# National Contingency Plan for *Tilletia contraversa*, the cause of Dwarf Bunt of Wheat

# Part I

# Pest Risk Assessment

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The National Contingency Plan for *Tilletia contraversa* has four manuals:

Part I: Pest risk assessment, with background information on the pathogen and disease
Part II: Preventative Measures, the current and recommended actions to reduce the risk of an incursion
Part III: Field Manual, for use by the incident manager and teams in the emergency response
Part IV: Diagnostic Protocol, for use by the diagnostic laboratories

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# EXECUTIVE SUMMARY

Dwarf bunt, also called –TCK smut", is one of five bunt and smut diseases that affect wheat throughout the world. None of these bunts and smuts is toxic to humans or animals but the bunts in particular can affect the appearance and smell of grain products. Three bunts and smuts occur in Australia and most other wheat growing countries: these are common bunt (caused by *Tilletia caries* and *Tilletia laevis*), loose smut (*Ustilago tritici*) and flag smut (*Urocystis agropyri*). The two that do not occur in Australia are Karnal bunt (*Tilletia indica*) and dwarf bunt (*Tilletia contraversa*). Karnal bunt and dwarf bunt have a restricted distribution and are subject to quarantine regulations by many countries. Karnal bunt is dealt with in a separate contingency plan.

Dwarf bunt is a serious disease for international trade because it reduces grain quality and has a restricted distribution, leading to some countries placing restrictions on the import of wheat from areas where the disease occurs. Dwarf bunt does not occur in Australia and so these trade restrictions do not apply to our wheat. However, because any report of dwarf bunt occurring in Australia could have implications for trade, the Grains Council determined that a contingency plan for dwarf bunt be developed.

The dwarf bunt contingency plan is divided into four parts:

Part I — Pest Risk Assessment, developed from background information on the pathogen and the disease

Part II — Preventative Measures, giving the current and recommended actions to reduce the risk of an incursion

Part III — Field Manual, for use by the incident manager and teams in the emergency response

Part IV — Diagnostic Manual, for use by the diagnostic laboratories

Part I — Pest Risk Assessment

Dwarf bunt only occurs in wheat growing areas where there is snow cover for some months on the wheat seedlings, and where the temperature on the soil surface under the snow is a little above freezing. Such conditions occur in small areas of North America, South America, Europe and the Near East; these conditions do not occur in the Australian wheat belt. A climate-matching model was developed based on the temperature and moisture conditions associated with dwarf bunt. This model was conservative and showed a larger area of North America and Europe met the conditions; this was probably because the model was not able to estimate snow cover. Nevertheless, these expanded conditions were not met in the Australian wheat belt.

Dwarf bunt is caused by the bunt fungus *Tilletia contraversa* Kühn, also known as *Tilletia controversa* Kühn. A bunt that occurs on barley grass (*Critesion* spp.) in Australia has spores that are similar in appearance to the wheat dwarf bunt and had been identified as *Tilletia contraversa*. Because of this, CAB International still lists Australia as having *Tilletia contraversa*. However, recent Australian work has shown that the barley grass bunt is another species, *Tilletia trabutii*.

Dwarf bunt can cause severe yield losses to susceptible wheats when not controlled. The bunt will also reduce quality. The combined yield and quality losses justify its listing as a major disease of wheat and efforts to contain its distribution.

The Pest Risk Assessment concluded that the risk of overall entry, establishment and spread in Australia is nil. The risks from dwarf bunt for Australia arise from the incorrect identification of another bunt admixed with wheat grain as dwarf bunt, or the contamination of an Australian cargo with dwarf bunt from a previous commodity transported in the vessel. If such a claim were made, it could have serious short-term economic consequences. This risk can be reduced by having an effective means to quickly identify the cause of the problem to internationally accepted standards.

Part II — Preventative Measures

Part II reviews the current measures in place to prevent the entry of dwarf bunt into Australia. Pathways for entry include admixture of dwarf bunt affected wheat with bulk commodities such as grain or fertilizer shipments, contaminated shipping containers and imported machinery, and on clothing and personal effects of travellers. Entry of wheat grain is presently closely controlled so this is an unlikely means of entry. Bulk commodities are subject to inspection, which reduces the risk.

Surveys of the wheat crop and harvested grain for bunt spores would provide a level of confidence that Australia is free of this pathogen. The survey proposed in the National Contingency Plan for Karnal Bunt of Wheat would also detect spores of dwarf bunt and other grass bunts. Thus, no further work would be required beyond that proposed for Karnal bunt.

Because conditions are unsuitable for development of dwarf bunt in Australia, no pro-active controls such as breeding for resistance or development of fungicidal controls are required.

Part III — Field Manual

Should dwarf bunt be detected or suspected in Australia, immediate action is required to contain the incursion and identify the pathogen. The field manual describes the actions required in the field while Part IV gives the laboratory protocol for identifying the pathogen. Sample collection from grain, harvesting equipment and machinery is described. Dispatch of samples to diagnostic laboratories must be done quickly and the laboratory notified that the sample has been sent. Part III gives the addresses of the diagnostic laboratories.

Trace back and trace forward procedures are given that will define the extent of the incursion based on likely scenarios for first detection.

Sample methodology is according to the International Seed Testing Association rules. Locations within equipment for sampling are given. Pictures are provided on the symptoms of dwarf bunt.

Part IV — Diagnostic Manual

Identification of *Tilletia contraversa* and similar *Tilletia* spp. requires examination of the spores by experienced taxonomists familiar with this group of organisms followed by nucleic acid sequencing. The protocol for these laboratory procedures is given in Part IV.

The procedure requires obtaining spores either from bunt balls or from washings of grain, microscopy to record the spore morphology, attempted germination of spores at a range of temperatures, growth of mycelial mats, extraction of DNA, and sequence analysis.

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# 1. INTRODUCTION

Dwarf bunt, also called –TCK smut", is one of five bunt and smut diseases that affect wheat throughout the world (Saari *et al.* 1996). 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 caries* and *T. laevis*), loose smut (*Ustilago tritici*) and flag smut (*Urocystis agropyri*). The two that do not occur in Australia are Karnal bunt (*T. indica*) and dwarf bunt (*T. contraversa*).

There are two spellings of the species name for the dwarf bunt fungus in use: –*controversa*" is in common use and is the name used by CAB International and most North American workers, while –*contraversa*" is the name advocated by Johnsson (1991) and Vánky (1994). We accept their arguments and use the name *Tilletia contraversa* J.G. Kühn in this Contingency Plan (see Section 2.1.1).

Dwarf bunt is a serious disease for international trade because it reduces grain quality and has a restricted distribution, leading to some countries placing restrictions on the import of wheat from areas where the disease occurs (see Section 4.2). Dwarf bunt is only found where wheat is grown with extended snow cover on the seedlings, occurring in higher elevations in the Near East and in areas of Europe and North America where such conditions occur (see Section 3.2). The world distribution recognised by CAB International shows a wider distribution, including some areas including Australia where wheat is not grown under snow cover. A recent taxonomic study casts doubt on the identification of –*T. contraversa*" described in such locations (Pascoe *et al*. 2005). All Australian records of –*T. contraversa*" are from barley grass (*Critesion* sp.) and are morphologically, physiologically and molecularly different from *T. contraversa* from wheat. Studies on the holotypes of bunt previously named from *Critesion* show that the correct name for the barley grass bunt is *Tilletia trabutii* Jacz. (Pascoe *et al*. 2005; Ian Pascoe, pers. comm.). Thus, there are no records of *T. contraversa* on wheat in Australia, and the true worldwide distribution and host range of *T. contraversa* requires revision.

Different *Tilletia* spp. cause bunt diseases on many grasses. Often, these different bunts have spores that are morphologically similar, making their identification difficult or impossible if the spores are found apart from their host. At harvest, weeds as well as wheat enter the harvester and any spores on these weeds can be spread onto the wheat. Thus, a wash test applied to wheat grain will collect spores that were harvested, not necessarily spores that have come from wheat. Wash tests of Australian wheat have found spores of several grass bunts including *T. ehrhartae* from *Ehrharta calycina* and *T. walkeri* from *Lolium* spp.

The known environmental requirements for *T. contraversa* show that it is highly unlikely to occur in Australian wheat (see Sections 3.2, 3.3.2). This contingency plan examines the taxonomy of *T. contraversa* and morphologically similar species that may occur admixed with wheat grain, the biology and likely world distribution of the wheat dwarf bunt fungus, and measures to respond to claims that *T. contraversa* is present in Australian wheat.

The wheat dwarf bunt contingency plan is divided into four parts:

Part I – Pest Risk Assessment, with background information on the pathogen and the disease and a risk assessment

Part II – Preventative Measures, giving the current and recommended actions to reduce the risk of an incursion

Part III – Field Manual, for use by the incident manager and teams in the emergency response

Part IV – Diagnostic Protocol, for use by the diagnostic laboratories

# 2. DWARF BUNT OF WHEAT

## 2.1 The pathogen and the disease

Pathogen: *Tilletia contraversa* J.G. Kühn

Synonyms: *Tilletia controversa* J.G. Kühn in Rabenhorst
*Tilletia brevifaciens* G.W. Fischer

Disease: Dwarf Bunt of Wheat

Other Names: TCK Smut, short smut, stunt bunt.

Notes on the species name

J.G. Kühn described the type specimen of this bunt from *Triticum repens* (= *Elymus repens*, *Agropyron repens*) collected near Halle, Germany in 1873 as *Tilletia contraversa*. The fungus was then listed by Rabenhorst in 1874 as "~~T~~*illetia controversa* J. Kühn". Kühn, however, continued to use the spelling "~~c~~*ontraversa*". Although the "~~o~~" spelling is widely used and is the one currently used by CAB International, Johnsson (1991) and Vánky (1994) strongly argue for the "~~a~~" spelling. We follow their view and use the spelling "~~T~~*illetia contraversa*" in this Contingency Plan.

On wheat, dwarf bunt was not recognised as a separate disease to common bunt caused by *Tilletia caries* until the mid 1900s. Several species names were proposed for the dwarf bunt fungus until Conners (1954) found that it was morphologically synonymous with Kühn's *T. contraversa*.

## 2.2 Symptoms

Plants affected by dwarf bunt are stunted, often with an unusually large number of tillers. Most or all tillers on a plant are affected. The heads have a more spreading appearance than normal heads due to the florets gaping. Usually, all florets contain bunt balls that look superficially like dark seeds. Bunt balls of dwarf bunt are rounder than those of common bunt. The bunt balls crush easily to release a grey mass of foul smelling spores.

At harvest, most bunt balls are broken with the many spores dispersed onto normal seeds. At high levels of infection, the harvested grain appears grey with black, frequently broken bunt balls admixed. The grain can have a foul fishy smell from the presence of trimethylamine.

The dwarf bunt fungus infects wheat at the two to three leaf/early-tillering stages. It may cause minor pale spots and streaks in the leaves. Stunting is noticed from stem elongation to maturity.

## 2.3 Host Range

*T. contraversa* was first described on *Triticum repens* (= *Elymus*, *Agropyron repens*). Bunts with the morphological characteristics of *T. contraversa* have been recorded on 68 species of Poaceae. The main hosts are:

Wheat (Triticum aestivum)
Barley (Hordeum vulgare)
Rye (Secale cereale)
Triticale (X Triticosecale)
Wheatgrass (Agropyron spp.)

The host list of the *Tilletia* sp. causing dwarf bunt of wheat needs revision following the finding by Pascoe *et al*. (2005; Pascoe, pers. comm.) that the bunt on barley grasses (*Critesion* spp., formerly *Hordeum* spp.) in Australia and Iran is *T. trabutii* and not *T. contraversa*. *T. trabutii* has spores that are slightly larger than *T. contraversa*, with reticulations that are slightly shorter. Its temperature for germination is higher. Nucleic acid sequencing shows that it belongs in a distinct clade intermediate between the *T. contraversa* / *T. caries* clade and the *T. bromi* clade (Pascoe *et al*. 2005; Pascoe, pers. comm.).

Part IV has the diagnostic protocol for distinguishing the two species.

# 3. EPIDEMIOLOGY

## 3.1 Disease Cycle

*T. contraversa* survives as spores for 3–10 years in soil (Smilanick *et al*. 1986). Spores on the soil surface can germinate under snow cover when the temperature is above freezing. These spores germinate with a short germ tube (promycelium) to form 14–30 primary sporidia of two mating types. Pairs of sporidia fuse and infection hyphae grow from these. Providing snow cover and temperature remain favourable for several weeks (see Section 3.2), the infection hyphae can infect tillers of winter wheat. The fungal mycelium develops in the growing point of the seedling and invades young spikelets, then the developing wheat kernel, replacing the kernel with a mass of spores.

At harvest, the bunt balls are broken and many spores fall to the ground, where they survive to infect subsequent wheat crops.

Dispersal to new areas can occur with bunt balls or spores admixed on wheat grain of contaminating machinery or other goods. Spores on seed do not infect the emerging coleoptile as occurs with common bunt. Rather, they contaminate the soil and can infect the wheat at tillering.

*T. contraversa* has one cycle of infection for each generation of the host plant.

Saari *et al*. (1996) provide a detailed description of the life cycle of *T. contraversa*.

## 3.2 Environmental conditions for infection

Dwarf bunt is largely restricted to areas with a prolonged snow cover where the temperature is above freezing at the soil surface under the snow (Goates 1996). Spores germinate on the soil surface after a preconditioning exposure to light and at least 3–5 weeks at about 5°C. The most favourable conditions for infection are temperatures of 3–8°C with high moisture, conditions that are provided by deep snow cover (Saari *et al*. 1996; Goates 1996). The upper limit for germination is <15°C and lower limit -2°C (Hoffmann 1982).

Johnsson (1992) found that the incidence of dwarf bunt in Sweden was positively correlated with the duration of snow cover and the number of days in autumn with the average temperature <0°C, and negatively correlated with the mean temperature in early winter.

## 3.3 Distribution

### 3.3.1 Known distribution

CABI (2007) provides the following world distribution:

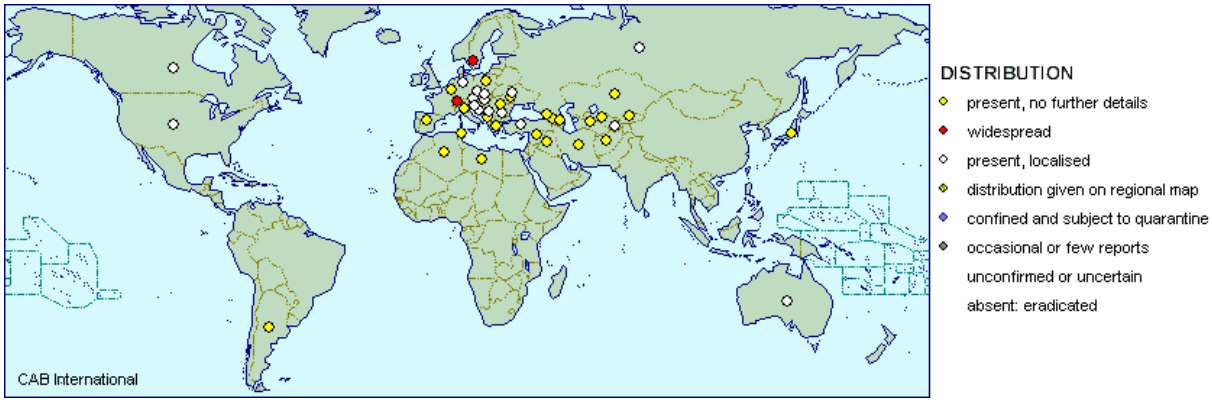


**Figure 1**: World distribution of *T. contraversa* (CABI 2007).

Asia

Afghanistan
Armenia
Azerbaijan
Georgia (Republic)
Iran
Iraq
Japan
Kazakhstan
Kyrgyzstan
Syria
Tajikistan
Turkey
Turkmenistan
Uzbekistan

Europe

Albania
Austria
Bulgaria
Croatia
Czech Republic
Denmark
France
Germany
Greece
Hungary
Italy
Lithuania
Luxembourg
Moldova
Poland
Romania
Russian Federation
- Central Russia
- Southern Russia
- Western Siberia

Serbia and Montenegro
Slovakia
Slovenia
Spain
Sweden
Switzerland
Ukraine

Africa

Algeria
Libya
Morocco
Tunisia

North America

Canada
- Alberta
- British Columbia
- Ontario

USA
- Colorado
- Idaho
- Indiana
- Montana
- New York
- Oregon
- Utah
- Washington
- Wyoming

South America

Argentina

Oceania

Australia
- New South Wales
- South Australia
- Western Australia

This distribution is doubtful and may be partly based on the incorrect identification of *T. contraversa* in many countries. For example, the Australian distribution is based on the bunt found on barley grasses (*Critesion* spp.), now recognised to be *Tilletia trabutii* (Pascoe *et al*. 2005; Pascoe, pers. comm.).

Another distribution map (Figure 2) was published by Saari *et al*. (1996) and shows a more restricted distribution, including the absence of *T. contraversa* from several countries including Australia.

Thus, the true distribution of *T. contraversa* requires a critical re-appraisal of the identity of the bunt pathogens based on the recent distinction of this species from similar species found on other grasses.

**Figure 2:** World distribution of *T. contraversa* (Saari *et al.* 1996) is shown with the black shading.

### 3.3.2 Potential Distribution

Dwarf bunt of wheat occurs in areas where there is persistent snow cover over autumn sown winter wheat, with the amount of infection related to the length of the snow cover (Johnsson 1992; Goates 1996). There are no areas of the Australian wheat belt where this occurs, so it is highly improbable that *T. contraversa* would establish in the Australia.

However, it is possible that trace amounts of disease could develop if temperatures were suitable during winter and there was an extended period of high moisture at the soil surface. To test this possibility, the potential distribution was modelled using the computer software program CLIMEX version 1.1 (Skarratt *et al*. 1995).

**Temperature parameters** for spore germination were based on those summarised by Hoffmann (1982): minimum temperature -2°C, optimum temperature 3–8°C, and maximum temperature <15°C. The values used in CLIMEX were: DV0 ~~li~~miting low temperature" = −2°C, DV1 ~~lo~~wer optimal temperature" = 3°C, DV2 ~~up~~per optimal temperature" = 8°C, and DV3 ~~li~~miting high temperature" = 10°C. At 5°C, most spores germinate in 6–8 weeks (Hoffmann 1982), showing that the day-degrees for germination are 210–280. Therefore, PDD ~~m~~minimum day-degrees" was set at 240.

ontraversa

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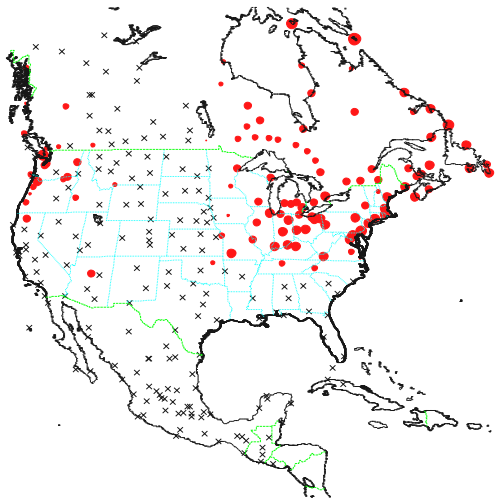
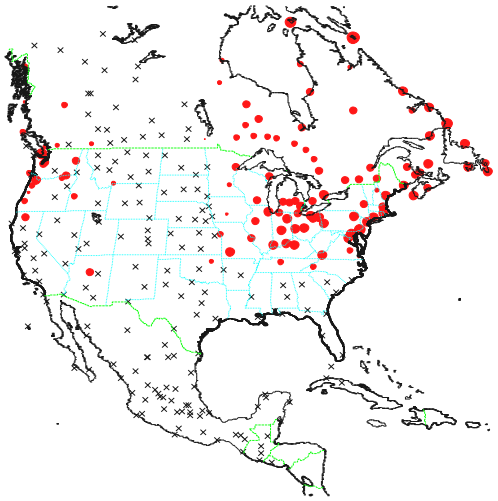


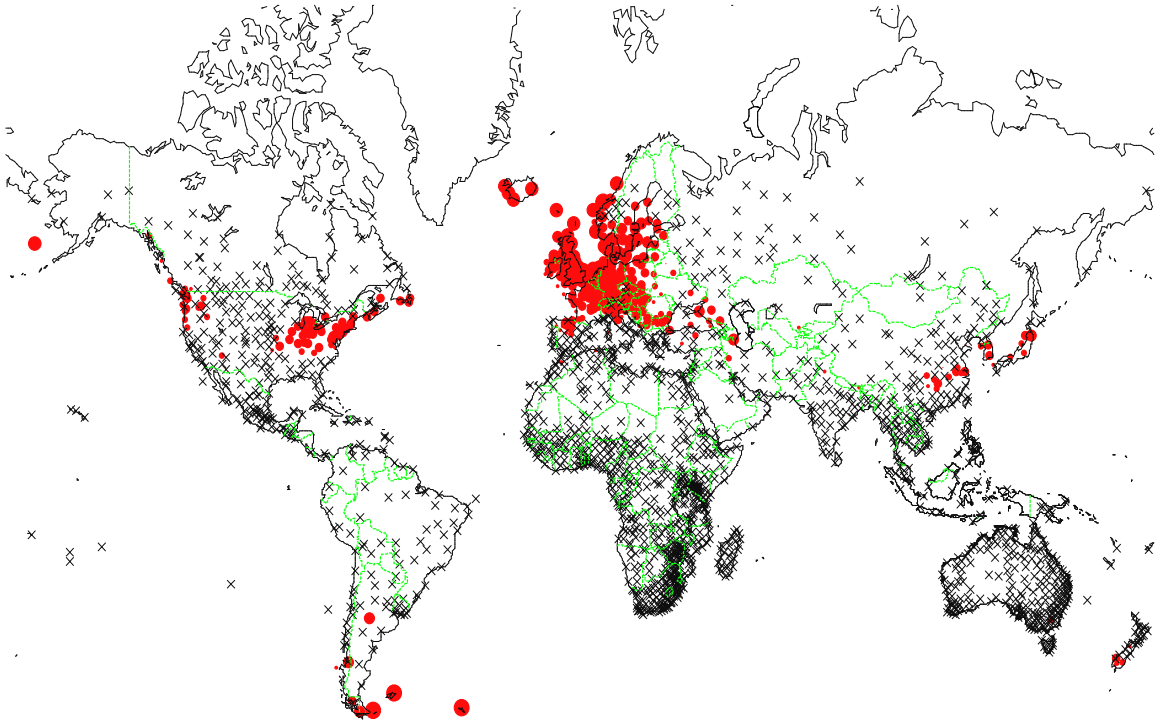
*contraversa* in North America based on temperature requirements for spore germination described in the text. Red circles show a match of the climate parameters with the model while ~~"x"~~ shows sites where no match occurs.

*contraversa* in North America based on temperature and moisture parameters described in the text. Red circles show a match of the climate parameters with the model while ~~"x"~~ shows sites where no match occurs.

**Cold stress parameters.** Cold stress may be a limiting factor for the distribution. The following parameters were adopted: TTCS ~~"c~~old stress temperature threshold" = −10; and THCS ~~"c~~old stress temperature rate" = 0.5. The addition of this factor gave a predicted distribution close to the known distribution in North America for part of the Pacific North West and for the area south of the Great Lakes (Figures 5 and 6).

This predicted distribution did not include the intermountain valleys in the eastern Pacific North West. This could be because the model does not predict snow cover. The prediction includes areas further south than the known distribution, possibly because these areas do not have prolonged snow cover.





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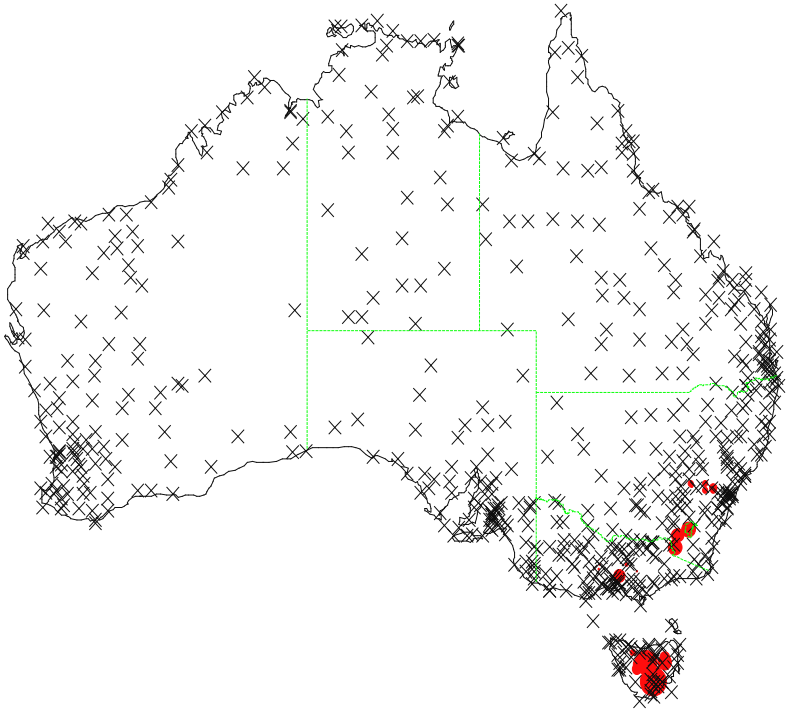

perature
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parameters are described in Section 3.3.2. Red circles show a match of the climate parameters with the model while ~~"x"~~ shows sites where no match occurs.

The environmental conditions of temperature and moisture are partly met in some locations on the Central Tablelands and Alpine areas of New South Wales, southern mountainous area of Victoria, and higher areas of Tasmania. However, no wheat is grown in these areas so conditions for establishment of *T. contraversa* are not met in Australia.

# 4. ECONOMICS AND MARKETING

## 4.1 Impact on production

### 4.1.1 Yield losses

Yield loss caused by dwarf bunt can be very high in susceptible varieties in a conducive environment. Infection levels over 70% of tillers have been recorded in the Pacific Northwest of the USA. Yield loss of about 0.8% for each 1% of infection (Goates 1996). In Germany, losses of up to 30% have been recorded (CAB International Crop Protection Compendium 2007). These high losses justify efforts to prevent the entry of *T. contraversa* to new areas that are suitable for its establishment.

Present yield losses in most countries where dwarf bunt occurs are minimal due to the effectiveness of current controls. These costs do not apply to Australia, as dwarf bunt will not establish (see Section 6.2).

### 4.1.2 Long-term contamination of contaminated land

Spores of *T. contraversa* survive for up to 10 years in soil. Thus, once a field is infested, normal crop rotations do not control the disease. This contamination imposes the costs of control measures required for wheat crops.

These costs do not apply to Australia because dwarf bunt will not establish (see Section 6.2).

### 4.1.3 Additional costs of field control treatments

In areas where dwarf bunt is endemic, farmers must apply controls by growing resistant varieties and using fungicide seed treatments. If the resistant varieties were lower yielding and had less quality than susceptible varieties, this imposes additional costs. Similarly, a systemic fungicide that will control dwarf bunt will potentially cost more than the standard treatment to control common bunt.

These costs do not apply to Australia because dwarf bunt will not establish (see Section 6.2).

### 4.1.4 Post-harvest effects on product quality and processing

Dwarf bunt has the same effect on wheat quality as common bunt. If visually detected, the wheat would be unacceptable for human consumption and thus be downgraded to feed quality. The difference in value depends on the related market value of the grades, but feed wheat usually trades at 30–50% of the value of human consumption grades (Part 1, Section 4.1.4 of Wright *et al*. 2006).

These quality effects justify the use of regulations designed to prevent the introduction of *T. contraversa* into new areas or countries. However, these costs do not apply to Australia because conditions for establishment of dwarf bunt do not occur (see Section 6.2).

### 4.1.5 Allied industries dependent on wheat

These are unlikely to be affected, as dwarf bunt would not establish in Australia (see Section 6.2). However, an unverified report that *T. contraversa* was present in Australia could affect exports of any commodities that use the same supply line as wheat.

## 4.2 Impact on the market for wheat

The additional loss from dwarf bunt over common bunt is the potential loss of markets. Some countries require that imported Australian wheat be free of *T. contraversa*. In 2007, the countries with this requirement as reported by the Australian Quarantine and Inspection Service (AQIS, Fiona Macbeth, pers. comm.) are:

India
Sri Lanka
China
Morocco
Vanuatu
Canada
New Zealand
Poland
South Africa
North Korea

There should be no loss of international markets for Australian wheat because conditions for establishment of dwarf bunt do not occur in the Australian wheat belt. However, any unverified reports that *T. contraversa* is present in Australia could result in market access issues. This could occur if bunt from a grass admixed with wheat grain was mis-diagnosed as *T. contraversa*, and guidelines need to be in place to ensure that any diagnosis is to internationally recognised standards. Diagnostic protocols are in Part IV.

## 4.3 Impact of regulatory controls

Two scenarios for *T. contraversa* incidents in Australia are likely. The first is a detection of *T. contraversa* on imported goods. The second is an erroneous identification on Australian grain.

### 4.3.1 Detection on imported goods.

Currently, bulk imports of wheat grain are heat processed at the port of entry. This would kill all spores and eradicate the pathogen at no additional cost.

However, the finding of *T. contraversa* in another bulk commodity such as fertilizer may result in rejection of a load, at significant cost to the importer and shipping company.

### 4.3.2 Erroneous detection in Australian grain

The erroneous identification of *T. contraversa* in Australian wheat would require work to test samples to an international standard. This could range from a single sample to the large scale testing of samples collected nationally. There would be no quarantine region defined.

# 5. CONTROL

Dwarf bunt is readily controlled by a combination of host resistance, fungicides and cultural methods.

## 5.1 Fungicides

*T. contraversa* infects the wheat plant at the 2–3 leaf stage under snow cover. Spores on the soil surface are the source of primary inoculum. Thus, chemical control from a seed treatment requires a systemic fungicide (Goates, 1996).

Spores of *T. contraversa* on wheat seed can be killed by sodium hypochlorite solution (1.25%) and several contact fungicides. These prevent dispersal of *T. contraversa* on infested wheat seeds.

## 5.2 Breeding

Selective breeding has been used to produce wheat cultivars with improved resistance to dwarf bunt. There is increasing interest in resistance because of concern over the widespread use of fungicides (Goates 1996). The same genes controlling resistance to dwarf bunt are effective against common bunt (causal pathogens *T. caries* and *T. laevis*) (Goates 1996). In nature, it has been observed that common bunt and dwarf bunt can be found in the same paddock, and evidence has shown that these different *Tilletia* species can hybridise with each other and form intermediate spore morphology, physiology and effect on the plant host (Goates 1996).

## 5.3 Cultural

Disease incidence is lower with deep sowing. Early or late planting to avoid the most susceptible plant stage coinciding with environmental conditions favouring infection can been used. However, these practices are not fully effective and may reduce yield potential (Goates 1996).

# 6. PEST RISK ASSESSMENT

## 6.1 Assessment of Likelihood

### 6.1.1 Entry potential

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

- Australia imports commodities such as bulk fertilizer, agricultural machinery and some bulk feed grains and has large numbers of travellers. These materials could be contaminated with spores. Entry through the imports of bulk grain or fertilizer appears to be the most likely means of entry for *T. contraversa*, based on an assessment that this would be the most likely means of entry for *T. indica* (Stansbury *et al*. 2002), whose spores are dispersed in a similar manner.
- There is a high frequency of travel between areas in Europe and the USA where the pathogen exists and Australian farming areas.
- The pathogen is difficult to detect by visual inspection unless seen as intact or broken bunt balls in grain. A washing technique followed by light microscopy is required to detect spores (see Part IV). Thus, increased surveillance of imported goods would be expected to detect *T. contraversa* more reliably.
- Spores of *T. contraversa* 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.
- There appear to be no interceptions of *T. contraversa* in Australia.
- There are no recent reports of spread of *T. contraversa* into new areas elsewhere in the world.

### 6.1.2 Establishment potential

Establishment potential is Nil, because environmental conditions in wheat growing areas of Australia are unsuitable (see Sections 3.2 and 3.3.2).

### 6.1.3 Spread potential

Nil, because there would be no establishment.

## 6.2 Overall entry, establishment and spread potential

The risk of overall entry, establishment and spread potential is ranked Nil.

## 6.3 Assessment of consequences

### 6.3.1 Economic impact

A) Nil - The economic impact of dwarf bunt is likely to be nil because environmental conditions in the Australian wheat belt will prevent establishment of the pathogen.

B) High - A mis-identification of another *Tilletia* spore admixed with wheat as *T. contraversa* could affect export of Australian grain. The loss would depend on the number of countries who would suspend imports of Australian grain until the spores were identified to an internationally acceptable standard. Countries that regulate wheat imports for presence of *T. contraversa* are listed in Section 4.2. The short-term economic impact of such mis-identification could be high.

### 6.3.2 Environmental impact

Negligible - The area affected by any detection of *T. contraversa* on imported grain or on commodities would be negligible and the treatments to eradicate the pathogen would have minimal environmental impact.

### 6.3.3 Social impact

Negligible There would be no disruption to normal community life by a detection of *T. contraversa*.

## 6.4 Combination of likelihood and consequences to assess risks

The qualitative risk analysis was used to assess the risk as follows:

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

# 7. CONCLUSIONS

## 7.1 Surveillance

There are economic advantages associated with market access to maintain and improve surveillance to validate Australia's claim for Pest Free Status for *T. contraversa*.

The national survey system recommended for *T. indica* (Wright *et al.* 2006) would also survey for *T. contraversa* at no additional cost.

Refer to Part II –Preventative Measures" for surveillance methodology.

## 7.2 Diagnostics

Samples with suspected *T. contraversa* would need to be identified quickly and accurately. Part IV of this contingency plan describes the methods of sample preparation, initial microscopic examination, and subsequent PCR tools used to confirm the identification of the pathogen.

## 7.3 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 dwarf bunt to maximise the likelihood of early detection (see Part III for training requirements for field officers and Part IV for training needs for diagnostics).

## 7.4 Research Options

There is a need to ensure that research capability and expertise in diagnostics is maintained and developed to ensure that the Australian wheat industry is prepared for any reports of *T. contraversa.* In particular, this applies to the development of molecular procedures that will enable the rapid and reliable identification of *Tilletia spp.* that could be admixed with grain. These bunts could come from wheat or from grass weeds in crops.

Research is needed to improve the methods used in diagnosis of the bunt fungi (see Part IV).

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# National Contingency Plan for *Tilletia contraversa*, the cause of Dwarf Bunt of Wheat

# Part II

# Preventative Measures

October 2007

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The National Contingency Plan for *Tilletia contraversa* has four manuals:

Part I: Pest risk assessment, with background information on the pathogen and disease. This part contains the Executive Summary
Part II: Preventative Measures, the current and recommended actions to reduce the risk of an incursion
Part III: Field Manual, for use by the incident manager and teams in the emergency response
Part IV: Diagnostic Protocol, for use by the diagnostic laboratories

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# 1. INTRODUCTION

Preventative measures for dwarf bunt involve firstly, actions to reduce the probability of entry and secondly, research into actions to control the disease should it establish. The preventative measures are based on the biology of *T. contraversa* outlined in Part I of this contingency plan. Part I also contains the Executive Summary for the Contingency Plan.

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

*Tilletia contraversa* can enter Australia by several pathways. These pathways are the same as for *T. indica* as discussed by Murray and Brennan (1998), while Stansbury and McKirdy (2000) estimated the probability of entry for these pathways for *T. indica*. The pathways include the following:

- The spores of *T. contraversa* 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 fertilizer shipments. Spores of *T. contraversa* 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 fertilizer.

- 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, Europe, and some parts of Central Asia 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.

- It can be carried by birds and animals, and it survives passage through the intestinal tract (Smilanick *et al*. 1986). However, infected seeds and spores would pass through the gut of migratory birds long before they reached Australia.

# 3. BORDER CONTROLS/QUARANTINE

## 3.1 Introduction

In order to prevent the possible introduction of the pathogen *T. contraversa*, 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.

## 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. contraversa*.

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

Small packets of seed (100 g) are visually inspected on arrival at the PEQ facilities. This method would fail to detect any spores of *T. contraversa* 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 spore germination. However, it is not 100 per cent effective.

Where possible, a spore wash test done before treating the seed will determine if any spores are present on the imported grain. Another wash test on the final grain harvest of the plants grown in the glasshouse may be required. However, careful inspection of the seeds in this situation should reveal presence of bunt balls.

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

## 3.3 Grain/fertilizer shipments

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

Random sampling of the bulk commodities (fertilizer, grain) and using a selective sieve wash test will determine if the spores of *T. contraversa* 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 for thorough testing and cleaning.

## 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 or be dry-cleaned before being worn out in 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 spores of *T. indica* (Dr Mui-KengTan, 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. contraversa* 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* and *T. contraversa* were not detected in any of the samples tested and supports the PFA status of Australia for this pathogen. As reported in Part I, the environmental conditions in the Australian wheat belt are not conducive for the development and the establishment of dwarf bunt.

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 dwarf bunt or to declare that the pathogen *T. contraversa* 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. 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).

## 4.2 Identification of *Tilletia contraversa*

*T. contraversa* is highly unlikely to occur in Australian wheat (see Part I of this contingency plan for the environmental requirements for development of dwarf bunt). However, it is morphologically similar to some other *Tilletia* spp. that occur on wheat and grasses. If a grass affected by one of these other bunts is growing as a weed in the wheat crop, its bunt spores will be spread onto the wheat grain at harvest. A wash test will recover these spores.

These other *Tilletia* spp. could be found in surveys or when wheat is tested for the presence of bunt spores in the laboratory.

Part IV of the contingency plan contains the diagnostic protocols required to identify *T. contraversa* and distinguish it from other *Tilletia* spp.

## 4.3 General surveillance for *Tilletia contraversa*

There are many sources of pest information. These include national and local government agencies, research institutions, universities, scientific societies (including amateur specialists), producers, consultants, museums, the 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 database needs to be developed to capture all the information that is collected. The development of such a database 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 to encourage that:

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

### 4.3.1 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, part of the general surveillance strategy would be to have the staff trained to look for dwarf bunt symptoms. 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 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.

### 4.3.2 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.

## 4.4 Specific surveys for *Tilletia contraversa*

Specific surveys may be detection, delimiting or monitoring surveys. These are official surveys and should follow a plan that 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.

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

In reality, this work would be covered by the survey for *T. indica*. Therefore, there is no requirement for a separate survey or strategy to be developed. Refer to the National Contingency Plan for Karnal bunt Part II (Preventative measures) (Wright et al, 2006), for details.

# 5. BREEDING FOR RESISTANCE

This work is not required, as the pathogen will not establish under Australian conditions.

# 6. REFERENCES

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# 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$^{o}$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

### Vitavax® 200 Flowable Fungicide

Use Vitavax® 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/m³ for each 5°C the temperature is expected to fall below 21°C, or subtract the 8 g/m³ 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/m³.

## 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/m³ for each 5°C the temperature is expected to fall below 21°C, or subtract the 8 g/m³ 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.

# National Contingency Plan for *Tilletia contraversa*, the cause of Dwarf Bunt of Wheat

# Part III

# Field Manual for Incident Management

October 2007

**Written and prepared by:**

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The National Contingency Plan for *Tilletia contraversa* has four manuals:

Part 1: Pest risk assessment, with background information on the pathogen and disease. This part contains the Executive Summary
Part 2: Preventative Measures, the current and recommended actions to reduce the risk of an incursion
Part 3: Field Manual, for use by the incident manager and teams in the emergency response
Part 4: Diagnostic Protocol, for use by the diagnostic laboratories

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# 1. INTRODUCTION

The National Contingency Plan for dwarf bunt of wheat is in four parts. Part I contains the background information and Executive Summary. This section, Part III, anticipates the decisions that would arise in a 'live' situation and describes the actions required. It is most important to effectively identify the pathogen at an internationally recognised standard and, if it is a pathogen of quarantine concern, to eradicate the pathogen. Recommendations and prescribed actions are 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 dwarf bunt are outlined. The actions required over one year aim to eradicate the causal agent. The decisions for these actions are based upon the information in Part I of the contingency plan. Diagnosis of any samples arising from actions outlined in Part III are covered by Part IV of the contingency plan, 'Diagnostic Protocols'.

Areas covered include:

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

First notification of suspected dwarf bunt in Australian wheat is likely to come from extension officers, agribusiness consultants or diagnostic laboratories of State Departments of Agriculture/Primary Industries. This notification is most likely to occur from a mis-identification of the pathogen infecting the grain.

The other likely report of dwarf bunt in Australian wheat grain may come from another country after testing of the imported grain. In this case, the rapid identification of the pathogen at an International Standard may be required to maintain market access for Australian grain.

The most likely "real" detection of *T. contraversa* would be from grain imported into Australia for breeding or for animal feed. Any samples submitted for identification will require swift follow-up action.

Early containment of infected grain is vital to minimise its spread and maximise opportunities for eradication.

This is especially important because dwarf 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 dwarf bunt is shown in Table 3.1.

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

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

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

| Time | Event | Action | Section |
|---|---|---|---|
| 0 | Preliminary identification of dwarf bunt.<br>Note: Confirmation of identification will take up to 6 weeks for 2 State laboratories - international confirmation will take longer. | Reported to the Chief Plant Health Manager (CPHM) of the state/territory agriculture department.<br>The process of identification is initiated and the relevant people and organisations are notified of the suspected detection.<br>Submit two samples to other State Laboratory for confirmation, and to Central Science Laboratory, York, UK, for confirmation.<br>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.<br>Commence trace-forward and trace-back procedures from infected premises.<br>Establish movement controls in Restricted Area and Control Area. | Identification of the EPP is confirmed and the outbreak is declared.<br>The Chief Plant Protection Officer (CPPO) convenes a meeting of the Consultative Committee on Emergency Plant Pests (CCEPP).<br>The CCEPP will determine the feasibility of eradication and make a recommendation to the National Management Group (NMG).<br>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).<br>The resource requirements needed to implement the response and costing for the eradication program will be identified.<br>The NMG will approve the EPP Response Plan and national cost sharing arrangements to fund the campaign | 3 and 5 |
| Weeks 2–6 | Continue activities of week 1<br>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.<br>The Lead Agency(s) will provide regular reports to the CCEPP on the progress of the campaign.<br>If relevant, a Scientific Advisory Panel (SAP) will evaluate the effectiveness of the response and its implementation.<br>If pathogen is confirmed as not being *T. contraversa* the decision to stand down may be made. | 3 and 5 |

| | | | |
|---|---|---|---|
| Months 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.<br>Clients in the Restricted Area and Control Area are notified regarding the eradication processes that are going to occur. | 3 and 5 |
| Months 7–11 | If needed, activities continue as in Week 1.<br>Surveying continues to ensure eradication has been successful.<br>Clients 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.<br>Clients in the Control Area are notified regarding cropping options, and are advised that restrictions will be lifted in month 12. | 3 |
| Month 12 | Surveying continues to ensure eradication has been successful.<br>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. | 3 |
| 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. | 6 and 7 |

# 2. INITIAL DETECTION AND CONFIRMATION OF DWARF 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 dwarf bunt requires urgent examination and should be 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 washed 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 these and 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.

- Grain found in the bottom of cargo containers. A sample of the grain should be collected in a plastic vial or bottle and enclosed in a plastic bag. The sample if possible should be approx. 50 g at least. Pack the grain in tightly to avoid movement of the sample during delivery, because this movement damages and destroys bunt spores on the surface of grain.

- Fertilizer contaminated with grain. Collect 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 fertilizer will need to be sampled to provide sufficient grain for at least a visual examination.

- Bulk grain of other commodities contaminated with wheat grain. Collect 1–2 kg so that there is at least 50 g 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 contraversa*' of this National Contingency Plan for Dwarf Bunt of Wheat. Diagnostic laboratories are located at the Elizabeth Macarthur Agricultural Institute and the Department of Agriculture and Food, 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
Facsimile: 08 9474 2658

Samples need to be packaged to prevent movement of the grain or plants as this damages the spores of 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 dwarf bunt on grain.

## 2.4 Actions on determination of positive detection

### 2.4.1 Scenarios for detection of dwarf bunt

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

| No. | Detection scenario |
|---|---|
| 1 | Imported grain at processing plant, mill or feedlot |
| 2 | Imported grain in sub-terminal sample |
| 3 | Imported grain in port sample |
| 4 | Imported grain on ship at destination |
| 5 | In imported material on entry to Australia |
| 6 | Misidentification of fungal spores in grain load |

### 2.4.2 Steps in the event of an incursion

1. Determine immediate source (load)
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 imported grain of wheat, rye, triticale and barley and many grass species. However, this host range will change as new information comes available with the use of sequencing and PCR techniques together with morphological taxonomy. These techniques are showing that some of the bunts on grasses formerly classified as *Tilletia contraversa* belong to other species. Refer to Appendix E for illustrated guides for identification of grains of cereal crops.

The most likely event is the misidentification of the dwarf bunt pathogen in grain exported from Australia. In recent work, Pascoe *et al* (2005) have shown that the species of *Tilletia* that infects barley grass is not *Tilletia contraversa* because it differs in morphology, temperature conditions for germination and molecular biology (see Part I of the contingency plan for more information). It is also possible for non experienced pathologists to confuse the identification of the wheat common bunt fungus *Tilletia caries* with *Tilletia contraversa*. Further, there are other grass bunts with spores similar to these that could also become admixed with wheat grain.

Contaminated grain is expected leave a trail of spores in 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 contraversa*. 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 where the contamination originated. The trace forward activity identifies possible further places where dwarf bunt 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 dwarf bunt is confirmed or presumed to exist. The Infected Premises includes the entire property on which dwarf 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 dwarf 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 fertilizer/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 km radius (as used for the Karnal bunt contingency plan) 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 contaminated grain, 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 dwarf bunt in a growing crop, as it is unlikely to establish in Australia.

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 grain including 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 grain including 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 are placed into the Restricted Area.

### 3.2.2 Contact Premises

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

### 3.2.3 Suspect Premises

- If premises are found positive, the property moves into the Restricted Area.
- If premises are 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 need 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. Every load delivered may need to be tested.

- Approximately 100 samples (dependent upon size of area being sampled) are required for a wash test, which represents a composite sample from an infected premises to further validate and detect at a lower level. If the wash test is positive this property then moves into the Restricted Area.

### 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 area.

- 1 kg in every 30 tonnes in restricted and control areas.
- 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 and unloading.

Grain and seed lots should be sampled according to the International Seed Testing Association (ISTA) rules, 2006 (refer to Appendix A for details). Note that as samples are taken from increasingly larger volumes (that is, as the sampling frequency declines), dilution is occurring, which 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 second hand machinery and equipment such as headers, seed cleaners, etc, can determine the presence of dwarf 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 dwarf 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 contraversa* ("Area freedom") are outlined in Dwarf Bunt Contingency Plan Part II, section 4. 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.

## 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 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, these premises become Suspect Premises.

#### *3.5.1.2 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, these premises become Suspect Premises.

#### *3.5.1.3 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, these premises become Suspect Premises.

### 3.5.2 Trace-back operations

#### *3.5.2.1 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.

#### *3.5.2.2 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.

#### *3.5.2.3 Detected in port / importing country*

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. Planning needs to recognise that it can take at least 6 weeks before the outbreak can be formally confirmed.

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

**Note:**

- Dwarf bunt is highly unlikely to occur or establish in Australia.
- Any identification of *T. contraversa* as the cause of dwarf bunt in Australia is likely to be incorrect.
- However, dwarf bunt could be present in wheat and other commodity shipments from the USA, mainland Europe and Central Asia (Near East).

# 5. IMMEDIATE QUARANTINE MEASURES WITHIN AFFECTED AREAS

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

## 5.1 Movement controls within affected areas in year of detection

### 5.1.1 Restricted Area

- Infested grain that is transported needs to be sealed in a secure manner.
- Machinery and equipment need to be decontaminated before being allowed to leave the Restricted Area.
- Where stock have eaten contaminated grain, need to pen animals before movement of stock, because the pathogen survives passage through the animal.
- Vehicles moving out of the Restricted Area need to be decontaminated.
- 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.

### 5.1.2 Control Area

- All imported grain loads from host crops need to be tested before delivery using the selective sieve wash test method (Part IV).

### 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).

## 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.

## 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 plant debris from equipment when it is moved between premises within the Restricted Area. This will not remove all viable spores of *T. contraversa* but is designed to reduce the numbers that potentially could be moved.

Washing down to remove 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% (a.i.) solution of sodium hypochlorite and allow to stand for 15 minutes, and 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.

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 |
|---|---|---|
| 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. contraversa* spores. | |

## 5.3 Treatment of contaminated grain in year of detection

This section applies within the Restricted Area and the Control Area. Appropriate biosecurity measures must 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 Contaminated grain on farm

- Grain with dwarf 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.

### 5.3.2 Contaminated grain in silo or terminals

This grain must not flow further along the normal supply chain. 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).

### 5.3.3 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 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.

## 5.4 Imported grain for Breeders

- This seed needs to be treated with bleach for 10 minutes (1.25% 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.
- It is most important that all equipment and facilities be disinfested and cleaned up following the guidelines listed in Section 5.1.

# 6. CONFIRMATION OF ERADICATION

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

## 6.1 Grain testing

Grain testing is the most reliable method to confirm presence or absence of the pathogen within the control zone and the restricted zone. This includes visual examination with the selective sieve wash test based on international standards (EPPO PM7/29(1)) (see Contingency Plan Part IV).

## 6.2 Announcement of confirmation of eradication

The formal requirements of the announcements of the conformation or eradication will be met by the CCEPP.

# 7 ACTIONS IF ERADICATION IS NOT UNDERTAKEN OR FAILS

This event is highly unlikely due to the fact that *Tilletia contraversa* will not establish in Australia. Most likely, the identification of the pathogen being present will be due to a misidentification. Hence, the diagnostic protocol in Part IV becomes very important in this instance.

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# 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

#### *2.2.1Seed lot*

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

#### *2.2.2Primary sample*

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

#### *2.2.3Composite sample*

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

#### *2.2.4Sub-sample*

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

#### *2.2.5Submitted sample*

A submitted sample is 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).

#### *2.2.6Duplicate sample*

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

#### *2.2.7Working 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.

### *2.2.8Sealed*

Sealed means that a container in which seed is held is closed in such a way, that it cannot be opened to 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.

### *2.2.9Self-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. 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

### *2.5.1Procedures/or sampling a seed lot*

#### 2.5.1.1 Preparation / a seed lot and conditions/or sampling

At the time of sampling, the seed lot shall be as uniform 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 determined 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 A.1 shall be regarded as the minimum requirement.

**Table A.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 A.1.

When sampling seed in containers of more than 100 kg, or from streams of seed entering containers the sampling intensity according to Table A.2 shall be regarded as the minimum requirement.

**Table A.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 A.1 and A.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 mm 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.

## *2.5.2Procedure 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.

### *2.5.3Storage 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.

### *2.5.4Conditions 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 International Seed Lot 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

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

**Augers, Straw 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: VISUAL EXAMINATION GUIDE FOR SEEDS FOR DWARF BUNT

Figure A.1 Grains of wheat and sori of common bunt (sori of dwarf bunt are similar but tend to be more round). Photograph: Gordon Murray.

# APPENDIX D. DWARF BUNT: DETECTION SCENARIOS

## Detection scenarios

(a) Detection in imported grain

1. At grain processing plant, mill or feedlot
2. In plant breeding material
3. In port sample
4. On ship at destination

(b) Misidentification of fungal spores

5. In plant breeding material
6. In port sample
7. On ship at destination
8. Detection in imported material

### *1. Detection in grain at processing plant, mill or feedlot*

| First response | Response - quarantine | Survey | Trace-back/forward |
|---|---|---|---|
| • Halt delivery of grain to plant<br>• Halt operations at plant<br>• Halt movement of grain products from plant | • Isolate plant<br>• Halt movement of trucks, machinery, etc, from plant | • Collect and analyse samples from plant<br>• Check nearby silos and other local outlets that imported grain delivered | • Seed source (recent years)<br>• Movement of plant materials<br>• Machinery used in previous and current seasons<br>• Trucks used for delivery from silo<br>• Shipments from plant |

### *2. and 5. Detection in plant breeding program*

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

### *3. and 6. Detection in Grain: running sample by handling authority - grain at port*

| First response | Response - quarantine | Survey | Trace-back/forward |
|---|---|---|---|
| • Halt delivery of grain<br>• Halt movement of grain from all silos in port zone | • Isolate silos in port zone<br>• Halt movement of grain from port<br>• Halt movement of trucks, machinery, etc, in port zone | • Collect and analyse silo samples<br>• Check port storages | • Seed source (recent years)<br>• Movement of plant materials<br>• Machinery used in previous and current seasons<br>• Shipments from silo<br>• Rail trucks |

### *4. and 7. Detection in Grain: Grain on ship at destination*

| First response | Response - quarantine | Survey | Trace-back/forward |
|---|---|---|---|
| • Halt movement from source port(s)<br>• Halt all ships from port "on the water"<br>• Halt delivery of grain to port(s) | • Assume port(s) of origin affected<br>• Isolate port(s)<br>• Isolate silos in port zone<br>• Halt movement of grain from port<br>• Halt movement of grain to silos in port zone | • Collect and analyse all silo samples<br>• Check port storages<br>• Analyse all silo running samples until silo(s) identified<br>• Check nearby silos | • Seed source (recent years)<br>• Movement of plant materials<br>• Machinery used in previous and current seasons<br>• Trucks used for delivery to silo<br>• Shipments from silo<br>• Rail trucks |

## *8. 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<br>• Trace source of infection |

# APPENDIX E. 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. TRACE FORWARD AND TRACE BACK DECISIONS

**Table 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 other machinery used for this grain been moved to other farms? | No | Not pathway | |
| | | Yes | Identify location now | Sample machinery<br>Careful check of next farm visited<br>Check other farms visited |
| | Have animals been grazed on this grain? | 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<br>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<br>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<br>Check trucks, rail trucks, loading equipment |
| | Has grain been loaded on board ship? | No | Not pathway | |
| | | Yes | Identify location now | Check all ships<br>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 |
| | Other possible pathways? | No | Not pathway | |
| | | Yes | | Follow up |
| **2. At silo** | Is detection confined to one bin or stack? | Yes | Notify other silos | Follow up Check load samples |
| | | No | | |
| **3. At sub-terminal** | Which silos have shipped to this sub-terminal? | | Silos delivering | Follow up all relevant silos (check running samples)<br>Follow up<br>Follow up all: Check load samples |
| **4. At port** | Which silos have shipped to this port? | | Silos delivering | Follow up all relevant silos (check running samples)<br>Follow up all:<br>Follow up all: Check load samples |
| | What routes has grain used to get to port? | | Supply chain | Check all links in supply chain for contamination |

# National Contingency Plan for *Tilletia contraversa*, the cause of Dwarf Bunt of Wheat

# Part IV

# Diagnostic Protocol

October 2007

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The National Contingency Plan for *Tilletia contraversa* has four manuals:

Part 1: Pest risk assessment, with background information on the pathogen and disease. This part contains the Executive Summary
Part 2: Preventative Measures, the current and recommended actions to reduce the risk of an incursion
Part 3: Field Manual, for use by the incident manager and teams in the emergency response
Part 4: Diagnostic Protocol, for use by the diagnostic laboratories

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Plant Health Australia funded the project that forms the basis of Part IV of the *Tilletia contraversa* contingency plan as part of their National Diagnostic Protocols Initiative. The protocol was developed out of a project on the taxonomy of *Tilletia contraversa*, funded by Grains Research and Development Corporation. That project was generously supported by advice from Dr. Lori Carris, Washington State University, and Dr Lisa Castlebury, USDA, Beltsville Maryland.

# 1. INTRODUCTION

The purpose of this manual, which is Part IV of the contingency plan for *Tilletia contraversa*, is to provide a nationally accepted, standardised protocol for the accurate detection of dwarf bunt of wheat caused by *Tilletia contraversa* Kühn. This fungus is a quarantinable pathogen in Australia. The plan is in four parts: Part I contains the background information and the Executive Summary; Part II contains the Preventative measures required and Part III is the field manual in the event of an incursion.

Part IV, the identification protocol, is designed for easy access to the relevant sections required to identify the pathogen. It contains the primary diagnostic protocols (spore germination and sequence analysis) and secondary confirmatory methods (spore morphology by light and electron microscopy), images of spore morphology and symptoms on wheat and references and appendices.

*Tilletia contraversa* is closely related to the common bunt fungi of wheat, *Tilletia caries* and *Tilletia laevis*, and a complex of similar grass smuts. Bunt fungi (*Tilletia* spp.) are smut fungi, members of the order Tilletiales of the Ustilaginomycetes. There is variation in spore morphology within each species so that it is often not possible to identify a single spore or a small number of spores by their appearance. Spores of bunts affecting grass weeds in wheat crops often occur mixed in wheat grain harvested from such crops. Some of these grass bunts have spores that morphologically are similar to bunts that affect wheat. In particular, *Tilletia trabutii*, which affects barley grasses (*Critesion* spp.), is morphologically very similar to *T. contraversa* and has been identified as such in the past. Part IV provides the protocol to distinguish *T. contraversa* from other *Tilletia* spp.

Wheat (*Triticum* spp.) is the principal host for *T. contraversa*. Winter wheats are more susceptible while the fungus is not known to occur in spring-sown wheat. *T. contraversa* has been reported on barley in northern Utah, USA (Dewey & Hoffmann, 1975) and from a range of other grasses (Goates 1996; Vánky 1994).

Historically, dwarf bunt has only been of economic concern in winter wheat in areas where a persistent snow cover occurs regularly. Incidence is high only after there is an early and persistent snow cover. Consequently, occurrence in these areas is sporadic. No outbreaks of the disease have occurred anywhere in the world in areas where persistent snow cover does not occur. No part of the Australian wheat belt meets these climatic requirements (see Part I of this contingency plan).

The requirement for persistent winter snow cover means that it is extremely unlikely that the fungus will ever occur in the Australian wheat belt. If it does occur, it is unlikely that it will be economically damaging to crops.

However, it is not clear whether conditions might exist which will make low levels of infection possible, in which case the disease might be considered a risk to market access. Part I of the contingency plan shows that temperatures in high elevations of southeast Australia and Tasmania are in the range for *T. contraversa*, although there is no wheat grown under snow cover in these areas.

Control is primarily by use of resistant varieties. Because spores of the fungus germinate and infect from soil rather than seed, some seed treatments used for control of common bunt are ineffective for dwarf bunt and soil fumigation is impractical. A higher cost seed treatment is registered for control of dwarf bunt in the USA.

Infection of wheat by *T. contraversa* originates almost exclusively from soil infestation. The fungus survives between crops as spores in the soil and on seed. Spores can remain viable in the soil for 3–10 years in the absence of wheat (Smilanick *et al*., 1986). Seedborne spores are only responsible for infection following contamination of soil by spores from seed surfaces. Grey *et al*. (1986) reported that at least 20,000 spores per seed would be required before infection of the resulting plant is likely to occur. This is far in excess of the usual contamination levels of intact seeds originating from heavily infected crops. Introduction and establishment of dwarf bunt in imported grain is therefore extremely unlikely.

Spores typically germinate following a pre-conditioning exposure to light and at least 3–5 weeks at about 5°C. The most favourable conditions for infection are temperatures of 0–8°C (maximum 10–12°C), as found under persistent snow cover. Spores do not germinate at 15°C or higher temperatures. Dwarf bunt tends to be localised at altitudes of 300–1000 m, and years with frequent snowfalls are usually associated with serious attacks. Soil compaction and shallow seeding promote dwarf bunt infection. Most infection occurs in the winter (December to February–April, northern hemisphere) when plants are forming susceptible stem buds. These requirements were used to estimate the potential distribution of *T. contraversa* in the world and Australia in Part I of this contingency plan.

Infection of winter wheat by *T. contraversa* does not occur during seed germination to seedling emergence but only after the seedling is well established. Following penetration, mycelium passes into the crown and keeps pace with the growth of the apex until the ear is formed. A smut ball (sorus) containing spores then forms in each ovary.

A number of races differing in pathogenicity exist and continue to be distinguished.

Figure 1 shows a flow diagram of the responsibilities and procedures required when a suspect sample is received. Refer to PLANTPLAN for further details.

## 1.1 Procedure

Figure 2 shows the order of steps/ procedures to be undertaken in the diagnostic process in a flow diagram. It is important that these steps and procedures be adhered to, as a misdiagnosis could happen.

## 1.2 Documentation

An electronic and a hard copy of this manual are maintained by the Senior Systematic Mycologist, Primary Industries Research Victoria (PIRVic), Dept. of Primary Industries-Knoxfield, Victoria and a copy is kept at PHA.

All hard copies and electronic copies are controlled documents. This means the methods cannot be changed without consultation with the confirmatory laboratories and in consultation with the Senior Systematic Mycologist at DPI, Victoria.

## 1.3 Records

The Recording sheets contained in Appendix 1 must be copied and filled in as appropriate for each sample received and kept together in a file marked ‘Dwarf bunt survey’. 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.

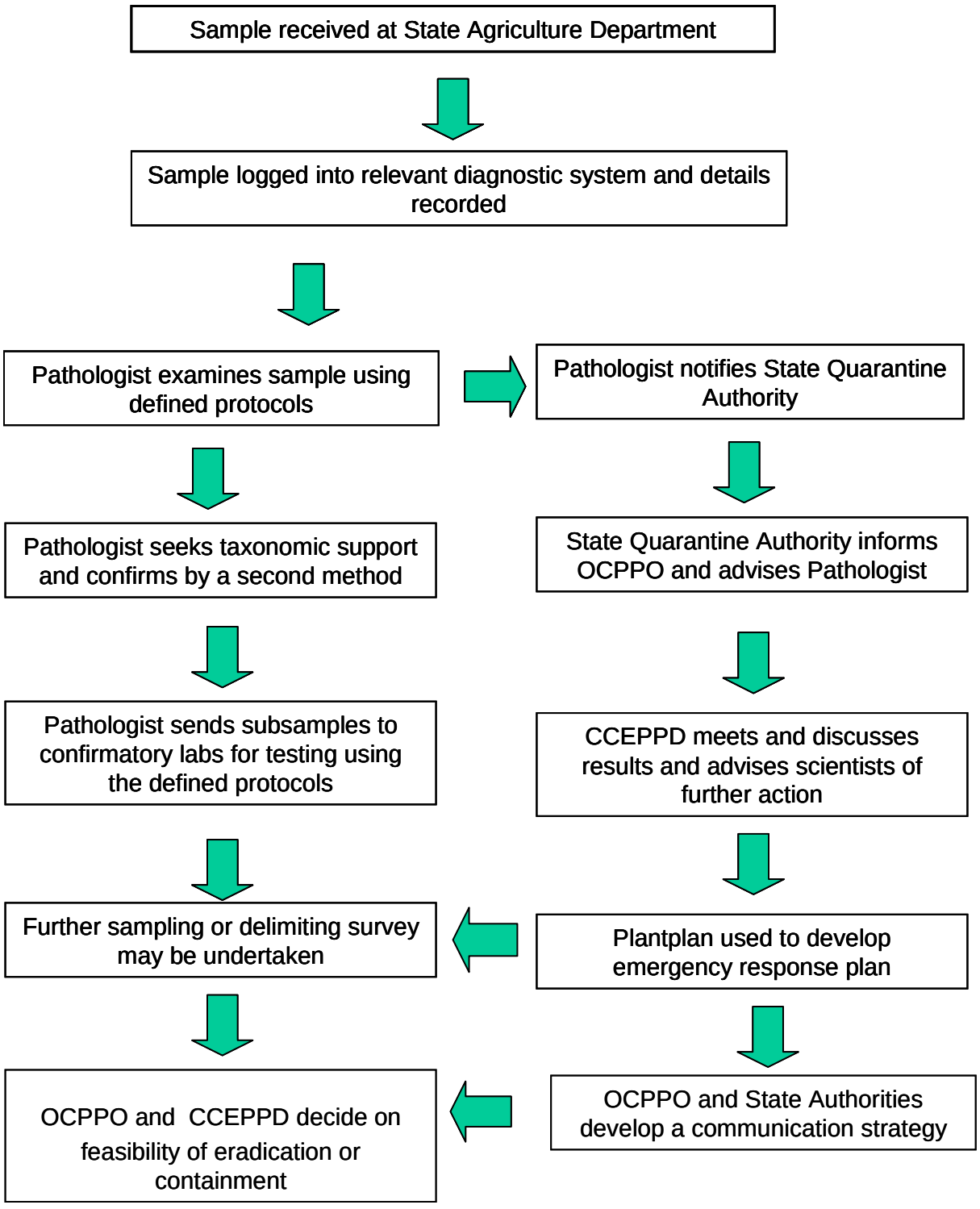


**Figure 1.1 Flow chart of the basic procedure and responsibilities of the relevant Departments if a suspect sample is received.**

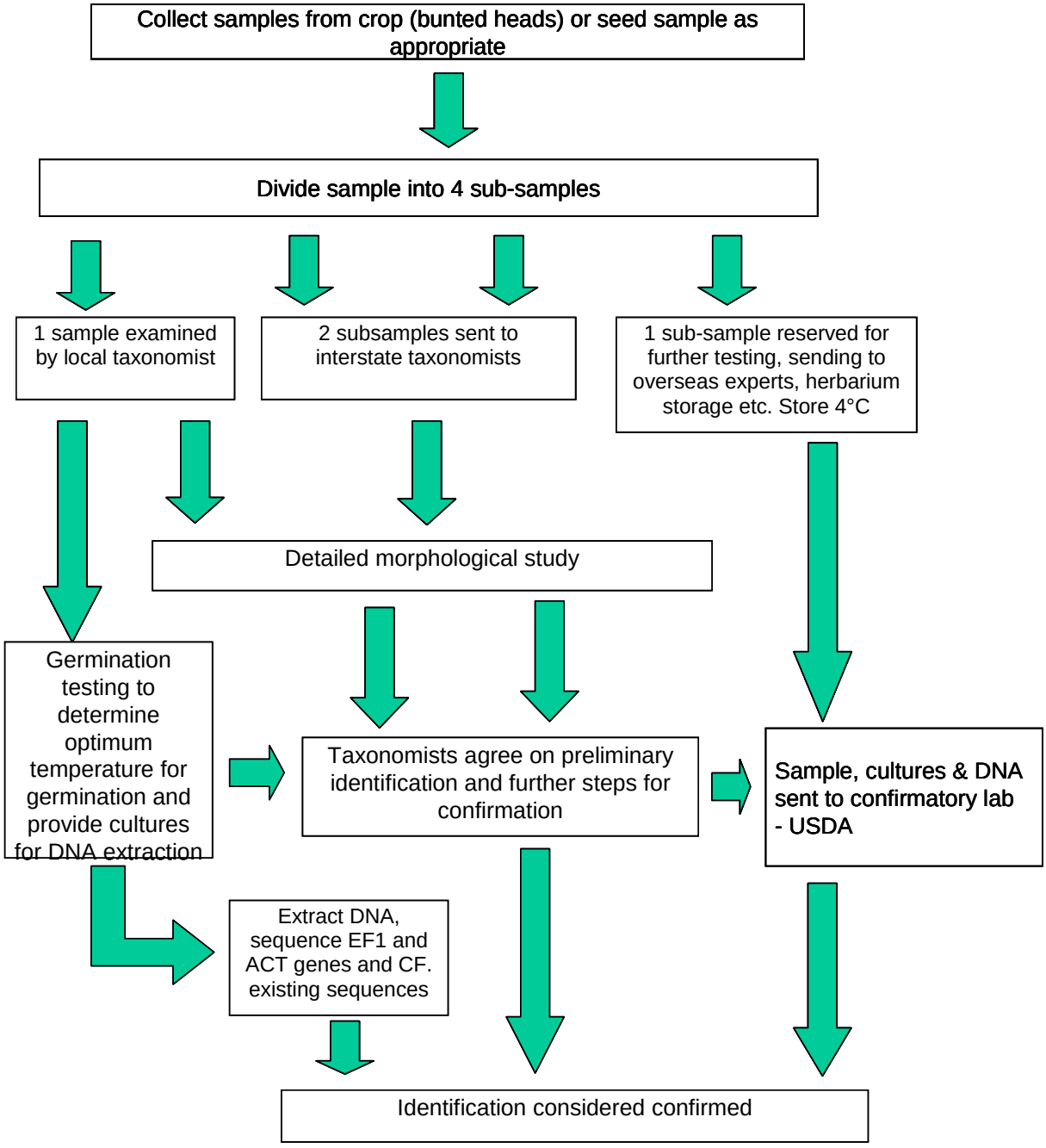


**Figure 1.2 Flow chart of protocols for the diagnosis of suspect dwarf bunt**

## 1.4 Identification

Identification is difficult because the fungus is quite difficult to distinguish from many of the *T. caries / T. contraversa* complex. Both the morphological characteristics of the causal organism and symptom expression of the host vary widely and overlap considerably with related species. The only consistent characteristics for identification are the long incubation period (at least 21 days) and the low temperature (<15°C, opt. 3–8ºC) required for spore germination. Attempts to develop rapid, reliable microscopic or biochemical techniques for identification of spores detected in routine screening of grain for export (e.g. Stockwell 1986) have had limited success and these techniques are no longer in use (B. Goates, USDA, pers. comm.).

Molecular sequencing of the ITS region and large subunit nuclear rDNA does not differentiate *T. contraversa* from related species including *T. bromi*, *T. caries*, *T. laevis*, *T. fusca*, *T. anthozanthi* and *T. holci* (Castlebury *et al*. 2005). It is necessary therefore to use combined sequences of the EF1, Actin and RPB2 genes to enable positive identification. DNA extraction is difficult from intact spores and it is necessary to germinate spores and grow the fungus in culture in order to extract sufficient DNA to carry out sequencing. Gemination of spores is therefore essential to demonstrate cardinal temperatures and time required, as well as providing cultures for DNA extraction.

Sori occur in the ovaries, usually infecting all of them in a head; mostly globose to broadly ellipsoid, covered by the pericarp; normally pulverulent when mature, but may be hard when immature; dark reddish-brown to almost black.

Spores are yellow-brown to red-brown (mature spores mostly much darker), globose or subglobose, mostly 19–24 µm (17–32 µm) diameter, mature spores are typically surrounded by a hyaline gelatinous sheath 1.5–5.5 µm thick. In median view, the exospore is reticulate, with relatively large, regular, polygonal areolae, 1.5–3 µm high and 3.5 µm diameter; areolae are occasionally irregular to subcerebriform. Morphological characters of spores vary with maturity. In particular, less mature spores are lighter in colour, the spore diameter is less, and the height of the walls of the areolae is greater. Thus, only the darkest spores of a sample should be measured.

Sterile cells are fewer and generally smaller than the spores, regularly globose, with smooth walls, hyaline or faintly greenish or brownish, sometimes encased in a hyaline, gelatinous sheath 2–4 µm thick; mostly 11–16 µm (9–22 µm) in diameter, including the sheath. For more information, see Duran and Fischer (1961).

## 1.5 Diagnostics

The relatively minor differences in spore morphology between species of the *T. caries / T. contraversa* complex make identification by morphology of spores almost impossible. Similarly the close relationships between taxa of this group means that sequencing of the ITS region cannot distinguish taxa. For these reasons, a multifaceted approach to diagnosis is essential. The following procedures are regarded as mandatory:

A) Morphological (light microscopy and SEM) – fine details of spore morphology are indicative but not diagnostic for *T. contraversa*. Principal morphological criteria include diameter of spore, height of reticulations and presence of a conspicuous sheath.

B) Germination – spore germination to determine cardinal temperatures for germination and to obtain axenic cultures of the fungus for use in molecular diagnostics. This is the most important and demanding step in the diagnostic process.

C) Molecular – multigene sequencing using EF1, Act and RPB2 genes.

D) Supplementary criteria include morphology of germination structures, symptoms and cultural characters, but these criteria are not accurately known for all related taxa.

## 1.6 Training

Training in spore extraction, morphological identification, germination procedures and culturing, and DNA extraction and sequencing can be provided using *T. caries* as a substitute for *T. contraversa*.

## 1.7 References


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10. Stockwell VO (1986) Distinguishing teliospores of *Tilletia controversa* from those of *T. caries* by fluorescence microscopy. *Plant Disease* **70**, 924–926.

11. Vánky K (1994) 'European smut fungi.' (Fischer Gustav Verlag GmbH & Co)

# 2. DETECTION OF *TILLETIA CONTRAVERSA* ON WHEAT GRAIN

## 2.1 Significance

*T. contraversa* causes the disease dwarf bunt of wheat (*Triticum* spp.), triticale (*X Triticosecale*), Rye (*Secale cereale*), barley (*Hordeum vulgare*), and wheatgrass (*Agropyron* spp.). 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 pathogens.

### 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.

#### *2.2.2.1 Sample Location*

Additional information including the detail of the sample date, location and site must be recorded on an accompanying sheet (Appendix D1), 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. It is important to record the precise location of all samples collected, preferably using GPS, or if this is not available, map references including longitude and latitude and road names should be recorded. Obtain meteorological data for the area from which the specimens came, as this is relevant to the identification.

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 may be 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
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

### 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 whichever is the longest. This is to allow further trace backs or retesting if required.

All samples received must be stored in a locked room and labelled as quarantine samples.

No special storage conditions are required for smut samples. The storage requirements will be different dependent upon the type of sample received. It is in fact preferable that they not be stored under refrigeration. The samples may be allowed to dry out, as this will not affect the accuracy of the diagnostic tests. Spores of *T. contraversa* retain viability in dried form for many years.

- Bunted heads can simply be allowed to dry in paper bags/envelopes and stored like this at room temperature.
- Whole plants should be pressed for permanent herbarium storage. Samples should not be stored in plastic bags as this may cause mould growth and adversely affect the spore germination procedure.
- Grain samples need to be stored in plastic bags or in calico bags, in dry conditions that are pest free.

If dwarf bunt or *T. contraversa* are not detected in the sample, the remainder of the sample needs to be stored in a separate box marked 'Quarantine, “not detected” dwarf 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. 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 being stored in a separate box marked 'Quarantine, “positive dwarf 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. Do not store seed with camphor. NB: the seed needs to be kept until the emergency response has been completed.

NB: 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 vitally important to record these details if further investigation is required.

### 2.2.4 Visual symptoms

Visual symptoms should be recorded and photos taken where possible. Provide a description of disease symptoms, if any, and provide images. Since the appearance of bunted heads is not particularly characteristic for dwarf bunt, most attention should be paid to recording symptoms of stunting in the wheat plants submitted.

## 2.3 Quality control

All media is quality controlled at the point of manufacture. Refer to the Work Instructions Section 3.

## 2.4 Documentation

It is important to note that proper documentation of samples and diagnostic procedures and results is initiated at this stage. See standard forms in (Appendices 1, 2, 3, 4, and specimen tracking protocol below (2.4.1).

### 2.4.1 Specimen Tracking Protocol

NB. The unique identification number for each sample is the identifier of each sample (i.e. its name) and MUST be on every piece of information and sub-sample belonging to that sample.

a) A number of samples are received. Sample details are entered into the database, unique identification numbers (labels) are printed, cover sheets are filled out, and all parts of 'the specimen' are compiled and handed to the seed examination team.

b) If the sample is grain without obvious bunt balls, each sample will require three unique identification numbers as it is split into three 50 g sub samples for further processing. Samples of bunt balls will not require division into sub samples.

c) The seed examination team splits and bags the sample into three $\times$ 50 g sub-samples and the rest is labelled 'remainder' and returned to the original bag, which must have an appropriate identification so that it can be matched with the other samples. Each sub-sample is provided with a set of labels to be applied to the bag and all paperwork, specimen tracking sheets, Petri dishes, microscope slides, etc. When examination (5.1) is completed, the three samples, associated paperwork, Petri dishes containing weed and bunted seed, and remainder are given back to the tracking team.

d) All paperwork and the presence of labels on appropriate forms, bags and Petri dishes are checked. A sample consists of the cover sheet, a sheet of labels, Petri dishes of weed/bunted seed, and the 50 g seed sub-sample all in a plastic bag.

Samples are compiled into 'one run' in a large bag (the number will depend on how many can be processed at one time) and handed to the Extraction Team.

e) Bags with the remainder are returned to the storage room and the next samples are collected.

f) Paperwork is kept in 'specimen tracking' folders at all stations. Labels are stuck on and the date and time of receival is entered. Once the sample has been appropriately processed, it is ticked and signed off by the team leader.

g) At the end of the day, a member of the specimen tracking team empties the daily work sheets from the specimen tracking folders for copying. The copy is returned to the folder and the tracking team retains the original for entry into the database.

## 2.5 Principle

The fungal pathogen is diagnosed by either morphological or PCR methods. There are two initial methods for detection of the spores:

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 spores.

## 2.6 Reagent

Unless otherwise stated all water used is sterile de-ionised water. .

(1) Water (Sterilised Tap water).

(2) Bleach (1.28%).

(3) Tween 20 Solution (Sigma Chemical Co. St Louis).

## 2.7 Equipment

Refer to operating manuals for general usage.

(1) Platform shaker.

(2) Compound microscope with 10, 20 and 40× objectives.

(3) Dissecting microscope up to 50× 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. The required speed for the centrifuge can be calculated from the following equation:

Equation for calculating Relative Centrifugal Force (× g) from Revolutions per Minute (RPM):

$$RCF = 1.12_{r\,max}(RPM/1000)^2$$

Where *r*max is the radius (mm) from the centre of the rotation to the bottom of the centrifuge tube.

(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. Labels. 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) 2x Large baby bath to hold flasks and sieves for washing up and soaking in bleach.

(15) .

## 2.8 Procedure

Both steps A and B are to be done if A is negative for bunt balls. If bunt balls are found in step A, spore extraction (step B) is not necessary because the spores from the bunt balls can go directly to microscopy (section 3.8) for identification.

### A: Direct examination of the grain

*Day 1*

Examine the submitted grain sample for bunted wheat seeds or other Poaceae seeds (for example, ryegrass). Assess the sample for symptoms of possible dwarf bunt disease. Record the presence of other seeds, lesions, such as shape, size, colour and specific patterning. Use photographs in Appendix A to compare and record possible symptoms.

(1) Enter 3 sample barcodes, the date and time in the logbook.

(2) Label a worksheet with the 3 sample barcodes, the date and time.

(3) Pour the grain sample carefully into a pile in the left-hand back corner of the plate of glass.

(4) Using the spatula, carefully move small quantities of grain towards you to the right-hand front corner of the glass, watching for any suspect grain as you go. Collect the inspected grain into the collecting tray provided.

(5) If you see any bunted or weed seeds, label the bases of 2 Petri dishes with all 3 barcode stickers. Pick out the suspect seeds with forceps and place them into the

Petri dishes — one for bunted seeds and another for weed seeds. (NB Bunted ryegrass seeds go into the bunted seed dish!). A dissecting microscope is provided if required.

(6) Record the number of bunted and weed seeds as you find them, and make any other notes that you think are appropriate.

(7) When you have visually inspected the whole sample, weigh out 3 × 50g of seed and put into the bags provided. Stick a barcode onto each bag. If any seed remains, return it to the original sample bag.

(8) Seal the Petri dishes containing bunted seeds and/or weed seeds with Parafilm.

(9) Hand everything, including all documentation, back to the designated member of the sample tracking team. The sample tracking team is responsible for returning excess seed to the storage room, and sending the samples onto to the next step for extraction procedures.

(10) Clean the work surfaces, spatula, weigh boat, etc. and spray with 70% v/v ethanol before processing the next sample.

(11) At the end of each day, the team leader must verify the logbook entries and sign. They must also sign the worksheets and have them copied. The duplicates are kept in the work area folder and the originals are to be returned to the tracking team to enter results in the database.

NB: The Team Leader is responsible for securing all samples in the storeroom and seeing that the room is locked at the end of the day.

## B: Sieve Wash Technique

Use this method if no bunt sori were found in step A.

(1) All equipment must be clean before use. Bleach sieves, funnels and flasks by immersion for 15 minutes in 1 per cent bleach.

(2) Rinse the equipment thoroughly with tap water to remove the bleach.

(3) Retrieve the samples from the specimen storage room. The original grain samples have already been weighed out into 50 g subsamples (hereinafter referred to as samples), each with its own identification number. It is not necessary to keep and process related subsamples together as they are now to be treated as independent samples.

(4) Record the sample barcodes, the date and time in the logbook and on a worksheet (see appendix 2).

(5) Pour the contents of the sample bag into a 250 mL Erlenmeyer flask.

(6) Repeat this step for the required number of samples being run concurrently. 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.

(7) Add 100 mL of Tween 20 solution to the grain in the flask and seal the flask with Gladwrap or Parafilm.

(8) 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.)

(9) 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).

(10) 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.

(11) Rinse the flask with approximately 100 mL of water and pour this into the corresponding sieve.

(12) Repeat step '11' twice.

(13) 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.

(14) Allow the sieves to drain fully. 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.

(15) Remove the top sieve, place the grain in a paper autoclave bag (labelled of course), and dry in an oven at 40°C for 24 hours. This is then to be collected and stored.

(16) Place the 53 µm sieves and the Erlenmeyer flasks into the washing up container that contains bleach. (NB, sieves need to be in a separate container as they break when placed into containers with flasks).

(17) Set up the centrifuge tubes with small funnels into a test tube rack.

(18) 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 Erlenmeyer flask to collect the water as it goes through the sieve (Figure 2.2).

(19) Wash the deposit into the centrifuge tube using as little water as possible (Figure 2.3).

(20) Alternatively, recover the suspension that collects at the edge of the sieve using a clean disposable Pasteur pipette and place into the centrifuge tube.

(21) 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.

(22) The solution left in the beaker is to be disposed into the waste disposal container for autoclaving and the flasks are to be placed into the washing up container with 1% available chlorine for at least 10 minutes.

(23) Centrifuge the tubes at 1000 × 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.

(24) Collect the tubes from the centrifuge and place in the test tube rack.

(25) 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.

(26) Leave the pellet in the labelled centrifuge tubes. Ensure the lids are on the tubes and place in the fridge ready for collection by a member of the microscopy team. Tick off the sample in the logbook.

(27) At the end of the day, the team leader must sign in the logbook that the records are correct. The duplicates stay in the lab folder and the originals are given to database administrator.

(28) At the end of each day, all waste is to be autoclaved. This can be left to run overnight. At the beginning of each day, waste is to be removed from the autoclave and disposed.

Note: The Team Leader is responsible for securing all samples and seeing that the room is locked at the end of the day.

**Figure 2.1 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 Erlenmeyer flask.**

**Figure 2.2 Washing the deposit on the membrane to one side of the 15-µm sieve.**

**Figure 2.3 Washing the deposit from the 15-µm sieve into the centrifuge tube.**

## 2.9 Results

Refer to next section on Morphological identification of teliospores.

**Units:**

All units used are SI units.

## 2.10 Validation

All techniques are standard, refer to references.

## 2.11. Reference ranges

N/A

## 2.12 Reporting

No reporting is done at this stage because the pellet needs to be examined by microscopy for presence of spores.

## 2.13 Notes

## 2.14 Glossary of terms

## 2.15 References

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

2. ISTA (2006) 'International rules for seed testing Edition 2006.' (International Seed Testing Association: Bassersdorf, CH-Switzerland)

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

4. Wright DG, Murray GM, and Tan MK (2003). 'National diagnostic protocol for the identification of *Tilletia indica* the cause of Karnal bunt.

# 3. MORPHOLOGICAL IDENTIFICATION OF SPORES

## 3.1 Significance

*Tilletia contraversa* causes the disease dwarf bunt of wheat (*Triticum* spp.), triticale (*X Triticosecale*), Rye (*Secale cereale*), barley (*Hordeum vulgare*) and wheatgrass (*Agropyron* spp.). 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 could be placed on the export of grain to other countries. However, climatic conditions in the Australian wheat belt are unsuitable for development of this pathogen: any finding of "*T. contraversa*" on Australian wheat is more likely to be an incorrect identification of another similar bunt from a grass. Nevertheless, true *T. contraversa* could be detected on imported grain or commodities.

Once the spores have been extracted from the wheat samples, they are delivered to the microscope laboratory as pellets in centrifuge tubes. Microscopists then mount the resuspended spore suspension on microscope slides. Each sample may require several (up to six) slides to ensure examination of the entire pellet. The microscopist examines the whole mount on each slide in a grid pattern so that all of the suspension on that slide is examined. Any objects resembling reticulate-ornamented spores are examined under higher magnifications, referred if necessary to a taxonomist and recorded. Any slide in which likely candidate spores are confirmed is stored for a more detailed examination and culturing. The process is described diagrammatically in Appendix C.

## 3.2 Specimen

### 3.2.1 Scope

Morphological identification of the spores 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. contraversa*. The more spores that are present in the sample after the selective sieve wash test, the more accurate the morphological identification becomes.

### 3.2.2 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 Primary Industries or The Department of Agriculture and Food, Western Australia for molecular confirmation. If the sample is found to be negative, the remainder of the sample needs to be stored 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.). This is vitally important if further investigation is required.

## 3.3. Quality control

All media are quality controlled at the point of manufacture. Refer to the Work Instructions Section 4.

## 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 spore 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. contraversa*, *T. caries* and *T. trabutii*.

## 3.5 Reagent

Unless otherwise stated all water used is sterile de-ionised water.

(1) Suspension from the Sieve wash test (Section 2.8).

(2) Distilled water.

(3) Shear's solution as an additional mountant, especially if more permanent mounts are required (e.g. for sending to taxonomist for confirmation). Because spores will be killed by Shear's solution, this should only be done if there are sufficient spores for other testing.

## 3.6 Equipment

Refer to operating manuals for general usage.

(1) Compound microscope with 10, 40 and 100× oil immersion objectives. The 100× oil immersion objective will be required for measurement of reticulation wall height if suspect *T. contraversa* is found (see section 3.9).

(2) Compound microscope with Nomarski differential interference contrast optics and/or phase contrast optics available with the 100× oil immersion objective.

(3) Dissecting microscope up to 50× magnification.

(4) Pipettes, micro (set to 20 µL) and Pasteur (long length, disposable).

(5) Microscope slides and cover slips (alternatively, a Sedgewick rafter cell can be used with a thin cover slip).

(6) Large waste disposal container containing bleach. Needs to be autoclavable.

(7) Labelled centrifuge tubes containing pellet from Section 2.8

(8) Foam holder for tubes.

(9) Containers (plastic take-away food boxes), lined with moistened paper towel, for storing examined slides pending culturing.

## 3.7 Procedure

(1) Samples arrive from extraction lab as labelled centrifuge tubes. Each tube contains the pellet of spores, some supernatant and other debris from the sieving process.

(2) Sample tubes are placed in refrigerator until microscopists ready to examine them.

(3) One sample tube only given to each microscopist. It is permissible for one sample tube to be shared between two microscopists, who will need to allocate slide numbers as they work.

(4) One slide preparation at a time is made from the tube (or two if two microscopists are working as a team).

(5) Microscopist applies label to microscope slide — stick label across slide and tear off ends.

(6) Microscopist writes initials on label, arrow pointing upwards (N direction on stage) to ensure coordinates for spores detected can be re-found.

(7) Each slide is expected to take at least 10 minutes to examine, but the examination must proceed as quickly as possible to avoid drying out of the slide. Once the slide starts to dry out the suspension starts to move and particles start to crowd together, obscuring some particles and making it likely that spores will be missed or unidentifiable.

### 3.7.1 Resuspension of the pellet

(8) Contrary to the original (Karnal bunt) protocol (Wright *et al*. 2003), it is not necessary to add extra water to the sample tube for resuspension because there is usually sufficient supernatant for examination to commence immediately.

(9) It is a good idea to stir the pellet and supernatant with the pipette tip to ensure an even suspension before withdrawing aliquots of suspension for slide preparation.

(10) Extra water may be needed if the pellet is particularly thick (to dilute the quantity of starch grains and other debris), or to resuspend the last small part of the pellet. This may be done by pipetting 20µL into the tube, stirring the pellet with the pipette tip and withdrawing another 20µL.

### 3.7.2 Slide preparation

(11) All microscopes should be set to bright field, which is the most suitable optical system for examination of spore suspensions.

(12) Place 20µL of suspension on slide and cover with a cover slip. It is important to examine every square millimetre of the suspension covered by the coverslip (if there is a meniscus of suspension outside the coverslip, check it too).

(13) Start examination in top corner of coverslip and move horizontally to the other side.

(14) Move down by one field of view, overlapping by at least 10–20% over the previous field of view, and proceed back to the other side. Repeat until the entire sample has been examined.

(15) Note every reticulate *Tilletia* spore seen at low magnification. It is almost impossible to differentiate *T. contraversa* from other reticulate *Tilletia* species at these magnifications and taxonomic assistance will be needed.

(16) Record the species seen on the slide worksheet, but only count spores of target species.

(17) Repeat steps 1 to 16 until all of the suspension has been examined.

(18) A nominated taxonomist must confirm every identification of the target species, and sign off the sample worksheet. The taxonomist will need to measure reticulation wall thickness at oil immersion magnifications in order to place the spore as a possible *T. contraversa* spore (see 5.4.1).

(19) Mark slides containing reticulate *Tilletia* spores with the abbreviation 'T'.

(20) Pass all suspect slides to taxonomist for measurement of spores and morphological characterisation.

(21) Place unmarked finished 'negative' slides in tray.

The term '**reticulate *Tilletia* spores**' is to be used to describe those spores which are similar in ornamentation to *T. contraversa*, and which require culturing to enable molecular characterisation.

### 3.7.3 Finalisation of specimen examination:

(22) Discard pipette tip into sharps container for disposal by incineration.

(23) Incomplete samples will be returned to the refrigerator for storage overnight.

(24) Return empty tube to "completed" rack on front desk for disposal by autoclaving.

(25) The taxonomist will place the suspect slides in a moist chamber to await culturing. Only slides from one sample should be placed in one chamber. The chamber must be labelled with the unique identification number for that sample.

(26) Moist chambers will be kept in the refrigerator until the culturing team is available to carry out culturing.

## 3.8 Examination of smutted grain samples:

(1) Smutted grain samples will be examined by a taxonomist for identification of spores.

(2) Results of this examination will be recorded on the sample worksheet.

(3) Any reticulate *Tilletia* seen will be identified to species by morphology, and sent to the culturing team for culturing. Taxonomists should note that accurate measurements of reticulation wall thickness can only be obtained from the most mature spores i.e. those that are darkest in colour.

(4) Smutted grain samples are returned to Petri dishes and resealed with Parafilm for storage and later disposal by autoclaving.

(5) All grain checked for bunt will be returned to and retained by the sample tracking team in case later examination is required.

NB: Reticulate teliospores detected in the wash tests of wheat grain are assumed to be *Tilletia contraversa*, *T. caries* (*T. tritici*) or *T. trabutii*. Other reticulate spored *Tilletia* species that infect grasses cannot be excluded as contaminants. Due to the size of the mesh used in the wash test, other pathogens may be 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.

## 3.9 Spore Morphology

While identification of *T. contraversa* is unreliable if based only on spore morphology, there are consistent morphological characters of spores, which can indicate a high probability of *T. contraversa*, or else eliminate *T. contraversa*. Accurate assessment of these characters requires measurement of at least 20 spores under the oil immersion objective. The most important of these characters are:

A) Presence of a conspicuous hygroscopic sheath, which extends beyond the outer limit of the spore ornamentation. This character helps to eliminate the closely related *T. caries* (*T. tritici*) which lacks a sheath. The character cannot be reliably assessed in lactic acid mounts, but is readily visible in water and optimal in Shear's solution. Phase contrast or Nomarski interference contrast microscopy will help in visualisation of the sheath.

B) Reticulate ornamentation in which the walls of the reticulations are mostly greater than 1.5 µm high in mature (dark-coloured) spores with a range of 1–2.5 µm and a median value of 1.8 µm. *T. trabutii* (from barley grass) has most reticulation heights between 1.0 and 1.3 µm. with a range of 0.6–1.8 µm and a median value of 1.1 µm.

C) Spore diameter (without ornamentation) mostly between 16.5 and 17.5 µm, with a range of 15–19.5 µm and a median value of 17µm. *T. trabutii* (from barley grass) has most spores between 17.5 and 19 µm, a range of 15.5–20.5 µm and a median value of 18 µm.

D) Ratio of spore diameter to reticulation wall height should be less than 15 for *T. contraversa* but greater than 15 for *T. trabutii*.

### 3.9.1 Method

(1) Locate candidate spores on microscope slide at 10 and 40× magnification.

(2) Change to 100× oil immersion and interference contrast or phase optics.

(3) Observe and measure the characters described above.

(4) Determine if the spores seen can be eliminated from identification as *T. contraversa* based on those characters.

(5) If the spores seen remain as possible *T. contraversa*, then continue with germination, culturing and/or molecular protocol (Section 3 and 4).

## 3.10 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 Appendix D. However, the literature has suggested that there are other reticulate-spored *Tilletia* species that are morphologically similar to those of *T. contraversa* (Pimentel *et al,* 1998).

**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 triticina* | 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 trabutii* | Barley grass bunt | |
| *Tilletia tritici* | Common bunt | **719** |
| *Tilletia walkeri* | Ryegrass bunt | |
| *Ustilago agropyri* | Flag smut | |

### 3.10.1 Symptoms

A) Spores obtained from stunted wheat plants

B) Sori more or less globose and disrupting the head so that the head looks broader than healthy heads.

### 3.10.2 Spore morphology

A) Spores have reticulate ornamentation

B) Spores have a conspicuous gelatinous sheath extending beyond reticulations when mounted in water

C) Reticulation walls of darkest, most mature spores are mostly higher than 1.5 µm

D) Ratio of spore diameter (without ornamentation) to height of reticulations is less than 15.

**Calculations:**

**Units:**

All units are SI units.

## 3.11 Validation

All techniques are standard, refer to references.

## 3.12 Reference ranges

The morphological features that distinguish *T. contraversa* and *T. trabutii* are in manuscript form and will be submitted for publication shortly as Pascoe *et al*.

## 3.13 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 results will be available in 25 working days.'

b) For negative results – 'The sample submitted was tested for *T. contraversa*. *T. contraversa* was not detected in the sample submitted'.
However, if other fungal spores such as rusts or flag smut were detected these must be reported.

## 3.14 Notes

N/A

## 3.15 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.

Reticulate: having a netlike pattern of ridges.

Tuberculate: having small rounded bumps or projections.

## 3.16 References

1. Inman AJ, Hughes KJD, Bowyer RJ (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 Dwarf bunt, ryegrass bunt and rice bunt.' www.nappo.org

3. Pimentel G, Carris LM, Levy L, Meyer R (1998) Genetic variability among isolates of *Tilletia barclayana*, *T. contraversa*, and allied species. *Mycologia* **90**, 1017–1027.

4. USDA (2002/3) 'Dwarf Bunt Manual.' (United States Department of Agriculture: Frederick, Maryland)

5. Wright DG, Murray GM, and Tan MK (2003). 'National diagnostic protocol for the identification of *Tilletia indica* the cause of Karnal bunt.

# 4. GERMINATION OF SPORES DETECTED AND MYCELIAL MAT PRODUCTION

## 4.1 Significance

*T. contraversa* causes the disease dwarf bunt of wheat (*Triticum* spp.), triticale (*X Triticosecale*), rye (*Secale cereale*), barley (*Hordeum vulgare*), and wheatgrass (*Agropyron* spp.). 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.

The procedure differs according to whether spores to be germinated have originated from grain samples (and are thus present in small numbers on microscope slides) or from bunted heads collected from a standing crop (and are thus present in very large numbers).

Germination of spores of *T. contraversa* is best done on carbon agar but water agar can also be used as a backup. The technique relies on rapid surface sterilisation of spores with sodium hypochlorite, and timing of this is critical. To be diagnostic for *T. contraversa*, germinations must be carried out at 5, 10 and 15°C under lights. Light is particularly important at the lower temperatures. *T. contraversa* should germinate best at 5°C but not at 15°C and is expected to germinate slowly, so that significant germination within 10 days would be unlikely. Once germination percentages have been assessed, the fungus is transferred to culture by placing pieces of the germination media with germinating spores, onto the lids of Petri dishes of PDA to allow spore-drop of basidiospores and/or ballistospores onto the agar surface. Subcultures of developing colonies are then made and used for DNA extraction.

## 4.2 Specimen

### 4.2.1 Scope

The spores 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. contraversa* and similar *Tilletia* spp. that can occur on grasses. 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 Primary Industries, Elizabeth Macarthur Agricultural Institute, Camden or AGWEST Plant Laboratories, Department of Agriculture, Western Australia.

### 4.2.3 Storage at laboratory

The initial sample needs to be labelled 'Quarantine pathogen' and stored in a secure place. All plates containing teliospores need to be marked in the same manner and kept in an incubator at 5°C that is labelled 'Quarantine Pathogens'.

## 4.3 Quality control

All media are quality controlled at the point of manufacture. Refer to the Work Instructions Section 4.

## 4.4 Principle

To identify the fungal pathogen, germination of spores is necessary both to obtain cardinal temperatures of germination and living cultures to allow identification by Sequencing or PCR methods. A method to directly detect and identify DNA of *Tilletia* spp. is being developed and will be incorporated in this manual when it becomes available.

## 4.5 Reagent

Unless otherwise stated all water used is sterile de-ionised water.

(1) Teliospores detected from examination of grain or selective sieve wash test.

(2) Water agar (WA) 1.5%

(3) Carbon agar (CA) prepared by addition of 1% activated charcoal to 2% WA prior to autoclaving (Boyd & Carris, 1998).

(4) Potato dextrose agar (PDA)

(5) Sodium hypochlorite at 0.3 per cent or acidified water

(6) Sterile distilled water

## 4.6 Equipment

Refer to operating manuals for general usage.

(1) Refrigerated incubators calibrated and tested at 5, 10, 15°C (±1.5°C) and provided with cool white fluorescent tubes, with timers running 12 hours light/12 hours dark.

(2) Petri dishes (90 x 15 mm, sterile)

(3) 1.5 mL Eppendorf tubes

(4) Vortex machine

(5) Benchtop centrifuge (calibrated so that speed necessary for 1200 × g is known)

(6) Needles, forceps

(7) Sterile spreader

(8) Parafilm

(9) Felt-tipped permanent marker pen

(10) Compound microscope with 10, 20 and 40× objectives

(11) Dissecting microscope up to 50× magnification

(12) Sieves 53 µm and 15 µm

(13) Centrifuge tubes (full set). Preferably, use Corning® Polypropylene tubes 15 mL with a conical bottom (not round).

(14) Test tube rack that will hold the centrifuge tubes.

(15) Small funnels. Need to fit into centrifuge tubes.

(16) Wash bottles.

(17) Pipettes, micro and Pasteur (long length, disposable).

## 4.7 Procedure

### 4.7.1 Germination of spores from microscope slides

This part of the procedure follows on from the microscopic examination of spores extracted from suspect grain (Sections 2 and 3). The procedure is difficult because in some cases there may by only one or two spores on a microscope slide and removal of these from the slide, surface sterilisation and culturing runs a high risk of losing the spores in the process. The procedure probably should not be attempted unless there are at least 20 spores on several different slides.

*Day1*

(1) Recover the suspect spores 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 '18 to 22') 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 spores and make the fungal and bacterial contaminants more susceptible to subsequent surface sterilisation.

**Note:** The next 3 steps involve surface sterilisation of spores to kill contaminating fungi and bacteria but there is a risk of killing the spores if they are surface sterilised for too long. It is important that steps 4and 5 are completed within 60 seconds in total, so that the spores are not exposed to undiluted NaOCl for long enough to reduce their viability. Speed, coordination and planning are essential. It may pay to practice on non-critical material (e.g. with a specimen of *T. caries*).

*Day 2*

(3) Centrifuge the sample for 3 minutes (1200 × 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.

(4) Then re-suspend the pellet in 10 mL of 0.3% bleach and immediately centrifuge for 1 minute (1200 × g). Quickly and aseptically, remove the supernatant using a disposable Pasteur pipette.

(5) Re-suspend the pellets in 1 mL of sterile distilled water and centrifuge for 5 minutes at (1000 × g) to wash the debris. Aseptically remove the supernatant.

(6) Repeat step '5'.

(7) Re-suspend the pellet in 1 mL of sterile distilled water. Vortex to ensure an even distribution of spores.

(8) Pipette 0.1 mL of the suspension alternately onto WA and CA plates until all suspension has been used (need at least 3 plates of each) and spread evenly using 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.

(9) Place unsealed plates upside-down in incubators at temperatures of 5, 10 and 15°C. If there are more than 3 plates from step 8, place additional ones at each temperature starting at 5°C. Set lighting to a 12h/12h light/dark cycle.

*Day 7*

(10) After 5 days incubation, remove excess condensed water from lids of upside-down plates by removing the lid and flicking the water out.

(11) Replace lids immediately and seal plates with Parafilm. Return to the incubator for another 7 days.

*Day 14*

(12) After one week, examine one replicate plate at each temperature every two days until germination is noted in any plate. Examine plates under the dissecting microscope, with the plates upright and the lid removed. Use at least 50× magnification and note any spores with clumps of basidiospores. These will be easily visible if they are upright, but harder to see if they are laying on the agar surface.

**Note:** Use only one replicate plate for this examination, as it will be necessary to remove the lid and expose the plate to contamination. Work quickly to minimise contamination risk. Keep the other plate clean and sealed up. Reseal the examined plate after each examination. Mark this plate so it can be checked again.

(13) Once germination is seen on one plate, examine all plates, counting percentage germination in randomly selected fields of view. You will need to count germinated and non-germinated spores simultaneously.

**Note:** If spores are very dense and germination is abundant, it may help to cut random discs with a cork borer (but without removing the discs) to clearly delimit an area to be counted and count the same discs each time, if necessary subdividing the discs into quarters with a scalpel blade to make counting easier.

(14) Repeat counts every 2–3 days for at least 2 weeks.

NB: For a diagnosis of *T. contraversa*, germination will probably not occur before 4 weeks have elapsed and germination will be highest at 5°C and will not occur at 15°C.

### 4.7.2 Germination of spores from bunted grains / heads

*Day 1:*

(1) Take a single intact sorus (bunt ball) from a bunted head, making sure if possible that this is from a stunted plant. Choose a sorus that has not had its outer membrane of host tissue broken and remove it gently from the spikelet with a pair of forceps.

(2) Soak the intact sorus in 1.0 mL of SDW in a 1.5 mL Eppendorf tube for at least 24h, preferably 48h. This helps to break dormancy and starts germination of contaminating fungi so that they can be more readily killed by surface sterilisation.

*Day 2 or 3:*

(3) After soaking, break the sorus wall with a needle and disperse the spore mass by gently agitating with the needle. Remove the sorus wall of host tissue with the needle and discard into a suitable container for autoclaving.

(4) Vortex the suspension for 10 seconds to ensure an even suspension and to break up any clumps of spores.

(5) Place the Eppendorf tube containing the spore suspension into the centrifuge and run at 1000 × g for 1 min.

(6) Pipette the supernatant off leaving a pellet of spores. The pellet will not be particularly solid and care must be taken to avoid sucking up part of the pellet.

**Note:** The next 7 steps involve surface sterilisation of spores to kill contaminating fungi and bacteria but there is a risk of killing the spores if they are surface sterilised for too long. It is important that steps 7–10 are completed within 60 seconds in total, so that the spores are not exposed to undiluted NaOCl for long enough to reduce their viability. Steps 11–13 must also be carried out as quickly as possible. Speed, coordination and planning are essential. It may pay to practice on non-critical material (e.g. with a specimen of *T. caries*).

(7) Add 0.5 mL aqueous 0.3% NaOCl and briefly vortex.

(8) Immediately centrifuge at 1000 × g for 30 sec.

(9) Immediately remove the supernatant by pipette, taking care not to remove any spores.

(10) Immediately resuspend in 1.0 ml SDW, briefly vortex and centrifuge at 1200 × g for 30 sec.

(11) Pipette off the supernatant, immediately resuspend again in 1.0 mL SDW, briefly vortex, and immediately centrifuge at 1000 × g for 30 sec.

(12) Pipette off the supernatant.

(13) Resuspend the pellet in 1.0 mL SDW.

(14) Briefly vortex to ensure an even distribution of spores in the suspension before pipetting 0.1 mL of the suspension alternately onto each of WA and CA plates until

all suspension has been used (need at least 3 plates of each) and spreading evenly using a sterile glass rod.

(15) Place **unsealed** plates upside-down in incubators at temperatures of 5, 10 and 15°C. Set lighting to a 12h/12h light/dark cycle.

*Day 7:*

(16) After 5 days incubation, remove excess condensed water from lids of upside-down plates by removing the lid and flicking the water out.

(17) Replace lids immediately and seal plates with Parafilm. Return to the incubator for another 7 days.

*Day 14:*

(18) After one week, examine one replicate plate at each temperature every two days until germination is noted in any plate. Examine plates under the dissecting microscope, with the plates upright and the lid removed. Use at least 50× magnification and note any spores with clumps of basidiospores. These will be easily visible if they are upright, but harder to see if they are laying on the agar surface.

**Note:** Use only one replicate plate for this examination, as it will be necessary to remove the lid and expose the plate to contamination. Work quickly to minimise contamination risk. Keep the other plate clean and sealed up. Reseal the examined plate after each examination. Mark the plate that was examined.

(19) Once germination is seen on one plate, examine all plates, counting percentage germination in randomly selected fields of view. You will need to count germinated and non-germinated spores simultaneously.

**Note:** If spores are very dense and germination is abundant, it may help to cut random discs with a cork borer (but without removing the discs) to clearly delimit an area to be counted and count the same discs each time, if necessary subdividing the discs into quarters with a scalpel blade to make counting easier.

(20) Repeat counts every 2–3 days for at least 2 weeks.

For a diagnosis of *T. contraversa*, germination will probably not occur before 4 weeks have elapsed and germination will be highest at 5°C and will not occur at 15°C. If germination occurs within two weeks and if germination is seen at 15°C, then the spores are almost certainly not *T. contraversa*. However, the rest of the procedure should be followed to get the fungus into culture for positive identification to species level.

### 4.7.3 Morphology of germination structures

(1) Locate germinating spores on the agar under the dissecting microscope.

(2) With a fine scalpel blade or a fine needle, excise a very small, shallow, piece of agar with germinating spores and place upright on a microscope slide.

(3) Add a coverslip **without** mountant to avoid disturbing the basidiospores. Do not apply heat. Examine under high magnification.

**Note:** On CA, it will be difficult to see structures clearly because of the carbon in the agar, which is why very thin preparations are advisable.

(4) Count the number of basidiospores per basidium — there should be 14–30 acicular (needle shaped) basidiospores, which conjugate forming H-shaped pairs (see Appendix B).

(5) Larger pieces of agar can also be mounted in lactic acid and heated — this will liberate the basidiospores and allow for measurement of individual basidiospores.

(6) The same technique can be used to observe structures (basidiospores, ballistospores and blastospores) on spore-fall plates.

### 4.7.4 Transfer of germinated spores to culture plates:

*Day 1*

(1) Excise several pieces of agar 5–10 mm square, bearing germinated spores from the CA plates and invert on the underside of a Petri dish lid and place over a Petri dish of PDA. Seal with Parafilm.

(2) Mark the 'drop-zone' on the underside of the plate with a felt-tipped pen so you know where to look for fallen spores.

(3) Incubate at 25°C for 3 days before examining.

(4) Rotate the lid daily to create a new drop-zone and mark the drop-zone.

*Day 4:*

(5) After 3 days incubation, examine each drop-zone daily to see if spore fall has occurred.

(6) When fallen basidiospores or ballistospores are seen and can be seen to be germinating, transfer individual spores (as many as possible up to about 20) with a fine needle to fresh PDA plates. Seal and incubate at 25°C.

**Note**

A) Colony formation will take several weeks before there is sufficient mycelium for DNA extraction. Colonies should be at least 3 mm diam. before attempting DNA extraction.

B) Remember to store any excess colonies in the culture collection for future reference.

## 4.8 Results

### 4.8.1 Spore germination

For a tentative identification of *T. contraversa*:

A) Significant germination not occurring at any temperature in less than 20 days

B) Optimum germination at 5ºC

C) No germination at 15ºC

### 4.8.2 Morphology of germination structures

Basidiospores fusing medially to form an H-shaped pair (Appendix B.3).

## 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. USDA (2002/3) 'Dwarf Bunt Manual.' (United States Department of Agriculture: Frederick, Maryland)

# 5. DNA EXTRACTION, MULTI GENE SEQUENCING AND SEQUENCE ANALYSIS

At the time of preparation of this manual, this protocol was not fully available. Staff of DPI Victoria at Knoxfield (Nigel Crump or James Cunnington) can provide assistance. The Systematic Botany and Mycology Laboratory, USDA, Beltsville (Section 6.1) can also provide assistance with molecular identification of cultures.

# 6. CONFIRMATION OF DIAGNOSIS

## 6.1 Confirmatory diagnostic test

Confirmation of provisional diagnosis made by Australian laboratory should be done by sending specimens and cultures to an internationally recognised authority. The USDA laboratories at Beltsville have the necessary expertise in morphological and molecular taxonomy to confirm diagnoses of bunts of similar morphology to *Tilletia contraversa*. The contact details are:

Dr Lisa A. Castlebury
Research Mycologist
USDA ARS Systematic Mycology and Microbiology Laboratory
Rm 304, Bldg 011A, BARC-West
10300 Baltimore Ave
Beltsville, MD 20705-2350
USA

E-mail: Lisa.Castlebury@ars.usda.gov

Telephone: +1 301 504 5270

Facsimile: +1 301 504 5810

# 7 REFERENCES AND WEBSITES

## 7.1 References

1. Boyd ML, Carris LM (1998) Enhancement of teliospore germination in wheat- and wild grass-infecting species of *Tilletia* on activated charcoal medium. *Phytopathology* **88,** 260–264.

2. Wright DG, Murray GM, Tan, M-K (2003) 'National diagnostic protocol for the identification of *Tilletia indica*, the cause of Karnal bunt. (Department of Agriculture, Western Australia)

## 7.2 Websites

1. Inman AJ, Hughes KJD, Bowyer RJ (2003) EU recommended protocol for the diagnosis of a quarantine pathogen, *Tilletia indica*. http://www.csl.gov.uk/science/organ/ph/diagpro/tipro.pdf

# APPENDIX A. PHOTOS FOR VISUAL EXAMINATION OF SEED

**Figure A.1 Grains of wheat and sori of common bunt (sori of dwarf bunt are similar but tend to be more round). Photograph: Gordon Murray.**

**Figure A.2 Ustilospores of *Tilletia* sp. on surface of wheat seed, resulting from breaking of bunt sorus during harvest (sorus could be from infected wheat or a grass weed so the spores could be from one or more different species of bunt). Photograph: Gordon Murray**

# APPENDIX B. USTILOSPORES OF *TILLETIA CONTRAVERSA* AND SIMILAR *TILLETIA* SPP.

**Figure B.1 Ustilospores of *Tilletia contraversa* from wheat by light microscopy (left) and scanning electron microscopy (right). Photographs: Ian Pascoe (left), Roger Shivas and Desley Tree (right).**

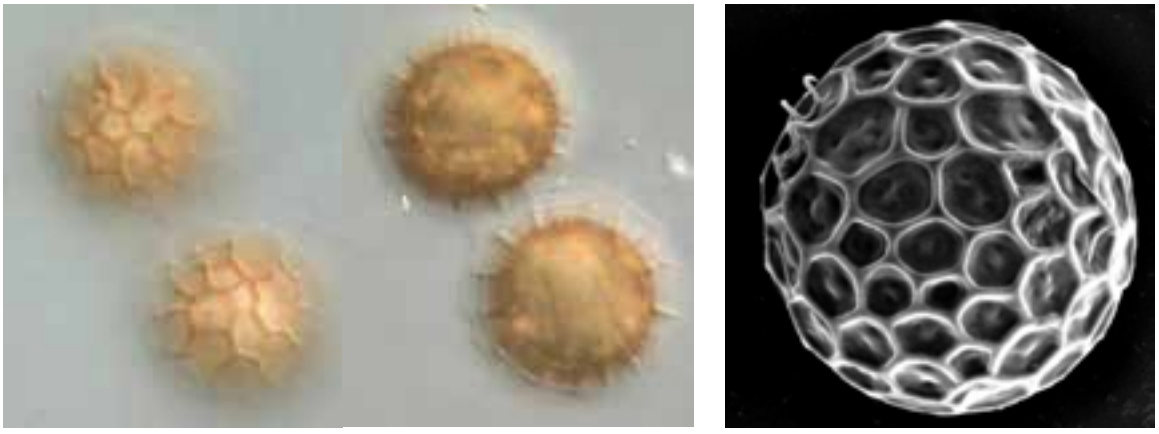


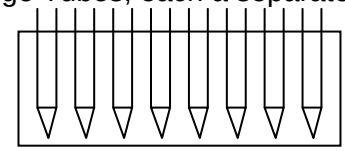
**Figure B.2 Ustilospores of *Tilletia trabutii* from barley grass (*Critesion* sp.) by light microscopy (left) and scanning electron microscopy (right). Photographs: Ian Pascoe (left), Roger Shivas and Desley Tree (right).**

**Figure B.3 Germination of ustilospores of *Tilletia trabutii* with conjugation of basidiospores. Photograph: Ian Pascoe.**

**Figure B.4 Three-week-old culture of *Tilletia trabutii*. Photograph: Ian Pascoe.**

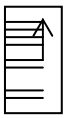
# APPENDIX C: PROTOCOL FOR MICROSCOPIC EXAMINATION OF USTILOSPORES EXTRACTED FROM GRAIN SAMPLES

(1) Centrifuge Tubes, each a separate sample, arrive from Extraction Lab.

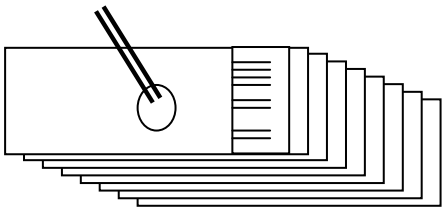


(2) One tube to each microscopist

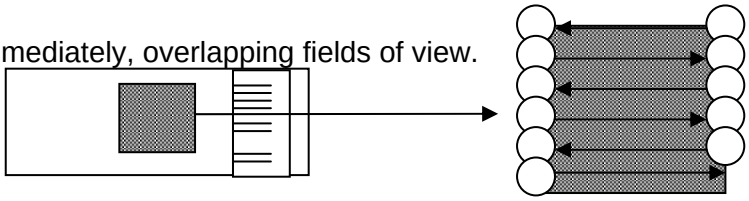
(3) Attach Barcode to slide. Place an upwards arrow in top right corner. Write slide number.



(4) Agitate with pipette tip and Pipette 20µl onto one slide.



(5) Add coverslip, examine immediately, overlapping fields of view.



(6) Identify and Record all smut/bunt ustilospores seen

(7) Reticulate Tilletia ustilospores seen??

**YES**

**NO**

(8) Examine with High power

(9). Mark slide

(10) Note stage coordinates

(11) Notify taxonomist, taxonomist to confirm ID and sign record sheet

(12) If *Tilletia contraversa*-like ustilospores seen, mark slide with 'T'

(13) Retain slide in moist chamber

(14) Refrigerate

(15) Return to 4. Repeat until all of sample examined.

(16). Negative slides retained until culturing complete.

(16). 'Positive' slides moved to culturing lab.

# APPENDIX D. WORKSHEETS FOR RECORDING DATA

Appendix D.1 Preliminary Information Data Sheet (PlantPlan 2004)

Appendix D.2 Recording Sheet for Sample Receival

Appendix D.3 Tracking Form, Visual Examination of Grain

Appendix D.4 Tracking Sheet, Extraction Technique

Appendix D.5 Ustilospore Germination, Tracking and Recording Sheet

## Appendix D.1 Preliminary Information Data Sheet (PlantPlan 2004)

Date: / / SUBJECT

Site details:

Ownership:

Location:

Map (lat. & long.):

GPS identifier:

Host plant location (clearly mark plant if necessary):

Winter weather conditions: (snow cover?)

HOST DETAILS

Species and variety:

Age:

Developmental stage:

DAMAGE

Description of symptoms:

Part of host affected:

Percent incidence:

Percent severity:

DETAILS OF WHEN AND WHERE THE PEST WAS FIRST NOTICED:

RECORDS OF PRODUCT MOVEMENT ON AND OFF DETECTION SITE:

SYMPTOMS / PHOTOGRAPHS:

FURTHER DETAILS OR COMMENTS:

## Appendix D.2 Recording Sheet for Sample Receival

| | | | |
|---|---|---|---|
| Barcode: | | Date of sampling: | |
| Host:<br>Botanical name: | | Host:<br>Common name | |
| Country of origin: | State (if applicable): | | |
| Quantity of consignment (kg, t) | | | |
| Consignment no./Lot or batch no.: | | | |
| Receiving Laboratory: | | State: | |
| Receiving Officer: | | Position: | |
| Sample size (g): | | Date sample examined: | |
| Symptoms and other comments: | | | |
| Results from microscopic examination of grain: | | | |
| Results from sieve wash test: (Number of reticulate Tilletia spores seen) | | | |
| Morphological identification of spores (attach working sheets): | | | |
| Results of spore germination test: | 5°C | 10°C | 15°C |
| Time until germination recorded (days) | | | |
| Germination percentage | | | |
| Culturing:<br>Number of colonies obtained | | No. of colonies to PCR: | |
| Results of DNA sequencing: | | | |
| Confirmed diagnosis/comments: | | | |
| Cultures sent to other lab? (name of lab, date, contact person) | | | |

## Appendix D.3 Tracking Form, Visual Examination of Grain

| Barcode | Date | Sign when complete | Suspect Bunted Seed? |
|---|---|---|---|
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# Appendix D.4 Tracking Sheet, Extraction Technique

| Barcode | Date Commenced | Date Completed | Signed |
|---|---|---|---|
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## Appendix D.5 Ustilospore Germination, Tracking and Recording Sheet

| | | | |
|---|---|---|---|
| Barcode: | | Ustilospores plated: (Date) | Signed: |
| Examined: | | | |
| Date: | Sign: | Date: | Sign: |
| Date: | Sign: | Date: | Sign: |
| Date: | Sign: | Date: | Sign: |
| First Germination Noted: | ºC | Date: | Sign: |
| Germination Counts: | | | |
| Date: | Germinated: | Ungerminated: | % |
| 5ºC | | | |
| 10ºC | | | |
| 15ºC | | | |
| Date: | Germinated: | Ungerminated: | % |
| 5ºC | | | |
| 10ºC | | | |
| 15ºC | | | |
| Date: | Germinated: | Ungerminated: | % |
| 5ºC | | | |
| 10ºC | | | |
| 15ºC | | | |