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**Rural Industries Research and
Development Corporation**

Development of a bacterial wilt test to facilitate the export of lucerne seed

**A report for the Rural Industries Research
and Development Corporation**

By Jan Gooden and Kathy Ophel Keller

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Researcher Contact Details

(Dr. Kathy Ophel Keller)
SARDI Field Crops Pathology Unit
Plant Research Centre
Waite Campus URRBRAE 5064

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

In submitting this report, the researcher has agreed to RIRDC publishing this material in its edited form.

RIRDC Contact Details

Rural Industries Research and Development Corporation
Level 2, Pharmacy Guild House
15 National Circuit
BARTON ACT 2600
PO Box 4776
KINGSTON ACT 2604

Phone: (02) 6272 4539
Fax: (02) 6272 5877
Email: rirdc@rirdc.gov.au
Website: <http://www.rirdc.gov.au>

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Foreword

The export trade in lucerne seed is worth \$8 to 10 million pa nationally. The major export markets for lucerne seed are Argentina, Saudi Arabia, North Africa and Europe. Importing countries require declarations of freedom from bacterial wilt. Bacterial wilt, caused by *Clavibacter michiganense* subsp. *insidiosus* (Cmi), is present in Victoria and along the lower Murray River and lower South East in South Australia. Surveys to support declarations of freedom in the seed producing areas are out of date. Importing countries now demand a reliable seed test.

There is currently no internationally accepted seed test for bacterial wilt. The most common tests are based on culturing the bacterium, which is slow and expensive. An internationally standardised, simple, rapid, reliable seed test for bacterial wilt, is required to support the export seed industry and facilitate trade.

This report covers development and validation of a seed test for bacterial wilt. The assay is antibody-based. This assay has been submitted to the International Seed Testing Association (ISTA) as part of an international comparison of antibody and plate based assays to determine a standardised international assay for bacterial wilt. This project is part of the RIRDC sub program Pasture Seeds, focusing on the quality of export seed.

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

This report is 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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Executive Summary

Development of a reliable, sensitive and internationally accepted test for bacterial wilt of lucerne is important to the Australian export lucerne seed industry as most major importing countries require a nil tolerance of bacterium, *Clavibacter michiganense* subsp. *insidiosus* (Cmi).

Antibody and DNA-based assays were considered as the basis for the Cmi seed test. The most widely used antibody assay, ELISA (enzyme-linked immunosorbent assay) is generally simple to perform, robust and internationally accepted, all important features for a routine diagnostic assay. An ELISA was developed for Cmi. The main task in developing an ELISA is to produce antibodies which detect specific proteins or polysaccharides on the target bacterium.

A rapid specific DNA assay was also developed, based on PCR (polymerase chain reaction) using primers developed by researchers in USA. The level of Cmi detected by this assay in artificially infected seed, where similar to that detected by ELISA.

The main limitation of PCR based assays is that the International Seed Testing Association (ISTA) has not supported such assays as accredited seed tests. One factor in this current view is that internationally the seed testing laboratories are not equipped to perform DNA assays. This seems likely to change in future.

No naturally infected seed could be sourced nationally or internationally during the course of this project, so artificially spiked seed was used to develop the test.

The Cmi ELISA test developed by this project can be completed in 2 days and is relatively simple to perform. The assay is currently being evaluated as part of an international program run by the International Seed Health Initiative (SAR 25-A). Initial feedback is positive, with the assay recently detecting Cmi in a naturally infected seed lot in Canada.

A final decision by ISTA could take several years. In the meantime the SARDI Diagnostic Centre will begin providing the test to industry on a cost-recovery basis from 30 June 2000..

1. Background and objectives

Bacterial wilt of lucerne is caused by the bacterium *Clavibacter michiganense* subsp. *insidiosus* (Cmi). The disease causes wilting, stunting and yellowing. In the field the symptoms of the disease are not severe until the second or third year when it begins to cause significant yield loss. Bacterial wilt is the most serious disease of lucerne in the US (EPPO Data sheet).

The bacterium can survive in dried plant material or seed for 8-10 years. In seed, it survives in the aleurone layer of the endosperm. The transmission rate to seed in the field is unknown. In recent glasshouse studies (Samac et al., 1998), 6.3-7.7% of diseased plants produced infected seed. When individual seeds from infected seed lots were examined, 2.5 to 8.7% of seed contained the bacterium.

Infected seed is the major source of international spread so phytosanitary regulations are in place in importing countries. In Europe the disease is not present in most lucerne growing areas so phytosanitary measures are emphasized.

Existing methods to detect the bacterium in infected seed rely on culturing the bacterium on semi-selective media. It grows slowly (7 days) and is unstable in culture. PCR primers were developed during the course of this project to identify cultured bacteria, but had not been used successfully on infected seed (Samac et al., 1998). A direct seed test for the bacterium remains the preferred option because the bacterium is difficult to culture.

2. Development of a rapid test

2.1 Methodologies

Antibody based assays, enzyme-linked immunosorbent assay (ELISA) and agglutination, and a DNA assay based on PCR (Polymerase Chain Reaction) were evaluated as direct seed tests for Cmi .

Antibody based assays:

ELISA based assays are used routinely for detecting plant viruses and bacteria. This type of assay is generally considered simple to use, robust and considered acceptable for use in seed testing laboratories.

Agglutination assays involve incubating bacterial suspensions with a specific antibody and determining visually if precipitation (clumping of bacterial cells) has occurred. The agglutination test is very rapid (10 minutes) but this approach was rejected because the bacterium must first be cultured. Cmi is notoriously difficult to culture. Also experience in our laboratory with an agglutination test for another plant pathogenic bacteria, *Pseudomonas syringae* pv *pisi*, had shown that the interpretation of results can be difficult in 5-10% of samples.

DNA Assays:

During the course of this project PCR primers specific to Cmi were published (Samac et al., 1998). PCR is now routinely used in medical diagnostics, and is generally considered to be more sensitive than ELISA. These primers were originally developed for use on cultured bacteria and not for use to detect bacteria in infected seed.

A PCR assay was developed in this project which could be performed on a seed soak of artificially infected seed. In the preliminary experiments the sensitivity of the test was similar to the ELISA developed by this project. The assay could probably be made more sensitive, but this work was not continued because ISTA has not yet accepted any PCR assays as accredited tests. This seems to be because few seed testing laboratories are equipped for such assays. While this will probably change soon, it was decided to use the PCR test only to confirm the ELISA results.

2.2 ELISA test development

A useful ELISA requires both polyclonal and monoclonal antibodies specific to Cmi.

Polyclonal antibodies were produced at SARDI. Two rabbits were immunised with Cmi bacterial cells. Antisera produced (R25/7 and R26/7) were screened against an extensive range of Cmi and non-Cmi *Clavibacter* strains and non-*Clavibacter* plant bacteria using direct ELISA (Table 1).

Both polyclonal antisera lines reacted strongly with all Cmi isolates tested. However, both also detected *C. michiganense nebraskense* (pathogen of corn) and *C. michiganense michiganense* (pathogen of tomato and capsicum). R25/7 also detected *Clavibacter rathayi* (Table 1).

Monoclonal antibodies, produced by cell cultures were produced in Dr Peter Macardle's laboratory at the Clinical Immunology Department, Flinders Medical Centre, Adelaide, SA. More than 100 cell lines were produced and screened for specificity to Cmi. Monoclonal antibodies produced by the best cell lines (B1 and D1) reacted strongly with all Cmi strains tested. However, they also detected some *Clavibacter tritici* strains and *C. michiganense* spp *michiganense* (Table 1).

Antiserum from the best anti-Cmi monoclonal line (B1) was then purified to obtain the immunoglobulin fraction (IgM). This purified fraction is then used in the assay.

Specificity of ELISA

The monoclonal antibody was used to coat the ELISA plate and trap the bacteria from the seedc soak. The polyclonal antibodies then bind to the bacterium trapped by the monoclonal antibodies and produce an ELISA specific to Cmi (Table 2). This ELISA detected all Cmi strains and did not detect *C. tritici* or *C. rathayi*.

Table 1. Specificity of polyclonal and monoclonal antibodies *Clavibacter michiganense* subsp. *insidiosus*¹

Isolate	Bacterium name	PAb R25/7	Pab R26/7	Mab B1	Mab D2
CS1	C toxicus	0	0	0	0
CS2	C toxicus	0	0	0	0
CS3	C toxicus	0	0	0	0
CS4	C tritici	0	0	0	0
CS5	C rathayi	2	0	0	0
CS6	C rathayi	1	0	0	0
CS7	C rathayi	1	0	0	0
CS8	Curtobacterium f pv flaccumfaciens	0	1	0	0
CS9	Curtobacterium f pv betae	0	1	0	1
CS10	Arthrobacter ilicus	1	1	0	0
CS11	C tritici	0	0	1	1
CS12	C tritici	0	0	>3	>3
CS13	C iranicum	0	0	0	0
CS14	C toxicus				
CS15	C rathayi	0	0	0	0
CS16	C tritici				
CS17	C tritici	0	0	0	0
CS18	C rathayi	0		0	0
CS19	Curtobacterium f pv pointsetiae	0	0	0	0
CS20	C michiganense insidiosus	>3	>3	>3	>3
CS21	C tritici	0	0	>3	>3
CS22	C tritici	0	0	>3	>3
CS23	C oortii				
CS24	C michiganense nebraskense	>3	2	0	0
CS25	C michiganense michiganense	>3	>3	1	1
CS26	C rathayi	0	0	0	0
CS27	Rhodococcus fascians	0	0	0	0

CS28	C toxicus	0	0	0	0
CS29	C toxicus	0	0	0	0
CS30	C toxicus	0	0	0	0
CS31	C toxicus	0	0	0	0
CS32	C toxicus	0	0	0	0
CS33	C toxicus	0	0	0	0
CS34	C toxicus	0	0	0	0
CS35	C agropyri	0	0	0	0
SE-1	C toxicus	0	0	0	0
SE-3	C toxicus	0	0	0	0
NSW-11	C toxicus	0	0	0	0
NSW-16	C toxicus	0	0	0	0
K110	P fluorescens	0	0	0	0
UQM551	P syringae pv pisi	0	0	0	0
L(3)	P syringae pv syringae	0	0	0	0
1087A	P coriandricola	0	0	0	0
1088B	P coriandricola	0	0	0	0
CS80	C michiganense insidiosus ex Aus	>3		3	
CS81	C michiganense insidiosus ex Aus	>3		>3	
CS82	C michiganense insidiosus ex Aus	>3		3	
CS83	C michiganense insidiosus ex Aus	>3		3	
CS84	C michiganense insidiosus ex NZ	>3		>3	
CS85	C michiganense insidiosus ex NZ	>3		3	
CS86	C michiganense insidiosus ex USA	>3		>3	
CS87	C michiganense insidiosus ex UK	>3		3	

¹ 0 = negative reaction, 1-3 =weak to strong reaction in ELISA assay. Pab = polyclonal, Mab = monoclonal antibody

Table 2. Specificity of triple antibody sandwich ELISA.¹

Isolate	Bacterium name	ELISA reaction
CS20	C michiganense insidiosus	>3
CS4	C tritici	0
CS5	C rathayi	0
CS6	C rathayi	0
CS7	C rathayi	0
CS8	Curtobacterium f pv flaccumfaciens	0
CS11	C tritici	0
CS12	C tritici	0
CS13	C iranicum	0
CS21	C tritici	0
CS22	C tritici	0
CS23	C oortii	0
CS26	C rathayi	0
CS35	C agropyri	0
CS80	C michiganense insidiosus ex Aus	>3
CS81	C michiganense insidiosus ex Aus	>3
CS82	C michiganense insidiosus ex Aus	>3
CS83	C michiganense insidiosus ex Aus	>3
CS84	C michiganense insidiosus ex NZ	>3
CS85	C michiganense insidiosus ex NZ	>3
CS86	C michiganense insidiosus ex USA	3
CS87	C michiganense insidiosus ex UK	>3

¹ 0 = negative reaction, 1-3 =weak to strong reaction in ELISA assay.

Assay protocol

The assay which has been developed is a triple antibody sandwich ELISA (tas-ELISA) comprising a coating antibody (monoclonal) to bind the bacterium to the ELISA plate, a detector antibody (polyclonal) to bind to the bacterium trapped by the coating antibody and an enzyme (horseradish peroxidase) conjugated anti-rabbit antibody which binds to the rabbit polyclonal antibody. The enzyme horseradish peroxidase converts the normally clear solution of the substrate TMB to a colour that can be read by a plate reader. This procedure takes 3 days to complete.

The detailed testing protocol submitted to the International Seed Health Initiative Herbage Group (ISHI-H) for evaluation, is attached (Appendix A). The ISHI-H is an initiative of the international seed industry federation (FIS) and the ISHI technical groups submit test protocols for approval to the International Seed Testing Association (ISTA) Plant Disease Committee.

2.3 Polymerase Chain Reaction (PCR)

Primers The PCR primers developed by Samac et al., (1998) produce a characteristic 127 bp band when used to amplify Cmi strains. The primers detected all Australian Cmi strains tested.

Direct seed PCR assay

Artificially infected seed extracts were centrifuged. Pelleted seed extract was suspended in a DNA extraction buffer and DNA was extracted using a bead shaking process. Extracted DNA was used directly in the PCR reaction.. The DNA band produced from Cmi is 127 base pairs (bp). No bands are produced by infected seed soaks.

2.4 Seed preparation and detection

We have been unable to obtain infected seed both nationally and internationally during the course of this project. Development of the assay has been based on lucerne seed spiked with Cmi bacterial cells. Two seed preparation methods were tested- grinding in a domestic blender and seed crushing.

Lucerne varieties Aurora, Aquarius , Sequel and Siriver have been used in the development of the assay. A subsample (5 g) was ground in a blender and suspended in extraction buffer. The ELISA test is then performed directly on the seed extract.

Known numbers of Cmi cells were added to ground seed extract and ELISA or PCR was performed on this extract. ELISA can detect *Clavibacter michiganense* subsp. *insidiosus* at a cell concentration in seed of 10^5 cells/ml. The PCR assay on seed soak gave a comparable level of sensitivity.

Table 3. Sensitivity of ELISA in detection of Cmi in seed.

Log bacterial cells/ml	Optical Density reading	
	Cmi cells only	Cmi cells in seed extract
0	0.103	0.132
2	0.121	0.155
3	0.124	0.162
4	0.151	0.189
5	0.255	0.307
6	0.638	0.877
7	1.617	2.483
8	2.433	2.5

Although naturally infected lucerne seed could not be sourced for this project, a small amount of infected seed was tested by the ELISA test as part of the international comparative testing (SAR 25-A) and was found positive. Therefore it appears promising that the test is sufficiently sensitive to detect bacteria in naturally infected seed.

3. Conclusions and recommendations

The ELISA assay developed shows good promise for routine detection of Cmi in lucerne. The assay is specific and does not cross-react with closely related bacteria or with lucerne seed. The assay is relatively sensitive but the absence of naturally infected seed makes it impossible to determine if the ELISA is more sensitive than plating tests.

The assay can be completed in 2 days. Evaluation of grinding methods in releasing the bacteria from seed cannot be tested without naturally infected seed but initial results from naturally infected seed tested in Canada as part of the international ring test suggested that pulverisation in a domestic blender was adequate.

This test is currently being compared with media plating methods as well as an immunofluorescence test as an international standard test. The Cmi ELISA developed in this project is being tested by four other laboratories this year as part of an international ring test (SAR 25-A).

3.1 Implications

The availability of the bacterial wilt test and the participation in the standardisation of the test in the International Seed Health Initiative will facilitate lucerne seed export by allowing seed exporters to access an internationally recognised seed test. The test is also beneficial in testing suspect plant material, in paddock inspections or area freedom surveys because current diagnosis and identification is difficult.

3.2 Recommendations

The results of the International Seed Health Initiative comparative testing will be collated in July 2000. This forms part of RIRDC project SAR 25-A (Determine international standards for disease testing of lucerne seeds). Initial feedback indicates that the test works well to detect Cmi. The test should be offered via the SARDI Diagnostic Centre and the availability of the test promoted through seed industry journals.

3.3 Intellectual Property

Intellectual property was developed in this project with respect to the antibody cell lines and the test protocol. It is proposed that the test protocol be in the public domain but SARDI retain control over the antibody cell lines. SARDI will have first commercial rights over the test and will offer the test on a national basis through the SARDI Diagnostic Centre. It is proposed that a royalty of 5% be payable to RIRDC and Flinders Medical Centre with a royalty split between RIRDC and Flinders of 80/20, based on their respective project contributions.

3.4 Communications Strategy

The test will be advertised through the Seed Industry Association magazine. It is proposed the test price is \$200. The availability of the test and test protocol will be communicated to AQIS.

4. Appendix

Protocol submitted to International Seed Health Initiative-Herbage Group for International Ring Test.

5. Bibliography/references.

European Plant Protection Organisation. Data sheets on Quarantine pests. *Clavibacter michiganense* subsp. *insidiosus*.

Samac, D.A., Nix, R.J. and Oleson, A.E. 1998. Transmission frequency of *Clavibacter michiganensis* subsp. *insidiosus* to alfalfa seed and identification of the bacterium by PCR. Plant Disease 82: 1362-1367.

APPENDIX

Protocol submitted to International Seed Health Initiative-Herbage group: Detection of bacterial wilt in lucerne seed by ELISA

Summary

A triple antibody sandwich ELISA (tas-ELISA) has been developed for the detection of *Clavibacter michiganense* sp *insidiosus*, (Cmi), the causal agent of bacterial wilt in lucerne. The ELISA is run over 2 days with an additional overnight coat of the trapping antibody making it a 3 day test detecting Cmi by direct extraction¹ from seed.

Purified² monoclonal antibody (IgM) against Cmi is used to trap Cmi onto solid phase (96-well ELISA plate). This antibody cross-reacts with some *Clavibacter tritici* strains as well as *C. michiganense* sp *michiganense*³.

Skim milk protein (5% in PBS) blocks uncoated areas of plate wells to minimise non-specific binding.

Uninfected lucerne seed (5g) is coarsely ground using a domestic blender for 10 seconds. Ground seed is suspended in 30ml extraction buffer (PBS, 0.05% Tween 20, 2% skim milk powder containing 37% protein) spiked with known numbers of Cmi bacterial cells.

Purified Ig from a polyclonal antiserum raised in rabbit against Cmi is used to detect the trapped antigen. This antiserum cross-reacts with some *C. rathayi* strains, *Cm* sp *nebraskense* and *Cm* sp *michiganense*³.

Donkey anti-rabbit IgG⁴ conjugated to horseradish peroxidase, followed by substrate (TMB) enables colorimetric determination by optical density at 450nm.

¹ Development of the assay has been based on bacteria-spiked lucerne seed extracts due to difficulty in obtaining naturally infected lucerne seed. The assay detects approximately 10⁵ bacterial cells directly from ground seed soak using a detection threshold of 3x background. Background is approximately 0.1 (optical density at 450nm) and is minimised by using milk protein (2% skim milk powder) in PBS-0.05% Tween 20 as the extraction buffer.

² Monoclonal antibodies in hybridoma cell supernatant are purified by ion exchange chromatography and gel filtration, concentrated, sterilized by filtration and stabilized with a chemical compatible with an horseradish peroxidase system. Further purification by culturing IgM-producing hybridomas in serum free medium will minimise background.

³ Although neither the monoclonal antibody or polyclonal antiserum is mono-specific, they each cross-react with different epitopes/antigenic sites. When used in a sandwich ELISA, specificity is enhanced.

⁴ Donkey anti-rabbit IgG is affinity purified and cross-absorbed to minimise species crossreaction with other proteins such as mouse immunoglobulin.

DETECTION OF BACTERIAL WILT IN LUCERNE SEED BY ELISA

Reagents

Unless otherwise stated all water used is de-ionised.

Refer to 'Reagents' manual for reagents not listed below.

Medium 523

Refer Media section of 'Reagents' manual.

Phosphate buffered saline (PBS)

Stock (x20)

To a 1L Schott bottle:

1. Add 900ml water
2. Add 160g Sodium chloride (NaCl)
3. Add 4.0g Potassium di-hydrogen orthophosphate (KH_2PO_4)
4. Add (28.4g anhydrous) Di sodium hydrogen orthophosphate (Na_2HPO_4)
(44.4g $\cdot 7\text{H}_2\text{O}$)
(58.0g $\cdot 12\text{H}_2\text{O}$)
5. Add 4.0g Potassium Chloride (KCl)
6. Dissolve solids. Gently warm on magnetic stirrer to completely dissolve.
7. Make to 1000ml.
8. Store room temperature (reagent shelves).

Working strength (x1) (0.01mol/L phosphate, 0.15 mol/L NaCl, 0.003 mol/L KCl)

To a 1L Schott bottle:

1. Add 50ml Stock PBS (x20)
2. Make to 1L with water
3. Adjust pH to 7.2 with HCl (**with care**)
4. Store 4°C

PBS-Tween (PBST) 0.05% Tween20 (Polyoxyethylene sorbitan laurate)

To 10L Working strength PBS:

1. Add 5ml Tween 20
2. Store at room temperature up to 1 week or at 4°C

Monoclonal antibody (Mab-Cmi-IgM) line MA1

Refer Antibodies section of 'Reagents' manual.

Monoclonal antibody directed against Cmi was produced in hybridoma cell supernatant and purified by ion exchange chromatography and gel filtration, concentrated, sterilized by filtration and stabilized with bronidox, a chemical compatible with an horseradish peroxidase system. Stored aseptically in 500ul aliquots, 4°C.

For stock Mab-Cmi-IgM, 1mg/ml, dilute stabilised concentrate 1/2 in sterile PBS. Store in 1 ml aliquots, 4°C. Discard after 2 months.

Coating antibody

Working strength Mab-Cmi-IgM was determined by titration against a positive control (10^8 cells/ml) to achieve OD_{450} of >2.5 .

For working strength dilute stock Mab-Cmi-IgM 1/100 in PBS. Prepare fresh.

Blocking solution (5% SMP in PBS)

Skim milk protein, (skim milk powder, 37.5% protein); 5% w/v in PBS. Prepare fresh.

Extraction buffer (2% SMP in PBST)

Skim milk protein, 2% w/v in PBST. Prepare fresh.

Bacterial isolate *Clavibacter michiganense* sp. *insidiosus*

Refer Bacterial cultures section of 'Reagents' manual.

Subculture Cmi reference isolate CS20 (ex NCPPB 1109) on medium 523 (modified).

Use after 5 days at 25°C.

Spiked seed standards**Prepare bacterial suspension**

1. Suspend bacteria from 5-day old culture in PBS to achieve a thick 'bulk' suspension.
2. Dilute 'bulk' suspension in 10ml PBS to achieve $OD_{600}=1.0$ ie. 10^8 cells/ml. Note dilution factor.
3. Prepare 25ml of bacterial cell suspension in extraction buffer using dilution factor to achieve 10^8 cells/ml.
4. Serially dilute tenfold in extraction buffer to 10^2 cells/ml to create 'standard curve'.
5. Transfer 10ml of each 'standard' to labelled 50ml tubes containing 20ml extraction buffer.
6. Include sample blank of 30ml extraction buffer only to complete 'standard curve'.

Prepare healthy seed for spiking

1. Weigh 8 x 5g healthy lucerne seed into leak-proof plastic sample bags.
2. Grind seed in domestic grinder, 10 seconds.
3. Return ground seed to individual sample bags.
4. Transfer ground seed to 50ml tube containing 20ml extraction buffer plus 10ml of bacterial suspension with known numbers of bacterial cells to create spiked seed standards.
5. Mix vigorously by inversion and allow to stand 30min;
6. Repeat mix and allow to settle up to 5min.
7. Transfer 100ul of ground seed extract to ELISA plate. Conform to established plate layout.

Sample preparation

The preparation of lucerne seed samples to be tested for the presence of bacterial wilt can only be evaluated when infected lucerne seed is available. The weight of seed per seed lot will be determined by ISTA. Seed sample size will be determined statistically to achieve maximum assay detectability. Extraction efficiency of the non-motile Cmi from ground seed will be maximised.

Polyclonal antiserum (Pab-IgG)

Refer Antibodies section of 'Reagents' manual.

Polyclonal antiserum (R25/7) directed against Cmi was purified by ammonium sulphate precipitation and filtration through DE22 cellulose column to obtain the IgG fraction. Yield was approximately 2mg/ml. Stored in 100ul aliquots at -20 °C.

For stock Pab-Cmi-IgG 1mg/ml, dilute frozen aliquot 1/2 in PBS.
Store at 4°C. Discard after 4 weeks.

Detecting antiserum

Working strength Pab-Cmi-IgG was determined by titration against a positive control (10⁸ cells/ml) to achieve OD₄₅₀ >2.5.

For working strength dilute Pab-Cmi-IgG 1/1000 in PBST; (=1ug/ml). Prepare fresh.

High salt diluent (HSD) (0.45 mol/L NaCl, 10% FBS, 1% Tween 20)

To a 500ml measuring cylinder:

1. Add 13.15g Sodium chloride (NaCl)
2. Add 50.0ml Foetal bovine serum (FBS)
3. Add 5.0ml Tween 20
4. Make to 500ml with water
5. Dissolve solids
6. Aliquot 50ml lots into 50ml plastic tubes. Store at -20°C.

Conjugate

Refer Conjugates section of 'Reagents' manual.

Commercial donkey anti-rabbit IgG conjugated to horseradish peroxidase, affinity purified and crossabsorbed. Store at 4°C.

Stock conjugate prepared by diluting commercial conjugate 1/10 in EPA buffer.
Store in dark, 4°C.

Working strength conjugate prepared by diluting stock 1/100 in HSD. Prepare fresh.

Substrate (TMB)

K-blue commercial substrate (ELISA Systems). Store at 4°C in dark bottle.

Aliquot 100ml into a 100ml dark bottle. Store at room temperature.

Stopper solution (1 mol/L H₂SO₄)

Caution; corrosive - wear rubber gloves and protective clothing (laboratory coat); dispense in fume hood with shield down or wear safety glasses.
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To a 1L measuring cylinder:

1. Add approximately 800ml deionised water.
2. Carefully pour 56ml of concentrated sulphuric acid (18.2 mol/L H₂SO₄) slowly with stirring.
3. Make up to 1litre with water. Store at room temperature.

DETECTION OF BACTERIAL WILT IN LUCERNE SEED BY ELISA

Equipment (refer to operation manuals for usage)

Balances

1. 0-200g, 3 decimal places
2. 0-1000g, 2 decimal places

Laminar Flow Unit

Spectrophotometer

Visible wavelength 600nm

Grinder

Domestic coffee grinder, Tiffany model 240 blender

Platform shaker

IKA Labortechnik, model KS250 basic. (or equivalent plate shaker)

Incubator

Thermostatically controlled 25°C

Incubator-shaker

Thermostatically controlled 37°C

ELISA plate reader

Multiskan spectrophotometer, 450nm wavelength filter

Refrigerator; 4°C

Freezer; -20°C

Electronic timer

Pipettes

Consumables

ELISA microtitre plates, Nunc maxisorb, 96 well

ELISA plate lids

Plastic sample bags, leak-proof

50ml plastic tubes with caps.

DETECTION OF BACTERIAL WILT IN LUCERNE SEED BY ELISA

Procedure

DAY 1

1. Antibody coating

Prepare working strength coating antibody Mab-Cmi-IgM; dilute stock 1/100 in PBS.
Coat microtitre plate with 100ul/well.
Place in sealed box and incubate overnight, 4°C.

DAY 2

NB. Prepare extraction buffer, bacterial suspensions, ground seed samples prior to blocking as this will take longer than the blocking step.

2. Wash

Wash x3, 3min/wash with PBST

3. Blocking

Prepare blocking solution; 5% skim milk powder in PBS.
Block plate with 300ul/well.
Place in sealed box and incubate 1hr, 25°C.

4. Wash

Wash x3 as in step 2.

5. Samples - test antigen and controls

Load plate with 100ul of sample extract / standard / negative control / positive control.

Observe plate layout to conform to plate reader program.

Seal plate with lid, place on shaking platform, 300rpm, and incubate overnight, 4°C.

DAY 3

6. Wash

Wash x3 as in step 2.

7. Detecting antiserum

Prepare working strength Pab-Cmi-IgG; dilute stock 1/1000 in PBST.
Add 100ul/well.
Seal plate with lid and incubate 1hr 30min, 37°C waterbath.

8. Wash

Wash x3 as in step 2.

9. Conjugate

Prepare working strength conjugate; dilute stock conjugate 1/100 in HSD.
Seal plate with tape and incubate 1hr, 37°C waterbath.

10. Wash

Wash x3 as in step 2.

11. Substrate

Add 100ul of TMB substrate to each well.
Incubate for 30 min at room temperature.

12. Stopper

Add 100ul of 1 mol/L H₂SO₄ and read immediately.

13. Reading

Read plate spectrophotometrically at 450nm using air as blank.

14. Calculation of results (based on subcultured individual colonies from seed extracts)

Calculate mean of replicate optical densities for blank, standards, samples.
Subtract background from standard and sample ODs.
Compare corrected sample OD with corrected standard OD to calculate number of bacterial cells/sample.

A result is deemed positive⁺ where the corrected OD of the sample is greater than 3x the OD of the background.

The background OD should not exceed 0.150 and preferably should less than 0.100.

The OD of the highest standard (10⁸cells) should be greater than 2.000 before background correction.

15. Example

Cell nos	OD rep 1	OD rep 2	Mean OD	Corrected OD
0	0.134	0.131	0.133	0
10 ²	0.161	0.149	0.155	0.022
10 ³	0.160	0.164	0.162	0.029
10 ⁴	0.192	0.186	0.189	0.056
10 ⁵	0.317	0.297	0.307	0.174
10 ⁶	0.907	0.847	0.877	0.744 ⁺
10 ⁷	2.500	2.466	2.483	2.350 ⁺
10 ⁸	2.500	2.500	2.500	2.367 ⁺

16. Controls

One negative seed control and two positive spiked seed controls included with each plate. To maximise stability, bulk healthy seed extract and extracts spiked with two different levels of bacteria were aliquotted and freeze dried. Duplicate controls to be included with each plate.

Test parameters

a. Accuracy

b. Limit of detection

To be determined.

The limit of detection is statistically based on the number of subsamples (groups) from a given batch of seed and the number of seeds within each subsample.

The weight of seed per seed lot will be determined by ISTA. Seed sample size and the number of subsamples will be determined statistically to achieve maximum assay detectability.

c. Precision

The assay precision based on the reproducibility at varying levels of infection will be reassessed when a source of infected lucerne seed is available.

Meanwhile the controls have been used to determine assay precision. (*Refer table*)

d. Sensitivity

The assay detects approximately 10^5 bacterial cells directly from spiked ground seed soak using a detection threshold of 3x background. Background is approximately 0.1 (optical density at 450nm) and is minimised by using milk protein (2% skim milk powder) in PBS-0.05% Tween 20 as the extraction buffer. (*Refer standard curves*)

Further reduction of the background is possible by additional purification of the monoclonal antibody.

e. Specificity

Although neither the monoclonal antibody or polyclonal antiserum is mono-specific, they each cross-react with different epitopes/antigenic sites. When used in a sandwich ELISA, specificity is enhanced (*Refer table*).

f. Interferences

Non-specific binding by glycoproteins and polysaccharides from seed exudates causes significant interference of antigen-antibody binding. This has been minimised by soaking dry-ground seed in a protein-rich buffer. Of the ten different varieties of lucerne seed tested, all gave consistently low backgrounds.

Antibody specificity to target epitopes/antigenic sites is enhanced when used together in a sandwich ELISA system.

Critical steps or parameters

Seed sample size, volume of extraction buffer, extraction time, time allowed for grounds to settle before bacteria when transferring seed soak for testing.

Comparison with other methods (if applicable)