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Development and Use of Diagnostic Tools for Subterranean Clover Red Leaf Disease

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Development and Use of Diagnostic Tools for Subterranean Clover Red Leaf Disease

by David Peck, Nuredin Habili, John Randles, Ramkrishnan Nair, Geoff Auricht and Carolyn de Koning

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Foreword

In the mid 2000s subterranean clover seed producers based in South Australia reported symptoms of red-leaf disease in their seed production fields. This outbreak affected seed production with estimates of up to 60% reduction in seed yield from affected stands. Red-leaf disease is now widespread in South Australia, and total annual losses are likely to be in the order of $4 m if half the stands are affected. Outside of seed production stands, red-leaf disease is likely to be causing significant losses in pasture herbage production across Australia's 30m ha of sub clover pastures. The project described in this report aimed to determine the causal agent/s of the red-leaf symptoms, develop diagnostic tools and develop integrated pest management protocols to control the red-leaf disease. Better understanding of the red-leaf disease and its control will benefit sub clover seed producers and also red meat and wool producers with sub clover pastures.

Seed production fields of sub clover and other annual legume pastures (eg. Persian clover and Balansa clover) were surveyed in 2008 and 2009 in the south-east of South Australia and western Victoria. The viruses found were *bean leaf roll virus* (BLRV), *alfalfa mosaic virus* (AMV) and *cucumber mosaic virus* (CMV). This project developed a set of molecular diagnostic tools that can detect the presence of these viruses and five other viruses known to infect sub clover. Results from this project also showed that bluegreen aphid (BGA, *Acyrthosiphon kondai*) can persistently transmit BLRV. This is a new finding as the scientific literature to date has indicated that BGA do not transmit BLRV. BGA is a widespread aphid of pasture legumes and is more active during the cool season than the other common aphids of pasture legumes and therefore more able to spread red-leaf disease during the cooler months.

The report contains recommendations to assist producers yo develop management strategies to minimise yield losses from red-leaf disease. A fact sheet for the integrated control of red-leaf disease in sub clover was developed from this project, and was distributed to seed producers via sub clover seed companies and on the internet. Recommendations to control viruses and aphids are given in the fact sheet. The main management strategies recommended are:

- Confirm the presence of virus by using the molecular markers developed by this project
- Reduce summer reservoirs of virus and aphids
- Reduce the spread of aphids and viruses by locating sub clover seed crops away from lucerne fields
- Strategically use insecticides to kill the aphid vectors.

This project was funded by RIRDC and Seedmark. The project was conducted by SARDI, The University of Adelaide and Seedmark.

This report is an addition to RIRDC's diverse range of over 2000 research publications and it forms part of our Pasture Seeds R&D program, which aims to maximise opportunities and minimise risks for a profitable and sustainable pasture seeds industry based on reputation for reliable supply, domestically and internationally, for a range of quality pasture species.

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Abbreviations

AMV	*Alfalfa mosaic virus*
BGA	Bluegreen aphid (*Acyrthosiphon kondai*)
BLRV	*Bean leaf roll virus*
BWYV	*Beet western yellows virus*
BYMV	*Bean yellow mosaic virus*
CMV	*Cucumber mosaic virus*
DIBA	Dot-immunobinding assay
ICARDA	International Center for Agricultural Research in the Dry Areas.
PSbMV	*Pea seed-borne mosaic virus*
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RIRDC	Rural Industries Research and Development Corporation
RNA	Ribonucleic acid
SARDI	South Australian Research and Development Institute
SCRLV	*Sub clover red leaf virus*, which is synonymous with SbDV
SbDV	*Soy bean dwarf virus* which is synonymous with SCRLV and the preferred name
SCSV	*Subterranean clover stunt virus*
Sub clover	Subterranean clover (*Trifolium subterraneum*)
TNA	Total nucleic acid

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Executive Summary

What the report is about

Sub clover seed production is estimated to be a $10-15 million dollar a year industry and seed producers from the mid 2000's have reported that an unknown red-leaf disease has been causing yield losses of up to 60% in their seed production crops. This report presents the results of a three year research project that investigated the causal agents of red-leaf disease in sub clover seed crops, developed diagnostic tools and developed a fact sheet on the control of red-leaf disease.

Who is the report targeted at?

Sub clover seed producers, pasture agronomists, sub clover pasture growers.

Where are the relevant industries located in Australia

Hassall and Associates (2001) provide a comprehensive overview of sub clover seed production in their report titled "A Study of the Costs of Production of lucerne, Medic and Clover Seeds in Australia". This report showed that South Australia is the leading state for sub clover production with production centred around Naracoorte to Bordertown and production in 1997/98 of 1400 t. Victoria produced 853 t in 1997/98, with production centred around Numurak, Dookie and Yarrawonga, while NSW produced 17 t and WA produced only 7 t, during the same period.

Sub clover is the dominant pasture legume species within Australia, and is grown on a wide range of soils and climates. Generally where soils are acidic, pastures are based on sub clover, however the subspecies *brachycalycinum* performs well on alkaline soils. While sub clover seed production is primarily restricted to areas with wet winters and hot summers, sub clovers are used as pastures in a much wider area. The sub clover seed industry underpins a much larger pasture industry which produces red meat and wool. While this project is focused on the sub clover seed industry, many of the findings will be applicable to the sub clover pasture industry.

Background

Aims/objectives

The findings of this project will benefit sub clover seed producers and sub clover pasture growers. The principal aim of the project was to determine what virus or viruses are causing the red-leaf disease, develop diagnostic tools for these viruses and develop a set of protocols for the integrated control of red-leaf disease.

Methods used

Commercial sub clover seed crops in the south-east of South Australia and western Victoria were surveyed for red-leaf disease in the spring of 2008 and 2009. It was then determined which viruses were present and a set of molecular marker diagnostic tools were developed. Transmission studies were conducted and a set of protocols was developed for control of red-leaf disease.

Results/key findings

Surveys of sub clover seed production fields found *bean leaf roll virus* (BLRV), *alfalfa mosaic virus* (AMV) and *cucumber mosaic virus* (CMV) but no cases of *sub clover red leaf virus* (SCRLV). Prior to conducting the surveys we speculated that SCRLV was the most likely cause of red-leaf disease as SCRLV is known to cause red leaves in sub clover. However in our surveys BLRV caused red-leaf symptoms. BLRV was also the most common virus found as well as the most widespread. On a world wide basis BLRV is known to be damaging to legumes, but is a relatively new virus in Australia, first reported in 1999 (Schwinghamer *et al.* 1999).

Bluegreen aphids (BGA) were shown for the first time to transmit BLRV. This is a significant finding as BGA are widespread on pasture legumes, pulses (except chickpeas) and lupins and are more active during the cool months than other legume aphids.

A fact sheet (Appendix 1) was developed that lists ten points for seed producers to consider when developing their management strategy to control red-leaf disease. The fact sheet will assist seed producers to lower the risk of BLRV and other sub clover viruses and hence lower the risk of seed yield losses.

Implications for relevant stakeholders for:

The identification of the causal agents of red-leaf disease, the development of diagnostic tools and the development of integrated disease management protocols will reduce the risk of crop failure for sub clover seed producers. This will assist farmers who produce sub clover seed and in turn assist the communities in which they live. Having a more reliable seed production system will assist seed companies and in turn red meat and wool producers who graze cattle and sheep on sub clover pastures.

Recommendations

The recommendations are aimed at sub clover seed producers but are also relevant to sub clover pasture producers. The recommendations are:

- Survey sub clover seed paddocks for plants with red-leaf symptoms and confirm the presence of virus by sending samples to Waite Diagnostics for molecular testing. This is an important step as leaf reddening alone is not diagnostic of plant viruses

- If sampling or past experience suggests that red-leaf disease is likely to occur seed producers should develop a management strategy to minimise the risk of viruses and their vectors being present

- In sub clover the viruses and aphid vectors die out over summer but are maintained in perennial legume species and legume volunteers. The best strategy is to minimise the virus and aphid vectors early in the season. Early in the sub clover season a producer should monitor for aphids (bluegreen aphids, cowpea aphids, pea aphids and green peach aphids) and consider the judicial use of aphicides to control aphid number and reduce the early spread of virus

- Producers should consider locating sub clover seed crops away from old lucerne stands as they are reservoirs of viruses and the aphids that transmit them.

Introduction

Sub clover (*Trifolium subterraneum*) is a self regenerating annual pasture legume suited to a wide range of soils and climates. Sub clover grows from autumn to spring and is the most widely sown pasture legume in southern Australia. Sub clover has excellent feed quality and fixes atmospheric nitrogen which is important for pasture production and also for cereal crops grown in rotation with sub clover. Sub clover has vigorous seedlings due to its large seed size and this trait is important for producing good winter feed. There are many cultivars of sub clovers and they are sown in areas with annual rainfall of 275-1200 mm with early maturing cultivars being sown in the dry areas and late maturing cultivars being sown in high rainfall zones. There are three subspecies of sub clover: ssp. *subterraneum* performs well on acidic to neutral soils; ssp *yanninicum* performs well on waterlogged soils; ssp. *brachycalycinum* performs well on mildly acidic to alkaline soils.

Underpinning the large sub clover pasture industry is a relatively small but important sub clover seed production industry. The value of the sub clover seed production industry in Australia is estimated to be 10-15 million dollars a year. Sub clover plants typically bury their seeds inside burrs in the top 1-5 cm of soil and require vacuum harvesters to harvest the seed. Seed production is concentrated in areas with low summer rainfall as dry conditions are needed to harvest the seed and also minimise deterioration in seed quality. South-east South Australia is the largest production area of sub clover seed (Hassall and Associates 2001).

In the mid 2000's sub clover seed producers based in South Australia reported symptoms of red leaves in their seed production fields and gave the generic name red-leaf disease to these symptoms. They reported that the red-leaf disease greatly affected their seed production and estimated a reduction in seed yield of up to 60% from affected stands. The seed company Seedmark raised the problem as of high priority to sub clover seed production.

Red leaves in plants are indicative of plant stress and are not specific to a single cause. However descriptions of the red-leaf symptoms and how it developed over time through sub clover seed production fields made SARDI and University of Adelaide researchers suspect that the symptoms are caused by a virus or viruses infecting sub clover plants. Several viruses, namely, *bean yellow mosaic virus* (BYMV), *subterranean clover stunt virus* (SCSV), *subterranean clover red-leaf virus* (SCRLV), *alfalfa mosaic virus* (AMV), *beet western yellows virus* (BWYV), subterranean clover mottle virus (SCMV) and *cucumber mosaic virus* (CMV) are known to affect subterranean clover. The symptoms of viral diseases in general include yellowing of the leaves, mottling of the leaves and stunting of the plants. SCRLV infection results in reddening of the leaves from the margin and eventual death of the plants (Kellock, 1971). SCRLV is indistinguishable from *soy bean dwarf virus* (SbDV) and SCRLV is now known in scientific literature as SbDV. Subterranean clover infected with BWYV develops systemic leaf reddening (Aftab *et al.* 2010b). A limited number of samples were sent to Agwest Plant Laboratories in 2005 and 2006 for virus testing, BYMV and a general Luteovirus was detected (Brenda Coutts, pers. comm.). The general Luteovirus was thought to be SbDV due to the red-leaf symptoms, however it could be any of the Luteoviruses. Virus infection of annual pasture legumes can cause losses in dry matter production and also seed yields. Jones 1992 reported that seed losses in sub clover were 71% with alfalfa mosaic virus (AMV) and 58-71% with bean yellow mosaic virus (BYMV). Both viruses were found to decrease seed size as well as seed yield.

Symptoms of virus infection can be confused with environmental factors (e.g. cold conditions, wet conditions, nutrient deficiency, herbicide damage or root diseases) and the presence of virus in plants needs to be confirmed by biological or molecular testing. Control of plant viruses is achieved by limiting the spread of the virus, or by resistance breeding. To achieve control a producer needs to accurately identify the virus causing the disease as each control measure needs to be tailored to the virus's epidemiology. If a virus of annual pastures (and its vector) become established in autumn, the virus can be expected to spread extensively in spring and cause significant losses in seed production.

Bean leaf roll virus (BLRV) is a destructive virus of food legume crops world wide and was first reported in Australia by Schwinghamer *et al.* 1999. BLRV has a wide host range in legumes and is considered to be a major viral disease of pulses. Several fact sheets have been written for grain growers in regards to BLRV, and while it is recognised that BLRV can infect sub clover (Aftab *et al.* 2010a) it is not recognised as an important virus of sub clover. For example NSW Primary Industries Agriculture has a fact sheet (Anon 2004) that gives a comprehensive overview of viruses of sub clover but make no mention of BLRV. Viruses in plants are transmitted by aphids and some viruses are also seed transmitted or mechanical transmitted. Viruses of sub clover have wide host range and the viruses and their vectors over-summer on perennial pastures such as lucerne and white clover, and can spread to annual pastures in autumn and then within the annual pasture during the growing season.

Pea aphid (*Acyrthosiphon pisum*) is a well known to persistently transmit BLRV, however bluegreen aphid (BGA, *Acyrthosiphon kondoi*), which is in the same genus as pea aphid, is not known to transmit BLRV. Schwinghamer *et al.* 2009 trapped aphids in northern NSW and while BGA was the most common aphid trapped, none of them caused any virus to develop in faba bean plants. Pea aphids, collected at the same time, resulted in BLRV, BYMV and SBDV infections. Since its arrival in the late 1970's BGA is a widespread aphid of pasture legumes, pulses (except chickpea) and lupins. During the 2009 survey of the south east region of South Australia BGA was the only aphid species present on lucerne plants adjacent to sub clover seed production plots (with sub clover plants displaying red leaf symptoms). Furthermore, a more virulent biotype of BGA has recently been recorded in South Australia (Humphries *et al.* 2010). For these reasons the virus transmission ability of BGA was investigated.

This project surveyed sub clover and other annual pasture seed production fields for red-leaf disease symptoms. Molecular markers were developed to be diagnostic tools for sub clover viruses, and a fact sheet on the control of red-leaf diseases was developed and distributed to sub clover seed producers and district agronomists.

Objectives

To improve seed production technologies of subterranean clover for maximizing yield, quality and processing efficiency by:

1) Developing molecular diagnostic tools for identification of subterranean clover red leaf virus (SCRLV) and other viruses known to infect subterranean clover. (Thereby providing a service to growers and industry and hence supporting both production and domestic and export sales of quality, certified sub clover seeds)

2) Establishing whether SCRLV is associated with the sub clover red-leaf disease currently prevalent in seed production stands

3) Assessing the incidence and severity of SCRLV in seed production regions

4) Determining the level of resistance to SCRLV in varieties of sub clover and selected genebank accessions

5) Determine the disease cycle leading to infection of the stands and develop integrated disease management (IDM) protocols

6) Compile the results from the project in a report for RIRDC to publish.

Methodology

Detection of virus

The reverse transcription polymerase chain reaction (RT-PCR) is used by Waite Diagnostics for the routine identification of plant viruses. The method uses a total nucleic acid extract (TNA) from leaf tissue and a pair of specific primers which hybridises with specific regions of the viral RNA which they are designed to detect. RT-PCR employs a reverse transcription step and a PCR step in a single tube and is named single step RT-PCR. This project developed RT-PCR tests for viruses listed in Table 1 and PCR tests for phytoplasmas as shown in Table 1.

Extraction of plant nucleic acids

Total nucleic acid (TNA) extracts were prepared using the silica capture method 2 of Foissac *et al.* (2005) with the following modifications: The extraction buffer contained 4 M guanidine hydrochloride in 0.2 M sodium acetate pH 5.0, 25 mM EDTA (Ethylene Diamine Tetraacetic Acid), 1% Na-metabisulphite (added fresh) and 2.5% PVP-40 (polyvinylpyrrolidone 40). The first wash of the silica matrix was in the extraction buffer without PVP-40 and Na-metabisulphite. Wash 2 was done in 75% ethanol and 25% Tris-EDTA-NaCl, pH 7.5. TNA was eluted in 70 µl 10 mM Tris pH 8.5 and stored at –20˚C until use.

Preparation of RT-PCR master mix for single step RT-PCR

The RT-PCR mix for single step RT-PCR (900 µl) contained 580 µl water, 20µl of 10 mM dNTPs (dATP, dGTP, dCTP, TTP), 100 µl 10 X Taq buffer (ex Invitrogen kit), 50 µl of 0.1M Dithiothreitol (DTT), 40 µL of 50 mM $MgCl_2$, 55 µl of forward primer (ex 10 µM stock), 55 µl of reverse primer (ex 10 µM stock).

Reaction Conditions

Per 0.2ml PCR tube: Master mix 9 µl; Taq DNA polymerase 0.4 units; Superscript III reverse transcriptase 7.2 units; Nucleic acid extract 1 µl TNA.

Thermocycler program:

Step 1: 50ºC for 45 min; step 2: 94 ºC for 2 min; step 3: 94 ºC for 30 sec; step 4: 52-56 ºC for 45 sec; step 5: 72 ºC for 1 min; step 6: go to step 3, 33 more times; step 7: 72 ºC for 5 min; step 8 END.

For the generic phytoplasma detection two step PCR (nested PCR) was carried out using primers P1 and P7 for the first step followed by a second step using R16f2n/m23SR as nested primers (Table 1). PCR mix of the first step was diluted 100 times and was used in the second PCR cycle. The master mix and the cycle steps were as described for the RT-PCR except that step 1 (RT step) was deleted. The MJ Research Peltier Thermocycler was used in all PCR runs.

Assay of RT-PCR products

Agarose gels (1.5%) in 0.5 X Tris-Borate EDTA (TBE) containing ethidium bromide were prepared. The electrophoresis run was for 45 min in 0.5 X TBE.

Table 1. List of virus and phytoplasma diagnostic primers used for the RT-PCR and PCR assays of pasture legume samples, together with the genes targeted and the sizes of the amplified products.

Virus/Phytoplasma	Primer name	Primer sequence (5'→ 3')	Tm (˚C)	Region amplified	Product size (bp)	Ref
AMV	AMV-U	GCGAGATTCCTCTACAGTTTTCTG	64	CP	422	Nair *et al*, 2008
	AMV-L	AACAGCCCCACAGTAATCAAAC	64			
BLRV	BLRV-3F	TCCAGCAATCTTGGCATCTC	65	CP	389	Prill *et al*. 1990
	BLRV-5R	GAAGATCAAGCCAGGTTCA	60			
CMV	CMV-F	GTAGACATCTGTGACGCGA	60	RNA3	535	De Blas *et al*. 1994
	CMV-R	GCGCGAAACAAGCTTCTTATC	65			
BWYV	BWYV CP-F	ATGAATACGGTCGTGGGTAC	61	CP	429	Kumari *et al* 2008
	BWYV CP-R	GATAGTTGAGGAAAGGGAGTTG	60			
BYMV	BYMV-F	GCAAAGCCAACATTCCGCC	70	CP	273	Mathews *et al* 1995
	BYMV-R	GTCTGTTCCAACATTGCCATC	64			
PSbMV	PSbMV-F	GTGTTGGAGGAATCACACCAGAAGAATGTG	74	Not described	1100	Torok and Randles 2001
	PSbMV-R	GCAGTTGCTACATCCATCATTGTTGGCCAT	76			
SbDV	SbDV CP-F	CGGTTAGCAATGTCGCAATAC	64	CP	415	AB038148 (YP strain)
	SbDV CP-R	GAGTAGCGATTGAATTTCGG	61			
SCSV	Nano F103	ATTGTATTTGCTAATTTTA	47	Not described	771-775	Kumari *et al*., 2008
	Nano R101	TTCCCTTCTCCACCTTGT	60			
All phytoplasmas	P1	AAGAGTTTGATCCTGGCTCAGGATT	64	Ribosomal RNA	~ 1800	
	P7	CGTCCTTCATCGGCTCTT	56			
All phytoplasmas	R16f2n	GAAACGACTGCTAAGACTGG	58	Ribosomal RNA	~ 1600	Gibb *et al*. (1999)
	m23SR	TAGTGCCAAGGCATCCACTGT	58			

AMV, *Alfalfa mosaic virus*; BLRV, *Bean leafroll virus*; BWYV, *Beet western yellows virus*; BYMV, *Bean yellow mosaic virus*; CMV, *Cucumber mosaic virus*; PSbMV, *Pea seed-borne mosaic virus*; SbDV, *Soybean dwarf virus*; SCSV, *Subterranean clover stunt virus*.

Mechanical transmission

Mechanical sap transmission of ten samples showing red-leaf symptoms onto indicator plants for viruses was conducted. The indicator plants were: *Chenopodium quinoa*, *Nicotiana benthamiana*, *N. clevelandii*, *N. glutinosa*, *Phaselous vulgaris* (cv Brown Beauty), *Pisum sativum* (cv Dundale), *Pisum sativum* (cv Green feast) and *Vicia faba.*

Serological Assays

Dot-immunobinding assay (DIBA) was conducted by Dr S Kumari (ICARDA, Syria) on all samples collected in 2008. Virus specific antibodies were used as well as generic potyvirus antibody and generic luteovirus antibody.

Survey of sub clover seed production areas

Initial observations on spaced plants

The SARDI sub clover breeding program grew individual spaced plants at the Waite campus (Adelaide) in 2008. The spaced plants were grown in strips of three rows with a 40cm space between plants and 2m gap between strips. The trial was located just below a single row of the perennial shrub *Medicago arborea* which in turn was adjacent to a small woodland of eucalyptus trees which had self regenerating sub clover and grasses growing beneath them. The occasional spaced plant developed symptoms of red-leaf disease and observations were made. The spaced plants had BGA on them and these were controlled with insecticide.

2008 field survey

A comprehensive field tour of seed production areas was undertaken in October 2008. This involved staff from SARDI, Waite Diagnostics and Seedmark and covered a wide area in the southeast of South Australia and western Victoria. Red-leaf disease incidence was considered to be light by seed producers and district agronomists. Thirty samples (28 expressing symptoms and 2 without symptoms) were taken from fifteen sites and the following species were sampled: sub clover (18); Persian clover (6); Balansa clover (6); faba beans (2); lucerne (1); crimson clover (1) (Fig. 1).

Figure 1. Sampling of a sub clover seed production field and typical red-leaf symptoms seen.

2009 field survey

A field survey of annual pasture seed production in the south-east of South Australia and western Victoria was undertaken on 19th and 20th August 2009 and an extra site was surveyed on the 22nd of October 2009. Samples were also taken from pasture plots on the Waite Campus (Adelaide) on the 20th October, Glenthorne (Adelaide) on the 1st October and Turretfield Research Centre on the 21st October. A total of ten sites were sampled and 63 samples collected. Leaf samples were taken back to Waite Campus and stored at -20 ºC before testing for viruses with the diagnostic tools developed by this project in 2008.

Bluegreen aphid transmission studies

Preliminary study

During the 2009 survey of the south east region of South Australia BGA was the only aphid species present on lucerne plants adjacent to sub clover seed production plots (with sub clover plants displaying red leaf symptoms) and this population was collected. This population was reared on two lucerne plants (transplanted into pots in a glasshouse), collected during the field survey. These plants were positive for the BLRV by RT-PCR method. After two weeks on the lucerne plants BGA were transferred onto individual healthy glasshouse grown plants of five sub clover cultivars and a Persian clover cultivar. The tops of individual plants with BGA were housed inside clear plastic cups with a lid with a fine mesh to allow for air transfer while keeping the BGA inside. The BGA were left on the plants for two weeks before the BGA were killed. Leaf samples from these plants were collected 21 days post-inoculation and stored at -20 ºC for 13 days. RT-PCR testing consisted of two RT-PCR controls, leaf samples from sub clover (cv Seaton Park) not exposed to BGA, sub clover (cv Seaton Park) collected fresh on the day of RT-PCR analysis and sub clover (cv Antas) with red-leaf symptoms collected from the field.

Persistence transmission study

As per the preliminary study, BGA were grown on lucerne plants with BLRV for 2 weeks. Sub clover cultivar Antas was used in this experiment and shoots and BGA were housed in plastic cups as previously described. Three Antas plants were inoculated with ten aphids on the first day and then the aphids were transferred onto another set of three plants daily for three occasions. Three weeks after the last transfer plants were scored for the presence or absence of red-leaf symptoms and leaves sampled for RT-PCR detection of BLRV.

Seed transmission of BLRV

A sub clover (cv Antas) plant which was showing red-leaf symptoms was successfully transplanted into a pot in the glasshouse and RT-PCR testing showed the plant was positive for BLRV. Seeds were collected from this plant and placed in 40ºC oven to break physiological dormancy. Seeds were then hand scarified by making a single knick with a scalpel blade and 120 seeds were planted. Three weeks after planting leaves were sampled and tested for BLRV by RT-PCR method.

Confirm BGA transmission study

We wanted to conduct a persistent transmission study on several cultivars. As a first step to this we wanted to establish several plants with BLRV on which we could later produce viruliferous (virus containing) BGA. We placed a population of BGA collected from Adelaide on the original lucerne plant with BLRV for seven days and then transferred ten aphids (adults and nymphs) immediately onto three Antas plants. The remaining aphids were placed in a petri dish with wet filter paper and a few leaves of Antas. After 24 hours starvation (to show persistence) we placed ten aphids (adults and nymphs) onto five Antas plants and onto three lucerne plants. Plants were tested for BLRV 21 days later by RT-PCR method, however none of the plants had BLRV. The original lucerne plant had old

woody stems and was not actively growing so we cut it back, potted it up into a bigger pot and allowed it to regrow. When the plant was actively growing we placed BGA onto it. The BGA had much better fecundity than the prior attempt and aphids were collected after two weeks. Ten adult aphids were placed directly onto three Antas plants and after 24 hours starvation onto five Antas plants and one lucerne plant.

Cultivar tolerance/resistance to BLRV

Turretfield

In 2009 a sub clover subspecies *brachycalycinum* field evaluation trial was sown at the Turretfield Research Centre, Rosedale South Australia as part of the SARDI sub clover breeding program. The trial consisted of two mid-late cultivars (Antas and Clare), two mid season cultivars (Rosedale and Mintaro) and four early-mid breeder's lines. Red-leaf symptoms were expressed in all the replicates and percent of plants with red-leaf symptoms within each plot was estimated. Leaf samples from plants displaying red leaf symptoms were taken across a diagonal transect in each plot for RT-PCR analysis of BLRV. Seed yield was determined by hand harvesting two 30 x 30cm quadrats per plot. Dry herbage residues and burrs were scraped from the soil surface within each quadrat. Samples were threshed by machine and cleaned by hand.

Kybybolite

Eight sub clover cultivars (subspecies *subterraneum* (2), *yanninicum* (2) and *brachycalycinum* (4)), three Persian clover cultivars and one Balansa clover cultivar were sown in four replicates on SARDI's Kybybolite research farm located just northeast of Naracoorte in the southeast of South Australia. In late spring the trial was scored for red-leaf symptoms and leaf samples taken for testing for BLRV.

Fact sheet.

Integrated disease management protocols were developed after reviewing project results, the scientific literature and fact sheets written for virus control in pulses. A fact sheet (Appendix 1) was produced which provides an overview of red-leaf disease, symptoms, disease transmission and lists ten management options for farmers to consider when developing their integrated disease management strategy. A list of seed companies that may be in direct contact with sub clover seed producers was compiled by visiting the web sites of the Australian Seed Federation and Pasture Australia. These seed companies were contacted and asked if they were willing to forward the fact sheet to their seed growers and/or sales and production staff.

Results

Development of molecular diagnostic tools

Objective 1 of this project was: Developing molecular diagnostic tools for identification of subterranean clover red leaf virus (SCRLV) and other viruses known to infect subterranean clover.

This project developed RT-PCR primers (Table 1) for the following viruses which are known to infect sub clover: *Alfalfa mosaic virus* (AMV); *Bean leafroll virus* (BLRV); *Beet western yellows virus* (BWYV); *Bean yellow mosaic virus* (BYMV); *Cucumber mosaic virus* (CMV); *Pea seed-borne mosaic virus* (PSbMV); and *Soybean dwarf virus* (SbDV, which is synonymous with *Sub clover red leaf virus* (SCRLV)) and *Subterranean clover stunt virus* (SCSV). Sub clover seed producers are encouraged to send samples to Waite Diagnostics to determine which virus, if any, are present in their sub clover plants.

Field Surveys

Initial observations on spaced sub clover plants 2008

The occasional plant developed symptoms of red-leaf disease while the surrounding plants had green leaves (Fig. 2). The strip of spaced plants closest to the *Medicago arboea* shrubs and the woodland with sub clover plants growing beneath them had the highest number of plants with red-leaf symptoms and each successive strip of spaced plants had fewer plants showing symptoms. Initially the plants with red-leaf symptoms had symptoms on the leaf margin of the lower leaves which then progressed to cover more of the leaf and then younger leaves. Initially the plants with symptoms were a similar size to the plants without symptoms, however when the symptomless plants started to grow rapidly in early spring the plants with symptoms did not put on this rapid growth. The plants with red-leaf symptoms set only a small amount of burrs and seeds. The sub clover plants had BGA on them which are likely to have come from the row of *Medicago arboea* shrubs adjacent to them and/or the sub clover plants growing beneath the eucalypt trees. The *Medicago arborea* shrubs act as a reservoir of BGA over the summer months and we speculated that they are also a reservoir of viruses. Later in the project (2009), when we had developed a diagnostic marker for BLRV, we sampled the *M. arborea* shrubs and they tested positive for BLRV. Testing of the *M. arborea* shrubs also revealed a strain of *Tomato big bud phytoplasma* and this was reported to the 9th Australasian Plant Virology Workshop in a paper by Habili *et al*. (2010) titled "A phytoplasma from subgroup 16Sr II is associated with little leaf of *Medicago arborea* (tree medic) in South Australia".

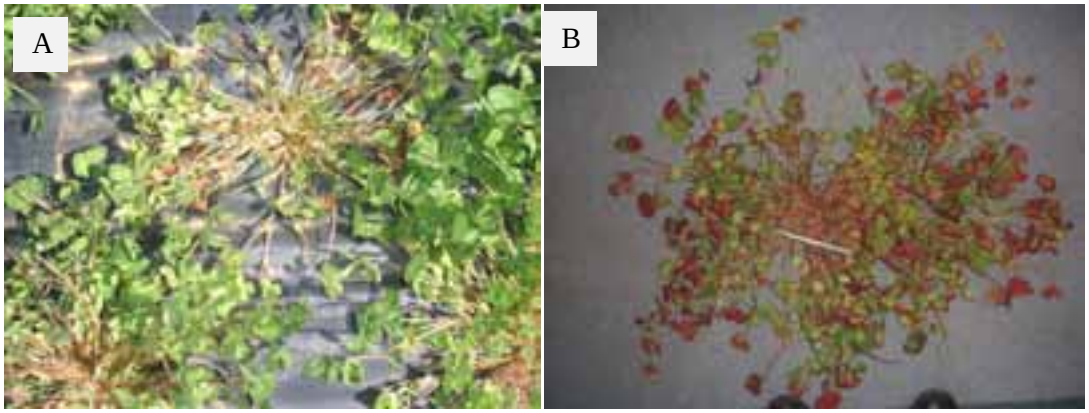

Figure 2. A. Red-leaf symptoms on spaced plants of sub clover with healthy plants around it. B. Sub clover plant showing advanced stage of leaf reddening.

Survey of seed production areas 2008

Pasture seed producers and district agronomists considered 2008 to be a low year for red-leaf disease. No phytoplasmas were detected and of the eight viruses tested, only three, AMV, BLRV and CMV were detected (Table 2). The results were confirmed by mechanical transmission to indicator plants and by serological assays performed at ICARDA, Syria. None of the 27 samples collected in 2008 from the south-east of South Australia tested positive with a generic potyvirus antibody, or with specific antibodies to *Soybean dwarf virus* (SbDV) or *Beet western yellows virus* (BWYV). The negative result on SbDV and BWYV was confirmed by RT-PCR. This is contrary to the results obtained by Agwest laboratories (Dr. Brenda Coutts, Western Australia) in 2005 and 2006 where BWYV was detected in both years from samples taken from south-east of South Australia. Two of the 2008 samples tested positive for a generic luteo antibody (ICARDA). The specific RT-PCR assays for BLRV, BWYV and SbDV on these two samples produced negative results.

Table 2. AMV, BLRV, CMV was detected in sub clover plants collected from the south east of South Australia as confirmed by mechanical transmissibility, DIBA antibody and RT-PCR testing.

Virus	Mechanical transmissibility	DIBA antibody test		RT-PCR
		Specific	Luteo generic	
AMV	+	+	-	+
BLRV	-	-	+	+
CMV	+	+	-	+

A different reddening disorder present both in Antas sub clover and in Persian clover tested negative to all the viruses listed in Table 1. This disorder could either be caused by an abiotic factor or by an as yet unidentified pathogen such as a virus. Work is required to characterise a possible new pathogen because a replicative form (dsRNA) of an unknown virus has been identified in red-leaf affected plants free of BLRV. Sequence analysis on a random PCR product of this virus is needed to identify this putative additional virus(es).

The most common virus detected was BLRV, which was detected in 11 samples collected from 7 sites. BLRV was detected in sub clover, faba bean and crimson clover. Sub clover and Balansa clover plants that tested positive for BLRV had reddening of leaves (Fig. 3A). The reddening started on the leaf

edges and then progressed to cover the whole leaf. Faba beans that tested positive for BLRV had yellowing of leaves, small leaves and distortion of leaves (Fig. 3B). However the crimson clover that had BLRV detected on it displayed no symptoms.

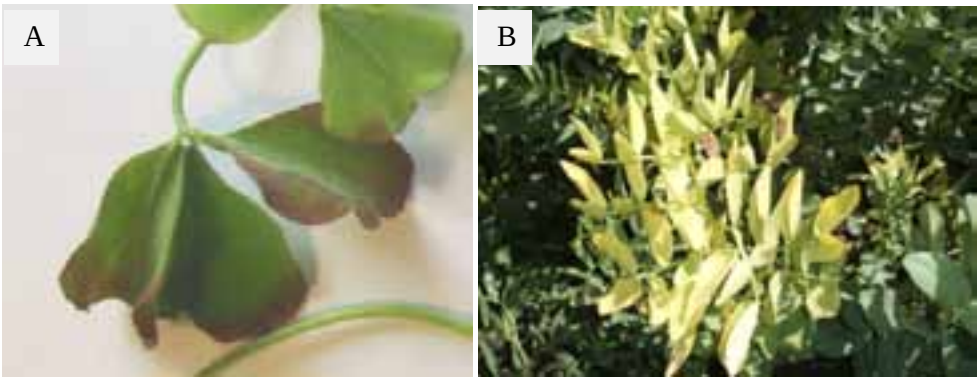

Figure 3. A. BLRV infected sub clover plant showing red-leaf symptoms. B. BLRV infected faba bean plant showing yellowing of leaves.

AMV was found in one sub clover plant, one lucerne plant and one persian clover plant. The sub clover plant with AMV had leaf reddening similar to the sub clover plants that tested positive for BLRV (Fig. 4). The only other virus found was CMV which was detected by itself in one sub clover sample and along with BLRV in one sub clover sample and one Balansa clover sample.

Figure 4. Sub clover plant that tested positive for AMV displaying red-leaf symptoms.

Objective 2 of this project was: "Establishing whether SCRLV is associated with the sub clover red-leaf disease currently prevalent in seed production stands". The 2008 field survey of sub clover seed production crops failed to find a single sample with SCRLV (now known as SbDV in the scientific literature). This suggests that the virus that we speculated as the most likely virus to cause the red-leaf disease epidemics in the mid 2000's was not the cause of the epidemics. However we regularly found BLRV in our samples of sub clover plants with red-leaf symptoms. BLRV was first reported in Australia in 1999 (Schwinghamer *et al.* 1999) and it is possible that it took some time to spread to major sub clover seed production areas and longer to experience an epidemic year. BLRV has a wide range of legume hosts and the Australian pulse industry recognise it as a major virus disease. It is highly likely that BLRV was the cause of the red-leaf epidemics in the mid 2000's. Like most plant diseases the level of viruses can fluctuate widely from one year to the next. For example Aftab *et al.* 2010a report that the incidence of BLRV in faba beans in South Australia from 2003-2007 ranged from 0 to 100%. It is possible that mild summers allowed more virus and vectors to over summer and the drought years of the mid 2000's may have allowed the virus to have a greater effect on plant growth and seed yield. While BLRV has been reported in pasture legumes (Aftab *et al.* 2010a) it has not been recognised by the industry as a major problem. Our survey results indicate that a management strategy to control BLRV should be adopted by pasture seed producers.

SCRLV has been recognised as a virus in sub clovers since 1971 (Kellock 1971) and while it is possible that a more virulent strain of SCRLV virus had developed it is probably more believable that the BLRV (a relatively new virus to Australia) is the cause of the red-leaf disease reported in the mid 2000's. McKirdy and Jones 1995 surveyed six districts of south west Western Australia for AMV and

SCRLV and concluded that infection with AMV and SCRLV is currently not a threat to sub clover pastures in the south-west of Western Australia. Our study failed to find SCRLV in sub clover and found AMV at low levels so our survey supports the conclusion of McKirdy and Jones 1995.

Some plants sampled with red-leaf symptoms had no virus detected in them. This is not a surprise as leaf reddening can be caused by a range of factors and tends to only indicate that the plant is under some stress. Similar to us, van Leur and Kumari 2011 only found virus in 42% of lucerne plants with virus like symptoms. Other than viruses the most likely causes of leaf reddening are cold conditions, wet conditions, nutrient deficiencies, herbicide damage and root diseases. Some farmers have reported that some red-leaf symptoms that occur early in the season have been alleviated with the application of fertiliser. As red-leaf symptoms can have a range of causes we recommend farmers and district agronomists make use of the diagnostic tools that this project developed to confirm the presence of sub clover viruses. If the test comes back positive the farmers can consult the fact sheet (Appendix 1) on the control of red-leaf disease and establish a control strategy. If virus tests come back negative the farmers can investigate other possible causes – such as using nutrient tissue analysis to see if a nutrient deficiency is present.

Survey of seed production areas 2009

Objective 3 was: "Assessing the incidence and severity of SCRLV in seed production regions". However no SCRLV was detected in the 2008 survey but rather survey results suggested that BLRV is the principal virus causing the red-leaf disease. For this reason our main objective in the 2009 survey of seed production areas was to determine the incidence and severity of BLRV.

In the 2009 surveys, twenty one of eighty pasture samples collected from ten sites tested positive for BLRV. These included sub clover (13 samples), lucerne (2), barrel medic (2), naturalised clovers (3) and *Medicago arborea* (1). All but one site sampled had at least one sample test positive for BLRV. Seven sites were located in the major sub clover seed production area of southeast of South Australia and Western Victoria, two sites were in the Adelaide metropolitan area and one in the lower mid-North of South Australia. The results show that BLRV is widespread and in geographical separate areas. All pasture species tested produced positive samples which indicate the wide host range of BLRV and highlights the many opportunities for virus spread. BLRV is also a well recognised virus in pulses which further shows the opportunity for it to spread. The 2009 survey results supports the conclusions of the 2008 field surveys that BLRV is likely to be a major cause of red-leaf disease in sub clover.

At the sites in Adelaide each sample tested positive for BLRV and consisted of sub clover, barrel medic, hop clover, *M arborea* and faba bean. However at the other sites many of the samples tested negative for BLRV. The Adelaide sites have a warmer climate than the other sites and it is likely that at the other sites some of leaf reddening is due to cold conditions.

It was noted that sub clover seed production paddocks located near to old lucerne stands had symptoms of red-leaf disease and incidence of plants with red-leaf symptoms decreased the further away from the lucerne field. The lucerne paddock was surveyed and we sampled plants with yellow leaves which tested positive to BLRV. Aftab *et al.* 2010a report that BLRV infection in lucerne is usually symptomless, but bright yellow vein clearing may be present. Van Leur and Kumari 2011 surveyed lucerne fields within 75 km of Tamworth (NSW) and found over half the fields surveyed had BLRV and they did not find a difference in virus infection levels between samples selected from poor growing plants with those from healthy looking ones. Van Leur and Kumari 2011 also report that old lucerne stands have higher incidence of BLRV than young stands. Since lucerne often carries BLRV without symptoms, a simple visual inspection is unable to tell a farmer whether a nearby lucerne stand is a threat. Lucerne stands also host the aphid species that are vectors of BLRV and hence old lucerne stands are a reservoir of both the virus and the aphid vectors. In the principal sub clover seed production areas many lucerne pastures and lucerne seed crops are present. Viruliferous (containing virus) aphids are able to migrate out of the lucerne stands into nearby emerging annual pastures. When selecting paddocks for sub clover seed production we recommend where possible that seed producers avoid planting adjacent to old lucerne stands.

Phytoplasmas

No phytoplasmas were detected in pasture plants sampled from the major sub clover seed production area of south east South Australia or western Victoria. *Medicago arborea* trees at Waite Campus Adelaide tested positive for a strain of *Tomato big bud phytoplasma*. This plant had a yellow and bushy appearance with reduced flowering. We have sequenced the 16 S rRNA gene of this phytoplasma strain. Other *M. arborea* plants showing witches' broom symptoms also tested positive for phytoplasma using two step nested PCR. Sequence analysis of the 16 S rRNA gene of the phytoplasma showed that it was also from a strain of *Tomato big bud phytoplasma*. This was confirmed by BLAST analysis which showed that it belonged to the 16Sr II group of phytoplasmas (Habili *et al*. 2010).

BGA transmission studies

Preliminary

BGA transmitted BLRV to three of the sub clover cultivars and the Persian clover cultivar tested (Fig. 5). The various controls behaved as expected: RT-PCR controls were positive (lanes 1 & 12); leaves collected from sub clover (cv Seaton Park) not exposed to BGA were negative (lane 4); Seaton Park leaves collected fresh (lane 2) were positive as was the Seaton Park leaves collected 13 days earlier and stored in -20ºC freezer (lane 3). SARDI Persian (lane 5), Antas (lane 6) and Trikkala (lane 7) tested positive for BLRV. Cultivar Dalkeith (lane 8) and Clare (lane 9) tested negative for BLRV. As this was an un-replicated preliminary trial the lack of these two cultivars having BLRV may just be due to not each aphid having virus in them and hence able to transmit the virus. A replicated trial would be required before we could say anything about the resistance of any sub clover germplasm to BLRV.

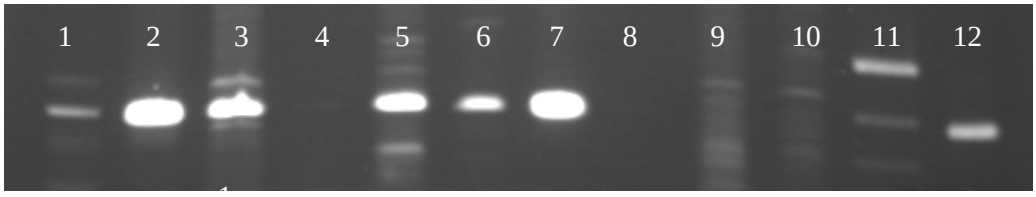

Figure.5. BLRV RT-PCR on plants inoculated with viruliferous aphids (AI) collected from a lucerne field adjacent to a sub clover field in the South East of South Australia. Lanes 1 & 12 positive controls; 2, symptomatic (red-leaf) Seaton Park sub clover sampled on 24/09/2009; 3, Seaton Park AI in the freezer since 11/09; 4, Seaton Park plant not exposed to AI; 5, SARDI Persian; 6, Antas; 7, Trikkala; 8, Dalkeith; 9, Clare; 10, Antas field sample showing reddening of leaves. 11, DNA size marker (Puc 19 Hpall cut).

Persistent transmission study of BGA

BGA persistently transmitted BLRV over four days (Table 3). However some of the sub clover plants not exposed to the BGA treatment also had BLRV symptoms (one of twelve plants) and tested positive for BLRV by RT-PCR (3 plants positive and 2 unsure). One explanation for this is that the glasshouse in which the plants were grown after the BGA treatment and prior to sampling for virus had a wild infestation of BGA. It is possible that they transmitted BLRV from BGA plants to plants that did not have BGA on as one of the treatments. Another explanation is that the BLRV is seed transmitted (see below).

Table 3. Bluegreen aphids (BGA) fed on BLRV infected lucerne plant persistently transmitted BLRV over four days to sub clover (cv Antas) plants as indicated by red-leaf symptoms and RT-PCR testing.

Rep	Exposed to BGA	Red-leaf symptoms				BLRV detected by RT-PCR			
		day1	day2	day3	day4	day1	day2	day3	day4
1	no	no	no	no	no	no	unsure	yes	no
	yes	yes	yes	yes	yes	yes	yes	yes	yes
2	no	no	no	no	yes	no	no	no	no
	yes	yes	yes	yes	yes	yes	yes	yes	yes
3	no	no	no	no	no	no	unsure	yes	yes
	yes	yes	yes	yes	yes	yes	yes	yes	yes

Confirm BGA transmission

None of the plants that had BGA from BLRV infected lucerne plants placed on them had BLRV detected on them. It is possible that due to the BRLV infected lucerne plants not actively growing, the virus was not being translocated through the plant and the BGA did not take up any BLRV. When we placed BGA onto the lucerne plant that was actively growing we achieved greater fecundity of the aphids. Two of the three plants that had BGA placed directly onto them became infected. The BGA that was starved for 24 hours infected two of five Antas plants and one of one lucerne plants and the three plants that did not have aphids on them tested negative for BGA. So this study confirms the preliminary study and the persistence transmission study that BGA can transmit BLRV. It also appears that the host plant needs to be actively growing in order for BGA to take up BLRV. Another possibility is that on the woody growth attempt we did not have enough aphids to get BLRV transmission. In that study we had ten aphids consisting of both nymphs and adult while in the later study we had ten adults. It is likely that the nymphs have not fed long enough to have a good chance of taking up BLRV.

In the persistence transmission study all plants that had BGA placed on them had virus in them while in the latter study only half the plants had BLRV transmitted to them. A possible explanation is that the persistent transmission study was conducted in winter while the last study was carried out in summer. We know from our screening trials for BGA resistant annual medics and lucerne that BGA do not perform the best in the glasshouse during the summer months as we have trouble keeping the temperature down to the optimal temperature for BGA activity. Another possibility is that the population of BGA collected from Adelaide does not transmit BLRV as effectively as the population of BGA collected from the south-east of South Australia does. However, this project has shown that two population of BGA collected from geographical separate areas are capable of transmitting BLRV.

BLRV is not seed transmitted

Growing 120 seeds collected from a BLRV infected sub clover plant failed to produce any plants with BLRV infection. This supports the literature that BLRV is not seed transmitted and therefore the explanation for BLRV in the plants that did not have our BGA treatment must be due to the wild population of BGA.

Resistance to BLRV

Objective 4 was: “Determining the level of resistance to SCRLV in varieties of sub clover and selected genebank accessions”. To determine level of resistance to red-leaf disease we planted a set of sub clover cultivars in the southeast of South Australia where red-leaf disease was reported in epidemic levels in the mid 2000’s and also studied SARDI sub clover breeding trials at Waite Campus in Adelaide and at the Turretfield Research Centre in the lower mid north. As stated earlier we do not

think that SCRLV is the causal agent of the red-leaf disease but rather we think that BLRV is the major causal agent.

Waite Campus

Cultivars Antas, Clare, Mintaro, Rosedale and Izmir were grown as cultivar checks when making single plant field selections from the SARDI sub clover breeding program. Red-leaf symptoms were low in this trial but each of these cultivars had a least one plant showing pronounced red-leaf symptoms.

Turretfield

Sub clover breeding line EB27 had a greater percent of plants with red-leaf symptoms than the other lines and cultivars (Table 4). EB27 had lower seed yields than lines EB1 and EB19 which had low levels of red-leaf disease. Due to the red-leaf symptoms in EB27 and its low seed yield this line was dropped from SARDI sub clover field evaluation program. The mid-late season cultivars Clare and Antas had low levels of red-leaf symptoms and low seed yields. The low seed yields of the mid-late cultivars is likely to be due to an abrupt finish to the season due to a prolonged hot spell (from the 7th November, 11 of the next 13 days had maximum temperature's > 35ºC including 8 days > 40ºC). However while the red leaves were present in most plots, only four plots tested positive to BLRV. Each of the positive plots was on the edge of the trial and consisted of two sets of adjacent plots. The location of the plots suggests that the BLRV has moved into the trial from the outside and spread locally. This agrees with van Leur and Kumari 2011 who reported BLRV had significant aggregation of infected plants and there was a tendency in paddocks with low level of BLRV for higher infections along the edge. The lack of BLRV in most of our plots suggests the leaf reddening is caused by other factors. A likely reason for the leaf reddening is cold conditions. While line EB27 was dropped due to having a higher score for leaf reddening than the other lines and cultivars, it is likely that the cause is something other than virus. The low seed yield of EB27 may be due to its genetic potential, since it has never had seed yields determined before and it is unknown whether its seed yield potential is as good as current cultivars.

Table 4. Percent of sub clover plants with red-leaf symptoms and seed yield (kg/ha) of four cultivars and four breeding lines at Turretfield Research Centre. Lines with the same letter are not significantly different.

Line/ Cultivar	%plants with symptoms	Seed Yield
EB 1	1.5 a	957 a
Mintaro	1 a	718 ab
EB19	0.25 a	717 ab
EB25	2.5 a	655 b
Rosedale	1 a	654 b
EB27	22.5 b	553 bc
Antas	3.5 a	508 bc
Clare	0.5 a	406 c

Kybybolite.

Red-leaf symptoms were highest in Persian clover and red-leaf symptoms were higher on the subspecies *brachycalycinum* than in the other two subspecies of subclover (Table 5). However RT-PCR testing showed only two of forty eight plots (2 of 12 entries) tested positive for BLRV. This shows that most of the leaf reddening is from other causes. The most likely reasons are cold and/or

wet conditions. As virus testing suggested the red-leaf symptoms were from other causes we did not determine seed yield.

Table 5. Score of red-leaf symptoms (0-10) sub clover, Persian clover cultivars and a Balansa clover cultivar. Scores followed by the same letter are not significantly different.

cultivar	red-leaf symptoms
Antas (Ssp. *brachycalycinum*)	3.8 c
Clare (Ssp. *brachycalycinum*)	3.6 c
Mintaro (Ssp. *brachycalycinum*)	1.3 e
Rosedale (Ssp. *brachycalycinum*)	1.6 d
Dalkeith (Ssp. *subterranean*)	0.4 e
Seaton Park (Ssp. *subterranean*)	0.1 c
Gosse (Ssp y*anninicum*)	0.3 a
Trikkala (Ssp *yanninicum*)	0.1 d
Kyambro (Persian clover)	3.3 d
Maral (Persian clover)	7 b
Sardi persian (Persian clover)	3.9 e
Frontier (Balansa clover)	1.9 e

Resistance summary

The project attempted to determine if sub clover cultivars differed in their resistance or tolerance to red-leaf disease. For this we relied on natural infection in areas likely to experience red-leaf disease. However the incidence of red-leaf disease was low during the years of study and only low levels of virus were detected in our field trials. We are unable to make any meaningful conclusions about levels of resistance or tolerance of different sub clover germplasm. Now that we have more knowledge on the relevant viruses and how they spread we could, in a future project, screen for resistance or tolerance to BLRV. This could be done by establishing a disease nursery consisting of infected lucerne plants planted in a grid pattern onto which we release aphids to feed on the infected plants and spread to sub clover germplasm that is growing between the lucerne plants. The lucerne plants would be infected with virus in the glasshouse as seedlings prior to transplanting in the field. Pulse Breeding Australia are breeding faba beans and field peas for resistance to BLRV and hence it is conceivable that sub clover germplasm with resistance or tolerance could be found.

Integrated disease management protocols

Objective 5 was: "Determine the disease cycle leading to infection of the stands and develop integrated disease management (IDM) protocols". The three viruses (BLRV, AMV and CMV) we found in our surveys are well known viruses and have been well studied in Australia on pulses. AMV has also been well studied in pastures. There is a lot of existing information about these viruses and we needed to adapt the information to pasture situations in general and sub clover seed production system in particular. The major output for this objective was a two page fact sheet titled "Control of red-leaf disease in sub clover seed crops" and can be found at http://www.sardi.sa.gov.au/pastures/annual_pastures/clover_red_leaf_disease. The fact sheet is written specifically for the viruses we found, namely BLRV, AMV and CMV. However the integrated disease management principles are relevant to virus control in annual pastures in general.

The seed companies we sent the fact sheet to were interested in the information and were willing to pass the fact sheet on to seed producers, grower groups, production staff, sales staff and/or

agronomists. The fact sheet has been well received, as shown by the following feedback: “This is exactly the information we need to get round our networks”. The fact sheet has been placed on SARDI pastures web site and an internet search with the words “red leaf disease sub clover” lists the SARDI site first.

The target audience of the fact sheet was sub clover seed producers. However much of the information in the fact sheet will be relevant to sub clover pasture growers, especially those in wetter environments which are more likely to have perennial legume pastures (lucerne and white clover) grown. In the drier areas virus and vectors are likely to survive the summer in lower numbers. The viruses have a wide host range and the information in the fact sheet will also be relevant to seed producers of other annual pasture legumes.

Minimising the early spread of viruses is important to reduce the risk of an epidemic developing in spring. If seed producers have experienced red-leaf disease problems they should develop a management strategy. An integrated approach should be taken to reduce the risk of virus infection in sub clover seed crops. The following list suggests some useful management strategies for sub clover seed producers to consider when developing their management strategy:

- Identification of red-leaf disease in prior years suggests that a management strategy should be developed

- The presence of red-leaf in itself does not indicate viruses are present. If viruses are suspected of being the cause of the leaf reddening it is worth getting this confirmed by molecular testing by Waite Diagnostics

- Summer rainfall and early breaks favour early increases in volunteer legumes that promote aphid and virus build-up

- Control of volunteer legumes and weeds in summer and autumn reduces the reservoirs of the viruses and aphids

- Sub clover seed crops should be located away from lucerne fields as aphids can migrate out of lucerne fields into sub clover fields. If possible remove old unproductive stands of lucerne

- Consideration should be given to delaying sowing in autumn as warm weather post planting increases the risk of aphid movement especially of cowpea aphid and green peach aphid

- Consideration could be given to treating seed with insecticide to control plant feeding insects

- Natural enemies of aphids should be monitored (e.g. lady birds, lacewings and parasitic wasps) as high numbers of these can indicate good aphid control

- If aphid numbers are high it may be necessary to spray with an insecticide. Only spray if necessary to avoid insecticide resistance. Where possible use registered aphicides rather than broad spectrum insecticides in order to limit damage to beneficial insects

- Since aphid infestations often start on the perimeter of a field it may be only necessary to spray the perimeters of fields

Implications

This project identified BLRV as the major cause of red-leaf disease in sub clover seed production areas. BLRV was shown to be widespread and for the first time we showed BGA to be a vector of BLRV transmission. BGA is a very common aphid of legume pastures, pulses and lupins. Surveys of field production also found plants infected with AMV and CMV. The project developed a set of diagnostic tools so that farmers or their agronomic advisers can send in samples and have the presence of a range of viruses (BLRV, AMV, CMV, SbDV, BWYV, BYMV, PSbMV and SCSV) confirmed. The project also developed a fact sheet on the control of red-leaf disease in sub clover which will assist producers in developing their own management strategies. The identification of the causal agents of red-leaf disease, the development of diagnostic tools and the development of integrated disease management protocols will reduce the risk of crop failure for sub clover seed producers. This will assist farmers who produce sub clover seed and in turn assist the communities in which they live. Having a more reliable seed production system will assist seed companies and in turn red meat and wool producers who graze cattle and sheep on sub clover pastures. The diagnostic tools and fact sheet are also relevant to sub clover pasture growers. We recommend sub clover pasture growers and their agronomic advisors make use of the diagnostic tools.

Recommendations

This project has developed a better understanding of the cause of red-leaf disease in sub clover seed production areas. The results of the surveys indicate that BLRV is likely to be a major cause of the red-leaf disease. This project developed a set of diagnostic tools that can detect the presence of sub clover viruses (BLRV, AMV, CMV, SbDV, BWYV, BYMV, PSbMV and SCSV) and also developed a fact sheet for control of sub clover red-leaf disease in sub clover seed crops. While this work concentrated on sub clover seed production many of the results and recommendations are likely to be relevant to sub clover pastures as well. Sub clover pasture growers are also encouraged to make use of the diagnostic tools developed by this project.

This project was unable to determine if sub clovers with resistance or tolerance exist. However now with a greater understanding of BLRV we could develop a much better screening method. This would consist of setting up a disease nursery with lucerne deliberately infected with BLRV being planted in a grid pattern and sub clover germplasm to be screened growing between the infected lucerne plants. A search for sources of resistance or tolerance would be warranted if reports of red-leaf disease continue to be received from sub clover pasture producers. Reports of red-leaf disease can now be assessed by the diagnostic tools developed by this project, and it can be determined whether the red-leaf symptoms are caused by virus. The fact sheet should be useful to any sub clover producers who report red-leaf disease in sub clover.

Surveys of seed production fields found two samples that tested positive to general Luterovirus serological test. A further two samples had a replicative form (dsRNA) of an unknown virus. It is possible that these unknown positive samples are new viruses to Australia. Further research would be required to determine what viruses they are and how widespread they are and what damage they are causing.

Appendices

Appendix 1 – Fact sheet

Control of red-leaf disease in subterranean clover seed crops.

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Overview

Subterranean clover (sub clover) seed producers have reported symptoms of reddening of leaves in sub clover over several recent seasons. Up to 60% reductions in seed yield have been estimated in affected stands. In 2008 the Rural Industries Research and Development Corporation (RIRDC) funded a collaborative project between the South Australian Research and Development Institute (SARDI), the University of Adelaide and Seedmark to survey seed production crops and to study the cause of the leaf reddening. Red leaf symptoms can be caused by a range of environmental factors and by virus infection. Viruses in pastures are transmitted by aphids, can result in large reductions in seed yield and have a wide host range among pasture and crop legumes. Viruses are first suspected when specific leaf symptoms appear and their identity is confirmed by biological and molecular testing. Viruses often spread before aphid infestation is noticed, and control of virus spread by insecticide applications is only successful in certain cases. If a virus and its aphid vectors become established in autumn the virus can be expected to spread extensively in spring and cause significant losses in seed production.

Symptoms

The general symptoms are reddening of leaves especially along the leaf margin of older leaves (figure 1). The symptoms can be easily confused with nutritional deficiency symptoms or physiological reddening caused by cold temperatures and/or water-logging. For these reasons it is worth collecting leaf samples and sending them to Waite Diagnostics where it can be determined which viruses, if any, are present.

Figure 1: Sub clover plants showing leaf reddening caused by viruses.

Main viruses affecting clover seed production.

Plants, like all organisms, can be infected with viruses. Over 1000 plant viruses have been described, and they differ greatly in their biology, epidemiology, molecular structure and composition. Unfortunately, plants do not recover from virus infection as mammals do, and there are no chemical therapeutants to eradicate them. Virus infected plants therefore become less productive or die. Control is achieved by attacking weak points in the way they spread, or by resistance breeding. To achieve control by these methods it is vital to accurately identify the virus causing a disease, as each control measure needs to be tailored to the virus's epidemiology.

Surveys conducted in the southeast of South Australia and western Victoria in the spring of 2008 and 2009 showed the principal viruses to affect sub clover were *Bean leaf roll virus* (BLRV), *Cucumber mosaic virus* (CMV) and *Alfalfa mosaic virus* (AMV). These three viruses have a wide range of legume hosts and produce different symptoms on different plant species. On pulses symptoms are interveinal chlorosis, yellowing, stunting and leaf rolling.

In annual pastures and crops the viruses and aphid vectors die out over summer but are maintained in perennial legume species and legume volunteers. The perennial species lucerne carries these viruses, often without symptoms or simply yellowing but no reddening. Lucerne also hosts the principal vectors of these viruses and in autumn aphids can migrate out of lucerne fields and green belts into new sowing of sub clover seed crops. The older the lucerne stand is, the greater the chance is of the lucerne plants being infected with virus.

Principal vectors

BLRV is persistently transmitted by aphids and is not seed transmitted, while CMV and AMV are non-persistently transmitted by aphids and are seed transmitted at low rates. Common aphids of pastures that transmit these viruses are bluegreen aphid (BGA, *Acyrthosiphon kondai*), pea aphid (PA, *Acyrthosiphon pisum*), cowpea aphid (CPA, *Aphis craccivora*) and green peach aphid (GPA, *Myzus persicae*). BGA, PA and CPA have a wide range of legume hosts (pastures and crops) and GPA have a wide host range. Correct identification of aphids will help determine if aphid control is required. The ideal time for controlling aphids is before colonies start to produce winged adults which can spread through the crop. Winged adults are produced when aphid colonies rapidly build up and start to overcrowd the host plant.

Management

An integrated approach should be taken to reduce the risk of virus infection in sub clover seed crops. The following list suggests some useful management strategies to consider when developing your management strategy:

1. Identification of red-leaf disease in prior years suggests that a management strategy should be developed.
2. The presence of red-leaf in itself does not indicate viruses are present. If viruses are suspected of being the cause of the leaf reddening it is worth getting this confirmed by molecular testing by Waite Diagnostics.
3. Summer rainfall and early breaks favour early increases in volunteers that favour aphids and virus build-up.
4. Control of volunteer legumes and weeds in summer and autumn reduces the reservoirs of the viruses and aphids.
5. Locate sub clover seed crops away from lucerne fields as aphids can migrate out of lucerne fields into sub clover fields. If possible remove old unproductive stands of lucerne.
6. Consider delaying sowing in autumn as warm weather post planting increases the risk of aphid movement especially of cowpea aphid and green peach aphid.
7. Consider treating seed with insecticide to control plant feeding insects.
8. Monitor for natural enemies of aphids (e.g. lady birds, lacewings and parasitic wasps) as high numbers of these can indicate good aphid control.
9. If aphid numbers are high it may be necessary to spray with an insecticide. Only spray if necessary to avoid insecticide resistance. Where possible use registered aphicides rather than broad spectrum insecticide in order to limit damage to beneficial insects.
10. Since aphid infestations often start on the perimeter of a field it may be only necessary to spray the perimeters of fields.

Molecular diagnostics

This project has developed a set of molecular diagnostic tools that can detect the presence of the causal viruses. For example the bright bands in figure 2 indicate that BLRV is present in plant samples 1, 2, 4, 7 and 8 but absent in the other plant samples. It is recommended that molecular diagnostics be used to confirm that a virus is the cause of leaf reddening. For pricing and sampling protocol please contact:

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Disclaimer

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Development and Use of Diagnostic Tools for Subterranean Clover Red Leaf Disease

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Sub clover seed production is estimated to be a $10-15 million dollar a year industry and seed producers from the mid 2000's have reported that an unknown red-leaf disease has been causing yield losses of up to 60% in their seed production crops. This report presents the results of a three year research project that investigated the causal agents of red-leaf disease in sub clover seed crops, developed diagnostic tools and developed a fact sheet on the control of red-leaf disease.

This report will be of interest to sub clover seed producers, pasture agronomists, sub clover pasture growers.

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