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Bacterial wilt of lucerne

A market access issue for lucerne seed growers

A report for the Rural Industries Research and Development Corporation

By Dr. Kathy Ophel Keller

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Researcher Contact Details
Dr. Kathy Ophel Keller
SARDI Field Crop Pathology Unit
GP Box 397
Adelaide SA 5001

Phone: 08 8303 9368
Fax: 08 8303 9393
Email: ophelkeller.kathy@saugov.sa.gov.au

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RIRDC Contact Details
Rural Industries Research and Development Corporation
Level 1, AMA House
42 Macquarie Street
BARTON ACT 2600
PO Box 4776
KINGSTON ACT 2604

Phone: 02 6272 4819
Fax: 02 6272 5877
Email: rirdc@rirdc.gov.au.
Website: http://www.rirdc.gov.au

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Foreword

The Australian export trade in lucerne seed is worth $22.2 million pa nationally. Over 90% of export lucerne seed is produced in South Australia. The major export markets for lucerne are Argentina, Saudi Arabia, North Africa and Europe. Overseas markets require phytosanitary declarations for bacterial wilt in lucerne. In addition, Western Australia has a legislative requirement for freedom from bacterial wilt of lucerne seed.

A survey for bacterial wilt was conducted in 2002 to update the area freedom status of major lucerne seed producing areas in South Australia and Victoria. Bacterial wilt was detected in the main lucerne seed production area of SA. This meant that the area freedom status was no longer applicable and that a system needed to be put in place to ensure that phytosanitary requirements of export markets with respect to bacterial wilt could still be met.

This project has developed phytosanitary inspection and testing protocols in collaboration with seed service providers, industry, government and regulatory authorities to ensure that lucerne seed producers can continue to access markets and meet international phytosanitary requirements.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report, an addition to RIRDC's diverse range of over 1500 research publications, forms part of our Pasture Seeds R&D program, which aims to facilitate the growth of a profitable and sustainable pasture seeds industry based on a reputation for the reliable supply, domestically and internationally, of a range of pasture species.

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Peter O'Brien
Managing Director
Rural Industries Research and Development Corporation

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- Alan McKay, SARDI Diagnostic Services
- David Cartwright, PIRSA Plant Health
- All industry and AQIS participants in workshops to develop recommendations.

Abbreviations

ASF – Australian Seeds Federation
AQIS- Australian Quarantine Inspection Service
Cmi- *Clavibacter michigenense* subsp. *insidiosus*, the causal agent of bacterial wilt of lucerne
ELISA- Enzyme Linked Immunosorbent Assay, antibody-based diagnostic technology
GCA – Grains Council of Australia
IPPC- International Plant Protection Convention
PCR- Polymerase Chain Reaction, DNA- based diagnostic technology
PIRSA – Primary Industries and Resources, South Australia

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Executive Summary

The Australian export trade in lucerne seed is worth $22.2 million pa nationally. The major export markets for lucerne are Argentina, Saudi Arabia, North Africa and Europe.

Overseas markets require phytosanitary declarations for bacterial wilt in lucerne. Argentina and Chile will accept a phytosanitary declaration based on a seed pathology test while the European Union, Tunisia and Uruguay will only accept phytosanitary declarations based on field inspections. In addition Western Australia has a legislative requirement for freedom from bacterial wilt of lucerne seed.

Field surveys in the 1960's and 1970's detected bacterial wilt in many of the lucerne growing areas adjacent to the river systems in NSW, Victoria and SA but not in the major seed production areas. Surveys to support declarations of area freedom in the seed producing areas were last conducted in 1978.

In 2002, a field disease survey of lucerne stands was initiated to update area freedom from bacterial wilt of lucerne in seed production areas in South Australia and Victoria. The survey was based primarily on testing of seed lots as well as a limited number of paddock inspections. Bacterial wilt was detected by field inspection in one seed crop paddock in the South East of SA.

After the detection of bacterial wilt, the lucerne seed industry recognised the need for phytosanitary inspections to maintain market access via area freedom on a place of production basis. A field inspection and sample testing protocol was developed, which has been adopted in SA and Victoria. These requirements meet International Plant Protection Convention and AQIS requirements. This was done for the first time in spring 2003 and the phytosanitary service incorporated inspection and testing for bacterial wilt, stem nematode, *Orobanche* spp. (branched broomrape and native broomrape species) and *Cirsium arvense* (perennial thistle).

Phytosanitary inspections for bacterial wilt occurred in spring 2003 in lucerne seed production areas in SA and western Victoria. Plant samples were collected by accredited inspectors, as part of the phytosanitary paddock inspection in October/November. A further 9 infected paddocks and 15 unconfirmed infected paddocks were detected in SA. The infected paddocks were growing the bacterial wilt susceptible lucerne cultivars Hunter River, Hunterfield and Sirosal. Stand life of infected paddocks was greater than 4 years old with the majority being greater than 15 years old.

At the same time, international acceptance of a seed test developed in a previous RIRDC project (SAR 15-A) is being pursued via the International Seed Testing Association (ISTA) process. Ideally, seed test standardisation should be based on inter-laboratory comparisons on naturally infected seed. Efforts to find naturally infected seed have not been successful, even from seed of infected lucerne plants grown in the glasshouse. Infected plants from paddocks where bacterial wilt was detected produce infected tillers but have not produced infected seed, indicating that transmission to seed is extremely low. This is likely influenced by environmental conditions. As a result, international seed test standardisation is proceeding with artificially infected seed. Ultimately, an internationally accepted seed test will provide lucerne seed growers with less restrictive export options now bacterial wilt is known to be present. An internationally accepted seed test is still a desirable aim but work is progressing slowly. As well as technical difficulties, the test must be accepted by EU authorities.

The measures to maintain export market access have been communicated directly to lucerne seed exporters with ASF taking a lead role. The approach being taken satisfies International Plant Protection Convention and AQIS requirements.

1. Bacterial Wilt of Lucerne in Australia

1.1 Lucerne Export Seed Industry

The Australian export trade in lucerne seed is worth $22.2 million pa nationally (2003-04) with 93% of certified lucerne seed exported from SA (6700 tonnes pa) annually. The remainder is exported from Victoria and New South Wales. The major export markets for lucerne are Argentina, Saudi Arabia, North Africa and Europe. Importing countries require declarations of freedom from bacterial wilt.

Field surveys in the 1960's and 1970's detected bacterial wilt in many of the lucerne growing areas adjacent to the river systems in NSW, Victoria and SA. However, field surveys specifically targeted at bacterial wilt had not been undertaken in major seed production areas since 1978.

1.2 Bacterial Wilt of Lucerne

Bacterial wilt is caused by the bacterium *Clavibacter michigenense* subsp. *insidiosus* (Cmi). The pathogen is North American in origin and has spread to southern Europe, South Africa, Australia and New Zealand but has a very limited recorded distribution in South America and Asia.

The disease is more prevalent in older lucerne stands, usually stands that are at least 3-4 years old. US experience suggests it can reduce stand life by 3-4 years. Wetter environments favour disease development where lucerne is grown on high rainfall, under irrigation and on poorly drained soils. Water, seed, hay and fodder, and machinery can spread the disease. The disease is not long lived in soil and lucerne can be safely replanted in paddocks, which have had a lucerne free break of two years.

Symptoms of the disease (stunting and yellowing) are more easily detected in active regrowth. Confirmation of the disease is possible via plating of the causal agent on semi-selective medium followed by confirmation by a DNA based test (PCR) or antibody-based testing (ELISA).

There is low disease transmission via seed with the literature indicating only 6-7% transmission to seed from diseased plants. A seed test was developed (SAR 15-A) to support export trade and substitute for area freedom declarations. This test has been used as part of an international program to standardise seed tests (SAR 25-A). However, progress in international test standardisation has been slow because it has been impossible to source infected seed for test comparison.

1.3 The lucerne phytosanitary certification process

Australia

AQIS is responsible for issuing Commonwealth Phytosanitary Certificates. However, where import conditions require the attachment of Additional Declarations stating disease, weed or insect freedom, AQIS relies upon appropriate state government agencies to provide these declarations. AQIS will not issue a Commonwealth Phytosanitary Certificate unless the exporter can provide an authentic Additional Declaration.

South Australia

In South Australia, where a particular weed, disease or insect has never been recorded an Additional Declaration will be issued for certified or uncertified seed on an area freedom basis.
Where a particular weed, disease or insect is known to occur in South Australia an Additional Declaration is only be issued for certified seed because certification requires crop inspection and linkage to the identified place of production.

Prior to 2002, SA provided pest free area (regional) area freedom declarations for bacterial wilt, *Orobanche* sp. and Perennial thistle in certified seed and uncertified seed for lucerne (in most areas except along or near River Murray), *Trifolium* and *Medicago* spp

In 2002, Crown Law advised PIRSA that area freedom declarations for bacterial wilt could not legally be defended because the declarations are based on an outdated 1978 field disease survey of lucerne stands. The provision of area freedom declarations for uncertified seed places a significant risk on government and seed exporters.

Victoria

Prior to 2002, as in SA, regional area freedom declarations were issued for bacterial wilt, *Orobanche* sp. and stem nematode in certified seed. No declarations are provided for uncertified seed.

NSW

NSW issues pest free production site (paddock) area freedom declarations for certified seed based on the paddock having been inspected at the correct time when pest/disease can be detected.

WA

WA does not export lucerne seed and bacterial wilt is not known to occur in WA.

1.4 Project Objectives

The objectives of this project were to:

1. Re-evaluate area freedom status for bacterial wilt of lucerne in seed production areas of Australia to export seed trade.
2. Develop protocols to maintain and facilitate export seed trade from lucerne seed-producing areas with bacterial wilt.

2. Survey of seed production areas for bacterial wilt

2.1 Recommendations of industry/government and regulatory groups

A meeting of industry, government and regulatory agencies was held in Canberra, June 6, 2002. The purpose of the meeting was to develop an action plan on bacterial wilt area freedom status for lucerne seed and other pasture species. In addition, additional phytosanitary requirements for lucerne and other pasture species (eg. *Orobanche* sp., perennial thistle, stem nematode and dodders) were discussed.

Attendance: Robert Rees Manager Industry Development, Field Crop Industries, PIRSA (Chairman); Vani Srungaram, Plant Pathologist, Plant BioSecurity, Biosecurity Australia, AFFA; Penny Hendy, Chairperson, Seed Section, GCA; Chris Melham, General Manager, SIAA; Peter Smith, Manager Seed Services SA, PIRSA; John Hannay, Principal Industry Consultant, Field Crop Industries, PIRSA; John Davidson, National Seed Manager, AgriQuality; Jamie Saunders, Business Manager, Agribusiness, AgriQuality; Richard Walker, Program Leader, Plant Protection Policy /Seeds, Agriculture NSW; Ric Cother, Principal Research Scientist, Agriculture NSW; Ray Elson, Assistant Program Manager, Grain Exports, AQIS; Alan McKay, Leader Plant Diagnostics, SARDI; Kathy Ophel-Keller, Leader Field Crops Pathology, SARDI; Jeff Davis, Research Manager, RIRDC; Mark Holland, Manager Plant Laboratories, AGWEST.

Prior to 2002, SA provided regional area freedom declarations for bacterial wilt in certified and uncertified seed for lucerne, *Trifolium* and *Medicago* spp. in most areas, except along or near River Murray. Freedom from *Orobanche* sp. and perennial thistle was based on field inspections conducted in February/March.

In 2002, Crown Law advised PIRSA that area freedom declarations for Bacterial wilt could not legally be defended in the future because the declarations are based on an outdated 1978 field disease survey of lucerne stands. This decision was significant as SA produces more than 90% of exported lucerne seed.

Options considered were renewal of bacterial wilt area freedom by a field survey or a seed survey, or maintenance of the status quo using field inspections primarily designed for seed certification.

It was agreed that it is essential that area freedom status is underpinned by robust scientific evidence and practices. AQIS advised that if bacterial wilt was detected by an importing country, in lucerne seed which had been given an area freedom declaration, it would probably result in Australia losing market access to that country. The methodology used to establish and maintain area freedom declarations must be sufficiently robust and accurate to detect the pest/disease if present.

The workshop recommended a seed survey of existing areas with a Pest Free Area status in SA and western Victoria. The survey was to include both certified and uncertified seed samples. Seed samples from ten percent of certified seed crops were to be tested and from 20% of uncertified seed crops. It was recommended to take a minimum number of samples from each Hundred in which lucerne seed crops were grown to ensure geographical coverage. The survey methodology was e based on International Standards for Phytosanitary Measures “Guidelines for Surveillance” (ISPM 6).

In areas where bacterial wilt is already known to be present such as NSW, individual farm based field survey in main NSW lucerne production areas will continue to establish Pest Free Places of Production.

Export lucerne seed has other phytosanitary requirements:

Stem nematode: Status quo to remain. Phytosanitary certificates to be issued on results of existing stem nematode seed test.

***Orobanche* sp.:** Phytosanitary certificates to be issued for certified seed based on field inspection at appropriate time. Advice on timing to be sought by each state from Animal and Plant Control Commission or equivalent.

Perennial thistle (*Cirsium arvense*): Status quo to remain Phytosanitary certificates to be issued for certified seed based on field inspection at appropriate time. Advice on timing to be sought by each state from Animal and Plant Control Commission or equivalent.

2.2 Area freedom survey 2002

A survey of lucerne was conducted in September-December 2002 to confirm area freedom from bacterial wilt of lucerne in major lucerne-growing areas of SA and Victoria.

As part of the 2002 survey, seed (120 samples) was tested using a new diagnostic assay developed by SARDI, with funding from RIRDC (SAR 15-A). The seed samples from SA and Victoria were all were found to be free of the disease. A small number of paddocks (15) were surveyed as well with a focus on higher risk older stands planted with susceptible cultivars, principally Hunter River.

Methodology

Seed survey:

All seed samples tested were from the 2002 harvest. SA seed was obtained as sub-samples from Seed Services Australia. One hundred certified seed samples were from South Eastern SA and mid-North with the distribution being roughly proportional to the areas sown to lucerne. A further 20 uncertified seed samples were tested from the same areas of SA.

Victorian seed was obtained from Agriquality, Victoria. Forty seed samples were tested from western Victoria.

Sub-samples of 50g of seed were ground, soaked overnight in sterile water and the extract plated on Cmi semi-selective media. Suspect colonies from the selective media were transferred to Cmi enrichment media, and tested by antibody-based ELISA. Isolates with results greater than background (cut-off 0.200) were tested by PCR.

Field survey:

Plant samples from the SA South East were collected by agronomic consultants (Wally Cole & Co., De Barro Agricultural Consulting, David Carter, Seed Services Australia, Raymond Christinat, Crop Monitoring Services) from paddocks selected by variety, age of stand and seed test. Further plant samples were collected by SARDI staff from lucerne sites in the Lake Alexandrina area, where bacterial wilt was found in 1978.

All plant samples received were examined visually within 24 hours of receipt for symptoms characteristic of bacterial wilt. Samples were sectioned from the crown and plated on Cmi semi-selective media.

Suspect colonies from the selective media were transferred to Cmi enrichment media, and tested by antibody-based ELISA. Isolates with results greater than background (cut-off 0.200 what is the unit) were tested by PCR. DNA was extracted from isolates with positive PCR results (1 only) together

with isolates selected on basis of ELISA results and visual morphology. DNA was sent to CSIRO Entomology (Canberra) for sequencing and confirmation as Cmi.

Results

No Cmi was isolated from any seed samples.

Two bacterial isolates, both from a plant collected from a paddock of Hunter River lucerne in the Keith area, were identified as Cmi by colony morphology, ELISA and PCR. Final confirmation as Cmi was done by sequence comparison over 500 base pairs of 16S ribosomal DNA sequence. They were an identical match to confirmed Cmi isolates from Australia, New Zealand, USA and UK.

Discussion

The policy of issuing Additional Declarations for bacterial wilt based on "regional" area freedom effectively ceased in December 2002 with the positive detection of bacterial wilt in the Keith district.

SA Government Crown Law advice to PIRSA was that Additional Declarations could only be issued for seed grown from crops that were specifically inspected for the absence of a particular disease or weed.

The main implication of the detection of bacterial wilt in the Keith area was that the export trade could no longer rely on area freedom declarations for export to countries /states which require freedom from this disease (Europe, Argentina, Uruguay, Tunisia, Western Australia). An industry-government meeting in January 2003 agreed to base future phytosanitary certificate production on inspection of paddocks for the disease in spring, when the disease is most easily seen in the field.

3. Maintenance of exports

3.1 Phytosanitary inspection process 2003

Seed Services Australia, in conjunction with PIRSA-SARDI, developed inspection protocols for paddocks for phytosanitary certification in spring 2003 for bacterial wilt, stem nematode and *Orobanche* spp. Agriquality used the same principles for their protocols, developed with assistance from PIRSA-SARDI. The development of these protocols was outside the scope of this RIRDC project.

SARDI developed the testing protocols for bacterial wilt and stem nematode to support the phytosanitary inspections. The cost of pathology testing is borne by the growers but the development of the testing protocol has been done within this project. As well, accredited Seed Services Australia and Agriquality inspectors have been trained by SARDI in the recognition of bacterial wilt, stem nematode and *Orobanche* spp.

As part of the 2003 phytosanitary inspection protocol, all paddocks with Hunter River stands > 7 years old were pathology-tested as well as suspect plants from any other paddocks.

Plants were collected by inspectors and sent to SARDI. To identify bacterial wilt, all plants submitted were screened using a DNA test based on DNA sequences unique to the bacterium. Crown sections from plants that the DNA test indicated were infected was then plated to grow out and isolate the bacterium. Identification of the bacterial wilt isolates was confirmed by a further DNA test followed by sequencing diagnostic ribosomal regions. Samples were considered infected only when a bacterial wilt isolate was cultured and conformed by a second stage DNA test.

For diagnostic purposes samples were classified as “highly suspect” when it was not possible to isolate the bacterium despite the sample returning a positive reaction to the first stage DNA test. The most likely explanation for this occurrence is because the bacterium is very difficult to isolate, it grows slowly on artificial media and is often overgrown by other organisms. While “highly suspect” samples have for this season been deemed negative for the purposes of issuing phytosanitary statements, there is a strong possibility the bacterium may be isolated from plants submitted from these paddocks in future seasons.

Although this process is lengthy it meets ISTA requirements for confirmatory testing. The protocol and testing support have been designed to meet international requirements, particularly those of the European Union, which has the most stringent conditions for seed acceptance.

The key principles of the SA phytosanitary inspection process are:

- Participation in the phytosanitary inspection program is voluntary as not all international markets require or specify phytosanitary certification
- The service is open to both certified and uncertified lucerne seed cropsThe spring phytosanitary inspection does not replace but is in addition to the ordinary seed certification pre harvest inspection usually conducted in the summer crop flowering period
- Crops are inspected at the ideal time to detect bacterial wilt, stem nematode and *Orobanche* spp.
- Crops ultimately confirmed as being positive for bacterial wilt or stem nematode remain eligible for seed certification; however access to certain international markets will be restricted due to the disease(s) detected
- Crops must be inspected in the period 1 September to 30 November. Crops, as an average, must have between 10 – 25 cm of growth to permit inspection

Pathology testing and inspection results

From the 2003 phytosanitary inspection process, 411 samples were submitted to SARDI for bacterial wilt / stem nematode diagnosis.

Of these, 10 crops were confirmed positive for bacterial wilt and 15 crops assessed as highly suspect but pathology testing unable to confirm presence of bacterium.
One crop was confirmed positive for stem nematode (in SA mid-North) and no crops containing *Orobanche spp* &/or *Cirsium arvense* were found.
All 10 infected crops (Table 1, includes crop confirmed in December 2002) and 15 highly suspect crops (Table 2) are located in the general Keith district (hundreds of Stirling, Wirrega, Pendelton, Willalooka and Coombe).

Table 1. Confirmed infected crops 2003

Cultivar	Infected Crops	Ha affected	Sown	Certification
Hunter River	6	122	1970-1987	Certified
Hunterfield	2	24	1983, 1986	Certified
Sirosal	2	28	?	Uncertified

Table 2. Highly suspect crops 2003

Cultivar	Infected Crops	Ha affected	Sown	Certification
Hunter River	7	189	1949-1996	Certified
Siriver	7	69	1992-1999	5 Certified , 2 Uncertified
Aurora	1	9	?	Uncertified

3.2. Phytosanitary Inspections 2004

SA and Victoria

The 2004 program will have two inspection options:

1. Full inspection service i.e. Bacterial Wilt, Stem Nematode, *Orobanche* spp & *Cirsium arvense*
2. Modified inspection service ie *Orobanche* spp & *Cirsium arvense* only (only for previously identified BW infected or suspect crops). Bacterial wilt freedom will need to be established for these crops via a pathology seed test acceptable for lucerne seed exports to Argentina + WA

The 2004 protocol will require mandatory plant collection from all cultivars entering their fourth spring. This is due to the 2003 detection of bacterial wilt in cultivars other than Hunter River and Hunterfield and the recognition that bacterial wilt is likely to be detected in susceptible varieties less than seven years of age. Some “suspect” crops of Siriver detected in 2003 were four years old.

Phytosanitary Inspections outside SA

Phytosanitary inspections for bacterial wilt of lucerne can be offered interstate by Seed Services Australia or Agriquality. SARDI Field Crop Pathology can provide training to other seed service providers.

Code of practice

A code of practice for lucerne seed growers is being developed by PIRSA and industry to isolate infected lucerne paddocks from paddocks where seed is being grown for export. This code of practice is based on principles of physical isolation via a buffer zone as well as restriction of water, plant material and machinery movement from infected to potential export seed paddocks.

3.3 Seed transmission

Efforts to find naturally infected seed for international testing of a seed test have not been successful, even when infected lucerne plants were grown in the glasshouse. Infected plants from paddocks where bacterial wilt was detected produce infected tillers but have not produced infected seed, indicating that transmission to seed is extremely low.

3.4 International seed test standardisation

Although the phytosanitary process allows continued export market access, an internationally accepted seed test would provide lucerne seed growers and marketers with greater flexibility, less restricted export options and overall lower cost as only the seed being exported to markets requiring a phytosanitary declaration for bacterial wilt would require testing..

The international acceptance of a seed test developed in a previous RIRDC project (SAR 15-A) is being pursued via the International Seed Testing Association (ISTA) process. Efforts to find naturally infected seed have not been successful. As a result, progress in kit distribution has been delayed but will proceed with artificially infected seed in 2005. At the same time, the ISTA Plant Health Committee, which coordinates this process, has recently been completely re-organised.

4. Discussion of Results

4.1 Implications

Impact of bacterial wilt on production

The disease is not impacting significantly on lucerne forage or seed production. Growers report that infected paddocks remain highly productive. The relatively drier environment and the mostly well-drained sandier soils in the upper South East do not favour Bacterial wilt disease development and expression or apparently seed transmission. However, it appears that the areas where infection is being found are often low-lying, less well-drained areas of a paddock.

Hunter River and Hunterfield appears to be particularly susceptible to the disease. Older stands of susceptible varieties are a reservoir for disease. Most of the Aurora and Siriver in the South East have been sown on older (20 years plus) Hunter River paddocks so detection of disease on younger Aurora stands may be related to inoculum carryover from older susceptible stands.

Impact of bacterial wilt on export markets

To date, the detection of bacterial wilt has not impacted on export markets as protocols have been put in place to meet phytosanitary requirements of export markets. Because the EU market is the most demanding in terms of their phytosanitary requirements, the phytosanitary inspection process has been targeted at the EU market. There are significant costs associated with this process (see below) and some growers are choosing not to participate. In the long term, this may impact on the availability of EU-eligible seed.

Costs

The cost of bacterial wilt phytosanitary inspections in 2003 was $5.50 per ha (inc. GST) plus inspection fees, bringing the total cost to approximately $12 per ha.

The proposed 2004 direct phytosanitary fees will be $6.60 per ha (inc.GST) for full inspection service i.e. bacterial wilt, stem nematode, *Orobanche* spp. and *Cirsium arvense* and $4.50 per ha for modified inspection service ie *Orobanche* spp. and *Cirsium arvense* only, not including inspection fees.

The bacterial wilt seed test costs $270 per sample (inc. GST). This means that phytosanitary field inspection based tests are a cheaper option, particularly for higher yielding irrigated crops.

4.2 Recommendations:

Strategies to reduce older stands of susceptible varieties, which may be acting as a reservoir for bacterial wilt, have been discussed with representatives from the lucerne seed industry.

Stand Life for Hunter River

Older stands of susceptible varieties appear to be a reservoir for bacterial wilt. The issue of introducing a stand life for Hunter River in line with other varieties has been discussed. The issue needs to be actively discussed with growers to seek agreement and would need to be phased in.
If a Hunter River stand is removed a two-year break is required to provide an effective disease break from bacterial wilt.

Higher Phytosanitary Inspection Fees for Bacterial wilt Susceptible Varieties

Higher phytosanitary inspection fees have been considered for older, more bacterial wilt susceptible varieties to reflect their greater risk to discourage growers from maintain older stands which may be acting as disease reservoirs. In the event that a differential charging approach was recommended, a national approach would be needed to achieve consistency between seed service providers. A national approach could only be realistically achieved through involvement by ASA in phytosanitary matters.

International Seed Test Recognition

This will continue to be pursued by SARDI in conjunction with ISTA Seed Health Committee. The ISTA Seed Health Group has recently undergone a complete change of structure and personnel, so it is hoped that this will streamline the seed test acceptance process, which is completely dependent on the participation of other labs.