DRAFT NATIONAL CONTINGENCY PLAN FOR KARNAL BUNT OF WHEAT

PART I

BACKGROUND AND IMPORTANCE

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1. INTRODUCTION

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

Karnal bunt is a serious disease for international trade because it reduces grain quality and has a restricted distribution, being limited to areas within the Indian subcontinent, neighbouring Middle East, Mexico, the south-western United States of America and South Africa (Fuentes-Davila 1996, Crous *et al.* 2001).

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

> Karnal bunt replaces part of the wheat seed with a black powder consisting of millions of teliospores. Bunted grain smells foul like rotting fish due to the presence of the volatile chemical trimethyline. Thus the disease reduces grain quality by discolouring and imparting an objectionable odour to the grain and products made from it. It also causes a small reduction in yield.

The disease cycle of Karnal bunt (Figure 1.1) differs from that of common bunt, loose smut and flag smut, so that the seed treatments that are highly effective for controlling these latter diseases are ineffective for controlling Karnal bunt. The introduction of *T. indica* to Australia would impose costs through disruption of export markets and the use of specific control measures to maintain the high quality of Australian wheat grain.

Murray and Brennan (1998) provided the first risk analysis for Karnal bunt for Australia, while Stansbury and McKirdy (2002) estimated the climate suitability for Karnal bunt in Western Australia, confirming the estimates of Murray and Brennan (1998) for that area. This analysis updates and provides additional details to these earlier ones.

Tilletia indica is listed as one of 28 fungal pathogens in the Threat Summary Table of Wheat Diseases compiled by Plant Health Australia. These pathogens are not present in Australia but they have been identified as possible threats to the wheat industry if they became established. This preliminary assessment considered that Karnal bunt posed an extreme economic threat to the industry. This has been borne out by the respondents to the Disease Threat Questionnaire on the Plant Health Australia web site (www.planthealthaustralia.com.au). This questionnaire had 33 responses by 29 October 2003, with the average disease rating being 62.2 (range 40.68), among the highest scores given to any plant pathogen.

T. indica is regarded as a high threat because:

- it reduces grain quality, producing masses of dark powdery spores that discolour the grain and grain products, and having an objectionable ‘dead fish’ smell;

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

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

2. EPIDEMIOLOGY OF *TILLETIA INDICA*

2.1 Disease cycle

The Karnal bunt disease cycle is the chain of events that lead from one occurrence of infected seed to the next occurrence of infected seed. Nagarajan *et al.* (1997) provides the most complete and recent description of the disease cycle, which is shown in Figure 1.1.

The sori develop in the growing seed in the heads of wheat plants. These sori contain masses of teliospores, the dark resting spores of *T. indica*. At harvest, many sori are broken up and vast numbers of teliospores fall to the soil surface. These spores, on and in the soil, are the ones most important for subsequent disease development in the infested area, and are the primary inoculum for the disease. Seeds with sori or contaminated with spores are important for dispersal of the pathogen to new areas (Nagarajan *et al.* 1997).

Survival of teliospores in soil is variable, and influenced by depth of burial, soil type, soil moisture content and temperature. In Karnal bunt areas, survival seems to be at least five years (Nagarajan *et al.* 1997).

Fresh teliospores typically germinate poorly. Better germination occurs in spores that are nine months old (McRae, 1932). Moisture and temperature influence germination. Teliospores germinate to produce a short germ tube (promycelium) with a cluster of basidiospores (primary sporidia) at the tip. For sporidia to be produced on the soil surface, the teliospores must germinate on or near the soil surface, since spores more than 2 mm deep are incapable of growing to the surface (Smilanick *et al.* 1985). On the soil surface, the sporidia germinate to form a hyphal mass. Secondary sporidia of two types develop on the hyphae: filiform sporidia similar to the primary sporidia, and allantoid sporidia (Nagarajan *et al.* 1997).

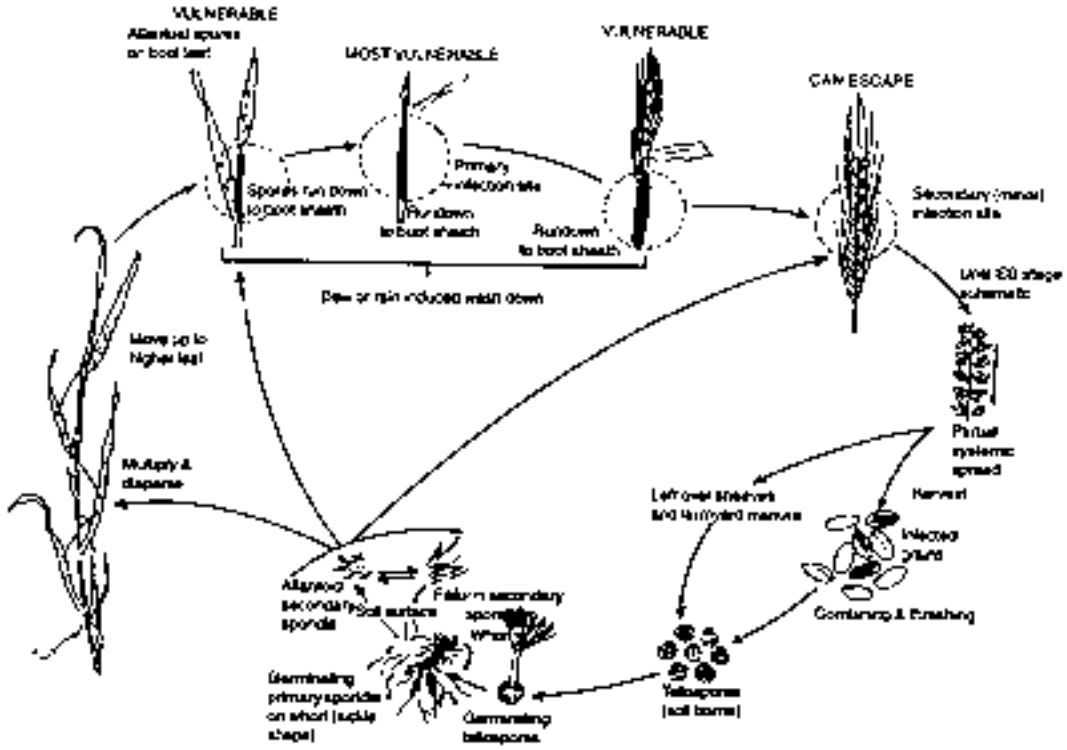

Figure 1.1 Disease cycle of Karnal bunt (from Nagarajan *et al.* 1997), reproduced with permission of CABI.

Primary sporidia, hyphae and secondary sporidia are short-lived and sensitive to desiccation and sunlight. Moisture and temperature influence their survival and development (Nagarajan *et al.* 1997).

Secondary allantoid sporidia are shot into the air and some lodge on wheat leaves and other surfaces. There they can germinate, producing superficial hyphal colonies from which more secondary sporidia can develop. In this way the sporidia reach the terminal or flag leaf of the wheat plant, where dew or rain can wash them into the boot just as the wheat head begins to emerge or becomes exposed (e.g. if the flag leaf surrounding the ear splits), or from where they can be rain-splashed onto the emerged head. Relative humidity, water and temperature within the crop canopy influence survival and growth of the secondary sporidia (Nagarajan *et al.* 1997).

For infection to occur, the timing of teliospore germination and subsequent development of sporidia must coincide with the phenologically susceptible stage of the crop. The results and data from other literature, e.g. Nagarajan *et al.* (1997), suggests that this is likely to be between growth stages (GS) 45 – 61 (although it is possible between GS 43 and 69) (see Appendix A for a detailed outline of the Zadoks Growth Stages). Some data suggests that this window of infection (range of susceptible phenological stages) may vary between cultivars. 'Booting' (GS 45) is when the wheat head is within the flag leaf sheath, and highest levels of infection are considered to occur when sporidia enter the boot cavity just as the head is about to emerge ('first awns visible', GS 49) (Nagarajan *et al.* 1997; Kumar and Nagarajan, 1998). Thus, teliospore germination to produce basidiospores (primary sporidia) must occur earlier, perhaps at or about flag leaf emergence (GS 37), for the sporidia to be available in high numbers at the susceptible period.

Sporidia in the boot can germinate and infect through stomata on the glumes. Once infection has occurred, the fungal hyphae grow to the rachilla and then to the ovaries of florets within the spikelet. Hyphae can also grow to the rachis and invade spikelets above and below the initial infection site. The hyphae invade the ovary before anthesis commences (GS 61). The sorus then develops in the seed to complete the disease cycle. Growth from glume infection to sorus development is most dependent on temperature, although relative humidity may also be important. The hyphae may grow superficially between the interspaces of the lemma and palea to reach the funiculus and directly enter the young ovary (Nagarajan *et al.* 1997).

2.2 Outline for a Karnal bunt model

Knowledge of the factors that control each step in the disease cycle would enable the disease cycle to be simulated in a mechanistic model that would be suitable for estimating the potential for disease development in new areas. However, current models are based on the correlation of disease development with climatic variables. Such methods provide useful models for the area in which they were developed but may not be reliable when used in another area. This would occur if, for example, one part of the disease cycle were usually supported in the present area where the pathogen occurred but was not supported in an area where the pathogen does not occur. Correlation techniques would not discover this relationship.

A complete model for the disease cycle must be able to: simulate teliospore germination in relation to the phenology of the wheat crop; simulate the production of sporidia that will survive and grow on leaves; simulate rain or heavy dew to wash sporidia into the boot or rain to splash the sporidia onto the emerged ear; simulate conditions favourable for sporidial germination and infection; and simulate growth of the fungus in the developing wheat head to produce sori in grain. If teliospores fail to survive in soil, or if they germinate at a time other than about early flag leaf emergence to heading of the crop, no Karnal bunt will develop. If

sporidia fail to survive and develop on wheat leaves between GS 43–69 or fail to spread to the upper leaves/head, no Karnal bunt will develop.

This report examines the relationships described between development of each step of the disease cycle and environmental factors, and then considers the reliability of the correlation-based models for their use to estimate the potential development of Karnal bunt in a new area.

2.3 Relationships between *Tilletia indica*, Karnal bunt development and meteorological factors

Development of Karnal bunt depends firstly on survival of *T. indica* in and on soil between susceptible crops, then on favourable weather conditions for germination of teliospores, infection and disease development from flag leaf emergence to the end of flowering (anthesis) of the wheat crop. Moderate temperatures, high relative humidity or free moisture, cloudiness, and rainfall during anthesis favour disease development (Fuentes-Davila, 1996). There is a range of sometimes-conflicting information available on how abiotic conditions during the rest of the year affect survival of the pathogen and development of Karnal bunt. Warham (1986) and Nagarajan *et al.* (1997) provide summaries of this information.

Until recently, Karnal bunt had a limited distribution, occurring in north western India, Pakistan and some mid-eastern countries of similar latitude, and in Mexico. This suggested that the pathogen had specific environmental requirements that limited its potential distribution. However, the recent occurrences in the south-western states of the USA, in South America (Brazil) and in South Africa show that there is potential for the pathogen to spread to new areas.

Currently, Karnal bunt occurs in areas of 24º-34º N and S latitudes, at low elevations with mild winters, hot summers and low rainfall. In most cases, these areas grow spring wheats that are sown in autumn and harvested in late spring or early summer. Frequently, the wheats are grown under irrigation (after Fuentes-Davila, 1996). Some winter wheat infection has been observed in Texas (G. Peterson, personal communication).

In India, relative humidity and maximum temperature during the 'heading' phase of the crop are the most important factors correlated with the level of disease in the Punjab (Mavi *et al.* 1992). Infection levels are increased with increased levels of nitrogen fertiliser (Aujla *et al.* 1981; Dhiman and Grewal, 1990) but the reason for this is unknown.

2.4 Role of teliospores

2.4.1 Introduction

Teliospores are the long-distance dispersal and survival structures of *T. indica*. At harvest, many fall onto the soil where they survive for one or more years in or on the soil. Most transmission of the disease occurs from teliospores that survive in the field where the wheat crop is grown. Teliospores can also be carried on grain and other materials to establish the pathogen in new areas. The teliospores must germinate at the appropriate time to continue the disease cycle successfully.

2.4.2 Teliospore survival

Survival of teliospores has been investigated under European conditions in an EU Project. Results showed that teliospores survived for at least 36 months buried at 5, 10 and 20 cm in soils of different types at single locations in the field in Italy (sandy clay loam), Norway (sandy loam) and the United Kingdom (clay); depth of burial did not affect survival. Thus, survival in soil does not seem to be a limiting factor for survival of *T. indica* between successive wheat crops in a range of European conditions.

Varying lengths of survival have been reported for teliospores. Viable spores were recovered from wheat seed stored for five years (Zhang *et al.* 1984) and from storage on laboratory shelves for 16 years (M. Bonde and G. Peterson, unpublished data). In India, teliospores survive soil flooding for irrigated rice grown in rotation with wheat. In Arizona, Karnal bunt developed in a wheat crop sown after four years of irrigated Medicago sativa that followed a diseased wheat crop (G. Peterson, personal communication) suggesting that teliospores had survived between wheat crops, unless there was another nearby source of inoculum.

Storage temperature affects survival. In India, teliospores survived for 54 months at room temperature and for greater than 60 months when refrigerated (Krishna and Singh, 1983). Babadoost *et al.* (2004) stored teliospores in a silty clay loam soil for 37 months at 22, 4, -5 and -18°C, recovering 1.6, 2.0, 5.7 and 11.3 per cent of the initial spores, respectively. Germination of the recovered spores was highest for those stored at -5°C.

Varying effects on teliospore survival have been reported for depth of teliospore burial, temperature, soil type and moisture content. In India, survival declined with depth of burial (Rattan and Aujla, 1990; Sidhartha *et al.* 1995); spores survived for 45 months on the soil surface, 39 months at 7.5 cm and 27 months at 15 cm burial (Krishna and Singh, 1983).

Babadoost *et al.* (2004) infested soils collected from four locations with teliospores: the soils were two silty clay loams, a loam, and a silt loam. These were placed in sealed tubes and buried in the field, which was a silty clay loam soil. Initially, the recovery of teliospores declined rapidly from 90.2 per cent on day 1 to 18.7 per cent on day 8, but thereafter remained relatively constant with 13.3 per cent being recovered after 32 months. Germination of the recovered teliospores similarly declined rapidly from 51.3 per cent on day 1 to 15.1 per cent on day 8, but remained at 16.5 per cent after 32 months. Recovery and survival were unaffected by depth of burial. However they found that teliospore recovery was greatest from a loam soil and least from a silt loam soil. Rattan and Aujla (1990) had earlier reported a similar effect of soil type on survival, with it being higher in loamy sand soil than in clay and sandy-loam soils.

Soil moisture content can affect survival. Smilanick *et al.* (1989) found that germinability of teliospores increased slightly after seven months burial in a sandy clay loam soil. However, only the germinability of spores buried in dry soil remained high after 22 months. Recent work by Bonde *et al.* (2004) has shown that survival rates vary between soils collected from different locations: during the first two years, viability declined more rapidly in fields in Kansas (silt clay loam) and Maryland (clay loam) than in Georgia (sand loam) or Arizona (sand loam) in the USA while after two years, viability declined nearly equally. In the laboratory over three years, viability decreased significantly more rapidly in dry soil from Kansas or Maryland than in dry soil from Georgia or Arizona, while pure teliospores remained unchanged (Bonde *et al.* 2004). The results of Bonde *et al.* (2004) show that soil type rather than other environmental factors influences the survival of teliospores at different locations.

Thus, the literature reports show that teliospores can survive for at least three years in most soils, and longer under more favourable conditions. Results from several experiments show that teliospores survive better in sandier soils than in clay soils. Overall, the results show that survival in soil does not seem to be a limiting factor for survival of *T. indica* between successive wheat crops in a wide range of conditions.

2.4.3 Germination of teliospores

Fresh teliospores are relatively dormant with only a low proportion capable of germination immediately on release from the sorus at harvest. *In vitro* studies have been conducted to investigate the effect of moisture and temperature on teliospore germination. Germination was shown to increase from a low level with fresh spores to a higher (but still low) level after nine months; presumably these spores were stored at uncontrolled room temperature in northern India (McRae, 1932). Dhiman and Bedi (1988) reported 1.93 per cent of fresh spores germinated at harvest and this rose to 10.25 per cent after one year of dry storage at 10°C. They also found that germination was abnormal, with a long, branched or unbranched promycelium, in spores up to four months old that were stored dry at 10°C. Exposure to dry heat and to blue light for 6 hours improved germination, but longer exposure of 14 hour was lethal (Rattan and Aujla, 1992). Germination of up to 50 per cent has been reported in one-year-old teliospores (Smilanick *et al.* 1985).

High water content of the substrate and air (> 82 per cent relative humidity, or better with free water) is required for germination. For example, Aujla *et al.* (1990) found that germination occurred in moist soil (> 15 per cent water content, soil type not known, but done at Ludhiana, India).

The effect of moisture and temperature on teliospore germination has been investigated in the EU project. A provisional experiment investigated germination in four soil types (sandy loam, clay loam, sandy clay loam and silty clay) at 5, 10, 15, 25 and 35 per cent (w/w) soil moisture after incubation at 5, 16, 25 and 36ºC for three weeks. Teliospores germinated in all four soil types at 16ºC at 15, 25 and 35 per cent soil moisture content. Detection of teliospore germination was observed at 25ºC in all but the silty clay soil. At 5ºC germination was only observed in the sandy soil at 25 per cent soil moisture. No germination occurred after incubation at 36ºC in any soil or at any soil moisture content

At high water availability, the optimum temperature for germination reported by many studies is 20°C, and occurs over the range 5-25°C, with slow germination occurring as low as 2ºC and up to 30ºC (Zhang *et al.* 1984). Smilanick *et al.* (1985) and Zhang *et al.* (1984) studied the time to commence germination and the rate of germination thereafter at a range of temperatures. From 5 to 25ºC, germination begins (1 per cent of spores germinated) after approximately 100 degree days (base 0ºC) as calculated from the published data in both studies. At 2ºC in the Zhang *et al.* (1984) study, the requirement was 84 degree days, close to the 100 degree days at higher temperatures. Bedi *et al.* (1990) reports the relationship between temperature and the start of germination over the range 5 to 25ºC. The 100 degree day requirement is approximately met at 10, 15 and 20ºC, but was 50 at 5ºC and 200 at 25ºC.

If the incubation of spores is interrupted by freezing or dry conditions, the spores will resume germination on return to higher moisture and temperatures within their germination range (Smilanick *et al.* 1985). Freezing seems to increase germination (Zhang *et al.* 1984).

Under optimum conditions, germination reached or approached 50 per cent of spores, but was reduced at 25ºC (Smilanick *et al.* 1985) and higher (Zhang *et al.* 1984).

There is the question as to what happens to the 50 per cent of spores that do not germinate. Are these available for germination later? Indu Sharma (personal communication) has observed that some spores may be immature and do not germinate, while others may germinate after a long time. Normally, she terminates observations when 30-50 per cent have germinated.

If teliospores are to play an effective role in disease development, it is likely that their germination must start to occur at least by about flag leaf emergence (GS 37). Some lower levels of disease can develop if germination to produce sporidia is timed for the end of anthesis (GS 69) (Nagarajan *et al.* 1997; and results from the EU project).

Thus, there appears to be sufficient data to develop a model for modelling germination of teliospores, providing moisture content and temperature at the soil surface under a plant canopy or on bare soil can be estimated. Degree days can be accumulated while moisture is not limiting, and this accumulation can resume when moisture again becomes favourable.

2.5 Role of primary and secondary sporidia

2.5.1 Introduction

The behaviour of primary (basidiospores) and secondary sporidia (soil surface to flag leaf) has been derived from the scientific literature.

Teliospores germinate with a promycelium that bears a large number (32-185) of basidiospores or primary sporidia in a whorl. This germination and production of primary sporidia occurs at the soil surface. The primary sporidia germinate to produce short hyphae on the soil surface, and secondary sporidia are produced. These secondary sporidia are of two types, allantoid and filiform. The allantoid sporidia are ejected into the air, and can be carried to leaf surfaces within the canopy. Sporidia can survive on several grass species apart from wheat (Rattan and Aujla, 1989), and possibly on other plant and inert surfaces. There the sporidia can germinate, producing short hyphae and then a new crop of secondary sporidia, which then continue to develop in the same manner (Nagarajan *et al.* 1997).

2.5.2 Primary (basidiospore) and secondary sporidial growth

Germ tube growth requires similar moisture conditions as for germination of the teliospores. The germ tube growth of secondary sporidia was studied by Smilanick *et al.* (1989) from 5 to 35ºC on potato dextrose agar (PDA). In the absence of studies of promycelia from teliospores and germ tubes from primary sporidia (basidiospores), it is assumed that their behaviour will be similar. In the Smilanick study, the rate of germ tube growth increased slowly from 5 to 10ºC, then approximately linearly to 25ºC, and declined rapidly to no growth at 35ºC.

There appear to be no studies of the rate of production of primary and secondary sporidia, and it must therefore be assumed that this will be similar to the growth rate of the germ tubes.

2.5.3 Behaviour of secondary sporidia

The production of secondary sporidia from primary sporidia requires light. The release of allantoid sporidia into the air shows diurnal periodicity. Most of these sporidia are released from 0200 to 0600 under high relative humidity and leaf wetness, with fewer released during the day (Sidhartha *et al.* 1995). Bains and Dhaliwal (1989) found most spores were released between 0500 and 0600 (just before sunrise) and that none were trapped between 1400 and

1800. Survival of sporidia increases with increasing relative humidity, but no spores survived for longer than 14 h (Smilanick *et al.* 1989).

2.5.4 Conclusion on the behaviour of sporidia

Relative rates of sporidial production can be estimated from temperature, assuming that relative humidity/moisture is not limiting. However, it is not known whether allantoid and filiform sporidia are produced similarly, or affected differently by temperature and other factors. The release of allantoid sporidia into the air will depend on time of day with most releases occurring shortly before sunrise. Their survival will depend on relative humidity. They will need to germinate and begin growing on leaf surfaces within 14 hours or all will have died. Survival of hyphae on soil and leaf surfaces has not been studied. It is presumed that they are more resistant to drying than sporidia, but would probably die in prolonged dry conditions.

2.6 Glume infection to sorus

2.6.1 Glume infection

It is known that infection can occur from GS (43) - 45 - 61 - (69) (Nagarajan *et al.* 1997). Glume infection requires free water on the flag leaf to wash sporidia into the boot as the awns begin to emerge from it (Nagarajan, 1991) or rain splash is needed for infection of the emerged ear. The most susceptible stage for infection and subsequent development of Karnal bunt is considered to be GS 49 (first awns visible), although infection can occur earlier in the boot, (GS 43) particularly with artificial inoculation by syringe, and later after head emergence up to about the end of anthesis (GS 69) (Singh and Krishna, 1982; Bains, 1994; Nagarajan *et al.* 1997; Kumar and Nagarajan, 1998). Inside the boot, the sporidia fuse to produce dikaryotic hyphae, which penetrate the glumes through stomata. Rain or heavy dew at GS 47–52 (flag leaf sheath opening to ¼ of inflorescence emerged) is required for inoculation of the boot with sporidia (Aujla *et al.* 1990). It is assumed that the rate of infection of the glumes and subsequent development of hyphae within the spikelet is related to temperature in a similar rate to that of germ tube development. Thus, the optimum temperature for infection would be about 20ºC.

2.6.2 Spikelet infection to formation of the sorus

Hyphae in the glumes grow to the rachilla, and then to the florets in the spikelet. Occasionally hyphae can grow to the rachis and then to other florets. From the rachilla, the hypha invades the ovary, where the fungus proliferates as mycelium within the space formed by the disintegration of the middle lamella of the parenchymatous cells of the pericarp. Here the mycelium produces the sorus containing the teliospores (Cashion and Luttrell, 1988; Goates, 1988; Nagarajan *et al.* 1997). Again, the rate of development from hyphal growth to sorus development will be related to temperature, probably with growth rates similar to those published for the development of the germ tubes. Low temperature (15ºC) before inoculation has been shown to predispose wheat to infection, while the optimum temperature for hyphal spread in the head was 18ºC (Sidhartha *et al.* 1995). Evidence from India suggests that higher temperatures during grain development restrict the size of the sorus (I. Sharma, personal communication).

2.7 Estimating 'favourableness' for seed infection

It appears that temperatures of 15-25ºC with rain and high humidity and perhaps clouds to reduce sunlight intensity are required for infection of the heads and development of sori in the developing seeds. Based upon Jhorar *et al.* (1992) and discussions with Dr Jhorar, the Humid Thermal Index (Section 3.3.1) is estimating the stage from sporidial production through infection and disease development. This index over the following growth stages; from boots just visibly swollen to medium milk in the grain ripening process (GS 43-75) has successfully predicted the extent of Karnal bunt development in the Punjab. Other models from India and Mexico show that rainfall at first awns visible to early (¼) head emergence (GS 49-53) is particularly favourable (Nagarajan *et al.* 1997).

3. MODELS

3.1 Introduction

Section 2 shows that teliospores will survive in soil under a variety of conditions. However, they need to germinate from flag leaf emergence to heading for infection to occur. Then conditions suitable for sporidial development, survival and infection, followed by development in the seeds, need to be suitable for Karnal bunt to occur. Models that either directly simulate development from environmental factors or correlate development from these factors are required to estimate the potential for Karnal bunt to develop in new areas. Such models will need to combine a model of crop phenological development with development of *T. indica.*

The HTI was applied to help assess the risk of establishment of *T. indica* in Europe from GS 37-65 (flag leaf just visible to mid-anthesis), the earlier stage being used to account for favourability of sporidial production from germinated teliospores (Figure 1.2).

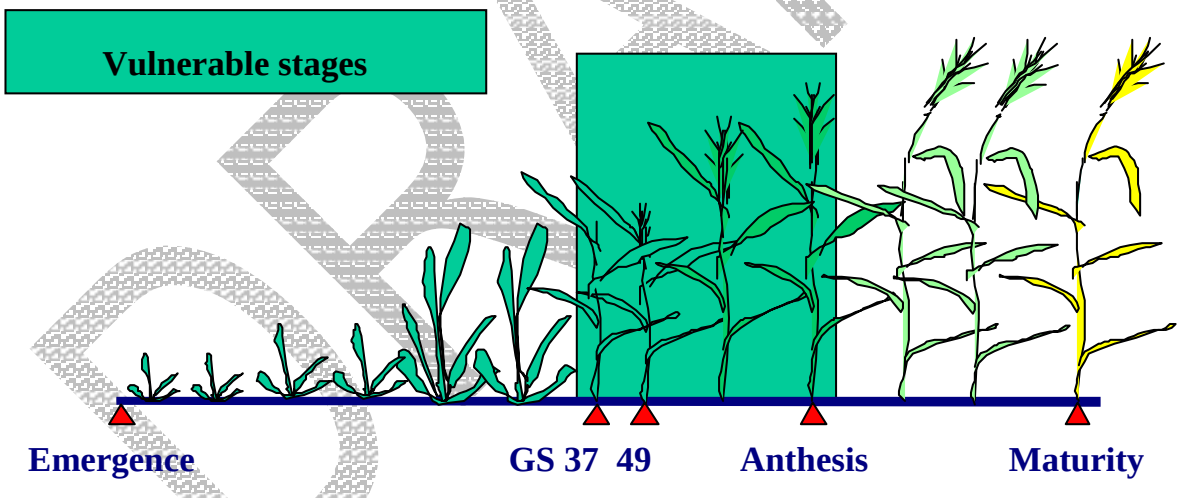

Figure 1.2 Stages of development of wheat over which teliospores must germinate, sporidia infect the head and colonisation of seed begin (GS 37-75).

3.2 Crop models

Development of the disease Karnal bunt requires key stages of development of the pathogen *T. indica* to occur at particular stages in the phenological development of wheat. Thus, any model of disease will require a wheat phenology model that will estimate sowing time and the subsequent key phenology stages of flag leaf emergence (GS 37-39), late boot (GS 49), commencement of ear/head emergence ('heading') (GS 51), end of heading (GS 59), anthesis (early - GS 61, mid - GS 65 and end - GS 69) and if possible mid-milk (GS 75). The EU project has used the bread wheat phenology models AFRCWHEAT and a durum model, IATA to estimate GS 37 to GS 65 from European climate data. The bread wheat model Sirius was used to parameterise some of the variety-dependent responses and to crosscheck the predictions from the AFRCWHEAT model.

3.3 Pathogen models

The ideal pathogen model would similarly estimate the sequential development of key stages of the life cycle of *T. indica*, taking into account wheat development, to simulate disease development. However, no such models exist.

The four models considered within this report are:

1. Humid Thermal Index or HTI (Jhorar *et al.* 1992);
2. 'Geophytopathology' Index (Diekmann, 1993);
3. Rainfall-Temperature Model (Smiley, 1997); and
4. Rainfall model (Nagarajan *et al.* 1997).

Models 1, 3 and 4 are derived from correlation relationships observed in the Indian Punjab between disease severity and weather factors. Multifactorial techniques analysing distribution data and average weather data were used to develop the second model.

3.3.1 The Humid Thermal Index

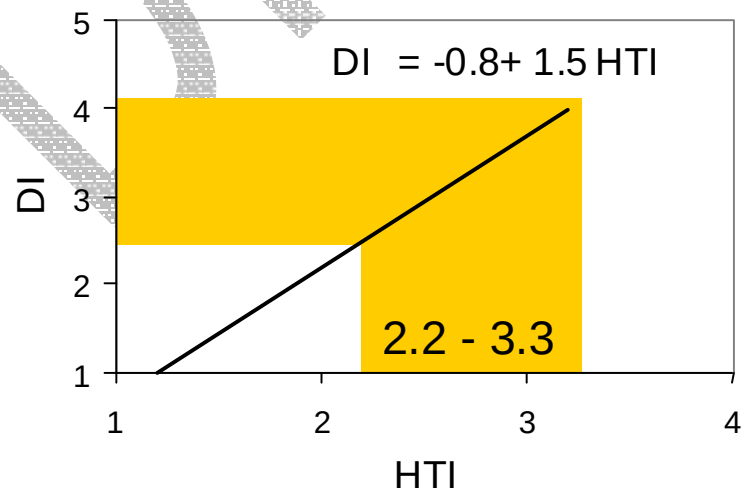

Figure1.3. Relationship between Karnal bunt Disease Index (DI) and the Humid Thermal Index (HTI). The zone where relative humidity and temperature are suitable for a Disease Index of 3 or 4 is shaded.

In India, disease development depends on weather conditions at the heading stage of wheat; Aujla *et al.* (1991) found that over five years with varying incidence and prevalence of Karnal bunt, relative humidity during heading and anthesis was most correlated with disease, while there was also less disease when the average temperature was above 20°C and below 16°C.

Mavi *et al.* (1992) compared Karnal bunt development with weather factors over 17 years, finding that relative humidity and maximum temperature were the most important factors in the Indian Punjab. The Disease Index used to rate the level of Karnal bunt in the Indian Punjab has four classes, defined by Mavi *et al.* (1992) as:

1 = < 2 per cent maximum disease intensity (MDI) and < 30 per cent disease prevalence (DP)

2 = 2 to 2.9 per cent MDI and 30 to 44.9 per cent DP

3 = 3 to 5 per cent MDI and 45 to 60 per cent DP

4 = > 5 per cent MDI and > 60 per cent DP

Mavi et al. (1992) developed a model based on the average maximum temperature during mid to late anthesis (-ve correlation), the 'evening relative humidity' (2:30 p.m. Punjab time or 3 p.m. standard time, (+ve correlation) and sunshine duration (-ve) during early to late anthesis, and the number of rainy days in early anthesis (+ve). This model has an r^2 of 0.89. These correlations need to be treated with caution because DI, the dependent variable, is ordinal rather than continuous with normal distribution. Thus, the probabilities associated with these correlations would not necessarily be those of normal data. Further, the model may not be directly portable to other locations for the following reasons:

- it is likely to be location specific due to the inclusion of sunshine hours
- afternoon relative humidity is usually negatively correlated with maximum temperature and sunshine hours. High correlation of factors usually means that deletion of any one or more of them is unlikely to alter the significance of the model.

Jhorar *et al.* (1992) used the data analysed by Mavi *et al.* (1992) to develop another model based on temperature and relative humidity. They found that the 3 p.m. relative humidity and maximum temperature from the 9th to 11th standard meteorological weeks (SMWs, i.e. weeks from the beginning of the calendar year), number of rainy days from the 9th to 11th SMWs and sunshine duration for the 9th SMW were highly correlated with the amount of Karnal bunt that developed. In the Punjab where this study was undertaken, wheat heads emerge during the 9th SMW and anthesis concludes during the 11th SMW.

Maximum temperature (r = -0.88) and sunshine duration (r = -0.73) were negatively related to disease severity, while evening relative humidity (r = 0.93) and number of rainy days (r = 0.71) were positively related. Regression analysis showed that evening relative humidity (RH) and maximum temperature (Tmax) could be incorporated into a disease model as independent variables in simple regression equations. A Humid Thermal Index (HTI = RH/Tmax) had the highest correlation with disease severity (r = 0.94) and was used for developing a forecasting model. Karnal bunt developed to reach a disease index of 3 or 4 when the HTI was between 2.2 and 3.3. When HTI was between 1.6 and 2.1, the disease index was 2 and when HTI < 1.6, the disease index was 1. Jhorar *et al.* (1992) concluded that when the HTI < 2.2, conditions were either too dry or too hot for disease to develop to severe levels, and when HTI > 3.3, conditions were either too wet or too cold (Figure 1.3).

The HTI model has the same difficulty as the Mavi *et al.* (1992) work in that it is based on an ordinal disease index. However, the finding that severe disease develops when the HTI lies between 2.2 and 3.3 is not affected by this.

The HTI model is used routinely in India to predict the likely levels of Karnal bunt at harvest each year in the Punjab (Indu Sharma, personal communication). The model has been reliable except in one season when very little disease developed although the model predicted a high level. In that season, teliospores germinated during prolonged rain in December, about one month earlier than usual, and the sporidia failed to survive to infect wheat at heading in February (Sharma and Nanda, 2003).

Sansford (1996, 1998) and Baker *et al.* (2000), Murray and Brennan (1998) and Stansbury and Pretorius (2001) have used the Jhorar *et al.* (1992) relationship to predict that conditions at heading would be suitable for Karnal bunt to develop in some areas of the United Kingdom, Australia and South Africa, respectively. These studies used long-term average monthly data on relative humidity and temperature broadly in the months of heading. This use differs from that in India where the model is applied to data within each year. The EU project has succeeded in combining crop phenology models with the HTI using climatic data on a year-by-year basis as well as evaluating the effect of sowing data and crop maturity class across Europe and at the country level for several European countries.

The general success of the HTI to predict Karnal bunt levels in the Punjab suggests that conditions at heading are the most important variables controlling disease development in that environment. However, the failure of the HTI to predict levels when another part of the disease cycle was not coordinated with crop development (Sharma and Nanda, 2003) suggests that a more refined model of the disease cycle is required to predict more accurately whether Karnal bunt can develop in other areas.

3.3.2 The Geophytopathology Index

Diekmann (1993) used 'geophytopathology' techniques to develop a relationship between Karnal bunt presence/absence and (i) the difference between the average maximum and minimum temperature in the month of sowing; (ii) the mean daily minimum temperature in the coldest month of the year; and (iii) the mean daily maximum temperature at anthesis. However, the method compared sites around the world where *T. indica* did and did not occur to develop the model. The presence or absence of disease did not take into account whether *T. indica* had been introduced to the area. If the method had been applied to areas of India and neighbouring countries where there had been considerable time for the pathogen to reach its climate limits, the model would be more reliable.

3.3.3 The Smiley Rainfall-Temperature model

Smiley (1997) used published information to assess whether Karnal bunt could develop in the Pacific Northwest of the USA, an area where the disease is yet to be found. He developed criteria for infection to occur based on published Indian data and relationships: (i) measurable rain (> 3 mm) had to occur on each of two or more successive days; (ii) at least 10 mm had to be collected within the two-day interval; and (iii) average daily relative humidity above the crop canopy must exceed 70 per cent during both days. However, his paper does not state how these relationships were derived. He computed the proportion of times that these conditions were met during the heading interval for several sites in the Pacific Northwest of the USA, and concluded, "it appears possible for *T. indica* to become established in selected regions".

The value of this model was its application to annual data to estimate the proportion of years that were favourable for Karnal bunt development. However, the model has not been validated for India or other locations where Karnal bunt is known to occur. Thus, its general applicability is unknown.

Stansbury and McKirdy (2002) compared the HTI model and their version of the 'Smiley' model in the Western Australian wheat belt. Their 'Smiley' model used the first two criteria but they were unable to obtain the relative humidity data to use Smiley's third criterion. Nevertheless, they found a close correlation between results from the two models.

3.3.4 Rainfall model

Rainfall during the booting stage and ear emergence stages (GS 45-59) is necessary to allow the sporidia to develop on leaves, be washed into the leaf sheath and infect the wheat head (Figure 1.1; Nagarajan *et al.* 1997). Total rainfall and number of rainy days during this two week period were highly correlated with the severity of Karnal bunt in north west India, allowing a model with R^2 of 0.89 to be developed (Nagarajan *et al.* 1997). Rainfall and rainy days during this stage of wheat development were also highly correlated with disease severity for areas of Mexico where Karnal bunt develops, allowing a model with R^2 of 0.91 to be developed (Nagarajan *et al.* 1997). However, the two models are location specific, containing different rainfall and rainy day parameters. In their present form they do not appear to be transferable to other locations.

3.4 Within-season predictive model

A within-season predictive model can be developed, based on the seasonal weather and the HTI, to identify areas that are most likely to be at risk from Karnal bunt. This modelling will identify the main regions to be targeted in the event of a possible outbreak.

3.5 Conclusions

Of the available published models, the Humid Thermal Index (Jhorar *et al.* 1992) appears the most suitable for use in estimating the potential for Karnal bunt to develop in Europe. It is best used with annual data to estimate the proportion of years that are suitable for sporidial production from germinated teliospores, infection and disease development.

The HTI should be computed for the time of the year when wheat is between flag leaf emerging (GS 37) through heading/flowering until to mid milk (GS 75). This time will vary with wheat maturation types and with seasonal conditions. The time will need to be estimated each year based on annual weather data.

The error in this model will arise from germination of teliospores outside the 'window' required for successful infection of wheat. Data suggest that this germination to produce infective sporidia should occur from flag leaf emergence to heading. Models to estimate the germination timing for teliospores are not yet available but it is likely that if teliospores are present on the soil surface they will germinate over a period of time (due to dormancy mechanisms) and some will germinate just prior to the susceptible period for infection leading to crop infection.

4. ECONOMICS AND MARKETING

4.1 Impact on production: yield, quality, and post-harvest issues

4.1.1 Yield losses

Karnal bunt causes only small yield losses (Singh 1986; Warham 1986; Brennan and Warham, 1990; Kehlenbeck *et al.* 1997). There appear to be no differences in yield impacts on bread, durum and feed wheats. The average weight loss in an infected grain is approximately, 25 per cent, so for each 1 per cent of infected grains there is a 0.25 per cent weight loss in harvested yield. Brennan and Warham (1990) examined Mexican data on infected samples from 1981 to 1988 in detail, and estimated that on average the yield loss where Karnal bunt is endemic averages 0.1 per cent per year. Sharma (pers. comm.) provided information on the Indian Punjab from 1994 to 2004 showing that 33 per cent of samples were infected and that the average infection level was 0.13 per cent, implying an average yield loss of approximately 0.03 per cent per year.

These two sources provide the following information:

		Mexico 81-88	Punjab 94-04
Incidence:	Average % of samples with infected grain	37%	33%
Infection:	Average level of infected grains per sample	0.4%	0.13%
Yield loss		0.1%	0.03%

The levels of infection and yield loss are expected to be similar tho those in Mexico and India if Karnal bunt were to be established in Australia. With state average yields varying from 1.3 t/ha to 1.9 t/ha (Table 1.1), and a national average yield of approximately 1.7 t/ha in recent years, those losses represent 0.4-2.5 kg per hectare, or $0.08 to $0.50 per ha. In higher-yielding regions, these values could reach $0.80 per ha, which is still a virtually insignificant loss, in terms of the gross value of the industry.

Table 1.1 Wheat data, by State[a]

	NSW	VIC	QLD	WA	SA	Australia
Area (000 ha)	3,379	1,315	701	4,675	2,001	12,080
Yield (t/ha)	1.86	1.85	1.34	1.54	1.77	1.69
Production (000 t)	6,295	2,432	938	7,222	3,547	20,457
Exports (000 t)	3,097	1,724	749	6,623	3,143	15,337
Domestic consumption	3,198	707	189	599	404	5,120
% exported	49%	71%	80%	92%	89%	75%
Gross value of production ($m)	$1,467	$566	$218	$1,677	$843	$4,777

[a] For detailed estimates by type of wheat, see Appendix Table 1.

4.1.2 Long-term contamination of productive land

Once a crop is infected with *Tilletia indica*, spores are scattered throughout the paddock. As these spores survive for several years in the soil and on the soil surface, the paddock in which the affected crop was grown is effectively contaminated with spores of KB fungus for several years. Any wheat crop grown in that paddock within the next 5-8 years will have spores that can infect the crop and lead to an outbreak of KB.

Once Karnal bunt was widespread in a region, all machinery (tractors, headers, trucks, trailers, cultivation machinery), equipment and storage facilities in that region would be contaminated with spores of KB fungus. All of these would need steam cleaning to prevent spores being further spread within the region, and all such equipment would also need cleaning before moving to other regions. The estimated costs for individual machines could be $30 to $200, but the total costs of the cleaning is likely to be in the order of $0.10 per hectare of crop in an affected region.

In addition, bags and other items used in handling the contaminated straw will be contaminated with spores, as well the straw of infected crops. While significant for particular loads and shipments, the cost is likely to be very small on a per hectare basis.

4.1.3 Additional costs of field control treatments

Once KB is detected in a crop, there are no management treatments or responses that can reduce the damage in that season, other than crop destruction.

In a situation where the disease became endemic, farmers in the affected region would be able to plant a more resistant variety. Some varieties have been found to have levels of resistance to KB (GRDC reports, CIM 0003, CIM 0008). However, those varieties are not necessarily the latest, highest-yielding varieties, so that farmers who were to grow them would effectively suffer a yield reduction from the best non-resistant variety. The size of that yield reduction would vary from region to region, and would be dependent on the relative yields of the most resistant variety and the highest-yielding non-resistant variety.

Where the disease was endemic, farmers growing wheat could also use additional applications of a fungicide, likely to cost approximately $80 per hectare, to reduce the likelihood of infection.

4.1.4 Post-harvest effects on product quality and processing

Direct quality losses occur when *infected* wheat is considered unsuitable for food uses and as a result is down-graded to feed wheat, where ‘Feed’ wheat is wheat suitable only for animal feed that is traded on the feed grains market. The economic cost associated with the loss of value of food wheat (both bread and durum) when it is down-graded to feed wheat, is highest where production is aimed at higher-priced premium grades (Murray and Brennan, 1998). If 37 per cent of samples have infected grains, then 37 per cent of production will be down-graded, as in Australia wheat infected with Karnal bunt would not be acceptable for food production, even though there are no human health concerns.

Where the presence of Karnal bunt was a marketing issue, and resulted in closure of some markets for Australian wheat, unaffected wheat from the affected region may still be down-graded.

Where wheat is down-graded in quality, the grower receives a lower price. The loss of value from down-grading is shown in the following table. Where production is down-graded, the loss of value, based on recent averages (Table1.2), would be: Australian Prime Hard (APH) $71/t, Australian Hard (AH) $54/t, Australian Premium White (APW) $43/t, and Australian Standard White (ASW) $35/t.

Table 1.2 AWB average pool payments, by grade (per tonne)

						Premiums lost when downgraded to feed			
Year	**APH[a]**	**AH[a]**	**APW[a]**	**ASW[a]**	**Feed**	**APH**	**AH**	**APW**	**ASW**
1995-96	289.00	262.30	254.40	249.40	219.30	70	43	35	30
1996-97	232.00	213.00	205.00	200.00	168.00	64	45	37	32
1997-98	230.00	205.50	198.00	193.00	163.00	67	43	35	30
1998-99	240.00	197.50	187.50	180.00	130.00	110	68	58	50
1999-00	233.00	193.00	181.00	178.00	145.00	88	48	36	33
2000-01	255.00	236.00	225.00	217.00	182.00	73	54	43	35
2001-02	265.00	247.50	233.00	225.00	190.00	75	58	43	35
2002-03	337.00	311.00	297.00	289.00	240.00	97	71	57	49
2003-04	243.50	232.00	224.00	212.00	190.00	54	42	34	22
2004-05 (p)	216.50	206.50	199.00	194.00	160.00	57	47	39	34
5 yrs to 2004	*263.40*	*246.60*	*235.60*	*227.40*	*192.40*	*71*	*54*	*43*	*35*

[a] APH - Australian Prime Hard; AH - Australian Hard; APW - Australian Premium White; ASW - Australian Standard White(p) preliminary, as at April 2005

Source: **AWB Ltd.**

The presence of Karnal bunt is also likely to exacerbate the differences between feed wheat prices and those for the food wheat grades. If quantities of wheat are shifted from the higher grades to feed grade, the prices of the premium grades are likely to rise, while the increased quantities of feed wheat are likely to reduce its price. Brennan, *et al.* (2004) found that these effects can be significant in the European Union. A similar analysis for Australia (Brennan unpublished) shows that prices for feed wheat can be expected to fall if large quantities of wheat are re-classified as feed. The extent of those changes depends on the elasticities of demand for feed wheat, and for feed grains in general because of the substitutability between the different feed grains.

4.1.5 Allied industries dependent on wheat

The majority of Australian wheat is exported unprocessed (Table1.3), though the proportion varies from as little as 49 per cent in NSW to 92 per cent in WA (Table 1.1). For the proportion exported, the “value adding” component involves handling, transport and storage of unprocessed grain from farm to port. As affected grain moves through this chain, the spores of the KB fungus contaminate the trucks, rail trucks, storages, augers and conveyor belts. All of these become contaminated, and are then liable to transfer those spores to other, unaffected grain taken through the same system subsequently.

Table 1.3 Supply and disposal of Australian wheat, 2000-01 to 2004-05

('000 tonnes)

	2000-01	2001-02	2002-03	2003-04	2004-05	Average 5 yrs to 04-05
Production	22,193	23,960	10,058	25,700	20,376	**20,457**
Domestic use						
- Human and industrial	2,185	2,208	2,418	2,443	2,487	**2,348**
- Feed	2,000	2,100	2,700	2,185	2,239	**2,245**
- Seed	519	503	558	540	530	**530**
- Other	11	71	0	-1	-29	**10**
Total	**4,715**	**4,882**	**5,676**	**5,167**	**5,227**	**5,133**
Exports	16,085	16,304	9,113	17,867	16,719	**15,218**
Total disappearance	20,800	21,186	14,789	23,034	21,946	**20,351**
Change in stocks	1,393	2,774	-4,731	2,666	-1,570	**106**
% exported	72%	68%	91%	70%	82%	**74%**
% domestic usage	21%	20%	56%	20%	26%	**25%**
% added to stocks	6%	12%	-47%	10%	-8%	**1%**

Source: **ABARE *Crop Report* (various).**

On average in the five years to 2004-05, approximately 5.1 million tonnes of wheat were consumed or processed domestically (Table 1.3). The main domestic uses of wheat are:

- flour-based products including bread, cakes and gluten products;
- wheat-based products such as breakfast foods;
- wheat for ethanol production;
- wheat for stockfeed; and
- wheat for seed.

The spores of KB are not toxic to humans and\or animals, so there are no direct human health issues. However, wheat with even moderate levels of infestation has an unpleasant 'fishy' odour that makes it unsuitable for use in food products (or animal feed at high levels of contamination). In an industry where quality assurance schemes 'from paddock to plate' are becoming widespread, the use of KB-infected grain in the human food chain is unlikely, even though there are no direct human health concerns. Thus the effect on flour mills and cereal-food processing would be significant if they used KB-infected wheat. The mill would be permanently contaminated, and the mill offal (bran and pollard), which contained the spores, would need to be carefully managed or heat-treated to avoid spreading the spores more widely.

Experience in the USA has shown that it is impossible to completely remove all spores from a complex handling chain, particularly handling and processing facilities. In the USA affected areas, some facilities are dedicated solely to KB-infected wheat, and are not available for use for unaffected grain.

Wheat used for stockfeed has two main pathways to consumption:

- Direct consumption by livestock.
- Grain processing though heat treatment (pelletisation, etc.).

Where wheat is fed directly to animals, such as chickens, the spores are not killed or sterilised by passing through an animal's gut. Thus, while the spores are not toxic to animals, the manure would be contaminated with live spores. The manure would need to be carefully managed or sterilised if the spores were not to be spread further though the manure. Where infected wheat is subjected to suitable heat treatment, the spores are killed. Thus, processes such as pelletisation, where the heat applied in the process is above that level, allow contaminated grain to be used without any risk of spreading spores or leading to further contamination. However, the processing plants would become contaminated with spores, at least in the sections where the infected grain was stored and handled prior to heat treatment.

Thus, the use of KB-infected grain as animal feed is feasible, especially grain processing involving heat treatment, but the presence of spores in the processing plant and in the manure of animals fed KB-infected grain, would impose major costs on those processing industries.

If the disease were to become endemic, industries based on the processing of contaminated grain for feed, and industries using feed wheat directly, could spring up within the affected region. One option is ethanol production. Rendell (2005) revealed plans to establish a series of medium-scale ethanol plants in the eastern wheat belt, with a view to using diseased or otherwise damaged wheat. Bunted grain would provide a good opportunity for such operations, and could provide a valuable outlet for contaminated grain in the event of an outbreak or if the pathogen became endemic.

If the controls were imposed to eradicate the disease, existing processing plants (for all end-uses) involved would be severely affected because of the difficulty of decontamination, and could have embargoes or strict decontamination regimes placed on them.

Since spores can also be contained in stubble and straw, industries relying on straw processing will also be affected by a Karnal bunt outbreak. Although any processing involving heat treatment is likely to destroy spores, the processing plants would become contaminated with spores if straw from affected crops were processed. If the policy were to eradicate Karnal bunt, these plants could be severely impacted by the policies, in terms of where they could source straw and/or decontamination costs if affected straw had already been processed.

4.2 Impact on the market for wheat

The presence of Karnal bunt in a country can lead to an embargo on exports from that country by some markets.

Many wheat-importing countries will not allow wheat to be imported unless it is certified as 'Karnal-bunt free'. On the first report of the discovery of Karnal bunt in a region, these countries suspend imports of all wheat from that country until the nature of the outbreak is clarified. As the nature and location of the outbreak is clarified by surveys and further testing, the embargo on wheat shipments is narrowed to shipments from the affected region(s). If the outbreak is detected in an isolated region, and the markets can be convinced that other parts of the country are not similarly affected, then the restrictions can be lifted on those unaffected parts.

In the Australian context, a detection in one State might initially lead to all Australian wheat shipments being regarded as suspect. If testing reveals no presence of spores in shipments from others states, the restrictions can be lifted on those states, and exports from them can

resume. As trace-back occurs within the affected state, the shipments to which restrictions apply may be reduced to those emanating from one port or one production region.

This has been the case for Karnal bunt in Arizona and Texas in the United States in recent years. In both cases, exports to sensitive markets have proceeded from the other production regions in the USA without restriction once it was shown that the wheat from those regions did not have Karnal bunt spores. Similarly, the suspected outbreak in Western Australia in 2004 for wheat being shipped to Pakistan meant that initially all Australian wheat was suspect, but in a short time the restrictions were lifted on wheat from the other states.

This distinction is less controversial where there is a clear geographical boundary between production regions, such as the Nullarbor Plain in the case of Western Australia. However, there are fewer natural barriers and boundaries within the contiguous wheat belt of Eastern Australia, so the difficulties of defining the extent of an outbreak would be greater in that situation. Nevertheless, although the 'wheat belt' extends from Central Queensland through New South Wales and Victoria to South Australia, there are some discontinuities in wheat-producing areas that allow the definition of some natural boundaries to regions (Figure 1.4).

Not all markets refuse to take wheat that has, or is suspected of having, Karnal bunt spores. There are a number of reasons for the differing attitudes to the possible presence of Karnal bunt:

1. countries that do not have their own wheat industry are less likely to be concerned about the possible spread of Karnal bunt;
2. the efforts of the USDA to convince markets that Karnal bunt is an unimportant disease means that there may be increasing numbers of countries prepared to accept that view;
3. countries that already have Karnal bunt may be less concerned about importing the pathogen (note, however, the Pakistan situation of March 2004); and
4. countries with low resources may be prepared to take Karnal bunt infected grain if it can be obtained at a lower price.

Rush *et al.* (2005) indicated that at the time of the initial discovery of Karnal bunt in the USA in 1996, 37 countries (accounting for nearly 50 per cent of US wheat exports) listed Karnal bunt as a quarantine pest. After the outbreak, the (US) APHIS could not issue a phytosanitary export certificate on the basis of national freedom from Karnal bunt. After negotiation, before they would import USA wheat, these countries required an Additional Declaration for Karnal bunt, declaring that "The wheat in this shipment originated in areas of the United States where *Tilletia indica* (Karnal bunt) is not known to occur". An additional 11 countries then sought to have that Additional Declaration for their wheat imports from the US. Eventually, all countries agreed to this declaration, and USA exports have continued to flow.

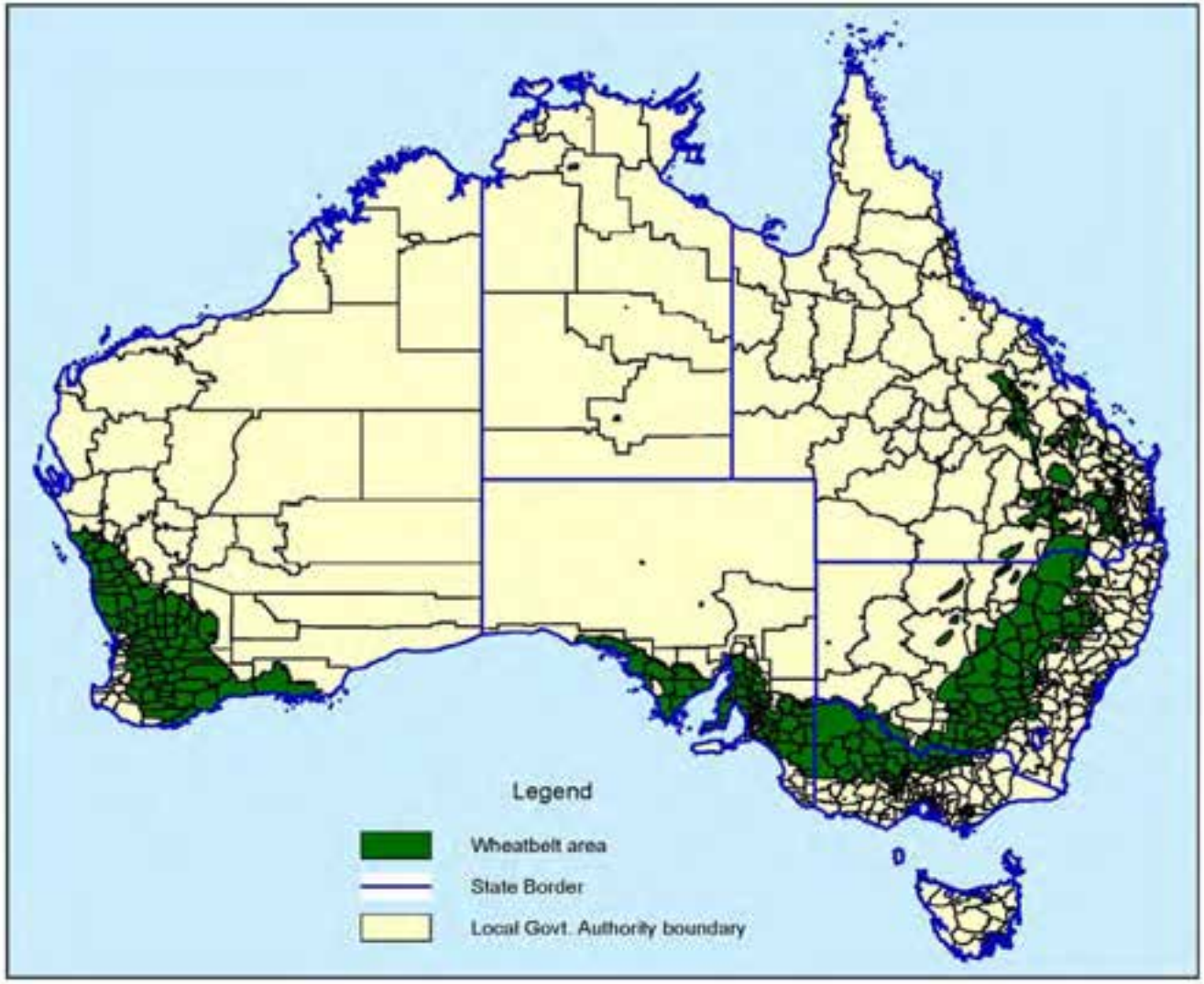

Figure 1.4 Map of Australia showing wheat-producing regions.

The response of different countries to the presence of Karnal bunt in wheat imports has been identified in three different sources:

1. Rush *et al.* (2005) list the countries that, before importing US wheat, require an Additional Declaration for Karnal bunt;
2. AQIS website lists countries that have restrictions on imports of wheat with Karnal bunt; and
3. Smith (2001) listed the countries that had specific restrictions on wheat in relation to Karnal bunt.

While no one list in these three sources is comprehensive for all countries to which Australia exports wheat, by combining the information in each list a comprehensive (though not complete) list is possible (see Appendix Table 2). In addition, there are some inconsistencies between the lists. Where there were inconsistencies in the lists, AQIS was taken as the most up-to-date authority for Australian wheat. On the basis that Rush (2005) is more current than Smith (2001), wherever they disagreed the Rush response was used. Where one source lists a country that is not on the other lists, its response is accepted. On that basis, the reactions shown in the "Restrictions" column of Appendix Table 2 are taken as the most comprehensive listing available. However, there are still gaps, notably with Japan and Pakistan listed as having no restrictions (despite the 2004 incident), and no listing for Iran, one of Australia's export markets. Countries producing 79 per cent of the world's wheat have restrictions on the entry of wheat from areas with Karnal bunt (Table 1.4).

Table 1.4 Reactions of wheat markets to presence of KB

World wheat production	**Production ('000 t)**	**% of total**
Countries with restrictions	440,299	79%
Countries without restrictions	108,078	19%
Total restrictions unknown	7,971	1%
Total	**556,349**	**100%**
Australian wheat exports (3 years to 2003-04)	**Quantity**	**% of total**
To countries with restrictions	3,336	22%
To countries without restrictions	8,424	55%
Total restrictions unknown	3,429	23%
Total	**15,188**	**100%**

From Table 1.4, 22 per cent of Australia's wheat exports in the three years to 2003-04 have been to markets that have restrictions on wheat with Karnal bunt, while 55 per cent have been to markets with no restrictions. A further 23 per cent has gone to countries for which the reactions are not identified in the above sources. Two key markets for Australian wheat,

Indonesia and Iraq, are both listed as not having restrictions on wheat with Karnal bunt, and a third (Iran) is believed to have no restrictions. Other countries such as Singapore, Malaysia and Papua New Guinea are also major markets with no restrictions. Australia's major markets with restrictions are Egypt, South Korea and New Zealand.

While these reflect the stated restrictions a present, it is likely that many countries without current restrictions would move to apply restrictions in the event of an outbreak in Australia, so that the loss of markets, at least in the short term, would be greater than indicated by the figures in Table 1.4.

4.3 Impact of controls

4.3.1 General

The control costs associated with an outbreak of KB (Brennan and Warham 1990; Kehlenbeck *et al.* 1997) are associated with the efforts that occur in an attempt to control and/or eradicate the disease.

If there were an outbreak of KB, widespread testing and surveillance programs would be undertaken, so that testing and surveillance costs would be incurred. The cost items to be considered here are not the already extensive current costs of surveillance at the border and the current regular grain testing costs, but rather the increase in costs of the additional testing that would be carried out in the event of an outbreak. In addition, the cost of any surveys to define the presence of the pathogen or to define the limits of its spread also needs to be incorporated into the cost estimates.

In addition, containment and/or eradication costs would be incurred in the event of an outbreak of KB. For example, it is likely that there would need to be fumigation of harvesting, transport and handling machinery and equipment, and there may be a need to treat mill by-products from the milling of infected grain, and possibly treatment for animal manure from animals fed KB-infected grain. If restrictions were placed on the crops that farmers could grow within the quarantine zone, or if seed treatments were required for seed sown within the zone (Brennan and Warham, 1990), such costs would also be containment and/or eradication costs. There are also likely to be costs of ensuring compliance with any regulations and policies introduced to control or eradicate KB. The costs of administering the controls and of ensuring compliance with any regulations are considered as control cost items.

The precise contingency plans for such control actions are needed before full costing can be undertaken, given an outbreak scenario. The control cost components identified are summarised in Table 1.5.

Table 1.5 Possible cost control components for an outbreak of Karnal Bunt

Survey and identification costs
Administrative – compliance costs
Cropping restrictions
Yield reduction from tolerant variety
Additional fungicide costs
Value of standing crop destroyed
Costs of destroying affected grain
Treatment of mill by-products
Grain processing costs (heat treatment)
Livestock industry costs
Machinery cleaning costs
Facility cleaning costs

4.3.2 Defining the affected quarantine region

In the event of an outbreak in Australia, the definition of the quarantine region depends on the point of detection (see Part III, section 3). However, the first step is to determine the port zone in which the initial detection occurs, and to determine whether other port zones are affected.

If the detection occurs at the port, the quarantine restrictions will depend on the port zone in which the detection occurs. Ports from each port zone vary widely in size and tonnage that is shipped from there (see Table 1.6 and Appendix Table 3). In five of the 19 zones, there are fewer than nine receival sites, while for another five zones there are up to 43 sites. However, for the larger port zones (Geelong, Port Kembla, Fremantle and Newcastle), more than 100 receival sites would be affected if the whole port zone was restricted. In terms of tonnages exported, the 10 smallest ports cover a total of 20 per cent of exports, the largest five cover 61 per cent of exports (with Fremantle accounting for 27 per cent of exports), so that if an outbreak occurred in one of more of these large port zones, the impact of the restrictions would be extremely high.

Table 1.6 Components of port zones in Australia[a]

(average of 2002-03 and 2003-04)

Port zone	Average ('000 t)	% of total	No. of receival sites	Tonne per receival site ('000 t)
Brisbane	476	3%	59	8
Gladstone	93	1%	12	8
Mackay	10	0%	7	1
QLD sub total	**579**	**4%**	**78**	**7**
Newcastle	992	7%	106	9
Port Kembla	987	7%	166	6
NSW sub total	**1,979**	**13%**	**272**	**7**
Geelong	544	4%	188	3
Melbourne	994	7%	79	13
Portland	377	3%	85	4
VIC sub total	**1,915**	**13%**	**352**	**5**
Port Adelaide	1,169	8%	83	14
Port Giles	242	2%	3	81
Port Lincoln	1,148	8%	31	37
Port Pirie	61	0%	4	15
Thevenard	118	1%	5	24
Wallaroo	205	1%	9	23
SA sub total	**2,943**	**20%**	**135**	**22**
Albany	1,307	9%	43	30
Esperance	683	5%	16	43
Fremantle	3,924	27%	123	32
Geraldton	1,426	10%	25	57
WA sub total	**7,340**	**50%**	**207**	**35**
Total	**14,755**	**100%**	**1,044**	**14**

[a] For more details, see Appendix Table 3.

Source: AWB Ltd.

5. CONTROL

5.1 Introduction

The options for control are quite limited. The best option is to prevent the disease from entering and establishing within Australia (Part II).

5.2 Fungicides

The European project tested five foliar fungicides were tested for their *in vitro* efficacy against mycelial growth and sporidial germination of *Tilletia indica.* Results from both types of *in vitro* tests indicated that azoxystrobin was the most effective of the five fungicides tested. Propiconazole, epoxiconazole and tebuconazole also showed good activity. Prochloraz was the least effective.

The project also examined the use of fungicides on inoculated wheat using both a standard variety grown and a highly susceptible Indian variety. The results showed that azoxystrobin acted as a protectant when applied at GS 39 or GS 49 and as an eradicant when applied at GS 65 or GS 71.

This investigation has shown that there are several fungicides that have potential for use against infection of wheat by *T. indica* and the development of Karnal bunt. Although there are no published reports on the efficacy of the strobilurin azoxystrobin for this purpose, it compares favourably to propiconazole, a well-established chemical with a long history of efficacy at reducing (but not eradicating) Karnal bunt when used as a foliar spray in countries where the pathogen is established. With the exception of prochloraz, the chemicals tested as part of this Project could have a significant role to play in disease management as part of normal farming practice for the wheat crop, should *T. indica* ever become established in the European Union.

Although no seed treatment is 100 per cent effective, several treatments that inhibit teliospore germination are available. These are shown in Appendix Table 4 (UC Davis, 2004). A summary of the ones that would be easy to get an emergency permit is shown below:

- Dividend
- Vitavax, other seed dressings (Raxil, Baytan, etc.).

There is the possibility of using a fungicide spray at heading:

- Propiconazole at 25 per cent heading and then 10 days later (South Africa recommendation).
- Azoxystrobin (EU recommendation).

This use of seed dressing would be useful, for controlling smuts but if grain was imported into Australia, and there was the risk of possible contamination due to an unclean cargo hold, the spores that maybe present on the seed would be killed with a seed dressing.

5.2.1 Impact of controls

- Seed dressing: impact should be minimal. Western Australian farmers very familiar with use of seed dressing. Maybe required for seed being imported into Australia to reduce the risk.
- Foliar sprays: withholding period and residues, need to be determined. If sprays are used in more northern region greater risk of withholding period due to faster finish of crop.

5.3 Breeding

Currently there is a project running at the International Wheat and Maize Improvement Centre (CIMMYT) in collaboration with Australia:

- KB resistance is a current breeding target at CIMMYT.
- Resistance in bread wheats is partial resistance (resistant lines express lower levels of infection).
- Some resistance in novel sources (some synthetic wheats) shows as immunity (resistant lines express no infection).

Initially there was another GRDC investment from 1997-2003 (CIM 0005) to access resistance identified at CIMMYT:

- CIMMYT resistance crossed into a limited number of Australian backgrounds and resistant material was returned to Australia.
- Subsequent penetration of this material back into Australian programs is reported to be low.
- A small number of Australian varieties were shown to have partial resistance.

Another current project:

- GRDC investment 2003-2006 (CIM 0008) towards marker assisted selection of resistance in breeding:
  - Molecular genetic studies on partial resistance in cv. Frame aims to identify molecular makers that can aid in selection of resistance in the absence of the disease
  - Develop new breeding material using immune resistance sources to enable subsequent studies on molecular markers for the improved resistance, work is in progress.

5.4 Cultural

Use of disease-free seed is essential. Resistant cultivars are being developed, but at present, no cultivars are immune. Durum wheat and triticale, however, are less susceptible than bread wheat.

In areas where the soil has become infested, rotate to crops other than wheat, durum wheat, and triticale for up to five years.

Mulching with polyethylene can be used to raise soil temperature and reduce teliospore germination.

Planting dates can also be adjusted so that heading does not occur under weather conditions conducive to infection.

5.4.1 Impact

Minimal - but hard to introduce into cropping system and there will be a delay waiting for resistant cultivars to be available.

6. PEST RISK ASSESSMENT

6.1 Part of plant or commodity affected

Seed.

6.2 Primary host range

Wheat (*Triticum aestivum*)
Durum (*Triticum durum*)
Triticale (*X Triticosecale*)

6.3 Current distribution

Figure 1.5 World distribution of Tilletia indica (CABI 2003).

Asia

Afghanistan
India - widespread
Bihar
Delhi
Gujarat
Himachal Pradesh
Haryana
Jammu and Kashmir
Madhya Pradesh
Indian Punjab
Rajasthan
Uttar Pradesh
West Bengal
Iran - restricted distribution
Iraq
Nepal
Pakistan - restricted distribution
Pakistan Punjab
North-West Frontier

Africa

South Africa present, few occurrences

North America

Mexico – restricted distribution
- Sonora
- Sinaloa
- Baja California Sur

USA – present, few occurrences
- Arizona
- California
- New Mexico
- Texas

South America

Brazil – absent, reported but not confirmed
- Rio Grade do Sul - present, few occurrences

6.4 Potential distribution in Australia

Murray and Brennan (1998) used the 'Humid Thermal Index' (Jhorar *et al.* 1992) to estimate the favourability of weather during heading and anthesis of wheat for development of Karnal bunt throughout the Australian wheat belt. Many locations in Western Australia, South Australia, Victoria, Tasmania and New South Wales had weather conditions suitable for Karnal bunt development. Conditions in Queensland and northern areas of the remainder of the wheat belt appeared too warm while some more southern areas within the wheat belt appeared either too cold or wet. Stansbury and McKirdy (2002) confirmed these estimates for Western Australia.

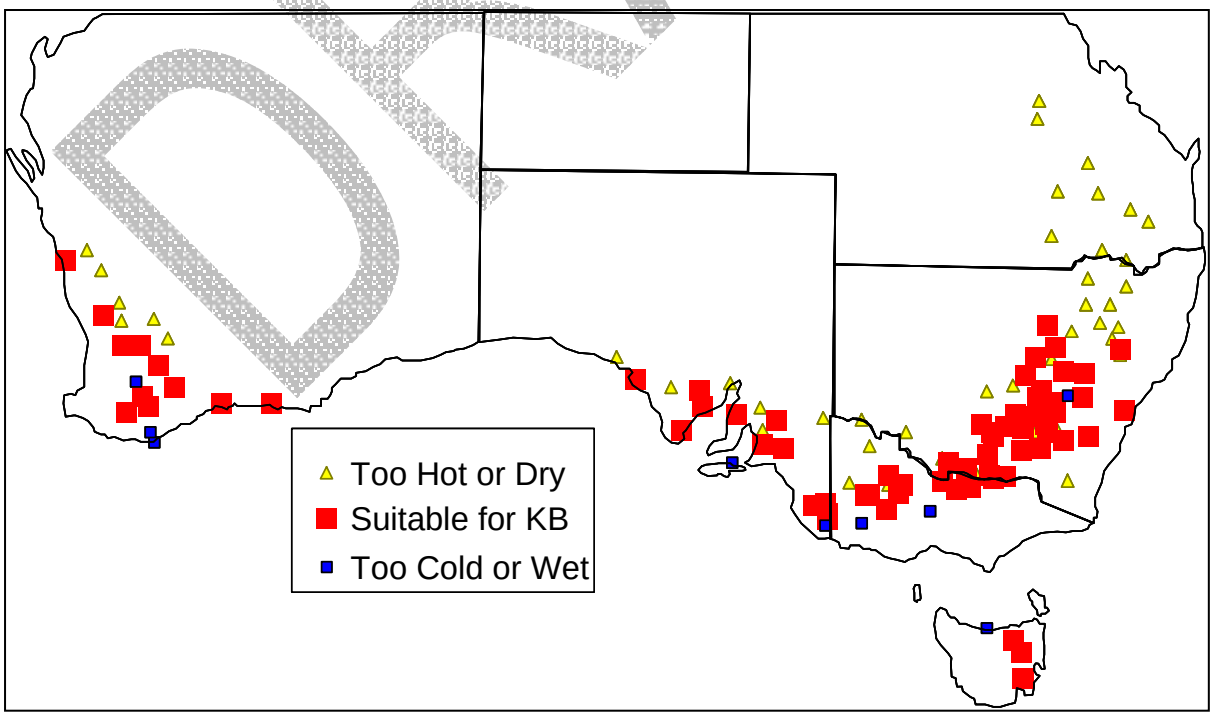

Figure 1.6 Estimated potential distribution of Karnal bunt in Australia (Murray and Brennan 1998).

6.5 Biology

6.5.1 Identification

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

Symptoms of the bunts are not readily seen in crops. When severe, they are readily seen and smelt in the harvested grain.

Formal identification of *Tilletia indica* is based on symptoms on seed, morphology of the teliospores, and detection of the unique DNA sequence by PCR techniques. These are covered in detail in later sections (Part IV) of this report.

6.5.2 Symptoms

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

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

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

6.5.3 Disease cycle

A pathogen maintains itself by continued re-infections over years. The 'disease cycle' is the detailed description of the chain of events that lead from one point in the development of the disease to the next occurrence of that point. The Karnal bunt disease cycle then is the chain of events that lead from one occurrence of infected seed to the next occurrence of infected seed. This description of the disease cycle is based on Nagarajan *et al.* (1997) and is shown diagrammatically in Figure 1.1.

The sori develop in the growing seed in the heads of wheat plants. These sori contain masses of teliospores, the dark resting spores of the Karnal bunt fungus. At harvest, many sori are broken up and vast numbers of teliospores fall to the soil surface. These spores, on and in the soil, are the ones most important for subsequent disease development in the infested area, and are the primary inoculum for the disease. Seeds with sori or contaminated with spores are important for dispersal of the pathogen to new areas.

Survival of teliospores in soil is variable, and influenced by depth of burial, soil type, soil moisture content and temperature. In Karnal bunt areas, survival seems to be at least five years.

Fresh teliospores typically germinate poorly. Better germination occurs in spores that are nine months old. Moisture and temperature influence germination. The teliospores germinate to produce a short germ tube (promycelium) with a cluster of basidiospores (primary sporidia) at the tip. For sporidia to be produced on the soil surface, the teliospores must germinate on or within 1 mm of the soil surface. On the soil surface, the sporidia germinate to form a hyphal mass. Secondary sporidia of two types develop on the hyphae: filiform sporidia similar to the primary sporidia, and allantoid sporidia.

Primary sporidia, hyphae and secondary sporidia are short-lived and sensitive to desiccation and sunlight. Moisture and temperature influence their survival and development. Secondary allantoid sporidia are shot into the air and some lodge on wheat leaves and other surfaces. There they can germinate, producing hyphae from which more secondary sporidia can develop. In this way the sporidia reach the terminal or flag leaf of the wheat plant, where dew or rain can wash them into the boot just as the wheat head begins to emerge, or onto the emerged head. Relative humidity, water and temperature within the crop canopy influence survival and growth of the secondary sporidia.

The timing of teliospore germination and subsequent development of sporidia must coincide with the development of the crop. Booting is when the wheat head is within the flag leaf sheath, and highest levels of infection occur when sporidia enter the boot just as the head is about to emerge (growth stage 49 on the Zadoks Scale). Thus, teliospore germination must occur earlier, perhaps at or about flag leaf emergence, for the sporidia to be available in high numbers at growth stage 49.

Sporidia in the boot can germinate and infect through stomata on the glumes. Once infection has occurred, the fungal hyphae grow to the rachilla and then to the ovaries of florets within the spikelet. Hyphae can also grow to the rachis and invade spikelets above and below the initial infection site. The hyphae invade the ovary before anthesis. The sorus then develops in the seed to complete the disease cycle. Growth from glume infection to sorus development is most dependent on temperature, although relative humidity may also be important. The hyphae may grow superficially between the interspaces of the lemma and palea and reach the funiculus (attached part to the rachilla) and directly enter the young ovary.

Only some ovaries in the spikelet will be infected. The fungus grows under the seed coat to produce the sorus in which new teliospores develop. Temperature during grain formation influences the size of the sorus: under mild conditions the sorus reaches maximum size and can replace most of the grain, while at high temperatures it may fail to develop or be pinpoint sized.

In India, weather conditions from flag leaf emergence to mid-milk formation have been correlated with the severity of Karnal bunt. Jhorar *et al.* (1992) developed the 'Humid Thermal Index' (ratio of average afternoon relative humidity to average daily maximum temperature from flag leaf to mid milk) to estimate when conditions were suitable for disease development. This ratio has been used to estimate whether areas in the United Kingdom, Europe and Australia are suitable for Karnal bunt development (Sansford 1998, Murray and Brennan 1998, Stansbury and McKirdy 2002).

6.5.4 Dispersal

The Karnal bunt fungus disperses as teliospores. Dispersal of teliospores can be:

- admixed or in sori on wheat seed and bulk grain;
- as contaminants of bulk commodities through contaminated containers, machinery, etc.:
  - on trash in second-hand machinery;
  - on clothing and personal effects of travellers;
  - windborne; and
  - carried by birds and animals, either on fur and feathers or in the intestinal tract (Murray and Brennan 1998).

The first four are possible means of trans-ocean spread while the last two would be additional means of movement within and between adjoining areas.

6.6 Assessment of likelihood

6.6.1 Entry potential

Entry potential is Low, but clearly possible given the expected combination of the following factors:

- Australia imports commodities such as bulk fertiliser, agricultural machinery and some bulk feed grains and has large numbers of travellers. These materials could be contaminated with teliospores (see Dispersal, section 6.5.4). Entry through the imports of bulk grain or fertiliser appears to be the most likely means of entry estimated at a probability of 0.023 per year, with entry by all modes being estimated at a probability of 0.042 per year into Western Australia (Stansbury *et al.* 2002).
- There is a high frequency of travel between areas in India and USA where the pathogen exists and Australian farming areas.
- Long distance dispersal by wind is unlikely to result in successful establishment, as there needs to be a high local concentration of spores for successful establishment. Infected seeds and spores would pass through the gut of migratory birds long before they could fly from an infested area to Australia. Thus, these two methods of dispersal are unlikely to provide entry.
- There appear to be no interceptions of *Tilletia indica* in Australia.
- Karnal bunt was first detected in the Mexico in 1972, presumably after introduction some years earlier from India. It was intercepted on Mexican material being imported into the USA many times before being found in the southwestern USA in 1996. The most recent incursion was in South Africa in 2000.

- Teliospores of *T. indica* are long-lived and survive extremes of temperature when dry. There is a high probability that they would survive admixed with bulk commodities or in contaminated agricultural machinery.

- The pathogen is difficult to detect by visual inspection unless seen fruiting on wheat seed. A washing technique followed by light microscopy is required to detect teliospores. Thus, increased surveillance of imported goods would be expected to detect *T. indica* more reliably.

6.6.2 Establishment potential

Establishment potential is Medium, as the pest has the potential to survive and become established in between approximately one-third and two-thirds of the wheat belt, since:

- Climate of much of the wheat belt of Australia appears suitable for infection of wheat heads by the pathogen (see Figure 1.6 and Murray and Brennan 1998). However, no study has been made of the climate suitability for survival of the pathogen in Australian soils or whether teliospore germination will be coordinated with wheat development so that they germinate only at flag leaf emergence to late booting of the crop.
- A limiting factor for establishment is that a large local concentration of teliospores is necessary for a high probability of infection. Successful establishment would be more likely if the entry was of a large number of sori into a small area. The entry of small widely dispersed numbers of spores is unlikely to result in successful establishment due to the Allee effect (Garrett and Bowden 2002).
- There is a history of establishment of *T. indica* in new environments overseas. The pathogen had successfully established in Mexico by 1972 and in the USA and South Africa in the last 10 years. New locations have been found in the USA after the Arizona detection in 1996.
- The pathogen is unlikely to be detected in the field. All detections have been on harvested grain. The earliness of detection will be related to the level of surveillance (Stansbury *et al.* 2002).
- The hosts of *T. indica* in Australia are wheat, durum, triticale and possibly cereal rye. Wheat is widely grown in all States.
- The pathogen has one reproductive cycle each year. Its rate of increase is unknown. Stansbury *et al.* (2002) have estimated the probability of establishment for various pathways of entry. The settings of the parameters in their model are largely arbitrary, but with low values set they estimated that there was a combined probability of 0.015 establishments per year.

6.6.3 Spread potential

The spread potential is High, as *T. indica* had potential for natural spread to all contiguous production areas because:

- Climate does not affect spread of spores on grain, contaminated machinery, etc.
- Its overseas history shows that the pathogen has spread to scattered sites in the southwestern USA in a wide area from southern California in the west to northern Texas in the east.
- There are no known vectors, although teliospores can survive passage through the gut of birds and animals and potentially be dispersed in this manner.

- There are no natural enemies of the pathogen.
- Its characteristic of forming sori containing masses of teliospores on wheat seeds, means that it is readily transported whole on seed while at harvest many spores are dispersed onto sound grain and throughout harvesting and grain handling equipment. These spores can contaminate clean grain that subsequently passes through such equipment.
- Grain quality can be controlled by grain inspection, hygiene, partial resistance to the disease and crop rotation to reduce spore loads in soil. Fungicides can reduce the level of seed contamination but seed treatments do not protect plants from infection of heads. However, the presence of the pathogen in an area may make all grain from that area unacceptable to some buyers or countries through quarantine regulations.
- The control measures for maintaining grain quality are largely compatible with existing control strategies. However, crop rotation requires at least a five-year break from wheat. Such rotations may be feasible in mixed pasture/cropping systems but would not currently be used in more intensive cropping systems.
- There is no evidence of successful eradication of the pathogen overseas.

6.7 Overall entry, establishment and spread potential

The risk of overall entry is then ranked Medium.

6.8 Assessment of consequences

6.8.1 Economic impact

Extreme - Presence of Karnal bunt in Australia has the potential to reduce grain quality and, through quarantine regulations, restrict the sale of Australian wheat to overseas buyers. If Karnal bunt spread to the extent of its estimated range, the combined cost of quality and sale restrictions was estimated to be 17 per cent of the value of Australian production ($490,900,000 per year) by Murray and Brennan (1998). Losses varied between States depending on the area likely to be affected, with 1 per cent loss in Queensland, 15 per cent in Victoria, 18 per cent in New South Wales and Western Australia and 23 per cent in South Australia. Using a different method of calculation, Stansbury *et al.* (2002) estimated the potential loss in Western Australia to range from 8 to 25 per cent of the value of production, comparable to the loss estimated by Murray and Brennan (1998).

6.8.2 Environmental impact

Negligible - There is no potential to degrade the environment or otherwise alter the ecosystems by affecting species composition or reducing the longevity or competitiveness of wild hosts.

6.8.3 Social impact

High - The large reduction in the value of production would be expected to cause major social impact with significant losses to the local and broader community. These losses have not been estimated. However, they are being researched by the European Community and by Plant Health Australia.

6.9 Combination of likelihood and consequences to assess risks

Qualitative risk analysis (McLeod) was used to assess the risks as follows:

- Economic risk: Extreme. Specific action is immediately required to reduce risk.
- Environmental risk: Low risk. Manage through routine procedures.
- Social risk: Extreme. Specific action is immediately required to reduce risk.

Stansbury *et al.* (2002) applied a quantitative analysis to the risk. Although the parameters used in the calculations are only estimates, they showed that the risk could be substantially reduced by increasing quarantine efforts and by increasing surveillance to detect the pathogen at an earlier stage.

6.10 Surveillance

It is clear from the estimates of Stansbury *et al.* (2002) that there are considerable economic benefits from improving quarantine procedures to reduce the risk of entry and crop surveillance to detect an outbreak of Karnal bunt as soon as possible after a successful establishment.

Evidence from the USA suggests that it takes several years after a successful establishment before Karnal bunt reaches obvious levels of affected grain. Improved surveillance to detect the disease at lower levels may result in successfully containing it to a smaller area, with the possibility of eradication. The ability to declare area freedom would mean that less of the grain harvest would suffer a value loss (Murray and Brennan 1998).

6.11 Diagnostics

Samples with suspected *Tilletia indica* would need to be identified quickly and accurately. Part IV of this Contingency Plan describes methods of sample preparation, initial microscopic examination, detailed microscopic examination, and the procedure for identification by molecular techniques. Detailed microscopic examination and a preliminary identification based on morphology can be done a suitably experienced and trained mycologist. This identification will need to be confirmed by another taxonomist and by molecular techniques. The molecular techniques will be available in New South Wales and Western Australia. See Part IV for the procedures.

6.12 Training

There is a need to provide on-going training of field officers and workers in the grain handling industry to be alert for symptoms of Karnal bunt to maximise the likelihood of early detection.

7. RESEARCH OPTIONS

7.1 Before detection

- There is a need for on-going screening of advanced breeding lines and common varieties for resistance to Karnal bunt
- There is a need to ensure that research capability, both in the research and in diagnostics to ensure that the Australian industry is prepared for any incursion.

7.2 Following detection of Karnal bunt

There is some research that needs to be carried out in parallel with the incident to provide information that will facilitate the management of the incursion.

- Sow spore-survival and germination trials (in contained trials) in the Restricted Area (refer to USDA and EU protocols).
- Use different soil types for the above experiments, as Bonde *et al.* (2004) have shown there are differences in survival due to soil types.
- Spore dispersal trials, using spore traps and trap plants. This will enable the appropriate-sized zone to be determined that will intercept spore movement with the greatest degree of confidence.
- Validation of seed treatment and foliar spray efficacy in the field, with a view to full label registration if required.

8. Bibliography

Anonymous (1998). EC Food and Veterinary Office Report on a Mission in Italy from 4 to 7 August 1998 on the interception of two consignments of durum wheat contaminated by *Tilletia indica*. http://europa.eu.int/comm/food/fs/inspections/pi/reports/italia/pi_rep_ital_1440-1998_en.pdf

Anonymous (2001). Update on the situation of *Tilletia indica* in the USA, EPPO Reporting Service, Issue 6, Item 2001/109.

Aujla SS, Gill KS, Sharma YR, Singh D, Nanda GS, 1981. Effect of date of sowing and level of nitrogen on the incidence of Karnal bunt of wheat. *Indian Journal of Ecology* **8:** 175–179.

Aujla SS, Kaur S, Gill KS, 1991. Forecasting Karnal bunt epidemic: a criterion based on analysis of weather profiles of epidemic and non-epidemic years. *Indian Journal of Ecology* **18:** 122–129.

Aujla SS, Sharma I, Gill KS, 1990. Effect of time and method of inoculation on Karnal bunt development. *Indian Phytopathology* **43:** 223–225.

Babadoost M, Mathre DE, Johnston RH, Bonde MR, 2004. Survival of teliospores of Tilletia indica in soil. *Plant Disease* **88:** 56–62.

Bains SS, 1994. Influence of wheat spikes maturity on susceptibility to infection and growth of sporidia of Neovossia indica. *Indian Journal of Mycology and Plant Pathology* **24:** 111-115.

Bains SS, Dhaliwal HS, 1989. Release of secondary sporidia of Neovossia indica from inoculated wheat spikes. *Plant and Soil* **115:** 83–87.

Baker RHA, Sansford CE, Jarvis CH, Cannon RJC, MacLeod A, Walters KFA, 2000. The role of climatic mapping in predicting the potential distribution of non-indigenous pests under current and future climates. *Agriculture, Ecosystems and the Environment* **82:** 57–71.

Beattie, B.R. and Biggerstaff, D.R. (1999), "Karnal bunt: A wimp of a disease, but an irresistible political opportunity", *Choices*, Second Quarter 1999, 4-8.

Bedi PS, Singh S, Singh PP, 1990. Effect of some physical factors on germination and sporidial potential index of teliospores of Neovossia indica, the pathogen of Karnal bunt of wheat. *Plant Disease Research* **5:** 38–46.

Bonde MR, Berner DK, Nester SE, Peterson GL, Olsen MW, Cunfer BM, Sim T, 2004. Survival of Tilletia indica teliospores in different soils. *Plant Disease* **88:** 316–324.

Brennan, J.P., Thorne, F., Kelly, P.W. and Murray, G.M. (2004), "Defining the costs of an outbreak of Karnal bunt of wheat", Contributed paper presented at the 48th Annual Conference of the Australian Agricultural and Resource Economics Society, February 2004.

Brennan, J.P. and Warham, E.J. (1990), *Economic Losses from Karnal Bunt of Wheat in Mexico*, CIMMYT Economics Working Paper 90/02, International Maize and Wheat Improvement Center (CIMMYT), Mexico.

Brennan, J.P., Warham, E.J., Byerlee, D. and Hernandez-Estrada, J. (1992), "Evaluating the economic impact of quality-reducing, seed-borne diseases: Lessons from Karnal bunt of wheat", *Agricultural Economics* **6:** 345-52.

Cardwell, K.F., Dowell, F., Mitchel, J., Riemenschneider, R., Spaide, R. and Vocke, G. (2003), "Phytosanitary versus regulatory risk of losses due to wheat Karnal bunt in the USA", Paper for the 8th International Congress of Plant Pathology, Christchurch, New Zealand February 2003 (Abstract).

Cashion NL, Luttrell ES, 1988. Host-parasite relationships in Karnal bunt of wheat. *Phytopathology* **78:** 75–78.

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

Dhiman JS, Bedi PS, 1988. Effect of storage period on germination behaviour of Neovossia indica causing 'Karnal' bunt of wheat. *Journal of Research Punjab Agricultural University* **25:** 576–580.

Dhiman JS, Grewal DS, 1990. Effect of row orientation and nitrogen level interaction on brown rust and Karnal bunt diseases of wheat. *Indian Journal of Ecology* **17:** 167–170.

Diekmann M, 1993. Epidemiology and geophytopathology of selected seed-borne diseases. Aleppo, Syria: ICARDA.

FAO (2005), Area, yield, production and trade data from FAOSTAT (http://apps.fao.org).

FAO/IGPRI, 1995. FAO/IGPRI Technical Guidelines for the safe movement of small-grain temperate cereals. FAO/IGPRI, Rome. (http://apps.fao.org)

Fuentes-Davila G, 1996. Karnal bunt. In: Wilcoxson RD, Saari EE, eds. Bunt and smut diseases of wheat: concepts and methods of disease management. Mexico, D.F: CIMMYT, 26–32.

Garrett, K.A., Bowden, R.L. (2002). An allee effect reduces the invasive potential of Tilletia indica. *Phytopathology* **92:** 1152.1159.

Goates BJ, 1988. Histology of infection of wheat by Tilletia indica, the Karnal bunt pathogen. *Phytopathology* **78:** 1434–1441.

GRDC (CIM5): Protecting the Australian wheat industry from karnal bunt - Component 1- CIMMYT (2003).

GRDC (CIM 0008) Protecting the Australian wheat industry from Karnal bunt through the development and implementation of molecular markers (2006).

Inman, A.J., Hughes, K.J.D., Bowyer, R.J. (2003). EU Recommended protocol for the diagnosis of a quarantine organism 'Tilletia indica'. Central Sciences Laboratory, York, UK.

Jhorar, O.P., Mavi, H.S., Sharma, I., Mahi, G.S., Mathauda, S.S., Singh, G. (1992). A biometeorological model for forecasting Karnal bunt disease of wheat. *Plant Disease Research* **7:** 204.209.

Kelly, P.W., Kinsella, A. and Brennan, J.P. (2002), "Assessing an optimal control policy for a quarantinable plant disease: the case of Karnal bunt of wheat", Paper presented at the European Association of Agricultural Economics Congress, Zaragoza, Spain

Kehlenbeck, H., Motte, G. and Unger, J-G. (1997), "[Analysis of the risk and consequences of *Tilletia indica* (Syn. *Neovossia indica*) introduction into Germany]", *Nachrichtenbl. Deut. Pflanzenschutzd.* **49:** 65-74.

Krishna A, Singh RA, 1983. Longevity of teliospores of Neovossia indica causing Karnal bunt of wheat. *Indian Journal of Mycology and Plant Pathology* **13:** 97–98.

Kumar J, Nagarajan S, 1998. Role of flag leaf and spike emergence stage on the incidence of Karnal bunt in wheat. *Plant Disease* **82:** 1368–1370.

Mavi HS, Jhorar OP, Sharma I, Singh G, Mahi GS, Mathauda SS, Aujla SS, 1992. Forecasting Karnal bunt disease of wheat – a meteorological model. *Cereal Research Communications* **20:** 67–74.

McLeod I (undated). Plant Health Australia Industry Biosecurity Planning Guide. Risk management guidelines for Australia's plant industries. Plant Health Australia: Canberra, ACT, Australia.

McRae W, 1932. Report of the Imperial Mycologist. Abstract. *Review of Applied Mycology* **11:** 424–426.

Mitra, M. (1931). A new bunt on wheat in India. *Annals of Applied Biology* **18:** 178–179.

Murray, G., Brennan, J. and Hare, R. (1996), "Karnal bunt: A concern for Australia?" *Australian Grain* 6(3) (August-September), 10-12.

Murray, G.M., Brennan, J.P. (1998). The risk to Australia from Tilletia indica, the cause of Karnal bunt of wheat. *Australasian Plant Pathology* **27:** 212.225.

Nagarajan S, 1991. Epidemiology on Karnal bunt of wheat incited by Neovossia indica and an attempt to develop a disease prediction system. CIMMYT, Mexico: Technical Report, Wheat Programme.

Nagarajan, S., Aujla, S.S., Nanda, G.S., Sharma, I., Goel, L.B., Kumar, J., Singh, D.V. (1997). Karnal bunt (Tilletia indica) of wheat - a review. *Review of Plant Pathology* **76:** 1207-1214.

Rattan GS, Aujla SS, 1989. Host range and survival of secondary sporidia of Neovossia indica. *Annals of Biology Ludhiana* **5:** 141–145.

Rattan GS, Aujla SS, 1990. Survival of Karnal bunt (Neovossia indica) teliospores in different types of soil at different depths. *Indian Journal of Agricultural Sciences* **60:** 616–618.

Rattan GS, Aujla SS, 1992. Ecology of germination of the teliospore of Karnal bunt pathogen (Neovossia indica). *Indian Journal of Agricultural Sciences* **62:** 228–231.

Rendell (2005) whose is this???

Rush, C.M., Stein, J.M., Bowden, R.L., Riemenschneider, R., Boratynski, T. and Royer, M.H. (2005), "Status of Karnal bunt of wheat in the United States 1996 to 2004", *Plant Disease* **89:** (3), 212-223.

Sansford CE, 1996. Karnal bunt (Tilletia indica) – An assessment of the current situation in the USA and the potential risk to the European Community. York, UK: Central Science Laboratory.

Sansford, C.E. (1998). An assessment of the significance of the initial detection of Tilletia indica Mitra in the USA in early 1996 and the potential risk to the United Kingdom (and the European Union). In Bunts and Smuts of Wheat: An International Symposium. (Eds V.S. Malik, D.E. Mathre), pp. 273.302. North American Plant Protection Organisation, Ottawa, Canada.

Sharma I, Nanda GS, 2003. Effect of prolonged period of fog/rain from sowing of wheat to pre-boot stage on Karnal bunt development. *Annals of Plant Protection Sciences* **11:** 93–95.

Sidhartha VS, Singh DV, Srivastava KD, Aggarwal R, 1995. Some epidemiological aspects of Karnal bunt of wheat. *Indian Phytopathology* **48:** 419–426.

Singh, D.V. (1986), "Bunts of wheat in India", In L.M. Joshi, D.V. Singh and K.D. Srivastava (eds) *Problems and Progress of Wheat Pathology in South Asia*, Malhotra, New Delhi.

Singh RA, Krishna A, 1982. Susceptible stage for inoculation and effect of Karnal bunt on viability of wheat seed. *Indian Phytopathology* **35:** 54–56.

Smilanick JL, Hoffmann JA, Royer MH, 1985. Effect of temperature, pH, light, and desiccation on teliospore germination of Tilletia indica. *Phytopathology* **75:** 1428–1431.

Smilanick JL, Prescott JM, Hoffmann JA, Secrest LR, Wiese K, 1989. Environmental effects on survival and growth of secondary sporidia and teliospores of Tilletia indica. *Crop Protection* **8:** 86–90.

Smiley RW, 1997. Risk assessment for Karnal bunt occurrence in the Pacific Northwest. *Plant Disease* **81:** 689–692.

Smith, D. (2001), "Regulations Governing the International Movement of Cereal Seed and Grain in Relation to the Plant Pathogen, *Tilletia indica*", Unpublished information paper, Central Science Laboratory, York.

Stansbury CD, Pretorius ZA, 2001. Modelling the potential distribution of Karnal bunt of wheat in South Africa. *South African Journal of Plant and Soil* **18:** 159–168.

Stansbury, C.D., McKirdy, S.J. (2002). Forecasting climate suitability for Karnal bunt of wheat: a comparison of two meteorological methods. *Australasian Plant Pathology* **31:** 81.92.

Stansbury, C.D., McKirdy, S.J., Diggle, A.J., Riley, I.T. (2002). Modelling 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.

Torarbi M, Mardoukhi V, Jaliani N, 1996. First report on the occurrence of partial bunt on wheat in the southern parts of Iran. *Seed and Plant* **12:** 8–9 (En), 59–60 (Pe).

USDA (2002/3). Karnal Bunt Manual. United States Department of Agriculture, Frederick, Maryland.

UC IPM Pest Management Guidelines (2004): Small Grains, UC ANR Publication 3466, Diseases R. M. Davis, Plant Pathology, UC Davis L. F. Jackson, Agronomy, UC Davis

Warham EJ, 1986. Karnal bunt of wheat - a literature review. *Tropical Pest Management* **32:** 229–242.

Wiese, M.V. (1998). Compendium of Wheat Diseases, 2nd Edition. APS Press, St Paul, USA.

Wilcoxson & Saari (1996) whose is this?

Ykema RE, Floyd JP, Palm ME, Peterson GL, 1996. First report of Karnal bunt of wheat in the United States (Disease Note). *Plant Disease* **80:** 1207.

Zhang Z, Lange L, Mathur SB, 1984. Teliospore survival and plant quarantine significance of Tilletia indica (causal agent of Karnal bunt) particularly in relation to China. *EPPO Bulletin* **14:** 119–128.

APPENDIX A. ZADOKS DECIMAL SCALE FOR GROWTH STAGES OF WINTER CEREALS

0 *Germination*
- 00 Dry seed
- 01 Start of imbibition (water absorption)
- 02 —
- 03 Imbibition complete
- 04 —
- 05 Radicle (root) emerged from caryopsis
- 06 —
- 07 Coleoptile (shoot) emerged from caryopsis
- 08 —
- 09 Leaf just at coleoptile tip

1 *Seedling growth*
- 10 First leaf through coleoptile
- 11 First leaf unfolded
- 12 2 leaves unfolded
- 13 3 leaves unfolded
- 14 4 leaves unfolded
- 15 5 leaves unfolded
- 16 6 leaves unfolded
- 17 7 leaves unfolded
- 18 8 leaves unfolded
- 19 9 or more leaves unfolded

2 *Tillering*
- 20 Main shoot only
- 21 Main shoot and 1 tiller
- 22 Main shoot and 2 tillers
- 23 Main shoot and 3 tillers
- 24 Main shoot and 4 tillers
- 25 Main shoot and 5 tillers
- 26 Main shoot and 6 tillers
- 27 Main shoot and 7 tillers
- 28 Main shoot and 8 tillers
- 29 Main shoot and 9 or more tillers

3 *Stem elongation*
- 30 Pseudostem (leaf sheath) erection
- 31 First node detectable
- 32 2nd node detectable
- 33 3rd node detectable
- 34 4th node detectable
- 35 5th node detectable
- 36 6th node detectable
- 37 Flag leaf just visible
- 38 —
- 39 Flag leaf ligule just visible

4 *Booting*
- 40 —
- 41 Flag leaf sheath extending
- 42 —
- 43 Boots just visibly swollen
- 44 —
- 45 Boots swollen
- 46 —
- 47 Flag leaf sheath opening
- 48 —
- 49 First awns visible

5 *Inflorescence (ear/panicle) emergence*
- 50 —
- 51 First spikelet of inflorescence just visible
- 52 —
- 53 ¼ of inflorescence emerged
- 54 —
- 55 ½ of inflorescence emerged
- 56 —
- 57 ¾ of inflorescence emerged
- 58 —
- 59 Emergence of inflorescence completed

6 *Anthesis (flowering)*
- 60 —
- 61 —
- 62 Beginning of anthesis
- 63 —
- 64 —
- 65 Anthesis half-way
- 66 —
- 67 —
- 68 —
- 69 Anthesis complete

7 *Milk development*
- 70 —
- 71 Caryopsis (kernel) water ripe
- 72 —
- 73 Early milk
- 74 —
- 75 Medium milk
- 76 —
- 77 Late milk
- 78 —
- 79 —

8 *Dough development*
- 80 —
- 81 —
- 82 —
- 83 Early dough
- 84 —
- 85 Soft dough
- 86 —
- 87 Hard dough
- 88 —
- 89 —

9 *Ripening*
- 90 —
- 91 Caryopsis hard (difficult to divide)
- 92 Caryopsis hard (not dented by thumbnail)
- 93 Caryopsis loosening in daytime
- 94 Over ripe, straw dead and collapsing
- 95 Seed dormant
- 96 Viable seed giving 50% germination
- 97 Seed not dormant
- 98 Secondary dormancy induced
- 99 Secondary dormancy lost

Source: Tottman, D.R. Makepeace, R.J., Broad, H. (1979). An explanation of the decimal code for the growth stages of cereal, with illustrations. *Annuals of Applied Biology* **93:** 221-234.

Appendix Table 1. Area, yield, production and exports, by State and by wheat type

(Average of 5 years to 2004-05)

Year	NSW	VIC	QLD	WA	SA	TAS	Australia
Area by type ('000 ha)							
Premium	1,904	194	663	346	299	0	3,406
APW	1,139	926	926	3,548	1,504	3	7,301
Feed	336	196	-143	779	196	4	1,371
Total	**3,379**	**1,315**	**701**	**4,675**	**2,001**	**8**	**12,080**
Yield by type (t/ha)							
Premium	1.86	1.85	1.34	1.54	1.77	0.00	1.08
APW	1.86	1.85	1.34	1.54	1.77	3.05	1.35
Feed	1.86	1.85	0.00	1.54	1.77	3.05	1.33
Total	**1.86**	**1.85**	**1.34**	**1.54**	**1.77**	**3.05**	**1.69**
Production by type ('000 t)							
Premium	3,547	358	887	537	531	0	5,860
APW	2,122	1,712	242	5,481	2,666	11	12,233
Feed	627	361	-191	1,204	351	13	2,365
Total	**6,295**	**2,432**	**936**	**7,222**	**3,547**	**23**	**20,457**
Exports by type ('000 t)							
Premium	1,745	254	709	492	470	0	3,670
APW	1,044	1,214	193	5,027	2,362	0	9,840
Feed	308	256	-153	1,104	311	0	1,827
Total	**3,097**	**1,724**	**749**	**6,623**	**3,143**	**0**	**15,337**
Consumption by type ('000 t)							
Premium	1,802	104	179	44	60	0	2,190
APW	1,078	498	49	454	304	11	2,393
Feed	318	105	-39	100	40	13	537
Total	**3,196**	**707**	**189**	**599**	**404**	**23**	**5,120**

Appendix Table 2. Countries with restrictions on wheat with Karnal Bunt

'Y' signifies restrictions, 'N' signifies no restrictions

Country	AQIS website	Rush *et al.*	Smith	Restrictions	Exports (000 t) *(3 years to 03-04)*
Albania			Y	Yes	0
Algeria		Y	N	Yes	0
Angola				Not known	0
Argentina			Y	Yes	0
Armenia		Y		Yes	0
Australia			Y	Yes	0
Austria		Y	Y	Yes	0
Azerbaijan		Y		Yes	0
Bahamas			N	No	0
Bahrain				Not known	2
Bangladesh	N		Y	No	135
Barbados			N	No	0
Belarus		Y	Y	Yes	0
Belgium		Y	Y	Yes	0
Bhutan				Not known	0
Bolivia		Y		Yes	0
Bosnia - Herzegovina		Y		Yes	0
Botswana				Not known	0
Brazil	Y	Y	Y	Yes	0
Bulgaria	Y	Y	Y	Yes	0
Burundi				Not known	0
Cambodia	N			No	0
Cameroon				Not known	0
Canada		Y	Y	Yes	0
Canary Islands			N	No	0
Chad				Not known	0
Chile		Y	Y	Yes	0
China	Y	Y	Y	Yes	280
Colombia	Y			Yes	0
Congo				Not known	0
Croatia			Y	Yes	0
Cyprus		Y	N	Yes	0
Czech Republic	Y	Y	Y	Yes	0
Denmark		Y	Y	Yes	0
Ecuador	Y	Y		Yes	0
Egypt	Y	Y		Yes	1,605
El Salvador				Not known	0
Eritrea				Not known	0
Estonia		Y	Y	Yes	0
Ethiopia			N	No	21
Falkland Islands			N	No	0
Fiji	N			No	115

'Y' signifies restrictions, 'N' signifies no restrictions

Country	AQIS website	Rush *et al.*	Smith	Restrictions	Exports (000 t) *(3 years to 03-04)*
Finland		Y		Yes	0
France		Y	Y	Yes	0
Georgia		Y		Yes	0
Germany		Y	Y	Yes	0
Ghana			N	No	0
Greece		Y	Y	Yes	0
Grenada			N	No	0
Guatemala	Y	Y		Yes	0
Honduras	Y	N		Yes	0
Hong Kong			N	No	0
Hungary		Y	Y	Yes	0
Iceland			N	No	0
India			N	No	1
Indonesia	Y	N	N	No	2,143
Iran				No	1,161
Iraq	N			No	1,464
Ireland		Y	Y	Yes	0
Israel			N	No	0
Italy	Y	Y	Y	Yes	0
Ivory Coast			N	No	0
Jamaica			N	No	0
Japan	N		N	No	1,193
Jordan			N	No	0
Kazakhstan		Y		Yes	0
Kenya	Y	Y	N	Yes	0
Korea DPR	Y			Yes	0
Korea S	Y	Y	N	Yes	1,011
Kuwait	N		N	No	221
Kyrgyzstan		Y		Yes	0
Latvia		Y	Y	Yes	0
Lebanon	N			No	0
Lesotho	Y			Yes	0
Libya				Not known	0
Lithuania		Y	Y	Yes	0
Luxembourg		Y	Y	Yes	0
Macedonia			Y	Yes	0
Madagascar		Y		Yes	0
Malawi			Y	Yes	0
Malaysia	N			No	596
Mali				Not known	0
Malta		Y	N	Yes	0
Mauritania				Not known	0
Mauritius	N			No	0
Mexico		Y		Yes	0
Moldova		Y	Y	Yes	0
Mongolia				Not known	0
Montenegro				Not known	0

'Y' signifies restrictions, 'N' signifies no restrictions

Country	AQIS website	Rush *et al.*	Smith	Restrictions	Exports (000 t) *(3 years to 03-04)*
Morocco	Y	Y	Y	Yes	0
Mozambique	Y			Yes	0
Muscat and Oman			N	No	0
Myanmar	N			No	0
Namibia		Y	N	Yes	0
Nepal				Not known	0
Netherlands		Y	Y	Yes	0
New Caledonia	N			No	0
New Zealand	Y	Y	Y	Yes	309
Niger				Not known	0
Nigeria			N	No	0
Norway		Y	Y	Yes	0
Oman	N			No	77
Pakistan			N	No	50
Palestine				Not known	0
Papua New Guinea	N			No	113
Paraguay		Y	Y	Yes	0
Peru		Y	N	Yes	0
Philippines	N		N	No	0
Poland	Y	Y	Y	Yes	0
Portugal		Y	Y	Yes	0
Qatar			N	No	0
Romania			Y	Yes	0
Russia	Y	Y	Y	Yes	0
Rwanda				Not known	0
Saudi Arabia			Y	Yes	0
Serbia-Montenegro		Y		Yes	0
Seychelles			N	No	0
Singapore	N		N	No	69
Slovakia		Y	Y	Yes	0
Slovenia			Y	Yes	0
Solomon Islands	N			No	0
South Africa	Y	Y	N	Yes	131
Spain		Y	Y	Yes	0
Sri Lanka	N	Y	Y	No	153
St Helena			N	No	0
St Lucia			N	No	0
St Vincent & Grenadines			N	No	0
Sudan	N			No	0
Swaziland				Not known	0
Sweden		Y	Y	Yes	0
Switzerland	Y	Y	Y	Yes	0
Syria			N	No	0
Taiwan	Y	N	N	Yes	0
Tajikistan		Y		Yes	0
Tanzania	Y	Y	N	Yes	0
Thailand	N	Y	N	No	369

'Y' signifies restrictions, 'N' signifies no restrictions

Country	AQIS website	Rush *et al.*	Smith	Restrictions	Exports (000 t) *(3 years to 03-04)*
Trinidad and Tobago			N	No	0
Tunisia		Y	Y	Yes	0
Turkey	N	Y	Y	Yes	0
Turkmenistan		Y		Yes	0
Uganda		Y	N	Yes	0
Ukraine		Y	Y	Yes	0
United Arab Emirates	N		N	No	188
United Kingdom		Y	Y	Yes	0
U.S.A.			Y	Yes	0
Uruguay		N	Y	No	0
Uzbekistan		Y		Yes	0
Venezuela	Y	Y		Yes	0
Vietnam	N			No	0
Yemen	N			No	353
Yugoslavia			Y	Yes	0
Zambia				Not known	0
Zimbabwe	Y	Y	N	Yes	0
- Sub-total					**11,761**
- Other				Not known	3,427
Total					**15,188**

'Y' signifies restrictions, "N" signifies no restrictions
'Restrictions' is an aggregation of the information from the three different sources.

Appendix Table 3. Exports by port zone and State, 2002-03 and 2003-04

		Port zone	2002-03	2003-04	Average	Percentage of total 2002-03	Percentage of total 2003-04	Percentage of total Average	Percentage of state 2002-03	Percentage of state 2003-04	Percentage of state Average
W	ALB	Albany	1,050,712	1,563,016	1,306,864	10%	8%	9%	20%	17%	18%
Q	BNE	Brisbane	227,945	723,980	475,963	2%	4%	3%	65%	89%	82%
W	ESP	Esperance	396,932	969,734	683,333	4%	5%	5%	8%	10%	9%
W	FRE	Fremantle	2,842,589	5,006,069	3,924,329	28%	26%	27%	54%	53%	53%
W	GER	Geraldton	975,666	1,876,049	1,425,858	10%	10%	10%	19%	20%	19%
V	GEX	Geelong	226,755	861,784	544,270	2%	4%	4%	26%	29%	28%
Q	GLT	Gladstone	116,131	68,924	92,528	1%	0%	1%	33%	9%	16%
V	MEL	Melbourne	431,547	1,556,060	993,804	4%	8%	7%	49%	53%	52%
Q	MKY	Mackay	4,358	16,129	10,244	0%	0%	0%	1%	2%	2%
N	NTL	Newcastle	637,824	1,345,285	991,555	6%	7%	7%	61%	46%	50%
S	PAD	Port Adelaide	1,043,121	1,294,590	1,168,856	10%	7%	8%	39%	41%	40%
S	PGI	Port Giles	198,769	285,220	241,995	2%	1%	2%	7%	9%	8%
N	PKE	Port Kembla	412,492	1,561,601	987,047	4%	8%	7%	39%	54%	50%
S	PLO	Port Lincoln	1,067,702	1,228,270	1,147,986	10%	6%	8%	40%	38%	39%
S	PPI	Port Pirie	87,810	34,393	61,102	1%	0%	0%	3%	1%	2%
V	PTL	Portland	226,641	526,614	376,628	2%	3%	3%	26%	18%	20%
S	THE	Thevenard	151,597	84,554	118,076	1%	0%	1%	6%	3%	4%
S	WAL	Wallaroo	142,567	267,314	204,941	1%	1%	1%	5%	8%	7%
			10,241,158	**19,269,586**	**14,755,372**	100%	100%	100%			
		Queensland	348,434	809,033	578,734	3%	4%	4%	100%	100%	100%
		New South Wales	1,050,316	2,906,886	1,978,601	10%	15%	13%	100%	100%	100%
		Victoria	884,943	2,944,458	1,914,701	9%	15%	13%	100%	100%	100%
		South Australia	2,691,566	3,194,341	2,942,954	26%	17%	20%	100%	100%	100%
		Western Australia	5,265,899	9,414,868	7,340,384	51%	49%	50%	100%	100%	100%
		- Australia	**10,241,158**	**19,269,586**	**14,755,372**	100%	100%	100%			

Appendix Table 4. List of seed treatments registered in the USA for the control of T. indica teliospores.

NB: These treatments are not 100% effective

Common name (trade name)		Amount/cwt	PHI+ (days)
The following materials are listed in order of usefulness in an IPM program, taking into account efficacy and impact on natural enemies.			
A.	CARBOXIN/THIRAM		
	(RtU-Vitavax-Thiram)	6.8 oz	
COMMENTS: Do not graze or feed livestock or treated areas for 6 weeks after planting.			
B.	PCNB	3 fl oz	
	(RtU-Vitavax-Thiram)		
C.	DIFENOCONAZOLE	3 fl oz	
	(Dividend)	0.5 fl oz	
COMMENTS: For use on wheat only. Do not use treated seed for feed or oil. Do not graze green forage for 55 days after planting. Do not plant any crop other than wheat within 30 days to fields in which treated seed was planted.			
D.	TEBUCONAZOLE/THIRAM	3 fl oz	
	(Raxil-Thiram)	3.5-4.6 fl oz	
COMMENTS: Do not use treated seed for feed, food or oil purposes. Do not graze barley, wheat or oat for 31 days after planting.			

Preharvest interval for grain and/or forage.
Cited from UC IPM UC Management Guidelines for Karnal Bunt of Wheat on Small Grains.htm (2004).

DRAFT NATIONAL CONTINGENCY PLAN FOR KARNAL BUNT OF WHEAT

PART II

PREVENTATIVE MEASURES

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Draft 1: February 2006

CONTENTS

1. INTRODUCTION

Preventative measures for Karnal bunt involve firstly actions to reduce the probability of entry and secondly, research into actions to control the disease should it establish.

This section considers the potential pathways of entry, the use of quarantine, surveys to establish area freedom and the ability to detect an incursion early, and breeding for resistance.

2. METHODS OF ENTRY AND PATHWAYS

There are a number of entry methods and pathways by which *T. indica* can enter Australia. Some of these pathways are discussed by Murray and Brennan, (1998), while Stansbury and McKirdy (2000) estimated the probability of entry for these pathways. The pathways include the following:

- The teliospores of *T. indica* can be dispersed through bulk grain or seed being imported into Australia
- Wheat seed with sori can be mixed in bulk grain and seed samples
- Both of the above examples can be contaminants in imported bulk grain, or fertiliser shipments. Teliospores of *T. indica* are long-lived and survive extremes of temperature when dry. There is a high probability of survival as a contaminant of machinery or in bulk grain or fertiliser.
- Shipping containers and machinery can be contaminated with the first two examples.
- The spores can be on clothing and personal belongings of travellers. There is a high frequency of travel between the USA, India and South Africa where the pathogen exists, and Australian farming areas.
- The spores can be windborne. However, long distance dispersal is unlikely to result in establishment of the pathogen, as high concentrations of spores are required for this to occur. This pathway has to be considered if the pathogen becomes established in Australia.
- Or can be carried by birds and animals, either on fur and feathers or in the intestinal tract (Murray and Brennan 1998). Infected seeds and spores would pass through the gut of migratory birds long before they reached Australia. However, this pathway has to be considered if the pathogen became established in Australia.
- The risk of bio-terrorism is an increasing issue. The main preventative action is being aware that it can exist and maintaining our quarantine regulations to a high standard to try and prevent the threat. Areas of concern include: a) the illegal importation of contaminated seed and this being introduced into an area where infection can occur; and b) the importation of animals that have recently been fed infected grain. *T. indica* survives passage through the intestinal tract and contaminates the manure of animal. The risk from this threat can be reduced by animals going through quarantine and manure being disposed of correctly.

3. BORDER CONTROLS/QUARANTINE

3.1 Introduction

In order to prevent the possible introduction of the pathogen *T. indica* it is important to adhere to quarantine guidelines and measures. These will involve the regulation of high risk commodities (such as cereal grain and seeds) being imported into Australia. The following sections discuss the most high risk pathways via which the pathogen can enter Australia.

It should be noted that the issue of bio-terrorism is not discussed in detail. It is thought to be difficult to prove that this had happened because of the length of time it would take, for the pathogen to establish to detectable levels. There are many fungal pathogens that can be potential agents and *T. indica* is considered to be one.

3.2 Seed

The current inspection methods for the importation of wheat seed for sowing are in Appendix A. These methods are currently used routinely. However, there are problems with the methodology and these pose a risk for failure to detect *T. indica*.

3.2.1 Entry of seed into Post-Entry Quarantine (PEQ)

Small packets of seed (100g) are visually inspected on arrival at the PEQ facilities. This method would fail to detect any teliospores of T. indica on the grain. However, the presence of bunted grain should be detected.

Currently the guidelines state that seed dressings are to be used before planting wheat in the glasshouse (Appendix A). The use of Thiram® or Vitavax® has been shown to inhibit teliospore germination. However, it is not 100 per cent effective.

Where possible, a spore wash test could be done before treating the seed to determine if any teliospores or other spores are present on the grain that has been imported. Another wash test on the final grain harvest of the plants grown in the glasshouse, maybe required. Although careful inspection of the heads in this situation should reveal if they are infected as a cursory glance is not appropriate.

Another option that could be investigated, is to examine the water used in the prewash test before the application of fungicides, in place of the above separate spore wash test.

3.2.2 Open quarantine

There is the current practice of new lines of wheat undergoing quarantine in NZ first and then coming into Australia for bulking up under open quarantine conditions. Large areas are planted and grown. Inspection of these crops would be difficult because of the size of the area grown, and the added complication that KB is difficult to detect in the field under most circumstances – unless there is a high infection level.

This system depends heavily upon the rigour of the inspection in NZ.

All grain entering the country should have a wash test conducted on it before treating the seed with a suitable fungicide before planting. Harvested grain needs to be inspected and have a wash test conducted on it before the grain is released on a commercial basis.

3.3 Grain/fertiliser shipments

Currently AQIS have guidelines that are followed in regards to the inspection of bulk commodities. Fertiliser shipments and other bulk commodities need to be inspected to ensure that there was no contamination with grain. It is necessary to have details of the shipping history.

Random sampling of the bulk commodities (fertiliser, grain) and using a selective sieve wash test will determine if the spores of T. indica are present. A visual inspection of the hull and bulk commodity will also show if there are any wheat grains contaminating the load.

3.4 Shipping containers and machinery

Currently AQIS have guidelines that are followed in regards to the inspection of shipping containers. It is necessary to have details of their shipping history.

The dust at the bottom of the shipping containers and in machinery that has had contact with wheat will need to be tested. In some cases this will mean the dismantling of second hand headers, to thoroughly test and clean.

3.5 Travellers

Due to the increase in travel throughout the world the risk of quarantinable disease entering Australia has increased. Many farming/grower groups travel to look at other farming methodology used throughout the world. It is important that awareness of the risks in bringing back spores unknowingly on clothing is increased. Clothing should be washed in hot water and detergent before being worn out in their own crops. It is also important that soil is not left on footwear.

Travellers need to have their awareness increased about the risks of bringing back undeclared seeds. Such introductions impact at the farm, community, state and national levels.

3.6 Other potential sources

There are numerous other potential sources for introduction. For example, food for consumption present on the ship. China has found that flour used in the galley of the ships has been contaminated with teliospores of *T. indica* (Dr Tan *pers. comm.*). This indicates that the origin of the ship is important, in terms of loading of food commodities and the risk of contamination. The implication of this finding is that food has to be disposed of in a way that ensures that the spores cannot be transported to wheat fields.

4. NATIONAL SURVEY TO DEMONSTRATE PEST FREE AREA STATUS

4.1 Introduction

Currently the pest status of *T. indica* in Australia is Absent: no pest records and Pest Free Area (PFA) status is claimed. The recent national survey conducted in relation to the Pakistan incident in 2004 showed that *Tilletia indica* was not detected in all of the samples tested and supports the PFA status of Australia for this pathogen.

It is important in light of this, that the "Pest Free Area" status remains and is backed by hard scientific evidence. This will require a regular survey of all wheat grain harvested within Australia. The sampling intensity is dependent upon the accuracy of the data required. The sampling intensity is also influenced by whether we want to declare Australia free of Karnal bunt or to declare that the pathogen *T. indica* is known not to occur.

International Sanitary and Phytosanitary Measures are developed by the International Plant Protection Convention and recognised by members of the World Trade Organisation (http://www.fao.org/ag/AGP/AGPP/PQ/En/Publ/ISPM/ispms.html). Survey protocols using these guidelines will be significant in terms of international market access.

Surveys should conform to the International Sanitary and Phytosanitary Measure (ISPM): ISPM 4 (1996) – requirements for the establishment of pest free areas; ISPM 6 (1997) – guidelines for surveillance; and to ISPM 8 (1999) – determination of pest status in an area.

ISPM 4 (EPPO) states that both data from general surveillance and from specific surveys are acceptable. The data collected from these different types of surveillance provide for different kinds or degrees of phytosanitary security.

- General surveillance is a process whereby information on particular pests for an area is gathered from many sources. This information is then collated and provided for use by the National Plant Protection Office (NPPO) (ISPM 4, 1996). In Australia the NPPO is the Office of the Chief Plant Protection Officer (OCPPO).
- Specific surveys are procedures by which NPPOs obtain information on pests of concern on specific sites in an area over a defined period of time. The verified information acquired may be used to determine the presence or distribution of pests in an area, or on a host or commodity, or their absence from an area (in the establishment and maintenance of pest free areas) (ISPM 4, 1996).

Specific surveys may be detection, delimiting or monitoring surveys. These are official surveys and should follow a plan which is approved by the OCPPO.

The survey plan should include:

- Definition of the purpose (e.g. early detection, assurances for pest free areas), and the specification of the phytosanitary requirements to be met.
- Identification of the target pathogen.
- Identification of scope (e.g. geographical area, production system, season).
- Identification of timing (dates, frequency, duration).
- Identification of hosts.

- Indication of the statistical basis, (e.g. level of confidence, number of samples, selection and number of sites, frequency of sampling, assumptions).
- Description of survey methodology and quality management including (sampling procedures (e.g. whole plant sampling, visual inspection, sample collection and laboratory analysis).
- The procedure would be determined by the biology of pathogen and/or purpose of survey, the diagnostic procedures and the reporting procedures.

The information gathered can be used to support declarations of pest freedom and to aid in the early detection of a new incursion. The information gathered can also aid in the compilation of host and pathogen lists and current distribution records.

4.2 General surveillance

There are many sources of pest information that can be gathered when required. These include national and local government agencies, research institutions, universities, scientific societies (including amateur specialists), producers, consultants, museums, the general public, scientific and trade journals, unpublished data and contemporary observations.

For Australia, the information gathered can be sourced from a number of places: a) the bulk handlers, as grain is inspected on delivery; b) from the Departments of Agriculture within each State, as they may have conducted surveys or have a diagnostic service that regularly inspects grain; and c) from the relevant quarantine inspection service, when grain is being loaded or unloaded.

For this information to be effective and available to use, a national data base needs to be developed to capture all the information that is collected. The development of such a data base will require all states, and stakeholders to be able to access and contribute to the data. Such a database would also be useful for the results of specific surveys to be collated.

The easiest and simplest general survey that all States can do is:

- All common bunt samples are submitted to local department of Agriculture for confirmation that it is either *T. laevis* or *T. tritici.* This ensures that a small number of samples are regularly checked on an annual basis for the presence of *Tilletia* species.

4.2.1 Inspection of deliveries

Training receival staff in the inspection of grain is a very important part of this strategy. As grain is generally inspected during the delivery, to have the staff trained to look for Karnal bunt symptoms would be part of the general surveillance strategy. This would then provide data on a yearly basis.

The requirement of any suspicious grains to be sent to the local Department of Agriculture, and that all common bunt samples are also submitted will help to fulfil the criteria required for general surveillance.

The use of photos provided in an inspection manual is very critical in training staff and having a reference guide on hand.

4.2.2 Inspection through marketing chain

Grain is sampled and inspected as it moves through the marketing chain. Similar procedures to those applying to deliveries should be implemented for those undertaking these inspections.

4.3 How to conduct specific surveys

To conduct a specific survey, we need to ask if Australia wants to maintain PFA status from Karnal bunt or from *Tilletia indica*. This has serious implications for the way a survey will be conducted.

In 2004, a national survey was conducted on all wheat loads for export after Pakistan suggested that Karnal bunt was present in Australia. The samples were examined and tested for the presence or absence of *T. indica*. *T. indica* was not detected in the samples tested. To continue to demonstrate that Australia is free from *T. indica* ongoing surveys of grain will need to be conducted.

4.3.1 Sampling strategy

Determining the sampling procedures required for the testing is quite difficult, because it is necessary that the correct number of samples are collected and tested to meet the confidence levels required for PFA status.

Internationally, there are currently two sampling strategies that could be used:

- The USDA currently selects an area (county) and determines the average production (t) over previous five years for that area. They then take composite samples from the receival sites of 2 kg per 27,000 tonnes. The areas to be surveyed are done in rotation. The USDA only certifies that Karnal bunt is not known to occur by State/County.

- The other option is to select specific areas and again determine the average production over five years. Composite samples per site within the area are then sampled using ISTA rules of 1 kg per 30 tonnes. This sampling regime increases the probability of detection, if *T. indica* was present in grain crops. However, as Australia has a current status of not known to occur, sampling at a greater intensity increases the justification of this statement.

The comparison of the number of samples that would be required to be tested under the different sampling regimes is shown in Table 2.1. The difference in the number of samples required is quite significant (approx a thousand times more samples from the second procedure). The more samples that are tested the greater the confidence in detecting a possible incursion.

The more samples that are collected the greater the confidence in saying that Karnal bunt / *T. indica* does not occur in Australia. This would be the more preferable option to take. However, this of course increases the amount of people required to do the testing and the length of time it would take to complete a survey for each state.

Table 2.1 An example of the production figures for wheat by State for Australia, and then their average production (t). The number of samples that would be required to be tested is then shown according to the USDA method and the ISTA methods

		Average for 5 years (1,000 tonnes)	No. of samples to be tested (2 kg samples USDA)	No. of samples to be tested (1 kg samples ISTA)
QLD	Prod	1,169	43	38,978
NSW	Prod	6,707	248	223,580
VIC	Prod	2,581	96	86,036
SA	Prod	3,491	129	116,385
WA	Prod	7,508	278	250,273
National	Prod	21,458	795	715,252

The next question that needs to be determined is how to break the sampling strategy down into smaller meaningful lots

i. State (as shown in Table 2.1, and Maps in Appendix B).

ii. Port Zone. This fits in best for export market samples. However, we run the risk of not sampling grain that is destined for the domestic market (such as flour mills, or feed). The number of samples would be more than if we sampled by State but there is a greater dilution affect, than if the sampling strategy was by District (as shown Table 2.2, and Maps in Appendix B).

iii. District. An example of this is shown in Table 2.2 for Western Australia. Most of the Bulk Handlers are able to break down the grain deliveries to this level.

iv. Shire. The number of samples by shire becomes very un-manageable and not necessarily an easy option for collection.

Table 2.2 A break down of sampling strategy for Western Australia. The sampling is done by districts and port zone

District	Tonnes (000)	No. of 2 kg Samples to be tested (USDA)	No. of 1 kg Samples to be tested (ISTA)
Morawa	818	30	27,288
Wongan Hills	1,054	39	35,143
Koorda	825	31	27,517
Avon	760	28	25,345
Merredin	942	35	31,391
Corrigin	578	21	19,267
Katanning	380	14	12,658
Lake grace	709	26	23,654
Geraldton	656	24	21,858
Mgc	123	5	4,096
Albany	76	3	2,549
Esperance	660	24	22,018
Total	**7,583**	**281**	**252,784**

The number of samples increases when the sampling strategy is broken down into smaller meaningful lots.

The best option would be to start with the port zone samples first (such as Geraldton, Albany and Esperance) and then if a possible detection was found check the district samples for that port zone.

4.3.2 Sample examination

A survey that examines grain visually for bunted grain or sori present on seed is a survey for Karnal bunt.

A survey that examines the wash samples by microscopy for the presence of teliospores is a survey for *T. indica.*

Both of these methods are described in detail in Part IV of the contingency plan.

The advantages of following the USA method (presence of Karnal bunt) are:

- Reduction of sample size by usingn image analysis to reduce the 2 Kg sample to approx, 200 g sample.
- The sample is then visually inspected visually for bunted grain samples.
- Much quicker process and less intensive for staff to do.

The disadvantage of following the USA method (presence of Karnal bunt) is:

- A good machine / program to do the image analysis is required. Image analysis in Australia at present has been investigated and been found not to be reliable. However, further investigations are continuing.

The advantages of using the International Standard of the selective sieve wash test (presence of *T. indica*) are:

- The results will be accepted internationally.
- The sample is visually inspected first before a selective sieve wash test is done.
- The confidence level is far greater for the detection of a positive sample, when looking for teliospores than looking for bunted grain.
- The wash test is more accurate than the visual inspection.

The disadvantages of using the Internation Standard of the selective sieve wash test (presence of *T. indica*) are:

- Very labour intensive to process samples.
- Need well trained staff to process samples.
- Results for a positive confirmation will take a minimum of 3 weeks.

4.3.3 Frequency of survey

In an ideal situation, a survey of grain during every harvest would demonstrate area freedom. However, the man power is not available for a survey of this size, as shown by the number of samples that would be required in Table 2.1.

Therefore the best option would be a regular survey that is conducted during harvest, and covers two or three states each year. Thus the whole country will be tested tri-annually to maintain its current pest free status.

- New South Wales and Queensland testing will be conducted in the same year
- Victoria, Tasmania and South Australia will be tested within the same year. Tasmania produces a very small quantity of grain compared to the other states.
- Western Australia will be tested separately.

Each State could be responsible for the testing, thus reducing the load on manpower and equipment to do the testing. Another option is to set up a specialist service that is solely responsible for the receiving and testing of the grain.

This still leaves the decision which sampling method to follow and how to examine the grain. The compromise could be to use the USA sampling method, do a visual inspection of the grain and then do the selective sieve wash test. The current protocol requires that three replicates of 50 g is taken from each sample. As the sample size has doubled, six replicates of 50 g would need to be taken to keep the accuracy of detection at a reasonable level.

5. BREEDING FOR RESISTANCE

5.1 Introduction

Under the GRDC project (CIM 5, 1997-2003) a collection of Australian varieties (both historic and recent) and advanced lines representing all the wheat breeding programs in Australia were evaluated for KB resistance.

A backcrossing program was initiated to transfer KB resistance into elite materials representing all Australian wheat growing regions. A total of 245 advanced lines based on these recurrent Australian parents, with KB resistance and in many cases enhanced rust resistance, were sent to Australian quarantine during the life of this project.

5.2 Status of current cultivars

A few (FRAME, PELSART & TAKARI) were found to be resistant to KB with levels of seed infection < 2 per cent.

All data collected in Mexico accompanied the germplasm. The University of Sydney (Frank Ellison) was responsible for the assessment and dissemination of the germplasm and data. A further 79 lines, developed under this project, were increased and sent to Australia in December 2003.

5.3 Current screening for resistance in new lines

In recent years two synthetic wheats with immunity to KB were identified. A project extension of one year was given to begin the development of doubled haploid (DH) populations, based on these immune sources, to facilitate the developent of molecular markers. These DH populations have been developed and will be assessed for KB resistance in a new GRDC funded project beginning July 2003 (GRDC CIM0008).

A mapping population FRAME/SILVERSTAR, segregating for KB resistance, was also evaluated during the one year extension. The results of this screening have been sent to Australia (MPBCRC and the Victorian Dep of Primary Industries have the results) and it is envisioned that molecular markers will be found for the FRAME based resistance.

6. REFERENCES

ISPM 4 (1996) requirements for the establishment of pest free areas. (http://www.fao.org/ag/AGP/AGPP/PQ/En/Publ/ISPM/ispms.html). International Sanitary and Phytosanitary Measures, International Plant Protection Convention and recognised by members of the World Trade Organisation

ISPM 6 (1997) guidelines for surveillance. (http://www.fao.org/ag/AGP/AGPP/PQ/En/Publ/ISPM/ispms.html). International Sanitary and Phytosanitary Measures, International Plant Protection Convention and recognised by members of the World Trade Organisation

ISPM 8 (1999) determination of pest status in an area. (http://www.fao.org/ag/AGP/AGPP/PQ/En/Publ/ISPM/ispms.html). International Sanitary and Phytosanitary Measures, International Plant Protection Convention and recognised by members of the World Trade Organisation

Murray, G.M., Brennan, J.P. (1998). The risk to Australia from Tilletia indica, the cause of Karnal bunt of wheat. *Australasian Plant Pathology* **27:** 212.225.

Stansbury, C.D., McKirdy, S.J. (2002). Forecasting climate suitability for Karnal bunt of wheat: a comparison of two meteorological methods. *Australasian Plant Pathology* **31:** 81.92.

Stansbury, C.D., McKirdy, S.J., Diggle, A.J., Riley, I.T. (2002). Modelling 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.

APPENDIX A. IMPORT CONDITIONS FOR WHEAT GRAIN

IMPORT CASE DETAILS – PUBLIC LISTING

Commodity:	*Triticum* spp. - other than *Triticum tauschii* and *Triticum juncellum*
Scientific name:	*Triticum* spp. - other than *Triticum tauschii* and *Triticum juncellum*
Country:	All countries
End use:	Seeds for sowing
Date printed:	31 March 2005

The information here covers AQIS quarantine requirements only and is current on the date of transmission but may change without notice. AQIS makes no warranties or representations with respect to the accuracy or completeness of that information and will bear no liability with respect to that information. Importers must satisfy quarantine concerns and comply with quarantine conditions applicable at the time of entry. The Commonwealth through AQIS is not liable for any costs arising from or associated with decisions of importers to import based on conditions presented here which are not current at the time of importation. It is the importer's responsibility to verify the accuracy and completeness of the information at the time of importation.

It is the importer's responsibility to identify and to ensure it has complied with, all requirements of any other regulatory and advisory bodies prior to and after importation including the Australian Customs Service, Therapeutic Goods Administration, Department of the Environment and Heritage, Australian Pesticides & Veterinary Medicines Authority and any State agencies such as Departments of Agriculture and Health and Environmental Protection authorities.

Importers should note that this list is not exhaustive. Importers should also note that all foods imported into Australia must comply with the provisions of the Imported Food Control Act 1992*, an Act which is administered by AQIS.*

Condition C10006

Seed for sowing conditions for wheat (Triticum spp. – other than *T. tauschii* and *T. juncellum*)

Importer's responsibilities
Quarantine procedures

Import Permit; material must be free of contaminants; post-entry quarantine facilities (PEQ); scheduled fees-PEQ; packaging

Importer's responsibilities

1. All consignments must be accompanied by a valid Import Permit or by means to allow the identification of the Import Permit.

2. It is the importers' responsibility to check the requirements of local, state or any other government organisation prior to importation.

3. A Quarantine Entry must be lodged for each consignment.

4. The importer must contact the AQIS regional office in port of entry prior to arrival, to confirm all arrangements for inspections and treatments of consignments.

5. The importer is responsible for payment of all associated AQIS fees and charges.

6. Seed must be free of live insects, soil, disease symptoms, prohibited seeds, other plant material (e.g. leaf, stem material, fruit pulp, pod material, etc.), animal material (e.g. animal faeces, feathers, etc.) and any other extraneous contamination of quarantine concern.

7. Each shipment must be packed in clean, new packaging, clearly labelled with the botanical name.

8. Seed must be grown in closed quarantine at either:

   - an Australian Government (AQIS) post-entry quarantine facility; or
   - a quarantine approved post-entry quarantine facility operating under a Compliance Agreement with AQIS.

9. The importer is responsible for contacting the post-entry quarantine facility to confirm all arrangements including space availability and number of seed lines, **prior** to the goods arriving in Australia.

10. There are scheduled fees associated with the growth of seed lines in an Australian Government (AQIS) post-entry quarantine facility. These fees are to be met by the importer. Refer to the AQIS website for further information on the fee schedule. State government post-entry quarantine facilities may have different fee schedules and importers are advised to contact the relevant state department for details.

11. Airfreight or mail shipments should have all documentation (e.g. permit or permit number, invoice, manufacturer's declarations and certification where applicable) securely attached to the outside of the package and clearly marked "Attention Quarantine". Alternatively, necessary documentation will need to be presented to AQIS at the time of clearance.

Quarantine procedures

Summary: inspections; closed quarantine; disposal of waste material

12. All documentation must be presented to a quarantine officer for examination on arrival.

13. All consignments must be inspected on arrival by a quarantine officer for freedom from live insects, soil, disease symptoms, prohibited seeds, other plant material (e.g. leaf, stem material, fruit pulp, pod material, etc.), animal material (e.g. animal faeces, feathers, etc.) and any other extraneous contamination of quarantine concern.

14. Following inspection, all seed must be contained and securely packaged to the satisfaction of a quarantine officer and forwarded directly to the quarantine approved premises for growth in quarantine and/or treatment.

15. All seed must be treated at the quarantine approved premises in accordance with **one** of the following methods:

a) Seed must be pre-soaked in water at ambient temperature for 4 - 5 hours on arrival. The seed must then be immersed in hot water at 54°C for 10 minutes (T9556) and then dried. Seed must then be dusted with Thiram® seed fungicide (T9420). (Note: Thiram must be applied as per the label instructions. All safety precautions must be followed as per the label instructions);

OR

b) The seed must be treated with Vitavax® 200 Flowable Fungicide (T9965). (Note: Fungicide must be applied as per the label instructions. All safety precautions must be followed as per the label instructions.

16. All treatments must be performed by either a quarantine officer or a quarantine approved person authorised under a Compliance Agreement with AQIS to perform quarantine activities.

17. Seed must be grown in closed quarantine at either:

- an Australian Government (AQIS) post-entry quarantine facility; or
- a quarantine approved post-entry quarantine facility* operating under a Compliance Agreement with AQIS.

(*Note: The quarantine officer must confirm with AQIS Plant Programs, Canberra Office that the post-entry quarantine facility is approved for growing *Triticum* spp.).

18. During growth in quarantine seed lines must be inspected at the following stages of growth:

a) seedling emergence;

b) halfway through the growing period;

c) heading and flowering; and

d) a final inspection of the harvested seed.

19. In the event that an exotic pest or disease is detected, AQIS Plant Programs, Canberra must be contacted immediately for further advice.

20. **Requirements for seed lines grown in a quarantine approved post-entry quarantine facility operating under a Compliance Agreement with AQIS.**

a) All seed lines must be inspected for pest and disease symptoms at the four mandatory growth stages and at regular intervals during the growing period by a quarantine approved person authorised under a Compliance Agreement with AQIS to perform quarantine activities

b) The quarantine approved premises operator must maintain records detailing:

- the results of all inspections including the name and position of the inspecting person, and dates of the inspections for each seed line; and
- all plant material / seed held and grown in the quarantine approved post-entry quarantine facility. These records may be subject to audit by a quarantine officer.

c) Release of material from quarantine is subject to provision of a "Release Request Form", detailing the name and position of the inspecting person, the dates and results of the four mandatory inspections of each seed line. Release Request Forms must be forwarded to the AQIS regional office for approval and notification of release from quarantine.

21. Seed from plants screened and found to be free of disease may be released from quarantine by a quarantine officer.

22. Once seed has been harvested, all plants, all residues, all derivatives and all materials that have been in contact with the imported material must be subjected to one of the following treatments:
    - autoclaving at 121^{o}C for 30 minutes; or
    - heat treatment at 160^{o}C for 2 hours; or
    - high temperature incineration; or
    - irradiation at 25kGray (2.5 Mrad); or
    - any other treatment approved by AQIS.

23. AQIS reserves the right, at any time to:
    a) apply further controls on the growth of prohibited seed lines; and
    b) rescind the right of quarantine approved premises operators to grow prohibited seed lines.

Treatment T9965

Vitavex® 200 Flowable Fungicide

Use Vitavex® 200 FF in accordance with label instructions.

Vitavax 200FF Seed Treatment is a suspension concentrate (flowable) seed dressing used for the treatment of various crops. The product contains carboxin, a systemic fungicide that is effective against a number of commonly occurring diseases of cereals and other crops, and thiram, a broad-spectrum surface contact fungicide. This combination increases the spectrum of diseases controlled than either fungicide used alone

Treatment T9044

Methyl bromide

48 g/m^3 for 2½ hours at 21°C at Normal Atmospheric Pressure (NAP).

Add 8 g/m^3 for each 5°C the temperature is expected to fall below 21°C, or subtract the 8 g/m^3 for each 5°C the temperature increases above 21°C during the fumigation. It is the minimum temperature during the course of the fumigation that is to be used for the calculation of the dose.

Treatment T9056

Methyl bromide – Khapra beetle rate

80 g/m^3 for 48 hours at 21°C at Normal Atmospheric Pressure (NAP) with an end point concentration at 48 hours of 20 g/m^3.

NOTE: The fumigation must undergo a monitor at 24 hours to ensure a minimum concentration of 24g/m^3.

Treatment T9072

Methyl bromide

48 g/m^3 for 2½ hours at 21°C with a minimum concentration of 32 g/m^3 under vacuum (660 mm vacuum).

Add 8 g/m^3 for each 5°C the temperature is expected to fall below 21°C, or subtract the 8 g/m^3 for each 5°C the temperature increases above 21°C during the fumigation. It is the minimum temperature during the course of the fumigation that is to be used for the calculation of the dose.

Treatment T9086

Phosphine

1.0-1.5 g/m^3 for 10 days at temperatures between 15°C-25°C.

1.0-1.5 g/m^3 for 7 days at temperatures above 25°C.

At the completion of the fumigation, the phosphine concentration must be at least 0.1 g/m^3

Phosphine should not be used at 15^{o}C, or below.

Aluminium phosphide is available as tablets, pellets or sachets.

1 g of phosphine = 1 tablet = 5 pellets = 1/11 sachet.

Commercial formulations include Phostoxin® and Gastoxin®.

Treatment T9556

Soak and hot water

Soak in water at ambient (surrounding) temperature for 4-5 hours and then immerse in hot water. Treat at 54°C for not less than 10 minutes, then dry.

A reasonably large volume of water should be heated to the required temperature with a heating unit capable of maintaining the temperature required. The seed should be tied loosely in open mesh cloth bags (such as cheesecloth) and suspended in the water for the treatment. The water should be constantly circulated throughout the duration of the treatment. The amount of seed treated at one time should not be such as to cause the temperature in the water bath to go more than one degree below the treatment temperature.

After treatment the seed should be immediately plunged into a cold water bath or cold running water and dried as quickly as possible.

When the seed is dry, a protective fungicidal dust treatment is desirable.

Treatment T9420

Thiram

Dust to excess.

Thiram® contains 50 to 80 per cent tetramethylthiuram disulphide.

APPENDIX B. Maps of receival sites for each state

Figure 2.1 Receival sites for Western Australia

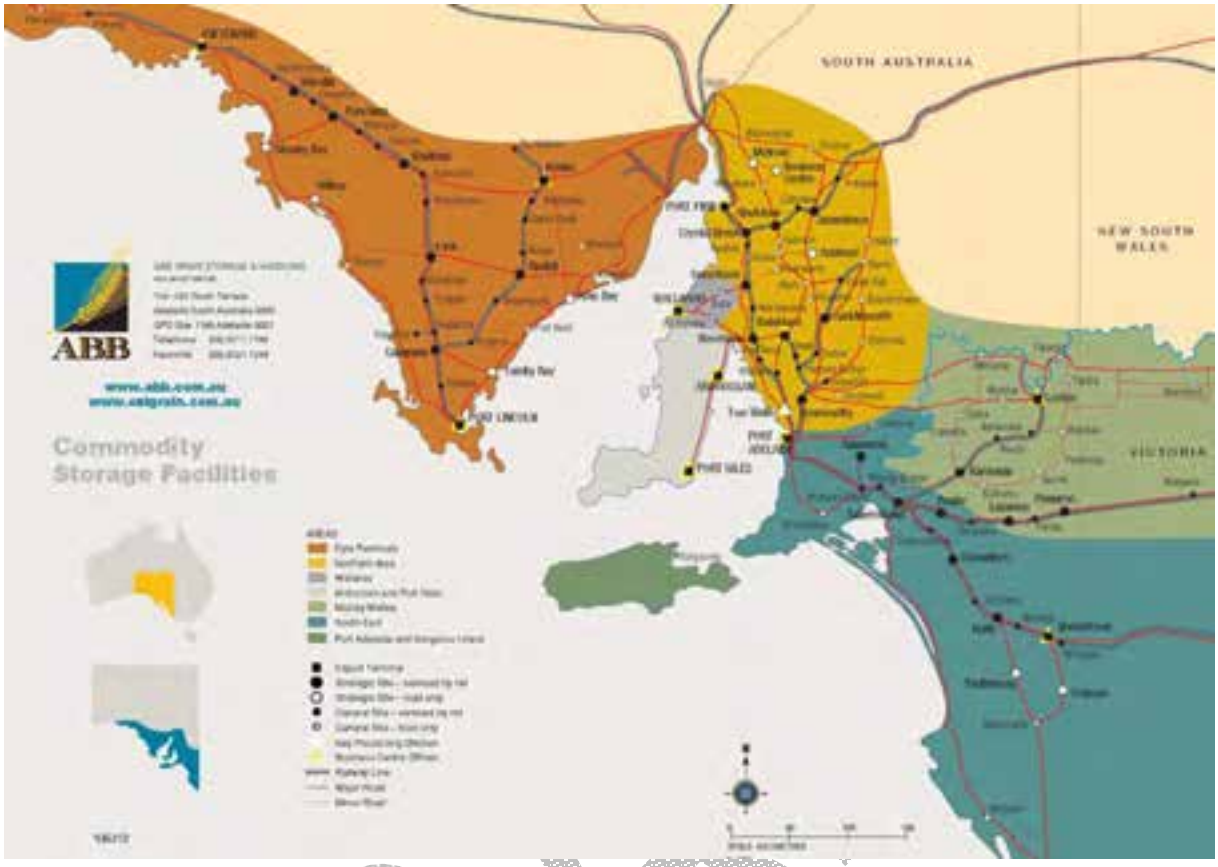

Figure 2.2 Receival sites for South Australia

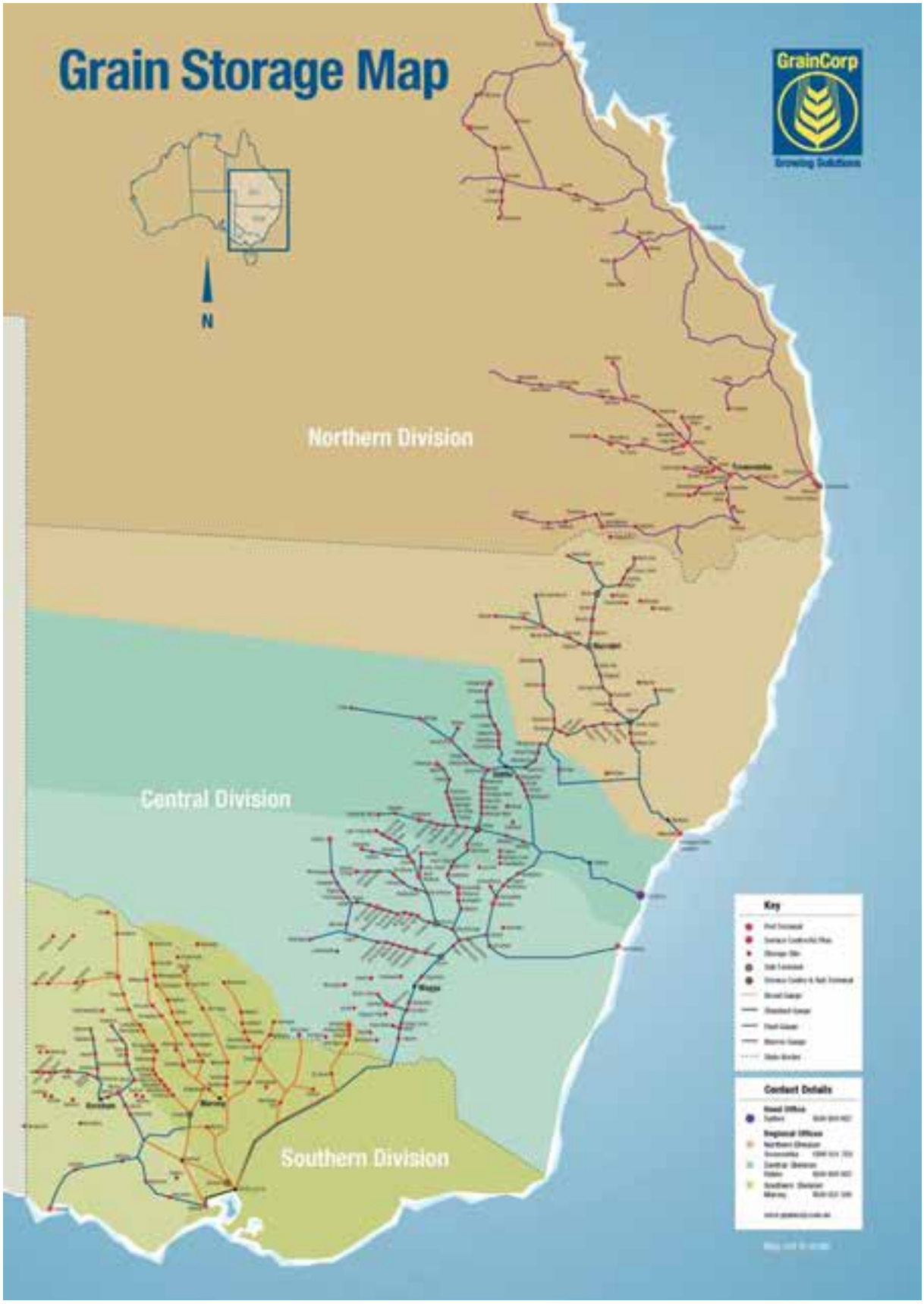

Figure 2.3 Receival sites for Eastern Australia, from Queensland to Victoria.

DRAFT NATIONAL CONTINGENCY PLAN

FOR KARNAL BUNT OF WHEAT

PART III

FIELD MANUAL FOR INCIDENT MANAGEMENT

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CONTENTS

1. INTRODUCTION

This section anticipates the decisions that would arise in a 'live' situation and describes the actions required to effectively eradicate or contain the disease. Recommendations and prescribed actions are intended as a guide to assist decision making by managers in Commonwealth and State regulatory agencies, industry and plant pathologists.

The issues, responsibilities and actions required initially over the first days, weeks and months following the report of a possible detection of Karnal bunt are outlined. The actions required over an extended period (up to ten years) that aim to eradicate or contain the causal agent are also outlined.

Areas covered include:

- field diagnosis of Karnal bunt
- establishment of quarantine controls
- methods of survey and sampling to define the affected area(s)
- treatments for the eradication and control of Karnal bunt
- management, coordination, communication

First notification of suspected Karnal bunt is likely to come from extension officers, agribusiness consultants or diagnostic laboratories of State Departments of Agriculture and Primary Industries. In most instances, a sample is submitted for identification and requires swift follow-up action.

Early containment of Karnal bunt is vital to minimise its spread and maximise opportunities for eradication or containment. This is especially important because Karnal bunt is easily spread by rain splash, machinery movement and grain and straw movement.

The time line associated with the detection, management and eradication of Karnal bunt is shown in Table 3.1. It demonstrates that the eradication of the disease is not a quick and easy process. The table also indicates the relevant sections of the document associated with each event.

> "Early containment of Karnal bunt is vital to minimise its spread and maximise opportunities for eradication or containment."

Under the national Cost-Sharing Agreements, the costs incurred are subject to agreements in the associated Deed (PLANTPLAN). Given that Karnal bunt has been categorised as an Emergency Plant Pest level 2, 80 per cent of the costs are met under the *Cost-Sharing Agreement* by government and 20 per cent by the industry.

Table 3.1 Timeline for events following detection of Karnal bunt, and associated section within this manual

Time	Event	Action	Section
0	Preliminary identification of Karnal bunt. Note: Confirmation of identification will take up to 3 weeks for 2 State laboratories - international confirmation will take longer.	Reported to the Chief Plant Health Manager (CPHM) of the state/territory agriculture department. The process of identification is initiated and the relevant people and organizations are notified of the suspected detection. Submit two samples to other State Laboratory for confirmation, and to Central Science Laboratory, York, UK, for confirmation. Action proceeds on assumption that preliminary identification is correct.	2
Week 1	Initiate wide area survey, and local area survey to identify pest free areas and Restricted Area and Control Area. Commence trace-forward and trace-back procedures from infected premises. Establish movement controls in Restricted Area and Control Area.	Identification of the EPP is confirmed and the outbreak is declared. The Chief Plant Protection Officer (CPPO) convenes a meeting of the Consultative Committee on Emergency Plant Pests (CCEPP). The CCEPP will determine the feasibility of eradication and make a recommendation to the National Management Group (NMG). If the NMG decides to proceed with the eradication campaign, the CCEPP will oversee the preparation of an EPP Response Plan by the Lead Agency(s). The resource requirements needed to implement the response and costing for the eradication program will be identified. The NMG will approve the EPP Response Plan and national cost sharing arrangements to fund the campaign	3 and 5
Week 2-4	Continue activities of week 1 Confirmation of positive or not- detected finding	The Lead Agency(s) in the state(s)/territory(s) in which the incursion occurs will implement and manage the EPP Response Plan overseen by the CCEPP. The Lead Agency(s) will provide regular reports to the CCEPP on the progress of the campaign. If relevant, a Scientific Advisory Panel (SAP) will evaluate the effectiveness of the response and its implementation.	3 and 5
Month 2-6	Continuation of activities in week 1	The Lead Agency(s) will provide regular reports to the CCEPP on the progress of the campaign. Growers in the Restricted Area and Control Area are notified regarding the cropping options and the eradication	3 and 5

		processes that are going to occur.	
Month 7-12	If needed, activities continue as in Week 1. Growers in Restricted Area and Control Area are audited to see if control/ eradication has been adopted.	The Lead Agency(s) will provide regular reports to the CCEPP on the progress of the campaign.	3, 6 and 7
Month 13-24	Surveying continues to ensure eradication has been successful. Growers in Restricted Area and Control Area are audited to see if control/ eradication has been adopted.	The Lead Agency(s) will provide regular reports to the CCEPP on the progress of the campaign. Growers in the Control Area are notified regarding cropping options, and are advised that restrictions will be lifted in month 25.	3, 6 and 7
Month 25	Restrictions are reduced / lifted for those in Control Area.	The Lead Agency(s) will provide regular reports to the CCEPP on the progress of the campaign. Growers in the Control Area are notified regarding cropping options, and are advised that restrictions will be lifted	3, 6 and 7
Ongoing	Continue actions for eradication and to determine pest free area status.	After the coordinated response is complete or if a review determines that eradication is not feasible, records of expenditure and technical reports are provided to PHA so that cost shares can be calculated.	8 and 9

NB: Anticipate potential flare ups in these years.

2. INITIAL DETECTION AND CONFIRMATION OF KARNAL BUNT

2.1 Detection of suspicious grain

All grain with potential defects needs to be examined carefully to determine its risk. Grain with potential plant disease issues needs to be examined by a qualified plant pathologist. Therefore, any sample that is suspected of having Karnal bunt should be treated as being in need of urgent examination and dealt with as quickly as possible.

2.2 Collection and dispatch of samples

Where possible samples should be dispatched on the same day as collection. Alternatively, they should be stored in a cool place until arrangements are made for mailing. Care is needed for clean up and disinfection protocols.

2.2.1 Types of samples

- In collecting samples, care must be taken to ensure that no spores from one sample contaminate another sample. All equipment, such as containers, spear samples etc, must be either new or thoroughly cleaned to remove any spores. While methylated spirits may kill spores, some may remain on the equipment and pose a source of contamination. Ideally, all equipment should be immersed in 10 per cent bleach for 15 minutes. If this is not practical or would damage the equipment, thorough washing with detergent is recommended. Methylated spirits can be used to dry cleaned equipment.
- Individual affected grains: Samples from individual grower deliveries that contain kernels exhibiting symptoms of affected seeds should be contained in a plastic vial or bottle and enclosed in a plastic bag. An associated bulk grain sample from which the affected seeds were collected should be included, as outlined below.
- Samples from bulk grain: Grain should be sampled according to the International Seed Testing Association (ISTA) rules (2006) (see Appendix A). Grain should be sampled to give a representative sample of a bulk consignment (1-2 kg is required). Place in double plastic bags, and then package tightly to prevent grain movement during transport.
- Dust samples from silos, machinery and equipment: A list of where to collect dust samples from within headers is in Appendix B. Place dust into a plastic sterile screw lid jar of approximately 250 mL. Use a suitable implement that has been cleaned to scrape the debris from the header or dust trap into a sterile jar. It is important to take the samples in such a way that minimises dust clouds and potential contamination of surveillance crew and equipment.
- Plant samples (field): Remove heads from crops with clean secateurs, that have been wiped with methylated spirits and dried. Place heads in paper bags (not plastic). Place paper bags into a box, to avoid damaging the samples.
- Fertiliser contaminated with grain: 1-2 kg or larger quantity so that there is plenty of grain in the sample. Place into double plastic bags and pack to avoid movement of grain during shipment. If the grain contamination level is low, a larger quantity of fertiliser will need to be sampled to provide sufficient grain for at least a visual examination.

- Bulk grain of other commodities contaminated with wheat grain: 1-2 kg so that there is at least 50 gm of wheat grain in the sample. Place into double plastic bags and pack to avoid movement of grain during shipment.

2.2.2 Preparation and transport of sample to laboratory

Two categories of laboratories are used:

- **Diagnostic laboratory**, with equipment and expertise to apply a range of morphological and molecular testing to confirm diagnosis. Procedure to be used is documented in Part IV 'Diagnostic Protocols for the Identification of *Tilletia indica*' of this National Contingency Plan for Karnal Bunt of Wheat. Diagnostic laboratories are located at the Elizabeth Macarthur Agricultural Institute and the Department of Agriculture, Western Australia (addresses below).
- **Field laboratory**, which is used for receipt, initial examination and repackaging of samples to the Diagnostic Laboratory. A Field Laboratory is located in each state, and has all necessary equipment for initial examination of the grain for bunted kernels.

Suspect samples should be marked "Plant Sample for Urgent Diagnosis" and sent to either of the National Diagnostic Laboratories:

Addresses of Diagnostic laboratories

Manager, Pest and Disease Diagnostics
NSW Department of Primary Industries
Elizabeth Macarthur Agricultural Institute
PMB 8
CAMDEN NSW 2570

Telephone: 02 4640 6333
Facsimile: 02 4640 6415

Broadacre Plant Pathologist
AgWest Plant Laboratories
Department of Agriculture WA
3 Baron-Hay Court
SOUTH PERTH WA 6151

Telephone: 08 9368 3875
Dacsimile: 08 9474 2658

Samples need to be packaged to prevent movement of the grain or plants as this damages the pathogen and makes detection and confirmation difficult. Samples need to be packed into a plastic container (preferably) or in a plastic bag tightly. Double bag the samples and wipe the outside of the bag with alcohol and allow to dry before dispatching the sample to the laboratory. If the grain is packed into a plastic container, wipe the outside of the container with alcohol and allow to dry before placing into a plastic bag. Also wipe the outside of the bag with alcohol to kill any spores that are attached.

Additional information including the detail of the sample date, location and site must be recorded on an accompanying sheet, together with all relevant paperwork. This information should be placed in a plastic bag, on which is also written the summary details of the sample and the address, and included with the samples that are dispatched.

All samples should be dispatched using an overnight courier service or Express Post.

Important: Prior to dispatch, the Manager of the laboratory to which the sample is being consigned should be advised by telephone (not e-mail - a more direct advice than e-mail is required) of the expected arrival date. Special arrangements may need to be made for weekends. If the receiving laboratory is in another state, then a permit from AQIS is required for the movement of seed into that State. Check with the State or Local Pest and Disease Control Headquarters that approval has been granted.

2.3 Guidelines for field diagnosticians to minimise risks of false positives

See Appendix C for a description and illustrations of symptoms of Karnal bunt on grain.

2.4 Actions on determination of positive detection

2.4.1 Scenarios for detection of Karnal bunt

The actions that need to be undertaken once a preliminary finding of a positive detection of Karnal bunt has been made depends on the point of initial detection. The different scenarios for the detection of Karnal bunt listed (see Appendix D for detailed actions associated with each scenario).

No.	Detection scenario
1	In field (growing crop) - crop green
2	In field (growing crop) - crop mature
3	Grain on farm at harvest
4	Grain at bulk handling authority (BHA) receival point at harvest
5	Grain at processing plant, mill or feedlot
6	Grain in running sample by BHA - grain at receival point
7	Grain in running sample by BHA - grain in transit
8	Grain in running sample by BHA - grain at port
9	Grain in sub-terminal sample
10	Grain in port sample
11	Grain on ship at destination
12	In breeder's plots or harvested grain
13	In imported material on entry to Australia

2.4.2 Steps in the event of an incursion

1. Determine immediate source (load, paddock, farm, etc)
2. Quarantine immediate source
3. Determine extent of initial affected region
4. Quarantine initial affected region
5. Begin trace-back for source of infection
6. Begin trace-forward for other possible pathways of spread from this source
7. Define additional affected regions as required
8. Determine extent of infection and spread of spores
9. Decide on eradication or containment
10. Put appropriate measures in place

3. DEFINING AFFECTED AND PEST-FREE AREAS

3.1 Introduction

The survey is restricted to wheat, durum and triticale, the only known hosts of *Tilletia indica*. The survey will be of crops and grain of these three cereals and associated equipment and facilities. Refer to Appendix E for illustrated guides for identification of grains and plants of cereal crops.

Karnal bunt is difficult to detect in standing crops unless the infection levels are extremely high. Staff should not inspect these while there are higher-priority sites for examination, such as harvested grain.

Contaminated grain from farms is expected leave a trail of spores in harvesters, trucks, silos, conveyors, augers and ship holds. These physical facilities all need to be identified for appropriate treatment (Part III, section 5) because they can contaminate subsequent lots of unaffected grain with spores of *Tilletia indica*. People who have been in contact with crops and grain can also carry spores on their clothing or shoes.

A trace back activity is designed to find out where the contamination originated. The trace forward activity identifies possible further places where KB has spread from its point of detection.

3.1.1 Definitions: Premises level

Premises are divided into three categories of risk, which provide the basis for quarantine controls under PLANTPLAN. Premises are defined to include farm property, receival bin, sub terminal, port, ship, feed-lot and mill (essentially anything associated with the movement of grain within the supply chain), as well as a plant breeding program.

- **Infected Premises** (IP): Premises (or locality) at which Karnal bunt is confirmed or presumed to exist. The Infected Premises includes the entire property on which Karnal bunt is found. Total movement control is imposed.
- **Contact Premises** (CP)*:* Premises (or locality) containing susceptible host crops and grain that are known to have been in direct or indirect contact with an Infected Premises. This includes neighbouring properties and nearby properties operated in conjunction with the Infected Premises. Total movement control is imposed.
- **Suspect Premises** (SP)*:* Premises (or locality) containing plants and grain that may have been exposed to Karnal bunt and which will be subject to quarantine and intense surveillance. Suspect Premises would include distant properties operated by the same farm operator as an Infected premises. Provided there is no evidence of infection and subject to a review of risk through movement of machinery or materials (including fertiliser/seed) the premises revert to normal status.

3.1.2 Definitions: Area level

Two categories of risk are identified to justify quarantine controls on an area basis:

- **Restricted area** (RA): Restricted areas will be drawn around all Infected Premises and Contact Premises. A high level of movement control and surveillance will apply to contain the pathogen and disease to preserve the pest free area status of unaffected wheat production regions.

- **Control area** (CA): Control areas will be imposed around the Restricted Area and include all remaining Suspect Premises. The purpose of the Control Area is to control movement of susceptible plant species or grain for as long as is necessary to complete trace back and epidemiological studies, and then to provide an area around the Restricted Area where potential further spread can be monitored Once the limits of the disease have confidently been determined, the CA boundaries will be reduced or removed.

The Control Area is initially determined by drawing a circle with a 5 kilometre radius (used by the UK and USA definitions) around the boundary of the Infected Premises. This is designed to cover all forms of natural spread from an initial point of infection. However, the boundaries can be modified as new information comes to hand. An additional factor that needs to be taken into consideration is the direction of the prevailing winds. The boundary of the Control Area should be drawn to include all of a property falling partly within the 5 km radius.

3.1.3 Surveys and sampling

Systematic surveys and sampling form the basis of locating outbreaks of Karnal bunt, and are equally important in defining pest free areas.

Two types of surveys are required:

- To define the Restricted and Controlled Areas that surround the outbreak site; and
- To check for additional outbreaks outside the RA and CA, and to provide a basis for future confirmation of pest free area status

International Sanitary and Phytosanitary Measures are developed by the International Plant Protection Convention and recognised by members of the World Trade Organisation (http://www.fao.org/ag/AGP/AGPP/PQ/En/Publ/ISPM/ispms.html). Survey protocols using these guidelines will be significant in terms of international market access.

Surveys should conform to the International Sanitary and Phytosanitary Measure (ISPM): ISPM 4 (1996) – requirements for the establishment of pest free areas; ISPM 6 (1997) – guidelines for surveillance; and to ISPM 8 (1999) – determination of pest status in an area.

3.2 What to survey

The survey is aimed at collecting representative grain samples for analysis to ensure the best possible chance for correctly identifying the scope and extent of the incursion. The samples obtained are then analysed, either by visual inspection or by laboratory analyses (see Part IV for the appropriate protocols associated with the analysis).

The survey is directed at grain and grain handling equipment and facilities rather than growing crops. It is highly unlikely that any sampling methods could detect the presence of Karnal bunt in a growing crop.

The survey needs to look at volunteer host (wheat, durum and triticale) plants and the extent of them in the Restricted and Control Areas.

A trace forward is required on all properties in the Restricted Area to determine the possible spread of the spores. This includes all movements of machinery, grain and people who have come in contact with the crops or grain including farm advisors, farm workers, visitors, and grain handlers. Premises identified by trace-forward are Contact or Infected Premises (see Section 3.5.1).

A trace back is required on all properties in the Restricted Area to determine where the spores originated from. This includes all machinery, grain and people who have come in contact with the crops or grain including farm advisors, farm workers, visitors, and grain handlers. Premises identified by trace-back are Contact Premises (see Section 3.5.2).

3.2.1 Infected Premises

- The original premises identified as having the positive sample is placed into the Restricted Area.
- All subsequent premises identified as having a positive sample is placed into the Restricted Area.

3.2.2 Contact Premises

- If premises is found to be positive, the premises is then placed into the Restricted Area.
- If premises is found 'not detected' the premises is defined as being tentatively in the Control Area.

3.2.3 Suspect Premises

- If premises is found positive, property moves into the Restricted Area.
- If premises is found "not detected" the property is defined as being tentatively in the Control Area.

3.2.4 Further considerations

- After the initial sampling, follow-up sampling of these properties is required to validate their status.
- A further 10 per cent of grain samples delivered per property needs to be visually examined to confirm current 'not detected' status.
- Standardised visual inspection procedures are required (see Guidelines in Contingency Plan Part IV).
- More samples are required to confirm the status of the Control Area. This will need to be repeated until a satisfactory level of sampling has been completed - may even need to test every load delivered.
- Approximately 100 samples (dependent upon size of area being sampled) are required for a wash test, which represents a composite sample from each farm, to further validate and detect at a lower level. If the wash test is positive this property then moves into the Restricted Area. Select a random sample to compare the results from the visual to the wash test, to validate the findings of the visual testing. CCPO will determine the level of sampling required.

3.2.5 Outside the control area

Samples will need to be collected from outside the Restricted and Control Areas to confirm area freedom for these areas.

- For receival bins outside the Restricted and Control Areas, use the BHA's running sample, sampling method. A wash test with 3 replications is required, rather than a visual test, to define PFA.

3.3 Sampling guidelines

3.3.1 Sampling frequency for grain

Every delivery needs to be examined visually for bunted grains at the receival bins (each truck load can range from 10 to 80 tonnes).

- 1 kg in every 30 tonnes in restricted and control areas.
- 1 kg in every 300 tonnes at the receival bins.
- 1 kg in every 3000 tonnes at the sub terminal.
- 1 kg in every 30,000 tonnes at the port.
- 1 kg in every 30,000 tonnes from ship – running sample collected during loading.

Grain and seed lots should be sampled according to the International Seed Testing Association (ISTA) rules, 2006 (refer to Appendix A for details). Need to recognise that as samples are taken from increasingly larger volumes (that is, as the sampling frequency declines), dilution is occurring, so that it reduces the probability of detection of a low level of infection.

3.3.2 Sampling frequency for machinery, equipment and facilities

Because spores accumulate in grain dust, collection of grain dust from key parts of machinery and equipment such as headers, seed cleaners, etc, can determine the presence pf Karnal bunt spores. These should be sampled on the following basis:

- One 250 mL sample from each suspect header.
- One 250 mL sample from each suspect seed cleaner.
- One 250 mL sample from each dust extractor at handling facilities.
- Other similar samples as required.

The best locations from which to collect samples from headers and other plant and equipment are identified in Appendix B.

3.3.3 Collection and dispatch of samples

Where possible, samples should be dispatched on the same day as collection. Alternatively, they should be stored in a cool place until arrangements are made for mailing.

- Samples of individual affected grains: Samples from individual grower deliveries that contain kernels exhibiting symptoms of possible Karnal bunt should be contained in a plastic vial or bottle and enclosed in a plastic bag.

- Samples from bulk grain: Grain should be sampled according to the International Seed Testing Association (ISTA) rules (2006) (see Appendix A). Grain should be sampled to give a representative sample of a bulk consignment (1-2 kg is required). Place in double plastic bags, and then package tightly to prevent grain movement during transport.
- Dust samples from silos, machinery and equipment: A list of where to collect dust samples from within headers is in Appendix B. Place dust into a plastic sterile screw lid jar of approximately 250 mL. Use a suitable implement that has been cleaned and dried with methylated spirits, to scrape the debris from the header or dust trap into a sterile jar. It is important to take the samples in such a way that minimises dust clouds and potential contamination of surveillance crew and equipment.

Samples should be marked "Plant Sample for Urgent Diagnosis" and sent to either of the National Diagnostic Laboratories:

Addresses of Diagnostic laboratories

Manager, Pest and Disease Diagnostics
NSW Department of Primary Industries
Elizabeth Macarthur Agricultural Institute
Woodbridge Road,
MENANGLE NSW 2568
PMB 8
CAMDEN NSW 2570

Telephone: 02 4640 6333
Facsimile: 02 4640 6415

Broadacre Plant Pathologist
AgWest Plant Laboratories
Department of Agriculture WA
3 Baron-Hay Court
SOUTH PERTH WA 6151

Telephone: 08 9368 3875
Facsimile: 08 9474 2658

Samples need to be packaged to prevent movement of the grain as this damages the pathogen and makes detection and confirmation difficult. Samples need to be packed into a plastic container (preferably) or in a plastic bag tightly. If necessary, use packing material within the box, to ensure tight packing of the samples. Double bag the samples and wipe the outside of the bag with alcohol and allow to dry before dispatching the sample to the laboratory. If the grain is packed into a plastic container, wipe the outside of the container with alcohol and allow to dry before placing into a plastic bag. Also wipe the outside of the bag with alcohol to kill any spores that are attached.

Additional information including the detail of the sample date, location and site must be recorded on an accompanying sheet, together with all relevant paperwork. This information should be placed in a plastic bag, on which is also written the summary details of the sample and the address, and included with the samples that are dispatched.

All samples should be dispatched using an overnight courier service or Express Post.

If the receiving laboratory is in another state, then a permit from AQIS is required for the movement of seed into that state. Check with the State or Local Pest and Disease Control Headquarters that approval has been granted.

3.4 Confirming affected or "pest free" areas

The activities to identify and define the areas that are free of *Tilletia indica* ("Area freedom") are outlined in Karnal Bunt Contingency Plan Part II, section 3. This information is used by a number of the committees in relation to national and international trade.

Data collected from the national survey, and previous suspicious grain samples submitted to Diagnostic Laboratories can help to confirm "pest free" areas. The national survey results are available from OCCPO.

International Sanitary and Phytosanitary Measures are developed by the International Plant Protection Convention and recognised by members of the World Trade Organisation (http://www.fao.org/ag/AGP/AGPP/PQ/En/Publ/ISPM/ispms.html). Survey protocols using these guidelines will be significant in terms of international market access.

Surveys should conform to the International Sanitary and Phytosanitary Measure (ISPM): ISPM 4 (1996) – requirements for the establishment of pest free areas; ISPM 6 (1997) – guidelines for surveillance; and to ISPM 8 (1999) – determination of pest status in an area.

ISPM 4 (EPPO) states that both data from general surveillance and from specific surveys are acceptable. The data collected from these different types of surveillance provide for different kinds or degrees of phytosanitary security.

- General surveillance is a process whereby information on particular pests for an area is gathered from many sources. This information is then collated and provided for use by the National Plant Protection Office (NPPO) (ISPM 4, 1996). In Australia the NPPO is the Office of the Chief Plant Protection Officer (OCPPO), DAFF.
- Specific surveys are procedures by which NPPOs obtain information on pests of concern on specific sites in an area over a defined period of time. The verified information acquired may be used to determine the presence or distribution of pests in an area, or on a host or commodity, or their absence from an area (in the establishment and maintenance of pest free areas) (ISPM 4, 1996).

Specific surveys may be detection, delimiting or monitoring surveys. These are official surveys and should follow a plan which is approved by the CPPO.

The survey plan should include:

- Definition of the purpose (e.g. early detection, assurances for pest free areas), and the specification of the phytosanitary requirements to be met.
- Identification of the target pathogen.
- Identification of scope (e.g. geographical area, production system, season).
- Identification of timing (dates, frequency, duration).
- Identification of hosts.
- Indication of the statistical basis, (e.g. level of confidence, number of samples, selection and number of sites, frequency of sampling, assumptions).
- Description of survey methodology and quality management including (sampling procedures (e.g. whole plant sampling, visual inspection, sample collection and laboratory analysis).
- The procedure would be determined by the biology of pathogen and/or purpose of survey, the diagnostic procedures and the reporting procedures.

Maps of BHA receival points are shown in Appendix F.

3.5 Trace-forward and trace-back

The immediate concern after an initial detection will be the contamination of grain to be marketed. Therefore, the first priority will be determining if grain has been moved from infected premises and where it is in the grain system (bin, sub-terminal, ship, importing country etc.) now, as well as its pathway to that location.

The next priority of the trace-forward will be to determine Contact and Suspect Premises from the pathway identified.

The third step is then to do a trace-back to determine the source of infection, and its subsequent Contact and Suspect Premises.

The process of determining trace-forward and trace-back decisions is outlined in Appendix G.

3.5.1 Trace-forward operations

3.5.1.1 Detected on farm

- Was grain delivered to a receival point?
- Which receival bins has the grain been delivered to?
- How was the grain delivered?
- Was seed or grain sold to other farmers?
- Has machinery used in this crop moved to another farm?
- If so, do you know where the machinery is now?
- Have straw or other wheat products moved from the farm?
- Movement of animals/livestock (if livestock have been in contaminated paddocks or been fed from contaminated hay or grain).

If grain from Infected Premises has been moved or sold to others, their premises become Suspect Premises.

3.5.1.2 Detected in silo

Determine destination of grain:

- Has grain been shipped to sub-terminal, or port or processor?
- When?
- How (truck/train transport)?
- Route?

If grain from Infected Premises has been moved or sold to others, their premises become Suspect Premises.

3.5.1.3 Detected in sub-terminal

Determine destination of grain:

- Has grain been shipped to port?
- Has grain been shipped to processor?

If grain from Infected Premises has been moved or sold to others, their premises become Suspect Premises.

3.5.1.4 Detected in port

Determine destination of grain:

- Has grain been loaded on ship?
- Has grain been shipped to processor?

If grain from Infected Premises has been moved or sold to others, their premises become Suspect Premises.

3.5.2 Trace-back operations

3.5.2.1 Detected on farm

Determine paddock and source:

- Was seed obtained off farm (past 5 years)?
- Was fertiliser purchased (past 5 years)?
- Has (contract) machinery come onto farm (past 5 years)? (i.e. who harvested the crop?)
- What are other possible pathways (past 5 years)?
- What is the paddock history (past 5 years)?

Follow up all possible pathways until the source of the infection has been determined.

3.5.2.2 Detected from contract harvester

Determine contact farm(s):

- Need a data base of contract harvesters to assist in trace-forward and trace-back
- What farms has the machinery been used on?
- Do any of the farms use same contractors?
- When were the crops harvested by the machinery
- What route did the machinery take when being moved between premises

Follow up all relevant farms to determine possible pathways and the source of the infection.

3.5.2.3 Detected in silo

Determine bins and stacks affected

- Is infection confined to one bin or stack?
- What other bins or stacks are affected?

Determine infected farm(s):

- What farms have delivered to silo?
- Do delivery patterns of farmers indicate other silos at risk?

Follow up all relevant farms to determine possible pathways and the source of the infection.

3.5.2.4 Detected in sub-terminal

Determine silos:

- What silos delivered to sub-terminal?

Follow up all relevant farms to determine possible pathways and the source of the infection.

3.5.2.5 Detected in port

Determine silos and/or sub-terminals:

- What silos deliver direct to port?
- What sub-terminals deliver to port?

Follow up all relevant farms to determine possible pathways and the source of the infection.

4. FEASIBILITY OF CONTAINMENT OR ERADICATION

The Quarantine Manager needs to start considering at this stage, as information is coming in on the number of premises that are infected, whether eradication is feasible. If the outbreak is contained to a small number of premises in secluded pockets, then it maybe possible to eradicate the disease. State and natural borders can be considered for containment.

The Consultative Committee on Emergency Plant Pests (CCEPP, PLANTPLAN) will determine the feasibility of eradication and make a recommendation to the National Management Group (NMG). An economic analysis of the consequences of containment and eradication policies, is needed to be undertaken to help determine the decision required. Need to recognise that it can take at least 3 weeks before the outbreak can be formally confirmed.

Given that Karnal bunt has been categorised as an Emergency Plant Pest level 2, 80 per cent of the costs are met under the *Cost-Sharing Agreement* by government and 20 per cent by the industry until the decision is made to aim for containment rather than eradication.

5. IMMEDIATE QUARANTINE MEASURES WITHIN AFFECTED AREAS

This section describes the immediate actions that need to be taken to prevent the spread of Karnal bunt from affected areas. These deal with movement controls, treatment of contaminated equipment and crops. It also considers breeders plots.

5.1 Movement controls within affected areas in year of detection

5.1.1 Restricted Area

- No host crops can be sown in the Restricted Area for five years (see Crop Options Section 6.3).
- There should be no host cereal seed production in the Restricted Area.
- Seed for all non-host cereal (barley and oats) crops should be sown with an approved seed dressing to minimise the risk of spreading spores on the seed surface.
- Infested grain that is transported off farm needs to be sealed in a secure manner and should only be transported to non-wheat producing areas (Section 6.2.6).
- Machinery and equipment need to be decontaminated before being allowed to leave the Restricted Area (Section 6.1).
- Straw or hay from infested farms needs to be sealed in a secure manner and should only be transported for disposal in non-wheat producing areas (Section 6.2.7).
- Where stock have grazed on stubble, need to pen animals before movement of stock after grazing, because the pathogen survives passage through the animal.
- For non-host cereals (barley, oats), there should be no movement from the Restricted Area in Year 1. The risk reduces in subsequent years when host crops are not planted. Seed cleaners could be used, but would become contaminated.
- Movement of non-cereal crops (lupins, canola, field peas, etc) will be severely restricted, because there may be wheat contamination and/or spore contamination from equipment.
- Vehicles moving out of the Restricted Area need to be decontaminated (see Section 6.1)
- Need increased vigilance for advisors and/or consultants who enter affected paddocks and farms in the Restricted Area. They should:
  - use disposable overalls;
  - not drive own vehicles into affected paddocks; and
  - apply general biosecurity rules.
- It is vital to remove all volunteer host plants from the Restricted Area, including crops, pastures, roadsides, shed areas, etc, for at least 5 years after an outbreak.

5.1.2 Control Area

- There should be no seed production for cereals in the Control Area for sale outside Control and Restricted areas.
- Seed for all cereal crops sown within the Control Area should be treated with an approved seed dressing.
- Host crops should use additional approved foliar fungicide application.
- All grain loads from host crops need to be tested before delivery using the selective sieve wash test method (Part IV).
- It is recommended that non-host crops be harvested before host crops.
- It is advisable to remove all volunteer host plants from the Control Area, including crops, pastures, roadsides, shed areas, etc.

5.1.3 Movement of vehicles and machinery

Movement of machinery out of the Restricted Area: The machinery will need to be cleaned and disinfected before it leaves this Restricted Area (see Section 5.2).

- Growers should minimise the number of vehicles taken into the affected paddocks. However, if this cannot be avoided, a wash-down is required.
- Restrictions on movement of vehicles from the Restricted Area excludes family and business vehicles that do not normally drive in affected paddocks. However, if they are taken into an affected paddock, they should be washed down before being moved out of the Restricted Area.
- Movement of machinery out of the Control Area: soil and plant material must be removed from harvesters (see Section 5.2).

5.1.4 Movement of harvesters, including contract harvesters

Movement of harvesting machinery (including contract harvesters) out of the Restricted Area:

- The machinery will need to be cleaned and disinfected before it leaves this Restricted Area (see Section 5.2).
- All further farms onto which the harvester is to move during the initial season need to be contacted and advised about the use of this harvester in Karnal bunt Restricted Area.
- Extra testing maybe required by that farm to confirm that no infection has occurred.

5.2 Treatment of contaminated equipment and facilities

In this section, the options available to clean and disinfect equipment and facilities are outlined. The preferred method to be used, based on the type of equipment, is then presented. Harvesters are addressed separately because of their complexity.

5.2.1 Methods for cleaning and disinfecting

A designated "wash-down" area must be provided where the following treatments are applied.

5.2.1.1 Wash down

Washing with water is designed to remove soil and plant debris from equipment when it is moved between fields or farms within the Restricted Area. This will not remove all viable spores of *T. indica* but is designed to reduce the numbers that potentially could be moved.

Washing down to remove soil and plant debris will also improve the efficiency of the remaining treatments for removing and killing spores.

5.2.1.2 Disinfection with sodium hypochlorite

Wet all surfaces to runoff with a 1.5 per cent (a.i.) solution of sodium hypochlorite and allow to stand for 15 minutes, then wash down all surfaces to remove the chemical solution. This treatment is highly corrosive and should be used with care. Hot water, steam cleaning or fumigation may be preferable for equipment likely to be damaged by bleach. Note that diluted chlorine is much more unstable than the concentrate and only enough diluted chlorine should be made up for the day's activity. Increased heat, organic contaminants and acidity increases the rate of chlorine (hypochlorous acid) decay.

5.2.1.3 Steam cleaning

Apply steam to all surfaces to the point of runoff and so the critical temperature of 82°C is reached at the point of contact.

5.2.1.4 Pressure hot water wash

Clean with a solution of hot water and detergent applied under pressure of at least 414 kPa at a minimum temperature of 82°C.

5.2.1.5 Fumigation

Tarp the equipment and fumigate with methyl bromide at the recommended rate for 96 hours. Note: State legislation requires that treatments should only be applied by licensed operators.

5.2.2 Preferred treatments

Quarantine managers will be required to quarantine contaminated equipment before it is disinfected or disposed of.

Harvesters and sowing equipment will be quarantined on farm until treated. Some disassembly of machinery may be required to remove all seed and trash.

Steam cleaning is suitable for cleaning bins and augers and harvesters (if required). This needs to be done on a designated 'wash down' area to minimise contamination of paddocks.

Augers at grain handling places - dilution, or eradication program in place. Bulk handlers will be responsible for organising the cleaning of equipment and silos within their jurisdiction.

Table 3.2 Summary of treatments for cleaning and disinfection

Article	Within Restricted Area	Moving out of Restricted Area
Tractors, farm vehicles	Wash down to remove soil and plant debris with/between farm movement (5.2.1.1).	Wash down (5.2.1.1), then disinfect with 5.2.1.3 or 5.2.1.4.
Sowing and tillage equipment	Wash down to remove soil and plant debris with/between farm movement (5.2.1.1).	Wash down (5.2.1.1), then disinfect with 5.2.1.3 or 5.2.1.4.
Farm tools	Wash down to remove soil and plant debris with/between farm movement (5.2.1.1).	Wash down (5.2.1.1), then disinfect with 5.2.1.2, 5.2.1.3 or 5.2.1.4.
Harvesters	See 5.1.4, then 5.2.3	See 5.1.4, then 5.2.3
Augers	Wash down to remove plant debris (5.2.1.1). If contaminated, disinfect with 5.2.1.3 or 5.2.1.4.	Wash to remove plant debris (5.2.1.1), then disinfect with 5.2.1.3 or 5.2.1.4.
Storage bins, grain transport trucks and rail wagons	Disinfect with 5.2.1.2, 5.2.1.3 or 5.2.1.4.	Disinfect with 5.2.1.2, 5.2.1.3 or 5.2.1.4.
Footwear	Wash, then disinfect with 5.2.1.2 or spray to runoff with methylated spirits: water (70:30).	Wash, then disinfect with 5.2.1.2 or spray to runoff with methylated spirits: water (70:30).
Clothing	Launder with disinfectant.	Launder with disinfectant.
Receival elevators, sub-terminals, terminals, mills, etc.	Disinfection of large facilities is most likely impractical because of their large size and complexity. Spore numbers can be reduced by running non-host or clean host grain through the facility. Any material passing through the facility could be contaminated with *T. indica* spores.	

5.2.3 Preferred treatment of harvesters

Soil and plant material must be removed from harvesters by:

- Running the harvester at maximum throttle to blow out as much debris as possible.
- Blowing out additional debris using a high pressure air hose.
- Thorough power-washing all reachable surfaces.
- Where there are known locations within specific harvesters where grain or debris cannot be removed using the above methods, some disassembly may be required to remove debris. (Refer to Appendix B for sampling of harvesters).

Harvesters leaving the Restricted Area require sanitation with one of the following: high-pressure steam to all surfaces (82°C), hot water power-washing at 30 psi [414 kPa], 82°C, or fumigation with methyl bromide for 96 hours. Any farm to which such a header is taken must be advised of its origin and treatment.

5.3 Treatment of crops within affected areas in year of detection

This section applies for all host cereal crops in Year 1 and Year 2 within the Restricted Area and the Control Area. Appropriate biosecurity measures need to be used at all times.

Where compensation or cost-sharing is applicable, the following steps are required:

- Detailed plans for treatment.
- Activities under the plan.
- Audit procedures to confirm processes and outcomes.

5.3.1 Green crop before grain fill

This applies to host crops sown before the paddock was declared to be in a Restricted Area. The aim is to prevent the crop getting to grain fill.

- Before stem elongation, crash graze and prevent regrowth by spraying with herbicide. If insufficient animals are available, cut for hay or silage and prevent regrowth.
- After stem elongation, crop can be cut for hay or silage, then spray with herbicide to prevent regrowth. Hay and silage must be used on farm. Silage has lower risk of having viable spores on it than hay.

Note: There is no PCR test that can be applied to growing crops to confirm infection of plants with *Tilletia indica*.

5.3.2 Green crop at grain fill

- Cut for hay or silage and spray paddock with herbicide to prevent regrowth.
- Hay and silage cannot be sold off farm. Silage has lower risk of having viable spores on it than hay.

5.3.3 Mature crop

- Cut for hay. Hay cannot be sold off farm. Need to spray paddock with herbicide to prevent regrowth.
- Swath crop, and windrow and then spray with an oil (or wetter) to minimise aerial dispersal of spores, then burn.
- In some cases harvesting is the best option. However this option poses the greatest risk. Thus, time of day for harvest becomes important and farmers downwind need to be considered.
    - Therefore, harvest when no wind, preferably when dew is highest (early morning) to reduce dust and spore dispersal.
    - No straw spreader on back of harvester.
    - Leave stubble *in situ*, spray with oil or wetter to reduce spore dispersal, burn stubble when weather conditions are suitable.

5.3.4 Harvested grain on farm

- This grain must not enter the normal supply chain or leave the Restricted Area, unless under the conditions below (see Section 6.2.6).
- Grain with Karnal bunt spores is not toxic to animals or humans. The aim is to use or dispose of the grain with a view to minimising its spread.
- Bury it, if not a too large an amount. If large amount, could be stored in sealed bunkers for a number of years until it can be disposed of elsewhere.
- Can be used on farm for feed. The ground where manure falls will be contaminated because the spores survive passage through the animals. It is recommended that it be fed out within 6 months. If not, the grain should be destroyed.
- Can be sold to other farms for feed use within the Restricted Area.
- This grain cannot be used for seed. Seed treatment is not sufficient for controlling *Tilletia indica*.

5.3.5 Harvested grain in silo or terminals

This grain must not flow further along the normal supply chain, unless under the conditions below (Section 6.2.6). However, it may be possible to move it through an alternative (contaminated) supply chain, involving processing or use as feed. If the grain is used for feed, the ground where manure falls will be contaminated because the spores survive passage through the animals. It is recommended that it be fed out within six months.

Any movement of contaminated or suspect grain from infected premises must be under strict conditions (see following section).

5.3.6 Delivery of contaminated and suspect grain from infected premises

As part of the eradication process, it may be appropriate to move grain from an infected premises, to allow the eradication to proceed. In moving the grain from the farm, silo or terminal, the following need to be addressed:

- Requires secure transport (ie, the load is covered, so that there is no leakage of grain or spores).
- Any premises that accepts the grain will be considered contaminated.
- Grain should not go to seed cleaners. Seed cleaning does not decontaminate the grain and causes the seed cleaning plant to be contaminated.
- Grain can be used for steam pelletisation. The pelletising plant will be contaminated by this grain. There may be the option of pelletising on farm, which would reduce the risk of cartage. Pellets are safe to be marketed anywhere providing they are not contaminated after manufacture, since the pelletising process kills the spores.
- Ethanol plants: grain can be used for ethanol production. The mash requires treatment, as it will be contaminated with spores.
- Grain can be used directly for livestock feed in non grain producing areas.
  Note: Manure will be contaminated and it is important that the livestock and untreated manure are not moved to grain producing areas.

- Contaminated grain could be blended to reduce the level of bunt for human consumption, but this would be difficult to market the flour. If this option is used, the mill will be contaminated. All mill offal (bran, pollard) will be contaminated with spores and needs careful control, including the manure of any animals using mill offal for feed. Not likely to be a practical option unless Karnal bunt becomes endemic.
- Contaminated grain could be sold to markets that will accept the grain (some export markets will accept contaminated grain), but the supply chain would be contaminated. Not likely to be a practical option unless Karnal bunt becomes endemic.

5.3.7 Movement of straw, hay or chaff from infected area

As part of the eradication process, it may be appropriate to move straw, hay or chaff from an Infected Premises. In moving the straw or hay from the farm, the following need to be addressed:

- Requires secure transport.
- Any premises that accepts the straw, hay or chaff will be considered contaminated.

5.4 Breeders plots

- Most seed has to be destroyed with the possible exception of small lots of genetically important lines may be kept. This seed needs to be treated with bleach for 10 minutes (1.25 per cent available chlorine as aqueous sodium hypochlorite) before going to be planted in closed quarantine glasshouse for one generation. This has to be managed under the supervision of the Quarantine Manager to ensure there is no survival of the spores.
- Affected and suspect plots should be fumigated with methyl bromide, and then planted with non-host crops for at least five years. A cereal crop cannot be planted in the first year after detection.
- It is most important that all equipment and facilities be disinfested and cleaned up following the guidelines listed in Section 6.1.

6. QUARANTINE MEASURES WITHIN AFFECTED AREAS IN LATER YEARS

This section describes the actions that need to be taken to prevent the spread of Karnal bunt from affected areas in later years. These deal with movement controls, treatment of contaminated equipment and crops.

6.1 Movement controls within affected areas

6.1.1 Restricted Area

- No host crops can be sown in the Restricted Area for five years (see Crop options Section 6.3).
- There should be no host cereal seed production in the Restricted Area.
- Seed for all non-host cereal (barley and oats) crops should be sown with an approved seed dressing to minimise the risk of spreading spores on the seed surface.
- Infested grain that is transported off farm needs to be sealed in a secure manner and should only be transported to non-wheat producing areas (Section 6.2.6).
- Machinery and equipment need to be decontaminated before being allowed to leave the Restricted Area (Section 6.1).
- Straw or hay from infested farms needs to be sealed in a secure manner and should only be transported for disposal in non-wheat producing areas (Section 6.2.7).
- Where stock have grazed on stubble, need to pen animals before movement of stock after grazing, because the pathogen survives passage through the animal.
- For non-host cereals (barley, oats), there should be no movement from the Restricted Area in Year 1. The risk reduces in subsequent years when host crops are not planted. Seed cleaners could be used, but would become contaminated.
- Movement of non-cereal crops (lupins, canola, field peas, etc.) will be severely restricted, because there may be wheat contamination and/or spore contamination from equipment.
- Vehicles moving out of the Restricted Area need to be decontaminated (see Section 6.1)
- Need increased vigilance for advisors and/or consultants who enter affected paddocks and farms in the Restricted Area. They should:
  - use disposable overalls,
  - not drive own vehicles into affected paddocks
  - apply general biosecurity rules
- It is vital to remove all volunteer host plants from the Restricted Area, including crops, pastures, roadsides, shed areas, etc, for at least 5 years after an outbreak.

6.1.2 Control Area

- There should be no seed production for cereals in the Control Area for sale outside Control and Restricted areas.
- Seed for all cereal crops sown within the Control Area should be treated with an approved seed dressing.
- Host crops should use additional approved foliar fungicide application.
- All grain loads from host crops need to be tested before delivery using the selective sieve wash test method (Part IV).
- It is recommended that non-host crops be harvested before host crops.
- It is advisable to remove all volunteer host plants from the Control Area, including crops, pastures, roadsides, shed areas, etc.

6.1.3 Movement of vehicles and machinery

Movement of machinery out of the Restricted Area: The machinery will need to be cleaned and disinfected before it leaves this Restricted Area (see Section 6.1).

- Growers should minimise the number of vehicles taken into the affected paddocks. However, if this cannot be avoided, a wash-down is required.
- Restrictions on movement of vehicles from the Restricted Area excludes family and business vehicles that do not normally drive in affected paddocks. However, if they are taken into an affected paddock, they should be washed down before being moved out of the Restricted Area.
- Movement of machinery out of the Control Area: soil and plant material must be removed from harvesters (see Section 6.1).

6.1.4 Movement of harvesters, including contract harvesters

Movement of harvesting machinery (including contract harvesters) out of the Restricted Area:

- The machinery will need to be cleaned and disinfected before it leaves this Restricted Area (see Section 6.1).
- All further farms onto which the harvester is to move during the initial season need to be contacted and advised about the use of this harvester in Karnal bunt Restricted Area.
- Extra testing maybe required by that farm to confirm that no infection has occurred.

6.2 Treatment of crops within affected areas after year of detection

This section applies for all host cereal crops in Year 2 onwards within the Restricted Area and the Control Area. Appropriate biosecurity measures need to be used at all times.

Where compensation or cost-sharing is applicable, the following steps are required:

- Detailed plans for treatment.
- Activities under the plan.
- Audit procedures to confirm processes and outcomes.

6.2.1 Delivery of contaminated and suspect grain from infected premises

As part of the eradication process, it may be appropriate to move grain from an infected premises, to allow the eradication to proceed. In moving the grain from the farm, silo or terminal, the following need to be addressed:

- Requires secure transport (i.e. the load is covered, so that there is no leakage of grain or spores).
- Any premises that accept the grain will be considered contaminated.
- Grain should not go to seed cleaners. Seed cleaning does not decontaminate the grain and causes the seed cleaning plant to be contaminated.
- Grain can be used for steam pelletisation. The pelletising plant will be contaminated by this grain. There may be the option of pelletising on farm, which would reduce the risk of cartage. Pellets are safe to be marketed anywhere providing they are not contaminated after manufacture, since the pelletising process kills the spores.
- Ethanol plants: grain can be used for ethanol production. The mash requires treatment, as it will be contaminated with spores.
- Grain can be used directly for livestock feed in non grain producing areas.
  Note: Manure will be contaminated and it is important that the livestock and untreated manure are not moved to grain producing areas.
- Contaminated grain could be blended to reduce the level of bunt for human consumption, but this would be difficult to market the flour. If this option is used, the mill will be contaminated. All mill offal (bran, pollard) will be contaminated with spores and needs careful control, including the manure of any animals using mill offal for feed. Not likely to be a practical option unless Karnal bunt becomes endemic.
- Contaminated grain could be sold to markets that will accept the grain (some export markets will accept contaminated grain), but the supply chain would be contaminated. Not likely to be a practical option unless Karnal bunt becomes endemic.

6.2.2 Movement of straw, hay or chaff from infected area

As part of the eradication process, it may be appropriate to move straw, hay or chaff from an infected premise. In moving the straw or hay from the farm, the following need to be addressed:

- Requires secure transport.
- Any premises that accepts the straw, hay or chaff will be considered contaminated.

7. FARMING OPTIONS WITHIN AFFECTED AREAS IN YEARS FOLLOWING DETECTION

Farming options available vary with time along the eradication process and are dependent upon if the premises are within the Restricted or Control Area. Table 3.3 gives a summary of options available to growers.

Table 3.3 Summary of options for farmers in Restricted Area and Control Area

Year		Restricted Area	Control Area
Year 1	Season when wheat on which detection is made is produced	Emergency restrictions	Emergency restrictions
Year 2	First crop sown after detection	No host crops, no cereals, minimum cultivation	No host cereal crops.
Year 3	Second year of restrictions	Non-host crops, including cereals, with cultivation	Host crops but careful monitoring
Year 4	Third year of restrictions	Non-host crops, including cereals	Host crops but careful monitoring
Year 5	Fourth year of restrictions	Non-host crops, including cereals	Host crops but careful monitoring
Year 6	Fifth year of restrictions	Non-host crops, including cereals	Host crops but careful monitoring
Year 7	Year to rest for eradication	Sentinel crops	Host crops but careful monitoring
Year 8	Year to confirm eradication	If sentinel clear, host crops but careful monitoring	If sentinel clear, remove from Control Area
Year 9	Year to confirm eradication	Host crops but careful monitoring	
Year 10	Year to confirm eradication	Host crops but careful monitoring	
Year 11	Year to confirm eradication	Host crops but careful monitoring	

7.1 Restricted Area

- It is important that all host volunteers are controlled throughout the incident.
- In Year 2, ground cannot be cultivated before planting with a non host crop, as spores that were on the surface would be buried where they can survive for more than five years. A rotation with a broadleaf crop is preferable because volunteer hosts can be controlled within this crop. An annual pasture can be planted, as long as herbicide is used to control host volunteers. Other cereal crops and perennial crops should not be planted in year 2.
- In Years 3 to 6: Non-host cereal crops can be grown. Cultivation is encouraged after Year 2 to help reduce the number of buried spores surviving in the soil. Perennial crops should not be planted. This is so that the ground is cultivated regularly to bring buried spores to the surface, so that they will be destroyed or will germinate without a host being present.

- Development of intensive animal production such as pigs, chickens or similar, for feeding the grain produced in the Restricted Area should be investigated with a business consultant.

7.2 Control Area

- It is important that all host volunteers are controlled throughout the incident.
- In Year 2, only non-host cereal, and broadleaf crops can be grown. A rotation with a broadleaf crop is preferable because volunteer hosts can be controlled within this crop. An annual pasture can be planted, as long as herbicide is used to control host volunteers. Other cereal crops and perennial crops should not be planted in Year 2.
- In Year 3, host crops can be grown, since it is clear that at that stage the risk of growing host crops is minimal since there has been no detection of spores despite close examination and testing. It must be sown with seed produced outside the Control Area. The harvested grain must be tested from every paddock before it can be moved off the farm. The analysis required is a wash test, at a sampling rate of 1 kg for every 30 tonnes produced. If found to be “not detected”, the grain can be delivered.
- Movement of machinery between the Restricted Area and the Control Area needs to be monitored. Movement within a Restricted Area or a Control Area is permitted. Movement of machinery from the Control Area to other areas is subject to steam cleaning and decontamination (see Section 5.2.3).

8. CONFIRMATION OF ERADICATION

International markets need statistically based evidence from an intensive and extensive survey to confirm eradication.

8.1 Soil testing

- Soil testing for spores is not accurate enough due to the difficulty of sampling and the availability of testing methods that can be used.
- There is no PCR test that can be used to confirm the presence of *Tilletia indica* spores in soil.

8.2 Sentinel crops

- In Year 7 (after five years of controls in the Restricted Area), sentinel crops should be planted within the restricted area, to confirm presence or absence of Karnal bunt. A variety with the highest known level of susceptibility should be grown.
- Sentinel plants should be sown on each of the farms in the Restricted Area. The number and size of sentinel plants is to be determined in discussion with a biometrician, and consider international protocols and market requirements.
- The sieve wash test (see Contingency Plan Part IV for description and protocols) should be used on samples from all sentinel crops.
- If sentinel plants are clear of *T. indica*, the former Restricted Area becomes part of the Control Area, and host crops may be grown (see Section 7.2), though there needs to be careful monitoring of all crops and loads for the next four years.
- If a sentinel plant is found to be infected then that farm continues in the Restricted Area (and reverts to Year 2 status).
- If a farm in the Control Area is found to have Karnal bunt, that is to be treated as a new outbreak, and requires new re-defined Restricted and Controls Areas, and the operations start back at Year 1 status.

8.3 Grain testing

- Most reliable method to confirm presence or absence of pathogen, within the control zone (all host crops are regularly tested) and the restricted zone (sentinel plants). This includes visual examination with the selective sieve wash test based on international standards (EPPO PM7/29(1)) (see Contingency Plan Part IV).

8.4 Announcement of confirmation of eradication

The formal requirements of the announcements of the conformation or eradication will be met by the CCEPP (PLANTPLAN, 2004).

9. ACTIONS IF ERADICATION IS NOT UNDERTAKEN OR FAILS

9.1 Activities needed

If the decision is made that eradication is not feasible or has failed, containment management is then undertaken in the affected area. This will need ongoing monitoring to confirm containment.

Once eradication is determined to have failed, or has been determined as inappropriate, State quarantine legislation replaces the Cost-Sharing Agreement.

If a large outbreak was detected, and the policy was to contain rather than eradicate the disease, it would be equivalent to having a separate wheat industry that has the disease. There would need to be separate marketing channels and separate wheat industries developed, one with Karnal bunt and one without.

9.2 Containment guidelines

9.2.1 Regulated area

- Scale is important: If a small area is involved, it may be possible to isolate the area and take it out of wheat production altogether. If a large area is involved, that will not be possible.
- The regulated area needs to be defined at least 5 km beyond the furthest known positive detection of Karnal bunt
- This area will be expected to grow in size with time through natural spread once controls are relaxed or removed.
- Wheat may be produced in rotation with broad-leaf crops to reduce population levels in paddocks that are known not to be infected with *Tilletia indica*.
- The supply chain will be different to, and entirely separate from, that of “not detected” Karnal bunt crops
- Grain can only be on-sold through a secure supply chain, but this supply chain will be deemed contaminated.
- New markets will need to be found or developed for this infested grain.

9.2.2 Borders

- Borders may be difficult to monitor if within a State. State borders are easier to regulate.
- Natural borders should be used where possible (Appendix H).
- Needs to be made as feasible as possible to define and maintain a border on the zone.
- Movement of machinery need to be regulated as in Section 5.1.

9.3 Level of surveillance

An on-going level of surveillance and other containment activities must be maintained by regulatory authorities (both government and the bulk handlers).

- National survey by State production, one sample for each 30,000 tonnes of production. This is to maintain Area Freedom for those States where Karnal bunt has not been detected.
- Survey of receival bins: Bins within the Karnal bunt infested area need to be monitored to assess the containment strategies for Karnal bunt.

9.4 Management

Karnal bunt is managed in countries where it is endemic by a combination of resistant varieties, grain inspection, crop rotation and fungicide spraying of crops. Further details are in the Karnal Bunt Contingency Plan Part II.

10. REFERENCES

I.Clayton, W.D., K.T. Harman, and H. Williamson. (2002 onwards). World Grass Species: Descriptions, Identification, and Information Retrieval. **http://www.kew.org/data/grasses–db.html**. [accessed 01 August 2005; 15:30 GMT]*.

http://www.hort.purdue.edu/newcrop/crops/wheat.html

http://www2.dpi.qld.gov.au/fieldcrops/2348.html,

http://www.raydw.com/grains.htm#Wheat

http://waltonfeed.com/self/triticale.html

www.missouriplants.com/Grasses/Triticum_aestivum_page.html

www.inspection.gc.ca/english/plaveg/bio/dir/dir9901e.shtml

Magness, J.R., Markle, G.M. and Compton, C.C. 1971. Food and feed crops of the United States. Interregional Research Project IR-4, IR Bul. 1 (Bul. 828 New Jersey Agr. Expt. Sta.).

NIAB. Seed identification handbook: agriculture, horticulture and weeds, 2nd edition, Steve Jones, Jane Taylor and Felicity Ash (eds), NIAB, Cambridge, UK, 2004.

Williams, J.T. 1995. Cereals and Pseudocereals. Chapman and Hall, London.

APPENDIX A. GRAIN SAMPLING RULES FROM INTERNATIONAL SEED TESTING ASSOCIATION

International Rules for Seed Testing Effective from 1st January 2006

Chapter 2: Sampling

2.1 Object

The object of sampling is to obtain a sample of a size suitable for tests, in which the probability of a constituent being present is determined only by its level of occurrence in the seed lot.

2.2 Definitions

2.2.1 Seed lot

A seed lot is a specified quantity of seed that is physically and uniquely identifiable.

2.2.2 Primary sample

A primary sample is a portion taken from the seed lot during one single sampling action.

2.2.3 Composite sample

The composite sample is formed by combining and mixing all the primary samples taken from the seed lot.

2.2.4 Sub-sample

A sub-sample is a portion of a sample obtained by reducing a sample.

2.2.5 Submitted sample

A submitted sampleis a sample that is to be submitted to the testing laboratory and may comprise either the whole of the composite sample or a sub-sample thereof. The submitted sample may be divided into sub-samples packed in different material meeting conditions for specific tests (e.g. moisture or health).

2.2.6 Duplicate sample

A duplicate sample is another sample obtained for submission from the same composite sample and marked "Duplicate sample".

2.2.7 Working sample

The working sample is the whole of the submitted sample or a sub-sample thereof, on which one of the quality tests described in these *ISTA Rules* is made and must be at least the weight prescribed by the *ISTA Rules* for the particular test.

2.2.8 Sealed

Sealed means that a container in which seed is held is closed in such a way, that it cannot be opened to gall access to the seed and closed again, without either destroying the seal or leaving evidence of tampering. This definition refers to the sealing of seed lots, as well as of seed samples.

2.2.9 Self-sealing containers

The 'valve-pack' bag is a specific type of self sealing container. It is filled through a sleeve-shaped valve which is automatically closed by the completion of"filling the bag.

2.2.10 Marked/labelled

A container of a seed lot can be considered as marked or labelled when there is a unique identification mark on the container, which defines the seed lot to which the container belongs. All containers of a seed lot must be marked with the same unique seed lot designation (numbers, characters or combination of both). Marking of samples and sub-samples must ensure that there is always an unambiguous link between the seed lot and the samples and sub-samples.

2.2.11 Coated seeds

Coated seeds are seeds covered with material that may contain pesticides, fungicides, dyes or other additives. The following types of coated seeds are defined:

Seed pellets. More or less spherical units, usually incorporating a single seed with the size and shape of the seed no longer readily evident.

Encrusted seed. Units more or less retaining the shape of the seed with the size and weight changed to a measurable extent.

Seed granules. Units more or less cylindrical, including types with more than one seed per granule.

Seed tapes. Narrow bands of material, such as paper or other degradable material, with seeds spaced randomly, in groups or in a single row.

Seed mats. Broad sheets of material, such as paper or other degradable material, with seeds placed in rows, groups or at random throughout the sheets.

Treated seed. Seeds with treatments, which have not resulted in a significant change in size, shape or addition to the weight of the original seed.

2.3 General principles

A composite sample is obtained from the seed lot by taking primary samples from different positions in the whole seed lot and combining them. From this composite sample, sub-samples are obtained by sample reduction procedures at one or more stages forming the submitted sample and finally the working samples for testing. For issuing *ISTA International Seed Analysis Certificates,* specific requirements have to be fulfilled as given under 2.5.4. Further help in interpreting this chapter may be found in the current *ISTA Handbook on Seed Sampling.*

2.4 Apparatus

Sampling and sample reduction must be performed using appropriate techniques and equipment that is clean and in good condition as described in 2.5.1 and 2.5.2.2.

2.5 Procedures

2.5.1 Procedures/or sampling a seed lot

"2.5.1.1 Preparation 0/ a seed lot and conditions/or sampling

At the time of sampling, the seed lot shall be as uniforn as practicable. If there is documentary or other evidence of heterogeneity, or the seed lot is found to be obviously heterogeneous, sampling must be refused or stopped. In cases of doubt heterogeneity can be deternined as described under 2.9.

Seed may be sampled in containers or when it enters containers. The containers must be fit for purpose, e.g. must not damage the seed, and must be clean to avoid cross contamination. The containers must be labelled or marked before or just after sampling is completed. The seed lot shall be so arranged that each part of the seed lot is conveniently accessible.

2.5.1.2 Sampling intensity

For seed lots in containers of 15 kg to 100 kg capacity (inclusively), the sampling intensity according to Table 2.1 shall be regarded as the minimum requirement.

Table 2.1 Minimum sampling intensity for seed lots in containers of 15 kg to 100 kg capacity (inclusively)

Number of containers	Minimum number of primary samples to be taken
1- 4 containers	3 primary samples from each container
5- 8 containers	2 primary samples from each container
9-15 containers	1 primary sample from each container
16-30 containers	15 primary samples from the seed lot
31-59 containers	20 primary samples from the seed lot
60 or more containers	30 primary samples from the seed lot

For seed lots in containers smaller than 15 kg capacity, containers shall be combined into sampling units not exceeding 100 kg, e.g. 20 containers of 5 kg, 33 containers of 3 kg or 100 containers of 1 kg. For seed mats and tapes, small packets or reels may be combined to sampling units of not exceeding 2,000,000 seeds. The sampling units shall be regarded as containers as described in Table 2.1.

When sampling seed in containers of more than 100 kg, or from streams of seed entering containers the sampling intensity according to Table 2.2 shall be regarded as the minimum requirement.

Table 2.2 Minimum sampling intensity for seed lots in containers of more than 100 kg, or from streams of seed entering containers

Seed lot size	Number of primary samples to be taken
Up to 500 kg	At least five primary samples
501- 3,000 kg	One primary sample for each 300 kg, but not less than five
3,001-20,000 kg	One primary sample for each 500 kg, but not less than 10
20,001 kg and above	One primary sample for each 700 kg, but not less than 40

When sampling a seed lot of up to 15 containers, regardless of their size, the same number of primary samples shall be taken from each container. Sampling intensity for coated seeds is as described in Tables 2.1 and 2.2.

2.5.1.3 Taking primary samples

When defining the number and/or the size of primary samples, the seed sampler needs to ensure (besides meeting the minimum sampling intensity) that the minimum amount of seed required for the requested test(s) is sent to the testing laboratory and enough seed remains available for obtaining duplicate samples if requested.

Primary samples of approximately equal size shall be taken from a seed lot, irrespective of where in the lot or container the primary sample is taken.

When the seed lot is in containers, the containers to be sampled shall be selected at random or according to a systematic plan throughout the seed lot. Primary samples shall be drawn from the top, middle and bottom of containers, but not necessarily from more than one position in any container, unless so specified in Tables 2.1 and 2.2.

When the seed is in bulk or in large containers, the primary samples shall be drawn from random positions.

Containers shall be opened or pierced for abstraction of primary samples. The sampled containers shall then be closed or the contents transferred to new containers.

When seed is to be packed in special types of containers (e.g. small, not penetrable, or moisture-proof containers), it should be sampled, if possible, either before or during the filling of the containers.

Sampling seed lots of seed tapes and seed mats should be done by taking packets or pieces of tape or mat.

The instruments being used must neither damage the seed nor select according to seed size, shape, density, chaffiness or any other quality trait. All sampling apparatus must be clean before use to prevent cross contaminations. Triers must be long enough so that the opening at the tip reaches at least half of the diameter of the container. When the container is not accessible from opposite sides, the trier must be long enough to reach the opposite side. Sampling seed lots may be done by one of the methods listed below.

a) *Automatic sampling from a seed stream.* Seed may be sampled by automatic sampling devices, provided that the instrument uniformly samples the cross section of the seed stream and the material entering the instrument does not bounce out again. It may be operated either under manual or automatic control. The intervals between taking primary samples should be constant but may also vary randomly.

b) *Manual sampling from a seed stream.* Seed streams may also be sampled by using manual instruments when fulfilling the requirements listed under (a).

c) *Sampling stick (synonym: stick trier; sleeve type trier).* The sampling stick consists of an inner tube which fits loosely inside an outer tube but tightly enough so that seed or impurities do not slip between them. The outer tube has a solid pointed end. Both tubes have slots cut into their walls so that the cavity of the inner tube can be opened and closed by twisting the tubes against each other. The sampling stick may be used horizontally, diagonally or vertically.

However, when used vertically the sampling stick must have partitions dividing the instrument into a number of compartments. The minimum inside diameter should be about 25 mm for all species.

When using the sampling stick, insert it in the closed position into the container, gently push it so that the point reaches the required position, open the sampling stick, agitate it slightly to allow it to fill completely, gently close and withdraw it and empty the primary sample into a container. Care should be exercised in closing the sampling stick so that seeds are not damaged.

d) *Nobbe trier :* The Nobbe trier (dynamic spear) is a pointed tube with an opening near the pointed end. Seed passes through the tube and is collected in a container. The minimum internal diameter of the Nobbe trier should be about 10 mm for clovers and similar seeds, about 14 rnm for cereals and about 20 mm for maize.

When using the Nobbe trier, insert it at an angle of about 30° to the horizontal plane with the opening facing down, push the trier until it reaches the required position and revolve it through 180°. Withdraw it with decreasing speed from the container, gently agitating the trier to help maintain an even flow of seed, and collect the seed sample coming from the trier in a suitable container.

e) Sampling by hand. This method is sometimes the most satisfactory method as in the following examples - Agropyron, Agrostis, Alopecurus, Anthoxanthum, Arrhenatherum, Axonopus, Bromus, Chloris, Cynodon, Cynosurus, Dactylis, Deschampsia, Digitaria, Elymus, Elytrigia, Festuca, Holcus, Lolium, Melinis, Panicum, Pascopyrum, Paspalum, Poa, Psathyrostachys, Pseudoroegneria, Trisetum, Zoysia.

Sampling by hand is also the most suitable method for seed that may be damaged by the use of triers e.g. in seed lots of large seeded legumes, for seed with wings or seeds which have a low moisture content or for seed tapes and seed mats.

For hand sampling seed in containers, all positions inside the containers must be accessible. Containers with layers which are not accessible from the regular opening may have to be cut open, sampled and repackaged. Containers may also be partially or completely emptied during the sampling process to gain access to all positions in the containers. For sampling by hand, clean the hand and roll the sleeve up if necessary, insert the open hand into the container to the required position, close and withdraw the hand, taking great care that the fingers remain tightly closed about the seeds so none may escape, and empty the hand into a receiving pan.

2.5.1.4 Obtaining the composite sample

If the primary samples appear uniform they can be combined to form the composite sample. If not, the sampling procedure must be stopped. When primary samples are collected directly into one container, the content of this container shall be regarded as the composite sample only if it appears uniform. If not, it must not be used for obtaining a submitted sample.

2.5.1.5 Obtaining the submitted sample

The submitted sample shall be obtained by reducing the composite sample to an appropriate size by one of the methods referred to in 2.5.2.2. Obtaining sub-samples such as for moisture testing must be carried out in such a way that changes in moisture content are minimal.

The composite sample can be submitted to the seed testing laboratory if it is of appropriate size or if it is difficult to mix and reduce the composite sample properly under warehouse conditions.

Duplicate samples, which were requested not later than at the time of sampling shall be prepared in the same way as the submitted sample.

2.5.1.6 Dispatch of the submitted sample

The submitted sample must be marked with the same identification as the seed lot. For an *ISTA International Seed Lot Certificate,* the sample must be sealed. The additional information required according to 17.4.2 and 17.4.3, as well as the name of any chemical treatment applied must be provided.

Submitted samples shall be packed so as to prevent damage during transit. Submitted samples should be packed in breathable containers.

Sub-samples for moisture testing, and samples from seed lots which have been dried to low moisture content, shall be packed in moisture proof containers which contain as little air as possible. Submitted samples for germination tests, viability tests and health tests may only be packed in moisture proof containers if suitable storage conditions can be assured.

Submitted samples shall be dispatched by the sampler to the seed testing laboratory without delay.

2.5.1.7 Storage of submitted samples before testing

Every effort must be made to start testing a submitted sample on the day of receipt. Storage of orthodox seeds, when necessary, should be in a cool, well-ventilated room. Non-orthodox (i.e. recalcitrant or intermediate) seeds should be tested as soon as possible after obtaining the submitted sample from the composite sample without any storage. Handling of the submitted sample and, if necessary, storage should be done under species specific optimum conditions.

2.5.2 Procedure for obtaining the working sample

2.5.2.1 Minimum size of working sample

Minimum sizes of working samples are prescribed in the appropriate chapter for each test. The working sample weights for purity analyses given in Table 2A are calculated to contain at least 2 500 seeds. These weights are recommended for normal use purity tests, see 3.5.1.

The sample weights in column 5 of Table 2A, Part 1, for counts of other species are 10 times the weights in column 4, subject to a maximum of 1 000 g.

Working samples of all coated seeds except those defined as treated seed in 2.2.11 shall contain at least the number of pellets, seeds or granules indicated in column 3 of Table 2B, Part 1 and Part 2. If a smaller sample is used, the actual number of pellets, seeds or granules in the sample shall be reported.

2.5.2.2 Sample reduction methods

If the seed sample needs to be reduced to a size equal to or greater than the size prescribed, the seed sample shall first be thoroughly mixed. The submitted/working sample shall then be obtained either by repeated halving or by abstracting and subsequently combining small random portions. The apparatus and methods for sample reduction are described in 2.5.2.2.1 to 2.5.2.2.4. One, two or more of these methods may be used in one sample reduction procedure. When using one of the dividers described for seed pellets the distance of fall must not exceed 250 mm.

Except in the case of seed health, the method of hand halving shall be restricted to certain genera listed in 2.5.2.2.4. Only the spoon method and the hand halving method may be used in the laboratory to obtain working samples for seed health testing where other samples or equipment may be contaminated by spores or other propagating material.

For seed tapes and mats take pieces of tape or mat at random, to provide sufficient seeds for the test.

After obtaining a working sample or half-working sample the remainder shall be re-mixed before a second working sample or half-working sample is obtained.

Sub-samples for moisture content determination may be taken in the following way: before taking the sub-sample, mix the sample by either stirring the sample in its container with a spoon or place the opening of the original container against the opening of a similar container and pour the seed back and forth between the two containers. Take at minimum three sub-samples with a spoon from different positions and combine them to the sub-sample of the required size. The seed may not be exposed to the air during sample reduction for more than 30 seconds.

2.5.2.2.1 Mechanical divider method

This method is suitable for all kinds of seeds except some very chaffy seeds. The apparatus divides a sample passed through it into two or more approximately equal parts.

The submitted sample can be mixed by passing it through the divider, recombining the parts and passing the whole sample through a second time, and similarly, a third time if necessary. The sample is reduced by passing the seed through repeatedly and removing parts on each occasion. This process of reduction is continued until a working sample of approximately, but not less than, the required size is obtained.

The dividers described below are examples of suitable equipment.

a) *Conical divider.* The conical divider (Boerner type) consists of a hopper, cone, and series of baffles directing the seed into two spouts. The baffles form alternate channels and spaces of equal width. They are arranged in a circle and are directed inward and downward, the channels leading to one spout and the spaces to an opposite spout. A valve or gate at the base of the hopper retains the seed. When the valve is opened the seed falls by gravity over the cone where it is evenly distributed to the channels and spaces, then passes through the spouts into the seed pans.

The following dimensions are suitable: About 38 channels, each about 25 mm wide for large seeds and about 44 channels, each about 8 mm wide for small free-flowing seeds.

b) *Soil divider* (synonym: riffle divider). The soil divider consists of a hopper with about 18 attached channels or ducts alternately leading to opposite sides.

A channel width of about 13 mm is to be suitable. In using the divider the seed is placed evenly into a pouring pan and then poured in the hopper at approximately equal rates along the entire length. The seed passes through the channels and is collected in two receiving pans.

c) *Centrifugal divider.* I n the centrifugal divider (Gamet type) the seed flows downward through a hopper onto a shallow cup or spinner. Upon rotation of the spinner by an electric motor the seeds are thrown out by centrifugal force and fall downward. The circle or area where the seeds fall is equally divided into two parts by a stationary baffle so that approximately half the seeds fall in one spout and half in the other spout.

The centrifugal divider tends to give variable results unless the spinner is operated after having poured the seed centrally into the hopper.

d) *Rotary divider.* The rotary divider comprises a rotating crown unit with 6 to 10 attached sub-sample containers, a vibration chute and a hopper. In using the divider the seed is poured into the hopper and the rotary divider is switched on so that the crown unit with the containers rotates with approx. 100 rpm and the vibration chute starts to feed the seed into the inlet cylinder of the rotating crown. The feeding rate and therefore the duration of the dividing operation can be adjusted by the distance between the funnel of the hopper and the chute and the vibration intensity of the chute. There are two principles: (i) The inlet cylinder feeds the seed centrally onto a distributor within the rotating crown distributing the seed to all containers simultaneously; and (ii) The inlet cylinder feeds the seed de-centrally into the inlets of the containers rotating underneath the inlet cylinder so that the seed stream is subdivided into a lot of sub-samples.

e) *Variable sample divider.* The variable sample divider consists of a pouring hopper and a tube underneath that rotates with about 40 revolutions per minute. The tube distributes the seed stream from the pouring hopper onto the inner surface of a further hopper, which is well fitted into a third hopper all being concentric. In the second and the third hopper there are slots that comprise 50 per cent of the perimeter of the hoppers. Fifty per cent of the seed will pass through the two hoppers into a collecting pan. The other 50 per cent will stay within the hoppers and will then go into a second collecting pan. The two hoppers can be twisted against each other resulting in more narrow slots. The effect is that a smaller percentage will pass through the slots. Either the smaller sample outside the hoppers or the bigger sample inside the hoppers can be used as the required sample. The position of the two hoppers in relation to each other can be adjusted accurately, resulting in pre-determined sub-sample sizes.

2.5.2.2.2 Modified halving method

The apparatus comprises a tray into which fits a grid of equal-sized cubical cells, open at the top and every alternate one having no bottom. After preliminary mixing, the seed is poured evenly over the grid. When the grid is lifted, approximately half the sample remains on the tray. The submitted sample is successively halved in this way until a working sample, of approximately but not less than the required size, is obtained.

2.5.2.2.3 Spoon method

The spoon method is recommended for sample reduction for seed health testing (7.4.1). For other tests it is restricted to species with seeds smaller than *Triticum* spp. A tray, a spatula and a spoon with a straight edge are required. After preliminary mixing, pour the seed evenly over the tray; do not shake the tray thereafter. With the spoon in one hand, the spatula in the other, and using both, remove small portions of seed from not less than five random places. Sufficient portions of seed are taken to constitute a sub-sample of the required size.

2.5.2.2.4 The hand halving method

This method is restricted to the chaffy seeds and to some tree and shrub seeds. For all other species it can be used only to obtain working samples in the laboratory for seed health tests (7.4.1).

For applying the hand halving method, pour the sample evenly onto a smooth clean surface, thoroughly mix the seed into a mound with a flat-edged spatula, divide the mound into half and halve each half again - giving four portions - and halve each portion again - giving eight portions - arrange the portions in two rows of four, combine and retain alternate portions: e.g. combine the first and third portions in the first row with the second and fourth in the second row, remove the remaining four portions. Repeat the procedure using the retained portions until obtaining the required sample size.

2.5.3 Storage of samples after testing

The primary aim of storage of samples after testing is to be able to repeat the original tests carried out on the submitted sample. Therefore, storage conditions should be such that changes in the seed quality traits tested are minimal. For example, in the case of the purity test or other seed count, the sample should be stored in such a way that the physical identity is kept. In the case of germination, viability or health test of orthodox seeds the sample should be stored under cool and dry conditions. For such tests in recalcitrant and intermediate seeds of tropical and sub-tropical species, long term storage is not possible. For such seed of temperate species storability depends on the fungal status and to some extent whether the seed is dormant or not. All factors pertaining to storage need to be determined on a species basis. Protection against insects and rodents may be necessary.

When a re-test in a different testing laboratory is required, a portion shall be drawn from the stored sample in accordance with 2.5.2.2, and submitted to the designated testing laboratory. The remainder shall be retained in store.

2.5.4 Conditions for issuing ISTA International Seed Lot Certificates

The sampling methods laid down in the *ISTA Rules* shall be followed when seed samples are drawn for the issue of *ISTA International Seed Lot Certificates.* Further conditions have to be fulfilled as listed below.

2.5.4.1 Seed lot size

The seed lot shall not exceed the quantity indicated in column 2 of Table 2A, subject to a tolerance of 5 per cent with the exception of:

(i) herbage and amenity seed being transported loose in bulk containers. The conditions under which this exception may be permitted are laid down in Appendix B; and;

(ii) seed pellets, seed granules, seed tapes or seed mats. The maximum number of seeds that a seed lot of seed pellets, seed granules, seed tapes or seed mats may contain is 1,000,000,000 (10,000 units of 100,000) except that the weight of the seed lot, including the coating material may not exceed 40000 kg subject to a tolerance of 5 per cent (42,000 kg). When seed lot size is expressed in units the total weight of the seed lot must be given on the *ISTA International Seed Lot Certificate.*

Maximum lot size for treated and encrusted seeds is defined by applying the quantities indicated in Table 2A to the seeds without coating material.

A seed lot in excess of the prescribed quantity shall be subdivided into seed lots not larger than the prescribed quantity, each of which shall be labelled or marked with a separate seed lot identification.

2.5.4.2 Marking/labelling and sealing of containers

The seed lot shall be in marked/labelled containers which are self-sealing, sealed (or capable of being sealed) or under the control of the seed sampler.

Where the seed lot is already marked/labelled and sealed before sampling, the seed sampler must verify marking/labelling and sealing on every container. Otherwise the sampler has to mark/label the containers and must seal every container before the seed lot leaves his/ her control.

The samplers are personally responsible for the seals, labels and bags supplied to them and it is their duty to ensure that primary, composite or submitted samples shall never be left in the hands of persons not authorised by the seed testing laboratory unless they are sealed in such a way that they cannot be tampered with.

2.5.4.3 Sampling from the seed lot

For sampling from the seed lot methods listed under 2.5.1.4.1 must be used. Automatic seed samplers must be approved by the ISTA seed testing laboratory.

An *ISTA IntemationalSeedLot Certificate* issued on a seed lot (see 2.2.1) is still valid after re-packaging the seed lot in new containers provided that:

a) The identity of the seed in the initial seed lot is preserved.

b) The seed lot designation (see 2.2.10) is not changed.

c) The moving of the seed into the new containers is done under the control of an ISTA seed sampler.

d) There is no processing of the seed during filling of the new containers.

2.5.4.4 Submitted sample

~ Minimum size of submitted samples are as follows:

a) For moisture determination, 100 g for species that have to be ground (see Table 9A) and 50 g for all other species. When moisture meters are to be used for testing, a larger sample size may be necessary. Contact the ISTA seed testing laboratory for specific instructions.

b) For verification of species and variety, as prescribed in Chapter 8.

c) For all other tests, at least the weight prescribed in column 3 of Table 2A. As long as a determination of other seeds by number is not requested, the submitted sample shall weigh at least the amount indicated for the working sample for purity analysis in column 4 of Table 2A. In the case of coated seeds, the submitted samples shall contain not less than the number of pellets or seeds indicated in column 2 of Table 2B, Part 1 and Part 2.

If the submitted sample is smaller than prescribed, the sampler shall be notified accordingly and analysis withheld until sufficient seed is received in a single submitted sample; except that in the case of very expensive seed, the analysis may be completed to the extent possible and the following statement inserted on the certificate: "The sample submitted weighed only …… g [or in the case of pelleted seeds contained only ….. pellets (seeds)] and is not in accordance with the International Rules for Seed Testing."

The submitted sample must be sealed and labelled or marked.

2.5.4.5 Sample reduction

For sample reduction, methods listed under 2.5.2.2 must be used.

2.5.4.6 Storage of submitted samples after testing

To provide for re-testing by the original or by another seed testing laboratory, submitted samples on which *ISTA International Seed Analysis Certificates* have been issued shall be stored for one year from the date of issue of the certificate. Only in the case of very expensive seed, the remainder of the submitted sample, except 25 seeds for assurance of identity, may be sent back to the applicant. The seed testing laboratory cannot be held responsible for any deterioration of the sample during storage.

2.6 Calculation and expression of results

No specific calculation or expression of results required except under 2.9 for heterogeneity tests.

2.7 Reporting of results

No specific calculation or expression of results required except under 2.9 for heterogeneity tests.

2.8 Tables for lot size and sample sizes

Table 2A

This table is referred to in various chapters of the *ISTA Rules* and indicates weights of lots and samples for different species, and the specific names to be used in reporting test results.

Each sample size is derived from a nominal 1,000-seed weight for each species which, on the available evidence, is expected to be adequate for the majority of samples tested.

Where a weight is not given in the table and a count of other species is requested, the submitted sample must contain a minimum of 25,000 seeds.

Names with an asterisk are not included in the list of scientific plant names stabilised by ISTA. Names without an asterisk are included in the list of scientific plant names stabilised by ISTA (but not the synonym which follows some of these names) or in the case of generic names (e.g. *Pyrus* spp.) conserved by the International Botanical

Congress and listed in the International Code of Nomenclature. Changes in the stabilised list agreed at the 2001 ISTA Congress and corrections made at the 2002 ISTA Extraordinary Meeting are included in this version of Table 2A. Where plant names have been changed, the old name is included with a cross reference to the new name. This applies only for 2001 Congress changes, and previous cross references have been removed.

Table 2A Part 1 agricultural and vegetable seeds

Species	Maximum weight of lot	Minimum sample weights		
		Sample submitted	Working sample for purity analysis	Working sample for count of other species
	Chapter 2 (kg)	Chapter 2 (g)	Chapter 3 (g)	Chapter 4 (g)
Triticosecale	30,000	1,000	120	1,000
Triticum aestivum L.	30,000	1,000	120	1,000
Triticum durum	30,000	1,000	120	1,000

APPENDIX B. LOCATIONS FROM WHICH TO COLLECT SAMPLES FROM HEADERS AND OTHER PLANT AND EQUIPMENT

Header

Header	Concaves	Screens
Bunk-out frame	Elevator shoes	Sickle
Bunk-out seed	Tank auger (seed)	Feeder
Gear box of straw spreader	Brackets in tank	Feeder paddle
		Stripper header

Augers, Staw carts, and Silos

Collect samples from where dust and particles tend to accumulate. This includes the bottom of the straw carts, silos etc.

APPENDIX C. PHOTOS FOR VISUAL EXAMINATION OF SEED

Figure C.1 Grains of wheat showing the different symptoms seen with different levels of infection of Karnal bunt. Photographs are the courtesy of Department of Agriculture, Western Australia.

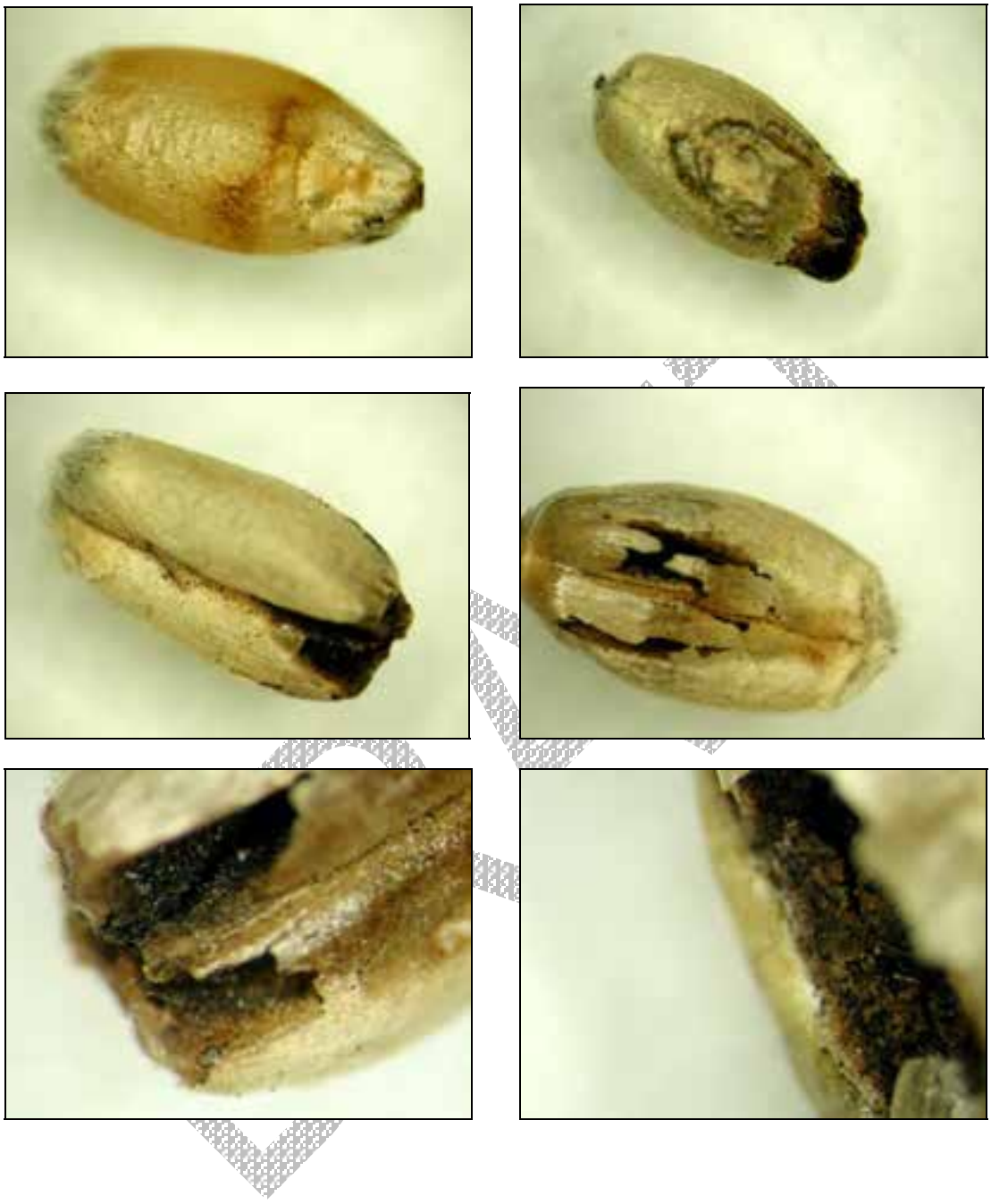


Figure C.2 Range of Karnal bunt symptoms seen on individuals grains of wheat. The embryo end of the seed is infected and will continue along the crease. The grain coat has a holey appearance when infection is severe. The teliospores will be evident where the grain coat has broken. Photographs are the courtesy of the Department of Agriculture, Western Australia.

APPENDIX D. KARNAL BUNT: DETECTION SCENARIOS

Detection scenarios

(a) Detection in crop
   1. Green crop
   2. Mature crop

(b) Detection in grain
   3. On farm at harvest
   4. At BHA receival point at harvest
   5. At grain processing plant, mill or feedlot

(c) Detection in running samples by BHA
   6. Grain currently at receival point
   7. Grain currently in transit
   8. Grain currently at port

(d) Detection in grain samples
   9. Sub-terminal sample
   10. Port sample

(e) Detection in grain on ship at destination (#11)

(f) Detection in plant breeding program (#12)

(g) Detection in imported material (#13)

1. Detection in field in green crop

First response	Response - quarantine	Survey	Trace-back/forward
• Desiccate crop • Bale crop and store on farm (decision on burial, etc. later)	• Assume whole farm is affected • Isolate farm and neighbours • Halt movement of materials from farms	• Initial farm and surrounding farms • Prepare for collection of harvest samples • Prepare for analysis of initial harvest samples	• Seed source (recent years) • Movement of plant materials • Machinery used in previous and current seasons

2. Detection in field in mature crop

First response	Response - quarantine	Survey	Trace-back/forward
• Bale crop and store on farm (decision on burial, etc, later) • Harvest can proceed, but store harvested grain on farm	• Assume whole farm is affected • Isolate farm and neighbours • Halt movement of grain from these farms • Halt movement of trucks, machinery, etc. from farms	• Initial farm and surrounding farms • Collect and analyse initial harvest samples	• Seed source (recent years) • Movement of plant materials • Machinery used in previous and current seasons

3. Detection in grain on farm at harvest

First response	Response - quarantine	Survey	Trace-back/forward
• Harvest can proceed, but store harvested grain on farm • Halt delivery of grain off farm	• Assume whole farm is affected • Isolate farm & neighbours • Halt movement of grain from these farms • Halt movement of trucks, machinery, etc, from farms Isolate silos to which deliveries have already been made from affected farm	• Initial farm & surrounding farms • Samples from neighbouring harvesters • Collect and analyse silo samples	• Seed source (recent years) • Movement of plant materials • Machinery used in previous and current seasons

4. Detection in grain at bulk handling authority receival point at harvest

First response	Response - quarantine	Survey	Trace-back/forward
• Halt delivery of grain to silo • Halt movement of grain from silo • Local harvest can proceed, but store harvested grain on farm	• Assume all farms delivering to silo are affected • Isolate silo • Isolate farms delivering to silo • Halt movement of grain from farms • Halt movement of trucks, machinery, etc, from farms	• Collect and analyse silo samples • Samples from local farms delivering to silo • Samples from harvesters • Check nearby silos and other local outlets	• Seed source (recent years) • Movement of plant materials • All farms delivering to silo • Machinery used in previous and current seasons • Trucks used for delivery to silo • Location of machinery and trucks used in local harvest • Shipments from silo

5. Detection in grain at processing plant, mill or feedlot

First response	Response - quarantine	Survey	Trace-back/forward
• Halt delivery of grain to plant • Halt operations at plant • Halt movement of grain products from plant • Local harvest can proceed, but store harvested grain on farm	• Assume all farms delivering to plant are affected • Isolate plant • Isolate farms delivering to plant • Halt movement of grain from farms • Halt movement of trucks, machinery, etc, from farms	• Collect and analyse samples from plant • Samples from local farms delivering to plant • Samples from harvesters • Check nearby silos and other local outlets	• Seed source (recent years) • Movement of plant materials • All farms delivering to plant • Machinery used in previous and current seasons • Trucks used for delivery to silo • Location of machinery and trucks used in local harvest • Shipments from plant

6. *Detection in grain: running sample by handling authority - grain at receival point*

First response	Response - quarantine	Survey	Trace-back/forward
• Halt delivery of grain to silo • Halt movement of grain from silo • Local harvest can proceed, but store harvested grain on farm	• Assume all farms delivering to silo are affected • Isolate silo • Isolate farms delivering to silo • Halt movement of grain from farms • Halt movement of trucks, machinery, etc, from farms	• Collect and analyse silo samples • Samples from local farms delivering to silo • Samples from harvesters • Check nearby silos and other local outlets	• Seed source (recent years) • Movement of plant materials • All farms delivering to silo • Machinery used in previous and current seasons • Trucks used for delivery to silo • Location of machinery and trucks used in local harvest • Shipments from silo

7. *Detection in grain: running sample by handling authority - grain in transit*

First response	Response - quarantine	Survey	
• Halt delivery of grain to silo • Halt movement of grain from silo • Local harvest can proceed, but store harvested grain on farm	• Assume all farms delivering to silo are affected, as well as down-stream pathways • Isolate silo • Isolate farms delivering to silo • Halt movement of grain from farms • Halt movement of trucks, machinery, etc, from farms • Halt movement of rail trucks	• Collect and analyse silo samples • Samples from local farms delivering to silo • Samples from harvesters • Check nearby silos and other local outlets	• Seed source (recent years) • Movement of plant materials • All farms delivering to silo • Machinery used in previous and current seasons • Trucks used for delivery to silo • Location of machinery and trucks used in local harvest • Shipments from silo

8. *Detection in Grain: running sample by handling authority - grain at port*

First response	Response - quarantine	Survey	Trace-back/forward
• Halt delivery of grain to silos in port zone • Halt movement of grain from all silos in port zone • Local harvest can proceed, but store harvested grain on farm	• Assume all wheat regions delivering to port are affected • Isolate silos in port zone • Isolate farms delivering to silos in port zone • Halt movement of grain from farms in port zone • Halt movement of grain from port • Halt movement of trucks, machinery, etc, from farms in port zone	• Collect and analyse silo samples • Samples from local farms delivering to silo • Samples from harvesters • Check nearby silos and other local outlets • Check port storages	• Seed source (recent years) • Movement of plant materials • All farms delivering to silo • Machinery used in previous and current seasons • Trucks used for delivery to silo • Location of machinery and trucks used in local harvest • Shipments from silo • Rail trucks

9. *Detection in grain: sub-terminal sample*

First response	Response - quarantine	Survey	Trace-back/forward
• Halt delivery of grain to silos in zone feeding sub-terminal • Halt movement of grain from all silos in zone feeding sub-terminal • Local harvest can proceed, but store harvested grain on farm	• Assume all wheat regions delivering to zone feeding sub-terminal are affected • Isolate silos in zone feeding sub-terminal • Isolate farms delivering to silos in zone feeding sub-terminal • Halt movement of grain from those farms • Halt movement of grain from sub-terminal • Halt movement of machinery, etc, from farms in zone feeding sub-terminal	• Collect and analyse sub-terminal samples • Collect and analyse silo samples from silos feeding sub-terminal • Samples from local farms delivering to silos feeding sub-terminal • Samples from harvesters on those farms • Check nearby silos and other local outlets • Check port storages downstream	• Seed source (recent years) • Movement of plant materials • All farms delivering to silos • Machinery used in previous and current seasons • Trucks used for delivery to silos • Location of machinery and trucks used in local harvest • Shipments from sub-terminal • Rail trucks

10. Detection in grain: port sample

First response	Response - quarantine	Survey	Trace-back/forward
• Halt delivery of grain to port • Halt delivery of grain to silos in port zone • Halt movement of grain from all silos in port zone • Local harvest can proceed, but store harvested grain on farm	• Assume all wheat regions delivering to port are affected • Isolate port • Isolate silos in port zone • Halt movement of grain from farms in port zone • Halt movement of grain from port • Halt movement of machinery, etc, from farms in port zone • Halt movement of grain to silos in port zone	• Collect and analyse all silo samples • Check port storages • Analyse all silo running samples until silo(s) identified • Samples from local farms delivering to silo(s) • Samples from harvesters • Check nearby silos	• Seed source (recent years) • Movement of plant materials • All farms delivering to silo • Machinery used in previous and current seasons • Trucks used for delivery to silo • Location of machinery and trucks used in local harvest • Shipments from silo • Rail trucks

11. Detection in Grain: Grain on ship at destination

First response	Response - quarantine	Survey	Trace-back/forward
• Halt movement from source port(s) • Halt all ships from port "on the water" • Halt delivery of grain to port(s) • Halt movement of grain from all silos in port zone • Harvest can proceed, but store harvested grain on farm	• Assume port(s) of origin affected • Isolate port(s) • Isolate silos in port zone • Halt movement of grain from farms in port zone • Halt movement of grain from port • Halt movement of machinery, etc, from farms in port zone • Halt movement of grain to silos in port zone	• Collect and analyse all silo samples • Check port storages • Analyse all silo running samples until silo(s) identified • Samples from local farms delivering to silo • Samples from harvesters • Check nearby silos	• Seed source (recent years) • Movement of plant materials • All farms delivering to silo • Machinery used in previous and current seasons • Trucks used for delivery to silo • Location of machinery and trucks used in local harvest • Shipments from silo • Rail trucks

12. Detection in plant breeding program

First response	Response - quarantine	Survey	Trace-back/forward
• Halt breeding activities • Halt distribution of material from breeding program	• Assume whole program affected • Isolate program and affiliates	• All seed sources • Affiliated programs	• Seed source (recent years) • Movement of plant materials • Machinery used in program • Bags, etc, used for grain • Recent visitors, travel destinations

13. Detection in Imported Material

First response	Response - quarantine	Survey	Trace-back/forward
• Reject shipment	• Halt shipments from similar source	• Check similar recent shipments	• Origin of material • Trace source of infection

APPENDIX E. FIELD GUIDE FOR THE IDENTIFICATION OF CEREAL HOST PLANTS

1. Wheat (*Triticum aestivum*)

Stems: to 1.2 m tall, erect, simple, multiple from base, forming tufts, typically glabrous, glaucous, annual. 5-7 nodes with 3 to 4 foliage leaves

Leaves: Leaf blades to +/- 30 cm long, +/- 9 mm broad, glabrous or somewhat strigose, entire, bluish-green. Auricles (when present) with a slightly wavy margin, scarious. Ligule a short membrane to +/- 1 mm tall. Leaf sheath open, glabrous or with the lower most pubescent.

Inflorescence: single terminal cylinder of spikelets to +10 cm long. Spikelets single at each node of the inflorescence, somewhat depressesd into the bent axis. Axis glabrous.

Flowers: spikelts light green with darker green lines, to 10 mm long, ovoid. Glumes either with a short tooth at the tip or sometimes with an awn up to 6cm long. Lemmas typically with an awn reaching 8 cm in length, sometimes without the awn and just with one or two short teeth. Terminal floral spike consisting of perfect flowers

2. Durum wheat (*Triticum durum* Desf.)

Stems are internally pithy and leaves are relatively broad. Spikes are intermediate in length and flattened. Awns are nearly always present and are long and coarse. They are white, yellow or black in colour

Figure E.1 Photo of Durum wheat in head. Note the conspicuous long awns or 'beard'. Photo courtesy of **http://www.hort.purdue.edu/newcrop/crops/wheat.html**

2. Triticale

The triticale plant is shorter than rye but taller than wheat. Triticale is distinguished from wheat as having an asymmetrical glume and a symmetrical lemma with distal converging veins (Williams, J T, 1995).

Figure E.2. Triticale head compared to wheat head. Photo courtesy of http://waltonfeed.com/self/triticale.html

APPENDIX E (a). GUIDE TO IDENTIFICATION OF CEREAL SEEDS

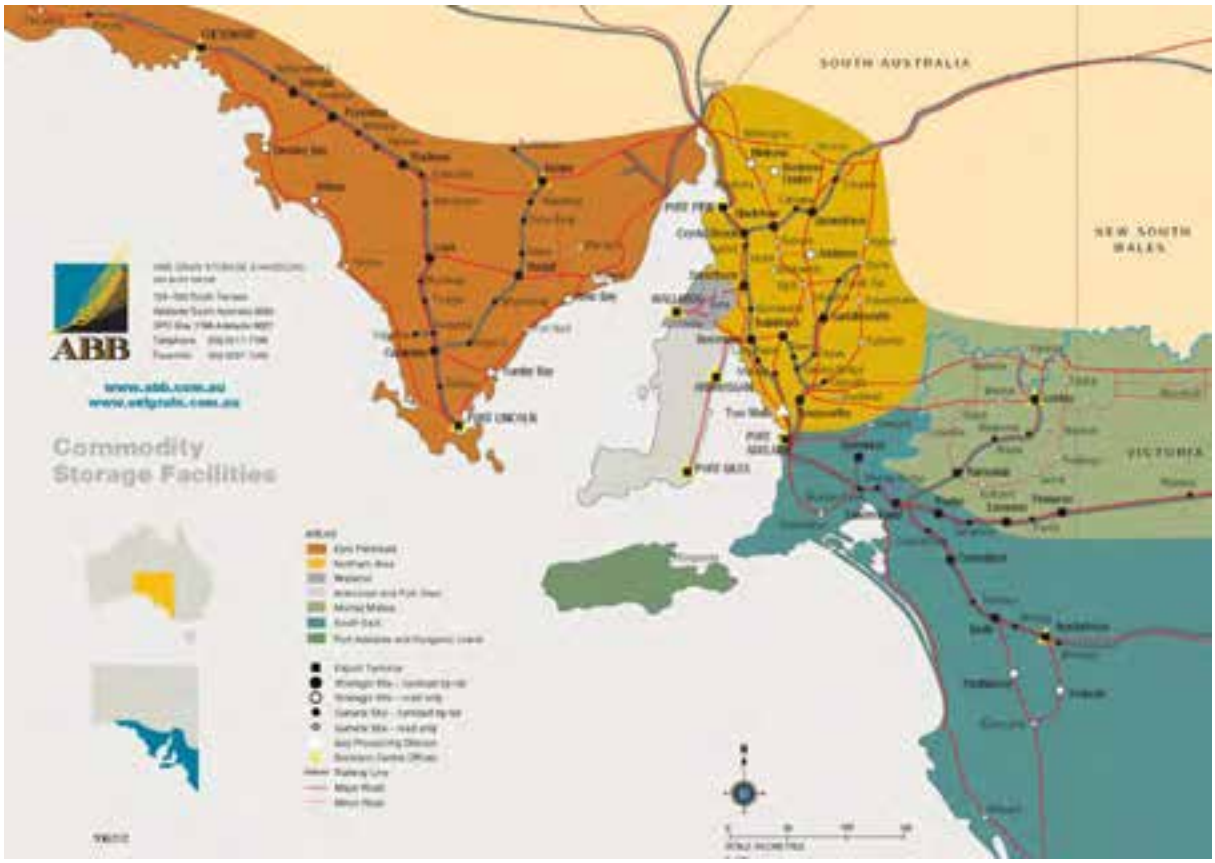
Table 1. Grain characteristics for wheat, durum and triticale

Characteristic	Wheat	Durum	Triticale
Size	5-9 mm	6-9 mm	7-8 mm
Shape	Oval, plump	Long, pointed	Irregular, rounded-angular
Colour	White or red	White or red	Yellow-buff, brown-yellowish red
Brush	Usually present on blunt apex	Usually absent	Varies, short to longer than wheat
Embryo	Steeply placed	Sharp ridge between embryo and rest of grain	
Other	Marked ventral groove	Very hard	Coat rough/uneven, heavily wrinkled on dorsal surface
Photo			

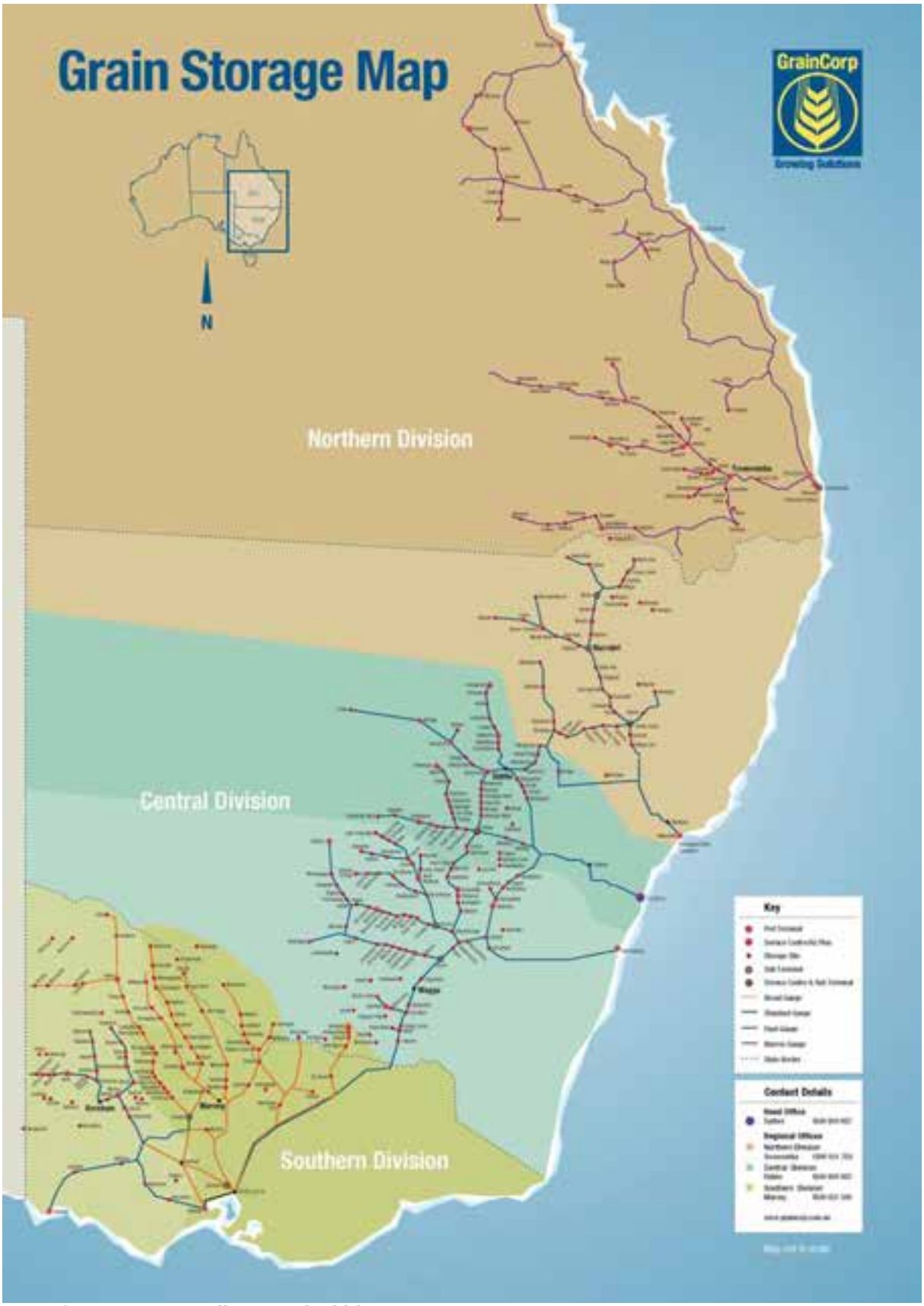
Photos courtesy of NIAB 2004.

APPENDIX F. MAPS OF RECEIVAL BINS IN AUSTRALIA

Map of receival bins in Western Australia.



Map of South Australia's receival bins.



Map of Eastern Australia's receival bins.

APPENDIX G. TRACE FORWARD AND TRACE BACK DECISIONS

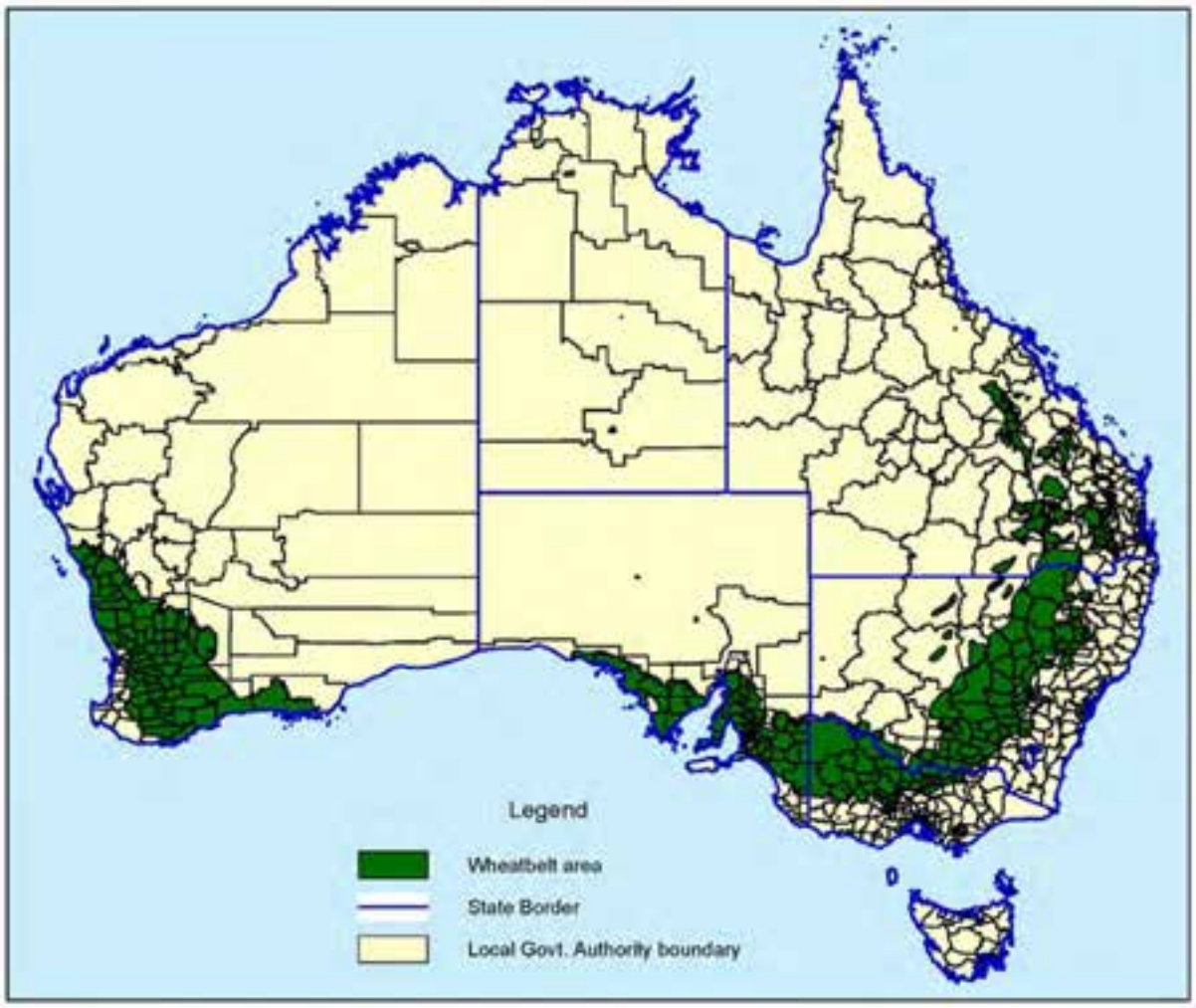
Tbale G.1: Trace forward

Detected	Pathway		Issue	Action
1. On farm	Has grain been delivered to silo?	No	Not pathway	
		Yes	Identify silos, receival bins	Check silos, receival bins
	Was seed or grain sold to other farms?	No	Not pathway	
		Yes	Identify location now	Check other farms
	Has straw from this crop been moved (off farm or within farm)?	No	Not pathway	
		Yes	Identify location now	Check destination
	Has contract harvester been used in this crop?	No	Not pathway	
		Yes	Identify location now	Sample harvester Careful check of next farm visited Check other farms visited
	Has other machinery used in this crop been moved to other farms?	No	Not pathway	
		Yes	Identify location now	Sample machinery Careful check of next farm visited Check other farms visited
	Have animals been grazed on this crop or stubble from this crop?	No	Not pathway	
		Yes	Identify location now	Check where animals moved to
2. At silo	Has grain been shipped from silo to sub-terminal, port or processor?	No	Not pathway	
		Yes	Identify location now	Check all locations Check trucks, rail trucks, loading equipment
3. At sub-terminal	Has grain been shipped from sub-terminal to port or processor?	No	Not pathway	
		Yes	Identify location now	Check all locations Check trucks, rail trucks, loading equipment
4. At port	Has grain been shipped from port to processor?	No	Not pathway	
		Yes	Identify location now	Check all locations Check trucks, rail trucks, loading equipment
	Has grain been loaded on board ship?	No	Not pathway	
		Yes	Identify location now	Check all ships Advise destinations, arrange sampling

Table G.2: Trace back

Detected	Pathway: past 5 years		Issue	Action
1. On farm	Seed obtained off farm?	No	Not pathway	
		Yes	Regular supplier?	Follow up
			Irregular supplier?	Explore
	Fertiliser obtained off farm?	No	Not pathway	
		Yes	Regular supplier?	Follow up
			Irregular supplier?	Explore
	Contract harvester used in this crop?	No	Not pathway	
		Yes	Location now?	Sample harvester, check previous farms
	Other possible pathways?	No	Not pathway	
		Yes		Follow up
	Paddock history for past 5 years: History of host crops?			Follow up possible pathways
2. In harvester	Which farms has harvester been used on during this season?			Follow up all farms from this season (most recent first) Check route travelled by this harvester for contamination
3. At silo	Is detection confined to one bin or stack?	Yes	Farms delivering to bin	Follow up all relevant farms: Halt harvest, check crops Follow up all relevant farms: Check load samples
		No	Farms delivering to silo	Follow up all relevant farms: Halt harvest, check crops Follow up all relevant farms: Check load samples
		Yes	Notify other silos	Follow up all relevant farms: Halt harvest, check crops Follow up all relevant farms: Check load samples
4. At sub-terminal	Which silos have shipped to this sub-terminal?		Silos delivering	Follow up all relevant silos (check running samples) Follow up all relevant farms: Halt harvest, check crops Follow up all relevant farms: Check load samples
5. At port	Which silos have shipped to this port?		Silos delivering	Follow up all relevant silos (check running samples) Follow up all relevant farms: Halt harvest, check crops Follow up all relevant farms: Check load samples
	What routes has grain used to get to port?		Supply chain	Check all links in supply chain for contamination

APPENDIX H. MAP OF WHEAT GROWING AREAS IN AUSTRALIA DEMONSTRATING NATURAL BORDERS



DRAFT NATIONAL CONTINGENCY PLAN FOR KARNAL BUNT OF WHEAT

PART IV

DIAGNOSTIC PROTOCOLS

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CONTENTS

1. INTRODUCTION

The purpose of this manual is that a nationally accepted standardised protocol is available for the accurate identification of *Tilletia indica,* the cause of Karnal bunt in wheat. *T. indica* is a quarantinable pathogen in many countries including Australia. However, it is endemic in many Asian and Middle Eastern countries and has been recently detected in a number of states of the United States. There is an increase in the likelihood of the disease occurring within the Australian wheat industry and it is important that protocols are available to identify the pathogen.

The pathogen *T. indica* is morphologically similar to the ryegrass bunt pathogen *T. walkeri* and to the rice bunt pathogen *T. horrida*. *T. walkeri* is present in Australia and in the United States. It is common for ryegrass to contaminate wheat seed at harvest or in storage and in some cases where rice is grown it is possible for rice to contaminate wheat seed at harvest or in storage. Due to the morphological similarity of these pathogens it is important that accurate identification of the different pathogens can be done to prevent either Karnal bunt entering the country or the accidental exclusion of a clean shipment of grain.

The laboratory manual is designed for easy access to the relevant sections required to identify the pathogens. The processes and protocols needed for the morphological identification of the possible pathogens and the molecular techniques used to confirm the identification are then described. All media recipes are provided in a separate section, as are the recording sheets to be used when handling a sample. Finally in the back section of the manual colour photos of the teliospore morphology are provided.

1.1 Procedure

Figure 4.1 shows the flow diagram for the order of the protocols that are to be followed for analysis of the suspect grain sample. It is most important that these protocols are adhered to otherwise the results obtained will not be valid. Direct examination of the grain for bunted kernels is not a reliable method on its own. Teliospores can be transported on harvesting equipment and contaminate another wheat load harvested using the same equipment.

The selective sieve wash test is the most efficient and rapid method for detecting teliospores in a grain sample. This method has an 82% efficiency of recovery (Inman, 2003) and only a few slide preparations are required to detect the presence of teliospores. The number of replicate samples required to detect teliospores has been tested and confirmed by both the USDA and CSL teams. Table 1 shows the number of replicate samples required to detect differing levels of teliospores.

Due to the morphological similarity between *T. indica, T. walkeri* and *T. horrida* it is important that the morphological identification of the spores is confirmed by the use of molecular techniques such as Conventional PCR or if available the use of the Direct real-time PCR or the Nested real-time PCR.

Table 4.1: The number of replicate 50 g sub-samples required to detect differing levels of teliospores. (Cited from Inman, 2003)

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

All of the procedures have been validated by the Central Sciences Laboratory, York, UK and by the US Department of Agriculture, Maryland (Inman 2003, USDA KB manual 2003).

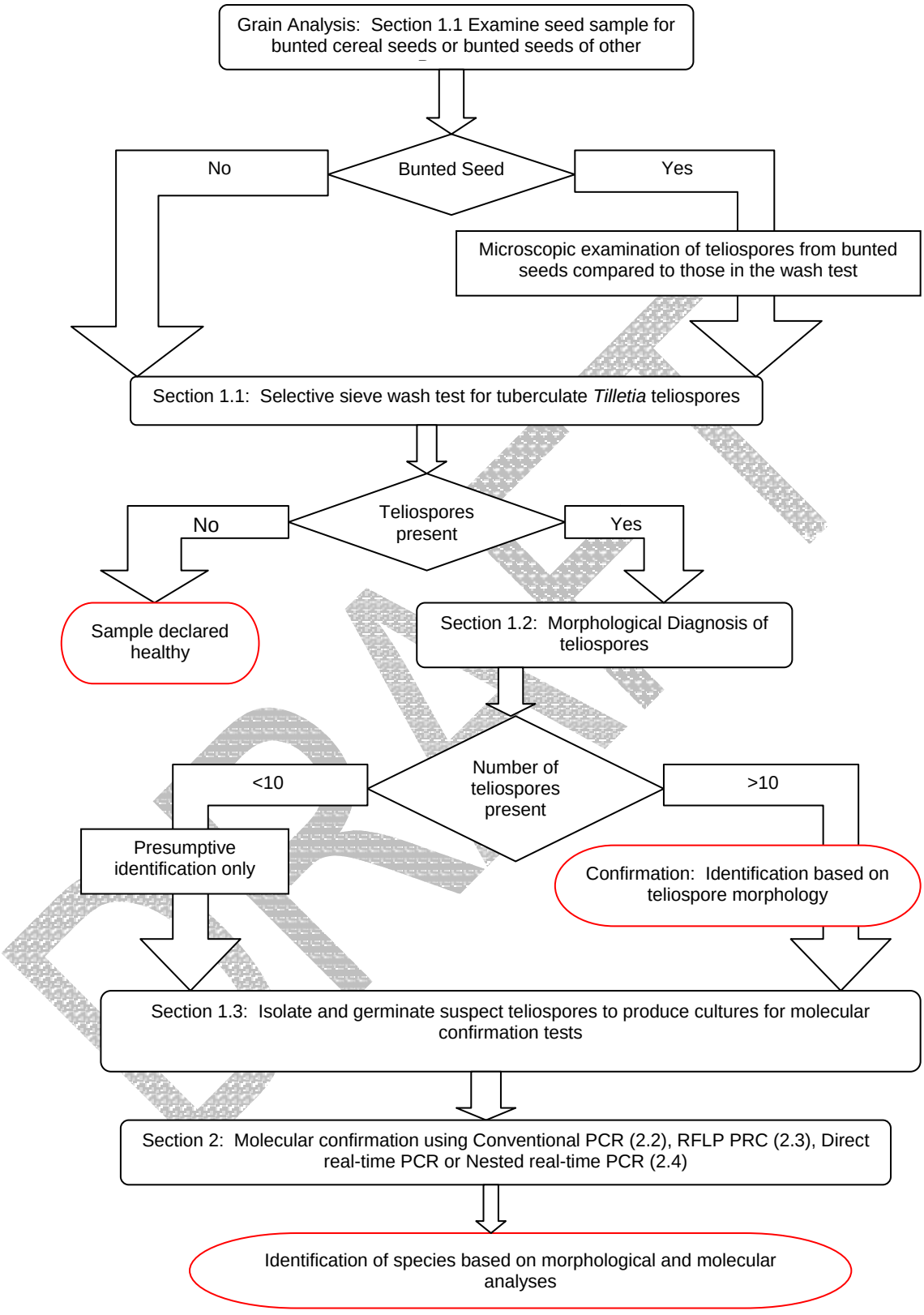


Figure 4.1 Flow diagram for the analysis of grain

1.2 Documentation

An electronic copy of the manual is kept by Plant Pathologist, Department of Agriculture, Western Australia and a copy is also kept by PHA on disc.

All hard copies and electronic copies are controlled documents. This means the methods cannot be changed without consultation with the two confirmatory laboratories and in consultation with CSL, UK.

1.3 Records

The Recording sheets contained in section 4 must be copied and filled in as appropriate for each sample received and kept together in a file marked 'Suspect KB grain samples'. All documents must also be copied and sent to confirmatory laboratories if the initial processing of the sample is conducted by the "State Laboratories".

Any data relating to the validation of a method must be kept for as long as the method is in use.

2. DETECTION OF TILLETIA INDICA ON WHEAT GRAIN

2.1 Significance

T. indica causes the disease Karnal bunt of wheat (*Triticum* spp.), triticale (*Triticum x secale*) and possible Rye (*Secale*). It is a quarantine plant pathogen in Australia and can have an economic impact on the marketability of wheat. If the disease were to occur within Australia major restrictions would be placed on the export of grain to other countries.

2.2 Specimen

2.2.1 Scope

Direct visual examination of the grain for bunted kernels or sori on kernels can be done. Generally this is not considered a reliable method for a quarantinable pathogen, as it would only detect high levels of bunt infection which may be caused by the endemic common bunt pathogen.

2.2.2 Transport sample to laboratory

Seed lots should be sampled according to the International Seed Testing Association (ISTA) rules (2006). Grain should be sampled to give a representative sample of a bulk consignment (1-2 kg minimum is required). (Refer to appendix A for further guidelines in regards to sampling requirements).

Suspect samples should be marked “Plant Sample for Urgent Diagnosis” and sent to the Plant Health Diagnostic Laboratory, NSW Department of Primary Industries, EMAI or AGWEST Plant Laboratories, Department of Agriculture, Western Australia (addresses below).

Samples need to be packaged to prevent movement of the grain or plants as this damages the pathogen and makes detection and confirmation difficult. Samples need to be packed into a plastic container (preferably) or in a plastic bag tightly (if there are not many “funny” grains pack normal grain in as well). If necessary, use packing material within the box, to ensure tight packing of the samples. Double bag the samples and wipe the outside of the bag with alcohol before dispatching the sample to the laboratory. If the grain is packed into a plastic container, wipe the outside of the container with alcohol, and place into a plastic bag. The outside of the bag is also wiped down with alcohol. If necessary, use packing material within the box, to ensure tight packing of the samples.

Additional information including the detail of the sample date, location and site must be recorded on an accompanying sheet, together with all relevant paperwork. This information should be placed in a plastic bag, on which is also written the summary details of the sample and the address, and included with the samples that are dispatched.

All samples should be dispatched using an overnight courier service or express post.

Important: Prior to dispatch the Manager of the laboratory to which the sample is being consigned should be advised by telephone (not email) of the expected arrival date. Special arrangements may need to be made for weekends. If the receiving laboratory is in another state, then a permit for the movement of seed into that state maybe required. Check with the State or Local Pest and Disease Control Headquarters that approval has been granted and that the sample can be received.

Addresses of Diagnostic Laboratories:

Manager, Pest and Disease Diagnostics
NSW Department of Primary Industries
Elizabeth Macarthur Agricultural Institute
PMB 8 3 Baron-Hay Court
CAMDEN NSW 2570

Telephone: 02 4640 6333
Facsimile: 02 4640 6415

Broadacre Plant Pathologist
AgWest Plant Laboratories
Department of Agriculture WA

SOUTH PERTH WA 6151

Telephone: 08 9368 3875
Dacsimile: 08 9474 2658

2.2.3 Storage at laboratory

The sample must be logged immediately upon arrival and processed as soon as practical. All samples are to be held until the emergency response has been completed and closed, or stored for at least 1 year after testing, or which is ever the longest. This is to allow further trace backs or retesting if required.

If Karnal bunt or *T. indica are* not detected in the sample, the remainder of the sample needs to be stored in the cold room in a separate box marked 'Quarantine, “not detected” Karnal bunt samples' until the client has been notified of the results. The client cannot be notified of the results until clearance has been given by the Quarantine Plant Pathologist. (Hire a cold room if need be for the emergency, do not store seed with camphor as this may kill spores and prevent identification).

Seed that has been tested and found to be positive, requires to be stored in the cold room in a separate box marked 'Quarantine, “positive Karnal bunt samples', until the client has been notified of the results. The client cannot be notified of the results until clearance has been given by the Quarantine Plant Pathologist. (Hire a cold room if need be for the emergency, do not store seed with camphor). NB: the seed needs to be kept until the emergency response has been completed.

NB: That all paperwork associated with the sample needs to be copied and sent with the sample. This should include the initial receiving laboratory’s unique sample identification number, the pathologist’s report, and all other information related to the sample (for example where it came from etc). It is really important that if further investigation is required that this can happen.

2.3 Quality control

All media is quality controlled at the point of manufacture. Refer to media instructions in Section 9.

2.4 Principle

The fungal pathogen is diagnosed by either morphological or PCR methods. There are two initial methods for detection of the teliospores: a) scraping the seed and sori present; and b) by selective sieve wash technique that washes the teliospores off the seed. This section is based on the morphological identification of the teliospores.

2.5 Reagent

Unless otherwise stated all water used is sterile de-ionised water. Refer to Work Instructions 1-9, in Section 9.

(1) Water (Sterilised Tap water).

(2) Bleach (1.28%).

(3) Tween 20 Solution (Sigma Chemical Co. St Louis).

(4) Shears Mounting Solution.

2.6 Equipment

Refer to operating manuals for general usage.

(1) Platform shaker.

(2) Compound microscope with 10, 20 and 40X objectives.

(3) Dissecting microscope up to 50X magnification.

(4) Sieves 53 µm and 15 µm. (A minimum of one each, however if labelled appropriately (e.g. 'A') can run as many samples as the centrifuge will hold.)

(5) Erlenmeyer Flasks (250 ml, 500 ml). Label as required.

(6) Centrifuge.

(7) Centrifuge tubes (full set). Label. Preferably use Corning® Polypropylene tubes 15 ml with a conical bottom (not round).

(8) Test tube rack that will hold the centrifuge tubes.

(9) Small funnels. Label. Need to fit into centrifuge tubes.

(10) Wash bottles.

(11) Pipettes, micro and Pasteur (long length, disposable).

(12) Microscope slides and cover slips (alternatively a Sedgewick rafter cell can be used with a thin cover slip).

(13) Large waste disposal container containing bleach. Needs to be autoclavable.

(14) Large baby bath to hold flasks and beakers for washing up and soaking in bleach.

(15) 5 ml sterile screw capped tubes with labels. Label corresponding run number and sample letter.

(16) Foam holder for tubes.

2.7 Procedure

Both step A and B are to be done regardless of the result found in the direct examination of the grain.

A: Direct examination of the grain

Day 1

(1) Examine the submitted grain sample for bunted wheat seeds or other Poaceae seeds (for example, ryegrass). Assess the sample for symptoms of possible Karnal bunt disease. Record the presence of other seeds, lesions, such as shape, size, colour and specific patterning. Use photographs in the Appendix A to compare and record possible symptoms.

(2) Examine some grain under the dissecting microscope and take a scraping of any suspicious blackening and place on a slide with water. Note if there are any sori present on the grain. If there are any fungal spores evident on the slide, confirm identification by referring to CMI descriptions and those in the manual. Refer to 'Results section' for possible fungal pathogens. Seal and label the slide to be sent to 'Expert' to confirm diagnosis. The grain sample is also to be included in the sample sent to the 'Expert' for confirmation.

(3) If there appears to be no sori, Tilletia spores or mycelia present on the sample proceed to the next step 'B'.

B: Sieve Wash Technique

(4) All equipment must be clean before use. Bleach sieves, funnels and flasks by immersion for 15 minutes in 1 per cent bleach.

(5) Rinse the equipment thoroughly with tap water to remove the bleach.

(6) Weigh 50 g of wheat into 250 ml Erlenmeyer flasks.

(7) Record the sample details on the recording sheet against its corresponding label and run number.

(8) Repeat this step until the required number of samples being run concurrently are weighed (label 'A-H'), i.e. if the centrifuge will hold 8 tubes then 8 samples can be run concurrently. If the centrifuge will only hold 4 or 6 tubes, then only 4 or 6 samples can be run concurrently.

(9) Add 100 ml of Tween 20 solution to the grain in the flask and seal the flask with gladwrap or Parafilm.

(10) Place the flasks on the shaker and set at 200 rpm for 3 minutes. This is to release the teliospores from the grain. (If the sample is on the shaker for longer than 3 minutes, there will be in an increase in the amount of starch grains present in the sample.)

(11) Set up the funnel and sieves on top of the corresponding 500 ml Erlenmeyer flask. The 53 µm sieve is placed on top of the 15 µm sieve, and then these are placed in the funnel (refer to Figure 2.1).

(12) Remove the flasks from the shaker and immediately pour off the solution and grain into the corresponding sieve. NB: It is important that the Tween 20 solution is not on the grain for more than 30 minutes.

(13) Rinse the flask with approximately 100 ml of water and pour this into the corresponding sieve.

(14) Repeat step '13' twice.

(15) Rinse the grain in the top of the sieve again using the wash bottle (approximately 100 ml). A total volume of 400 ml should be in the flask.

(16) Allow the sieves to fully drain. You may find that the sieves block up, and this can be fixed by slowly lifting an edge of the top sieve from the bottom sieve. This breaks the air lock. Alternatively, holding the beaker and the sieves, slowly lower the bottom of the beaker onto a vortex mixer (touch mode) and press down. This will cause the surface of the sieve to vibrate and the water to pass quickly through.

(17) Remove the top sieve and place the grain in a paper autoclave bag (labelled of course) and dry in an oven at 40°C for 24 hours. The remaining grain from the sample is also to be kept in case there is a need to examine the grain directly for disease symptoms.

(18) Place the 53 µm sieves and the Erlenmyer flasks in to the washing up container that contains bleach.

(19) Set up the centrifuge tubes with small funnels into a test tube rack.

(20) Tilt the 15 µm sieve to an angle of 30-45° and then using a wash bottle gently wash the deposit on the membrane, to one side of the sieve. Keep the sieve over the funnel and Erlenmyer flask to collect the water as it goes through the sieve (Figure 4.2).

(21) Wash the deposit into the centrifuge tube using as little water as possible (Figure 4.3).

(22) Alternatively: Recover the suspension that collects at the edge of the sieve using a clean disposable Pasteur pipette and place into the centrifuge tube.

(23) Repeat step '20 and 21' until the sieve appears to be clean. There should be approximately 3-5 ml suspension in the centrifuge tube. Check the sieve under the dissecting microscope to see if there are any teliospores remaining on the sieve.

(24) The solution left in the beaker is to be disposed into the waste disposal container and the flasks are to be placed into the washing up container.

(25) Centrifuge[1] the tubes at 1000 x *g* for 3 minutes. Make sure that the level of solution in each tube is the same (i.e. that they are balanced). If debris is seen to adhere to the inside walls of the centrifuge tubes, re-suspend in Tween 20 solution and repeat the centrifugation.

(26) Collect the tubes from the centrifuge and place in the test tube rack.

(27) Carefully remove the supernatant using a disposable Pasteur pipette. Take care not to disturb the pellet. Discard the removed supernatant into a disposable waste vessel for autoclaving.

(28) Place the pellet into labelled small screw capped tubes. Re-suspend the pellet using 50-100 µL of distilled water. NB: If warm laboratory conditions cause water preparations on slides to dry out quickly then Shear's solution can be used. However, the Shear's solution will start to kill the teliospores within a few minutes and will not

[1] Equation for calculating Relative Centrifugal Force (RCF (x *g*)) from RPM: RCF = 1.12 $r_{max}(RPM/1000)^2$, where r_{max} is the radius (mm) from the centre of rotation to the bottom of the centrifuge tube.

germinate after 1 hour exposure thus slides should be immediately examined (within 10 minutes) and any spores from the slides should be recovered from the slides (Section 3.) and washed in water to allow for germination and molecular confirmation. Store the samples in the fridge.

(29) Using a micropipette and tip, place a 20 µl drop onto a microscope slide and cover with a cover slip (22 x 22 mm). Examine the slide immediately (slide can quickly dry out) under the microscope at 100-400 X magnification. Label slide with sample number and run number.

(30) Assess the characteristics of any teliospores found (Section 1.2) and record the results on the recording data sheets.

(31) Repeat steps 28, 29, 30 and 31 until all of the suspension has been examined.

(32) If suspect teliospores are found, refer to and follow the morphological diagnostic protocol (Section 1) and the general diagnostic scheme. It is also important to examine the seed for bunted kernels, and germinate the teliospores for molecular identification.

(33) Bleach all equipment used for 15 minutes and rinse with water before re-using.

Figure 4.2 The set up for the selective sieve wash test. Two sieves (50 µm and 15 µm) are placed within funnel on top of the 500 ml Erlenmyer flask.

Figure 4.3 Washing the deposit on the membrane to one side of the 15 µm sieve.

Figure 4.4 Washing the deposit from the 15 µm sieve into the centrifuge tube.

2.8 Results

Refer to next section on Morphological identification of teliospores.

Calculations:

Equation for calculating Relative Centrifugal Force (x g) from Revolutions Per Minute:

$$RCF = 1.12_{r\max}\left(RPM/1000\right)^2$$

Where rmax is the radius (mm) from the centre of the rotation to the bottom of the centrifuge tube.

Units:

All units used are SI units.

2.9 Validation

All techniques are standard, refer to references.

2.10. Reference ranges

N/A

2.11 Reporting

Only a negative result can be reported at this stage, where no teliospores were detected in the direct examination of the grain and in the selective sieve wash method.

For negative results – 'The sample submitted was tested for possible fungal pathogens. No fungal pathogens were detected in the sample submitted'. If other fungal spores such as rusts or flag smut were detected these must be reported.

2.12 Notes

N/A

2.13 Glossary of terms

2.14 References

1. Inman, A.J., Hughes, K.J.D., Bowyer, R.J. (2003). EU Recommended protocol for the diagnosis of a quarantine organism '*Tilletia indica*'. Central Sciences Laboratory, York, UK.
2. ISTA, 2003. International rules for seed testing Edition 2003. International seed testing Association, Bassersdorf, CH-Switzerland.
3. USDA (2002/3). Karnal Bunt Manual. United States Department of Agriculture, Frederick, Maryland.

3. MORPHOLOGICAL IDENTIFICATION OF TELIOSPORES

3.1 Significance

T. indica causes the disease Karnal bunt of wheat (*Triticum* spp.), triticale (*Triticum* x *secale*) and possible Rye (*Secale*). It is a quarantine plant pathogen in Australia and can have an economic impact on the marketability of wheat. If the disease were to occur within Australia major restrictions would be placed on the export of grain to other countries.

3.2 Specimen

3.2.1 Scope

Morphological identification of the teliospores recovered from the previous section on the grain analysis can be done. However, it is not conclusive as there are other Tilletia species that are morphologically similar to *T. indica*. The more teliospores that are present in the sample after the selective sieve wash test, the more accurate the morphological identification becomes.

3.2.2 Transport sample to laboratory

Seed lots should be sampled according to the International Seed Testing Association (ISTA) rules (2003). Grain should be sampled to give a representative sample of a bulk consignment (1-2 kg minimum is required).

Suspect samples should be marked 'Suspect exotic plant disease' and sent to the nearest Department of Agriculture Diagnostic Laboratory within the State or can be submitted to Plant Health Diagnostic Laboratory, NSW Department of Agriculture, Wagga Wagga or AGWEST Plant Laboratories, Department of Agriculture, Western Australia.

3.2.3 Storage at laboratory

The sample must be processed on the day of arrival, or within 24 hours if the sample arrives in the late afternoon. If, after examination of the sample, the sample is found to be positive, the remainder of the sample, slides from the sample and teliospores plated onto water agar, the tube containing the extraction are to be sent to the 'Experts' at either, The NSW Department of Agriculture or The Department of Agriculture, Western Australia for molecular confirmation. If the sample is found to be negative, the remainder of the sample needs to be stored in the cold room in a separate box marked 'Quarantine' until the client has been notified of the results. The client cannot be notified of the results until clearance has been given by the Quarantine Plant Pathologist.

NB: That all paperwork associated with the sample needs to be copied and sent with the sample. This should include the initial receiving laboratory's unique sample identification number, the pathologist's report, and all other information related to the sample (for example where it came from, etc.). It is really important that if further investigation is required that this can happen.

3.3. Quality control

All media is quality controlled at the point of manufacture. Refer to the Work Instructions Section 9.

3.4 Principle

The fungal pathogen is diagnosed by morphological methods and then confirmed using the PCR methods in the preceding sections. The morphological identification is based on the teliospores size, type of ornamentation and the colour of the spores. All of these characteristics are important as a group and cannot be used alone to distinguish between *T. indica, T. walkeri* or *T. horrida*.

3.5 Reagent

Unless otherwise stated all water used is sterile de-ionised water. Refer to Work Instructions NDPW 1-9, Section 9.

(1) Suspension from the Sieve wash test (Section 2.1).

(2) Shears Mounting Solution.

(3) Distilled water.

3.6 Equipment

Refer to operating manuals for general usage.

(1) Compound microscope with 10, 20 and 40X objectives.

(2) Dissecting microscope up to 50X magnification.

(3) Pipettes, micro and Pasteur (long length, disposable).

(4) Microscope slides and cover slips (alternatively a Sedgewick rafter cell can be used with a thin cover slip).

(5) Large waste disposal container containing bleach. Needs to be autoclavable.

(6) Two ml or 5 ml sterile screw capped tubes with labels. Label corresponding run number and sample letter.

(7) Foam holder for tubes.

3.7 Procedure

(1) Using a micropipette and tip, place a 20 µl drop onto a microscrope slide and cover with a cover slip (22 x 22 mm). Examine the slide immediately (slide can quickly dry out) under the microscope at 100–400X magnification. Label slide with sample number and run number.

(2) Assess the characteristics of any teliospores found and record the results on the recording data sheets (Section 5, Worksheet 2).

(3) If tuberculate teliospores are found in the wash test, record the morphological characteristics of the teliospores using Worksheet 3, Appendix C.

NB: Tuberculate teliospores detected in the wash tests of wheat grain are assumed to be *Tilletia indica*, *T. walkeri* or *T. horrida*. Other tuberculate spored Tilletia species that infect grasses cannot be excluded as contaminants, but have not been previously found to be contaminating wheat. Due to the size of the mesh used in the wash test other pathogens maybe detected, these are listed in the Results section of the protocol along with their CMI reference number. The presence of these pathogens should be recorded on the recording sheet.

(4) After recording the teliospores detected and a presumptive diagnosis has been made, either seal slide and/or plate out teliospores for germination (Section 2.3).

(5) Re-examine the wash grain sample to see if any bunted seed had been missed.

(6) If bunted ryegrass seeds are found, but no bunted wheat seeds, confirm *T. walkeri* by microscopic examination of the teliospores. If confirmed, compare the teliospores from the seed with those found in the wash test. If the teliospores are identical make a diagnosis. Send the slides and sample to the 'expert' as molecular confirmation of the pathogen is still required.

(7) If bunted wheat seed is found and no bunted ryegrass, confirm *T. indica* by microscopic examination of the teliospores. If confirmed, compare the teliospores from the seed with those found in the wash test. If the teliospores are identical make a diagnosis. Send the slides and sample to the 'expert' as molecular confirmation of the pathogen is still required.

(8) If wheat seeds infected with *T. indica* or ryegrass seeds with *T. walkeri* are not found, make a presumptive identification of teliospores found in the wash test: Use Worksheet 3, (Appendix C) in conjunction with the following reference ranges (adapted from NAPPO, 1999., Inman *et al.* 2003):

   a. Samples with teliospores all < 36 µm, with curved spines, are most likely to be *T. horrida.*

   b. Samples with teliospores > 36 µm are most likely to be *T. indica.*

   c. Samples with teliospores mostly (28-35 µm), translucent brown, never black/opaque, very spherical, with blunt spines with distinct gaps between are most likely to be *T. walkeri*. These gaps are more obvious in profile after bleaching. Further, this assumption can be made if the grain is from areas where ryegrass is grown, and if there are ryegrass seeds present in the sample.

   d. Samples with mature, dark teliospores less than 25 µm are most likely to be *T. horrida* not *T. indica* or *T walkeri*.

   e. Samples with some black, opaque teliospores are most likely *T. indica.* This is because *T. walkeri* teliospores are never opaque, black; *T, horrida* teliospores can be dark, but are semi-opaque.

(9) If relatively large numbers of teliospores are present (> 10), it may be possible to identify the teliospores morphologically if all morphological criteria (size range, mean size, colour, ornamentation) clearly confirm to any one species (see Table xx). However, molecular confirmation is still recommended if bunted wheat seeds are not found in the sample.

(10) If only a few teliospores are detected (< 10) or morphological characteristics are not conclusive, then molecular methods are recommended for confirmation of any presumptive diagnosis (Appendix C).

3.8 Results

The following table lists the possible pathogens that may be detected in the sieve wash test. If these pathogens are detected they should be recorded on the results sheet (Section 5). The morphology of the teliospores of the *Tilletia* species must be recorded on the results sheets in Section 5 (page 3). However, the literature has suggested that there are other tuberculate-spored *Tilletia* species that are morphologically similar to those of *T. indica* (Pimentel *et al,* 1998) but these are not known contaminants of wheat (Inman *et al*, 2003).

Table 4.2: List of pathogens that may be detected in the selective sieve wash test

Pathogen	Common Name	CMI reference, Photos in Appendix, and slide collection
Puccinia graminis f.sp. *tritici*	Stem rust	
Puccinia recondita	Leaf rust	
Puccinia striiformis	Stripe rust	**291**
Tilletia contraversa	Dwarf bunt	**746**
Tilletia indica	Karnal bunt	**748**
Tilletia horrida	Rice bunt	**75**
Tilletia laevis	Common bunt	**720**
Tilletia tritici	Common bunt	**719**
Tilletia walkeri	Ryegrass bunt	
Ustilago agropyri	Flag smut	

Calculations:

Units:

All units are SI units.

3.9 Validation

All techniques are standard, refer to references.

3.10 Reference ranges

Refer to Table xx in Section 5 and the following guidelines from NAPPO (1999) and Inman *et al.* (2003).

a. Samples with teliospores all < 36 µm, with curved spines, are most likely to be *T. horrida.*

b. Samples with teliospores > 36 µm are most likely to be *T. indica.*

c. Samples with teliospores mostly (28-35 µm), translucent brown, never black/opaque, very spherical, with blunt spines with distinct gaps between are most likely to be *T. walkeri*. These gaps are more obvious in profile after bleaching. Further, this assumption can be made if the grain is from areas where ryegrass is grown, and if there are ryegrass seeds present in the sample.

d. Samples with mature, dark teliospores less than 25 µm are most likely to be *T. horrida* not *T. indica* or *T walkeri*.

e. Samples with some black, opaque teliospores are most likely *T. indica.* This is because *T. walkeri* teliospores are never opaque, black; *T, horrida* teliospores can be dark, but are semi-opaque.

3.11 Reporting

Results are to be reported to the Quarantine Plant Pathologist only:

a) For positive results (tentative diagnosis) – 'The fungal pathogen (state the full name of the pathogen) was detected in the sample submitted'. This is currently being confirmed by the use of molecular methods. The final results will be available in 15 working days.

b) For negative results – 'The sample submitted was tested for possible fungal diseases. No fungal pathogens were detected in the sample submitted'. However, if other fungal spores such as rusts or flag smut were detected these must be reported.

3.12 Notes

N/A

3.13 Glossary of terms

Cerebriform: with folds, bends and undulations which give a brain-like appearance.

Coralloid: Having the appearance of coral, because of the manner of branching.

Echinulate: with spines or bristles but are smaller and less rigid than those that are described as echinate.

Polygonal: having many angles

Tuberculate: having small rounded bumps or projections.

3.14 References

1. Inman, A.J., Hughes, K.J.D., Bowyer, R.J. (2003). EU Recommended protocol for the diagnosis of a quarantine organism '*Tilletia indica*'. Central Sciences Laboratory, York, UK.

2. NAPPO (1999). NAPPO Standards for Phytosanitary Measures: A harmonised procedure for morphologically distinguishing teliospores of Karnal bunt, ryegrass bunt and rice bunt. www.nappo.org

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

4. USDA (2002/3). Karnal Bunt Manual. United States Department of Agriculture, Frederick, Maryland.

4. GERMINATION OF TELIOSPORES DETECTED AND MYCELIAL MATTE PRODUCTION

4.1 Significance

T. indica causes the disease Karnal bunt of wheat (*Triticum* spp.), triticale (*Triticum x secale*) and possible Rye (*Secale*). It is a quarantine plant pathogen in Australia and can have an economic impact on the marketability of wheat. If the disease were to occur within Australia major restrictions would be placed on the export of grain to other countries.

4.2 Specimen

4.2.1 Scope

The teliospores from *Tilletia* species can be identified morphologically. However, this method is only accurate when a large number of teliospores are present due to the crossover in size and ornamentation and colour between *T. indica*, *T. walkeri* and *T. horrida*. Thus, the teliospores detected in the sieve wash test need to be germinated for molecular testing to be conducted on them to confirm their identification.

4.2.2 Transport sample to laboratory

Suspect samples should be marked 'Suspect exotic plant disease' and sent to the nearest Department of Agriculture Diagnostic Laboratory within the State or can be submitted to Plant Health Diagnostic Laboratory, NSW Department of Agriculture, Wagga Wagga or AGWEST Plant Laboratories, Department of Agriculture, Western Australia.

4.2.3 Storage at laboratory

The initial sample needs to be labelled 'Quaratine pathogen' and stored in the cold room. All plates containing teliospores need to be marked in the same manner and kept in an incubator at 19°C that is labelled 'Quarantine Pathogens'.

4.3 Quality control

All media is quality controlled at the point of manufacture. Refer to the Work Instructions Section 9.

4.4 Principle

To identify the fungal pathogen by PCR methods, the teliospores detected need to be germinated and then a mycelial matte is produced for DNA extraction to occur.

4.5 Reagent

Unless otherwise stated all water used is sterile de-ionised water. Refer to Work Instructions NDPW 1-9, Section 9.

(1) Teliospores detected from examination of grain or selective sieve wash test.

(2) Two per cent water agar plates (Difco Laboratories) (plus 100 mg of ampicillin (or penicillin-G (Na salt) and streptomycin sulfate/L).

(3) Potato dextrose broth (Difco Laboratories).

(4) Sodium hypochlorite at 0.5 per cent.

4.6 Equipment

Refer to operating manuals for general usage.

(1) Compound microscope with 10, 20 and 40X objectives

(2) Dissecting microscope up to 50X magnification

(3) Sieves 53 µm and 15 µm

(4) Centrifuge

(5) Centrifuge tubes (full set). Preferably use Corning® Polypropylene tubes 15 ml with a conical bottom (not round).

(6) Test tube rack that will hold the centrifuge tubes.

(7) Small funnels. Need to fit into centrifuge tubes.

(8) Wash bottles.

(9) Pipettes, micro and Pasteur (long length, disposable).

(10) Petri dishes (90 x 15 mm, sterile).

4.7 Procedure

Day1

(1) Recover the suspect teliospores from both the microscope slide and cover slip by washing them with distilled water over a clean 15 µm sieve. Recover the spores from the sieve (refer to Protocol 2.1, Steps '20 to 23') into the centrifuge tubes. Make up the final volume to 3-5 ml with water.

(2) Incubate the teliospore suspension overnight at 21°C to hydrate the teliospores and make the fungal and bacterial contaminants more susceptible to subsequent surface sterilisation.

Day 2

(1) Centrifuge the sample for 3 minutes (1200 x *g*). Tip off the supernatant or use a disposable Pasteur pipette, taking care not to disturb the pellet. Pipette the supernatant into a suitable waste bottle for autoclaving and quarantine disposal.

(2) Then re-suspend the pellet in 10 ml of 0.5 per cent bleach and immediately centrifuge for 1 minute (1200 x *g*). Quickly and aseptically remove the supernatant using a disposable Pasteur pipette.

(3) Re-suspend the pellets in 1 ml of sterile distilled water and centrifuge for 5 minutes at (1200 x *g*) to wash the debris. Aseptically remove the supernatant.

(4) Repeat step '5'.

(5) Re-suspend the pellet in 1 ml of sterile distilled water.

(6) Transfer 200 µl of the suspension onto the water agar plates, and spread with a sterile spreader. Plates should be quite dry (this can be done by placing in a lamina flow for approx 15 minutes (without their lids on) before using).

(7) Incubate plates (unsealed) at 21°C (12 hr light) for 5 days.

Day 7

(1) Seal the plates with parafilm or place them in clear plastic bags and leave for another five days at 21°C (12 hr light).

(2) The plates can then be sent to the 'expert' laboratories at this stage for molecular confirmation of the Tilletia species.

Day 12

(1) Examine the plates for germinated teliospores bearing a tuft of filiform basidiospores (primary sporidia, Appendix B, Figure 8.).

(2) Take a 1 cm^2 plug from each plate. Make sure it contains a germinating teliospore. Transfer this to a sterile lid of a Petri dish containing sterile potato dextrose broth (5-10 ml). One plug per plate.

(3) Incubate the plates at 21°C for 2 to 3 days.

Day 14 or 15

(1) Examine the plates for a floating mycelial matte (0.5 cm–1.0 cm diameter).

(2) Remove the mycelial matte with a sterile needle and then touch to a piece of filter paper to remove excess media.

(3) Transfer this matte to a 1.5–2.0 ml microcentrifuge tubes.

(4) At this stage the sample can either be used for PCR testing or can be frozen (-80°C) for future use at a later date.

4.8 Results

Tilletia indica and *T. walkeri* produce similar cultures and that of *T. horrida* is quite different. On potato dextrose agar (PDA) after 14 days at 19°C (12 hour light cycle) white to cream-coloured slow growing colonies are produced. These appear to be irregular in shape and slightly crusty. The colonies tend to be 4-6 mm in diameter (Appendix B, Figure 7) (*Inman et al* 2003). In comparison, *T. horrida* produces an even more slow growing culture (2-3 mm, diameter) at this temperature because it prefers a higher temperature. The cultures also have a reddish-purple pigment both on PDA and potato dextrose broth (Appendix B, Figure 7) (*Inman et al*, 2003).

Calculations:

Equation for calculating Relative Centrifugal Force (x g) from Revolutions Per Minute:

$$RCF = 1.12_{r\max}\left(RPM/1000\right)^2$$

Where rmax is the radius (mm) from the centre of the rotation to the bottom of the centrifuge tube.

Units:

All units used are SI units.

4.9. Validation

All techniques are standard, refer to references.

4.10 Reference ranges

N/A

4.11 Reporting

No results to be reported at this stage.

4.12 Notes

N/A

4.13 Glossary of terms

4.14 References

1. Inman, A.J., Hughes, K.J.D., Bowyer, R.J. (2003). EU Recommended protocol for the diagnosis of a quarantine organism '*Tilletia indica*'. Central Sciences Laboratory, York, UK.

2. USDA (2002/3). Karnal Bunt Manual. United States Department of Agriculture, Frederick, Maryland.

5. EXTRACTION OF DNA FROM TILLETIA SPECIES TELIOSPORES

5.1 Significance

T. indica causes the disease Karnal bunt of wheat (*Triticum* spp.), triticale (*Triticum x secale*) and possible Rye (*Secale*). It is a quarantine plant pathogen in Australia and can have an economic impact on the marketability of wheat. If the disease were to occur within Australia major restrictions would be placed on the export of grain to other countries.

5.2 Specimen

5.2.1 Scope

Once a possible detection of the pathogen *T. indica* has occurred this needs to be confirmed by the use of PCR. However, before this can be done, DNA extraction needs to occur on the germinated teliospores. Refer to Section 4.3 on 'Germination of teliospores and Production of mycelial mat'.

5.2.2 Transport sample to laboratory

Suspect samples should be marked 'Suspect exotic plant disease' and sent to Plant Health Diagnostic Laboratory, NSW Department of Agriculture, Wagga Wagga or AGWEST Plant Laboratories, Department of Agriculture, Western Australia. The suspect sample needs to include the original grain sample, slides of the teliospores, and plates containing the germinated teliospores.

5.2.3 Storage at laboratory

Plates containing the teliospores need to be stored at 19°C with alternating light (12 hr) until they are producing sporidia from the basidium. These should be in a marked Quarantine incubator and enclosed in a plastic bag marked Quarantine.

NB: It is very important that all paper work concerning the sample is kept and proper records are kept so if further investigation is required this can be done.

5.3 Quality control

All media is quality controlled at the point of manufacture. Refer to the Media Preparation Section 9

NB: Chemical resistant gloves should be worn, with laboratory coat and safety glasses. Designated pipettes and tips should be used for each step in the PCR process and for either the conventional PCR or PCR-RFLP at least two replicates should be set up per dilution used.

5.4 Principle

The identification of the fungal pathogen is confirmed by PCR methods. Firstly DNA needs to be extracted from germinated teliospores before the PCR methods can be used. Previous methods that tried to extract DNA from ungerminated teliospores have not been successful.

5.5 Reagent

Unless otherwise stated all water used is sterile de-ionised water. Refer to Work Instructions in Section 9.

(1) Potato broth containing mycelial growth of *Tilletia* isolates to be tested.

(2) Two per cent tap water agar plates with colonies of isolates.

(3) Sterile distilled water.

5.6 Equipment

Refer to operating manuals for general usage.

(1) Beadbeater.

(2) 100 µl pipettes and sterile tips.

(3) Scalpel handles and blades.

(4) Screw cap Treff tubes containing O-ring (2 ml).

(5) Microcentrifuge tubes (0.2 ml).

(6) Glass beads (0.5 mm).

(7) Petri dishes (35 mm).

(8) Parafilm.

(9) QIAquick spin column.

(10) Microcentrifuge.

(11) Qiagen® DNAeasy Kit.

5.7 Procedure

A: DNA extraction

(1) Pick off between five and seven colonies from the potato broth cultures or the water agar plates and wash these in two 10 µl drops of distilled water to remove any of the growth medium. This can be done by aseptically placing the colonies into a sterile Petri dish (little ones) containing the sterile distilled water, and then transferring to another Petri dish.

(2) Then place the colonies in a sterile 0.2 ml microcentrifuge tube, containing sterile 0.5 mm beads (⅓ full) and 50 µl of distilled water.

(3) Seal the tube with parafilm and wedge into a 2 ml screwcap centrifuge tube.

(4) Place the tube in the Beadbeater and turn on at a reduced power level (¼) for 5 minutes.

(5) Remove the tube from the Beadbeater and allow the sample to stand for 30 seconds.

(6) Dilute the 10 µl aliquot from the homogenised sample with 90 µl of sterile distilled water.

(7) The sample can then be stored at -20°C until required.

B: Alternative DNA extraction method using QIAGEN DNA extraction kits

(1) Follow the instructions supplied within the extraction kits.

5.8 Results

A homologous sample containing DNA should be available after the extraction.

Calculations:

Equation for calculating Relative Centrifugal Force (x g) from Revolutions Per Minute:

$$RCF = 1.12_{r\max}\left(RPM/1000\right)^2$$

Where rmax is the radius (mm) from the centre of the rotation to the bottom of the centrifuge tube.

Units:

All units are SI units.

5.9 Validation

All techniques are standard, and have been validated by the authors in the references.

5.10 Reference ranges

N/A

5.11 Reporting

No results are reported at this stage of the process. This is to occur after the PCR process has been completed.

5.12 Notes

5.13 Glossary of terms

5.14 References

1. Inman, A.J., Hughes, K.J.D., Bowyer, R.J. (2003). EU Recommended protocol for the diagnosis of a quarantine organism '*Tilletia indica*'. Central Sciences Laboratory, York, UK.

6. CONVENTIONAL PCR FOR THE IDENTIFICATION OF TILLETIA SPECIES

6.1 Significance

T. indica causes the disease Karnal bunt of wheat (*Triticum* spp.), triticale (*Triticum x secale*) and possible Rye (*Secale*). It is a quarantine plant pathogen in Australia and can have an economic impact on the marketability of wheat. If the disease were to occur within Australia major restrictions would be placed on the export of grain to other countries.

6.2 Specimen

6.2.1 Scope

Once a possible detection of the pathogen *T. indica* has occurred this needs to be confirmed by the use of PCR. This can be done using a number of alternative methods including; traditional PCR using species specific primers, confirmation by PCR using Taqman or Corbett Real time PCR machine, and by confirmation using restriction enzyme analysis. The PCR process is performed on germinated teliospores. Refer to Section 4.3 on “Germination of teliospores, and Production of mycelial matte”, and Section 5.1 “DNA extraction process”.

6.2.2 Transport sample to laboratory

Suspect samples should be marked 'Suspect exotic plant disease' and sent to Plant Health Diagnostic Laboratory, NSW Department of Agriculture, Wagga Wagga or AGWEST Plant Laboratories, Department of Agriculture, Western Australia. The suspect sample needs to include the original grain sample, slides of the teliospores, and plates containing the germinated teliospores.

6.2.3 Storage at laboratory

The extracted DNA should be kept frozen and marked Quarantine Sample and stored in a separate container that is labelled appropriately and away from other DNA samples.

NB: It is very important that all paper work concerning the sample is kept and proper records are kept so if further investigation is required this can be done.

6.3 Quality control

All media is quality controlled at the point of manufacture. Refer to the Work Instructions Section 9.

A positive and negative control needs to be included in each PCR run. The positive control is extracted DNA imported from the USA that is stored under Quarantine conditions. The negative control is the use of extracted DNA from *Tilletia tritici*. It is also important that primers for both *T. indica* and *T. walkeri* are used on the same sample at the same time.

NB: Chemical resistant gloves should be worn, with laboratory coat and safety glasses. Designated pipettes and tips should be used for each step in the PCR process and for either the conventional PCR or PCR-RFLP at least two replicates should be set up per dilution used.

6.4 Principle

The identification of the fungal pathogen is confirmed by PCR methods (Frederick *et al*. 2000). A few sets of primers were published for the identification and differentiation of *T. indica* and *T. walkeri*. A pair of *T. indica*-specific primer (Tin3/Tin10) and a pair of *T. walkeri*-specific primer (Tin11/Tin10) were used in the conventional PCR method.

6.5 Reagent

Unless otherwise stated all water used is sterile de-ionised water. Refer to Work Instructions PPW xx-xx.

(1) Ethyl alcohol (95%) or Industrial Methylated Spirit.

(2) Sterile Distilled Water.

(3) 10x PCR buffer (Invitrogen).

(4) Taq polymerase (Invitrogen).

(5) $MgCl_2$.

(6) Primer 1[a].

(7) Primer 2[b].

(8) 10mM dNTP mix.

(9) Extracted DNA.

(10) Agarose gel.

(11) TBE buffer (NDPW 7, Section 4).

(12) Loading Buffer (NDPW 8, Section 4).

(13) Molecular weight marker.

(14) Ethidium bromide stain.

(15) Tap water.

NB: For amplification of *T. indica* use:
[a] Tin3 (5'CAA TGT TGG CGT GGC GGC GC 3')
[b] Tin10 (5'AGC TCC GCC TCA AGT TCC TC 3')

For amplification of *T. walkeri* use:
[a] Tin11 (5'TAA TGT TGG CGT GGC GGC AT 3')
[b] Tin10 (5'AGC TCC GCC TCA AGT TCC TC 3')

NB: Ethidium bromide is a carcinogen and appropriate gloves, safety glasses and laboratory coat should be worn at all times.

6.6 Equipment

Refer to operating manuals for general usage.

(1) Thermocycler and loading rack.

(2) Microcentrifuge.

(3) 100 µl pipettes and sterile tips.

(4) Screw cap Treff tubes containing O-ring (2 ml).

(5) Waste solvent bottle.

(6) Microcentrifuge tubes (0.2 ml).

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

(8) Electrophoresis tank, electrodes and power pack.

(9) Marked container for Ethidium bromide.

(10) UV transiluminator.

(11) Camera.

6.7 Procedure

A: Amplification

(1) In the laminar flow cabinet, aseptically prepare enough of the reaction mix described (Table 4.3) below for the number of test samples plus 10 per cent.

Table 4.3. PCR reaction mixture for one sample for amplification of conventional PCR protocol

Reagent	Volume for one sample
10x PCR buffer (Invitrogen)	2.5
$MgCl_2$ (50 mM)	0.75
10mM dNTP mix (10 mM)	0.5
Taq polymerase (Invitrogen) (5 U/µl)	0.1
Primer 1[a] (10 µM)	0.5
Primer 2[b] (10 µM)	0.5
Sterile distilled water	19.15
TOTAL	**24.0**

NB: For amplification of *T. indica* use: [a] Tin3 (5'CAA TGT TGG CGT GGC GGC GC 3')
[b] Tin10 (5'AGC TCC GCC TCA AGT TCC TC 3')

For amplification of *T. walkeri* use: [a] Tin11 (5'TAA TGT TGG CGT GGC GGC AT 3')
[b] Tin10 (5'AGC TCC GCC TCA AGT TCC TC 3')

(1) Into separate 200 µl reaction tubes aliquot 24 µl of the reaction mix.

(2) Moving away from the lamina flow and working on a laboratory bench, add 1 µl of the DNA test extracts to each of the reaction tubes.

(3) Place the reaction tubes into a thermal cycler and run program.

(4) Parameters for NSW Department of Ag EMAI thermal cycler: The temperature profile is 94°C denaturation for 1 min, 33 cycles of 94°C 15 sec, 68°C 30 sec and 72°C 30 sec, followed by an extension step of 72°C 6 min.

(5) Parameter for DAWA: Not determined as yet.

(6) Store the cycled products at 4°C or lower.

B: Resolution of amplified PCR products

(1) Dissolve 1.5 per cent agarose in working TBE buffer. Pour melted agarose into gel tray and then insert comb with appropriate number of wells. The wells should be large enough to hold 12 µl samples and molecular markers. Allow agarose to set (for a minimum of 30 minutes). Remove comb.

(2) Fill the electrophoresis tank with working TBE buffer, ensuring that the entire gel is covered by at least 3 mm of buffer.

(3) Aliquot 2 µl of loading buffer onto Parafilm for each sample to be loaded.

(4) Aliquot 10 µl of the cycled product onto Parafilm and mix with the 2 µl of loading buffer already on the parafilm.

(5) Separately on the Parafilm mix 2 µl of 100 bp molecular weight marker with 8 µl of sterile distilled water and 2 µl of loading buffer.

(6) Place gel into the tank and load the 12 µl aliquots from each sample on the Parafilm into their respective wells.

(7) Record on the template which well corresponds to which sample.

(8) Place lid on tank and run small gel at 80 volts or large gel at 160 volts approx for 1 hour or until the marker is 40 mm from the edge of the gel.

NB: Ethidium bromide is a carcinogen and appropriate gloves, safety glasses and laboratory coat should be worn at all times during the rest of the process.

(9) Place gel in aqueous ethidium bromide stain (0.05 µg/ml) and agitate on a shaker for 30 minutes.

(10) Lift gel from stain and destain in tap water for 5 minutes.

(11) Remove gel from water and place on UV transiluminator.

(12) View illuminated gel through transilluminator system and follow the instructions to print the result.

6.8 Results

a) The Tin3/Tin10 primer pair will produce a fragment of 212 bp for *T. indica* and a negative result for *T. walkeri*.

b) The Tin11/Tin10 primer pair will produce a fragment of 212bp for *T. walkeri* and a negative result for *T. indica.*

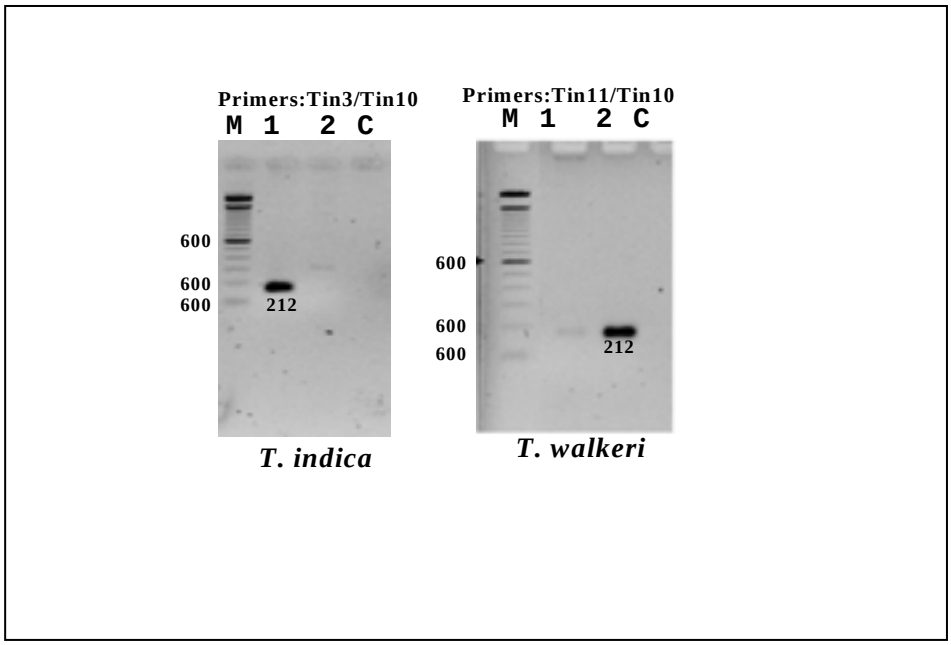


Figure 4.5 Result from Conventional PCR using Tin3/Tin10 primers and Tin11/Tin10 primers for the identification of *T. indica* and *T. walkeri*

Calculations

1. Rehydration of lyophilised primers:

For each primer, dilute lyopphilised primer pellet in 1 ml filtered and autoclaved molecular grad water to obtain the [pmol/µl] given on the tube label.

Calculate the required primer volume to give 1 µM per 50 µl reaction as follows:

Primer vol (µl) to give 1 µM = 50/[pmol/µl]

2. Equation for calculating Relative Centrifugal Force (x g) from Revolutions Per Minute:

$$RCF = 1.12_{r\max}\left(RPM/1000\right)^2$$

Where rmax is the radius (mm) from the centre of the rotation to the bottom of the centrifuge tube.

Units:

All units used are SI units.

6.9 Validation

All techniques are standard, refer to references.

6.10. Reference ranges

N/A.

6.11. Reporting

Results are to be reported as:

a) For positive results – 'the fungal pathogen (state the full name of the pathogen) was detected in the sample submitted. This causes the disease known as (state the disease common name). No other fungal pathogens were detected in the sample submitted'.

b) For negative results – 'the sample submitted was tested for possible fungal diseases. No fungal pathogens were detected in the sample submitted'.

6.12 Notes

N/A.

6.13 Glossary of terms

6.14 References

1. Inman, A.J., Hughes, K.J.D., Bowyer, R.J. (2003). EU Recommended protocol for the diagnosis of a quarantine organism '*Tilletia indica*'. Central Sciences Laboratory, York, UK.

2. Frederick, R.D., Snyder, K.E., Tooley, P.W., Berthier-Schaad, Y., Peterson G.L., Bonde, M.R., Schaad, N.W., and Knorr, D.A. (2000). Identification and differentiation of *Tilletia indica* and *T. walkeri* using the polymerase chain reaction. *Phytopathology* **90:** 951-960

3. Smith, O.P., Peterson, G.L., Beck, R.J., Schaad, N.W., and Bonde, M.R. (1996). Development of a PCR-based method for identification of *Tilletia indica.* *Phytopathology* **86:** 115-122.

4. USDA (2002/3). Karnal Bunt Manual. United States Department of Agriculture, Frederick, Maryland.

7. RFLP - PCR FOR THE IDENTIFICATION OF TILLETIA SPECIES

7.1 Significance

T. indica causes the disease Karnal bunt of wheat (*Triticum* spp.), triticale (*Triticum x secale*) and possible Rye (*Secale*). It is a quarantine plant pathogen in Australia and can have an economic impact on the marketability of wheat. If the disease were to occur within Australia major restrictions would be placed on the export of grain to other countries.

7.2 Specimen

7.2.1 Scope

Once a possible detection of the pathogen *T. indica* has occurred this needs to be confirmed by the use of PCR. This can be done using a number of alternative methods including; traditional PCR using species specific primers, confirmation by PCR using Taqman or Corbett Real time PCR machine, and by confirmation using restriction enzyme analysis. The PCR process is performed on germinated teliospores. Refer to Section 4 5 and 6 on Germination of teliospores, Production of mycelial mat, and DNA extraction process.

7.2.2 Transport sample to laboratory

Suspect samples should be marked 'Suspect exotic plant disease' and sent to Plant Health Diagnostic Laboratory, NSW Department of Agriculture, Wagga Wagga or AGWEST Plant Laboratories, Department of Agriculture, Western Australia. The suspect sample needs to include the original grain sample, slides of the teliospores, and plates containing the germinated teliospores.

7.2.3 Storage at laboratory

The extracted DNA should be kept frozen and marked Quarantine Sample and stored in a separate container that is labelled appropriately and away from other DNA samples.

NB: It is very important that all paper work concerning the sample is kept and proper records are kept so if further investigation is required this can be done.

7.3 Quality control

All media is quality controlled at the point of manufacture. Refer to the Media Preparation Manual.

A positive and negative control needs to be included in each PCR run. The positive control is extracted DNA imported from the USA that is stored under Quarantine conditions. The negative control is the use of extracted DNA from *Tilletia tritici*. It is also important that primers for both *T. indica* and *T. walkeri* are used on the same sample at the same time.

NB: Chemical resistant gloves should be worn, with laboratory coat and safety glasses. Designated pipettes and tips should be used for each step in the PCR process and for either the conventional PCR or PCR-RFLP at least two replicates should be set up per dilution used.

7.4 Principle

The identification of the fungal pathogen is confirmed by PCR methods. The RFLP-PCR method produces a single amplicon from the positive controls (*T. inidca* and *T. walkeri*) and from the unknown sample using the universal primers ITS1 and ITS4. These products are then 'cut' using the restriction enzymes *Taq* 1 and *Sca* 1. Identification of the putative *Tilletia* species is based on the restriction profiles of the ITS fragment amplified by ITS1 and ITS4.

7.5 Reagent

Unless otherwise stated all water used is sterile de-ionised water. Refer to Work Instructions NDPW 1-9.

(1) Sterile distilled water.

(2) PCR buffer (Perkin Elmer) containing 15 mM $MgCl_2$.

(3) AmpliTaq® polymerase 5 U/µl (Perkin Elmer).

(4) Primer ITS1.

(5) Primer ITS4.

(6) 10mM dATP.

(7) 10mM dCTP.

(8) 10mM dGTP.

(9) 10mM dTTP.

(10) BSA (10 µg/µl).

(11) Restriction enzyme Taq 1.

(12) Restriction enzyme Sca 1.

(13) Restriction enzyme buffer.

(14) Extracted DNA.

(15) Agarose gel.

(16) TBE buffer (NDPW 7, Section 4).

(17) Loading Buffer (NDPW 8, (Section 4)).

(18) Molecular weight marker.

(19) Ethidium bromide stain.

(20) Tap water.

NB: Primer ITS1: (5' TCC GTA GGT GAA CCT GCG G 3')
Primer ITS4: (5' TCC TCC GCT TAT TGA TAT GC 3')

NB: Ethidium bromide is a carcinogen and appropriate gloves, safety glasses and laboratory coat should be worn at all times.

7.6 Equipment

Refer to operating manuals for general usage.

(1) Thermocycler and loading rack.

(2) Microcentrifuge.

(3) 100 µl pipettes and sterile tips.

(4) Incubator set at 37°C.

(5) Waste solvent bottle.

(6) Microcentrifuge tubes (0.2 ml).

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

(8) Electrophoresis tank, electrodes and power pack.

(9) Marked container for Ethidium bromide.

(10) UV transiluminator.

(11) Camera.

7.7 Procedure

A: Amplification

(1) In the laminar flow cabinet, aseptically prepare enough of the reaction mix described (Table 4.4) below for the number of test samples plus 10 per cent.

Table 4.4 PCR reaction mixture for one sample for amplification step of RFLP protocol

Reagent	Volume for one sample
10x PCR Buffer (Perkin Elmer) containing 15 mM $MgCl_2$	5.0
Primer ITS1	0.2
Primer ITS4	0.2
dATP (10 mM)	0.5
dCTP (10 mM)	0.5
dGTP (10 mM)	0.5
dTTP (10 mM)	0.5
AmpliTaq polymerase 5 U/µl (Perk Elmer)	0.2
Sterile distilled water	41.4
TOTAL	**49.0**

(1) Into separate 200 µl reaction tubes aliquot 49 µl of the reaction mix.

(2) Moving away from the lamina flow and working on a laboratory bench, add 1 µl of the DNA test extracts to each of the reaction tubes.

(3) Place the reaction tubes into a thermal cycler and run program.

(4) Parameters for NSW Department of Ag EMAI thermal cycler: The temperature profile is 94°C denaturation for 2 min, 33 cycles of 94°C 30sec, 54°C 60 sec and 72°C 60sec, followed by an extension step of 72°C 10 min.

(5) Parameter for DAWA: Not determined as yet.

(6) Store the cycled products at 4°C or lower.

B: Restriction

(1) Pipette into a 0.2 ml tube for each sample the following (Table 4.5):

Table 4.5 PCR reaction mixture for one sample for restriction step of RFLP protocol

Reagent	Volume for one sample (µl)
Sterile distilled water	7.3
Restriction enzyme buffer (10x)	2.0
BSA (10 µg/µl)	0.2
Amplified PCR mix from Step A	10.0
Restriction enzyme Taq 1 or Sca 1	0.5
Total volume	**20.0**

(2) Gently flush the mixture using a pipette.

(3) Incubate the mix for 3 hours at 37°C.

C: Resolution of amplified PCR products

(1) Dissolve 1.5 per cent agarose in working TBE buffer. Pour melted agarose into gel tray and then insert comb with appropriate number of wells. The wells should be large enough to hold 12 µl samples and molecular markers. Allow agarose to set (for a minimum of 30 minutes). Remove comb.

(2) Fill the electrophoresis tank with working TBE buffer, ensuring that the entire gel is covered by at least 3 mm of buffer.

(3) Aliquot 2 µl of loading buffer onto Parafilm for each sample to be loaded.

(4) Aliquot 10 µl of the restriction mixes onto Parafilm and mix with the 2 µl of loading buffer already on the parafilm.

(5) Repeat Steps '13' and '14' using unrestricted PCR products (from **A:** Step 7).

(6) Separately on the Parafilm mix 2 µl of 100 bp molecular weight marker with 8 µl of sterile distilled water and 2 µl of loading buffer.

(7) Place gel into the tank and load the 12 µl aliquots from each sample on the Parafilm into their respective wells.

(8) Record on the template which well corresponds to which sample.

(9) Place lid on tank and run small gel at 80 volts or large gel at 160 volts approx for one hour or until the marker is 40 mm from the edge of the gel.

NB: Ethidium bromide is a carcinogen and appropriate gloves, safety glasses and laboratory coat should be worn at all times during the rest of the process.

(10) Place gel in aqueous ethidium bromide stain (0.05 µg/ml) and agitate on a shaker for 30 minutes.

(11) Lift gel from stain and destain in tap water for 5 minutes.

(12) Remove gel from water and place on UV transiluminator.

(13) View illuminated gel using the gel documentation system.

(14) Refer to instructions on how to use the gel documentation system.

7.8 Results

Both *Tilletia* species have a restriction site in the ITS2 region for Taq 1 while only *T. walkeri* has a restriction site for Sca 1 which is in the ITS1 region. This is shown in the gel in Figure 4.6.

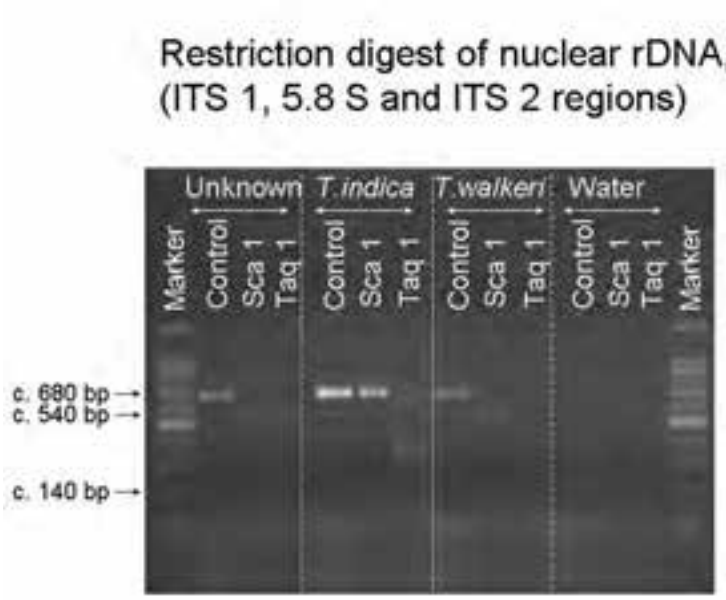


Figure 4.5 Results from the RFLP PCR for *T. indica* and *T.walkeri*.

Calculations:

1. Rehydration of lyophilised primers:

For each primer, dilute lyopphilised primer pellet in 1 ml filtered and autoclaved molecular grad water to obtain the [pmol/µl] given on the tube label.

Calculate the required primer volume to give 1 µM per 50 µl reaction as follows:

Primer vol (µl) to give 1 µM = 50/[pmol/µl]

2. Equation for calculating Relative Centrifugal Force (x g) from Revolutions Per Minute:

$$RCF = 1.12_{r\max}\left(RPM/1000\right)^2$$

Where rmax is the radius (mm) from the centre of the rotation to the bottom of the centrifuge tube.

Units:

All units used are SI units.

7.9 Validation

All techniques are standard, refer to references.

7.10 Reference ranges

N/A

7.11 Reporting

Results are to be reported as:

(a) For positive results – 'the fungal pathogen (state the full name of the pathogen) was detected in the sample submitted. This causes the disease known as (state the disease common name). No other fungal pathogens were detected in the sample submitted'.

(b) For negative results – 'the sample submitted was tested for possible fungal diseases. No fungal pathogens were detected in the sample submitted'.

7.12 Notes

N/A

7.13 Glossary of terms

7.14 References

1. Inman, A.J., Hughes, K.J.D., and Bowyer, R.J. (2003). EU recommended protocol for the diagnosis for a quarantine organism '*Tilletia indica'*. Central Sciences Laboratory, Sand Hutton, York, UK.

2. Levy L., Meyer R.J., Carris L., Peterson G. and Tschanz A.T. (1998). Differentiation of *Tilletia indica* from the underscribed Tilletia species on Ryegrass by ITS sequence differences. *Proceedings of the 12th Biennial Workshop on Smut fungi*, p29.

3. Smith, O.P., Peterson, G.L., Beck, R.J., Schaad, N.W., and Bonde, M.R. (1996). Development of a PCR-based method for identification of *Tilletia indica. Phytopathology* **86:** 115-122.

8. DIRECT OR NESTED-REALTIME - PCR METHOD FOR THE IDENTIFICATION OF TILLETIA SPECIES

8.1 Significance

T. indica causes the disease Karnal bunt of wheat (*Triticum* spp.), triticale (*Triticum x secale*) and possible Rye (*Secale*). It is a quarantine plant pathogen in Australia and can have an economic impact on the marketability of wheat. If the disease were to occur within Australia major restrictions would be placed on the export of grain to other countries.

8.2 Specimen

8.2.1 Scope

Once a possible detection of the pathogen *T. indica* has occurred this needs to be confirmed by the use of PCR. This can be done using a number of alternative methods including; traditional PCR using species specific primers, confirmation by PCR using Taqman or Corbett Real time PCR machine, and by confirmation using restriction enzyme analysis. The PCR process is performed on germinated teliospores. Refer to Section 4.3 on “Germination of teliospores,and mycelial matte production”, and Section 5.1 on “DNA extraction process”.

8.2.2 Transport sample to laboratory

Suspect samples should be marked 'Suspect exotic plant disease' and sent to Plant Health Diagnostic Laboratory, NSW Department of Agriculture, Wagga Wagga or AGWEST Plant Laboratories, Department of Agriculture, Western Australia. The suspect sample needs to include the original grain sample, slides of the teliospores, and plates containing the germinated teliospores.

8.2.3 Storage at laboratory

The extracted DNA should be kept frozen and marked Quarantine Sample and stored in a separate container that is labelled appropriately and away from other DNA samples.

NB: It is very important that all paper work concerning the sample is kept and proper records are kept so if further investigation is required this can be done.

8.3 Quality control

All media is quality controlled at the point of manufacture. Refer to Section 9.

A positive and negative control needs to be included in each PCR run. The positive control is extracted DNA imported from the USA that is stored under Quarantine conditions. The negative control is the use of extracted DNA from *Tilletia tritici*. It is also important that primers for both *T. indica* and *T. walkeri* are used on the same sample at the same time.

NB: Chemical resistant gloves should be worn, with laboratory coat and safety glasses. Designated pipettes and tips should be used for each step in the PCR process and for either the conventional PCR or PCR-RFLP at least two replicates should be set up per dilution used.

8.4 Principle

The identification of the fungal pathogen is confirmed by PCR methods. The published protocol (Frederick *et al*. 2000) used a direct real-time PCR approach to distinguish *T. indica* and *T. walkeri*. In the nested PCR approach reported here, *T. indica* and *T. walkeri* are more related to each other than to the other *Tilletia* species which were first diagnosed using the primer pair, Ti1 and Tin4. Both *T. indica* and *T. walkeri* will give a positive fragment of 1320 bp whereas a negative result will be observed with the other *Tilletia* species. Real–time PCR is then employed to distinguish between *T. indica* and *T. walkeri*.

8.5 Reagent

Unless otherwise stated all water used is sterile de-ionised water. Refer to Work Instructions in Section 9.

(1) Sterile Distilled Water.

(2) 10x PCR buffer (Invitrogen).

(3) Taq polymerase (Invitrogen).

(4) $MgCl_2$ (50 mM).

(5) Primer 1[a].

(6) Primer 2[b].

(7) 10 mM dNTP mix.

(8) Dual-labelled fluorescent probe (10 µM).

NB: For amplification of *T. indica* use:
[a] Tin3 (5'CAA TGT TGG CGT GGC GGC GC 3')
[b] Tin10 (5'AGC TCC GCC TCA AGT TCC TC 3')

For amplification of *T. walkeri* use:
[a] Tin11 (5'TAA TGT TGG CGT GGC GGC AT 3')
[b] Tin10 (5'AGC TCC GCC TCA AGT TCC TC 3')

NB: For nested PCR: Primer 1: Ti1 (5' TGGGCTGAGTCTGAGATGC 3')
Primer 2: Tin4 (5'CAACTCCAGTGATGGCTCCG 3')

8.6 Equipment

Refer to operating manuals for general usage.

(1) Thermocycler and loading rack.

(2) Microcentrifuge.

(3) Real time PCR machine.

(4) 100 µl pipettes and sterile tips.

(5) Waste solvent bottle.

(6) Microcentrifuge tubes (0.2 ml).

8.7 Procedure

There are two alternative methods for the real time PCR; a direct and a nested approach and a standard method. The nested PCR approach may give a more distinct separation of the two species.

A: Standard direct method

(1) Refer to Section 9 for primer optimization before commencing this section.

(2) In the laminar flow cabinet, aseptically prepare enough of the reaction mix described (Table 4.6) below for the number of test samples plus 10 per cent.

Table 4.6 PCR reaction mixture for one sample for amplification step of standard method for real-time PCR

Reagent	Volume for one sample
10x PCR Buffer (Invitrogen)	2.0
MgCl2 (50 mM)	2.0
10mM dNTP mix (10 mM)	0.4
Taq Polymerase (Invitrogen) (5 U/µl)	0.1
Primer 1 Forward (10 µM)	1.8
Primer 2 Reverse (10 µM)	0.2
Dual-labelled Fluorescent Probe (10 µM)	0.5
Sterile distilled water	12.0
TOTAL	**19.0**

Note: Each sample will be assayed with 2 different pairs of primers, Tin3/Tin10 *(T. indica* specific) and Tin 11/Tin10 (*T. walkeri* specific).

(1) Into separate 200 µl reaction tubes aliquot 19 µl of the reaction mix.

(2) Moving away from the lamina flow and working on a laboratory bench, add 1 µl of the DNA test extracts to each of the reaction tubes.

(3) Place the reaction tubes into the Real time PCR Machine.

(4) Parameters for NSW Department of Ag EMAI rotogene (Corbett Research) run temperature cycling program (94^{o}C denaturation for 2 min, 45 cycles of 94^{o}C 15 sec, 68^{o}C 30 sec (data acquiring) and 72^{o}C 30sec).

(5) This maybe different for DAWA – (not determined as yet).

(6) Generate reports of real-time fluorescence data using the applicable software.

B: Nested PCR method

The nested PCR method is a 2-step process. Step I is a conventional PCR protocol and Step II is the real-time PCR protocol as in the direct method above but the template used is the amplification product from Step I.

Step I

(1) In the laminar flow cabinet, aseptically prepare enough of the reaction mix described (Table 4.7) below for the number of test samples plus 10 per cent.

Table 4.7. PCR reaction mixture for one sample for amplification step of Nested PCR protocol

Reagent	**Volume for one sample** (µl)
10x PCR Buffer (Invitrogen)	2.5
MgCl2 (50 mM)	0.75
10mM dNTP mix (10 mM)	0.5
Taq Polymerase (Invitrogen) (5 U/µl)	0.1
Primer 1 (10 µM)	0.5
Primer 2 (10 µM)	0.5
Sterile distilled water	19.15
TOTAL	**24.0**

Note: Primer 1: Ti1 (5' TGGGCTGAGTCTGAGATGC 3').

Primer 2: Tin4 (5'CAACTCCAGTGATGGCTCCG 3').

(1) Into separate 200 µl reaction tubes aliquot 24 µl of the reaction mix.

(2) Moving away from the lamina flow and working on a laboratory bench, add 1 µl of the DNA test extracts to each of the reaction tubes.

(3) Place the reaction tubes into the thermocycler and run program.

(4) Parameters for NSW Department of Ag EMAI thermal cycler: The temperature profile is 94^{o}C denaturation for1 min, 33 cycles of 94^{o}C 15 sec, 68^{o}C 30 sec and 72^{o}C 30 sec, followed by an extension step of 72^{o}C 6 min. This maybe different for DAWA – (not determined as yet).

(5) Parameters for DAWA has not been determined yet.

(6) Store the cycled products at 4°C.

(7) Run 1/10th of the reaction volume on a gel (Section 3.2, page 7) to check that a fragment of 1320 bp is observed.

(8) Dilute the product 5 X to be used in the Step II real-time PCR reaction.

Step II

(1) In the laminar flow cabinet, aseptically prepare enough of the reaction mix described (Table 4.8) below for the number of test samples plus 10 per cent.

Table 4.8. PCR reaction mixture for one sample for nested PCR protocol

Reagent	Volume for one sample
10x PCR Buffer (Invitrogen)	2.0
MgCl2 (50 mM)	2.0
10mM dNTP mix (10 mM)	0.4
Taq Polymerase (Invitrogen) (5 U/µl)	0.1
Primer 1 Forward (10 µM)	1.8
Primer 2 Reverse (10 µM)	0.2
Dual-labelled Fluorescent Probe (10 µM)	0.5
Sterile distilled water	12.0
TOTAL	**19.0**

Note: Each sample will be assayed with 2 different pairs of primers, Tin3/Tin10 *(T. indica* specific) and Tin 11/Tin10 (*T. walkeri* specific).

(1) Into separate 200 µl reaction tubes aliquot 19 µl of the reaction mix.

(2) Moving away from the lamina flow and working on a laboratory bench, add 1 µl of the 5x dilution of PCR product from Step I (Section 3.4, page 19).

(3) Place the reaction tubes into the Real time PCR Machine.

(4) Parameters for NSW Department of Ag EMAI rotogene (Corbett Research) run temperature cycling program (94^{o}C denaturation for 2 min, 45 cycles of 94^{o}C 15 sec, 68^{o}C 30 sec (data acquiring) and 72^{o}C 30 sec).

(5) This maybe different for DAWA – (not determined as yet).

(6) Generate reports of real-time fluorescence data using the applicable software.

8.8 Results

Result from the first step of the Nested PCR real-time assay is shown in Figure 4.6.

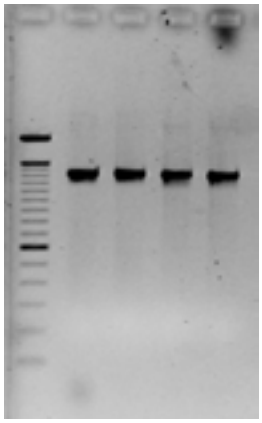


Figure 4.6 Result from the amplification step I shows 1320 bp amplified from both *T. indica* and *T. walkeri* using primers Ti1 and Tin4. Other *Tilletia* species e.g. *T. horrida* and *T. tritici* gave negative result.Results from the RFLP PCR for *T. indica* and *T.walkeri*.

Calculations:

Equation for calculating Relative Centrifugal Force (x g) from Revolutions Per Minute:

$$RCF = 1.12_{r\max}\left(RPM/1000\right)^2$$

Where rmax is the radius (mm) from the centre of the rotation to the bottom of the centrifuge tube.

Units:

All units used are SI units.

8.9 Validation

All techniques are standard, refer to references.

8.10 Reference ranges

N/A

8.11 Reporting

Results are to be reported as:

a) For positive results – 'the fungal pathogen (state the full name of the pathogen) was detected in the sample submitted. This causes the disease known as (state the disease common name). No other fungal pathogens were detected in the sample submitted'.

b) For negative results – 'the sample submitted was tested for possible fungal diseases. No fungal pathogens were detected in the sample submitted'.

8.12 Notes

N/A

8.13 Glossary of terms

8.14 References

1. Inman, A.J., Hughes, K.J.D., Bowyer, R.J. (2003). EU Recommended protocol for the diagnosis of a quarantine organism '*Tilletia indica*'. Central Sciences Laboratory, York, UK.

3. USDA (2002/3). Karnal Bunt Manual. United States Department of Agriculture, Frederick, Maryland.

9. MEDIA RECIPES

9.1 Bleach solution (1.25%)

1 part bleach (pool chlorine (NaOHCl))

9 parts distilled water

NB: Always add the bleach to the water.

Mix the water and bleach into a suitable container and label as “Bleach solution (1.25%)”, date of preparation, and your initials. Store in the fridge.

9.2 tween 20 solution

2 ml	Tween 20
3 L	Distilled water

Mix the Tween 20 into the water in a large Schott bottle. Label the bottle “Tween 20 Solution”, date of preparation, and your initials. Store in the fridge.

9.3 Shear's solution (600 ml)

19.45 ml	0.2M Na_2HPO_4 (Refer to NDPW 4, page 4)
0.55 ml	0.1M citric acid (Refer to NDPW 5, page 5)
6 g	Potassium acetate
280 ml	Distilled water
120 ml	Glycerine
180 ml	Ethyl alcohol (95%)

Using a pipette, add 19.45 ml of 0.2M Na_2HPO_4 into a 1L beaker. Then add 0.55 ml of the 0.1 M citric acid solution. Add the potassium acetate and the distilled water to the same beaker. Stir with a magnetic stirrer until all of the solids are dissolved and the solution is uniform in appearance. Continue stirring and add the glycerine and ethyl alcohol to the beaker.

When the solution is uniform in appearance, transfer this to a 1L Schott bottle and label as "Shear's solution", date of preparation, and your initials. Store in the fridge.

9.4 0.2 M Na_2HPO_4

38.39 g Na_2HPO_4 (anhydrous)

1L Distilled water

Mix in a 2L beaker with a magnetic stirring rod until fully dissolved. Transfer into a 1L Schott bottle, label as "0.2 M Na_2HPO_4," date of preparation and your initials. Store in the fridge.

9.5 0.1 M citric acid

21.01 g citric acid (monohydrate)

1L Distilled water

Mix in a 2L beaker with a magnetic stirring rod until fully dissolved. Transfer into a 1L Schott bottle, label as “0.1M Citric acid solution”, date of preparation, and your initials. Store in the fridge.

9.6 2% Tap water agar plates with antibiotics

20 g	Agar Technical No. 3 (Oxoid)
60 mg	Penicillin-G (Na salt)
200mg	Streptomycin sulphate
1 L	Tap water

In a 2L Schott bottle mix the agar with the distilled water and autoclave for 40 minutes. When agar is cool add antibiotics. Pour plates in the laminar flow and leave to dry overnight. Label the plates appropriately.

9.7 10x TBE Buffer

108 g Tris-base

55 g Boric acid

9.3 g $Na_2EDTA \times 2\ H_20$

1 L Distilled water

Mix in a 2L beaker with a magnetic stirring rod until fully dissolved. Transfer into a 1L Schott bottle, label as “10x TBE Buffer”, date of preparation, and your initials. Store in the fridge.

9.8 6x loading buffer

30% (v/v)	Glycerol
0.05%	Bromophenol blue
0.05%	Xylene cyanol

In a fume hood mix in a beaker the above ingredients with a magnetic stirring rod until fully dissolved. Transfer into a Schott bottle, label as “6x Loading Buffer”, date of preparation, and your initials.

9.9 Primer Optimization

This work instruction needs to be completed so that the final primer concentrations to be used in Sections 6, 7 and 8 are correct for the equipment being used.

9.9.1. NSW OPTIMISATION

	Primer 2	Primer 2	Primer 2
Primer 1	100nM/100nM	300nM/100nM	900nM/100nM
Primer 1	100nM/300nM	300nM/300nM	900nM/300nM
Primer1	100nM/900nM	300nM/900nM	900nM/900nM

Amount of 10 μM primer needed in a 20 μl reaction:

For 100nM, use 0.2 μl of 10μM stock

For 300nM, use 0.6 μl of 10μM stock

For 900nM, use 1.8 μl of 10μM stock

Reagent	**Volume for one sample** (μl)
10x PCR Buff (Invitrogen)	2.0
$MgCl_2$ (50mM)	2.0
10mM dNTP mix (10mM)	0.4
Taq Polymerase (Gibco) (5U/μl)	0.1
Dual-labelled fluorescent probe	0.5
Sterile distilled Water	15.0
TOTAL	**20.0**

Temperature cycling program in Rotorgene : 94°C denaturation for 1 min, 45 cycles of 94°C 15sec, 68°C 30sec (data acquiring) and 72°C 30sec.

Primer optimisation has estimated the optimum primer concentration of Primer 1 (Forward primer) and Primer 2 (Reverse primer) for the Rotorgene to be 900nM and 100nM respectively. In the published protocol (Frederick et al. 2000), the template DNA is the total fungal DNA extract. The Australian team has validated the protocol using both total fungal DNA extract and the amplified PCR fragment from step 1.

9.9.2. USA

	Primer 2	Primer 2	Primer 2
Primer 1	50nM/50nM	300nM/50nM	900nM/50nM
Primer 1	50nM/300nM	300nM/300nM	900nM/300nM
Primer1	50nM/900nM	300nM/900nM	900nM/900nM

Amount of 10 μM primer needed in a 25 μL reaction:

For 50nM, use 1.25 μl of 10μM stock

For 300nM, use 0.75 μl of 10μM stock

For 900nM, use 2.25 μl of 10μM stock

Reagent	**Volume for one sample** (μl)
2X Universal Master Mix	12.5
Dual-labelled fluorescent probe	1.0
Sterile distilled Water	11.50
TOTAL	**25.0**

APPENDIX A. PHOTOS FOR VISUAL EXAMINATION OF SEED

Figure A.1 Grains of wheat showing the different symptoms seen with different levels of infection of Karnal bunt. Photographs are the courtesy of Department of Agriculture, Western Australia.

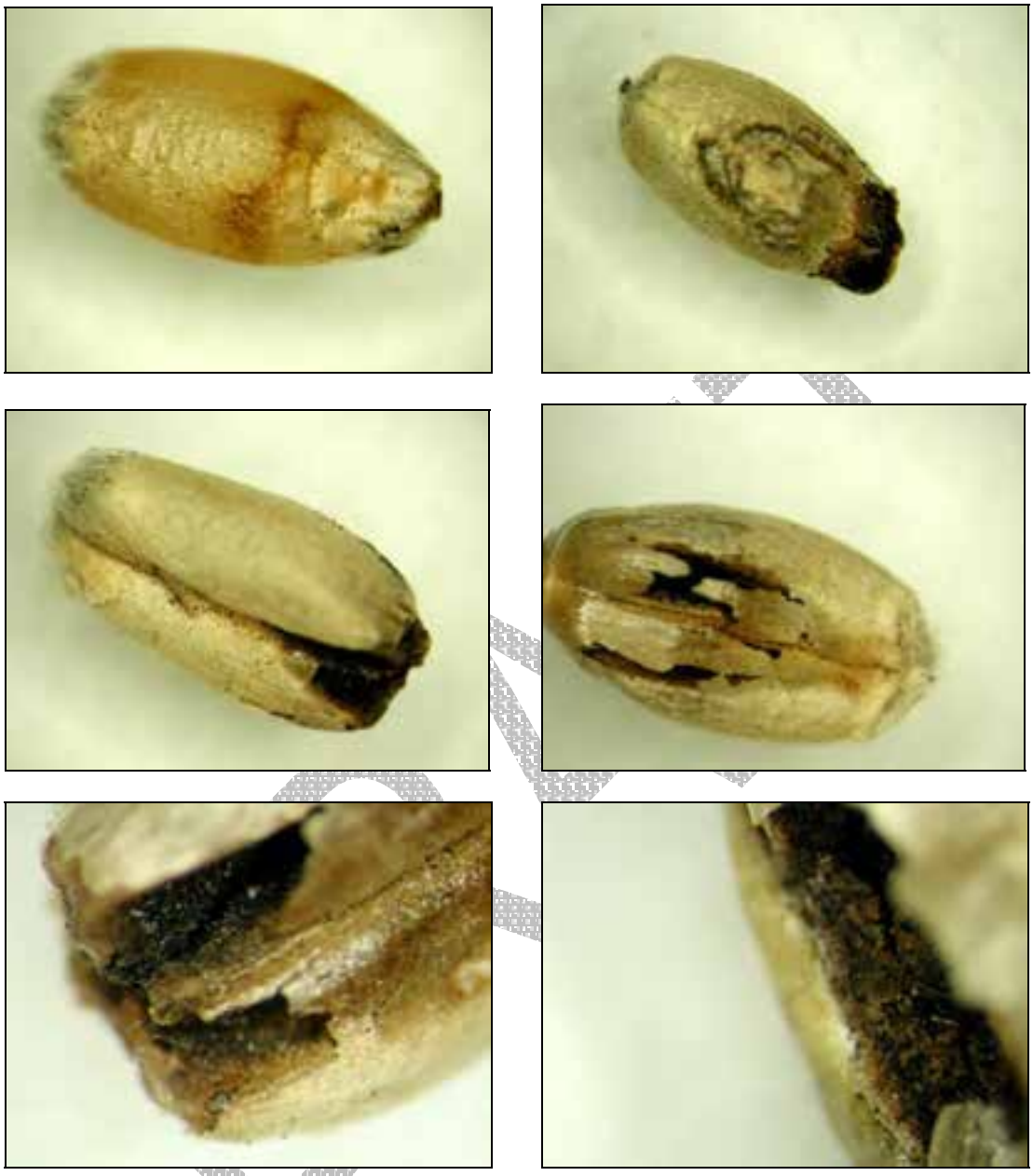


Figure A.2 Range of Karnal bunt symptoms seen on individuals grains of wheat. The embryo end of the seed is infected and will continue along the crease. The grain coat has a holey appearance when infection is severe. The teliospores will be evident where the grain coat has broken. Photographs are the courtesy of the Department of Agriculture, Western Australia.

APPENDIX B.1: *TILLETIA INDICA* TELIOSPORES

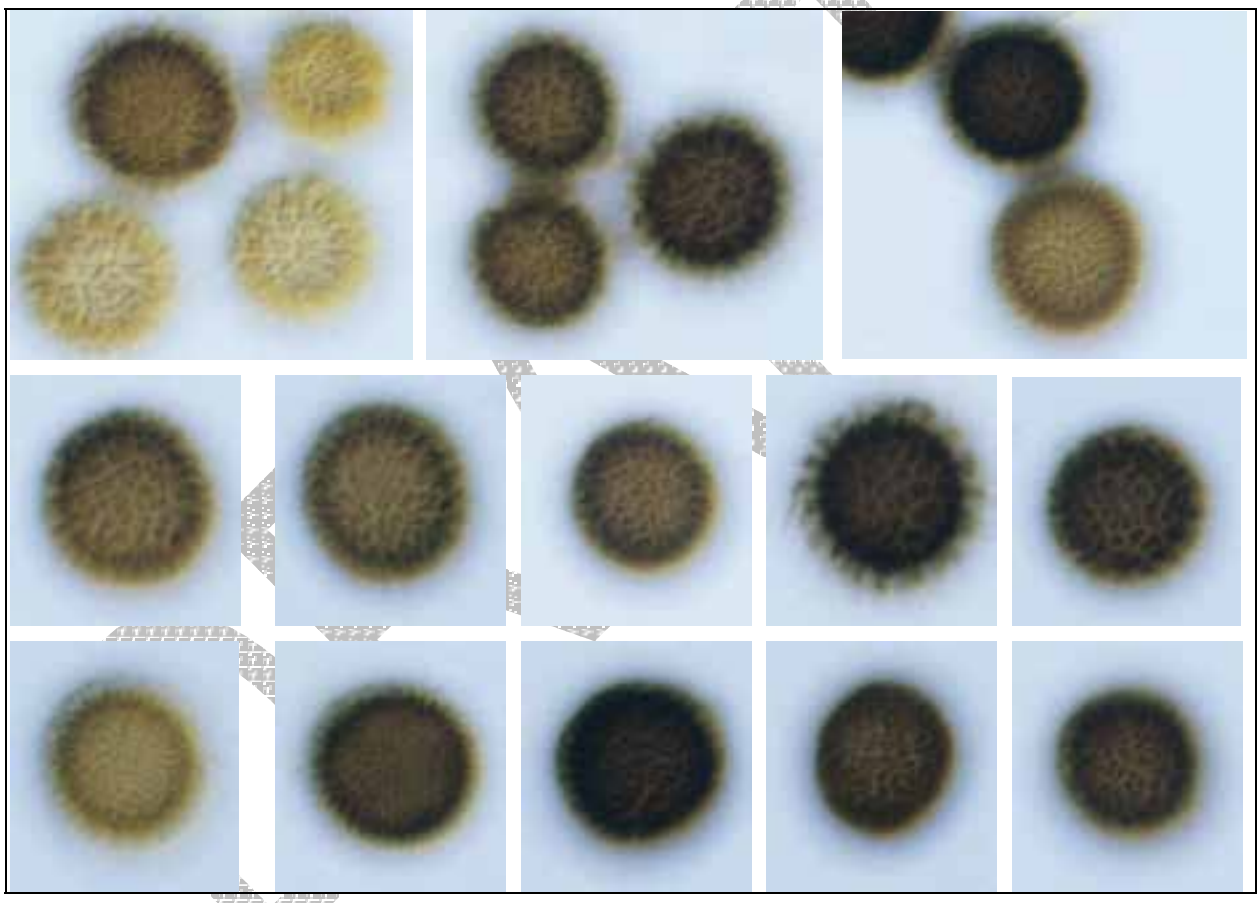


Figure B.1 Teliospores of *Tilletia indica* showing surface ornamentation patterns. Spines are densely arranged, either individually (densely echinulate) or in closely spaced, narrow ridges (finely cerebriform). Scale: 10mm = 17 µm. Photographs are the courtesy of Dr Alan Inman, Central Sciences Laboratory, York.

APPENDIX B2: *TILLETIA WALKERI* TELIOSPORES

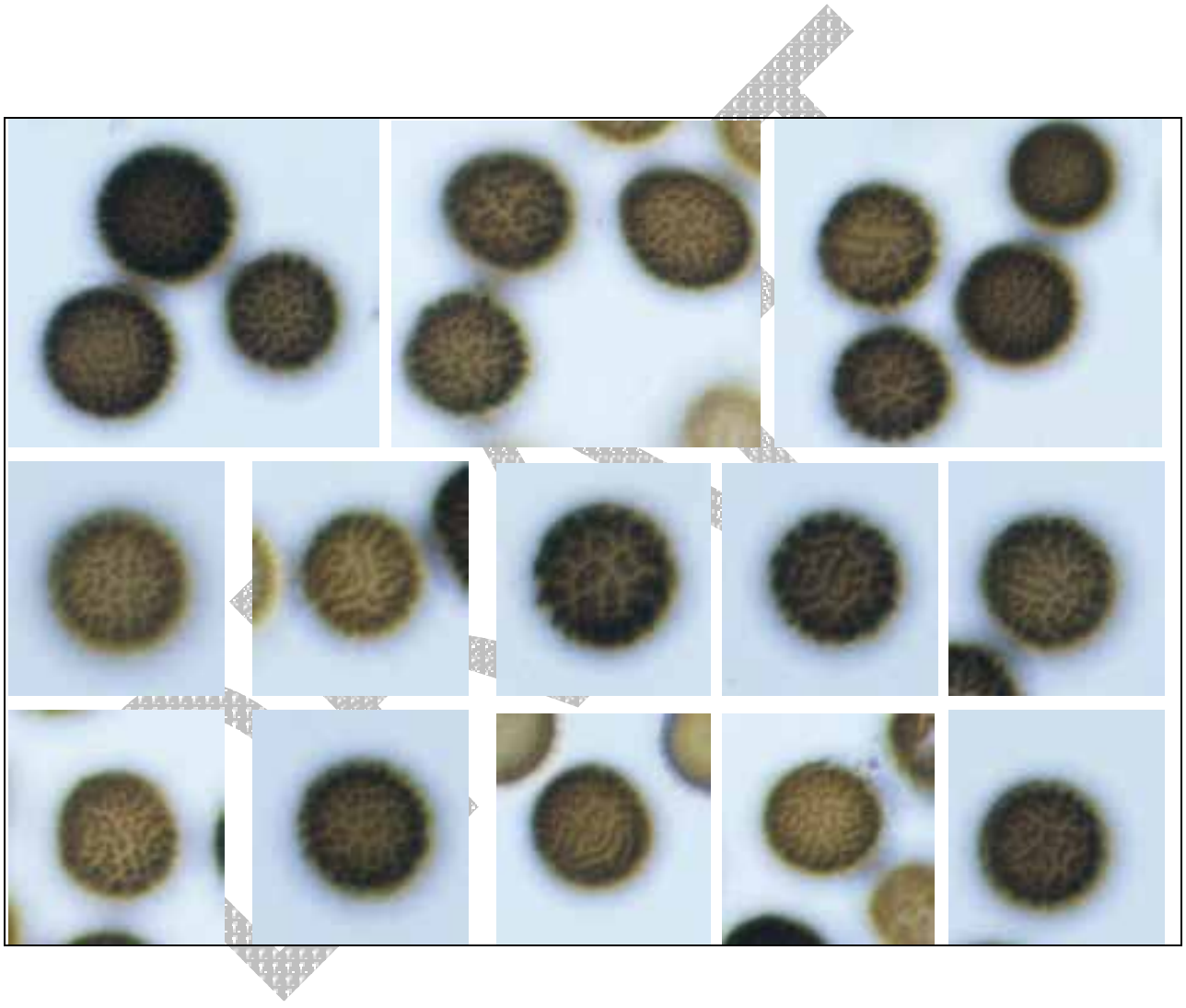


Figure B.2 Teliospores of *Tilletia walkeri* showing surface ornamentation patterns. Spines are coarsely arranged and forming wide, incompletely cerebriform to coralloid ridges or thick clumps. Scale: 10mm = 17 µm. Photographs are the courtesy of Dr Alan Inman, Central Sciences Laboratory, York.

APPENDIX B.3: TILLETIA HORRIDA TELIOSPORES

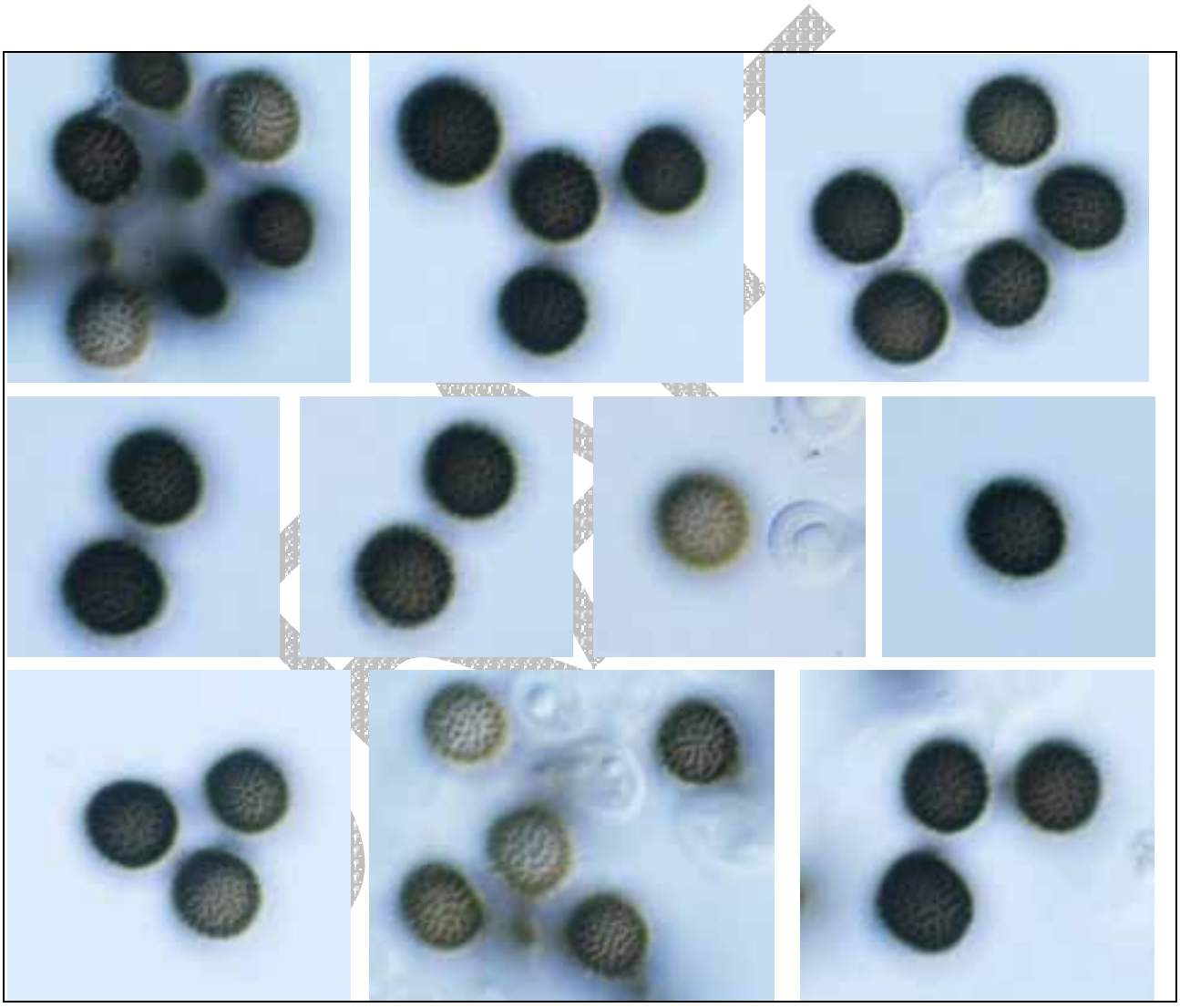
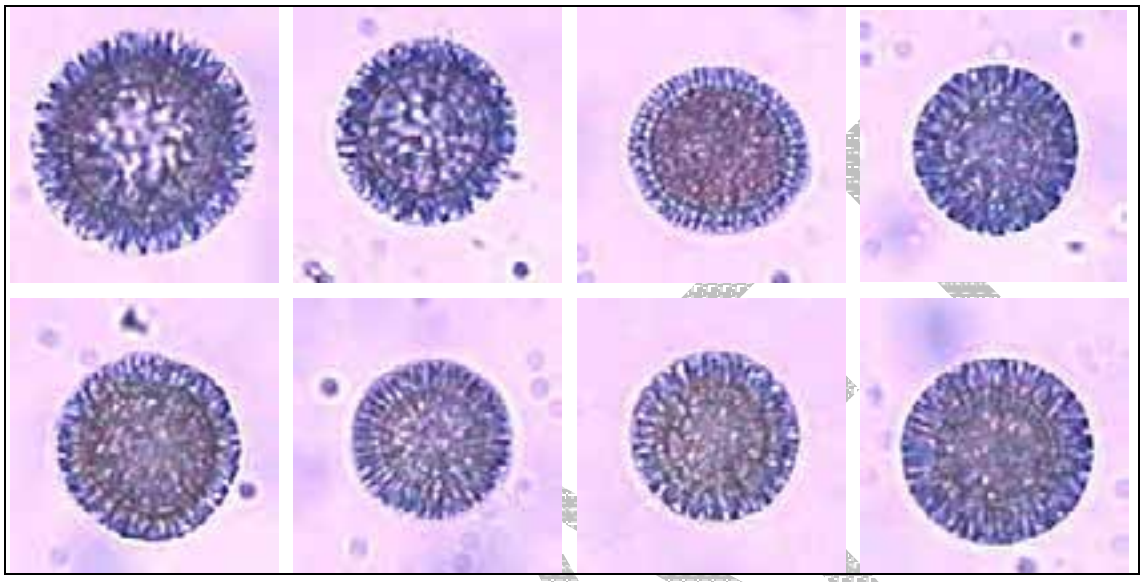


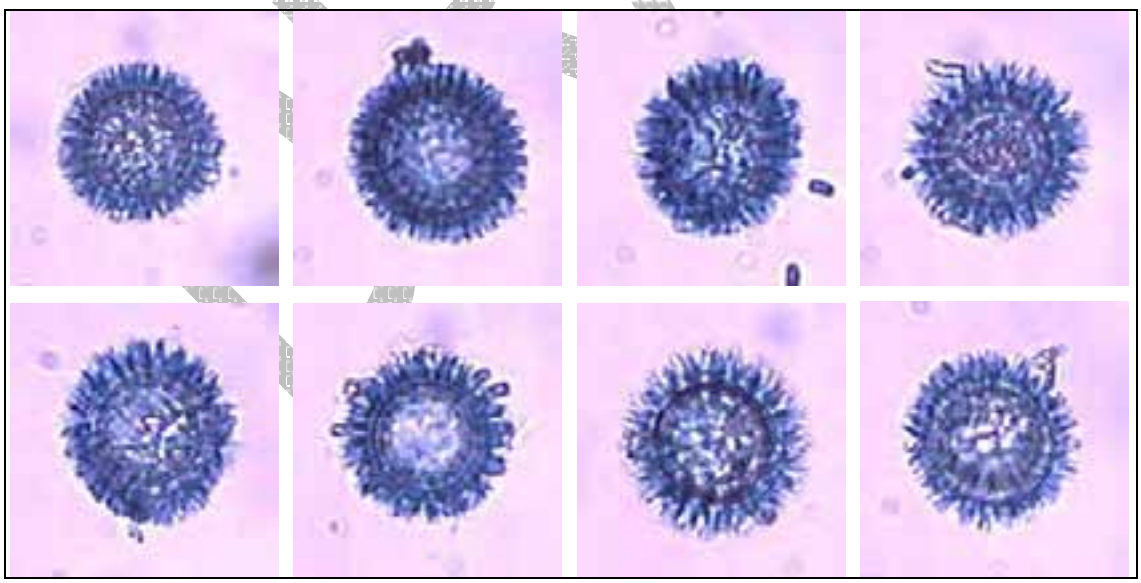
Figure B.3 Teliospores of *Tilletia horrida* showing surface ornamentation patterns. Polygonal scales or, occasionally, with cerebriform ridges. Scale: 10mm = 17 µm. Photographs are the courtesy of Dr Alan Inman, Central Sciences Laboratory, York.

APPENDIX B.4: TILLETIA INDICA AND WALKERI TELIOSPORES IN MEDIAN VIEW

A: *Tilletia indica*



B: Tilletia walkeri



FigureB.4 Teliospores of *Tilletia indica* (top) and *T. walkeri* (bottom) showing teliospore profiles in median view after bleaching and then staining with lactoglycerol-trypan blue. Note: The smoother outline on *T. indica* teliospores compared to the more irregular outline of *T. walkeri* teliospores with more obvious gaps between spines. Photos courtesy of Dr Alan Inman, Central Sciences Laboratory, York.

APPENDIX B.5: TILLETIA INDICA TELIOSPORES IN MEDIAN VIEW

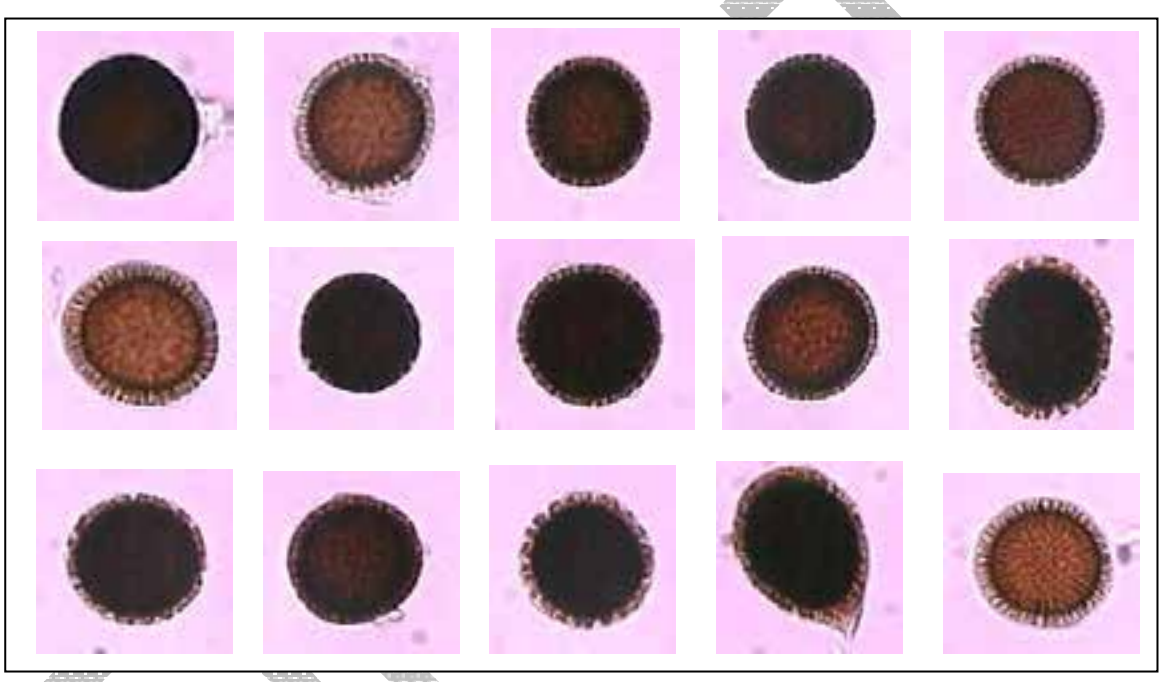


Figure B.5 ***Tilletia indica*** **teliospores in median view (20–50 μm diameter; mean 35–41 μm). Photos are the courtesy of Dr Alan Inman, Central Sciences Laboratory, York.**

APPENDIX B.6: COMPARISON OF *T. INDICA*, *T. WALKERI* AND *T. HORRIDA* TELIOSPORES

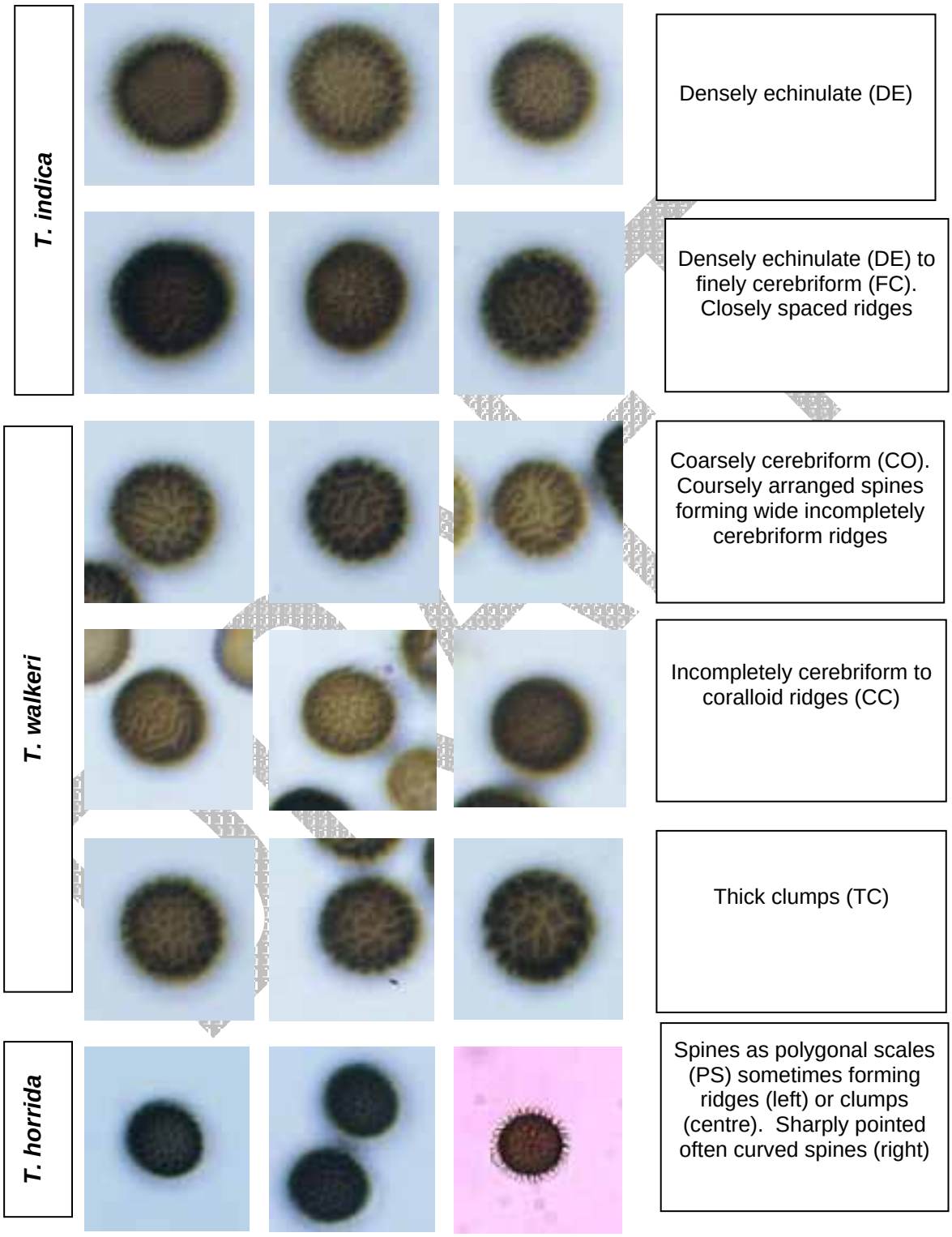


Figure B.6 Pictorial key to teliospore ornamentation. Use in conjunction with Worksheet 2, Section 5. Photographs courtesy of Dr. Alan Inman, Central Science Laboratory, York.

APPENDIX B.7: GROWTH OF TILLETIA SPP. COLONIES

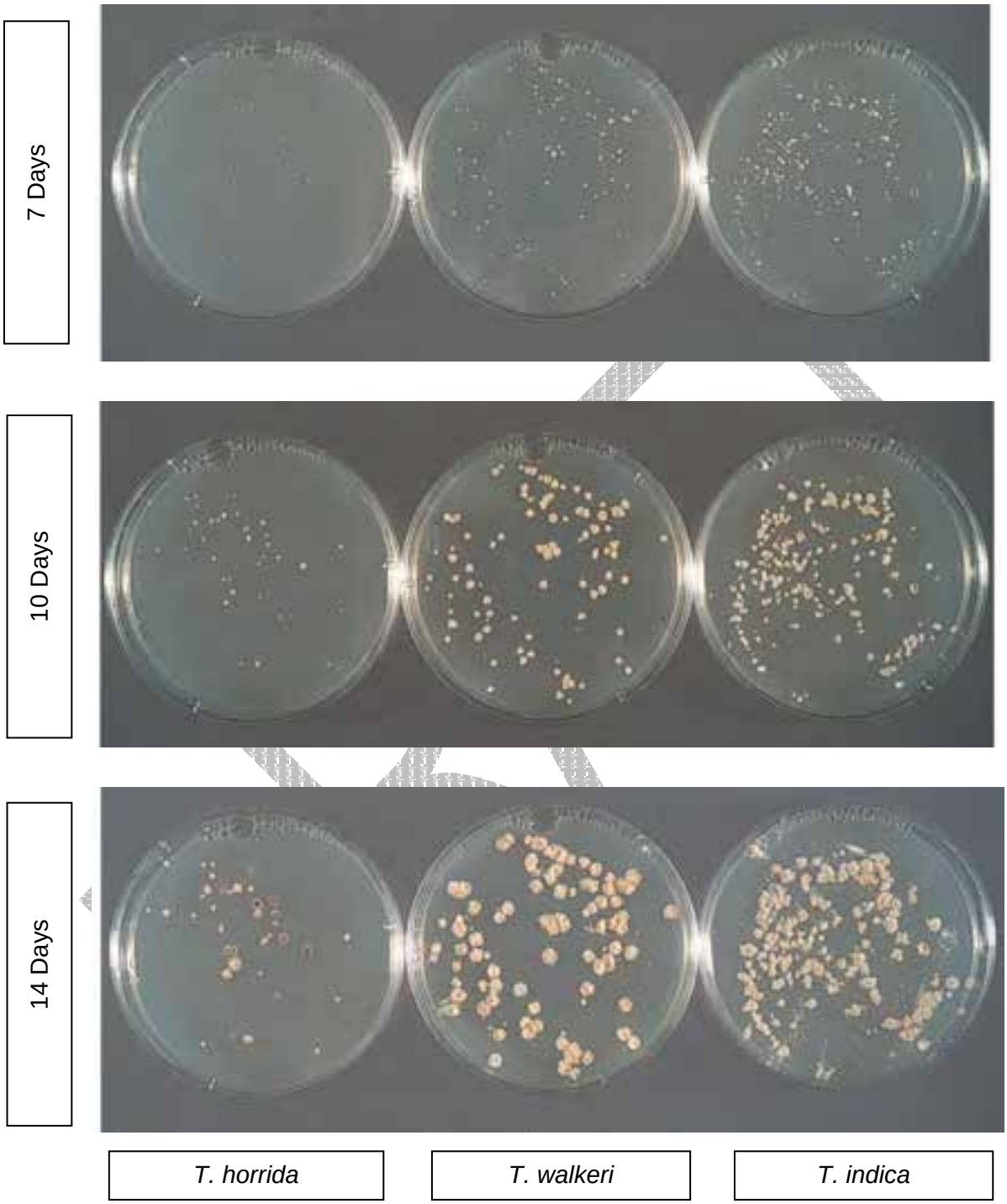


Figure B.7 Colonies of *Tilletia indica* (right), *T. walkeri* (centre) and *T. horrida* (left) after 7 days (top), 10 days (centre) and 14 days (bottom) on PDA at 19°C and a 12 hour dark/light cycle. Note: slower growth, and purple pigmentation after 14 days, for *T horrida* colonies. Photos courtesy of Dr. Alan Inman, Central Sciences Laboratory.Flow diagram for the analysis of grain

APPENDIX B.8: *T. INDICA* TELIOSPORES GERMINATING

Figure B.8 Photograph of *Tilletia indica* teliospores germinating on water agar after 10–14 days, producing a tuft of primary sporidia (basidiospores) at the apex of the promycelium. Primary sporidia germinate *in situ* to produce small colonies which produce secondary sporidia of two types: further filiform sporidia; allantoid sporidia which are forcibly discharged onto the agar. Photo courtesy of Dr Alan Inman, Central Sciences Laboratory, York.

APPENDIX C. WORKSHEETS FOR RECORDING DATA

Appendix C.1: Recording sheet for sample receival.

Appendix C.2: Recording sheet for teliospore characteristics.

Appendix C.3: Recording sheet for morphologically distinguishing teliopsores

Appendix C.4: Recording template for agarose gels.

Appendix C.1: Recording sheet for sample receival.

Sample number:	**Date of sampling:**

Host:
Botanical name:
Common name:

Country of origin:	**State (if applicable):**

Quantity of consignment (kg/t):
Consignment number/Lot or batch number:

Receiving laboratory:	**State:**

Receiving officer:	**Position:**

Sample size:		**Date sample examined:**
Symptoms: And any other comments		
Results from microscopic examination of grain		
Results from sieve wash test: (Teliospores detected or not detected)		
Results from morphological identification of teliospores: (Attach working sheets)		
Results from germination of teliospores: (Colony morphology, pigmentation etc)		
Results from PCR confirmation tests: (Attach copies of results)		

Appendix C.2: Recording sheet for teliospore characteristics detected in wash tests.

Sample number:	**Run letter:**	**Date:**

Teliospore number	**Size µm** (diam.)	**Colour** (see codes below)	**Ornamentation** (see codes below**)**	**Notes**
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				
26				
27				
28				
29				
30				
31				
32				
33 etc				

Size range	**Mean ± s.d**

Provisional Identification	

Colour code examples		**Ornamentation code examples**	
BO	Black/opaque	DE	Densely echinulate (spines densely and individually arranged).
RB	Reddish - brown	FC	Finely cerebriform (spines forming closely spaced narrow ridges).
CB	Chestnut – brown	CC	Coralloid (ridges much branched).
P (PY, PO, PB)	Pale (yellow/orange/brown)	CO	Coarsely cerebriform (spines coarsely arranged forming wide, incompletely cerebriform ridges).
		TC	Thick clumps (spines forming thick clumps).
		PS	Polygonal scales (curved in profile).

Appendix C.3: Recording sheet for morphologically distinguishing teliopsores of *Tilletia indica, T. horrida and T. walkeri* (cited from Inman *et al*. 2003)

	Max size (diam., µm)			Mean size (diam., µm)			Colour			Spines (ornamentation) in surface view and median profile		
Description of spores	< 36	> 36- < 45	> 45–50+	24–28	30–31	35–41	Pale yellow to mostly light or dark chestnut-brown (to semi opaque).	Pale yellow to mostly reddish-brown (never opaque).	Pale orange but mostly dark reddish-brown to opaque black.	Echinulate, polygonal scales in surface view; occasionally cerebriform ridges or rarely clumps. Sharpely pointed becoming truncate, occasionally curved.	Course; broad, incompletely cerebriform ridges (to collaroid), or thick clumps. Conical to truncate (gaps between spines obvious in profile after bleaching).	Dense; echinulate or closely spaced narrow ridges (finely cerebriform) Sharpley pointed to truncate, occasionally curved (few or no gaps between spines after bleaching).
Sample (place a tick in reloxes)												
T. horrida												
T. walkeri												
T. indica												

By placing a tick under the relevant description listed in the first row of the table, this will then line up with a shaded box below indicating the possible identification of the pathogen.

Appendix C.4: Recording template for agarose gels dependent upon Number of samples to be tested.

Sample wells on agarose gel						
Lane identification 1-7						
100bp molecular weight marker	Positive control	negative control	Sample 1	Sample 2	Sample 3	Sample 4