Potential exotic virus threats to lucerne seed production in Australia

by Ralf Georg Dietzgen
February 2020
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Foreword

Plant viruses cause significant economic losses to agriculture through reduced yields and quality. Lucerne is an important silage crop for nutritious biomass production for livestock and is a highly valued commodity. In 2010, a high incidence of severe dwarfing disease of lucerne (alfalfa dwarf disease (ADD)) was reported in Argentina and Uruguay, where yield was reduced by up to 30%. Due to its potential economic impact in Australia, ADD is listed as a high-risk pathogen in the Australian Fodder Biosecurity Plan.

This collaborative research project brought together the complementary expertise of Australian and Argentinian researchers to further study ADD in Argentina and to determine its potential risk for the Australian lucerne industry.

The research project concluded that there is no evidence that ADD is currently present in Australia. In addition, the project found that the alfalfa leaf curl virus, which has emerged in Europe and the Middle East, is a significant contributor to the severe disease symptoms seen in Argentina, together with the alfalfa mosaic virus (AMV). Industry, state departments of agriculture, and biosecurity agencies should work together to prevent this virus from entering Australia. To assist with this collaboration, a draft contingency plan has been developed for consideration and potential implementation.

The project identified a new enamovirus that had previously been described only on chickpea. The new lucerne virus likely warrants further investigation of its importance and prevalence. A number of persistent viruses known to infect lucerne globally were also identified.

This project was funded by AgriFutures Australia, and was jointly supported by the Queensland Department of Agriculture and Fisheries, and the University of Queensland through the Queensland Alliance for Agriculture and Food Innovation (QAAFI). This research was conducted in consultation and with the support of Lucerne Australia.

This report for the AgriFutures™ Pasture Seeds Program adds to AgriFutures Australia’s diverse range of more than 2,000 research publications. It forms part of our Pasture Seeds Program, which aims to increase production and processing efficiency, and sustainability.

Most of AgriFutures Australia’s publications are available for viewing, free downloading or purchasing online at: www.agrifutures.com.au.

John Smith
General Manager, Research
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About the author

Dr Ralf Dietzgen is a Principal Research Fellow and Associate Professor in Virology in the Queensland Alliance for Agriculture and Food Innovation at the University of Queensland (UQ). Professor Dietzgen has more than 35 years of research experience in the study of plant virus diseases and molecular interactions of viruses with their plant hosts and insect vectors. Before joining UQ in 2010, he was a principal researcher and Science Leader in Emerging Technologies in the Queensland Department of Agriculture and Fisheries.

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This research would not have been possible without the financial support of AgriFutures Australia, and the generous assistance of Lucerne Australia, lucerne growers, colleagues and international collaborators.

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This research was jointly supported by the QLD Department of Agriculture (QDAF) and the University of Queensland (UQ) through the Queensland Alliance for Agriculture and Food Innovation, and by AgriFutures project PRJ-009751.

This project was completed in collaboration with researchers at the Instituto de Patologia Vegetal (IPAVE), Centro de Investigaciones Agropecuarias, Instituto Nacional de Tecnologia Agropecuaria (INTA) at Cordoba, Argentina. My special thanks go to Dr Nicolas Bejerman who has been instrumental in fostering this long-distance collaboration and in achieving project outputs. I would like to acknowledge the support and sharing of data prior to publication of many INTA colleagues who are involved in various aspects of alfalfa dwarf disease research in Argentina, namely Drs Veronica Trucco, Fabian Giolitti, Soledad de Breuil and Sergio Lenardon.

UQ PhD student Samira Samarfard was supported by a living allowance scholarship from AgriFutures Australia and a tuition fee scholarship from UQ Graduate School. She was advised by Drs Ralf Dietzgen, Nicolas Bejerman and Murray Sharman (QDAF). Murray deserves a special mention for his expert critical comments to improve the ADD Contingency Plan.
Abbreviations

ADD    alfalfa dwarf disease
ADV    alfalfa dwarf virus
AEV    alfalfa enamovirus
ALCV   alfalfa leaf curl virus
AMV    alfalfa mosaic virus
ARaV   alfalfa ringspot-associated virus
BLRV   bean leaf roll virus
cDNA   complementary DNA
CP     coat protein
CpCAV  chickpea chlorosis Australia virus
DNA    deoxy-ribonucleic acid
ds     double-stranded
ELISA  enzyme-linked immunosorbent assay
INTA   Instituto Nacional de Tecnologia Agropecuaria
MsAV   *Medicago sativa* amalgavirus
MsAPV  *Medicago sativa* alphapartitivirus
N      nucleoprotein
NCBI   National Center for Biotechnology Information
NGS    next-generation sequencing
PCR    polymerase chain reaction
QDAF   Queensland Department of Agriculture and Fisheries
RIRDC  Rural Research and Development Corporation
RNA    ribonucleic acid
RT     reverse transcription
TBIA   tissue blot immunoassay
UQ     University of Queensland
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Executive summary

This report outlines the rationale, methods, results and outcomes of a research project funded by AgriFutures Australia (PRJ-009751) from June 2015 to March 2019.

This research aimed to identify whether the viruses associated with alfalfa dwarf disease (ADD), a severe disease syndrome first reported in Argentina in 2010, also occur in Australia, and whether they might pose potential threats to Australia’s lucerne seed production. Based on these findings, a contingency plan was developed to help guide appropriate responses to an incursion of the exotic viruses of main concern found associated with the disease.

Background

The lucerne seed industry is mainly centred around the South Australian towns of Keith, Naracoorte, Tintinara and Bordertown. Some lucerne seed is also produced in the Riverland in SA and in the Lachlan Valley, NSW. Most lucerne hay is produced in NSW, VIC, QLD and WA. The lucerne seed industry is made up of more than 250 individual seed production farms in irrigated and non-irrigated production systems. Seed is produced for the domestic and overseas markets. The lucerne seed industry will most benefit from this research.

In 2010, a severe disease of lucerne was reported from Argentina. Due to the economic importance of lucerne in Argentina and Australia, and the sudden impact of ADD in Argentina, a collaborative research proposal was developed in consultation with AgriFutures™ Pasture Seeds Program to investigate the risks that ADD might pose to the Australian lucerne industry.

Aims/objectives

This research aims to protect the Australian lucerne seed and hay industry from the exotic alfalfa dwarf disease (ADD), which has major negative impacts on production in Argentina. ADD is associated with five viruses; this project led to the development of sensitive diagnostic assays for these viruses and their use to survey Australian seed and hay crops. Collaborative research in Argentina aimed to determine ADD epidemiology and genetic diversity of the viruses involved. Based on the knowledge gathered during this project, a draft contingency plan was developed to help keep Australia free of the disease.

Methods used

Molecular diagnostic assays based on the polymerase chain reaction were developed to detect the five known viruses associated with ADD. The assays were used for limited, targeted surveys of Australian lucerne crops. Other RNA and DNA viruses infecting lucerne in Australia were identified using next-generation sequencing and rolling circle amplification. Genetic diversity of ADD-associated viruses was determined by sequence comparison of representative isolates. Seed transmission, alternative hosts and insect vectors were investigated in Argentina, and a contingency plan was developed from the collected biological data.

Results/key findings

The current study found that:

- There is no evidence of ADD being present in Australia
- A co-infection of lucerne with alfalfa leaf curl virus and alfalfa mosaic virus appears to be the main cause of the severe alfalfa dwarf disease in Argentina
- Molecular diagnostic assays for the five viruses associated with ADD were developed and validated
- Alfalfa mosaic virus and bean leaf roll virus, which are known to be present in Australia, were the only viruses associated with ADD that were detected in Australia.
- A contingency plan for ADD was developed from the epidemiological data collected in Argentina.
- A new virus, tentatively named alfalfa ringspot-associated virus, was identified as infecting lucerne in South Australia and Victoria.
- Chickpea chlorosis Australia virus was, for the first time, found to be infecting lucerne.

Implications for relevant stakeholders

Based on our limited, targeted surveys of lucerne crops, there is no evidence of alfalfa dwarf virus in Australia. There is a risk of accidental introduction of the major exotic virus involved in the disease. Biosecurity measures should be put in place to prevent the introduction of alfalfa leaf curl virus (ALCV) into Australia. Grower vigilance and early detection will be key to keeping the industry safe from ADD.

Recommendations

1. For ADD, biosecurity measures should focus on keeping Australia free from ALCV. Additional input by biosecurity agencies into the draft contingency plan that was developed as part of this project would be useful and welcome to update the information as new data come to hand. The question of potential low-level ALCV seed transmission is still unresolved; this should be a research priority.

2. Alfalfa ringspot-associated virus should be further investigated to determine its biology, prevalence and effect to determine whether it poses a risk to the Australian lucerne industry.

3. The potential impacts of chickpea chlorosis Australia virus on lucerne production should also be investigated.
Introduction

Plant viruses cause millions of dollars in crop damage each year, and pose a threat to global food production and food security. A devastating dwarfism disease causing >30% yield reduction was observed with >70% incidence in commercial lucerne fields in Argentina in 2010, with estimated annual losses of US$700 million (Bejerman et al., 2011) (Figure 1).

*Alfalfa dwarf virus (ADV)/Cytorhabdovirus* (-)ssRNA

*Alfalfa mosaic virus (AMV)/Alfamovirus* (+)ssRNA

*Bean leaf roll virus (BLRV)/Luteovirus* (+)ssRNA

*Alfalfa enamovirus-1 (AEV-1)/ Enamovirus* (+)ssRNA

*Alfalfa leaf curl virus (ALCV)/Capulavirus* ss DNA

*Figure 1: Characteristic ADD symptoms include severe leaf puckering (A); leaf enation (B); severely dwarfed plants (C, right white circle); leaf curling (D); and dwarfed plants (left) compared to uninfected plants (E).*

*Figure 2: Viruses associated with alfalfa dwarf disease in Argentina.*
Alfalfa dwarf disease (ADD) was found to be associated with five diverse viruses: alfalfa dwarf virus (ADV), alfalfa mosaic virus (AMV), bean leaf roll virus (BLRV), alfalfa enamovirus-1 (AEV-1), and alfalfa leaf curl virus (ALCV). ADV is an enveloped bacilliform virus with a negative-sense, single-stranded (ss) RNA genome; AMV, BLRV and AEV-1 have spherical particles enclosing a positive-sense ssRNA genome, while ALCV has a DNA genome and geminate particles (Figure 2).

Surveys of lucerne fields in Australia in 2004 and 2011 have identified high incidences of AMV and luteoviruses in WA and northern NSW (Jones, 2004; Van Leur and Kumari, 2011). However, because diagnostic assays for the majority of ADD-associated viruses were not available at this time, the presence or absence of these viruses in Australian lucerne was unknown. Because of its potential impact on the Australian lucerne industry, ADV has been listed as a risk pathogen in the Australian Fodder Biosecurity Plan.

Alfalfa dwarf disease was discovered as recently as 2010. At the start of this research project, there were no peer-reviewed published scientific reports about longer-term impact and detailed epidemiology of the disease. The first and only scientific report at the time was a “Disease Note” published in the international journal Plant Disease (Bejerman et. al 2011). According to the latest unpublished information from Argentina, testing of lucerne crops in regions adjacent to the initial sites of infection indicates that the disease continues to spread into new areas. Epidemiological research to identify alternative plant hosts and vectors as well as knowledge of genetic diversity of the associated viruses was urgently needed. To achieve this goal, diagnostic assays needed to be developed and validated.

Due to the economic importance and sudden impact of ADD in South America, Dr Dietzgen, a globally renowned expert on plant rhabdoviruses at the University of Queensland, was contacted in 2011. As a result, the UQ Queensland Alliance for Agriculture and Food Innovation (QAAFI) research institute in collaboration with the Instituto Nacional de Tecnologia Agropecuaria (INTA), Ministry of Agriculture of Argentina embarked on a project to characterise one of the new lucerne pathogens, ADV, commencing in 2012. Dr Bejerman, who first reported the disease, was funded by his home institute in Argentina to spend two years at UQ in Dr Dietzgen’s laboratory in Brisbane to conduct this research. That study determined the complete genome sequence of ADV, demonstrated the intracellular localisation of its encoded proteins by transient expression in Nicotiana benthamiana leaves, and established a viral protein interaction map. This molecular work was published in the international journal Virology (Bejerman et al., 2015).

The scientific knowledge and molecular tools developed during the earlier collaborative research formed a solid basis on which to build diagnostic and epidemiological research. It also placed the international research team in an excellent position to successfully achieve the industry-targeted research objectives proposed for this AgriFutures Australia project, including the development and application of sensitive diagnostic assays for ADD-associated viruses. There are two major benefits of this research: determining whether ADV and other novel ADD-associated viruses occur in Australian lucerne growing regions; and developing a contingency plan based on improved knowledge of the biology of these viruses, including insect and seed transmissibility and natural host range. The research plan was also designed to facilitate the education of a higher-degree-by-research student.

The AgriFutures Australia research project will determine whether the economically highly damaging viruses associated with ADD identified in South America are also present in Australian lucerne. We predicted that Australia was free of the viruses that cause severe ADD symptoms. If, however unexpectedly, severe ADD symptom-causing viruses were to be detected, immediate biosecurity measures could be implemented in an attempt to contain and eradicate the disease. The diagnostic assays developed can also be used for early detection of incursions of ADD-associated viruses in post-entry quarantine or field situations. These assays can be added to those already used to detect other viruses infecting lucerne.
Through collaboration with researchers in Argentina, another beneficial outcome of the research will be detailed scientific knowledge about the genetic diversity and epidemiology of ADD-associated viruses, where they are known to occur. This information will be used to develop biosecurity strategies, including adopting the diagnostic assay to help prevent ADD from entering Australia.

In the longer term, Australian-certified lucerne germplasm may be screened for resistance to ADD. If suitable varieties are found, they could yield a source of added-value resistant seed for export to countries where this virus syndrome is a problem. That could also serve as an insurance policy against accidental introduction into Australia.

Objectives

1. Development of a sensitive molecular diagnostic assay for ADV
2. Application of this assay to survey Australian lucerne crops
3. Collaborative research in Argentina to study ADV genetic diversity and epidemiology to identify the range of natural insect vectors, potential for seed transmission, and alternative crop and weed hosts
4. Risk analysis and development of a biosecurity plan and integrated control measures based on this combined new knowledge.

Expanded project objectives

At the time of writing the project proposal and at the beginning of this research, ADV was considered as an important contributor to ADD in Argentina. It was initially used as a marker for the disease. As our research progressed, the importance of other viruses associated with ADD became clearer. The objectives were broadened to include all five ADD-associated viruses in the development of diagnostic assays, survey of lucerne crops in Australia, genetic diversity and epidemiological studies.

Indeed, one of the ADD-associated viruses – the aphid-transmitted ALCV – was found to cause severe disease symptoms in lucerne, not just in Argentina, but also throughout the Mediterranean and Middle East region. This led to the project team’s involvement in a large-scale international study of this emerging virus to determine its evolutionary history and increasing spread.

The identification of only two different viruses in lucerne samples collected in 2015-17 in Australia (AMV and BLRV) prompted questions about the potential causes of the diverse virus-like symptoms seen in our samples. This led to the inclusion of an additional project objective (no. 5) to identify putative other viruses associated with Australian lucerne plants.
Methodology

1. Virus molecular diagnostic assays

Nucleic acid-based assays were developed using polymerase chain reaction (PCR) DNA amplification technology, due to its specificity, sensitivity, relatively high throughput and reasonable cost. Information on virus genome sequences that are a requirement of this technique were available on the National Centre for Biotechnology Information (NCBI) database online and through our collaborators at INTA.

Total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Germany). DNA was extracted using a published cetyltrimethyl ammonium bromide method.

Detection of RNA viruses necessitated the conversion of RNA into complementary DNA, using reverse transcription (RT) as a first step. For ease of use and speed, we used a commercial one-step RT-PCR kit and virus-specific primers that facilitated both steps in succession using a thermal cycler. (RT)-PCR products, also called amplicons, were visualised and their size determined following electrophoresis through an agarose gel and fluorescent staining.

We developed two duplex RT-PCR assays to detect AMV and ADV, and BLRV and AEV-1; in each case, the unique-sized products indicated which virus was detected. Positive and negative control samples were included in each experiment. Details of the materials and methods have been published in Samarfard et al., (2018a).

2. Survey of Australian lucerne crops for ADD-associated viruses

Lucerne leaf samples showing diverse virus-like symptoms were collected between December 2015 and March 2017 from seeds crops (with a focus on older paddocks to detect accumulated viruses) near Keith, SA, and from fodder crops at Gatton, QLD, and at Hamilton and Edenhope, VIC. Sampling in SA and QLD was done across fields and a representative range of symptoms was collected; each sample consisted of 5 or 6 symptomatic leaves taken from an individual plant. Photos of the collected leaves were taken and GPS coordinates recorded. Bulked samples from several plants were provided by Dr Mohammad Aftab (Agriculture Victoria), and sub-samples were pooled for testing.

3. Identification of novel RNA and DNA viruses in lucerne in Australia

Ten lucerne leaf samples representative of the diversity of observed virus-like symptoms were analysed by next-generation sequencing (NGS) of tagged cDNA libraries for the presence of any RNA viruses. To enrich viral sequences before library preparation, double-stranded (ds) RNA (representing mostly viral sequences) was captured using dsRNA-specific monoclonal antibodies, as described by Blouin et al. (2016). DsRNA was reverse transcribed, amplified and barcoded, and the 10 samples combined for sequencing by GENEWIZ (China) using an Illumina MiSeq and 2x250 base-paired ends. Viral sequence contigs were assembled and analysed for sequence identities with NCBI database. The identity of viral sequences detected by NGS was validated by RT-PCR and sequencing.

Total nucleic acid extracts of 34 leaf samples with leaf curling, rolling and leaf enation symptoms were subjected to rolling circle amplification with TemPlPhi (GE Healthcare) to detect any single-
stranded DNA viruses, such as ALCV. Amplified long DNA was cleaved by restriction endonucleases, and linearised fragments were cloned into a bacterial plasmid for sequencing.

Detailed materials and methods for this research will be published in the PhD student thesis and a scientific publication that is in preparation.

4. Genetic diversity of ADD-associated viruses

From the 13 isolates of ADV collected from different lucerne-growing districts in Argentina, the nucleoprotein (N) gene was amplified, cloned and sequenced (Samarfard et al., 2018b). Similarly, the coat protein (CP) gene of several isolates of AMV and BLRV from our lucerne surveys was amplified, cloned and sequenced. Isolates of ALCV from the Mediterranean, Iran and Argentina were collected, and 120 complete genome sequences recovered from 10 countries were analysed. Total DNA was extracted, amplified, cloned and sequenced, as detailed by Davoodi et al. (2018).

Genetic diversity of the different viruses was determined by pairwise sequence distance, phylogenetic and recombination analyses using standard bioinformatics software (Samarfard et al., 2018a, b; Davoodi et al., 2018).

5. Epidemiology of ADD-associated viruses

Seed transmission was determined in 500 progeny seeds collected from virus-infected mother plants.

Biological virus transmission assays to healthy lucerne plants were done in insect-proof cages under glasshouse conditions using aphids collected from ADD-affected paddocks. Plants were scored by symptoms and (RT)-PCR.

Plant species with virus-like symptoms were collected from areas surrounding ADD-affected lucerne fields and tested for all five ADD-associated viruses.

6. Development of an ADD contingency plan

Based on the knowledge obtained about ADD during this research project and parallel projects in Argentina, a draft contingency plan was developed. It considered potential pathways for incursion of the main causal viruses, the risk that this may occur, potential impacts and measures for early detection and potential control. This was done as a desktop study, with contributions from INTA and the QLD Department of Agriculture and Fisheries.
Chapters

1. Development and validation of molecular diagnostic assays

#Results of this chapter address Objective 1.

PCR-based molecular diagnostic assays were developed for all five ADD-associated viruses: alfalfa mosaic virus (AMV); alfalfa dwarf virus (ADV); bean leaf roll virus (BLRV); alfalfa enamovirus-1 (AEV-1); and alfalfa leaf curl virus (ALCV). These assays were validated under laboratory conditions using positive and negative control samples. To reduce consumable costs and save time, two duplex assays were developed to specifically detect two RNA viruses in each assay, based on unique assay products that could be differentiated by size. Briefly, a duplex RT-PCR was developed for simultaneous detection of ADV and AMV using a cloned non-infectious ADV RNA fragment as positive control. Similarly, the presence of BLRV and AEV-1 was determined by duplex RT-PCR, and ALCV by PCR. The amplification product sizes were 670 base pairs (bp) for ADV, 363 bp for AMV, 955 bp for BLRV, and 572 bp for AEV-1. The ALCV diagnostic amplification product size was 700 bp. All diagnostic reactions yielded single products of the expected size. Details of this research have been published in the journal Australasian Plant Pathology by Samarfard et al., 2018a (see reference list for the complete citation).

2. Presence or absence of ADD-associated viruses in Australian lucerne

#Results of this chapter address Objective 2.

Twelve lucerne seed paddocks were surveyed in 2015, and 11 in 2017 near Keith, SA (Figure 3). Samples from three lucerne paddocks in Gatton in QLD, and seven paddocks in VIC (Hamilton and Edenhope) were also collected, and assayed for AMV and ADV (as an initial marker for ADD) (Figure 3). Only AMV and BLRV were detected in Australian lucerne seed paddocks. AMV was found to be widespread, especially in older paddocks used for seed production (Table 1, Table 2).

Figure 3: Map of Australia showing lucerne collection sites for this research in South Australia (SA), Victoria (VIC) and Queensland (QLD) (http://www.google.com.au/maps).
Table 1: Virus-testing data for lucerne samples collected in seed paddocks around Keith, SA. (Samarfard et al., 2018a)

<table>
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<th>Paddock Identifier 2015</th>
<th>Age (years)</th>
<th>Variety</th>
<th>No. of Samples tested</th>
<th>AMV</th>
<th>ADV</th>
<th>BLRV</th>
<th>AEV-1</th>
<th>AMV + BLRV</th>
<th>AMV (%)</th>
<th>BLRV (%)</th>
<th>AMV + BLRV (%)</th>
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<th>Variety</th>
<th>No. of Samples tested</th>
<th>AMV</th>
<th>ADV</th>
<th>BLRV</th>
<th>AEV-1</th>
<th>AMV + BLRV</th>
<th>AMV (%)</th>
<th>BLRV (%)</th>
<th>AMV + BLRV (%)</th>
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<td>12.5</td>
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Table 2: Virus-testing data for lucerne samples collected in hay paddocks in Victoria and Queensland. (Samarfard et al., 2018a)

<table>
<thead>
<tr>
<th>Victoria</th>
<th>Age (years)</th>
<th>Variety</th>
<th>No. of Samples tested</th>
<th>AMV</th>
<th>ADV</th>
<th>Location</th>
<th>AMV (%)</th>
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<tr>
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<td>6</td>
<td>3</td>
<td>0</td>
<td>Hamilton</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>–</td>
<td>6</td>
<td>3</td>
<td>0</td>
<td>Hamilton</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>–</td>
<td>6</td>
<td>5</td>
<td>0</td>
<td>Hamilton</td>
<td>83</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>–</td>
<td>8</td>
<td>5</td>
<td>0</td>
<td>Edenhope</td>
<td>62</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>–</td>
<td>8</td>
<td>7</td>
<td>0</td>
<td>Edenhope</td>
<td>87</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>–</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>Edenhope</td>
<td>12.5</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>–</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>Edenhope</td>
<td>16.6</td>
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<table>
<thead>
<tr>
<th>Queensland</th>
<th>Age (years)</th>
<th>Variety</th>
<th>No. of Samples tested</th>
<th>AMV</th>
<th>ADV</th>
<th>Location</th>
<th>AMV (%)</th>
</tr>
</thead>
<tbody>
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<td>Sequel</td>
<td>12</td>
<td>9</td>
<td>0</td>
<td>Gatton</td>
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<tr>
<td>9</td>
<td>3–4</td>
<td>SARDI 7</td>
<td>15</td>
<td>8</td>
<td>0</td>
<td>Gatton</td>
<td>53.5</td>
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</table>
3. Genetic diversity of ADD-associated viruses

Results of this chapter address Objective 3.

Knowledge of the genetic diversity of viral genomes is important to optimise diagnostic assays to detect all variants and to predict evolutionary changes.

In this research project, we determined the coat protein (CP) gene sequences of selected Australian lucerne isolates of AMV and compared them to sequences from global isolates, especially isolates associated with ADD in Argentina. AMV isolates from lucerne in Australia and Argentina (ADD-associated) are closely related, and differ by only 1–2% in CP nucleotide sequence (Samarfard et al., 2018a).

The CP gene of BLRV was analysed in a similar fashion. Genetic diversity of all known isolates deposited on GenBank is very low; BLRV CP gene sequences from Australia and Argentina are 98.3–100% identical (Samarfard et al., 2018a; Trucco et al., 2016).

ADV is a new virus and has been reported only from Argentina. The genetic diversity of 13 isolates of ADV collected across all lucerne-growing regions of Argentina was determined from the complete nucleocapsid (N) gene. All isolates are closely related and have not diverged more than 1% in this gene, despite geographical separation across Argentina (Samarfard et al., 2018b). Phylogenetically, the closest known relatives of ADV are strawberry crinkle virus and persimmon virus A.

AEV-1 is a new enamovirus reported only from Argentina as associated with ADD. The complete genome sequence of the Manfredi isolate has been determined that will enable future studies of its genetic diversity (Bejerman et al., 2016).

ALCV is classified in the genus Capulavirus, family Geminiviridae. Capulaviruses have a non-segmented circular single-stranded DNA genome, and are transmitted by the widespread aphid species Aphis craccivora. The original ALCV isolate from ADD-affected lucerne in Argentina is 83.2–92.6% identical in genome sequence to European isolates, and appears to be a recombinant of two European strains (Bejerman et al., 2018).

In this AgriFutures Australia project, we investigated the genetic diversity of 35 ALCV isolates from different lucerne-growing regions in Argentina. Total nucleic acids were extracted, and complete ALCV genomes amplified and sequenced. These sequences were compared to isolates from France, Italy, Spain, Iran, Syria and Jordan. All ALCV isolates from Argentina were most closely related to strain B isolates from the northern hemisphere. They appear to have emerged following recombination events between strains A and B, and have diverged by up to 5%, likely indicating a recent introduction into Argentina.

The results of our research were subsequently incorporated into a much larger international study that was published in the international open access journal Viruses. ALCV isolates could be placed into four genotypes (A-D) that showed frequent recombination events. The evolutionary history of ALCV suggests that this virus emerged and diversified in the Middle East, then spread to the western Mediterranean basin and Argentina (Davoodi et al., 2018). The Argentinian isolates belong to group D and can be considered as a distinct strain.
4. Epidemiology of ADD-associated viruses in Argentina and a contingency plan for ADD incursion

#Results of this chapter address Objectives 3 & 4.

Epidemiology of ADD-associated viruses in Argentina is described in the ADD Contingency Plan (Appendix 1).

In summary, the viruses that appear to be the main contributors to severe ADD symptoms in lucerne are the exotic ALCV in co-infection with the endemic AMV that is widespread in Australia. Both viruses are known to be efficiently transmitted by the cowpea aphid, *Aphis craccivora*. AMV is also naturally seed transmissible in lucerne. None of the other ADD-associated viruses is known to be seed transmissible in lucerne, but only 500 seeds from ALCV-infected plants were tested in initial experiments. A much larger number of seeds will need to be tested to detect potential low-level ALCV transmission through seed.

ALCV is known to occur in Argentina, the Mediterranean basin and the Middle East. Potential pathways of ALCV incursion into Australia are by viruliferous aphids or infected host plants. Limited studies have shown that ALCV infects only lucerne and related subspecies. The likelihood of ALCV entry into Australia is considered to be low, whereas the potential for establishment, spread and economic impact is high. The risk of ALCV incursion and establishment of ADD can be minimised by appropriate biosecurity measures.

The establishment and spread of ALCV will be dependent on the prevalence and movement of the major known vector species *A. craccivora*, and the availability of suitable susceptible host plants in the field at the time of incursion. The most effective control will be through the combined control of the vector aphids and timely identification and removal of symptomatic lucerne plants. ALCV strain D detected in ADD-affected paddocks in Argentina is often found as mixed infections, so it appears likely that this virus will also co-infect with AMV and BLRV that are widely distributed in Australian lucerne.

Diagnostic assays for ALCV and the other ADD-associated viruses were developed during this project. Suspect samples can be tested in diagnostic laboratories using the published PCR primers and assay conditions. Research to identify potentially ADD-resistant lucerne germplasm is continuing in collaboration with colleagues at INTA, Argentina.
5. Novel viruses in Australian lucerne

#Results of this chapter address Objectives 1 & 5.

Over the years, several viruses have been reported to infect lucerne crops in Australia (Table 3). Of these viruses, we used diagnostic assays for detection of AMV and BLRV, since they are associated with ADD in Argentina.

However, many of the lucerne leaf samples collected showed diverse virus-like symptoms. Therefore, the question was posed: which virus(es) could be infecting these lucerne plants? Instead of using assays for individual known lucerne viruses, we used high-throughput next-generation sequencing to detect all sequences of RNA viruses (the RNA virome) of a plant sample in one assay.

Eleven selected samples with diverse virus-like symptoms (Figure 4) identified the following viruses:

- AMV
- BLRV
- Alfalfa ringspot-associated virus (ARaV) – a new emaravirus not described before
- *Medicago sativa* alphapartitivirus 1
- *Medicago sativa* amalgavirus 1.

No emaraviruses (order *Bunyavirales*) have been previously reported to infect lucerne. The virus we identified appears to be new to science. Sequences related to ARaV were detected in samples 2, 3, 5 and 9 (Figure 4) collected in SA and VIC (Table 4). Partial ARaV genome sequences matching three emaravirus segments encoding the polymerase, nucleocapsid and matrix proteins were obtained. Emaravirus genomes of up to six segments have been reported in the literature. Further study of ARaV may be warranted to determine its complete genome, how widespread this virus is, how it is transmitted, and what effect, if any, it has on lucerne hay and seed yield. The genome segment sequences we obtained can be used to develop a diagnostic assay for this new virus.

Unlike acute or chronic viruses, persistent viruses are known to occur at low levels and to be transmitted vertically through the gametes. The viruses we detected have been reported globally from lucerne; their presence in Australian varieties was expected but shown here for the first time (Table 4).

The ssDNA virus *chickpea chlorosis Australia virus* (genus *Mastrevirus*, family *Geminiviridae*) was identified in a lucerne sample collected in SA (Table 4); lucerne represents a new, alternative host for this legume virus in addition to bean. The complete genome was sequenced and found to be >97% identical to a chickpea isolate of this virus from NSW.

Except for AMV and BLRV, none of the viruses listed in Table 3 that had previously been detected in lucerne in Australia were detected in the selected samples analysed.
Table 3: List of viruses shown to infect lucerne pastures in Australia, their symptoms, vectors, and mode of transmission. Source: (modified from Jones, 2013); *syn. soybean dwarf virus (SbDV)

<table>
<thead>
<tr>
<th>Virus</th>
<th>alfalfa mosaic virus (AMV)</th>
<th>bean leaf roll virus (BLRV)</th>
<th>subterranean clover red leaf virus (SCRLV)*</th>
<th>beet western yellows virus (BWYV)</th>
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</thead>
<tbody>
<tr>
<td>Transmission Mode</td>
<td>Non-persistent by aphids, pollen, seed, and mechanically</td>
<td>Persistent by aphids</td>
<td>Persistent by aphids</td>
<td>Persistent by aphids</td>
</tr>
<tr>
<td>Vector</td>
<td>Blue-green aphid, pea aphid, spotted alfalfa aphids and cowpea aphid</td>
<td>Pea aphid, green pea aphid, potato aphid</td>
<td>Pea aphid, foxglove aphid, and potato aphid</td>
<td>Green peach aphid, cowpea aphid, foxglove aphid and potato aphid</td>
</tr>
<tr>
<td>Genome</td>
<td>+ssRNA</td>
<td>+ssRNA</td>
<td>+ssRNA</td>
<td>+ssRNA</td>
</tr>
<tr>
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<td>Bromoviridae</td>
<td>Luteoviridae</td>
<td>Luteoviridae</td>
<td>Luteoviridae</td>
</tr>
<tr>
<td>Genus</td>
<td>Alfamovirus</td>
<td>Luteovirus</td>
<td>Luteovirus</td>
<td>Polerovirus</td>
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<tr>
<td>Symptom</td>
<td>Yellow leaf mottle and interveinal chlorosis Subtle, light green vein banding or mottle of leaves, or symptomless</td>
<td>Symptomless</td>
<td>Symptomless</td>
<td>Symptomless</td>
</tr>
<tr>
<td>Location reported</td>
<td>TAS, WA, VIC, QLD, SA, ACT, NSW</td>
<td>NSW, VIC, WA</td>
<td>WA, VIC</td>
<td>TAS, VIC, NSW, WA</td>
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<td>Detection method</td>
<td>ELISA, TBIA</td>
<td>ELISA, TBIA, RT-PCR</td>
<td>ELISA, TBIA</td>
<td>ELISA, TBIA RT-PCR</td>
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<tr>
<td>Control measures</td>
<td>Sowing new pastures with healthy seed stock, AMV-resistant and aphid-resistant cultivars, reduction of aphid population, Mixed planting, and planting barrier crops</td>
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<td>No studies</td>
<td>No studies</td>
</tr>
<tr>
<td>Virus</td>
<td>lucerne Australian symptomless virus (LASV)</td>
<td>lucerne transient streak virus (LTSV)</td>
<td>lucerne Australian latent virus (LALV)</td>
<td>cucumber mosaic virus (CMV)</td>
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<tr>
<td>-------</td>
<td>------------------------------------------</td>
<td>--------------------------------------</td>
<td>--------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td><strong>Transmission Mode</strong></td>
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<td>No seed transmission reported sap-transmissible</td>
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<td>Seed-borne in lucerne; Non-persistent</td>
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<tr>
<td><strong>Vector</strong></td>
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<td>Unknown</td>
<td>Unknown</td>
<td>Lucerne blue green aphid, cowpea aphid, and foxglove aphid</td>
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<tr>
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<td>(+) ss RNA</td>
<td>(+) ssRNA</td>
<td>(+) ssRNA</td>
</tr>
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<td>Bromovirida</td>
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<td><strong>Genus</strong></td>
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<td>Sobemovirus</td>
<td>Nepovirus</td>
<td>Cucumovirus</td>
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<tr>
<td><strong>Symptoms</strong></td>
<td>Symptomless</td>
<td>Chlorotic streaking along lateral leaf veins; leaf distortion</td>
<td>Symptomless or bright yellow vein clearing</td>
<td>1) yellow mosaic and reduction in leaf size 2) bright leaf mosaic</td>
</tr>
<tr>
<td><strong>Location reported</strong></td>
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<td>NSW, VIC, WA</td>
<td>SA, VIC, WA</td>
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<td>1972</td>
<td>1971</td>
<td>1988</td>
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<td>Serology</td>
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<td>ELISA, TBIA</td>
<td>TBIA</td>
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<td><strong>Control measures</strong></td>
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<td>No studies</td>
<td>Sowing healthy seed stock</td>
<td>No studies</td>
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Figure 4: Australian lucerne samples showing virus-like symptoms used for virome analyses.
Table 4: Symptoms observed in collected lucerne samples (see Figure 3 above), RNA viruses detected, and molecular methods used for detection.

<table>
<thead>
<tr>
<th>Sample</th>
<th>State</th>
<th>Symptoms</th>
<th>Detected RNA viruses and detection methods</th>
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<td></td>
<td></td>
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<td>AMV</td>
</tr>
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<td>VIC</td>
<td>Yellow mosaic</td>
<td>A, B, C</td>
</tr>
<tr>
<td>2</td>
<td>VIC</td>
<td>Ringspot and yellow mosaic</td>
<td>A, B, C</td>
</tr>
<tr>
<td>3</td>
<td>SA</td>
<td>Ringspot</td>
<td>B, C</td>
</tr>
<tr>
<td>4</td>
<td>VIC</td>
<td>Yellow patches &amp; mild ringspot</td>
<td>B, C</td>
</tr>
<tr>
<td>5</td>
<td>VIC</td>
<td>Ringspot</td>
<td>A, B, C</td>
</tr>
<tr>
<td>6</td>
<td>SA</td>
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<td>B, C</td>
</tr>
<tr>
<td>7</td>
<td>VIC</td>
<td>Reddening</td>
<td>A, B, C</td>
</tr>
<tr>
<td>8</td>
<td>VIC</td>
<td>Leaf rolling, enation</td>
<td>B, C</td>
</tr>
<tr>
<td>9</td>
<td>SA</td>
<td>Ringspot, yellow patches</td>
<td>B, C</td>
</tr>
<tr>
<td>10</td>
<td>SA</td>
<td>Leaf curling</td>
<td>A, B, C</td>
</tr>
<tr>
<td>11</td>
<td>SA</td>
<td>Leaf rolling, yellow mottle</td>
<td></td>
</tr>
</tbody>
</table>

Symptoms of samples, as shown in Figure 4.
Viruses were detected by A: duplex RT-PCR; B: next-generation sequencing (NGS); and C: single RT-PCR. (-): not detected.
(*) Cowpea chlorosis Australia virus (CpCAV) detected by rolling circle amplification.
Discussion of results

As set out in objective 1, specific molecular diagnostic assays for the five viruses known to be associated with ADD in Argentina were developed and validated.

As set out in objective 2, these assays were used to survey lucerne seed paddocks around the town of Keith, SA in 2015 and 2017. Mostly older stands of lucerne were visited (up to 29 years old) based on the likelihood of accumulation of insect-transmitted viruses over several years, and only leaf samples were collected that showed virus-like symptoms, in particular, symptoms similar to those associated with ADD. Samples from three hay paddocks in QLD and seven paddocks in VIC were also tested.

Only two viruses were detected that are known to be endemic in Australia, and known to infect lucerne and several other plant species, namely AMV and BLRV. The other ADD-associated viruses ADV, AEV-1 and ALCV did not appear to be present in Australia.

The lucerne leaf samples that were collected showed diverse virus-like symptoms. Therefore, objective 5 was added, to investigate which viruses are associated with these symptomatic plants in Australian seed (SA) and hay (VIC) paddocks. Using a generic high-throughput sequence detection method for RNA viruses, we identified as expected AMV (in all samples) and BLRV (sample #10), but also a novel emaravirus and two persistent viruses (Table 4). The emaravirus appears to be new to science and should be studied further for complete genome sequence, prevalence, epidemiology and potential effects on lucerne production. Using a method specific for detection of ssDNA viruses, such as ALCV, a different geminivirus was detected that was originally described infecting chickpea in Australia. The potential effects of this virus on lucerne and as a reservoir for infection of other legume crops should be further investigated.

As set out in objectives 3 & 4, collaborative research with INTA Argentina studied genetic diversity of ADV and ALCV, and epidemiology of ADD-associated viruses. These data on virus genetic diversity, seed and insect transmission, and alternative hosts allowed for the development of a draft contingency plan for ADD (Appendix 1).

This research concluded that the ADD-associated viruses ADV, AEV-1 and ALCV do not appear to be present in Australian lucerne. Alfalfa dwarfism symptoms as described in Argentina have not been reported in Australia thus far. ALCV and AMV appear to be the main contributors to the severe dwarfism disease. The main objective of any biosecurity measures must therefore be to keep ALCV out of Australia.
Implications

1. Based on our limited, targeted surveys, there is no evidence – neither typical symptoms nor exotic ADD-associated viruses detected – that alfalfa dwarf disease occurs in Australia.

There are currently no indications that the severe alfalfa dwarf disease that occurs in Argentina also occurs in Australia. However, growers will need to remain vigilant and watch for any severe symptoms like those described for ADD overseas. Confirmation of ADD-associated viruses is now possible based on the diagnostic assays developed during this project.

2. ALCV appears to be the major factor that leads to severe alfalfa dwarf disease.

Based on the biological research in Argentina thus far, ALCV appears to be the main factor for the severe dwarf symptoms in lucerne, especially when plants are co-infected with the widespread AMV. ALCV occurs in the Mediterranean basin, the Middle East and Argentina, but has not been reported from Australia. Accidental entry of ALCV into Australia must be prevented to keep Australia free of ADD.

3. There is a risk of accidental introduction of ALCV in viruliferous aphids, infected plant material and potentially in seed, assuming a low seed-transmission rate (that still has to be proven).

As detailed in the draft ADD Contingency Plan, the potential routes of accidental introduction of ALCV based on its biological properties should be considered by biosecurity agencies. A considerably larger number of seeds (~5,000) from lucerne plants infected with ALCV and showing ADD symptoms should be tested in Argentina to detect potential low-level seed transmission, because if confirmed, it may lead to undetected ALCV introduction in imported seed lots.

Recommendations

1. Biosecurity measures should focus on keeping Australia free from ALCV.

2. The draft contingency plan for ADD should be consulted and appropriate actions initiated if ALCV were to arrive in Australia. The plan should be updated annually as new information about ADD emerges. Biosecurity authorities should comment on the draft plan so it can be appropriately refined.

3. The question of potential low-level ALCV seed transmission is still unresolved; it should be a research priority in Argentina.

4. The alfalfa ringspot-associated emaravirus should be further investigated to determine its complete genome sequence, biology, prevalence, and potential risk to the Australian lucerne industry.

5. The distribution and potential effect of chickpea chlorosis Australia virus on lucerne and other pasture seed crops should be investigated.
References


Appendices

Appendix 1: Alfalfa dwarf disease – draft contingency plan

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1. Executive summary

Alfalfa dwarf disease (ADD) is a devastating disease of lucerne in Argentina. Alfalfa leaf curl virus (ALCV) is the most likely key virus, leading to severe symptoms and yield losses. ALCV is known to be transmitted by the aphid *Aphis craccivora*. It is found in South America, the Mediterranean region and the Middle East, and infects lucerne (*Medicago sativa*). The potential pathways of ALCV entry into Australia are by viruliferous aphids or infected host plants. Although limited studies have been done, ALCV is only known to infect lucerne and related sub-species. The risks of ALCV incursion and establishment of ADD can be minimised by biosecurity measures offshore, at the border, and onshore.

2. Purpose and background

This contingency plan delivers the information available as of October 2018 on disease epidemiology, virus diagnostic methods, and potential control measures to assist with an appropriate biosecurity response in case of an ADD incursion into Australia. ADD is caused by a mixed infection of lucerne with up to five different viruses that may pose a potential threat to the Australian lucerne seed and hay industry. This contingency plan includes recommendations for the steps that should be considered when an ADD response plan is required. Any response plan developed from this contingency plan should follow the steps detailed in the Plant Health Australia 2018 PLANTPLAN ([www.planthealthaustralia.com.au](http://www.planthealthaustralia.com.au)) and be confirmed by the National Management Group ([www.directory.gov.au/portfolios/agriculture-and-water-resources/national-management-group](http://www.directory.gov.au/portfolios/agriculture-and-water-resources/national-management-group)) before implementation.

3. Lucerne production in Australia

The Australian lucerne (*Medicago sativa* L.) seed industry is one of the major economic contributors to agricultural communities in regional and remote SA, relying on lucerne seed production for employment and global trade. In 2008, about 83% of Australian lucerne seed was produced predominantly across the upper and mid-south-east of SA, around the towns of Keith, Naracoorte, Tintinara and Bordertown, including more than 16,000 hectares of both irrigated and dryland areas (Carter and Heywood, 2008). Australian lucerne seed production also occurs in small areas in the
Riverland of SA, and around Forbes in the Lachlan Valley of NSW (Figure 5) (Carter and Heywood, 2008). Lucerne hay accounted for 17% of the Australian hay produced, with NSW contributing 40%, VIC 25%, QLD 18%, WA 10%, and the remainder produced in SA, TAS and NT (Figure 5) (Martin, 2009). The lucerne seed industry in Australia is made up of more than 250 individual seed production farms. This figure is based on the number of farmers submitting lucerne seed for certification by the Australian Seeds Authority (ASA), including non-irrigated and irrigated production systems (Carter and Heywood, 2008). In 2015, up to 11,000 million tonnes (mt) of lucerne seed was shipped from Australia to overseas markets, mainly to feed livestock and dairy in Saudi Arabia. However, lucerne seed exports from Australia decreased to around 9,257 mt and 6,349 mt in 2016 and 2017, respectively, because there was a global oversupply. Demand from the major market, Saudi Arabia, was reduced (Myall, 2017). The lucerne seed industry in Australia is capable of producing low dormancy varieties through to highly winter-active varieties. The seed market for dormant varieties (dormancy 3-6) was mostly affected by the recent global oversupply situation. However, dormant seed varieties in Australia are only a minor market for seed production (Myall, 2017). The lucerne seed industry in Australia was valued at about A$95 million per year in 2008. Exports accounted for about A$30 million, domestic sales A$8.7 million, and the remainder is due to value-added industries (Carter and Heywood, 2008).

![Figure 5: Lucerne seed and hay production regions in Australia. Source: https://earth.google.com (Carter and Heywood, 2008; Martin, 2009). Bold circles show regions with highest hay production. WA, SA, NSW, QLD, NT and VIC](image)

To establish sustainable and productive lucerne farming, there are two key steps: 1) choose varieties that suit the farm management system and environment; and 2) consider the local and global markets. Currently, more than 100 lucerne varieties are available for Australian markets, including winter-active, winter-dormant, resistance to, or tolerance of pests and diseases, and grazing-tolerant varieties. The lucerne breeding program in Australia is managed by the South Australian Research and Development Institute (SARDI: [http://pir.sa.gov.au/home](http://pir.sa.gov.au/home)) that has pioneered development of new Australian-bred varieties and has strong industry links. The lucerne breeding program aims to introduce varieties that are uniquely suited to the Australian environment (Heritage Seeds, 2018).
According to Heritage Seeds public information (Heritage Seeds 2018), SARDI lucerne-breeding program objectives are:

- A wide range of winter activity and dormancy
- High yield and strong growth after cutting
- Pest and disease resistance
- Adaptability to different climates and soil types
- High fodder nutritional quality
- High grazing tolerance.

Recently, a lucerne-breeding program in Australia has focused on developing unique traits, such as acid tolerance for cultivation in regions where the soils have low pH, and also glyphosate herbicide tolerance (Heritage Seeds 2018; Wigley et al. 2018).

4. Alfalfa dwarf disease

4.1 General information

In 2010, ADD, a severe dwarfism disease of lucerne, was first observed in numerous commercial fields in Argentina. Disease incidence reached more than 70%, and up to 30% seed weight reduction in the diseased plants was reported (Lenardon et al., 2010). In 2017, ADD was reported for lucerne fields in Argentina with an incidence of about 86.4%. In some cases, an annual loss of seed weight up to 38% was observed for plants showing ADD symptoms, specifically when both AMV and ALCV co-infection occurred, as confirmed by available diagnostic tests (Trucco, 2018). High throughput sequencing of the diseased plants revealed the presence of five different viruses: alfalfa dwarf virus (ADV); alfalfa mosaic virus (AMV); bean leaf roll virus (BLRV); alfalfa enamovirus 1 (AEV-1); and alfalfa leaf curl virus (ALCV) (Bejerman et al., 2011; Trucco et al., 2014; Trucco et al., 2016; Bejerman et al., 2015, 2016, 2018). Symptoms similar to ADD were previously observed in lucerne-growing regions in the Mediterranean basin between the late 1950s and 1980s (Alliot et al., 1972; Blattný, 1959; Cook and Wilton, 1984; Leclant et al., 1973; Rodriguez Sardiña and Novales Lafarga, 1973). In 2010, samples collected from lucerne paddocks in France and Spain that were co-infected with AMV, ALCV and a rhabdovirus showed similar symptoms to ADD-affected lucerne plants in Argentina (Bernardo et al., 2016). More recently, in 2018, ALCV-like symptoms were reported in lucerne pastures in some Middle Eastern countries, including Jordan, Lebanon, Syria and Tunisia (Kumari et al., 2018), and different regions of Iran (Davoodi et al., 2018a).

4.2 Lucerne industry in Argentina

In 2007, Argentina was the second largest producer in the world, with almost 4.7 million hectares of lucerne-growing regions (Basigalup and Ustarroz, 2007). The current lucerne cultivation in Argentina is almost 3.2 million ha that is mainly (80%) focused in the Pampa Region (provinces of Buenos Aires, La Pampa, Santa Fe, Entre Ríos and Córdoba) where more than 90% of the area devoted to lucerne is dedicated for direct grazing for beef and dairy production. The rest of the lucerne cultivation regions (20%) in Argentina (irrigated regions, mainly in western provinces), is for seed and hay production (Basigalup and Odorizzi, 2017, personal communication). Lucerne seed production in Argentina is located in 12 provinces supported by advanced irrigation and agricultural systems (P. Lavignolle, Director of Certification of Seed Production, INASE personal communication) (Figure 6). In 2016, Australia was the main supplier of certified lucerne seed to Argentina, providing about 48% of imported seeds, followed by the United States with 29%, and the rest from Canada, France, and Italy (Basigalup, personal communication).
4.3 ADD symptomatology

ADD-affected plants are characterised by shortened internodes (bushy appearance), leaf puckering, and varying sized vein enations on abaxial leaf surfaces, shortening of internodes, which severely decreases size and deforms the leaflets, chlorosis and the appearance of papillae and buds in the ribs of the underside of the leaves (Figure 7) (Bejerman et al. 2011; Trucco, 2018). Recent studies have shown that whenever ADD infection rate is above 50% in a lucerne-growing area (paddock), it can lower yield in hay dry mass by up to 30% (INTA-Informa, 2010).

*Figure 6: Lucerne hay and seed production regions in Argentina. Lucerne hay production regions are indicated by black circles, while seed production regions are shown in colour. Different colours indicate different yields (kg/ha). Source: P. Lavignolle, Director of Certification of Seed Production, Instituto Nacional de Semillas - República Argentina (INASE).*
Figure 7: Characteristic ADD symptoms include severe leaf puckering (A); leaf enation (B); severely dwarfed plants (C, right, white circle); leaf curling (D); and dwarfed plants (left) compared to uninfected plants (E).

4.4 Geographic distribution and symptoms of ADD in Argentina

ADD is widespread in Argentina and possibly some neighbouring countries (Trucco, 2018). The disease was identified in 17 out of the 23 provinces of Argentina, with a prevalence of 86.4%. The five known ADD-associated viruses were detected in all 17 provinces, except for BLRV that was not detected in Chaco province. Lucerne seed production regions in Argentina are mainly located in the western part of the country. Annual seed loss of up to 10% had been previously reported as due to ADD (Odorizzi, 2015). However, according to a recent survey by Instituto Nacional de Tecnología Agropecuaria (INTA), a decrease in seed production of up to 38% was recorded for plants with ADD symptoms. Seed yield losses were especially high and symptoms were severe when lucerne plants were co-infected with AMV and ALCV, indicating that mixed infection with these two ADD-associated viruses leads to severe ADD symptoms (Trucco, 2018).

4.5 Global distribution of ADD-associated viruses

AMV and BLRV are globally distributed, and their genetic diversity is low, with 1% or less nucleotide sequence diversity in the coat protein gene (Samarfard et al, 2018b). The cytorhabdovirus ADV from Argentina has been characterised (Bejerman et al., 2011; 2015; Samarfard et al., 2018a). A rhabdovirus named lucerne enation virus (LEV) was reported in Europe some time ago (Alliot et al., 1972), but there is no genome information and no preserved isolate to compare with ADV. Recently, INTA researchers detected the complete ADV genome in the NCBI database by analysing the Sequence Read Archive library generated from lucerne germplasm exported from the United States to China (N. Bejerman, personal communication). This suggests that the non-symptomatic ADV was exported to China undetected in infected lucerne plants, and that ADV may occur undetected in the USA; more research will be needed to validate this finding and to determine the true global distribution of this virus. A strain of the enamovirus AEV-1, named alfalfa enamovirus 2 (AEV-2), was detected in lucerne-growing regions in Sudan. AEV-2 differed by only 4.7% from AEV-1 at full
genome nucleotide sequence level (Nemchinov et al., 2017). So far, four different strains of ALCV have been characterised through an international collaboration with researchers from several countries (Davoodi et al., 2018b). Two different isolates of ALCV referred to as “strains A and B” were initially identified from leaf curl-exhibiting lucerne plants collected in a large-scale survey in southern France, and then in lucerne samples collected from Spain in a mixed infection with AMV and a rhabdovirus (Bernardo et al., 2016). In 2018, ALCV strain A was detected in lucerne plants exhibiting leaf roll and leaf curling, stunting, mottling, leaf thickening, leaf chlorosis and leaf malformation symptoms. These plants were collected from lucerne in several Middle Eastern countries, including Iran, Jordan, Lebanon and Syria and also from the western Mediterranean basin, including Italy, Greece, Spain and Tunisia (Kumari et al., 2018; Davoodi et al., 2018a; Davoodi et al., 2018b). However, ALCV strain B isolates have not been detected beyond the borders of France and Spain. ALCV strain C isolates have, to date, been found only in Iran in lucerne samples with leaf curling, marginal leaf chlorosis and leaf malformation. Strain D isolates have been identified only in ADD-affected lucerne paddocks in Argentina (Davoodi et al., 2018b; Bejerman et al., 2018).

4.6 Seed transmission of ADD-associated viruses

AMV is seed-transmitted in lucerne at a rate of 3.75% (Trucco, 2018). This virus is also seed-transmitted in other plant species, including soybean, lentils, chickpea, and faba bean, at rates of 5-9%, 0.1-5%, 0.1-1% and 0.04%, respectively (He et al., 2010; Jones and Coutts, 1996; Latham et al., 2004). ADV, AEV-1, ALCV and BLRV are not seed-transmissible in lucerne (no seed transmission detected in 500 seeds collected from infected mother plants) (Trucco, 2018).

4.7 Vector transmission of ADD-associated viruses

Biological virus transmission assays in insect-proof cages under glasshouse conditions using aphids collected from ADD-affected paddocks have demonstrated that ADD-associated viruses are transmitted from ADD-infected plants to healthy plants by Aphis craccivora (cowpea aphid, black legume aphid). They are possibly at different efficiencies, titres and incubation periods, with the exception of ADV, where A. craccivora remains unconfirmed as a vector (Trucco, 2018). Leaf enation and yellowing were recorded in 1 of 40 plants onto which were placed five aphids per plant, and in 3 of 30 plants with 10 aphids per plant, indicating a transmission rate of 2.5% and 10%, respectively. The aphids were shown to transmit three (AEV-1: BLRV: ALCV or AMV: BLRV: ALCV) or two (BLRV: ALCV) of the ADD-associated viruses simultaneously. However, aphids carrying only ALCV have also been collected from ADD-affected paddocks (Trucco, 2018). LEV, a rhabdovirus infecting lucerne plants in France in the 1970s, is known to be transmissible by the black legume aphids, A. craccivora (Leclant et al., 1973). ADV has been detected by RT-PCR in A. craccivora collected in the field from plants with ADD symptoms, but ADV transmission by these aphids has not been demonstrated (Trucco, 2018). This may be due to the fact that either A. craccivora is not a vector of ADV, or the virus latency period was not met (Hogenhout et al., 2008). All plant rhabdoviruses are known to be transmitted by their insect vectors in a persistent-propagative manner characterised by long accession feeding and latency periods before transmission (Hogenhout et al., 2003; Jackson et al., 2005; Mann and Dietzgen 2014).

4.8 Alternative plant hosts and insect vectors

INTA staff collected different plant species with ADD-like symptoms, including white clover (Trifolium repens), red clover (Trifolium pratense), mouse ear (Dichondra repens), sweet clover (Melilotus spp.), sowthistle (Sonchus oleraceus) and fameflower (Talinum paniculatum). These plants were collected from areas surrounding ADD-affected lucerne fields in the provinces of Buenos Aires, Córdoba, Santa Fe and Santiago del Estero, and were tested for all five ADD-associated viruses. The samples displayed various virus-like symptoms, and AMV was detected in all tested plant species (Table 5), with the exception of T. paniculatum, which was negative for all five viruses. ADV and AEV-1 were detected only in white clover, and ALCV and BLRV were not detected in any of the samples (Table 5). Other insects including blue alfalfa aphid (Acythosiphon kondoi), pea aphid (A.
pisum), spotted alfalfa aphid (Therioaphis trifolii) and leafhoppers were also observed in ADD-affected fields, but A. craccivora was by far the dominant species.

Table 5: Potential alternative plant host species tested for ADD-associated viruses (Trucco, 2018).

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>No. plants</th>
<th>Symptoms</th>
<th>Viruses detected by diagnosis assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AMV</td>
</tr>
<tr>
<td>Trifolium repens</td>
<td>2</td>
<td>A</td>
<td>+</td>
</tr>
<tr>
<td>Trifolium pratense</td>
<td>1</td>
<td>B</td>
<td>+</td>
</tr>
<tr>
<td>Dichondra repens</td>
<td>4</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td>Melilotus spp.</td>
<td>5</td>
<td>D</td>
<td>+/-</td>
</tr>
<tr>
<td>Sonchus oleraceus</td>
<td>2</td>
<td>E</td>
<td>+</td>
</tr>
<tr>
<td>Talinum paniculatum</td>
<td>2</td>
<td>F</td>
<td>-</td>
</tr>
</tbody>
</table>

A - Yellowing, foliar deformation and leaf enation on the abaxial leaf surface; B - leaf yellowing; C - mosaic, foliar deformation and keel papillae in the ribs of the abaxial side and small leaves; D - chlorotic mosaic, foliar deformation and in some cases, stunting; E - intense calyx; F - mild mosaic with chlorotic rings.

5. ALCV is an exotic, high-risk ADD-associated virus that significantly contributes to alfalfa dwarf disease

Based on the information provided in section 4, we conclude that: 1) ADD is associated with five diverse viruses; and 2) that the main contributors to the severity of symptoms are AMV, which is present globally and widespread in Australia in combination with the exotic ALCV. We will therefore concentrate the remainder of this contingency plan on ALCV.

6. Potential pathways for incursion of ALCV into Australia

ALCV has been detected in diverse climatic zones, including temperate oceanic and subtropical (Argentina and France), mountainous (Italy), continental (France), and cold/hot semi-desert (Iran). Due to the global distribution of the virus vector A. craccivora and the distribution of ALCV in these climatic zones, it is possible that ALCV could spread worldwide (Commonwealth Institute of Entomology, 1983). ALCV was found to infect several subspecies in the Medicago sativa species complex, including M. sativa subsp. sativa, and probably M. sativa subsp. falcata populations, giving it a broad host base across lucerne varieties (Davoodi et al., 2018b).

Importation of live lucerne plants or alternative hosts (flowers or ornamental plants) of ALCV that may also be contaminated with A. craccivora may be a potential pathway for incursion. Cowpea aphids have a large host range (Blackman and Eastop, 2011), and it may be feasible that ALCV-infected aphids associated with non-ALCV host plants from a region where ALCV occurs could be inadvertently imported.

ALCV is widely distributed in Argentina, and the virus population differs by only about 5% at the nucleotide sequence level. The Argentine ALCV population (strain D) appears to have descended from the same recombinant ancestor, implying that ALCV was first introduced into Argentina and subsequently spread through all lucerne-growing regions due to the efficient, large-scale and long-
range transmission by *A. craccivora* (Davoodi et al., 2018b). Previous studies on the subterranean clover stunt virus (SCSV; family *Nanoviridae*), which is also transmitted by *A. craccivora*, revealed that in Australia this aphid species can move hundreds of kilometres from the coastal areas to cause SCSV infection of pastures in dry regions of South-East Australia (Gutierrez et al., 1971).

Although seed transmission of capulaviruses, including ALCV, has not been confirmed, recent studies have demonstrated that some geminiviruses belonging to *Begomovirus* and *Curtovirus* are seed transmissible (Anabestani et al., 2017; Kil et al., 2016). Therefore, although there is no evidence for seed transmission in 500 seeds tested, it is possible that ALCV may be seed-transmitted in lucerne at a lower rate. If this were the case, then ALCV could be introduced into Australia directly or indirectly in contaminated seed. This scenario could occur if ALCV-infected lucerne seed were inadvertently imported from a region where ALCV has not yet been reported, and a mix of Australian and imported varieties were grown in Australia (Raymond, 2017).

7. **Notification process for ALCV incursion in Australia**

ALCV incursion and the associated potential to lead to ADD in Australian lucerne may increase costs for control of insect vectors, and reduce lucerne hay yields and seed quality. Timely detection and reporting might ease eradication and reduce the long-term impact of an incursion into Australia. This process generally includes the following steps:

1) Recognition of a symptomatic plant by growers, consultants, research personnel, university staff, agribusiness, or the general public

2) Validation of the presence of the virus by a diagnostic facility

3) Informing relevant State Department of Agriculture through the Exotic Plant Pest Hotline (1800 084 881) or contact the department directly.

8. **Eradication or containment process**

If ALCV is detected in Australia, it will be subject to eradication and/or containment processes. The decision to eradicate should consider the potential economic impact for hay and seed production, the cost of eradication and technical viability. Eradication costs must factor in long-term surveys to validate the success of the eradication program. A minimum period of time with no detection of the pathogen will be essential before pest-free status can be declared. The no-detections period must be determined from the size of the infected area, the ALCV host range in the incursion zone, and the extent of surveillance and intensity of monitoring for the virus. If there was an incursion and establishment in a growing region, ALCV may become established in hosts other than lucerne, which will make eradication more unlikely. However, more research into the potential host range of ALCV is needed to determine the level of this risk.

9. **Risk assessment for entry and establishment pathways and potential impacts**

**Likelihood of entry: Low**

ALCV has been reported from several Middle Eastern countries (Iran, Jordan, Syria, Lebanon and Tunisia), Europe (France, Italy, Greece and Spain), North Africa (Sudan) and Argentina. Entry of this virus could theoretically occur via seed, other hosts or infective aphids. Seed transmission of ALCV has not been shown to date, but cannot be excluded, considering the small number of seeds tested. Entry in other hosts, may be ornaments, may be possible if imports occur from ALCV-infected regions. Because countries with ALCV are far away from Australia, it is highly unlikely that winged aphids could carry this virus to Australia. Unless ALCV arrives in near-neighbour countries, such as Papua New Guinea, Timor-Leste (maybe Indonesia) or New Zealand, the risk of natural movements...
of aphids to Australia is very low. However, ALCV-infected cowpea aphids could enter Australia on other plant species (e.g. ornamentals) that are grown near ALCV-infected fields overseas.

**Establishment potential: High**

The aphid vector (*A. craccivora*) and lucerne are present at all times of the year in all subtropical and temperate regions of Australia. *A. craccivora* is known to rapidly disperse and move large distances.

**Spread potential: High**

The aphid vector (*A. craccivora*) and known susceptible plant host species are present in all lucerne-growing regions of Australia (Heritage Seeds 2018; Kamphuis et al., 2012). Therefore, the potential of spread is considered to be high.

**Economic impact: High**

ALCV is considered the most important ADD-associated virus affecting lucerne crops in Argentina (Trucco 2018). ADD is considered the most damaging complex viral disease in lucerne globally, specifically in mixed infections of ALCV and AMV. The reported 38% seed weight reduction caused by ADD in Argentina (Trucco 2018) resulted in a US$1.1 million loss (Giolitti, personal communication). AMV is common and widely distributed in Australian lucerne paddocks (Samarfard et al., 2018b) and in various other hosts. In an ALCV incursion, dual infection with AMV and ALCV will likely lead to ADD-like severe symptoms and severe losses in lucerne pastures.

**Overall risk: Medium**

The establishment and spread of ALCV will be dependent on the prevalence and movement of the major known vector species *A. craccivora*, and the availability of suitable susceptible host plants in the field at the time of incursion. The most effective control will be through the combined control of the vector aphids, and timely identification and removal of symptomatic lucerne plants. ALCV strain D detected in ADD-affected paddocks in Argentina is often found as mixed infections, so it appears likely that this virus will also co-infect with AMV and BLRV that are widely distributed in Australian lucerne.

**Impact on native vegetation, weed hosts and home garden plants**

The host range of ALCV has not been studied well. However, it is possible that ALCV may affect some native and home garden plants that are colonised by the vector aphids, although their susceptibility is unknown. Areas adjacent to affected lucerne crops could be most affected by migrating virus-carrying aphids.

**10. ALCV management**

The following sections provide a summary of proposed actions to be taken as part of a response plan to counteract an incursion of ALCV into Australia. Given ALCV is known to be transmitted by an aphid species, it is recommended that the Contingency Plan for Aphid-Transmitted Viruses (Plant Health Australia, 2011) also be consulted in any response to ALCV.

**10.1 ALCV survey**

Information given in Section 4 provides a framework for the development of early detection and delimiting surveys for ALCV presence. Sources of ALCV within the seed and hay production areas may provide an opportunity for aphids to spread the virus to other susceptible plants outside that area. To limit movement of both the vector and virus-infected plant material, farmworkers should avoid moving plant material or anything potentially contaminated with live aphids between lucerne farms.
Shoes, tools and vehicle tyres should be decontaminated of plant material. Extra precautions, including disposable over-boots that may be used and disposed of onsite, should be undertaken.

**10.2 Important information required for surveys**

Before surveys for the presence and distribution of ALCV and the aphid vector, the following key points will provide basic biological knowledge that is essential for developing an appropriate survey strategy:

- There is a high risk of aphid movement on machinery, equipment and personal effects, which may cause distribution of infected aphids and the virus between crops, farms or regions.
- Adult aphids (winged forms) can travel large distances when propelled by wind, so some control measures should consider the direction of prevailing winds or strong wind events (i.e. storms).
- *A. craccivora* have a wide summer and winter host range.

**10.3 Sampling procedure**

As a priority, plants showing virus-like symptoms and severe symptoms resembling ADD should be sampled. Capulaviruses, including ALCV, have no specific tissue tropism. The virus might be distributed in different plant parts, potentially with different range of titre. Ideally, collection of a short branch of about 20 cm with terminal growth of the youngest leaves with obvious, severe symptoms will provide good-quality material for diagnostic testing.

**10.4 Number of samples required for ALCV diagnosis**

Five to ten samples of symptomatic plants (Figure 7) per paddocks should be collected for initial detection of the virus. If a survey to determine the incidence of disease within a crop or geographic area is required, then a statistically based sampling strategy should be employed like the one proposed for the whitefly-transmitted cotton leaf curl disease (Gambley and Grundy 2013) based on the statistical recommendations of Cannon and Roe (1982) and MacDiarmid (1988). This strategy would also be suitable for ALCV. Essentially, it involves the inspection for typical ADD symptoms from at least 300 plants within a lucerne paddock. This will provide 95% confidence of detection of a randomly distributed virus that occurs at an incidence of 1% in the crop. It assumes a 100% detection rate (i.e. every infected plant within the 300 plants will be detected). If a lower rate of 50% detection is assumed, then it is advisable to inspect at least 600 plants for the same level of confidence. The distribution of ALCV-infected plants is unlikely to be random within the crop, so inspection of plants should be focused on the edge of the crop most likely to be subjected to influx of virus-carrying aphids.

**10.5 How to collect plant samples**

- Recording the precise location of all collected samples (GPS coordinates) is essential. For labelling and packaging procedures for suspect plant material and insects, consult PLANTPLAN (Plant Health Australia, 2010).
- Ensure only symptomatic parts are collected when symptoms are distributed unevenly on the plant. In cases where latent or mild symptoms are observed, sample at least three small parts of terminal branches, each from a different growing point where possible.
- The collected samples should be kept cool (but not frozen) and out of direct sunlight from the time of collection. A chiller box containing a freezer brick wrapped in a thick towel is ideal for field collections, and then at 5°C (e.g. fridge) for longer storage in laboratory. Adding a lightly moistened paper towel to a plastic sample bag before transport can help to reduce sweating or drying.
- Fresh samples should be stored at 5°C for up to one week only. Samples can be stored for
longer periods if frozen at -80°C, or dried using an appropriate method, such as freeze drying or drying over silica gel or calcium chloride.

10.6 ALCV diagnostic laboratories

The preferred diagnostic laboratory for suspect ALCV-infected samples is that of the Plant Virology group, Queensland Alliance for Agriculture and Food Innovation (QAAFI), the University of Queensland.

The preferred point of contact is Associate Prof Ralf Dietzgen (r.dietzgen@uq.edu.au), who has work experience in field and lab diagnostics for ALCV. Mark samples ‘Attention of A/Prof Ralf Dietzgen’ and send via Australia Post or by courier to the address below. For a known or suspected incursion, send samples only by Express Courier to:

Queensland Alliance for Agriculture and Food Innovation (QAAFI)
Queensland Bioscience Precinct [Bld#80], Level 3 (South)
306 Carmody Road, St Lucia, The University of Queensland, St Lucia QLD 4072

A second suitable diagnostic laboratory for independent diagnosis is that of the plant virology group of the QLD Department of Agriculture and Fisheries.

Dr Murray Sharman (murray.sharman@daf.qld.gov.au)
Address: Q-DAF, 2C-West, Ecosciences Precinct, Dutton Park, QLD 4102.

A suitable international expert for ALCV is Dr Nicolas Bejerman with the following contact details:

Instituto de Patología Vegetal – Centro de Investigaciones Agropecuarias – Instituto Nacional de Tecnología Agropecuaria (IPAVE-CIAP-INTA), Camino 60 Cuadras Km 5,5 (X5020ICA), Córdoba, Argentina

Email: nicobejerman@gmail.com

10.7 Communication of consignment details and sample storage

Before sending samples, please notify the laboratory contact. Include details of the expected arrival time, courier, and consignment reference number. Also include details on the crop sampled, such as recent spray applications and presence of potential vectors. When the samples arrive at the laboratory, the field officer will check that the shipment package details match the information supplied at dispatch.

11. Potential control methods

In lucerne pastures of the south-west of Australia, the main aphid vector species found was the bluegreen aphid, although the presence of cowpea aphid or black aphids (Aphis craccivora Koch) and pea aphids have been also reported (Jones, 2004). Disease severity and secondary spread is largely determined by the extent of the primary infection due to migration of viruliferous aphids into young lucerne crops. In ADD-affected paddocks in Argentina, an upward trend of disease incidence has been observed as the crop matures, specifically in summer, which seems to indicate in-field transmission by aphids. This varies slightly between lucerne cultivars, but no resistant cultivars have been identified so far (Samarfard et al., 2018b).
The Australian lucerne breeding program has an emphasis on improving lucerne cultivars for Australian farming systems. Led by the South Australian Research and Development Institute (SARDI), the program operates in conjunction with their commercial partner, Heritage Seeds Pty Ltd. Accordingly, there are two types of lucerne bred in Australia: winter-active lucerne and winter-dormant lucerne. Both types have similar activity over summer (Stanley et al., 2002). One of the key traits for improving lucerne cultivars in Australia is sowing cultivars that are resistant to aphids, including bluegreen, spotted alfalfa, and pea aphids. However, the selection and evaluation process for new lucerne cultivars is slow, taking between seven and 10 years, with three rounds of selection. The selection process can be reduced to five years if the breeders can introgress resistance with a trait already present within the breeding program (Slattery and Taylor, 2013).

11.1 Chemical controls for aphids – immediate

If an incursion occurred and it was decided to contain or eradicate, aphids (and any other potential insect vector) should be chemically controlled. Managing the risk of insecticide resistance should also be an important consideration for control. Relevant recommendations for chemical control of aphid species can be found in the Contingency Plan for aphid-transmitted viruses (Plant Health Australia, 2011).

For current best-practice recommendations for distribution and chemical control of black aphids, refer to the QLD Department of Agriculture and Fisheries website: https://www.daf.qld.gov or the guidelines available as a PDF: http://www.pir.sa.gov.au/__data/assets/pdf_file/0003/275826/Cowpea_Aphid.pdf

11.2 Identification and introduction of resistant germplasm – medium term

QAAFI is collaborating with INTA to identify Australian lucerne varieties with a degree of resistance or tolerance to ADD and black cowpea aphid. This collaboration can be considered an insurance policy against accidental introduction of ALCV into Australia. AgriFutures Australia supports this continuing research.
12. References


Davoodi Z., Bejerman N., Richet C., Filloux D., Kumari S.G., Chatzivassiliou E.K., Galzi S., Julian


13. Protocols

A: Total nucleic acid extraction

1. Grind 100 mg of plant tissue with 1 mL of 2% CTAB buffer
2. Transfer solution to a 2 mL Eppendorf tube
3. Centrifuge solution at 10,000 rpm for 5 minutes at room temperature
4. Transfer supernatant to a clean 2 mL tube
5. Incubate at 60°C for 30 minutes
6. Place tube on ice for 5-10 minutes
7. Add an equal volume of chloroform-isoamyl alcohol (24:1 v/v) and mix well by shaking to form an emulsion
8. Centrifuge at 14,000 rpm for 5 minutes at 4°C
9. Carefully remove the aqueous phase (on top) with a pipette, taking care not to suck up any of the middle or chloroform phases, and place it in a clean 2 mL tube
10. Repeat steps 7 to 9.
11. Add an equal volume of isopropanol, mix by inversion. Incubate at 4°C for 15 minutes
12. Centrifuge at 14,000 rpm for 10 minutes at 4°C
13. Discard supernatant carefully, add 500 μL of cold 70% ethanol to the pellet and mix by inversion
14. Centrifuge at 14,000 rpm for 10 minutes at 4°C
15. Discard supernatant and allow the pellet to dry on the bench for no longer than 10 minutes
16. Resuspend dry pellet in 50 μL of ultrapure water, and incubate for 30 minutes at 37°C
17. Store dissolved total nucleic acid at -80°C.

B: 2% CTAB buffer

100 mM Tris-HCl pH 8.0
1.4 M NaCl
20 mM EDTA
2% CTAB (cetyltrimethyl ammonium bromide)
2% PVP
0.2% 2-mercaptoethanol
C: Diagnosis PCR primers

**Table 6: Oligonucleotide primers for use in PCR assays for detection of alfalfa dwarf disease-associated viruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Target</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMV</td>
<td>AMV-F: ATAGATGCCGGTTCTCCAAGGAT</td>
<td>AMV-R: GACTTCATACCTGACCTTAATCCAC</td>
<td>CP gene</td>
<td>884bp</td>
</tr>
<tr>
<td>AMV</td>
<td>AMV CP 214F: GCGAGATTCCCTCTACAGTTT</td>
<td>AMV CP 578R: GACCCAAACTTCGTTGAATC</td>
<td>CP gene</td>
<td>363bp</td>
</tr>
<tr>
<td>ADV</td>
<td>ADV-F3: ATCAGCTTTGACCTGTGGCTGTT</td>
<td>ADV-R3: TCTTCCAGATGGACCCCTGCTCA</td>
<td>N gene</td>
<td>394bp</td>
</tr>
<tr>
<td>ADV</td>
<td>ADV-N-654F: TACCCTAICAGATCAAGCTATG</td>
<td>ADV-N-1323R: GGTAGCTTGATGATCTGATG</td>
<td>N gene</td>
<td>670bp</td>
</tr>
<tr>
<td>AEV-1</td>
<td>AEV-F: CAGAGTGATAATGCCGACGAA</td>
<td>AEV-R: CGTTCCCTTCAGAGATATACGG</td>
<td>CP gene</td>
<td>716bp</td>
</tr>
<tr>
<td>AEV-1</td>
<td>AEV1-F: CATGGCTATCCCCCTTAA</td>
<td>AEV1-R: AAACCATTTCCTTCCCAGGTT</td>
<td>CP gene</td>
<td>572bp</td>
</tr>
<tr>
<td>ALCV</td>
<td>ALCV-F: 5’-GGAACGTGATGGATT-3’</td>
<td>ALCV-R: 5’-GFTACATGACCATCT-3’</td>
<td>CP gene</td>
<td>690bp</td>
</tr>
<tr>
<td>BLRV</td>
<td>BLRV-F: 5’-TAGGTTCCTCATTCCAAG-3’</td>
<td>BLRV-R: 5’-CCTCAATATCGTCCAGTGTC-3’</td>
<td>CP gene</td>
<td>955bp</td>
</tr>
</tbody>
</table>

D: Commercially available ELISA kits

Commercial antisera and kits are available for the detection of:

- AMV for double-antibody sandwich ELISA (LOEWE Biochemica GmbH, Germany)
- BLRV for triple-antibody sandwich ELISA (Leibniz-Institut DSMZ GmbH, Braunschweig, Germany).

For detection of ADV, AEV-1 and ALCV, there are no commercial serological reagents available at this time.

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Potential exotic virus threats to lucerne seed production in Australia

by Ralf Georg Dietzgen
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