	0.136033347	1	4	4	4
H	3	0.176327502	1	4	0	2
H	4	0.201112507	1	0	4	3
H	5	0.277063129	1	0	4	3
H	6	0.357120823	1	4	4	4
H	7	0.402347913	1	10	10	5
H	8	0.436818239	0	0	0	1
H	9	0.469900385	1	10	10	5
H	10	0.617782401	1	4	0	2
H	11	0.762149407	1	4	4	4
H	12	0.804552764	1	4	0	2
H	13	0.829826378	1	10	10	5
H	14	0.882512513	0	0	0	1
H	15	0.95801626	1	0	4	3
I	1	0.049058206	0	0	0	1
I	2	0.142108801	1	4	0	2
I	3	0.197154023	1	0	4	3
I	4	0.286283532	1	4	4	4
I	5	0.294454787	1	4	4	4
I	6	0.338829511	1	10	10	5
I	7	0.378124918	1	4	0	2
I	8	0.388099724	1	4	4	4
I	9	0.567548356	0	0	0	1
I	10	0.569200222	1	10	10	5
I	11	0.596543176	1	10	10	5
I	12	0.600608469	0	0	0	1
I	13	0.628781225	1	4	0	2
I	14	0.667701911	1	0	4	3
I	15	0.801717329	1	0	4	3
J	1	0.004182207	1	10	10	5
J	2	0.186310433	0	0	0	1
J	3	0.258151498	1	4	4	4
J	4	0.263481934	1	0	4	3
J	5	0.274154136	1	10	10	5
J	6	0.322116146	1	10	10	5
J	7	0.409826358	1	4	0	2
J	8	0.543496468	1	0	4	3
J	9	0.576411733	1	4	4	4
J	10	0.771001345	1	0	4	3
J	11	0.846479084	1	4	0	2
J	12	0.881903994	0	0	0	1
J	13	0.929591022	0	0	0	1

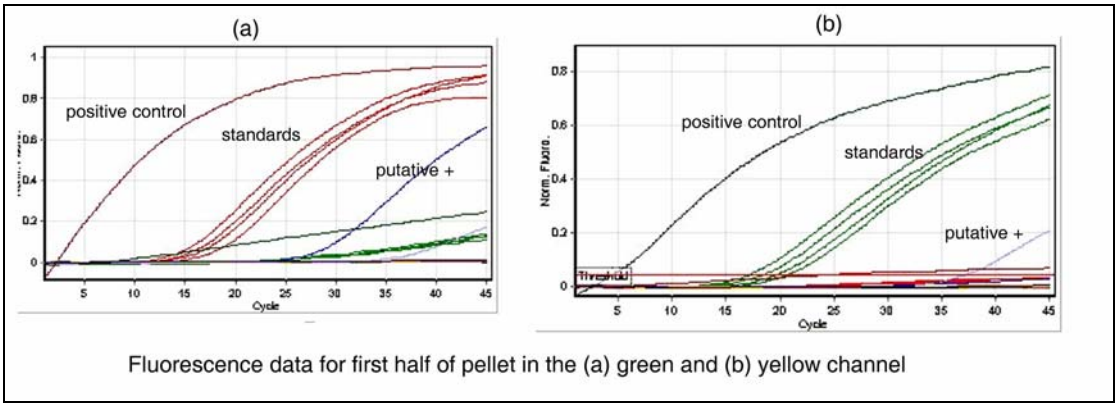
J	14	0.986984567	1	4	4	4
J	15	0.995100258	1	4	0	2

Note: The *T. indica* spores were from bunted grains supplied by Dr. I. Sharma, Punjab Agricultural University. The bunted grains were autoclaved at 121^0C and 15 psi twice, first in India and then on entry into Australia. The *T. walkeri* spores were kept in Shear's solution for more than 5 years.

10.2 Lab H Results Sheet

Lab Letter (on your tubs of grain)		H					
		Detected in 1st half pellet		Individual spores detected in second half of pellet		Confirmation of id for individual spores detected	
Sample Number	Treatment level	T. indica	T. walkeri	T. indica	T. walkeri	T. indica	T. walkeri
1	1 (0,0)	0	0	0	0	0	0
8	1 (0,0)	0	0	0	0	0	0
14	1 (0,0)	0	0	0	0	0	0
3	2(4,0)	0	0	0	1	0	0
10	2(4,0)	0	0	2	1	0	0
12	2(4,0)	0	0	2	1	0	**positive**
4	3 (0,4)	0	0	0	1 broken	0	0
5	3 (0,4)	0	0	0	0	0	0
15	3 (0,4)	0	0	0	0	0	0
2	4(4,4)	0	0	1	1	**positive**	0
6	4(4,4)	0	0	1	1	0	0
11	4(4,4)	0	0	1	2	0	0
7	5 (10, 10)	0	0	1	2	0	0
9	5 (10, 10)	weak positive	0	3	0	0	0
13	5 (10, 10)	0	0	1	3	0	0

Results for the 1st half of pellet



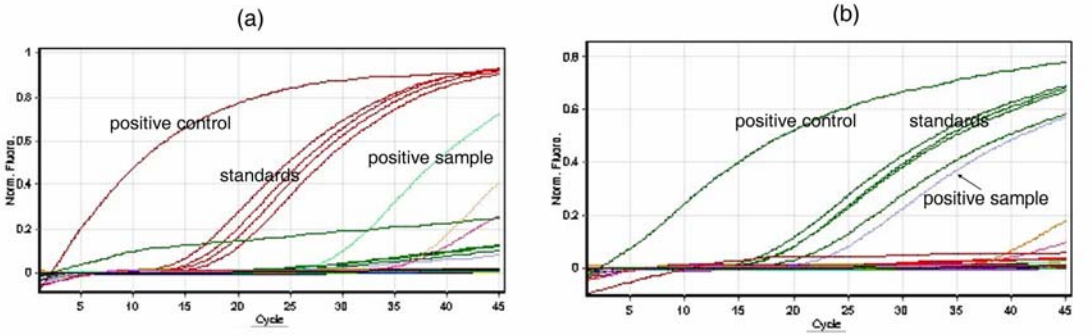
Fluorescence data for first half of pellet in the (a) green and (b) yellow channel

Standards used for Quantifications	CT value
T.indica 10ng/ul	20.8
T.indica 5ng/ul	22.02

Calc Conc (ng/ul)	R2 value
10.22	0.98928
4.56	

T.indica 2.5ng/ul	22.74		2.82	
T.indica 1.25ng/ul	24.03		1.19	
T.indica Positive control from enrichment step	6.68		123,329.43	
H9	34.95		weak positive	
T. walkeri 10ng/ul	17.23		10.18	0.99902
T. walkeri 5ng/ul	18.45		4.95	
T. walkeri 2.5ng/ul	19.67		2.41	
T. walkeri 1.25ng/ul	20.75		1.28	
T.walkeri Positive control from enrichment step	4.59		17,146.42	

Results for the single spore detected



Fluorescence data for single spore analysis in (a) green and (b) yellow channel of a real-time PCR machine

Standards used for Quantifications	CT value		Calc Conc (ng/ul)	R2 value
T.indica 10ng/ul	21.68		10.6	0.99252
T.indica 5ng/ul	23.08		4.47	
T.indica 2.5ng/ul	23.94		2.62	
T.indica 1.25ng/ul	25.14		1.26	
T.indica Positive control from enrichment step	7.64		60,034.67	
H2	35.75		weak positive	
T. walkeri 10ng/ul	19.8		8.79	0.8223
T. walkeri 5ng/ul	21.08		4.5	
T. walkeri 2.5ng/ul	21.06		4.55	
T. walkeri 1.25ng/ul	24.21		0.87	
T.walkeri Positive control from enrichment step	6.18		11,244.86	
H12	26.07		0.33	

Description of single spore detected from International Ring Testing samples

Sample ID	Spore No.	Spines	Ornamentation	Notes
H1	0			
H2	1	coursely arranged	Not very clear	picked
	1	fine and close	fine cerebriform	picked

H3	1	coarsely arranged	Not very clear	picked
H4	1 broken	coursely arranged	thick clumps	picked
H5	0			
H6	1	fine and dense	Not very clear	picked
	1	thick clumps	coarse cerebriform	picked
H7	2	thick and wide ridges	incomplete cerebriform	picked
	1	fine	fine cerebriform	picked
H8	0			
H9	2	fine and close	Not very clear	picked
	1	long and defined	fine cerebriform	picked
H10	2	fine slightly coarse	thick clumps but complete cerebriform	2 picked in same tube
	1	not very dense	Not very clear	pecked
H11	1	fine	complete cerebriform	Not picked
	1	coarse	thick clumps and ridges	picked
	1	coarsely arranged	incomplete cerebriform	Not picked
H12	1	not very coarse	some thin clumps	picked
	1	thick ridges	thick clumps	picked
	1	close but thick	Not very clear	picked
H13	1	wide	thick clumps	picked
	1	thick	thick clumps	picked
	1	fine and individually arranged	fine cerebriform	Not picked
	1	wide	thick clumps	picked
	1	fine and close	narrow ridges	picked
H14	0			
H15	0			

N.B Not all volume of the 2nd half pellet was examined under microscope.

10.3 Lab C Results Sheet

Lab Letter (on your tubs of grain)						C			
		Detected in 1st half pellet				Individual spores detected in second half of pellet		Confirmation of id for individual spores detected	
		Rep 1	Rep 2	Rep 1	Rep 2				
Sample Number	Treatment label	T. indica	T. indica	T. walkeri	T. walkeri	T. indica	T. walkeri	T. indica	T. walkeri
5	1 (0,0)	34.61	32.83	0	0	0	0		
7	1 (0,0)	31.47	0	0	0	0	0		
10	1 (0,0)	0	0	0	0	0	0		
1	2(4,0)	0	0	0	0	0	0		
4	2(4,0)	34.16	36.78	0	0	0	0		
8	2(4,0)	31.47	31.4	0	0	2	0		
2	3 (0,4)	0	0	0	0	0	1		lost
3	3 (0,4)	0	33.26	0	0	0	0		
13	3 (0,4)	0	35.37	0	0	2	0		
9	4(4,4)	0	0	0	0	1	0	38.28	
12	4(4,4)	35.55	0	0	0	0	1		
15	4(4,4)	33.91	38.57	0	0	2	3	35.88	
6	5 (10, 10)	34.93	32.53	0	0	1	0		
11	5 (10, 10)	0	32.41	0	0	0	1		
14	5 (10, 10)	33.03	0	0	0	0	5		

10.4 Lab I Results Sheet

Lab Letter (on your tubs of grain)							I
Date of Testing						26/05/2009	
		Detected in 1st half pellet		**Individual spores detected in second half of pellet**		**Confirmation of id for individual spores detected**	
			2/06/2009				11/06/2009
Sample Number	**Treatment label**	***T. indica***	***T. walkeri***	***T. indica***	***T. walkeri***	***T. indica***	***T. walkeri***
1	1 (0,0)	Weak +	Weak +	0	0	none tested	none tested
9	1 (0,0)	Weak +	Neg	1 spore (not identified)		5 suspect spores tested, all negative	
12	1 (0,0)	Neg	Neg	Nil (1 bleached *T. indica* observed)			
2	2(4,0)	Weak +	Neg	1 spore (not identified)		1 tested negative	
7	2(4,0)	Weak +	Med +	5 spores (not identified)		4 tested, all negative	
13	2(4,0)	Neg	Weak +	4 spores (not identified)		4 tested, 1 medium positive indica,	
3	3 (0,4)	Neg	Neg	3 spores (not identified)		2 tested, both negative	
14	3 (0,4)	Weak +	Med +	4 spores (not identified)		5 suspect spores tested, 1 weak positive walkeri,	
15	3 (0,4)	Weak +	Weak +	4 spores (not identified)		4 spores tested, all negative	
4	4(4,4)	Neg	Neg	1 Tilletia 'type' spore		Positive	Weak positive
5	4(4,4)	An accident during the sieve wash phase caused sample to be lost					
8	4(4,4)	Neg	Neg	0	0	None tested	
6	5 (10, 10)	Neg	Weak +	9 spores (not identified)		3 tested, 1 positive for indica, 2 negative	
10	5 (10, 10)	Neg	Neg	Nil			
11	5 (10, 10)	Neg	Neg	1 T. indica (no PCR result as spore was lost)			

Notes on *Tilletia indica* Ring test from Lab I **11/06/09**

1) Selective sieve wash

One sample (No.5) met with an unfortunate accident so no data is available for this.

2) DNA extraction from ground pellet

250ul from result of split of sieve wash, was placed into a 1.5ml microtube. This was centrifuged @ 1000g for 3 mins. The supernatant was removed using a 1ml pipettor.

Pellet was very small and almost non-existent in some samples. Pellets were ground by hand using a disposable mini grinding pestle in a 1.5ml microtube. There was no evidence of the pellet becoming a smooth paste as there was insufficient pellet to see this. I was unsure of how much force to use and how long to grind the pellet for as I have little experience of extracting spores in this manner, and I am concerned that I may not have ground some of the pellets sufficiently to release the DNA from any spores present.

DNA extraction was carried out using a Qiagen DNeasy Plant Mini kit (50) according to the manufacturers instructions.

3) Enrichment assay

Platform used was the ABI Veriti. For both enrichment assays test and confirmation, dNTPs (bioline) 10µm was replaced by dNTPs (ABgene) 20µm at half the volume. The missing 10µm was made up in the reaction mix by water.

After enrichment, bands were observed for positive controls *T. caries*, *T. indica* and *T. walkeri*, but no bands were observed for any of the extracted samples.

4) Real-time PCR results analysis 1st half of pellet

Platform used was ABI 7900 (96 well format). All reactions were performed in duplicate. *T. indica* had a FAM label and *T. walkeri* a JOE label.

One point on the *T. indica* standard curve was omitted as no DNA had been added, but there was still another valid duplicate result.
T. indica: Set to manual threshold and baseline.
Threshold: ΔRn 597.7
Baseline set between 3 to 10 Ct's.
Slope of standard curve -3.300
R^2 = 0.989

T. walkeri: Set to manual threshold and baseline.
Threshold: ΔRn 39.8
Slope of standard curve -3.456
R^2 = 0.990
No strong amplifications were observed for any sample.

5) Microscopic examination of 2nd half of pellet

A few spores were identified as being *Tilletia* 'type' and occasionally a halo was observed around the spore but it was impossible to visually distinguish between *T. indica* and *walkeri* spores.

Samples which gave a negative result from the real-time assay have also been examined for the presence of spores. One *Tilletia* 'type' spore has been found in a negative sample and subsequently gave a positive result for *T. indica* on confirmation extraction/assay. 3 samples remain to be examined and my colleague will forward those results later.

6) Spore sandwich

Placing spores onto the small piece of microscope slide and making a sandwich did get easier with practise. A couple of suspect spores were lost due to inexperience with this practice.

I found that $1mm^2$ were impossible to create so opted for something in the area of $4mm^2$. I also reduced the volume from 1µl to 0.5µl TE as the larger volume caused the spores to squirt out from between the glass pieces and be lost.

One PCR tube was pierced by glass while I was trying to crush the glass square using a pipette tip. I replaced the tube and transferred the contents as best I could (Sample 4). Is there an alternative to glass?

7) Real-time PCR results analysis confirmation of spores

T. indica: Set to manual threshold and baseline.
Threshold: ΔRn 3.91
Baseline set between 3 to 6 Ct's.
Slope of standard curve -3.99
$R^2 = 0.991$

T. walkeri: Set to manual threshold and baseline.
Threshold: ΔRn 1.03
Slope of standard curve -3.31
$R^2 = 0.940$

Stronger amplifications were observed in a couple of spore reactions.

10.5 Lab B Results Sheet

Lab Letter (on your tubs of grain)		B					
		Detected in 1st half pellet		Individual spores detected in second half of pellet		Confirmation of id for individual spores detected	
		Date of testing 07/07/2009		Date of testing 13/07/2009		Date of testing 15/07/2009	
Sample Number	Treatment No	T. indica	walkeri	T. indica	T. walkeri	T. indica	T. walkeri
3	1	nd	nd	nd	nd	np	np
6	1	positive	negative	nd	nd	np	np
14	1	negative?	positive	nd	nd	np	np
1	2	negative	negative	nd	nd	np	np
4	2	negative?	positive	nd	nd	np	np
13	2	negative?	positive	nd	nd	np	np
11	3	nd	nd	nd	nd	np	np
12	3	nd	nd	nd	nd	np	np
15	3	positive	positive	nd	nd	np	np
5	4	nd	nd	nd	nd	np	np
7	4	negative?	positive	7-1 positive	negative	negative	negative
				7bis/7-2 ?	7bis/7-2 ?	negative	negative
8	4	positive	positive?	nd	nd	np	np
2	5	negative	negative	nd	nd	np	np
9	5	positive?	positive	9A positive	nd	negative	negative
10	5	positive?	positive	10 positive	negative	negative	negative
				10Y positive?	10Y positive?	negative	positive

*	ABI 7000 prism not allowed us to read samples during the touch down cycles (first 5 cycles)
nd	not detected: negative samples with no growing curve necessary to be analysed under ROX passive references, threshold 0.2 and baseline 6 -15
	(correspond 1-10 in our instrument*)
negative?	negative in the report but growing curve present and close to/under the threshold limit
positive?	positive in the report as little amount and growing curve close to/over the threshold limit
np	not performed
7bis/7-2 ?	probable Tilletia sp. spore, but two operatore consider it not T. i. or T.w.
10Y	two spores, one or both juveniles not clearly identifcable by morphology with unsure diagnosis by operators: one operator believe both juvenile spores of *T. indica*, another one operator believe both T.indica, with some probability of T. w. regarding the smaller one; one operator believe both spores belonging to the same species but unable to identify which T. species.
10	*T. indica* spore in glass 10-1

PERFORMANCE OF RING TEST, RESULTS, COMMENTS AND SUGGESTIONS

For Mui Keng Tan

In the ring test we use one probe provided by you (FAM-BHQ1 for *T.indica*) and one prepared by MWG according your works.

However, we performed many pre-Ring test analysis using also CALFLO probe always provided by you. Since these analysis are out of the RING TEST protocol they will be given to you upon your request.

Finally, here below you find some observations for the different phase of the ring test:

WASH TEST:

at the end of the wash test we observed the lack of visible pellet in many samples.

DNA EXTRACTION:

we used samples after the obtainment of wash test pellet leaving 20-30 □l of supernatants in order not lost some material since many samples do not give any appreciable pellet;

DNA PURIFICATION

we used the elution volume of 20ml once as you suggest in your e-mail modifying the DNA easy mini kit protocoll;

ENRICHMENT OF SAMPLES

the enrichment was performed with success as we saw in the gel electrophoresis for the std DNA, moreover by using DNA stds in the Real time we were able to quantify that samples increased their amount of about 1 million;

REAL TIME ANALYSIS 1ST HALF

we performed the analysis on ABI 7000 prism by fixing FAM, JOE as detector, ROX as passive reference, threshold 0,2 6-15 baseline;

baseline was modified to 1-10 cycles checking the passive reference ROX at threshold 0,2 since ABI prism software did not allow monitor fluorescence during the touchdown cycles;

we performed inference on data results also by removing ROX reference and evaluating results with two different threshold one for *T. walkeri* detector and one for *T. indica*, the results indicated an affinity of FAM probe versus *T.walkery* DNA but less efficient in comparison with *T. indica* DNA so that we were able to distinguish both species; this did not happened with JOE probe versus *T. indica*; for the above observations fixing two different thresholds seems to be a necessary step to be performed in the analysis

INDIVIDUAL SPORES DETECTED IN 2ND HALF PELLET

observation were performed after the results of 1st analysis (one week later);

despite than what we expected we did not find so many spores in the samples and moreover we observed many debris, especially in some samples, that would be considered part of disrupted spores but with no clear evidences;

Except the two spore of sample 10Y all the other appeared broken in one or more point of the episporial surface;

COLLECTION OF SPORE

Spore pictures were taken in order to compare the molecular results as you required;

Pick-up and crash of spores were performed and glass sandwich dimension was a little bit higher than your indication and not perfectly squared, but it worked well;

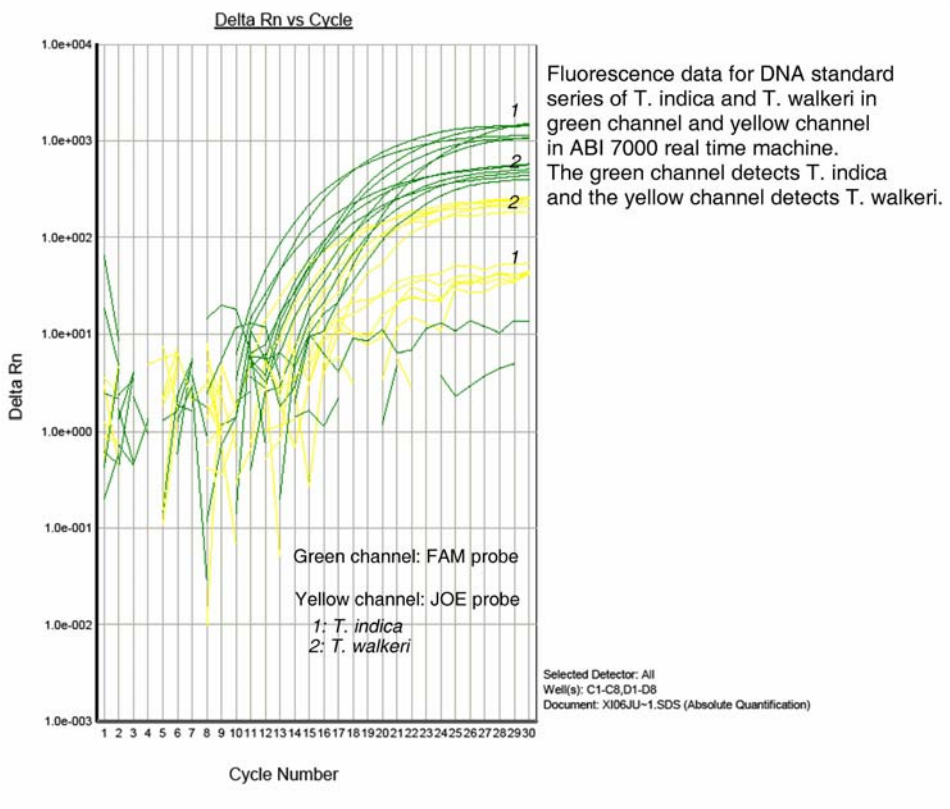
ENRICHMENT PCR

AS ABOVE, as above nothing to report;

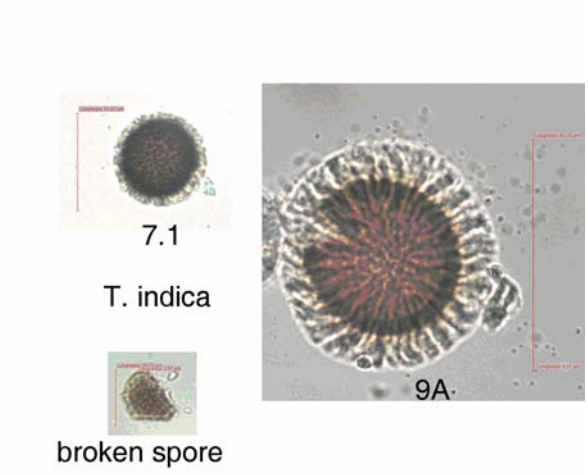
REAL TIME ANALYSIS 2ND HALF

AS ABOVE, nothing to report;

PICTURES



Fluorescence data for DNA standard series of T. indica and T. walkeri in green channel and yellow channel in ABI 7000 real time machine.
The green channel detects T. indica and the yellow channel detects T. walkeri.



Spores present in the 2nd half of pellet from grain washings.
7.1 and 9A are T. indica spores found.
Broken spores were present too.

RESULTS, COMMENTS, SUGGESTIONS

We were not able to detect and identify *Tilletia* species from disrupted single spores by the molecular analysis while we were able to detect two integral spores in combination.

A great question point is opened on the result of the 1st half pellet since I found many positive samples but we were not able to see so many spores in the samples with the morphological approach in the 2nd half analysis. Moreover, the maintenance of the sample before the analysis and in TE buffer for one week before the observations could be a weak point. In fact, many presumable spore debris were found in samples and the few spores

we found were corrupted. Finally, I am not in the position to decide if this results is due to the lost of spores in the conservation or to an invalid result in the first real time. I hope to know the samples, and what are the outcomes of the other colleagues which are involved, will help us to clear the results of this work and the contribute to the ring test.

10.6 Lab G Results Sheet

Lab Letter (on your tubs of grain)	G
Date of Testing	03/07/2009

		Detected in 1st half pellet		Individual spores detected in second half of pellet		Confirmation of id for individual spores detected	
Sample Number	Treatment label	T. indica	T. walkeri	T. indica	T. walkeri	T. indica	T. walkeri
6	1 (0,0)	0	0	0	0	not performed	not performed
7	1 (0,0)	0	0	0	0	not performed	not performed
13	1 (0,0)	0	0	0	0	not performed	not performed
1	2(4,0)	0	0	1	0	not performed	not performed
2	2(4,0)	0	0	0	1	not performed	not performed
15	2(4,0)	0	0	0	0	not performed	not performed
4	3 (0,4)	0	0	0	0	not performed	not performed
5	3 (0,4)	0	0	0	0	not performed	not performed
8	3 (0,4)	0	0	0	0	not performed	not performed
10	4(4,4)	0	0	2	1	not performed	not performed
12	4(4,4)	0	0	1	0	not performed	not performed
14	4(4,4)	0	0	0	0	not performed	not performed
3	5 (10, 10)	0	0	0	1	not performed	not performed
9	5 (10, 10)	0	0	0	1	not performed	not performed
11	5 (10, 10)	0	0	0	3	not performed	not performed

10.7 Lab F Results Sheet

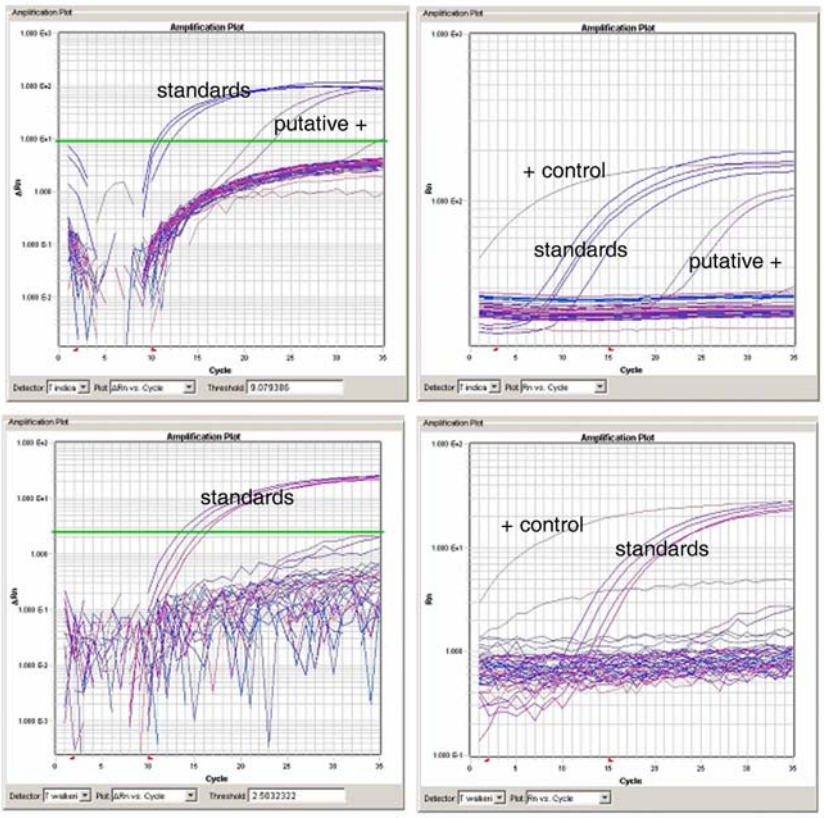
Lab Letter (on your tubs of grain)									F
			Detected in 1st half pellet		Individual spores detected in second half of pellet		Confirmation of id for individual spores detected		#spores detected
Sample Number	Treatment label	Date of Testing	T. indica	T. walkeri	T. indica	T. walkeri	T. indica	T. walkeri	
6	1 (0,0)	28th, May							0
7	1 (0,0)	28th, May							0
15	1 (0,0)	2th,June							0
4	2(4,0)	27th, May							1
8	2(4,0)	28th, May							1
13	2(4,0)	2th,June							2
1	3 (0,4)	27th, May							2
3	3 (0,4)	27th, May							2
10	3 (0,4)	2th,June							0
5	4(4,4)	28th, May							3
9	4(4,4)	28th, May							2
14	4(4,4)	2th,June							0
2	5 (10, 10)	27th, May							4
11	5 (10, 10)	2th,June							5
12	5 (10, 10)	2th,June							2

10.8 Lab S Results Sheet

This test was performed in the national ring testing (April 2008)

PCR No	Sample No	Task Name	Reporter Used	Ct Value	Quantity (fg/PCR)	Reporter Used	Ct Value	Quantity (fg/PCR)
			Tilletia indica			*Tilletia walkeri*		
1	1A	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
2	1B	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
3	1C	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
4	2A	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
5	2B	Unknown	FAM	23.0	+	VIC	Undetermined	0
6	2C	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
7	3A	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
8	3B	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
9	3C	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
10	4A	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
11	4B	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
12	4C	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
13	5A	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
14	5B	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
15	5C	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
16	6A	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
17	6B	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
18	6C	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
19	7A	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
20	7B	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
21	7C	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
22	8A	Unknown	FAM	20.8	+	VIC	Undetermined	0
23	8B	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
24	8C	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
25	9A	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
26	9B	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
27	9C	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
28	10A	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
29	10B	Unknown	FAM	34.4	Weak +	VIC	Undetermined	0
30	10C	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
31	11A	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
32	11B	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
33	11C	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
34	12A	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
35	12B	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
36	12C	Unknown	FAM	Undetermined	0	VIC	Undetermined	0
Ntc PCR		NTC	FAM	Undetermined	0	VIC	Undetermined	0
Pos T indica		Unknown	FAM	Undetermined	+	VIC	Undetermined	0
Pos T walkeri		Unknown	FAM	Undetermined	0	VIC	Undetermined	+
10000 pg/ul		Standard	FAM	10.4		VIC	13.5	

5000 pg/ul		Standard	FAM	10.8		VIC	14.4	
2500 fg/ul		Standard	FAM	12.0		VIC	15.6	
1250 fg/ul		Standard	FAM	Omitted		VIC	16.3	
Water		Unknown	FAM	Undetermined	0	VIC	Undetermined	0
Slope			-2.7			-3.2		
Y-Intercept			21.0			26.3		
R^2			0.9			1.0		



Fluorescence data for T. indica (green channel) and
T. walkeri (yellow) channel in ABI 7900 real time machine