ACKNOWLEDGMENTS

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Figure 8. *Sitona lineatus*, vector of BBTMV.
1.0 Introduction

**Broad bean true mosaic comovirus** (BBTMV) is characterised by a very narrow host range in the Fabaceae. The natural host of BBTMV is faba bean (*Vicia faba*), although Quantz (1953) and Gibbs *et al.* (1968) found that some pea varieties (*Pisum sativum*) became infected in the field and by artificial mechanical inoculation. Other species in the Fabaceae are susceptible (Brunt *et al.*, 1997). BBTMV is one of a number of viruses which are seedborne in faba beans and it is also seedborne in vetch (*V. sativa*) but not in peas.

BBTMV occurs mainly in Europe and has been reported as the cause of significant virus disease problems in faba bean production in Great Britain and western and eastern European countries. It has also been reported in virus surveys in north Africa, but generally at low incidence and has been reported from China. BBTMV has not been found in surveys of a number of Middle East countries and is not reported from the United States of America.

BBTMV is one of a number of seedborne viruses, which infect temperate pulse crops and cause mottle/mosaic symptoms. BBTMV was first described in *Vicia faba* crops in Germany in 1953 (Quantz, 1953) and was then found in England in 1959 and 1960 (Gibbs *et al.*, 1968). In 1960, Gibbs *et al.* (1968) found a second virus in faba bean crops, which they named broad bean stain virus (BBSV), which was only distinguishable from BBTMV by the symptoms it caused on infected seed. BBTMV and BBSV are both comoviruses but are not serologically related. They have similar isometric particles, cause indistinguishable symptoms on broad beans (*Vicia faba L. major*) and usually in field beans (*Vicia faba L. minor*) and are both transmitted through seed and by adult weevils of the genera *Apion* and *Sitona* (Cockbain *et al.*, 1975). Both BBTMV and BBSV may be distinguished from other viruses which cause mosaic leaf symptoms by the telltale chewing of the leaf margins by their weevil vectors.

A number of strains of BBTMV have been reported, based on symptoms. Symptoms range from infected leaves developing a mild green mottle or mosaic or irregular patches of chlorotic tissue and malformations with infrequent mottle stuntning to severe dwarfing and deformity of broad bean, pea and summer vetch plants. All isolates of BBTMV appear to be serologically closely related (Jones and Mitchell, 1994). BBTMV is serologically related to the yellow strain of cowpea mosaic virus (CpMV), glycine mosaic virus (GMV), and bean curly dwarf mosaic virus (BCDMV) (Section 3.9.3).
The standard detection methods for BBTMV are serological tests. A number of different ELISA procedures have been found to be satisfactory (Kumari, Makkouk, 1993). A second serological test, tissue blot immunoassay (TBIA) (Makkouk, Comeau, 1994), has been used for field surveying for BBTMV (eg. Makkouk et al., 2003, Tadesse et al., 1999, Ouizbouben, Fortass, 1997). Sequence data are not available for BBTMV, therefore PCR is not used as a diagnostic test. Due to the limited host range of BBTMV herbaceous indicator plant tests are also not used as a diagnostic test. BBTMV particles are morphologically indistinguishable from BBSV, therefore electron microscopy is only suitable as a confirmatory test unless immunosorbent electron microscopy is used.

Control of BBTMV in pulses is undertaken by sowing healthy seed and undertaking crop management practices which reduce alternate sources of the virus (eg weed control, proximity to other crops, etc) and control of the vector.
2.0 National Diagnostic Protocol Procedure

2.1 Purpose and scope of diagnostic protocol
The purpose of this manual is to provide a nationally accepted, standardised protocol for the accurate detection of broad bean stain virus in temperate pulses. BBTMV is a quarantinable pathogen in Australia and is routinely tested for in the post-entry quarantine program at the DPI Temperate Pulse Quarantine Station, Horsham, Victoria, using ELISA. The manual is designed for easy access to the relevant sections required to identify the pathogen. The manual contains the Pest Risk Analysis for broad bean true mosaic virus for Australia, the primary diagnostic protocols (ELISA) and tissue blot immunoassay (TBIA), secondary confirmatory methods (electron microscopy), images of virus symptoms on host plants and seeds, and virus particle morphology and references and appendices.

2.2 Responsibility
Figure 1 shows a flow diagram of the responsibilities and procedures required when a suspect sample is received. The responsibilities are also listed quite clearly in the following points:

A: State/territory agriculture departments receiving suspect plant sample:
- Receiving scientists will record details of the sample so that a trace back can occur if required.
- Receiving scientists will examine the sample and provide diagnostic services (in this case, conducting the serological tests ELISA and/or TBIA) to identify the pathogen.
- Receiving scientists will notify the State Quarantine Authority (eg. DPI-Victoria Plant Standards Branch) of the suspect sample.
- The State Quarantine Authority will examine the evidence and inform the Office of the Chief Plant Protection Officer (OCPPO) and AQIS and advise scientists of required action.
- The State Quarantine Authority will participate in the Consultative Committee on Exotic Plant Pests and Diseases (CCEPPD), chaired by the Chief Plant Protection Officer and decisions made and actions required will be passed onto state scientists for action.
- Scientists may be requested to provide expert advice to the CCEPPD.
- Scientists will conduct a second type of diagnostic test (secondary confirmatory test) as advised by the State Authority.
- Scientists will send part of the sample to the interstate confirmatory laboratories for repeat of the primary diagnostic test as advised by the State Authority.
• Under direction from the State Authority, state scientists will undertake delimiting surveys if required and undertake diagnostics on survey samples.
• The State Authority will liaise with industry representatives.
• The State Authority will develop communication strategies in conjunction with the CCEPPD.
• The State Authority will report to all interested parties (OCPPO, CCEPPD, AQIS, national bodies and industry) as required.
• The State Authority will keep up to date with the processing of the suspect sample and will notify the clients of the final result and the corresponding decision for that result.
• The State Authority will handle all correspondence with clients. This is very important and is to be made clear to other personnel involved with handling the sample that they are not to correspond with the client.

• **B: Interstate agriculture departments**
  • Scientists will re-examine the suspect sample.
  • Scientists will repeat diagnostic tests and confirm diagnosis.
  • Scientists may be requested to provide expert advice to the CCEPPD.
  • State Quarantine Authority will inform the Chief Plant Protection Officer and the CCEPPD and will implement their decisions.

• **C: Office of the Chief Plant Protection Officer (OCPPO)**
  • OCPPO will convene the CCEPPD and all decisions regarding the steps involved in handling and diagnosing the original sample will be made by the committee.
  • The CCEPPD will determine whether or not the incursion requires a national response or involves only one state and will determine the need for delimiting surveys.
  • Information from each state will be provided to the CCEPPD to enable national decisions to be made.
  • OCPPO will provide media releases to the public and interested parties.
  • OCPPO and the CCEPPD will determine whether or not the pathogen can be eradicated, contained or will be declared endemic.

**2.3 Diagnostic Procedure**

Figure 2 shows the order of steps/procedures to be undertaken in the diagnostic process in a flow diagram.
2.4 Documentation
An electronic and a hard copy of this manual are maintained by the Senior Virologist, Primary Industries Research Victoria (PIRVic), Dept. of Primary Industries-Horsham, Victoria and PHA.

2.5 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 “Suspect broad bean true mosaic virus samples”.
Figure 1. Flow chart of the basic procedure and responsibilities of the relevant Departments if a suspect sample is received.
Collect samples from crop (shoot or leaf) or seed sample as appropriate

Divide sample into 3 subsamples

Long term storage
Freeze or dry

Store sample at 4°C until processed

Sample sent to confirmatory lab

Identify initial samples using ELISA and/or TBIA
Confirm diagnosis using EM

Test survey samples using ELISA or TBIA

Figure 2. Flow chart of protocols for the diagnosis of suspect BBSV-infected plants
3.0 Pest Risk Analysis

3.1 Background
Broad bean true mosaic comovirus is listed on the Australian Quarantine and Inspection Service (AQIS) ICON Import Conditions database as a quarantinable pathogen in Australia. BBTMV is tested for in post-entry quarantine in all hosts in which it is seedborne (Vicia species) as required in the regulations listed on ICON.

3.2 Species name
Broad bean true mosaic virus (Genus Comovirus, Family Comoviridae)

3.3 Synonyms
Echtes Ackerbohnenmosaik-Virus, true broad bean mosaic virus, Vicia virus 1, Viciavirus varians

3.4 Common names
None.

3.5 Host range

<table>
<thead>
<tr>
<th>Host</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vicia faba var major</em> (broad bean)</td>
<td>Edwardson JR, Christie RG (1991)</td>
</tr>
<tr>
<td><em>Vicia faba var minor</em> (tick or field bean)</td>
<td>Cockbain <em>et al.</em> (1976), Jones AT (1977),</td>
</tr>
</tbody>
</table>
Edwardson JR, Christie RG (1991)

*Vicia sativa* (vetch) Cockbain *et al.* (1976)

### 3.6 Distribution

#### 3.6.1 Australian status

Exotic

#### 3.6.2 Current distribution

<table>
<thead>
<tr>
<th><strong>Regions:</strong></th>
<th><strong>Countries:</strong></th>
</tr>
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<tbody>
<tr>
<td>Africa, Europe, Middle East, Asia</td>
<td>Brunet <em>et al.</em> (1997)</td>
</tr>
<tr>
<td><strong>Countries:</strong></td>
<td></td>
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<tr>
<td>Austria</td>
<td>Wodicka B (1984)</td>
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<tr>
<td>China</td>
<td>Liang XY (1986)</td>
</tr>
<tr>
<td>Ethiopia</td>
<td>Abraham <em>et al.</em> (2000)</td>
</tr>
<tr>
<td>Egypt</td>
<td>El Shaieb <em>et al.</em> (1981)</td>
</tr>
<tr>
<td>Hungary</td>
<td>Simay El, Beczner L (1993)</td>
</tr>
<tr>
<td>Italy</td>
<td>Gallitelli <em>et al.</em> (1978)</td>
</tr>
<tr>
<td>Morocco</td>
<td>Fischer HU, Lockhart BE (1976)</td>
</tr>
<tr>
<td>Poland</td>
<td>Blaszczak W, Jamrog-Janicka K (1972), Blaszczak W, Fiedorow Z (1979)</td>
</tr>
<tr>
<td>Tunisia</td>
<td>Najar <em>et al.</em> (2000)</td>
</tr>
<tr>
<td>UK</td>
<td>Bailiss KW, Senanayake S (1984)</td>
</tr>
</tbody>
</table>
3.6.3 Potential distribution in Australia

As per host plants- temperate and sub tropical grain belt (See Figure 3 below).

Figure 3. Potential distribution of BBSV in Australia
### 3.7 Plant parts affected

#### 3.7.1 Vegetative

All

#### 3.7.2 Seedborne

<table>
<thead>
<tr>
<th>Plant</th>
<th>Authors</th>
</tr>
</thead>
</table>

### 3.8 Disease features

Broad bean true mosaic virus is one of a number of seedborne viruses, which infect temperate pulse crops and cause mottle/mosaic symptoms. BBTMV was first described in *Vicia faba* crops in Germany in 1953 (Quantz, 1953) and was then found in England in 1959 and 1960 (Gibbs *et al.*, 1968). In 1960, Gibbs *et al.* (1968) found a second virus in faba bean crops, which they named broad bean stain virus (BBSV), which was only distinguishable from BBTMV by the symptoms it caused on infected seed. BBTMV and BBSV are not serologically related but have similar isometric particles, cause indistinguishable symptoms on broad beans (*Vicia faba* L. *major*) and usually in field beans (*Vicia faba* L. *minor*) and are both transmitted through seed and by adult weevils of the genera *Apion* and *Sitona* (Cockbain *et al.*, 1975). The natural host of BBTMV is *Vicia faba*, although Quantz (1953) and Gibbs *et al.* (1968) found that some pea varieties became infected in the field and by artificial mechanical inoculation. Other species in the Fabaceae are susceptible (*Cicer arietinum, Lens culinaris, Lupinus albus, Melilotus albus, Phaseolus vulgaris, Pisum sativum, Trifolium incarnatum, Trifolium repens, Vicia faba, Vicia sativa, Vicia villosa*) (Brunt *et al.*, 1997).

### 3.9 Biology

#### 3.9.1 Identification

Identification of the virus is based on serological reactions. BBTMV has frequently been confused with another exotic pulse comovirus, BBSV, which is indistinguishable by symptoms and particle morphology but which is serologically distinct (Cockbain *et al.*, 1972,
Cockbain et al., 1976, Fischer HU, Lockhart BE, 1976). Mixed infections may occur and careful diagnosis is required. BBTMV is not serologically related to a number of other comoviruses (bean-pod mottle virus, radish mosaic virus, red clover mottle virus, squash mosaic virus, clover mild mosaic virus) (Gibbs et al., 1968, Gerhardson, 1977) and is serologically related to one of the two strains of cowpea mosaic virus (CpMV). No sequence data for BBTMV is listed on the Genbank database and there do not appear to be any published PCR tests for BBTMV diagnosis. BBTMV has a very limited host range restricted to the Fabaceae and no diagnostic indicator plants are known.

3.9.2 Virus strains

Jones and Mitchell (1994) found a strain of BBTMV (SB) in Scotland which differed from other strains in a number of characteristics including infecting some non-leguminous hosts (several Nicotiana and Chenopodium species) but it was found to be serologically identical to the other strains.

3.9.3 Serological relationships

Although indistinguishable in other characteristics, BBTMV and BBSV are not serologically related (Cockbain et al., 1975, Pospieszny H, 1983, Jones AT, Mitchell MJ, 1994). Jones and Mitchell (1994) found that a coat protein variant isolate of BBTMV was serologically indistinguishable from other isolates from England, Scotland and Germany, suggesting little serological variation within BBTMV. Jones and Barker (1976) found that BBTMV and BBSV were both related to strains of cowpea mosaic virus (CpMV). CpMV has two distinct strains: a yellow strain and a severe strain (Agarwal, 1964). Jones and Barker (1976) found that BBTMV was related to the yellow strain and not related to the severe strain of CpMV and that BBSV was related to the severe strain but not to the yellow strain. Glycine mosaic virus (GMV) is a comovirus from Australian native Glycine species, and although it has not been found naturally occurring in legume crops, it was transmitted to a number of leguminous species by mechanical inoculation and reacts with antisera to BBSV, BBTMV, and squash mosaic virus (Bowyer JW et al., 1980). Meiner et al. (1977) found that bean curly dwarf mosaic virus (BCDMV), a comovirus of common bean (Phaseolus vulgaris) found in El Salvador, was serologically related to BBTMV. BBTMV is not serologically related to a number of other comoviruses (bean-pod mottle virus, radish mosaic virus, red clover mottle virus, squash mosaic virus, clover mild mosaic virus) (Gibbs et al., 1968, Gerhardson, 1977).
3.9.4 Symptoms
Gibbs et al. (1968) and Cockbain et al. (1976) reported that, on broad beans (Vicia faba var. major) and field beans (Vicia faba var. minor), BBTMV infected leaves develop a mild green mottle or mosaic, other leaves may be malformed with irregular patches of chlorotic tissue or remain symptomless. Stunting was very rare and Cockbain et al. (1976) surmised that the few very stunted plants which occurred may not survive. Symptoms are more severe during cold weather and may disappear in hot weather and symptoms also tend to be cyclical during the life of the plant. Blaszczyk (1972) reported two strains of BBTMV in Poland, which caused severe dwarfing and deformity of broad bean, pea and summer vetch plants. Gibbs et al. (1968) reported that infected Pisum sativum plants develop systemic chlorotic mottling and mosaic, occasionally necrosis, wilting and stunting. Blaszczyk et al. (1985) found that ten pea cultivars were all susceptible to BBTMV and although the effect on plant height, plant weight and number of plants producing seed varied between cultivars, BBTMV always greatly reduced seed yield but was never seed transmitted.

BBTMV appears to be seedborne only in Vicia species and seeds infected with BBTMV do not develop the necrotic patches or stains on the seed coat associated with BBSV infection (Gibbs et al., 1968). The Rothamstead Experimental Station (1985) reported frequent infections of BBTMV in seedlots (47% and 32% of broad bean and field bean seedlots tested). Jones (1978) reported that losses in yield from BBTMV infection in V. faba were largely due to fewer seeds per pod whereas Bailiss and Senanyake (1985) found yield loss was due to decreased seed size rather than number. They also found that seed yield was decreased or abolished when plants were infected with BBTMV pre- or early bloom. Mali et al. (2003) found that BBTMV was seed transmitted in a range of faba bean and vetch cultivars, at rates of up to 28% of seed. They were also able to inoculate common bean (Phaseolus vulgaris) with BBTMV but the virus was never seed transmitted.

3.9.5 Disease cycle
BBTMV is an obligate plant pathogen, surviving in host plants and seed. Its host range is very narrow and limited to the Fabaceae and it is only seedborne in Vicia species. It appears most likely to survive between cropping seasons in infected Vicia seed (Cockbain et al., 1975, Cockbain et al., 1976). BBTMV has been reported to be transmitted by the weevils Apion vorax and Sitona lineatus and A. vorax is a much more efficient vector than S. lineatus (Cockbain et al., 1975, Jones AT, 1978). The adult weevils feed on plant tissue and spread the virus as they move from plant to plant.
3.9.6 Dispersal
BBTMV has been reported to be transmitted by the weevils *Apion vorax* and *Sitona lineatus* (Edwardson JR, Christie RG, 1991). Cockbain *et al.* (1975) found that *Apion vorax* and *Sitona lineatus* were the most common vectors of BBTMV and BBSV but that *A.vorax* was the more efficient vector. They found that five other species of *Apion* transmitted the viruses infrequently or not at all and that the viruses were not transmitted by aphids (*Aphis fabae* and *Acyrthosiphon pisum*) or pollen beetles (*Meligethes* spp.).

3.10 Assessment of likelihood

3.10.1 Entry potential
HIGH: BBTMV is seedborne in *V. faba* and *V. sativa*.
Cockbain *et al.* (1976) found that of 12,600 *V. faba* seeds sown in the glasshouse between 1972 and 1974, 0.44% produced BBTMV infected seedlings and in other experiments with *V. faba* they found up to 3% seed transmission. Vorra-Urai and Cockbain (1977) reported experimental seed transmission rates of up to 5.3% in broad bean (*V. faba var. major*) when 4 week old seedlings were inoculated in the glasshouse. Jones (1978) inoculated field bean plants (*V. faba var. minor*) 3, 5, 7 and 11 weeks after emergence and found 0.5%, 2.1%, 0.6% and 0% BBTMV infected seed at harvest. Mali *et al.* (2003) found that BBTMV was seed transmitted in a range of faba bean and vetch cultivars, at rates of up to 28% of seed. They also found that BBTMV was not seed transmitted in common bean (*Phaseolus vulgaris*).

3.10.2 Host range potential
LOW: BBTMV has a limited host range in a small number of closely related hosts in the Fabaceae family. El-Shaieb *et al.* (1981) tried to mechanically inoculate a wide range of plant species but were only successful in transmitting the virus to ten leguminous species. Thirty six other species in seven families were not susceptible. Gallitelli *et al.* (1978) were also only successful in mechanically inoculating BBTMV to a small number of leguminous species.

3.10.3 Establishment potential
HIGH: High seed transmission rates have been reported in major crop species (*V. Faba* and *V. sativa*) therefore establishment is likely (Edwardson JR, Christie RG (1991), Vorra-Urai S, Cockbain J (1977) Mali *et al.* (2003).

3.10.4 Spread potential
LOW: The pest has potential for natural spread locally. The known vectors are not present in Australia. The main source of introducing this virus into disease-free areas is through infected seed (Naumann I (1993). It is expected that only localised infection would occur where infected seed is sown, with no secondary spread in the field. Further spread would depend on the rate of seed transmission and distribution of the harvested seed. However, if the *Apion vorax* or *Sitona sp.* vectors were also brought into Australia, the spread potential would be much higher. There are no data on the only endemic *Sitona* species, *Sitona discoideus*, as a potential vector of BBSV. However, the preferred hosts of *S. discoideus* are lucerne (*Medicago sativa*) and burr medic (*M. polymorpha*), which are not hosts of BBTMV.

**3.11 Overall entry, host range, establishment and spread potential**

The overall pest rating is MEDIUM (ratings based on PHA Industry Biosecurity Planning Guide) or LOW based on Biosecurity Australia ratings.

**3.12 Assessment of consequences**

**3.12.1 Economic impact**

LOW: There is minor impact on yield, host longevity, production costs or storage. The data below show the potential impact of BBTMV on crop production, when the vector is present, which can be very high. However, the vectors do not occur in Australia, therefore the maximum yield loss is based on the maximum level of seed transmission (28%, Mali *et al.* 2003). If the beetle vectors entered Australia with infected seed then the economic impact would be much higher, as indicated in the following research. Cockbain (1972) found that BBSV and BBTMV were indistinguishable in broad bean crops examined in England in 1969-1970 and that their combined within crop incidence ranged from 2-92% of plants and that early infection by either virus can decrease yield by up to 70%. Blaszczak and Jamrog-Janicka (1972) found that the plant weight and seed yield of broad bean (*V. faba* L. *major*) plants infected with BBTMV were reduced by 44% and 60% respectively. Blaszczak (1972) reported two strains of BBTMV in Poland, which caused severe dwarfing and deformity of broad bean, pea and summer vetch plants and reduced plant weights by 52%, 88% and 57% respectively. Vorr-a-Urai and Cockbain (1977) found that faba bean plants infected with BBTMV through seed had pod yield reduced from 11.9 to 1.6 pods/plant and seed yield reduced from 14.6 to 1.7 g/plant. Jones (1978) found that yield losses in broad bean plants manually inoculated before flowering were up to 20% but were much greater in plants infected through seed. Cockbain and Bowen (1976) found that in the field bean cultivar Maris Bead infected through seed with either BBTMV or BBSV yielded on average 69% fewer pods...
and 76% less weight of seed than did plants without symptoms at the end of flowering. Plants which were initially virus-free but which developed symptoms before or during flowering yielded, respectively, 42 and 14% fewer pods and 52 and 24% less seed than plants without symptoms. Blaszczak and Jamrog-Janicka (1972) found that infection with BBTMV reduced plant weight and seed yield in broad bean by 44% and 60% respectively. Blaszczak (1972) found, in glasshouse experiments, that infection with two strains of BBTMV caused severe dwarfing and deformity of broad bean, pea and summer vetch and reduced plant weights by 52, 88 and 57% respectively.

3.12.2 Environmental impact
NEGLIGIBLE: There is no potential to degrade the environment or otherwise alter the ecosystems by affecting species composition or reducing the longevity or competitiveness of wild hosts. BBTMV has no effect on human or animal health.

3.12.3 Social impact
NEGLIGIBLE: There is no potential to affect the social environment.

3.13 Combination of likelihood and consequences to assess risks
The pest risk is MEDIUM, the economic impact is LOW, the environmental and social impacts are NEGLIGIBLE. Therefore the economic risk rating is MEDIUM, the environmental risk rating is LOW and the social risk rating is LOW (Risk ratings based on PHA Industry Biosecurity Planning Guide).

3.14 Surveillance
BBTMV is a quarantinable pathogen and is actively tested for in post-entry quarantine. Regular surveys of pulse crops for endemic viruses could easily be extended to include screening of samples for BBTMV using ELISA or TBIA.

3.15 Diagnostics
Samples suspected of being infected with BTMV would need to be identified quickly and accurately. The accompanying report describes methods for sampling and diagnosing BBTMV using ELISA and TBIA and other confirmatory methods. The ELISA procedure for BBTMV detection is used on a regular basis at the DPI Post-entry Quarantine Station for temperate Pulses at DPI-Horsham, Victoria, and could be undertaken by any trained virologist. TBIA is used at the Quarantine Station to confirm positive ELISA results. The initial diagnosis would need to be confirmed by another virologist and by a second method. This is
particularly important as BBTMV has frequently been confused with another exotic pulse comovirus, broad bean stain virus, which is indistinguishable by symptoms and particle morphology but which is serologically distinct (Cockbain et al., 1972, Cockbain et al., 1976, Fischer, HU Lockhart BE, 1976). BBSV is also spread by the same vectors as BBTMV. Mixed infections may occur and careful diagnosis is required.

3.16 Training
There is a general need for industry training in biosecurity and awareness of the potential impact of exotic diseases. Due to the similarity of symptoms of a range of pulse viruses, including BBSV, on pulse hosts, training and education need to be of a general nature. Training in the recognition of seed symptoms is likely to maximise the likelihood of early detection.

3.17 References
See Section 8.
4.0 Diagnostic protocol

4.1 The diagnostic test/s and diagnostic sequence

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) is the recommended primary test for detection of BBTMV (Clark MF, Adams AN, 1977). There are no published sequence data for BBTMV therefore molecular tests such as polymerase chain reaction (PCR) cannot be used for routine diagnostics. There are extensive published data on serological detection of BBTMV, including the serological relationship between BBTMV and other comoviruses (Section 3.9.3) and differences between strains of the virus (Section 3.9.2). Tissue-blot immunoassay (TBIA) is included as an alternative primary diagnostic test, because although less widely used than ELISA it has proved to be the test of choice under some circumstances (Makkouk KM, Comeau A, 1994).

ELISA is the most commonly used serological method for BBTMV detection and ELISA using polyclonal antisera has been reported for detecting BBTMV in leaf material from the field and from surveys in a number of countries (eg. Rothamstead Experimental Station 1985, Makkouk et al. 1988, Makkouk et al. 1992, Simay EI, Beczner L, 1993). The Rothamstead Experimental Station (1985) reported that ELISA was a reliable method for detecting BBTMV in seedlots. Haack (1990) used ELISA to show that BBTMV and BBSV were easily detectable after artificial inoculation of faba bean seeds. Both ELISA (Simay EI, Beczner L, 1993) and TBIA (Abraham et al., 2000, Makkouk et al., 2001, El-Muadhidi et al., 2001) have been used to detect BBTMV in legume samples collected during field surveys.

4.2 The initial samples

4.2.1 Sample handling and subsampling

It is important that the samples are entered onto sample reference sheets (Appendix 1) which contain sufficient information to enable revisiting of the site, describe symptoms and other relevant information and recording of diagnostic test results. It is vital that information is provided here to ensure that samples are handled correctly, that sub samples are taken as reference samples and so that material can be sent to other experts for confirmation.

4.2.2 Sample storage

As soon as the diagnostician becomes aware that the sample submitted for diagnosis may be an exotic or emergency pathogen, the diagnostician has the responsibility to seek expert advice from State Plant Standards or equivalent or AQIS or the Office of the Chief Plant protection officer (OCPPO) on the appropriate manner/location in which the sample should be stored and appropriate further testing/action. It is not appropriate for the diagnostician to
continue tests without informing the proper authorities. In Victoria, suspected BBSV-infected plants can be stored in the DPI pulse quarantine station's AQIS registered storage area for quarantine samples. Reference material from the original sample should always be kept: for virus samples, material should be dried and/or frozen, and if possible nucleic acid extractions conducted.

4.2.3 Visual symptoms on the sample
Visual symptoms should be recorded and photos taken where possible.

4.2.4 Documentation
It is important to note that proper documentation of samples and diagnostic procedures and results is initiated at this stage.

4.3 Further samples
4.3.1 Sample collection, transport and storage
It is important that samples are collected and stored correctly as deteriorating plant samples may be unsuitable for diagnostic tests. Leaf samples should be placed in labelled sealed plastic bags and stored in the field in a cooled, insulated container (Esky). Samples should then be transferred to a refrigerator if they are to be tested within a week of collection. If there are to be delays in testing then samples for use in ELISA tests should be frozen. This practice is not recommended as freezing is likely to reduce the sensitivity of the test and should only be resorted to if there is some impediment to rapid receipt and processing of a batch of samples. If samples are to be used in TBIA tests then samples should be blotted onto multiple nitrocellulose membranes and the remaining tissue frozen or dried. If a survey is being conducted and a team of people is assembled to assist, pictures of plants with virus symptoms will help in sample selection. Advice on phytosanitary measures required to prevent disease spread in the field should be provided (Appendix 2).

4.3.2 Sample locations
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.

4.4 Confirmation of diagnosis
It is important that all diagnoses of suspected exotic and emergency pathogens are undertaken according to the following parameters: the diagnostician has expertise in this
form of diagnosis, the test is undertaken as described in this manual, the results are confirmed by diagnosis in another recognised laboratory or another diagnostician and where possible diagnosis is confirmed by a second method. Methods suitable for confirming the primary diagnosis are described in Section 6 (eg. electron microscopy to confirm presence of the correct size virus particle).
5.0 Identification of pathogen

5.1 Enzyme-linked immunosorbent assay (ELISA)

5.1.1 Introduction
Direct ELISA (e.g. Clark and Adams, 1977) is used with polyclonal antisera but indirect ELISA (e.g. Torrance and Pead, 1986) is normally employed with monoclonal antibodies. Both procedures are given below

5.1.2 General items required
1. Samples - leaves, shoots, seedlings (germinated seeds).
2. Microtitre plates (e.g. Nunc maxisorp plate) and lids.
3. Mortars and pestles (sterile and kept cool), or sap extractor machine or plastic sample bags and rolling pins.
4. 200 µl pipettes, 0.5-200 µl pipettes, 100-200 µl multistepper pipettes and sterile tips.
5. Balance (that weighs to at least two decimal places) and weighboats.
6. pH meter and magnetic stirrer.
7. Plate reader and computer.
8. Incubator kept at 37°C.
9. 1 litre graduated glass bottles with lids, volumetric flasks, flasks, beakers, tube racks and screw cap 5 ml plastic specimen storage bottles, plastic wash bottles.
10. Disposable gloves, paper towels, plastic reservoir.

5.1.3 Specific items
1. BBTMV antisera and conjugate.
The antiserum recommended for this protocol was produced at the International Centre for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria. It has been used extensively by Dr Khaed Makkouk and Dr Safaa Kumari (previous and current Manager of the Virology Laboratory, ICARDA, respectively) in survey and research work throughout the Middle East and North Africa (as cited in this protocol). It has been used at the DPI pulse quarantine station, DPI, Horsham, Victoria, for post-entry quarantine testing for BBTMV for ten years. The BBTMV antiserum is conjugated with alkaline phosphatase for use in DAS-ELISA. It is used successfully in both DAS-ELISA and TBIA. 1ml of the ICARDA BBTMV antiserum has been purchased and is stored at DPI, Horsham, for use in an incursion. Dried and frozen BBTMV- infected leaf tissue is also stored in the locked quarantine -18°C storage facility at the quarantine station. A stock of BBTMV antiserum is held at the station for use in post-entry quarantine testing and additional antiserum could be made available for use in an incursion. Additional BBTMV- alkaline phosphatase conjugate would be prepared when required.
NB. DSMZ have recently added BBTMV to their list of commercially available antisera. The antiserum is polyclonal and produced from the original isolate of Quantz (Quantz, 1953), catalogue number DSMZ-AS-0152 (check the DSMZ Website). If further antisera has to be purchased from overseas an AQIS permit is required. Dr Angela Freeman, manager DPI pulse quarantine station holds a current permit to import the antiserum and virus infected tissue.

5.1.4 Buffer recipes

5.1.4.1 Coating Buffer (pH = 9.6)

<table>
<thead>
<tr>
<th></th>
<th>1 l</th>
<th>500 ml</th>
<th>250 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂CO₃</td>
<td>1.59 g</td>
<td>0.79 g</td>
<td>0.39 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.93 g</td>
<td>1.46 g</td>
<td>0.73 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled water, adjust pH to 9.6 with HCl and make up to required volume.

5.1.4.2 Phosphate Buffered Saline (pH 7.4) = PBS

<table>
<thead>
<tr>
<th></th>
<th>5 l</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>40.00 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.00 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>5.75 g</td>
</tr>
<tr>
<td>KCl</td>
<td>1.00 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled water, adjust pH to 7.4 with NaOH and make up to 5 litres.

5.1.4.3 Wash Buffer: Phosphate Buffered Saline + Tween (pH 7.4) = PBST

<table>
<thead>
<tr>
<th></th>
<th>5 l</th>
<th>25 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tween 20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5.1.4.4 Standard Extraction Buffer: PBST + 2% PVP (pH 7.4)

<table>
<thead>
<tr>
<th></th>
<th>1 l</th>
<th>500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBST</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVP-40</td>
<td>20.00 g</td>
<td>10.00 g</td>
</tr>
<tr>
<td>(PolyVinyl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrolidine-40)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dissolve PVP in PBS solution on a magnetic stirrer.
5.1.4.5 Conjugate Buffer: PBST + 2% PVP + 0.2% ovalbumin (pH 7.4)

<table>
<thead>
<tr>
<th></th>
<th>1 l</th>
<th>500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBST +2% PVP</td>
<td>1 l</td>
<td>500 ml</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>2.0 g</td>
<td>1.0 g</td>
</tr>
</tbody>
</table>

Dissolve ovalbumin in PBST-PVP solution on a magnetic stirrer.

5.1.4.6 Substrate buffer  (pH = 9.8)

<table>
<thead>
<tr>
<th></th>
<th>1 l</th>
<th>500 mL</th>
<th>250 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diethanolamine</td>
<td>106.7 g</td>
<td>53.35 g</td>
<td>26.65 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled water, adjust pH to 9.8 with HCl and make up to required volume.

5.1.4.7 Extraction buffer for indirect ELISA  (pH = 9.6)

<table>
<thead>
<tr>
<th></th>
<th>1 L</th>
<th>500 mL</th>
<th>250 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂CO₃</td>
<td>1.59</td>
<td>0.79</td>
<td>0.39</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.93</td>
<td>1.46</td>
<td>0.73</td>
</tr>
<tr>
<td>PVP</td>
<td>20.00</td>
<td>10.00</td>
<td>5.00</td>
</tr>
</tbody>
</table>

Dissolve in distilled water, adjust pH to 9.6 with HCl and make up to required volume.

5.1.5 Methods

5.1.5.1 Direct double antibody sandwich (DAS) ELISA
1. Coat microtitre plate with polyclonal antibody diluted at recommended rate in coating buffer using 200 μl/well. Coat 60 internal wells only. Put 200 μl distilled water in each of the outside wells.

2. Cover microtitre plate and incubate at 37°C for 4 hours.

3. Extract samples individually in extraction buffer (1:5 or 1:10 w/v) using a fresh mortar and pestle for each sample, or a sap extractor with crusher rollers that are washed thoroughly in between each sample or a plastic bag and rolling pin. Collect extract and store in labelled 5ml plastic specimen bottles.

4. Wash coated plate with wash buffer (PBST) at least three times (3-5 times), using a wash bottle, allowing a three minutes soak for each wash.
5. Pipette sample extracts and control extracts into paired wells (200 μl/well) on the washed, coated plate. Controls should consist of four wells each of extraction buffer (blank), healthy plant extract (negative) and extracts of at least one known BBSV-infected plant (positive).

6. Cover microtitre plate and incubate in refrigerator at 4°C overnight. Although overnight incubation is recommended, 4 hours at 37°C is satisfactory.

7. Remove microtitre plate contents and rinse with PBST, using pressure with the wash bottle, to remove traces of plant material.

8. Wash plate with wash buffer (PBST) at least three times (3-5 times), using a wash bottle, allowing a three minutes soak for each wash and ensuring no plant material remains.

9. Dilute conjugate (BBSV antibody conjugated with alkaline phosphatase enzyme) to recommended rate in conjugate buffer, and add to the 60 inner wells of the plate at 200 μl/well. Put 200μl distilled water in each of the outside wells of the plate.

10. Cover microtitre plate and incubate at 37°C for four hours.

11. Remove microtitre plate contents. Wash plate with wash buffer (PBST) at least three times (3-5 times), using a wash bottle, allowing a three minute soak for each wash.

12. Just prior to usage, prepare p-nitrophenyl phosphate (PNP) substrate by dissolving PNP tablets (Sigma 104-105) in substrate buffer to make a 1 mg/ml solution.

13. Add 200 μl/well of freshly prepared PNP substrate to all the wells of the plate. Incubate at room temperature for 1-2hr or until unambiguous reactions are obtained.

14. Read the absorbance values (OD values) of each well at a wavelength of 405 nm (A_{405}) using a plate reader after 30-60 minutes and again after two hours.
5.1.5.2 Indirect ELISA

1. Extract samples individually in indirect-ELISA extraction buffer (1:5 or 1:10 w/v) using a fresh mortar and pestle for each sample, or a sap extractor with crusher rollers that are washed thoroughly in between each sample or a plastic bag and rolling pin. Collect extract and store in labelled 5ml plastic specimen bottles.

2. Pipette sample extracts and control extracts into paired wells (200 µl/well) of the plate. Fill the 60 internal wells only. Put 200 µl distilled water in each of the outside wells. Controls should consist of four wells each of extraction buffer (blank), healthy plant extract (negative) and extracts of at least one known BBSV-infected plant (positive).

3. Cover microtitre plate and incubate in refrigerator at 4°C overnight.

4. Remove microtitre plate contents. Wash plate with wash buffer (PBST) at least three times (3-5 times), using a wash bottle, allowing a three minute soak for each wash.

5. Dilute monoclonal antibody to recommended rate in conjugate buffer, and add to the 60 inner wells of the plate at 200 µl/well. Put 200µl distilled water in each of the outside wells.

6. Cover microtitre plate and incubate at 37°C for four hours.

7. Remove plate contents. Wash plate with wash buffer (PBST) at least three times (3-5 times), using a wash bottle, allowing a three minute soak for each wash.

8. Dilute anti-species conjugated antibody (eg goat anti-mouse) to the recommended rate in conjugate buffer and add 200 µl/well to the 60 inner wells. Put 200 µl distilled water into each of the outside wells.

9. Cover microtitre plate and incubate at 37°C for four hours.

10. Remove microtitre plate contents. Wash plate with wash buffer (PBST) at least three times (3-5 times), using a wash bottle, allowing a three minute soak for each wash.

11. Just prior to usage, prepare p-nitrophenyl phosphate (PNP) substrate by dissolving PNP tablets (Sigma 104-105) in substrate buffer to make a 1 mg/ml solution.
12. Add 200 µl/well PNP substrate to all the wells of the plate.

13. Read the absorbance values (OD values) of each well at a wavelength of 405 nm ($A_{405}$) using a plate reader after 30-60 minutes and again after two hours.

5.2 Tissue-blot immunoassay (TBIA)

5.2.1 Introduction
Lin et al. (1990) found that TBIA was suitable for detection of viruses in the cucumovirus, luteovirus, potexvirus, potyvirus and tospovirus groups and from a range of tissue types. Hsu and Lawson (1991) compared both direct blotting of tomato spotted wilt virus plant tissue with dot-blot immunoassay and ELISA, both of which involve extracting the virus into buffer. TBIA was found to be sensitive, reliable and rapid and had the added advantages of simplicity and convenience. Makkouk et al. (1994) found that TBIA was a simple, sensitive and quick method for barley yellow dwarf virus detection. Makkouk and Comeau (1994) looked at a range of modifications to this method and their modifications form the basis of the method described in this manual.

5.2.2 General items required
1. Nitrocellulose membrane (NCM) (CN. 0.45 um) from Schleicher & Schuell, Cat No. 401.196 BA85
2. Goat Anti-Mouse-alkaline phosphatase conjugate (from Bioreba Cat No. 1031-04 or Sigma Cat No. A-5153)
3. Goat Anti-Rabbit- alkaline phosphatase conjugate (from Bioreba Cat No. 4050-04 or Sigma Cat No. A-8025)
4. BCIP (from Sigma Cat No. B-8503 or ROCHE Cat no. 1017373)
5. NBT (from Sigma Cat No. N-6876 or ROCHE Cat No. 1087479)
6. Poly Vinyl Alcohol (PVA)
7. Small plastic, flat-bottomed containers with lids (eg empty pipette tip boxes or food storage boxes).
8. Shaker

5.2.3 Specific items
1. BBTMV polyclonal antiserum form ICARDA (See ELISA section)
5.2.4 Buffer recipes

5.2.4.1 Phosphate Buffered Saline (pH 7.4) = PBS

<table>
<thead>
<tr>
<th></th>
<th>5 l</th>
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</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>40.00 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.00 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>5.75 g</td>
</tr>
<tr>
<td>KCl</td>
<td>1.00 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled water, adjust pH to 7.4 with NaOH and make up to 5 litres.
1.0g NaN₃, a carcinogen used as preservative, may be added per 5 litres. As an alternative, store at –4°C instead.

5.2.4.2 Wash Buffer: Phosphate Buffered Saline + Tween (pH 7.4) = PBST

<table>
<thead>
<tr>
<th></th>
<th>5 l</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>5 l</td>
</tr>
<tr>
<td>Tween 20</td>
<td>2.5 ml</td>
</tr>
</tbody>
</table>

5.2.4.3 Standard Extraction Buffer: PBST + 2% PVP (pH 7.4)

<table>
<thead>
<tr>
<th></th>
<th>1 l</th>
<th>500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBST</td>
<td>1 l</td>
<td>500 ml</td>
</tr>
<tr>
<td>PVP-40 (PolyVinyl Pyrrolidone-40)</td>
<td>20.00 g</td>
<td>10.00 g</td>
</tr>
</tbody>
</table>

Dissolve PVP in PBS solution on a magnetic stirrer.

5.2.4.4 Conjugate Buffer: PBST + 2% PVP + 0.2% ovalbumin (pH 7.4)

<table>
<thead>
<tr>
<th></th>
<th>1 l</th>
<th>500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBST</td>
<td>1 l</td>
<td>500 ml</td>
</tr>
<tr>
<td>Ovalbumin (Sigma A-5253)</td>
<td>2.0 g</td>
<td>1.0 g</td>
</tr>
</tbody>
</table>

Dissolve ovalbumin in PBST-PVP solution on a magnetic stirrer.
5.2.4.5 Substrate Buffer: Tris 0.1 M, NaCl 0.1 M (pH 9.5)

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris (0.1 M)</td>
<td>12.1g</td>
</tr>
<tr>
<td>NaCl (0.1 M)</td>
<td>5.8g</td>
</tr>
<tr>
<td>MgCl$_2$ (5 mM)</td>
<td>0.4g</td>
</tr>
</tbody>
</table>

5.2.4.6 Substrate stock solutions: NBT and BCIP

**Tube 1.** Prepare a p-Nitro Blue Tetrazolium (NBT) stock solution of 75 mg/ml in 70% diemethylformamide. Store at -20°C.

**Tube 2.** Prepare a 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) stock solution of 50 mg/ml in undiluted diemethylformamide. Store at -20°C.

5.2.5 **Method for tissue-blot immunoassay (TBIA) on nitrocellulose membranes**

1. It is important to select a type of nitrocellolose membrane which will optimise the result. The preferred membrane is the Schleiger and Schultz Protran 55 μm membrane. Do not use nylon membranes as they give high backgrounds. Prepare nitrocellulose membrane by stamping a 10 x 10 grid or use a standard 5 x 10 cm sheet and underline every fifth row of blots to keep straight. Mark top left corner with an asterisk (*). Handle the membranes with blunt ended forceps. N.B. If blotting faba bean samples, which tend to oxidise and go brown/purple on the membrane, pre-treat membrane by first dipping in 1% sodium sulphite.

2. It is much easier to blot petioles than leaves, which have to be rolled into a cigar shape before blotting, therefore collect leaves with petioles intact if possible. If thin tissues such as leaves are used, roll them into a tight core or cigar and secure with masking tape.

3. Wearing gloves, hold tissues in one hand and cut petiole or cigar with a scalpel blade with the other hand to obtain a single plane cut surface. It has been found that it is not necessary to wash the blade between the cutting of each sample.

4. Press the newly cut surface with a firm but gentle force onto one of the cells of the grid on the nitrocellulose membrane (NCM).
5. **Always include blotted positive and negative controls in the test.** These can be blotted at the same time as the samples or a large number of controls can be blotted in advance and small pieces of NCM with controls can be added to the dish for processing.

6. If TBIA tests are not used regularly it is advisable to run through the procedure outlined below using only the positive and negative controls to ensure that the test is working well. If results are not clear, optimise the test by increasing the concentration of antiserum and checking the viability of the conjugate and possibly increasing its concentration. Some monoclonal reactions can be improved by incubating overnight in the fridge. This does not work for polyclonals and gives a high background. The colour development varies with different conjugates. It may be worth comparing the results with a couple of different brands of conjugate.

7. Prior to commencing the processing, prepare polyclonal antisera by reacting out antibodies to healthy plant proteins. First grind 1g fresh healthy host tissue in 20mls PBS-Tween (or equivalent 1:20 extraction if larger volume needed). Strain to remove particulate matter. Dilute polyclonal antiserum 1/2000 or as required in the extract and incubate 2 hrs in the oven at 37 C.

8. Place all membrane/s in one dish by carefully lowering them, making sure the back of the membranes are wet first. Wash 3 times with PBS-Tween at 5 minute intervals on a small shaker.

9. Block NCM in 1 µg/ml Polyvinyl Alcohol (PVA) in PBST and incubate for 1 minute at room temperature. Save PVA as it is reused many times. Mark date on record sheet on back of bottle

10. Wash 3 times with PBS-Tween at 5 minute intervals.

11. Place membranes into separate, labelled dishes.

12. Remove prepared polyclonal antisera from oven and prepare monoclonal antisera by diluting 1/2000 or as required in conjugate buffer (PBST +2% PVP + 0.2% ovalbumin). Add diluted antiserum to each dish and incubate for 1 hour at room temperature on a
small shaker. Save antiserum to be reused and mark record sheet on bottle (may be used up to 10 times).

13. Wash 3 times with PBS-Tween at 5 minute intervals.

14. Add anti-rabbit alkaline phosphatase conjugate (dilution 1/2000 in conjugate buffer) to membranes processed with polyclonal antisera or anti-mouse alkaline phosphatase conjugate to membranes processed with monoclonal antibodies and incubate for 1 hour at room temperature on shaker. N.B. Membranes can be processed together at this stage. Save conjugate to be reused, mark date on record sheet on back of bottle (may be used up to 10 times).

15. In preparation for Step 16 remove frozen substrate buffer and substrate solutions tube1 and tube 2 from freezer and thaw.

16. Wash as instep 8.

17. Prepare substrate solution. First check pH of substrate buffer- it is essential that the pH is exactly 9.5. The reaction will be much fainter if the pH is not optimum. For each 5 ml substrate buffer add 20 µl tube 1 (NBT) and 20 µl tube 2 (BCIP).

18. N.B. The substrate solution must be prepared fresh just prior to usage. Add substrate solution to the dish (all membranes may be processed together as long as there is sufficient movement between sheets and they do not stick together thus impeding development). Incubate for 5 minutes on shaker.

19. To stop reaction, wash with deionised H₂O.

20. To stop reaction, wash with deionised H₂O. Dry membranes and view under a dissecting microscope. Phloem restricted viruses will show as a clear dark purple staining of the phloem. Viruses which invade the whole plant will be seen as clear dark purple staining of the whole petiole cross section.
5.2.6 Reagent suppliers

Schleicher & Schell
BioScience GmbH,
P.O. Box 4
Hahnestrasse 3
Dassel 1, D-3354
Germany
e-mail: bioscience@s-und-s.de
Internet: http://www.s-und-s.de

ROCHE Diagnostics GmbH
Dept. GD-F
68298 Mannheim
Germany

Bioreba AG
Chr. Merian-Ring 7
CH-4153 Reinach BL1
Schweiz
e-mail: admin@bioreba.ch
6.0 Confirmation of diagnosis
6.1 Electron microscopy
6.1.1 Introduction
BBSV has angular, isometric particles of about 25 nm diameter (Gibbs AJ, Smith HG, 1970). BBSV particles are morphologically indistinguishable from BBTMV, therefore direct examination of plant sap using the electron microscope (sap dip) is only suitable as a confirmatory test for the presence of isometric particles. Immunosorbent electron microscopy (trapping or decoration) with virus-specific antibodies enables trapping of particles of the target virus and offers a more definitive test result. However, like ELISA, this is a serological test, which means that both these forms of diagnosis/virus detection depend on the quality of the antiserum. It is preferable to use a confirmatory test which depends on other properties of the virus.


6.1.2 General items required
1. Samples - leaves, shoots or washed roots.
2. Electron microscope grids: copper 400 mesh, Formvar coated, then carbon coated.
3. Glass microscope slides, waxed glass microscope slides, plastic wells, pasteur pipettes, filter papers, fine forceps.
4. Distilled water, 0.1M sodium phosphate buffer, pH 7.0.
5. Freshly prepared stains: 2% phosphotungstic acid (PTA) and 2% uranyl acetate (UA) dissolved in distilled water, adjusted to pH 7.0 with NH₃.
6. Freshly prepared dilutions of appropriate virus antibodies in 0.07 M sodium phosphate buffer, pH 6.5.

6.1.3 Method
6.1.3.1 Sap dip (negative staining) method
1. Using the scalpel blade, cut approximately 3 mm² of the test plant material and place it on a clean microscope slide (if the test material has any suspicious virus symptoms, take the tissue from this area).

2. Place a 3 mm diameter drop of PTA next to the piece of plant material and thoroughly crush the plant material into the PTA. If necessary add an extra drop of PTA.
3. Pick up a coated grid with forceps and touch it, coated-side down, onto the drop of PTA and plant sap mixture.

4. After 2-3 seconds, drain the excess droplet of the grid by touching its edge with a piece of torn filter paper.

5. Allow the grid to dry for approximately 2 minutes then observe grids for virus particles using an electron microscope.

6.1.3.2 Immunosorbent electron microscopy

6.1.3.2.1 Trapping method

1. Pipette 30 μl drops of antiserum diluted 1:100, 1:1,000 and 1:10,000 in normal saline onto waxed glass slides.

2. Float a carbon-coated grid, film-side down, on each drop and incubate for 2-3 hours at 37°C.

3. Wash grids five times in normal saline or place grids in 0.1 M sodium phosphate buffer, pH 7.0, in plastic wells, agitate at intervals for 10 minutes, transfer to a second plastic well containing buffer and leave for a further 10 minutes and then drain.

4. Extract plant material in normal saline at 1:5 wt/vol and then centrifuge at 8,000 g for 10 minutes.

5. Float grids, film-side down, on 30 μl drops of sample sap extract and incubate for 2 hours at room temperature or for 3-36 hours at 4°C.

6. Wash grids five times in normal saline or place grids in plastic wells, containing normal saline, agitate at intervals for 10 minutes, transfer to a second plastic well containing normal saline and leave for a further 10 minutes and then drain.

7. Stain grids with 2% PTA and/or UA by floating grids on the stain for 10 minutes then drain grids by touching the edge with torn filter paper.

8. Observe grids for virus particles using an electron microscope.

6.1.3.2.2 Decoration method

1. As an additional step just prior to examination of the prepared grid using the electron microscope, in either of the above procedures, add a drop of suitably diluted antiserum to the prepared grids, incubate for 3 hours at 37°C and drain.

2. Observe grids using an electron microscope for antibody halos surrounding virus particles. Such halos indicate the specific binding of the virus-specific antibody to the
trapped virus particles on the grid and therefore provide evidence of the true identity of the virus, based on the specificity of the antiserum used.
7.0 Images

7.1 BBTMV symptoms on Host plants

Figure 4. BBTMV symptoms on faba bean including patchy mosaic and vein clearing. (Photo courtesy: Dr S Kumari, ICARDA)

Figure 5. BBTMV symptoms on faba bean including large mosaic spots connected by malformations.
7.2 BBTMV vectors and vector symptoms

Figure 6. *Sitona lineatus*, the vector of BBTMV and BBSV, feeding on leaf (note characteristic leaf notching).
(Photo courtesy: Dr S Kumari, ICARDA, from Agrevo)

Figure 7. Faba bean shoot showing characteristic leaf notching caused by *Sitona lineatus* feeding.
(Photo courtesy: Dr S Kumari, ICARDA)
Figure 8. *Sitona lineatus*, vector of BBTMV and BBSV.

(Photo courtesy: Dr S Kumari, ICARDA, from Agrevo)
8.0 References and websites

8.1 References


Ackerbohne (*Vicia faba* L.); Mitteilungen aus der Biologischen Bundesanstalt fur Land- und Forstwirtschaft Berlin-Dahlem:31-37.


Cockbain AJ (1972). Epidemiology and control of weevil-transmitted viruses in field beans. Proceedings of the Sixth British Insecticide and Fungicide Conference, 15th-18th November,
1971, Hotel Metropole, Brighton, England. Vols 1,2 and 3; pp302-306; Publisher: London, British Crop Protection Council; UK, 7 ref.


Schmidt HE (1981). Principles of plant resistance to diseases and pests; pp25-33; Publisher:Research Institute for plant protection; Bucharest, Romania.


8.2 Websites

AAB-CMI Descriptions of plant viruses http://www.dpvweb.net/index.php

All the virology on the WWW http://www.virology.net/garryfavwebplant.html

CABI Crop Protection Compendium http://www.cabicompendium.org/cpc/home.asp

Plant viruses online- descriptions and lists from the VIDE database http://image.fs.uidaho.edu/vide/refs.htm

DSMZ http://www.dsmz.de/nf-plvirus/index.html

American Type Culture Collection http://www.atcc.org/

International Committee on the Taxonomy of Viruses Databases http://www.ictvdb.rothamsted.ac.uk/index.htm
Appendix 1: Preliminary Information Data Sheet (Plantplan, 2004).

SUBJECT:
Site details:
Ownership:
Location:
Map (lat. & long.):
GPS identifier:
Host plant location (clearly mark plant if necessary):

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 2 Personnel Hygiene

On **entering** the paddock, personnel **must**:

- Wear protective overalls and rubber boots.
- Prepare footbath of bleach, and spray bottles of methylated spirits brew (95% metho, 5% water) for use following completion of the inspection.
- Conduct inspections by foot (refer to **Appendix 3 Machinery Hygiene** for vehicle access).

On **leaving** the paddock, personnel **must**:

- Wash boots in footbath of disinfectant (solution of household bleach 10%) and remove adhering material, ie soil, with a suitable brush (ie domestic scrubbing brush).
- Spray boots with methylated spirits brew until soaked.
- Remove overalls and place into a bag and seal.
- Exterior of sample bags to be sprayed/swabbed with methylated spirits brew.
- Spray hands with methylated spirits brew irrespective of whether disposable gloves have been worn.

You **must** decontaminate before leaving the paddock **always**.

Overalls must be washed and allowed to completely dry before being used again. If disposable overalls are used, they can be either washed, or if disposed, sent to land fill or burnt.
Appendix 3 Machinery Hygiene

- No machinery, including vehicles, are to enter paddock without prior approval from the applicant. Approval to use vehicles in paddock must be included with the application for access.
- Decontamination procedures **must be followed immediately** before leaving the site at the area identified for decontamination.
- Decontaminate the machinery by removing all visible lupin trash and wash down with a high pressure spray using detergent, paying particular attention to the underside, axles, wheels and tyres. This also includes all hand held tools such as hoes and shovels.
- Personal decontamination procedures must follow the decontamination of machinery.
- It is recommended that any machinery or vehicle that has entered the paddock is not to be taken into another green lupin crop this season.

Harvest Machinery

- In addition to the above requirements, machinery will be cleaned of all seed and trash remaining. This material will be destroyed in a manner approved by the relevant State Authority (ie, landfill within quarantine boundary or similar).