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Australian Lucerne Yellows Disease

– Pathogen, vector and control

A report for the Rural Industries Research and Development Corporation

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Foreword

Lucerne is a perennial, deep-rooted pasture legume of increasing world-wide significance due to its use in managing aspects of environmental sustainability, such as rising water tables and soil salinity. The production of lucerne seed is an important sector of Australia's pasture seed industry but is affected by the disease Australian lucerne yellows (ALuY).

This disease has a severe effect on seed production, frequently causing death of plants and reduced vigour in those that survive. The disease also has led to the cutting or ploughing-under of seed crops, resulting in estimated losses of $7m annually to the Australian lucerne seed industry.

The aim of the present work was to undertake the first comprehensive study of the disease in order to develop an understanding of the pathogen and how it is transmitted. Such knowledge is important in underpinning rational disease management approaches and several of these approaches have been tested.

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

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

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Peter O'Brien
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Abbreviations

ALuY – Australian lucerne yellows
TBB - tomato big bud
DNA – deoxyribonucleic acid
PCR – polymerase chain reaction
RFLP – restricted fragment length polymorphism

Contents

Executive Summary

Foliar and root symptoms are described for 'Australian lucerne yellows' (ALuY), a disease common in Australian lucerne seed crops. Using electron microscopy and molecular methods, a phytoplasma was detected in plants exhibiting symptoms but not in symptomless lucerne plants. DNA testing found the ALuY phytoplasma to be distinct from that responsible for another lucerne disease, tomato big bud (TBB). The ALuY phytoplasma fell within the Faba bean phytoplasma group or phytoplasma group '16srII'. Fungal, bacterial and viral causes were ruled out.

Three newly-sown lucerne stands in the Mid Lachlan Valley region of New South Wales, Australia were sampled over 50 weeks for ALuY disease distribution and severity. Leafhopper populations were also monitored. Symptoms developed in all three stands within 32 weeks of sowing. There were statistically significant spatial patterns in the density of symptomatic plants for two dates at two sites. Two possible insect vectors, the spotted leafhopper (*Austroagallia torrida*) and the large green jassid (*Batracomorphus angustatus*) were more numerous in some sections of crop-margins at two sites. These two species and a third possible insect vector, the common brown leafhopper (*Orosius argentatus*) each had a statistically significant spatial and temporal correlation with symptomatic plant numbers for at least one site-date. The same leafhoppers were present in South Australia.

Two border treatment experiments evaluated the effect of crop-margin treatments on leafhopper movement into and from the stand. The second border treatment experiment examined also the treatment effect on ALuY disease incidence. Treatment with insecticide or herbicide significantly reduced the overall movement of leafhoppers. In addition, the insecticide treatment lowered the incidence of disease expression in adjacent lucerne. Results suggest that there is scope for management of this plant disease by reducing immigration of leafhopper vectors into lucerne from non-crop vegetation.

The spotted leafhopper, large green jassid and the common brown leafhopper were used in transmission tests to determine their vector status for the phytoplasmas associated with ALuY. Caged, seed-grown lucerne plants were monitored for foliar symptom expression after feeding by leafhoppers transferred from ALuY symptomatic lucerne plants. Twelve of 25 plants developed phytoplasma disease-like symptoms including stunting and yellowing. The most pronounced foliar symptoms were displayed by five plants that had been fed on by the common brown leafhopper and four plants that had been fed on by spotted leafhopper. Only one plant, fed on by the common brown leafhopper, showed the distinctive root symptoms of ALuY. Molecular tests detected a phytoplasma in one plant each from the batches that had been fed upon by each leafhopper species. The phytoplasma detected from the plant that had been fed on by spotted leafhopper was shown by DNA analysis to be tomato big bud (TBB), a separate phytoplasma that causes witches' broom and phyllody in lucerne. Electron microscopy detected an unidentified phytoplasma in two plants fed on by the common brown leafhopper and one by spotted leafhopper. Molecular tests failed to detect phytoplasma DNA from any of the leafhopper species used in transmission tests or from specimens sampled from lucerne crops. The common brown leafhopper is considered the most likely vector for the ALuY pathogen though the large green jassid cannot be ruled out. The spotted leafhopper is shown to be a vector for TBB phytoplasma.

Diseased plants within an established irrigated lucerne stand in the mid Lachlan Valley were used to assess the effect of several treatments on Australian lucerne yellows (ALuY) disease. Treatments included applications of supplementary water, multi-nutrients, potash, tetracycline antibiotic and a nil control. After 12 weeks, the plants in five of the ten blocks were harvested and fresh weight, dry weight and symptom severity were recorded. Two weeks later, the remaining plants were harvested and the same parameters, except fresh weight, as well as seed yield and seed germination rate were measured. Supplementary water resulted in a modest but statistically significant increase of seed yield though effects on other measures of plant health were not significant. Other treatments had no

significant effect so the scope for effective management of this disease via symptom alleviation or antibiotic treatment appears limited.

1. Detection and identification of a phytoplasma from lucerne with 'Australian lucerne yellows' disease

Introduction

Australian lucerne yellows (ALuY) is one of several major lucerne diseases in New South Wales (Stovold, 1983; McDonald *et al.*, 2003) and is attributed to a phytoplasma (Fletcher, 1980; McGechan, 1980). The disease has a severe effect on seed production, frequently causing death of plants and reduced vigour in those that survive (Stovold, 1981). The disease also causes a reduction in seed yield and has led to the cutting or ploughing-under of seed crops, resulting in estimated losses of $7m annually to the Australian lucerne seed industry (Pilkington *et al.*, 1999).
Symptoms associated with ALuY include a discoloration of leaves ranging from yellow to red (Stovold, 1983) that affects the entire foliage (Pilkington *et al.*, 1999). Roots of affected plants have a characteristic yellow-brown discoloration immediately under the periderm of the taproot (Stovold, 1983; Pilkington *et al.*, 2002).

Phytoplasmas have been detected in 38 plant species in Australia (Schneider *et al.*, 1999b) including lucerne. The TBB and sweet potato little leaf strain V4 (SPLL-V4) phytoplasma have been detected in lucerne (Gibb *et al.*, 2000; Wilson *et al.*, 2001; Gibb, Northern Territory University, Darwin, personal observation).

'Yellows' symptoms have been recorded in Australian lucerne since the early 1950s (Anonymous, 1953). During the 1970s, yellowing of lucerne was reported to be very common and considered responsible for decline in the density of lucerne stands in many areas (Anonymous, 1975). Hellemere (1972) discussed possible causes and ruled out bacterial wilt and nutrient disorders. The symptomatology of the disease indicated a pathogen that was either a "mycoplasma-like organism" or a virus (Hellemere, 1972; McGechan & Stovold, 1976).

The aim of the present study was to analyse plants with and without symptoms i) for the presence of phytoplasmas and ii) presence of bacterial and fungal pathogens reported on, as are characterisations of phytoplasmas detected in ALuY symptomatic lucerne using molecular techniques.

This paper describes the finding and characterization of phytoplasmas in yellow-affected lucerne plants. A preliminary report on the aetiology of Australian lucerne yellows was published earlier (Pilkington *et al*., 2002).

Materials and Methods

Source of material
Lucerne plants with and without symptoms of ALuY were collected from each of four certified seed crop sites numbered one to four in the Lachlan Valley of central New South Wales (NSW), Australia. Wet paper towelling was placed around the roots to reduce stress and samples were transported to the laboratory at 4°C in a 12-volt car refrigerator. Individual plants were selected initially on their foliar symptoms and ALuY confirmed by root examination (Pilkington, *et al.*, 1999). There are no known diseases of lucerne that express similar foliar and root symptoms although care was taken to ensure the stele of the taproot was not discoloured which may have indicated bacterial wilt (Harvey, 1982). Five plants each with and without symptoms were selected
at random from sites one and two. An additional set of two plants each were collected from site three in the Lachlan Valley, NSW and used for fungal examinations, whilst another set of 10 plants, each with and without symptoms of ALuY were selected at random from site four in the Lachlan Valley, NSW for bacterial examination. A tomato plant exhibiting symptoms of TBB disease was collected

from and cultivated in the laboratory. During the course of the study, this plant was used as a source of the TBB phytoplasma for comparative purposes.

Fungal isolations from roots

A segment of the tap-root, approximately five centimetres long, was cut from each plant and washed thoroughly in tap water then in sterile distilled water and dried with paper towelling. A small section of the root cambium was removed using standard aseptic techniques. A thin sliver of tissue, approximately 2 x 2 x 0.5 mm, was removed from the inner side of the exposed cambium layer and four pieces of this tissue from each root were placed onto one quarter strength potato dextrose agar (¼PDA) supplemented with 100 μg mL^{-1} novobicin to inhibit bacterial growth. Isolation plates were placed on the laboratory bench in natural light at 22°C (± 3°C).

Fungal isolations were examined after five days of incubation and the leading edge of each individual colony was sub-cultured onto ¼PDA and maintained under the conditions described above.

Bacterial isolations from roots, stems and leaves

Sections from the root and young shoots from each plant were examined with a light microscope for evidence of bacterial ooze. Bacterial isolations were then made from the roots of five plants with symptoms and one plant without symptoms. Roots were washed thoroughly in sterile distilled water and a segment (approximately 1 x 1 x 1 cm) was removed from the taproot leaving the cambium layer intact. This section was surface-sterilised for two minutes in 1% sodium hypochlorite, agitating every 30 seconds, then rinsed twice in sterile distilled water for 2 minutes.

All exterior surfaces of the root section were removed aseptically using standard sterile techniques. Discoloured tissue from ALuY affected plants, and matching tissue from symptomless plants, were sliced into fine pieces (approximately 1 x 1 x 0.5 mm) and teased out. The slices were placed in 10 ml sterile distilled water for one hour.

Stems of five plants with symptoms and one symptomless plant were selected and four young stems and petioles were aseptically removed from each plant, rinsed twice in sterile distilled water for two minutes, the pieces (approximately 1 x 1 x 1 mm) aseptically cut, roughly macerated and placed into 10 ml of sterile distilled water for one hour. The suspension was streaked out with a one millimetre loop onto each of four plates of sucrose peptone agar (SPA), SPA + 250 ppm glycohexamide and nutrient agar (NA) Oxoid (Oxoid Ltd, Basingstoke Hampshire, England). Plates were sealed with Parafilm (American Can Company Greenwich, C.T., USA) and placed in an incubator at 25°C. After three days, cultures were examined and individual colonies were sub-cultured onto the same medium from which they had been isolated.

Eleven colonies were selected and submitted for fatty acid analysis (Agilent Technologies 6890N Network GC System Machine) at Orange Agricultural Institute, New South Wales, Australia. Cultures identified as *Clavibacter michiganense* subsp. *insidiosus* by fatty acid analysis were then retested by enzyme linked immunosorbent assay (ELISA) using specific antibodies at the South Australian Research and Development Institute, South Australia, Australia.

Detection of phytoplasmas

DNA Extraction

DNA was extracted as described by Dellaporta, *et al*. (1983) from 0.5g combined leaf midribs, stems and roots from lucerne plants with and without symptoms of ALuY within 12 hours of arrival in the laboratory. DNA was extracted twice from 130 individual ALuY affected plants to give a total of 260 DNA samples. Single extractions were made from 30 symptomless lucerne plants to give a total of 30 samples. Ethanol-precipitated DNA pellets were each re-suspended in 50 μl 1 x TE buffer (10 mM Tris-HCl, 1 mM EDTA) and stored at –20°C until used.

Primers and PCR protocols
Template DNA samples were diluted to 1:1, 1:10, 1:50 and 1:100 with sterile distilled water prior to using 1 μL aliquot of each in a PCR reaction. Each 50 μL PCR reaction mixture consisted of 1.25 units of *Taq* polymerase, buffer consisting of 1.5 mM $MgCl_2$, 0.4 μM of each primer and 0.1 mM of each dNTP (all components listed supplied by GeneWorks, Adelaide, SA, Australia).

The primers P1 (Deng & Hiruki, 1991) and P7 (Kirkpatrick *et al.*, 1994), fU5 (Lorenz *et al.*, 1995) and m23sr (Padovan *et al.*, 1995) were used in PCR and nested PCR assays. PCR cycling conditions were as follows: denaturation for one minute (two minutes for first cycle) at 95°C, annealing temperature of 55°C for one minute and an extension time of 1.5 minutes at 72°C for 35 cycles (9.5 minutes on final cycle). TBB phytoplasma DNA and sterile distilled water (SDW) were used for positive and negative controls, respectively. Sixteen nested PCR assays were conducted each consisting of sixteen ALuY DNA samples, two symptomless lucerne DNA samples, one TBB sample and one SDW sample using the universal primers P1/P7. One μL of each P1/P7 PCR cocktail was then subjected to re-amplification using the primer pair fU5/m23sr and the same cycling conditions. After each nested PCR assay, 2 μL of PCR product were analysed by electrophoresis on a 1.0% agarose gel and stained with ethidium bromide prior to being visualised with a UV transilluminator.

PCR inhibitors
Eighteen samples from ALuY affected plants that tested negative by PCR were analysed for the presence of PCR inhibitors. One μL of DNA from each ALuY symptomatic plants was combined with an equal volume of the control (TBB) DNA and subject to PCR using primers P1/P7.

Restriction fragment length polymorphism analysis
Nested PCR products from ten ALuY affected lucerne plants and six TBB phytoplasma controls were subjected to RFLP analysis. Following the manufacturer's (New England Biolabs, Inc., Beverley, MA, USA) instructions, 5 μL of each PCR product was digested separately with each of the following enzymes: *Mse*I, *Alu*I, *Rsa*I and *Hpa*II. The products from these digestions were then subjected to electrophoresis through a 5% polyacrylamide gel then stained with ethidium bromide and visualised by UV transillumination.

Sequence analysis
The entire PCR product obtained from a DNA sample extracted from a single ALuY lucerne plant that tested positive for phytoplasma by PCR was purified using the QIAquick PCR purification kit (Qiagen, Clifton Hill, NSW, Australia). Sequencing of products was performed at the Australian Genome Research Facility (St Lucia, Queensland, Australia). Sequencing primers consisted of P3 (Schneider, *et al.*, 1995), rP3 (reverse and complement of P3), 16R723f, r723SEQ (the reverse and complement of 16R723f), rU3 (Lorenz *et al.*, 1995), fsLYa (5' CAAACCACGAAAGTTGGC 3'), fsLYb (5' AAAAACAGTCCCAGTCCG 3'), fU5 (Lorenz *et al.*, 1995) and M23sr (Padovan *et al.*, 1995). The ALuY 16S rDNA sequence was compiled using CodonCode Assembler version 0.000918 (CodonCode Corporation, Dedham, MA, USA) available through BioNavigator (Entigen Corporation, Sunnyvale, CA, USA). ALuY phytoplasma 16S rDNA was aligned with other phytoplasmas using ClustalW (Thompson *et al.*, 1994) (Table 1). A phylogenetic tree was prepared using DNAdist and Neighbour (Felsenstein, 1989) and phylodendron (D. G. Gilbert & BioNavigator, Entigen Corporation). Pairwise comparisons between ALuY phytoplasma and several closely related phytoplasmas (Table 1) were conducted using the programme GAP (Accelrys, San Diego, CA, USA). *Acholeplasma palmae* and *A. laidlawii* were used as outgroups.

Electron microscopy
Leaf midribs from six ALuY affected and two unaffected lucerne plants were dissected into approximately $1mm^3$ pieces containing phloem tissue. Samples were fixed with standard methods (Bozzola & Russell, 1992). Specimens were infiltrated with 100% acetone/Spurrs resin (1:1) overnight at room temperature on rotators, transferred to 100% Spurrs resin overnight on rotators and embedded in fresh Spurrs resin and polymerised at 60°C overnight. Specimens were then cut into ultra-thin (80nm) sections and viewed in a Philips Biofilter CM120 (120kV) electron microscope.

Table 1 Phytoplasma names, abbreviations and EMBL accession numbers

Phytoplasma	Abbreviation	Accession No.
Sweet potato witches' broom	SPWB	L33770
Sweet potato little leaf	SPLL	X90591
Tomato big bud	TBB	Y08173
Faba bean phyllody	FBP	X83432
Bonamia little leaf	BoLL	Y15863
Clover phyllody	CPh	L33762
Oenothera aster yellows	OAY	M30970
American aster yellows	AAY	X68373
Australian grapevine yellows	AGY	X95706
Phormium yellow leaf	PYL	U43571
Stolbur disease	STOL	X76427
Peanut witches' broom	PnWB	L33765
Sunhemp witches' broom	SUNHP	X76433
Vergilbungskrankheit	VK	X76428
Sugarcane white leaf	SCWL	X76432
Bermuda grass white leaf	BGWL	Y14645
Rice yellow dwarf	RYD	L26997
Pigeon pea witches' broom	PPWB	L33735
Clover yellow edge	CYE	L33766
Coconut lethal yellowing	LY	L27030
Loofah witches' broom	LfWB	L33764
Ash yellows	AshY	L33759
Clover proliferation	CP	LL33761
Elm yellows	EY	L33763
Flavescence dorée	FD	X76560
Spartium witches' broom	SPAR	X92869
Omani alfalfa witches' broom	OaWB	AF438413
Papaya yellow crinkle	PPYC	Y10095
Papaya mosaic	PPMz	Y10096
Pear decline	PD	X76425
Acholeplasma palmae		L33734
Acholeplasma laidlawii		M23932

Results

Fungal isolations

Eighteen distinct taxa of fungi were isolated from symptomatic and asymptomatic plants. *Fusarium solani* was isolated from three of the twelve diseased plants examined. Several other fungi e.g. *Phoma medicaginis; Colletotrichum trifolii*, were identified less commonly from plants both with and without symptoms. No consistent association between any fungus and ALuY symptoms was apparent.

Bacterial isolations

No bacterial ooze was evident in any prepared sample. Seven isolated species of bacteria were identified using fatty acid analysis. Two were known pathogens of lucerne. Rhodococcus fascians was isolated only from symptomless plants whilst C. michiganense subsp. insidiosus was a likely identity of two isolates from AluY affected plants. In one of these cases, the fatty acid analysis Similarity Index (SI) (Anonymous, 2002) for C. michiganense subsp. insidiosus of 0.702 was lower than that for the alternative identification of Leifsonia aquatica (0.780 SI), a non-lucerne pathogen. Both isolates tentatively identified as C. michiganense subsp. insidiosus were, however, negative when tested by ELISA.

Detection of phytoplasmas in lucerne

No bands were amplified by simple PCR of either AluY or symptomless plants, but in all assays the TBB phytoplasma control was positive and amplified a 1.6kb band. In nested PCR using primers P1/P7 followed by fu5/m23sr, the TBB phytoplasma positive controls gave a product of 1.1kb while water controls gave no amplified product. Of the 260 ALuY samples tested from 130 individual yellows affected plants, 63 gave a product of 1.1kb when amplified in nested PCR assays. No positive signal was observed with DNA extracted from the 30 symptomless plant samples. A 1.6kb PCR product was observed when 18 ALuY DNA samples that had tested negative was spiked with TBB phytoplasma DNA and subjected to single round PCR. A representative PCR result for 16 ALuY affected plants and two symptomless plant samples is shown in Fig.1.

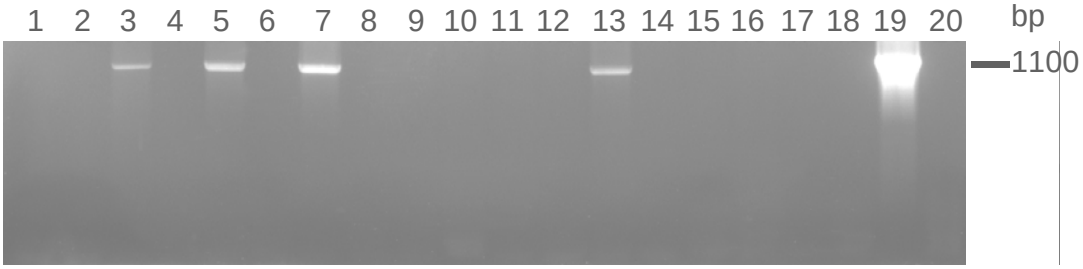

Fig. 1 Polymerase chain reaction amplification of phytoplasma DNA from AluY affected lucerne using the primer pairs P1/P7 and fU5/m23sr. Lanes 1 – 16 ALuY plants; 17 and 18 symptomless lucerne; lane 19 TBB; lane 20 water control. Size markers indicated on the right hand side of the gel were used to determine the size of the PCR products.

RFLP

When 10 PCR products amplified from 10 separate ALuY plant samples were digested with the restriction enzymes *Mse*I, *Alu*I, *Rsa*I and *Hpa*II, all resulting RFLP profiles for each enzyme were identical, but differed from the patterns of the TBB digests. In all ALuY RFLP profiles for *Alu*I and *Hpa*II enzymes, extra bands were present that were absent from TBB profiles. These extra bands result in a total fragment size larger than 1.1kb. Representative RFLP profiles of ALuY and TBB phytoplasmas are shown in Fig. 2.

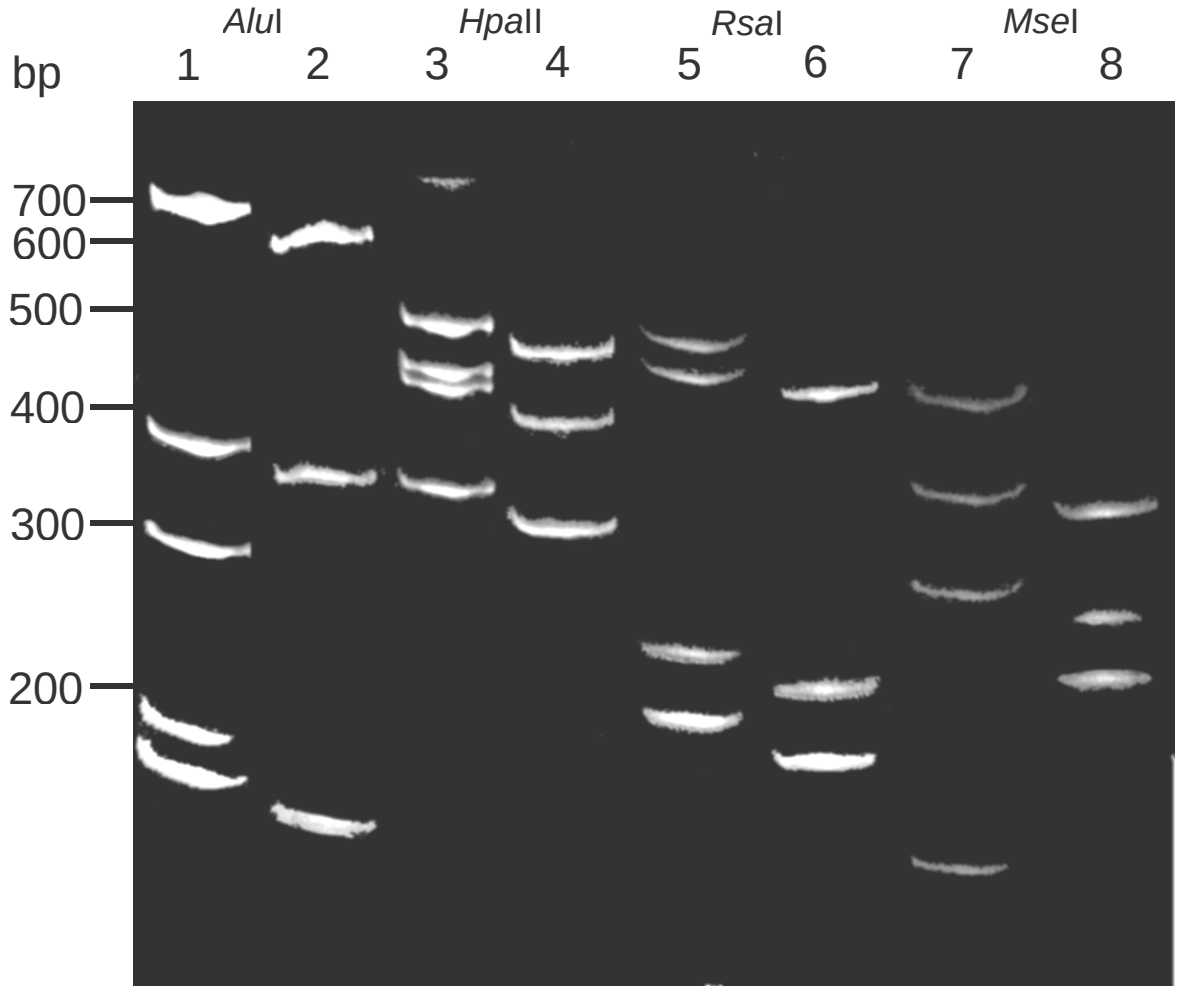

Fig. 2 RFLP profiles of 16s rDNA amplified by nested PCR from the phytoplasma associated with ALuY and TBB phytoplasma. Lanes 1, 3, 5 and 7 ALuY DNA digested with *Alu*I, *Hpa*II, *Rsa*I and *Mse*I respectively. Lanes 2, 4, 6 and 8 TBB DNA digested with *Alu*I, *Hpa*II, *Rsa*I and *Mse*I respectively.

Sequence Analysis

The entire PCR product of approximately 1.1kb amplified from a DNA sample extracted from an ALuY diseased lucerne plant was sequenced. The region sequenced included the 16S rRNA gene and the entire 16S/23S spacer region (SR). The 16S/23S spacer region (accession number AJ315966) was 241 bases long which is consistent in size with other phytoplasmas (Cronje *et al.*, 2000; Tran-Nguyen *et al.*, 2000). The 16S rRNA region (accession number AJ315965) represents a partial sequence (position 520 to the start of the spacer region at position 1480).

Pairwise sequence comparisons indicated that the phytoplasma associated with ALuY disease is most similar to peanut witches' broom (PnWB) with a similarity of 99%, Omani witches' broom (OaWB) (99%), papaya yellow crinkle (PpYC) (99%), papaya mosaic (PpMz) (99%), sunhemp phytoplasma (SUNHP) (99%) and TBB (99%) (Table 2). A phylogenetic tree (Fig. 3) showing the relationship between the phytoplasma associated with ALuY disease and other phytoplasma species indicates that the former is associated with the FBP phytoplasma (16srII) group (Lee *et al.*, 1998; Seemüller *et al.*, 2002).

Table 2 Sequence similarity (%) matrix of the partial 16s region (approximately 5' 520 to the start of the IGS 5' 1480) of several phytoplasma species from the FBP group rounded to the nearest decimal point percentage.

	ALuY	OaWB	BoLL	FBP	PpMz	PpYC	TBB	PnWB	SUNHP	SPLL	SPWB	
ALuY												ALuY
OaWB	99											OaWB
BoLL	97	98										BoLL
FBP	97	98	99									FBP
PpMz	99	100	99	98								PpMz
PpYC	99	100	99	98	100.0							PpYC
TBB	99	99	98	98	99	99						TBB
PnWB	99	100	98	98	100	100	100					PnWB
SUNHP	99	99	98	98	100	100	99	100				SUNHP
SPLL	98	99	98	98	99	99	98	99	98			SPLL
SPWB	98	99	97	97	99	99	99	99	99	98		SPWB
	ALuY	OaWB	BoLL	FBP	PpMz	PpYC	TBB	PnWB	SUNHP	SPLL	SPWB	

Electron Microscopy

Examination of ultrathin cross-sections of leaf midrib from AluY affected plants showed numerous phytoplasmas (200-400nm diameter) in the phloem of four of the plants. The structures were spherical to ovoid, were enclosed by a single unit membrane and contained dark structures centrally located that were consistent in appearance to bundles of DNA (Fig. 4). Some phloem cells were completely occluded with phytoplasmas. No phytoplasmas were evident in sieve tube sections of two symptomless plants examined.

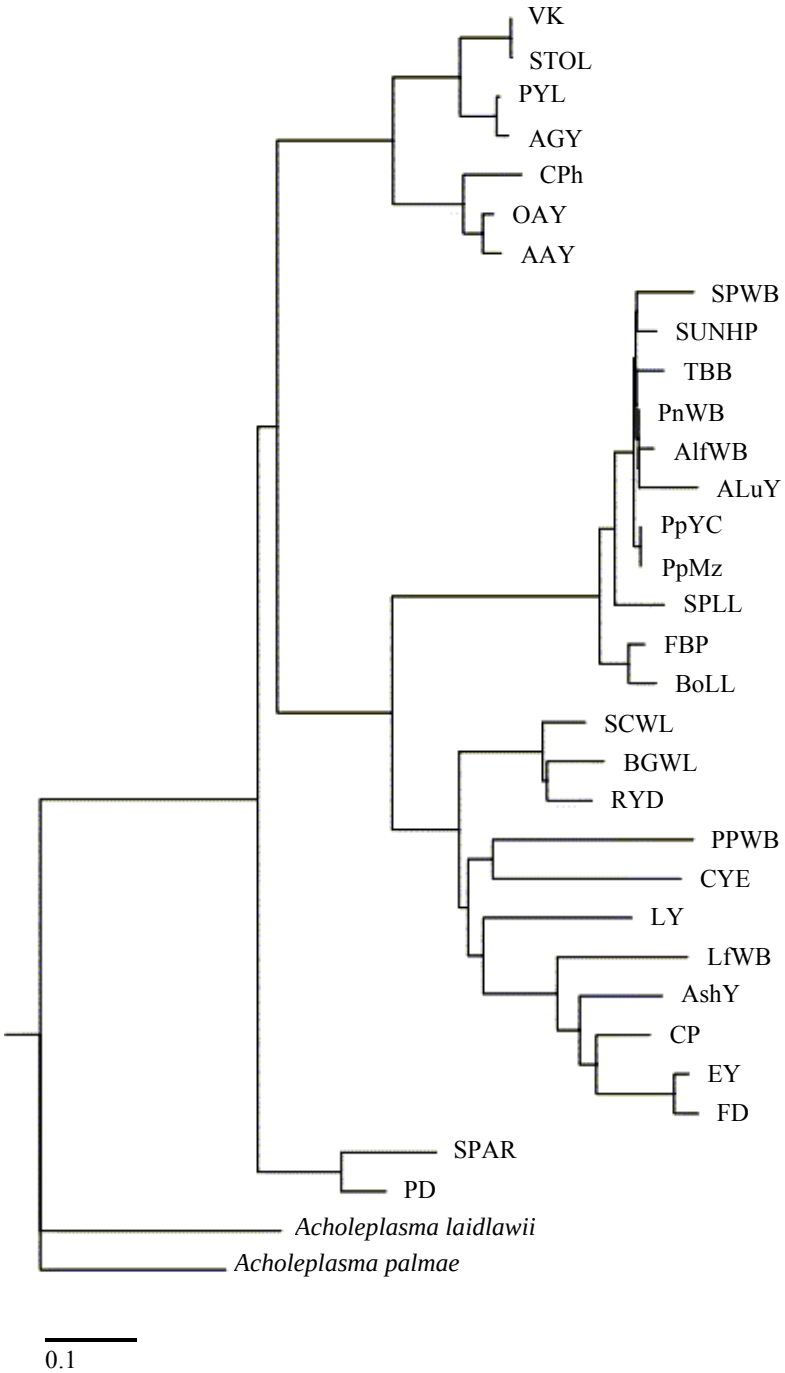

Fig. 3 Phylogenetic tree of the 16srRNA gene sequence of the phytoplasma associated with ALuY (partial sequence of 960bp) and other selected phytoplasma 16srRNA sequences. *Acholeplasma laidlawii and Acholeplasma palmae* were used as outgroup. The bar represents a phylogenetic distance of 10%. Phytoplasma strains are given in Table 1.

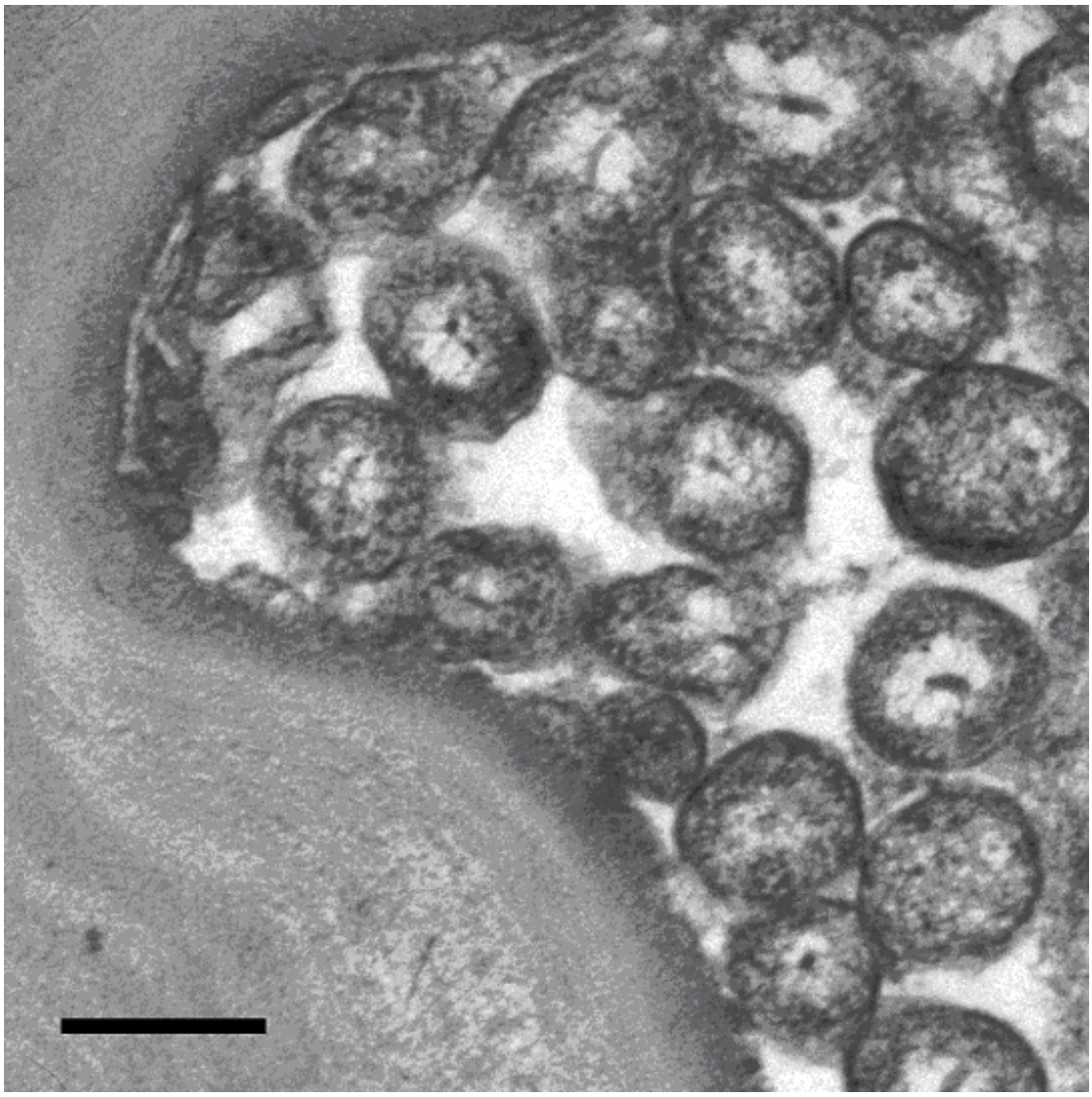

Fig. 4. A phloem cell of a lucerne plant affected with ALuY showing phytoplasma bodies (bar = 0.30μm).

Discussion

Lucerne with ALuY symptoms was tested for the presence of potential pathogens including fungi, bacteria and phytoplasmas. No apparent association was found between symptoms and any individual fungus. Five of the 12 fungal species isolated from AluY affected plants were also isolated from symptomless plants and six other species were recovered solely from symptomless plants. *Fusarium solani was* the most frequently isolated fungus from yellows affected plants and previously has been associated with crown and root rots of lucerne (Leath & Kendall, 1978; Nikandrow, 1990)

Other known lucerne fungal pathogens including *Phoma medicaginis*, the cause of black stem, and *Colletotrichum trifolii*, the cause of crown rot (Stuteville & Erwin, 1990) were inconsistently isolated from plants with and without symptoms. The symptomatology associated with all three fungi, however, are inconsistent with ALuY disease.

Two known bacterial plant pathogens were tentatively identified by fatty acid analysis: *R. fascians* is known to cause fasciation in many plant hosts (Crespi *et al.*, 1994; Stange *et al.*, 1996) but no evidence indicates that it causes a disease in lucerne, whilst *C. michiganense* subsp. *insiodosum* causes bacterial wilt of lucerne but its tentative identification was not supported by subsequent, more detailed, ELISA studies. Bacterial wilt causes yellowed and stunted leaves in lucerne and symptoms are most apparent immediately after cutting or grazing (Stovold, 1983). Root symptoms of bacterial wilt are a yellow to brown discoloration throughout the stele of the tap-root thus are distinct from the symptoms seen in plants infected with ALuY (Stovold, 1983) in which discolouration occurs directly beneath the cambium layer of the tap-root (Pilkington *et al.*, 1999). Aside from differences in symptoms, no obvious association with *C. michiganense* subsp. *insidiosus* could be inferred because like *R. fascians* it was isolated from only one of the 12 AluY affected plants tested. The involvement of a culturable bacterial pathogen with lucerne yellows has also been ruled out in previous studies (Hellemere, 1972).

There are three viruses reported to cause diseases in lucerne in Australia: *Alfalfa mosaic virus* (AMV*), Lucerne latent nepovirus* (LALV) and *Lucerne transient streak sobemovirus* (LTTV) (Blackstock, 1978; Johnstone & Barbetti, 1987). Symptoms of AMV include mild to severe mosaic, leaf stunting and rolling, chlorotic vein-banding and leaf reddening (Hajimorad & Francki, 1988). There are no expressed symptoms for LALV in naturally infected lucerne plants (Blackstock, 1978). Lucerne plants infected with LTTV typically develop chlorotic streaks around the main lateral veins of leaflets and necrotic and chlorotic lesions, none of which are expressed in summer (Blackstock, 1978). Variations of LTTV have been found in Australia but symptoms are similar (Dall *et al.*, 1990). As these symptoms are distinct from those of ALuY, a viral cause is unlikely.

Several phytoplasmas have been reported in lucerne. Alfalfa witches' broom (AWB) is distributed world-wide (Khan*, et al.*, 2002). Others include the stolbur phytoplasma from lucerne in Italy (Marzachi *et al.*, 2000); little leaf phytoplasma in India (Suryanarayana *et al.*, 1996); and aster yellows phytoplasma in Wisconsin (Peters *et al.*, 1999). Lucerne has been implicated as being a reservoir for phytoplasma diseases such as canola yellows (Wang & Hiruki, 2001a). The most common phytoplasma, AWB, is associated with several different phytoplasma groups depending on geographical location. AWB has been associated with phytoplasmas from the faba bean phyllody (FBP) group (Marcone *et al.*, 1997; Khan *et al.*, 2002), the clover proliferation (CP) group (Wang & Hiruki 2001b) and the aster yellows group (Valiunas *et al.*, 2000).

In this study, a phytoplasma was detected in ALuY symptomatic lucerne plants using PCR and electron microscopy but both methods failed to detect phytoplasmas in symptomless plants. An association of 24.2% between phytoplasma detection and ALuY disease symptoms was achieved by nested PCR using primers P1/P7 and fu5/m23sr. A nested PCR approach is often needed for detection of phytoplasmas (Schneider & Gibb, 1997) because they often occur at low levels in plants and are unevenly distributed making direct detection difficult (Goodwin *et al.*, 1994; Andersen *et al.*, 1998). Poor or unreliable amplification of target DNA by PCR is sometimes attributed to inhibitors present in host plant tissue (Cheung *et al.*, 1993; Schneider & Gibb, 1997). TBB phytoplasma DNA was, however, amplified successfully in the presence of DNA extracted from AluY affected lucerne. This suggests an absence of PCR inhibitors in lucerne tissue.

RFLP analysis is useful for differentiating phytoplasmas (Gundersen *et al.*, 1996) and has been used to classify phytoplasmas into a series of groups or subgroups for taxonomic purposes (Schneider*, et al.*, 1993). RFLP profiles for ALuY phytoplasma that were digested with the enzymes *Alu*I and *Hpa*II produced extra bands and the total fragment size was therefore greater than the 1.1kb fragment expected. Phytoplasmas contain two 16SrRNA operons (Schneider & Seemüller, 1994) and these can sometimes be resolved as double bands in agarose gel electrophoresis of PCR products (De La Rue *et al.*, 2001). Whilst only a single band was consistently amplified from ALuY DNA samples, it cannot be ruled out that the extra bands in the RFLP analysis may have resulted from slight differences in the 16SrRNA gene sequences from each operon. Although these differences may be so slight that the PCR product co-migrates on an agarose gel (Schneider & Seemüller, 1994; Liefting*, et*

al., 1996), any sequence differences that affect restriction enzyme recognition sites will result in different inter-operon banding patterns that can be resolved on an acrylamide gel. An alternative explanation for the additional RFLP bands is that ALuY diseased plants are subject to a mixed phytoplasma infection though this is unlikely as the RFLP patterns observed were consistent across all samples. PCR products amplified from individual ALuY affected plants gave consistent RFLP patterns that differed from those of the positive control, the TBB phytoplasma. Such a finding indicates that the phytoplasma detected in ALuY-diseased lucerne is distinct from the widespread TBB phytoplasma (Davis*, et al.*, 1997; Schneider*, et al.*, 1999a) and on this basis it is now referred to as the Australian lucerne yellows phytoplasma (ALuY).

A large number of phytoplasmas have been taxonomically characterised using sequence analysis of the 16S rDNA and 16S/23S spacer region (Davis & Sinclair, 1998; Seemüller *et al.*, 2002). In this study, the phylogenetic positions of several phytoplasmas were compared with the ALuY phytoplasma. It was most closely related to the FBP phytoplasma group (Schneider *et al.*, 1999b) or phytoplasma group 16srII (Lee *et al.*, 1998). The similarity of ALuY to TBB and SPLL was not unexpected given the wide variety of plant species in which these phytoplasmas occur throughout Australia and Southeast Asia (Padovan *et al.*, 1996). Although placed in group 16srII, the ALuY phytoplasma is not identical to any other known phytoplasma and represents a new strain, possibly endemic to Australia.

2. Reducing the immigration of suspected leafhopper vectors and severity of Australian lucerne yellows disease

Introduction

Lucerne (*Medicago sativa* L.) has been used for grazing, conserved fodder and the production of value added products (cubes for example) in Australia since the mid 1920s (Fitzgerald *et al.* 1980). Lucerne is now considered to be the most important forage crop in the world (Lolicato and Lattimore 1998). Australian lucerne yellows (ALuY) is a serious disease, impacting heavily on the Australian lucerne seed industry, causing an estimated annual loss of $7M to the industry (Pilkington *et al.* 1999). The disease has been reported in lucerne since the early 1950s (Anon. 1953) yet little is known of its etiology. Symptoms of ALuY include yellow discolouration of foliage and a distinctive yellow to dark brown discolouration immediately under the periderm of the taproot (Pilkington *et al.* 2002).

Recent work has shown that a phytoplasma is associated with ALuY disease (Pilkington *et al.* In Press). This group of plant pathogenic mollicutes is associated with over 300 plant diseases around the world (Davis *et al.* 1988). Phytoplasmas are transmitted exclusively by insects (Hanboonsong *et al.* 2002); specifically leafhoppers (Cicadelloidea), planthoppers (Fulgoroidea) and psyllids (Psylloidea) (Tsai 1979; Ploaie 1981). More than 30 species from these superfamilies have been identified in Australian (Bishop and Holtkamp 1982; Osmelak *et al.* 1989) and American lucerne stands (Sulc *et al.* 2001).

Preliminary surveys of the above insect taxa in the study area showed the presence only of: *Austroagallia torrida* (Evans); *Batracomorphus angustatus* (Osborn); *Orosius argentatus* (Evans); *Balclutha incisa* (Matsumura) and/or *B. saltuella* (Kirschbaum); *Austroasca viridigrisea* (Paoli) and/or *A. alfalfae* (Evans); and *Zygina zealandica* (Myers) (Pilkington unpublished data).

As phytoplasmas are restricted to the phloem of infected plants (Guthrie *et al.* 2001), it follows that their vectors feed on phloem (McCoy 1979). *Balclutha* sp., which feeds exclusively on grasses (Knight 1987), and the known parenchyma feeding species, *Austroasca* sp. and *Zygina* sp. (Carver *et al.* 1991), were discounted as possible vectors of ALuY. *O. argentatus* has been shown to be responsible for transmission of lucerne witches' broom and has also been implicated in many other phytoplasma diseases, including Australian grapevine yellows (AGY) (Padovan *et al.* 1996). *A. torrida* is a known vector of viral and bacterial plant diseases (Grylls 1979) and both *A. torrida* and *B. angustatus* have also been suggested as possible vectors of AGY (Osmelak *et al.* 1989). Their presence in ALuY-symptomatic lucerne stands suggests that they are possible vectors of this disease.

Information on spatial and temporal appearance of symptoms is important in understanding the epidemiology of any disease and, when combined with data on densities of insect species is likely to identify potential vectors (Lindblad and Areno 2002). Many plant diseases have a clear association with an insect vector because of their presence in high numbers or a spatial and/or temporal relationship (Zhang *et al.* 2000) such as is seen in the association of alfalfa witches' broom in lucerne with high levels of the three leafhopper species *Aceratagallia* sp., *Neokolla hieroglyphica* (Say) and *Cuerna septentrionalis* (Walker) (Khadhair *et al.* 1997). Lindblad and Areno (2002) found that a high over-wintering population of *Psammotettix alienus* (Dahlbom) in non-crop vegetation was associated with subsequent high levels of wheat dwarf virus. Higher densities of *Delphacodes kuscheli* Fennah were associated with extremely high levels of maize rough dwarf virus (Grilli and Gorla 1998). Correlations between potential insect vectors and distribution of disease symptoms can,

therefore, provide significant clues to help identify principal components of the disease transmission process.

To assist studies of disease-vector relationships it is also useful to consider information such as the spatial distribution of symptomatic plants (Arnò *et al.* 1993) and combine this with the spatial distribution of potential vectors (Ioannou and Iordanou 1985; Grilli and Gorla 1998; Lindblad and Areno 2002). In many disease systems where the vector is a leafhopper, disease incidence declines with distance from the source of the vector (Purcell 1974) and proximity to host plants of the vector also increases disease incidence (McClure 1980; Grilli and Gorla 1998). Correlating temporal incidence of insect populations with disease expression is also useful in studies of disease-vector relationships and can identify potential vectors (Groves *et al.* 2001; Elder *et al.* 2002) as a higher incidence of insect numbers prior to a disease outbreak is common (Mann *et al.* 1996; Lindblad and Areno 2002). Removing insect vectors from non-crop vegetation adjacent to crops before they are able to transmit the pathogen offers a means to reduce disease incidence (Grilli and Gorla 1998) though some diseases require almost total eradication of the insect vector to successfully manage the disease (Holt *et al.* 1999). Limiting the movement of vectors into a crop may, however, lower the incidence of disease (Chancellor *et al.* 1996; Lindblad and Areno 2002) and this approach presents an opportunity to develop a management strategy for ALuY. In order to establish potential management strategies, an understanding of the biology of the insects involved is essential (Osmelak 1984).

The aims of this study were first to survey three lucerne stands over 12 months to capture, for the first time, information on the etiology of ALuY disease. Second, data on the spatial and temporal appearance of disease symptoms were correlated with equivalent data on incidence of the three most common leafhopper species to provide a preliminary indication of the vector status of each leafhopper species. The third aim of this study was to utilise pesticide treatments to crop-margin vegetation aimed to measure the extent of disease management that may be achieved by reducing vector immigration.

Materials and Methods

Symptom and leafhopper survey

Three newly sown certified lucerne (cv. Aurora) seed stands were selected in the Mid Lachlan Valley region of New South Wales, Australia. All were less than six months old at the commencement of monitoring and had a density of 20 to 40 plants per square metre. These irrigated stands were separated by a minimum of 20km. The area of each stand ranged from 12 to 15 hectares. Vegetation adjacent to each field included exotic weeds, native grasses, trees and crops.

Each stand was divided into either 99 or 104 sub-regions using a grid format with width and length divided into intervals such that, on a power transformation, the sub-regions were of equal size (Fig. 1). This power transformation was chosen so that, when back-transformed and dimensions used in each stand of lucerne, sub-regions nearer the boundaries, where it was anticipated greater precision in spatial sampling would be required, were smaller than sub-regions closer to the centre of the stand, where greater homogeneity was likely.

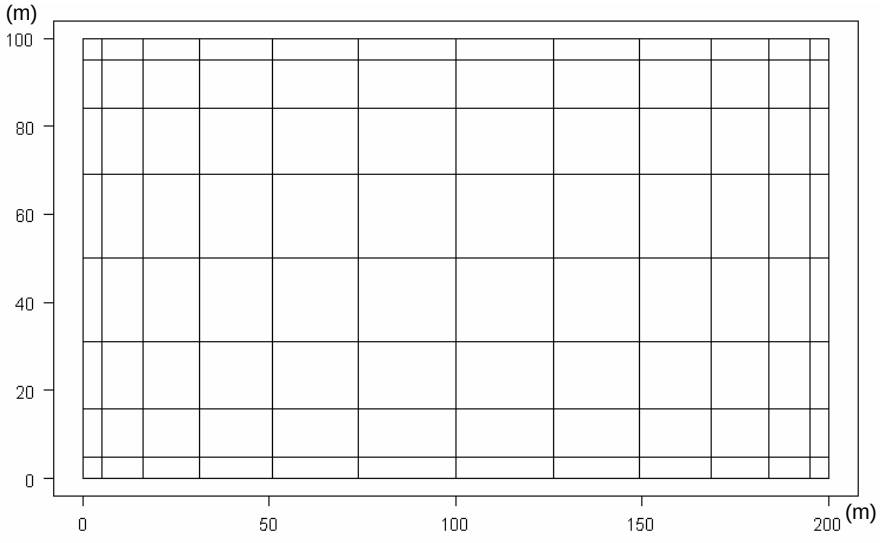

Fig. 1. Example of division of a site into sub-regions.

On each sampling date for insect distribution (details in following section) evidence of symptom expression was monitored. Plant disease surveys were initiated at each site at the first appearance of ALuY symptoms. For each of the three sites, disease data were recorded from each sub-region on the following occasions. Site one was sampled monthly on three occasions after symptoms appeared on 4 January 2001; site two was sampled monthly on four occasions after ALuY symptoms appeared on 22 January 2001 and; site three was sampled monthly on five occasions after symptoms appeared on 23 January 2001. The first sample dates for each site were within 32 weeks of the date of sowing for each stand.

On each sampling occasion, a small ball was cast into each sub-region, the nearest 100 lucerne plants identified and the area these plants occupied was measured. The number of plants showing ALuY symptoms was recorded and the severity of the symptoms for each plant rated on a scale of one to five (Table 1). Where there was more than one symptomatic plant, within the sample of 100, the distance from each symptomatic individual to its nearest symptomatic neighbour of 100 was recorded. Numbers of symptomatic plants, symptom severity scores and numbers of symptomatic plants per square metre were initially mapped to identify factors with marked spatial trends that merited further analysis. Regression analysis was subsequently made of numbers of ALuY symptomatic plants versus the two dimensions of the lucerne stand using Genstat 6th edition statistical software package (GenStat Committee).

Insect distribution survey

Leafhoppers were surveyed at all three sites fortnightly in summer and monthly in winter for 12 months commencing on the 8 November 2000, including the dates on which symptom data was recorded at each site. The sample position within each sub region was determined by casting a small ball into each sub-region and then taking a random number of steps, between ten and twenty, in a random direction so as not to cross the original path of the throw or roll of the ball. This method was adopted to ensure minimal disturbance of the insect population in the immediate sampling area. A circular area of 0.2 m² was then delineated by placing a plastic garbage bin from which the bottom had been removed. Insects were collected from this area with a motorised vacuum sampler as described by Hossain *et al*. (1999). Samples were stored in a portable 12V car refrigerator at 9°C and returned to the laboratory for identification and counting.

Table 1 ALuY symptom severity assessment scale.

Symptom level	Symptom description
1	Healthy plant
2	Slight yellowing of foliage No discolouration or drying of stems
3	Severe yellowing of foliage, some reddening Slight yellowing of some stems Drying of leaves or stems
4	Severe reddening Severe drying of foliage or stems
5	Death of plant (root symptom verification)

On each sampling date, leafhoppers were also sampled from non-crop vegetation adjacent to each lucerne stand. This was done with the vacuum sampler, but not the bin, because the vegetation included large shrubs. Plants at positions approximately 50m apart were randomly selected along each crop-margin when the plant community was a monoculture and if not a representative of each plant species was sampled along the entire border. At each point, individual plants (if large), or plant community (if consisting of smaller individuals) was sampled for 60 seconds. Plant species were identified in the field or samples collected for subsequent identification.

Regression analysis of numbers of leafhoppers caught versus row distance and column distance was performed using Genstat 6th edition statistical software package (GenStat Committee) to model the spatial distribution of each species within the stand. On two occasions (20 November 2000 and 29 December 2000), a two dimensional model (insect numbers and distance from a stand edge) was used as this maximised the variance accounted for by the regression model. On all other dates, three dimensional models (that included insect numbers and distance from stand edge in two dimensions) were used.

Border treatment experiment

Experiment 1. Two certified lucerne (cv. Aurora) seed stands, separated by two kilometres, were established in the Mid Lachlan Valley. Both of these irrigated stands had adjacent vegetation consisting of *Echium plantagineum* L. (Paterson's curse), *Trifolium repens* L. (white clover), *Cynodon dactylon* (L.) Pers. (couch grass), *Silybum marianum* (L.) Gaertn.(variegated thistle), *Onopordum acanthium* L. (Scotch thistle) and *Chenopodium album* L. (white goosefoot). At each site, a 180m-long and 10m-wide strip was marked along one boundary of the lucerne field. This strip was chosen so that each end of the strip was at least 50 metres from the ends of the selected boundary and the vegetation within it was relatively homogenous for botanical composition and vigour.

The strip on each of the two sites was divided into nine 20m by 10m plots (three blocks, each with three treatments). The allocation of treatments to plots was undertaken using Spades (Coombes and Gilmour 1999) to generate nearest neighbour designs and were randomly allocated to treatments of herbicide (1.5 L/ha 360 g/L glyphosate), insecticide (0.465 L/ha 300 g/L dimethoate) and a control, where no application was made. To ensure sufficient replication to generate neighbour balance, the experiment was designed on the assumption that the strips on each of the two sites were contiguous.

Bi-directional sticky traps were used to measure insect movement from the neighbouring vegetation into the lucerne field and vice versa. Each trap ($0.0637m^2$) was constructed from ten 90mm-diameter Petri dishes mounted on a 1800mm-tall wooden stake. The inner surface of each Petri dish base was coated with a thin layer of Tanglefoot sticky trap glue (Australian Entomological Supplies, Bangalow, Australia). Five Petri dishes (total area of $0.0318m^2$) faced the stand and five on the opposite edge of the stake faced the non-crop vegetation. The Petri dishes on each face of the stake were arranged vertically with their edges touching. The centre of the lowest dish was 300mm from the soil surface and the centre of the top dish was 690mm from the soil surface. Each of these was

nested, with its sticky surface outermost, within the lid of the Petri dish that was fixed to the stake by a drawing pin. The Petri dish lids had previously been sprayed with three coats of yellow paint (Carnival Yellow, Dulux, Clayton, Australia). The coloured lids remained attached to the stakes whilst the sticky bases were collected twice weekly between 21st September 2001 and 5th November 2001. This period was the 45 days immediately following the application of treatments to the non-crop vegetation. For each plot, a single trap was placed on the boundary of the lucerne field/non-crop vegetation, equidistant from the plot's edges. For each collection date, leafhoppers on each trap were identified and counted using a binocular microscope (10X). Catches of each leafhopper species were pooled over all dates. Analysis of variance using Genstat 6th edition statistical software package (GenStat Committee) was used to test for effects of pesticide treatment, direction of flight and trap height following a square root transformation ($\sqrt{(x+0.5)}$) on all data.

Experiment 2. Four certified lucerne (cv. Aurora) seed stands, separated by a minimum of ten kilometres, were established in the Mid Lachlan Valley. These irrigated stands had adjacent vegetation consisting of *E. plantagineum*, *S. marianum*, *O. acanthium*, *Marrubium vulgare* L. (horehound), *Cucumis myriocarpus* E. Mey. ex Naud. (paddy melon) and *Heliotropium europaeum* L. (heliotrope). At each site, a strip of at least 200m in length and 10-20m in width was marked along the entire length of opposite boundaries of the field. This strip was chosen so that the vegetation within it was relatively homogenous for botanical composition and vigour. Each boundary was divided into four plots of at least 50m in width. Each plot was assigned randomly to a different treatment: herbicide (1.5 L/ha 360 g/L glyphosate), insecticide (0.465 L/ha 300 g/L dimethoate), a combination of both insecticide and herbicide at the above rates, and a control where no applications were made. Treatments were reapplied 34 days after the initial application. A total of eight replicate blocks were used (i.e. two blocks per site, four sites).

Insect movement into and out of each lucerne stand was monitored using bi-directional yellow sticky traps (total area of 0.0254m^2) placed on the crop-margin. Traps were constructed as described in experiment 1 using two Petri dishes facing in each direction, the centre of the bottom dish being 300mm from the soil surface and the centre of the top dish being 390mm from the soil surface. Two traps were placed in the lucerne stand on the boundary with non-crop vegetation, five metres either side of the mid point of the plot. The traps were changed weekly (12 November 2002 to 23 December 2002) and, for each collection date, leafhoppers on each trap were identified and counted using a binocular microscope (10X). Catch data were pooled across all collection dates following a square root transformation ($\sqrt{(x+0.5)}$). Analysis of variance using Genstat 6th edition statistical software package (GenStat Committee) was used to test for effects of pesticide treatments, directions of flight and trap height for each leafhopper species.

Disease severity was assessed in experiment 2 by delineating an arc with a radius of 30m in the lucerne adjacent to each border treatment plot using a string attached to the midpoint of each plot's edge. This was done on 30 January 2003 when observations indicated the appearance of ALuY symptoms. Within each arc, counts were made of all symptomatic lucerne plants.

Results

Symptom and leafhopper survey

ALuY symptoms developed at site one 29 weeks after sowing and after 31 weeks at sites two and three. Maximum numbers of symptomatic plants on any given date at all sites were relatively low and dates that had a symptomatic plant count maximum of less than five were excluded from analyses. Separate three dimensional maps of symptomatic plant numbers, symptom severity and symptomatic plant density showed no significant differences within sample dates so regression analysis was performed on symptomatic plant numbers only. Regression analysis of data from two dates from site one (4 January 2001 and 8 February 2001) indicated statistically significant spatial effects (Fig. 2) with regression models counting for up to 20.6 percent of the variability in 300 symptomatic plants.

On both dates, symptom incidence was significantly higher in some parts of the crop-margin than in other parts of the margin.

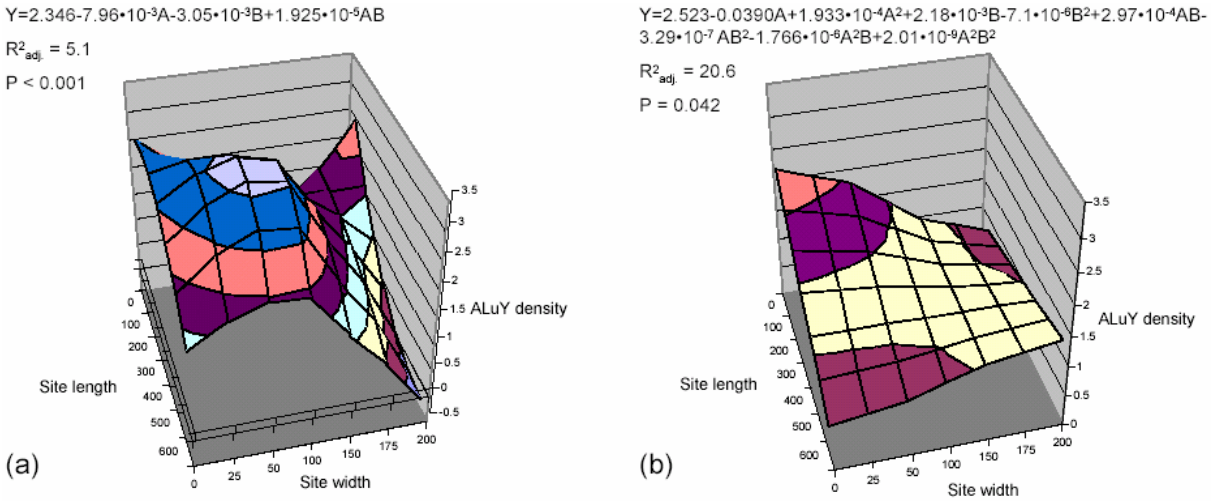

Fig. 2. Fitted models representing spatial distribution of ALuY disease severity for site 1 on (a) 4 January 2001 and (b) 8 February 2001.

Distribution of both *A. torrida* and *B. angustatus* showed a significant edge effect at sites one and three on at least one date (Fig. 3 and Fig. 4, respectively). Whenever a significant spatial effect was found, catches of leafhoppers were highest in one or more sections of the crop-margin and lower in the stand interior though catches were not consistently high in all margins. At site one, symptomatic plant numbers on 8 February 2001 were positively correlated with *O. argentatus* distribution, nine days earlier, on 31 January 2001 (r=0.195, P=0.05). Symptomatic plant numbers at site three showed a correlation on 20 February 2001 with the spatial distribution of *B. angustatus*, 54 days earlier, on 28 December 2000 (r=0.318, P<0.05). Similarly, there was a significant correlation between the symptomatic plant numbers on 4 January 2001 with the spatial distribution of *A. torrida* on 11 April 2001 at site one (r=0.300, P<0.05)

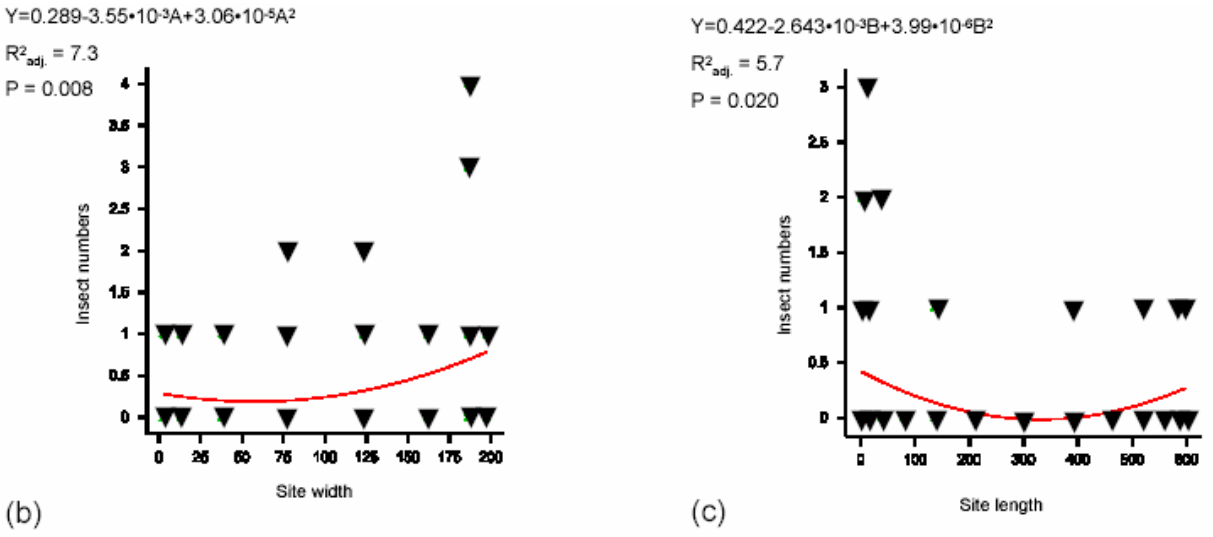

Fig. 3. Fitted models representing spatial distribution of *B. angustatus*. (a) three dimensional distribution on 28 December 2000 at site 3; (b) two dimensional distribution on 20 November 2000 at site 1; (c) two dimensional distribution on 29 December 2000 at site 1.

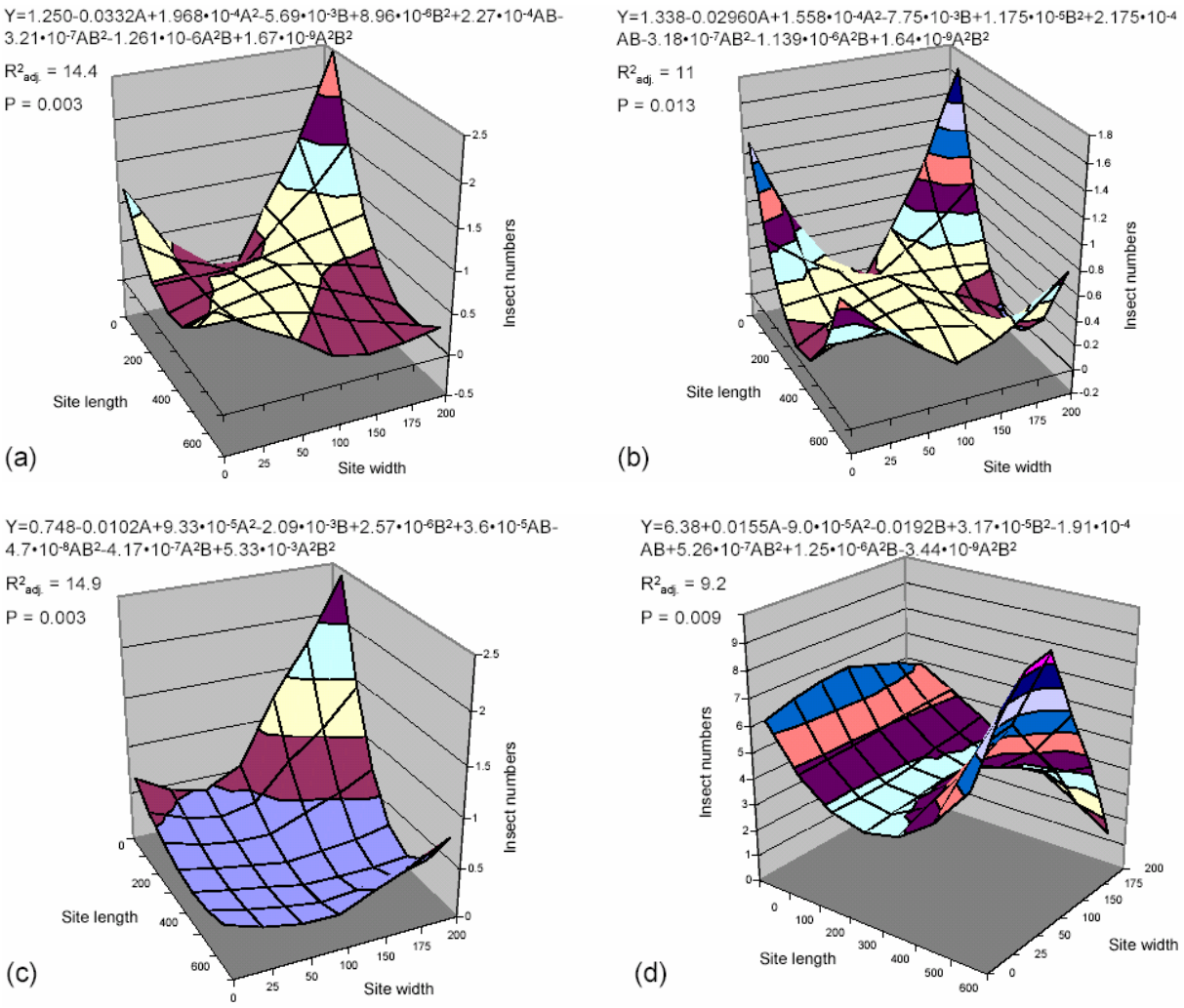

Fig. 4. Fitted models representing insect numbers at site 1. (a) *A. torrida* on 29 December 2000; (b) *A. torrida* on 19 January 2001; (c) *A. torrida* on 31 January 2001; and (d) *A. torrida* on 13 February 2001.

Leafhopper species were found on plants adjacent to the monitored lucerne stands at all three sites. *A. torrida* was common on *M. vulgare*, *Polygonum aviculare* (Hogweed), *C. album* and *H. europaeum*; *O. argentatus* was common on *H. europaeum*, *M. vulgare*, *P. aviculare* and *C. album*. *B. angustatus* was less abundant than other leafhoppers but most common on *P. aviculare*, *C. myriocarpus* and *H. europaeum*. Trends in the distribution of leafhoppers in non-crop vegetation were non clear-cut and were not correlated with symptomatic plant numbers within the stand.

Border treatment experiment

Experiment 1

Only nine *B. angustatus* were caught in all treatments over the course of the experiment so data for this species were excluded from analyses. Border treatments did not significantly affect catches of either

A. torrida and *O. argentatus* but catches were strongly affected by trap height with catches declining as trap height increased (Fig. 5a, b).

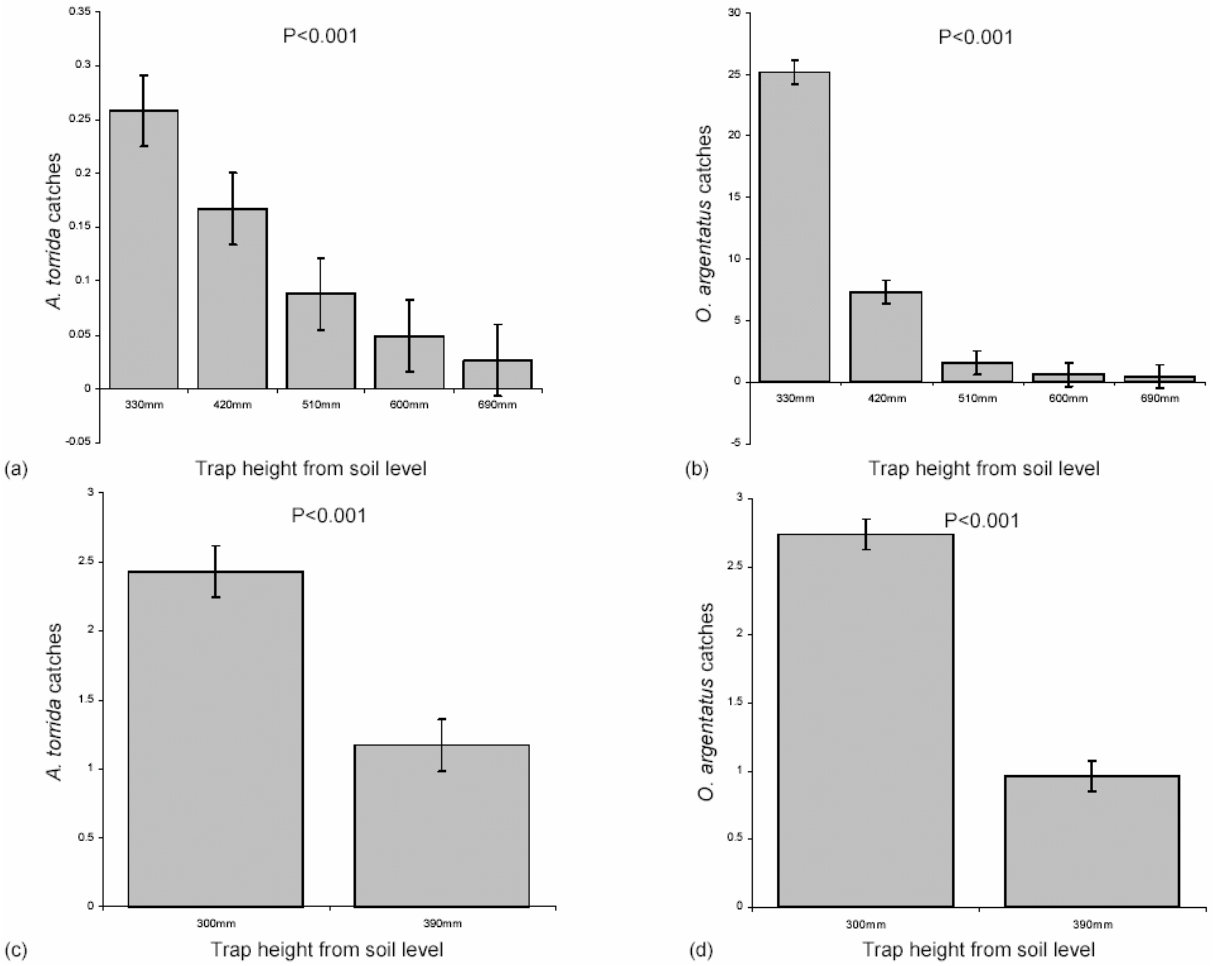

Fig. 5. Effect of trap height on catches of *A. torrida* and *O. argentatus* in experiment 1 (a and b) and; experiment 2 (c and d).

Experiment 2

No statistically significant spatial effects were found in numbers of *B. angustatus*. Irrigation at one of the sites ceased in early November 2002 due to the grower's reduced water allocations during drought conditions. This site was excluded from analyses due to desiccation of the stand and non-crop vegetation leading to low insect catches for all treatments at that site. For the remaining three sites, pooled counts of *A. torrida* and *O. argentatus* were significantly higher in the lower traps (Fig 5c, d). Herbicide treatment reduced *A. torrida* migration into the lucerne as well as overall (i.e. pooled immigration and emigration) catches to a statistically significant (P=0.02 and P=0.005, respectively) extent compared with the control treatment (Table 2). Similarly, catches of immigrating *O. argentatus* were reduced significantly by herbicide treatment (Table 2). Catches of emigrating *O. argentatus* were reduced significantly by insecticide treatment. Throughout all treatments there were higher numbers of *O. argentatus* and *B. angustatus* migrating into the stand than from the stand.

Table 2 Effect of border treatments on catches of leafhoppers on lucerne borders when compared with the control.

				Comparison with control	
Leafhopper	Treatment	Flight Direction	Mean catches	l.s.d.	P value
Austroagallia torrida					
	Herbicide and insecticide				
		Immigration	1.25	0.201	0.223
		Emigration	1.19	0.157	0.519
		Pooled	1.22	0.127	0.176
	Herbicide				
		Immigration	1.15	0.190	0.020
		Emigration	1.10	0.166	0.105
		Pooled	1.12	0.126	0.005
	Insecticide				
		Immigration	1.26	0.211	0.311
		Emigration	1.19	0.167	0.558
		Pooled	1.23	0.135	0.247
	Control				
		Immigration	1.37		
		Emigration	1.24		
		Pooled	1.30		
Orosius argentatus					
	Herbicide and insecticide				
		Immigration	1.63	0.173	0.098
		Emigration	1.33	0.135	0.378
		Pooled	1.48	0.112	0.069
	Herbicide				
		Immigration	1.33	0.158	0.061
		Emigration	1.10	0.166	0.105
		Pooled	1.26	0.103	0.029
	Insecticide				
		Immigration	1.33	0.153	0.056
		Emigration	1.13	0.119	0.024
		Pooled	1.23	0.004	0.098
	Control				
		Immigration	1.48		
		Emigration	1.26		
		Pooled	1.37		

The numbers of plants expressing ALuY symptoms was significantly reduced (P=0.007) in plots treated with insecticide (mean 4.50) when compared with the control (mean 6.33). The reduction in disease levels achieved by herbicide treatment (mean 5.00) fell just outside of 95% confidence limits of significance (P=0.052) when compared with the control.

Discussion

At all three sites, ALuY symptoms appeared within 32 weeks of the stand being sown, showing that losses may be experienced even in the first season of seed stands. The period between inoculation and expression of symptoms known for other phytoplasma diseases is approximately 40 – 60 days in the case of the eastern peach X-Mycoplasma like organism (Chiykowski and Sinha 1988) and as low as 16 – 25 days in other phytoplasma pathosystems (Chiykowski and Sinha 1990). The period between sowing and disease expression in the present study allows for the possibility of an insect vector of ALuY.

Leafhoppers are known vectors of several phytoplasma diseases (Davis and Sinclair 1998) such as sugarcane white leaf (Hanboonsong *et al.* 2002), aster yellows (Beanland *et al.* 1999) and European stone fruit yellows (Carraro *et al.* 2001) . Reservoirs of the pathogen in crop-margin vegetation are suspected in other pathosystems to constitute a source of inoculum (Wilson *et al.* 2001) and may also be hosts to vectors (Lee *et al.* 2001). If such a scenario were indicated for ALuY, management of non-crop vegetation may reduce disease severity by minimising the pathogen reservoir and/or limiting vector immigration as observed for strawberry mottle virus by Raworth and Clements (1990).

The spatial distribution of leafhoppers on some dates was significantly correlated with symptomatic plant numbers on other dates. On 28 December 2000 the distribution of *B. angustatus* at site three was significantly correlated with the distribution of symptoms 54 days later. The length of this incubation period is consistent with that known for other phytoplasma diseases (Chiykowski and Sinha 1988). Whilst the distribution of *O. argentatus* at site one was correlated with disease incidence only nine days later, the immigration of the vectors into the stand may have happened up to 12 days earlier when the preceding sample was taken, placing the disease incubation time within the range known for other phytoplasma diseases (Chiykowski and Sinha 1990). Catches of *A. torrida* prior to emergence of ALuY symptoms may have been too highly variable to allow the detection of a statistically significant relationship with symptoms though a significant relationship between symptoms and later densities of *A. torrida* was found.

Caution is required in interpreting correlations between insect catches and symptoms because of the danger of a type I statistical error resulting from the large number of combinations that were used. Three significant correlations were detected from a total of 38 insect species/symptom relationships tested. Despite this risk, and the fact that correlations do not constitute evidence for causality, the spatial results are consistent with the hypothesis that *O. argentatus*, *B. angustatus* and, to a lesser extent, *A. torrida* are vectors for ALuY. Evidence from other experiments is useful to further test this hypothesis for each species.

The significant edge effects evident in field surveys for *A. torrida* and *B. angustatus* is consistent with the finding that leafhopper catches were greater in lower traps than in identical traps placed further from the ground. This suggests that the leafhoppers do not undergo long-range dispersal to reach newly-sown lucerne stands but enter by trivial, short-range movement from adjacent vegetation. There were no significant spatial patterns detected for the leafhopper *O. argentatus*, though casual observation and marginally non-significant patterns (not presented) suggest the presence of higher numbers in the crop-margins than in the stand interior.

In the present study, the use of herbicide reduced the overall catches of *A. torrida* and *O. argentatus* and the migration of *A. torrida* into the stand, and insecticide reduced the migration from the stand of *O. argentatus*. In experiment two, reduced leafhopper movement was associated with a reduction in the disease incidence adjacent to the plots treated with insecticide. Taken with the spatial trends and correlations, this suggests that *A. torrida* and *O. argentatus* are vectors of ALuY, though transmission tests or molecular studies are required to verify this and the possibility that *B. angustatus* is a vector cannot be ruled out. The result also suggests that disease management

strategies involving the limiting of immigration of leafhopper species into the stand from non-crop vegetation may be successful on a larger scale.

The use of an ALuY management strategy based on heavy pesticide inputs to large areas of non-crop vegetation is unlikely to be acceptable to farmers, regulatory authorities or the broader community. A narrow strip of treated non-crop vegetation, however, may suffice to give useful levels of disease suppression. A normal application rate would require 4.9kg of dimethoate to treat a 35ha stand of lucerne in comparison with 660g of dimethoate to treat a 20 m wide band of the non-crop vegetation bordering the stand. It would seem that removal of host species with an effective weed control strategy might have the most potential and greatest acceptability to farmers.

Further work may show that pesticides are not required in management strategies developed for ALuY. During the study by Schaber *et al*. (1990) it was seen that physical barriers such as farm access roads or irrigation channels limited the movement of all insects with a short flight pattern similar to the leafhoppers examined in this study. Given that the results from this study suggest that the leafhoppers are moving only short distances, physical structures such as shade-mesh barriers mounted on existing fences may provide an adequate barrier against vector immigration.

Results indicate that the successful management of ALuY disease may be achieved by limiting the movement of leafhoppers into lucerne stands though further studies need to be undertaken. The reduction of disease incidence in relation to the lowered movement of *O. argentatus* and *A. torrida* into the lucerne stand is the best indication to date that one or both of these leafhopper species is a vector of ALuY.

3. The phytoplasma vector status of three leafhopper species

Introduction

Insects that have been shown to be responsible for the vectoring of phytoplasmas are leafhoppers (Membracoidea), planthoppers (Fulgoromorpha) and psyllids (Psylloidea) (Ploaie 1981, Hill & Sinclair 2000). Fletcher (1980) conducted insect trapping studies in order to identify the vectors responsible for the spread of ALuY and analysis of the occurrences of the 26 cicadellid leafhopper species caught indicated that 23 were not associated with the disease. The planthopper family Delphacidae and the cicadellid subfamily Deltocephalinae contain the most species with the documented ability of transmitting phytoplasma pathogens (Fletcher 1984). To date, however, there has been no direct experimental evidence to implicate any of the species as ALuY vectors.

The disease named tomato big bud (TBB) by Samuel *et al.* (1933) was first reported in Australia in 1902, being described on tomato plants (Cobb 1902). TBB, originally thought to be caused by a viral pathogen (Bowyer 1974), was shown to be caused by a mycoplasma-like organism (now known as a phytoplasma) by Bowyer *et al.* (1969) and further work, including the treatment of symptoms with antibiotics, was continued (Bowyer & Atherton 1972, Osmelak 1986). The leafhopper, *O. argentatus,* was shown to be a vector for the pathogen (Hill 1943) which is widespread in a range of plant species throughout Australia (Davis *et al.* 1997). *O. argentatus* is responsible for the transmission of witches' broom in lucerne and has been implicated as a vector of many other phytoplasma pathogens (Tsai 1979, Osmelak *et al.* 1989, Padovan *et al.* 1996).

A. torrida is a known vector of viral and bacterial plant diseases (Grylls 1979) but no literature could be found indicating vector status for TBB or any other phytoplasma. *A. torrida* as well as *B. angustatus* have, however, both been suggested as possible vectors of Australian grapevine yellows (Osmelak *et al.* 1989).

Transmission testing by relocating a candidate vector from symptomatic plants to a known 'clean' plant and subsequently examining the plant for symptom expression has been used extensively to test insects for phytoplasma vector status (Blanche *et al.* 1999, Gatineau *et al.* 2001, Jarausch *et al.* 2001). Insects are often allowed to cycle through several generations on the symptomatic plant to maximise the chances of the insect acquiring the pathogen (Carraro *et al.* 2001a). Field-collected insects have also been used based on the assumption that some will have been feeding on symptomatic plants, may have acquired the pathogen and will be capable of transmitting the pathogen (Maeso Tozzi *et al.* 1993, Carraro *et al.* 2001a, Jarausch *et al.* 2001).

Leafhoppers must be given enough time on symptomatic plants to acquire the phytoplasma and also be given time to let the pathogen replicate within the insect before transmission is possible (Bowyer 1974). The acquisition time is generally 1-2 days (Carraro *et al.* 2001b), whilst the latent period of phytoplasma diseases within insect hosts can be as long as 40-60 days (Chiykowski & Sinha 1988).

In the past, insect DNA extraction techniques involved grinding the whole leafhopper, including the gut contents (Maixner *et al.* 1995, Charles *et al.* 2002) and subjecting this DNA to PCR analysis to test for the presence of a phytoplasma. This leads to the possibility that the insect has simply fed on an infected plant, contains phytoplasmas in its gut, and does not establish that the insect is capable of vectoring the phytoplasma. Despite this, no reports could be found of removing the abdomen, leaving only the head containing the salivary glands of the insect, therefore implicating the insect more reliably as the possible vector. The only evidence of dissecting the leafhopper prior to DNA extraction was by Weber and Maixner (1998) who cut the leafhopper longitudinally to facilitate PCR assay on one half and ELISA assays for the other half.

The aims of this study were to identify vectors for the disease ALuY using field-collected and laboratory-reared leafhoppers in caged transmission tests. Plants were to be assayed using PCR techniques and monitored for symptom expression. A supplementary aim was to identify other possible phytoplasma pathogens that may be transmitted by field-collected insects.

Materials and methods

Source of ALuY symptomatic lucerne

ALuY symptomatic plants were collected from certified lucerne seed crops, cv. Aurora, growing in the Lachlan Valley region of central New South Wales, Australia in September 2002. Symptomatic plants were provisionally identified by foliar symptoms and each plant was carefully removed from the ground, removing the soil surrounding the bulk of each plant's root system. A lateral root was exposed, and a check for root symptoms (Pilkington *et al.* 2002) was performed to confirm the plant's disease status. Each plant was transferred to a 16.5L pot that was back filled with a simple potting mix (Pot'n'peat, Envirogreen, Castlereagh, Australia) and, thereafter, watered regularly. The foliage was sprayed with insecticide (active constituents 0.4g/L pyrethrins, 1.6g/L piperonyl butoxide applied as a fine mist over the entire plant) and rinsed thoroughly with water 24 hours later prior to introduction of leafhopper colonies to ensure that all other insect species were removed.

Transmission tests

A. torrida and *B. angustatus adults and nymphs* were obtained by sweep netting certified lucerne seed crops, cv. Aurora, growing in the Lachlan Valley region of central New South Wales, Australia in September 2002. Leafhoppers were removed from the sweep net and placed, with some green lucerne foliage, into a container stored in a 12V car refrigerator set at approximately 9°C for transport back to the laboratory.

In the laboratory, leafhoppers were anaesthetised with carbon dioxide and sorted into species. A total of 40 – 50 individuals of *A. torrida* were placed onto two individually caged ALuY symptomatic plants. A total of 30 – 40 individuals of *B. angustatus* were placed onto two individually caged ALuY symptomatic plants. After seven days, the groups from each individually cages plant were moved to caged individual seed-grown lucerne plants. *B. angustatus* were placed on eight caged clean lucerne plants, and *A. torrida* were placed on nine caged clean lucerne plants. The insects were allowed to feed on these plants for 50 days.

Specimens of *O. argentatus* were sourced from a laboratory culture maintained at Northern Territory University as only very low numbers were present in the field at the time other species were captured. *O. argentatus* suffered high mortality on lucerne plants in previous culturing attempts so were placed on two seed-grown, caged, faba bean (*Vicia faba* L.) plants at 28°C on which they bred readily. Approximately 50 individuals were removed from the beans and transferred to individual leaflets on two ALuY symptomatic plants in batches of two or three insects per clip cage. They were allowed to feed on the lucerne for seven days before being transferred back to faba bean for 30 days to allow any pathogen to replicate and the leafhoppers time to become infective. The insects were then transferred to eight "clean" seed-grown lucerne plants and placed on individual leaflets, each plant having three clip cages with batches of two to three leafhoppers which were allowed to feed for seven days.

After exposure of "clean" plants to leafhoppers as described above, insects were removed, placed in a -20°C freezer for later PCR analysis and the plants were transferred from the pots and planted in the field on The University of Sydney, Orange campus farm in November 2002.

Symptom expression

Lucerne plants were monitored weekly for expression of foliar symptoms consistent with phytoplasma infection. After seven months, the plants were removed from the ground, their roots

washed thoroughly with water and the periderm carefully exposed to check for expression of ALuY root symptoms on each plant.

PCR assays of transmission plants

DNA was extracted from two batches of three leaf midribs collected from two locations from each transmission plant using standard DNA extraction techniques (Dellaporta *et al.* 1983). Each 50 µl PCR reaction mixture contained 1 µl of DNA, 1.25 units of Taq polymerase, Taq buffer consisting of 1.4 mM $MgCl_2$, 0.4 µM of each primer and 0.1 mM of each dNTP (all components listed supplied by GeneWorks, Adelaide, Australia). Universal phytoplasma primers P1 (Deng & Hiruki 1991) and P7 (Kirkpatrick *et al.* 1994) were used in first round PCR and primers fU5 (Lorenz *et al.* 1995) and m23sr (Padovan *et al.* 1995) were used in nested PCR assays. PCR cycling conditions were as follows: denaturation for one minute (two minutes for first cycle) at 95°C, annealing temperature of 55°C for one minute and an extension time of 1.5 minutes at 72°C for 35 cycles (9.5 minutes on final cycle). TBB phytoplasma DNA and sterile distilled water (SDW) were used for positive and negative controls. After each nested PCR assay, 2 µl of PCR product were analysed by electrophoresis on a 1.0% agarose gel and stained with ethidium bromide prior to being visualised with a UV transilluminator.

PCR assays of leafhoppers

The heads of all three leafhopper species were removed and DNA was extracted, in batches of five to ten individuals, using the techniques outlined in the previous section. Individuals of laboratory reared *O. argentatus* were fed on disease free faba bean to remove pathogens from the insect's gut. For the purposes of consistency, they were processed in the same manner as the other insect species. Insects originated from transmission tests and also field-collected insects. Insects were collected from three newly sown certified lucerne (cv. Aurora) seed stands in the Mid Lachlan Valley region of New South Wales, Australia between December 2000 and February 2001. Insects were collected with a motorised vacuum sampler as described by Hossain *et al*. (1999) and were transported to the laboratory in a portable 12V car refrigerator at 9°C before being placed in a freezer at a constant -20°C. DNA was extracted from 50 leafhoppers of each species from transmission tests. Field-collected insects included 115 *B. angustatus,* 145 *A. torrida* and 155 *O. argentatus*. PCR protocols were identical to those used for plant DNA.

RFLP of PCR product

Amplified products from PCR assays, including the TBB control, were subjected to restriction digestions. Five µl of each positive PCR product were digested separately, following the manufacturer's instructions (New England Biolabs, Inc., MA, USA), with enzymes *Alu*I, *Hpa*II and *Rsa*I. The RFLP products were then subjected to electrophoresis through a 5% polyacrylamide gel, the gel stained with ethidium bromide and then visualised by UV transillumination.

Electron microscopy

Leaf midribs from all transmission plants were dissected into approximately 1 mm^3 pieces containing phloem tissue and were fixed with standard methods (Bozzola & Russell 1992). Specimens were infiltrated with 100% acetone/Spurrs resin (1:1) overnight at room temperature on rotators, transferred to 100% Spurrs resin overnight on rotators and embedded in fresh Spurrs resin and polymerised at 60°C overnight. Specimens were then cut into ultra-thin (80 nm) sections and viewed in a Philips Biofilter CM120 (120 kV) electron microscope.

Results

Symptom expression

Six out of the eight plants fed on by field collected *B. angustatus* showed no symptoms of phytoplasma infection. The remaining two plants had mild stunting of leaves on some shoots near the crown of the plant. None of the plants fed on by *B. angustatus* exhibited root symptoms of any kind.

Three of the nine lucerne plants fed on by field collected *A. torrida* showed no symptoms. One showed leaf chlorotic vein banding and leaf reddening, symptoms that are consistent with alfalfa mosaic virus. Two plants showed leaf stunting and some yellowing of leaves. The remaining three plants fed on by *A. torrida* showed severe stunting of leaves and stems and in one case die back of stems. None of the plants fed on by *A. torrida* exhibited root symptoms of any kind.

One of the eight plants fed on by *O. argentatus* showed no symptoms. Two plants showed some stunting of leaves limited to a few shoots. Three plants showed more extensive stunting of leaves and shoots with a further two plants showing signs of yellowing. One plant, showing severe stunting and yellowing of its foliage, had a dark discolouration immediately under the periderm of its taproot (Fig. 1). This discolouration did not extend into the stele of the taproot typical of bacterial wilt, caused by *Clavibacter michiganensis* subsp. *insidiosus* (McCulloch), and was consistent with root symptoms expressed by lucerne plants with ALuY disease (Pilkington *et al.* 2002). Another plant, with some minor stunting of leaves, had a light discoloration under the periderm of its taproot. This discolouration extended slightly into the stele of the taproot.

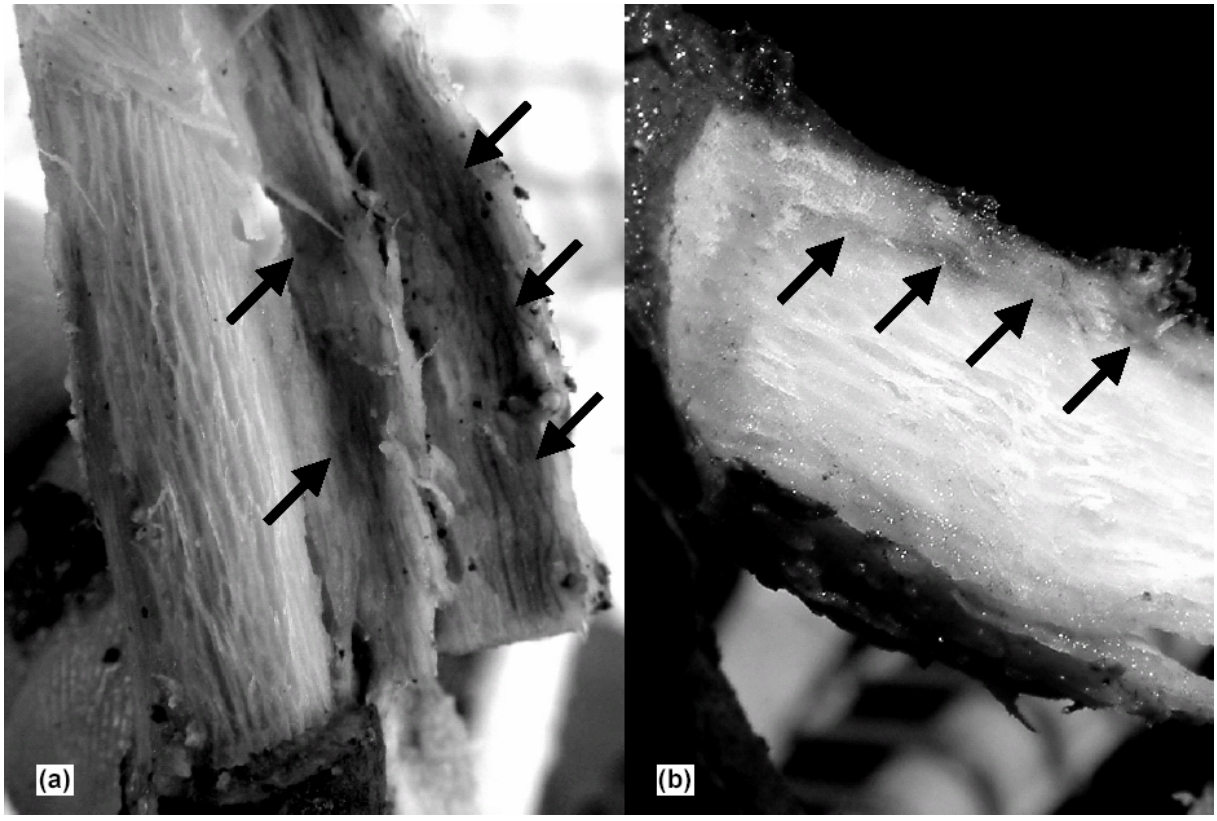

Fig. 1. Dark discolouration of the root periderm of a lucerne plant exposed to *O. argentatus*.

PCR assays of transmission plants

Two faint positive signals (Fig. 2, a and b) were amplified from DNA extracted from a plant fed on by *O. argentatus* that was expressing stunting of leaves and a plant fed on by *B. angustatus* that showed no symptoms. A third, very strong, PCR signal was amplified from a plant that had been fed on by *A. torrida* (Fig. 2, c).

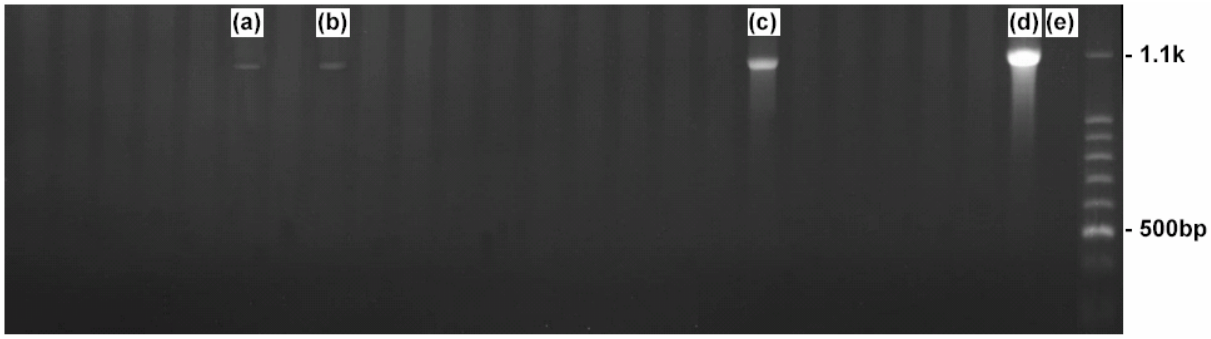

Fig. 2. Agarose gel of lucerne transmission plants in nested PCR with primers P1/P7 followed by Fu5/M23sr. Lanes (a) and (b) amplified from lucerne plants fed on by *Batracomorphus augustatus* and *Orosius argentatus* respectively; lane (c) amplified from lucerne fed on by *Austroagallia torrida*; lane (d) tomato big bud positive control; and lane (e) sterile distilled water negative control.

PCR assays of leafhoppers

No PCR products were detected in DNA extracted from leafhoppers used in transmission tests or detected in DNA extracted from leafhoppers collected from the field.

RFLP of molecular positives

When the strong PCR product amplified from the plant fed on by *A. torrida* was digested with the restriction enzymes *Alu*I, *Hpa*II and *Rsa*I, all resulting RFLP profiles for each enzyme were identical to the patterns from the TBB digests (Fig. 3). The PCR products from the two faint positives were subjected to RFLP assays but no digestion products were evident in the subsequent gels.

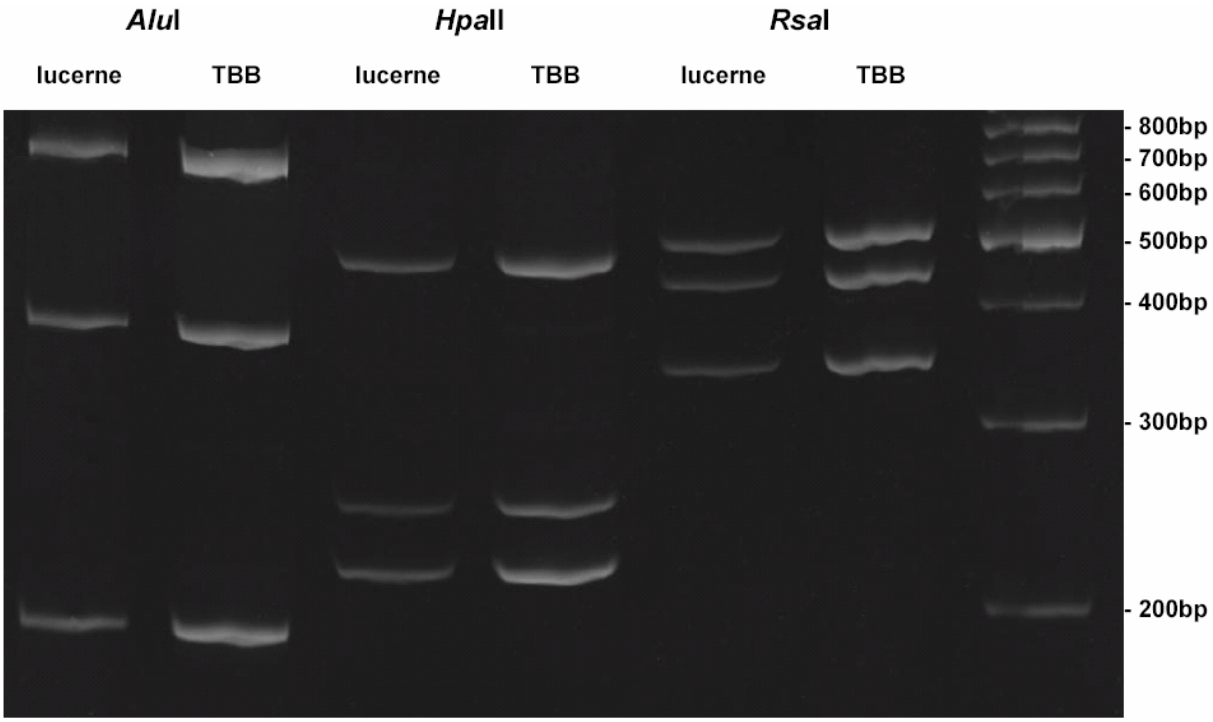

Fig. 3. Polyacrylamide gel of RFLP digestions of amplified product from lucerne plant fed on by *Austroagallia torrida* ("lucerne") and tomato big bud positive control ("TBB") using digestions enzymes *Alu*I, *Hpa*II and *Rsa*I.

Electron microscopy

Examination of ultra-thin cross-sections of leaf midrib from transmission test plants showed numerous phytoplasmas (200-400nm diameter) in the phloem of the plants (Fig. 4). Structures were found in one of the two plants that showed a faint phytoplasma positive in PCR assays that had been fed on by *O. argentatus*, the lucerne plant, fed on by *A. torrida*, that amplified a strong TBB product and in the plant displaying distinct ALuY root symptoms that had been fed on by *O. argentatus*. No phytoplasma bodies were visualised in other plants The structures were consistent with those found in lucerne plants affected with ALuY disease (Pilkington *et al.* In Press).

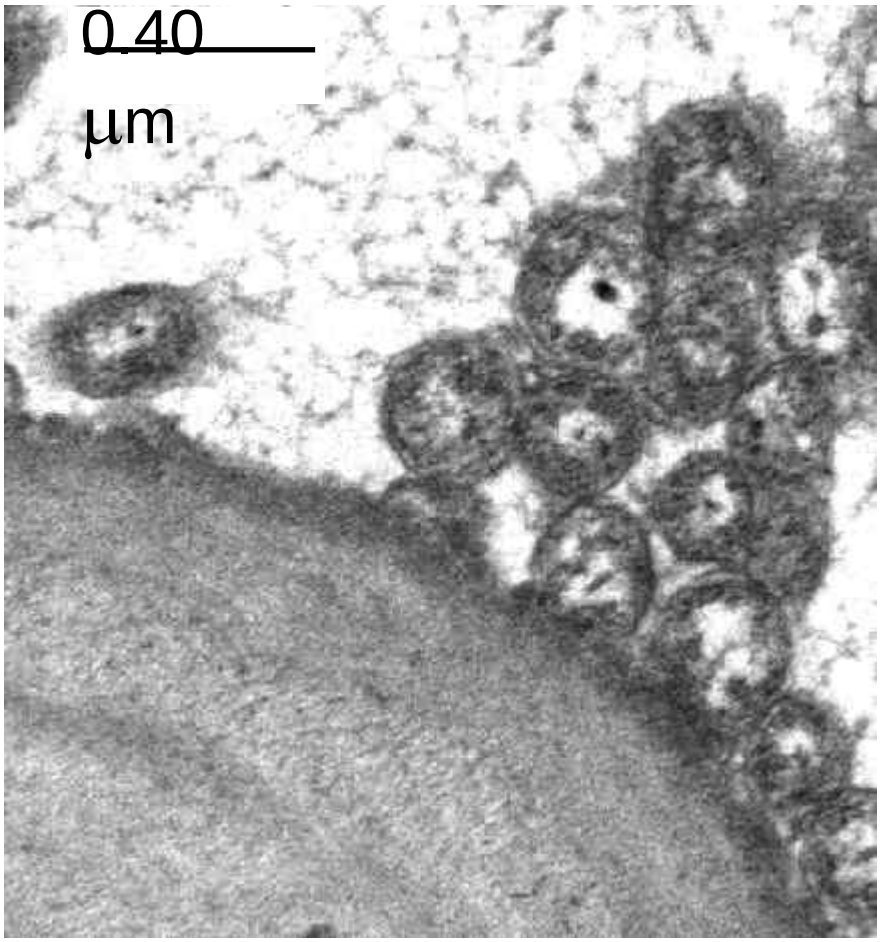

Fig. 4. A phloem cell from a lucerne plant showing phytoplasma symptoms fed upon by *O. argentatus* in transmission tests.

Discussion

The lucerne plants that were fed on by *B. angustatus* showed little or no symptoms, six plants being symptomless and the remaining two plants showing very slight stunting on some shoots and leaflets. There were no root symptoms to show incidence of ALuY infection. A PCR band of a size that was consistent with phytoplasma DNA was amplified from one of these symptomless plants. Attempts to conduct RFLP assays on this PCR product failed, probably due to the small amount of amplified DNA present. *B. angustatus* insects were collected from the field to be used in transmission tests and therefore it is possible they were carrying a range of phytoplasma pathogens. Without confirming the identity of the phytoplasma amplified in PCR assays using RFLP analysis, and no supporting implication of *B. angustatus* as a vector of any phytoplasma, little information may be inferred with the positive PCR result.

The plant fed on by *O. argentatus* that showed a positive PCR results and was not confirmed by RFLP analysis carries a far stronger implication of the ALuY vector status of *O. argentatus*. Very few vectors of phytoplasmas have been shown to be capable of transovarial transmission (Chiykowski 1981) and not capable of transmitting to the next generation of the insect vector (Kawakita *et al.* 2000). This will allow a colony to persist on known healthy plants thus ensuring

that phytoplasma pathogens are not carried within that leafhopper colony. *O. argentatus* was laboratory reared for more than 20 generations on plants known not to contain phytoplasmas and therefore it is unlikely that they carried pathogens other than those acquired from ALuY symptomatic plants.

Of the eight plants fed on by *O. argentatus,* one exhibited distinct, dark discolouration under the periderm of the taproot. The foliar symptoms expressed by all but one of the plants fed on by *O. argentatus* were broadly consistent with a phytoplasma infection and two of the plants expressed symptoms that were consistent, in particular, with ALuY disease (Pilkington *et al.* 1999, Pilkington *et al.* 2002) including distinct root symptoms in one plant. This result suggests that *O. argentatus* is a vector for the ALuY phytoplasma. The husbandry of plants used in transmission tests (for example, pot grown and then transferred to the field) may have affected the normal symptomology of the disease, preventing the development of ALuY-characteristic root symptoms in some plants.

Five lucerne plants fed on by *A. torrida* showed significant stunting of leaves and some stems and three lucerne plants fed on by *A. torrida* showed no symptoms. One plant showed symptoms consistent with alfalfa mosaic virus, a seed borne disease common in Australian lucerne crops (Hajimorad & Francki 1988). The TBB phytoplasma amplified from one plant, identified with RFLP, demonstrates for the first time, that *A. torrida* is capable of vectoring this phytoplasma, an important pathogen in Australian lucerne seed crops (McDonald *et al.* 2003).

The most conclusive results are the distinctive ALuY root symptoms displayed by a plant fed on by *O. argentatus* and the TBB positive from *A. torrida* fed lucerne plant. The 13% and 11% rates of infection are broadly consistent with equivalent rates in other studies of leafhopper vector-phytoplasma systems. Transmission rates as high as 50-100% have been reported in transmission tests with chrysanthemum yellows phytoplasma (Palermo *et al.* 2001, Tanne *et al.* 2001), though more commonly, transmission is less frequent. Jarausch et al. (2003) reported 10% (one of ten test plants) and 18% (seven of 40 test plants) transmission rates in PCR assays conducted with apple proliferation phytoplasma and its suspected vector *Cacopsylla picta* (Foerster). These results are confirmed by electron microscopy results in which phytoplasma bodies were seen in plants displaying symptoms and amplifying phytoplasma DNA but not in asymptomatic plants.

Rates of symptom expression in test plants are similarly often reported to be low. The vector of pear decline phytoplasma caused symptoms in 17 out of 56 test plants (30%) and was considered a 'highly active vector' (Carraro *et al.* 2001a), and Jarausch *et al*. (Jarausch *et al.* 2001) demonstrated a less than 1% (one of 50 test plants) transmission success in trials between *C. pruni* (Scopoli) and apricot seedlings infected with European stone fruit yellows phytoplasma. A 14-18% (four to five of 25 test plants) rate of successful transmission of symptoms was demonstrated in trials involving *Nephotettix virescens* (Distant) and rice yellow dwarf transmission (Rajappan *et al.* 1999).

Alma *et al*. (2000) concluded from modest symptom expression rates in transmission tests (32 of 125 test plants or 26% symptom expression), that *Euscelis incisus* (Kirschbaum) was a vector. The transmission rate in that study reflected cyclamen (*Cyclamen persicum* L.) being a dead-end host (Alma *et al.* 2000). Potatoes and peaches, for example, are both affected by phytoplasma diseases yet are highly inefficient sources of inoculum for the pathogens causing these diseases (Purcell 1982). The vector does not need to breed on the diseased plant, or prefer to feed on it, to be capable being a vector (Garat *et al.* 1999). Many vectors of virus pathogens make little use of the crop, neither feeding on the plants or using them for oviposition (Holt *et al.* 1999). Lucerne may itself be a dead end host for ALuY, with the inoculum either hard to acquire or the plant not being the preferred host for the insect.

The RFLP assay conducted on the PCR product obtained from an *A. torrida* fed plant was identical to that obtained for the TBB positive controls used in the assay. That plant exhibited severe stunting and dieback, though these symptoms are not typical of TBB in lucerne. Symptoms for a given phytoplasma can vary widely within, as well as between, host plant species (Wilson *et al.* 2001).

The TBB phytoplasma band amplified from plants fed on by *A. torrida* was strong and clear when compared with very faint positives that were amplified from lucerne plants fed on by *O. argentatus* and *B. angustatus*. Positive bands obtained from PCR assays often differ in intensity depending on the time of sampling and titer of the pathogen in the plant (Bertaccini *et al.* 1996). Often TBB DNA will produce bands of a considerably greater intensity during gel electrophoresis than ALuY DNA when using the same primers and protocols (Pilkington, unpublished data). It is possible that the two positive bands, seen in these results that are of less intensity (Fig. 2, a and b), are ALuY phytoplasma DNA. The presence of the expected sized band in electrophoresis, estimated by the fragment of DNA that is isolated in the primer design process, is enough for identification of a phytoplasma infection (Kaminska *et al.* 1999), however, the lack of RFLP characterisation makes it impossible to confirm its identity.

The lack of phytoplasma products in PCR assays cannot consistently rule out phytoplasma infection as titers may be low or the distribution uneven (Bertaccini *et al.* 1997). The inherently uneven distribution of phytoplasmas in plant tissue often makes detection unreliable (Gundersen & Lee 1996) and many other factors can adversely effect the efficiency of PCR (Davis *et al.* 1997, Andersen *et al.* 1998). This unreliability, as in this study, sometimes leads to clearly symptomatic plants not yielding positive results in PCR tests (Bertaccini *et al.* 1997).

PCR products were not amplified from DNA extracted from the heads of leafhoppers used in transmission tests and leafhoppers collected from the field. This is the first time that an attempt to amplify phytoplasma DNA from only the heads of leafhoppers has been made, effectively isolating the area of the leafhopper that contains the salivary glands and excluding the gut. Sampling a small section of the individual insect, however, compounds the inherent problem of low concentration of phytoplasma DNA within leafhoppers. By using up to 10 leafhopper heads in each DNA extraction, it was hoped that this would overcome this difficulty.

The field-collected insects had been stored for over 12 months prior to analysis. Storage was at -20°C and the DNA, when ran through agarose gel at 105V and examined for clear peaks and banding, was of high quality when tested prior to PCR assays. It is unlikely that storage was the cause of the negative PCR results.

The results presented in this study are also supported by spatial and temporal survey data of the three leafhopper species examined in the current study that were found to be more prevalent in lucerne stand margins (Pilkington *et al.* 2003b). Further, herbicide and insecticide treatments of non-crop vegetation adjacent to lucerne stands resulted in statistically significant reductions in overall movement of *O. argentatus* and herbicide treatments resulted in statistically significant reductions in overall movement of *A. torrida* (Pilkington *et al.* 2003a). Insecticide treatment of the non-crop vegetation successfully lowered ALuY disease incidence adjacent to the treatment (Pilkington unpublished data). These findings strongly support the results presented in this study and offer scope for developing disease management strategies in future studies.

This study provides evidence that *O. argentatus* is a vector for the ALuY pathogen and *A. torrida* has the ability to transmit the TBB phytoplasma. This is the first report of the vector status of these two leafhoppers with two important Australian phytoplasma pathogens.

4. Management of Australian lucerne yellows disease by water, nutrient and antibiotic treatments

Introduction

Management strategies for plant diseases range from preventative measures prior to sowing to the drastic destruction of an entire crop to prevent further spread into other paddocks (Chand *et al.* 1987). The management options for lucerne diseases vary widely. These commonly include practices such as the selection of free-draining sites and efficient irrigation throughout the growing season, as in the case of the alleviation of Phytophthora root rot symptoms, using resistant varieties of lucerne for Anthracnose crown rot and stem nematode and cutting or grazing to reduce infected material in disease systems such as common leaf spot, rust and pepper spot fungus (McDonald *et al.* 2003). The application of supplementary water, for example in the disease management of Leucostoma canker of stone fruit trees caused by either of the two fungal pathogens *Leucostoma persoonii* Hohn or *Leucostoma cincta* (Fr. ex Fr.) Hohn, increases the succulence of the host plant, often decreasing symptom severity (Agrios 1997). The alleviation of disease symptoms culminating in the avoidance of yield losses, given that in many plant disease systems the disease may not lead to the death of the plant but to an overall qualitative or quantitative reduction in yield (Jones 1987), would be a highly desirable outcome in terms of disease management.

The nutrition of plants is recognised as a method of managing plant diseases by improving the general health of the plant and lowering the effect of disease symptoms (Prasad 1979) thereby increasing yields. Potash has been used by Australian lucerne growers for the treatment of ALuY, anecdotally reducing foliar symptoms and increasing the overall vigour of the stand (Gilkin, P. 2000, pers. comm., 1 Dec.). This observation by an Australian lucerne grower suggests that application of potash may have the desired effect of lowering symptom severity in ALuY affected lucerne but has not previously been tested experimentally.

Tetracycline compounds inhibit the activity of phytoplasmas (Bowyer and Atherton 1972) and was first demonstrated with the suppression of phytoplasma symptoms of mulberry dwarf disease in mulberry (*Mulberry* spp.) plants (Ishiie *et al.* 1967). The use of such compounds inhibits the pathogen and also often results in a marked increase of vigour and health in the host plant (McCoy 1982). Application of tetracycline compounds several times a week may be unaffordable in lucerne production but a less frequent application regime may give economic control.

No current advice for the management ALuY is available to growers and this has resulted in destructive practices such as ploughing the infected crop in and using a crop rotation or use of insecticides on the (untested) assumption that insects vector the pathogen (Pilkington *et al.* 1999; McDonald *et al.* 2003). This situation requires studies of the scope for symptom alleviation or suppression of the pathogen to improve plant health and increase seed yield for producers.

The aim of this study was to assess in the field the effects of possible disease management treatments on symptomatic plants. Water, a blend of nutrients, sulphur of potash alone, and tetracycline hydrochloride were compared with a nil control treatment.

Materials and methods

Study Site

The study site was in mid Lachlan Valley, New South Wales. The study took place on an established, irrigated certified cv. Aurora lucerne seed stand located 50 km west of Forbes.

Experimental design

One hundred ALuY-symptomatic plants were selected based on foliar symptoms rather than destructive root inspections in December 2001. Each plant was marked with a 2m tall, 5mm diameter fibreglass poles driven into the ground within five centimetres immediately to the north of the plant crown. Individual plants were at least 20 m apart and were selected for uniformity of symptom expression. Soil type, drainage and irrigation patterns were consistent across the study area. The experimental design was a randomised complete block in which five treatments were replicated 20 times.

Treatments

Treatments were (i) nil control, (ii) 3L water applied once a fortnight and a woven synthetic fabric mulch placed around the base of the lucerne plant to reduce evaporation, (iii) 20 g of prilled sulphate of potash (Incitec Pivot Limited, Melbourne, Australia) applied in pellet form to $1m^2$ around the plant and watered into the soil with three litres of water on one occasion at the outset of the experiment, (iv) 30 g of prilled MultiGro (Incitec Pivot Limited, Melbourne, Australia), N:P:K 10:3:6, applied $1m^2$ around the plant and watered into the soil with three litres of water on one occasion at the outset of the experiment, and (v) 0.1L of 0.1g/L tetracycline hydrochloride (Sigma Aldrich, Castle Hill, Australia) antibiotic applied as a fine mist to all foliar parts of the plant once a fortnight.

Measurements

The plants in five blocks were destructively sampled on 10 February 2002, 12 weeks after initial application of treatments. Remaining plants were sampled on 25 February 2002, two days before host farmer harvested seed from the entire stand in which the experiment was located. Immediately prior to each destructive sampling, symptom severity of individual plants was measured on a scale of one to five (Table 1). Individual plants were then cut just above the crown of the plant, labelled and placed in a brown paper bag for transport to the laboratory. Fresh weight was recorded immediately then plants were dried to constant weight in a dehydrator at 30°C. Seeds from the second batch of plants were extracted using a small threshing machine, plant debris was removed and the seed weight recorded.

Table 1. ALuY symptom severity assessment scale.

Symptom level	Symptom description
1	Healthy plant
2	Slight yellowing of foliage No discolouration or drying of stems
3	Severe yellowing of foliage, some reddening Slight yellowing of some stems Drying of leaves or stems
4	Severe reddening Severe drying of foliage or stems
5	Death of plant (root symptom verification)

The germination rate of the collected seed was measured following protocols developed by the International Seed Testing Association (Anon. 1993). Thick paper towel was placed on the bases of shallow stainless steel trays. 100 seeds from each plant were placed on the paper towel and covered with another layer of paper towel. 250ml of distilled water was slowly added to each tray, which was

then covered with a glass plate and held at 5° for 7 days. The trays were then placed in an incubator at 20°C. Germination was assessed after 10 days.

Analysis of variance using Genstat 6th edition statistical software package (GenStat Committee) was used to test for effects of treatments following a logarithmic transformation ($\log_{10}(x+0.5)$) on fresh weight, dry weight and seed weight. Seed germination rate and symptom expression was not transformed prior to analysis of variance.

Results

There was no significant treatment effect on fresh weights or dry weights though the effect of potash treatment on the latter fell just outside the threshold for statistical significance (P = 0.059) (Fig. 1).

Water treatment significantly (P = 0.040) increased seed yield compared with the control. No statistically significant differences were found for the remaining treatments though potash and nutrient blend treatments numerically increased seed weight (Fig. 2).

There were no significant treatment effects on seed germination rate (Fig. 3) or on symptom severity though the potash treatment had numerically lower disease severity score (Fig. 4).

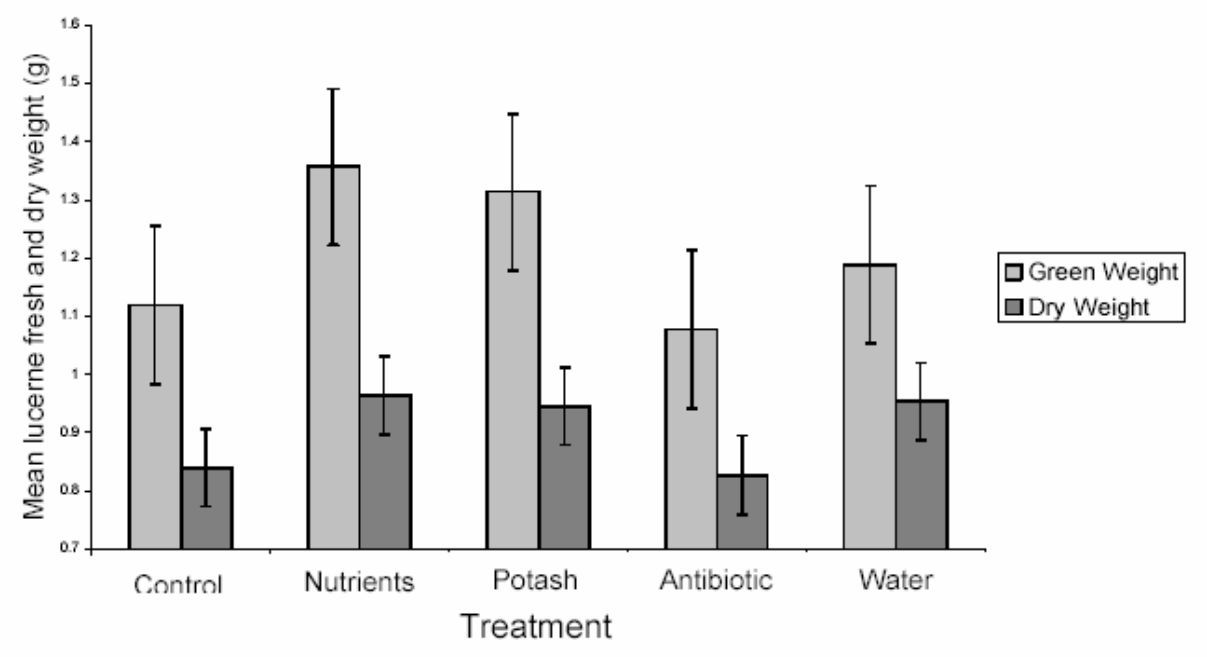

Fig. 1. Effect of treatments on fresh and dry weight of ALuY infected lucerne. Error bars indicate standard error of means.

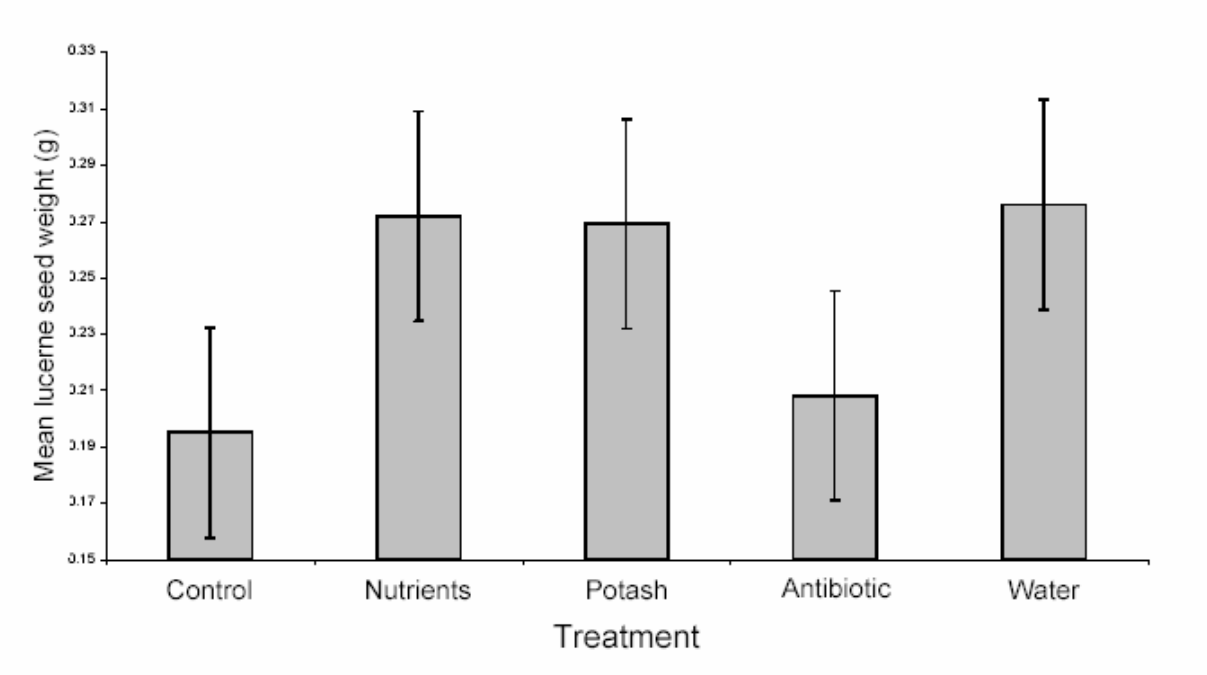

Fig. 2. Effect of treatments on seed yield of ALuY infected lucerne. Error bars indicate standard error of means.

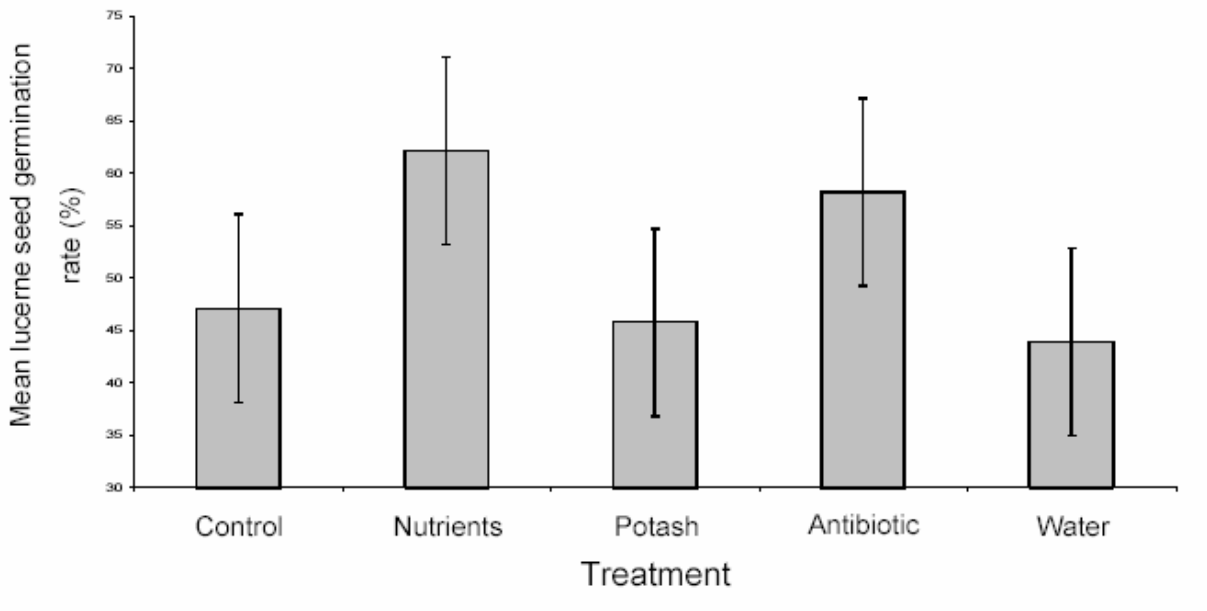

Fig. 3. Effect of treatments on seed germination rate of ALuY infected lucerne. Error bars indicate standard error of means.

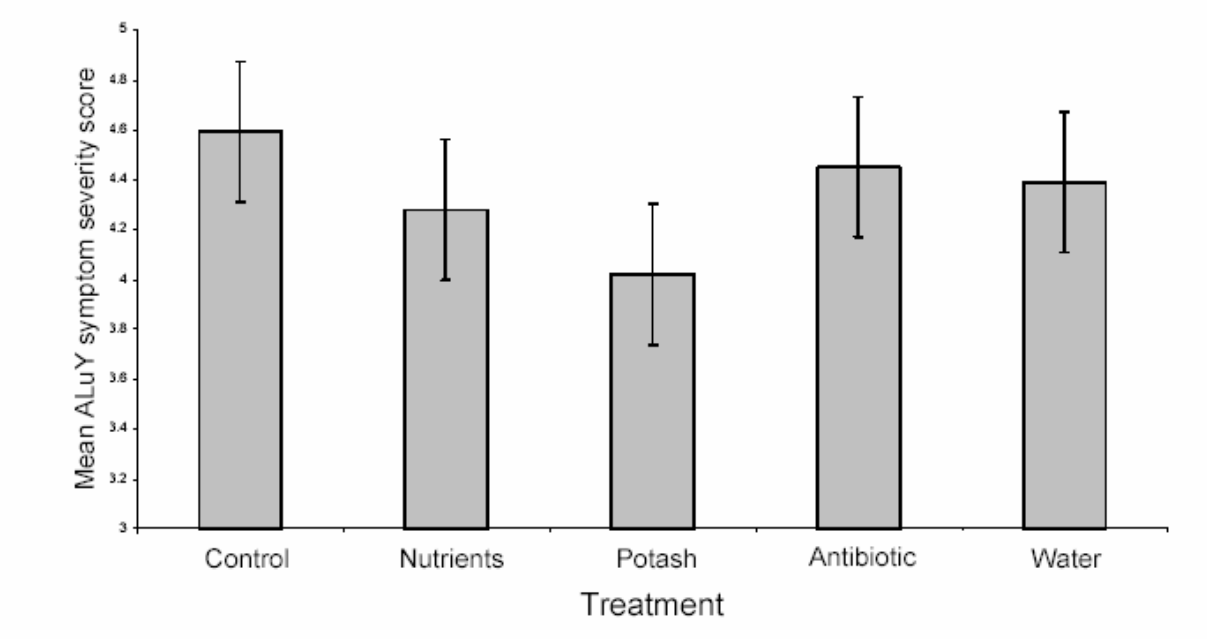

Fig. 4. Effect of treatments on symptom severity of ALuY infected lucerne. Error bars indicate standard error of means.

Discussion

The results obtained in this study suggest sufficient scope to manage ALuY disease to merit future studies. These will need to test treatments on a range of sites and over several years. Dose-response studies also are required before recommendations can be made to lucerne growers. The effect of water on seed yield was the only statistically significant response though potash had a marked effect on several measured of plant health.

Tetracycline treatment led to no effect though it is known that plant response to tetracycline compounds varies according to plant age, symptom severity, method of treatment and the type of tetracycline used (McCoy 1982). Coconut palms (*Cocos nucifera* L.), for example, showed a marked decrease in symptom expression in early stages of lethal yellows infection and failed to improve when the disease was at a more advanced stage (Cha and Tattar 1993). The plants selected in the present study were selected on the basis of moderate to severe symptom severity and this factor may explain the lack of effect. Commencing treatment earlier in the season when plants are less severely affected should be tested in future studies.

Applications of tetracycline were applied fortnightly, the extended period between applications in line with several other studies using antibiotic applications to reduce symptom expression (Greber and Gowanlock 1979; McCoy 1982). Other studies of tetracycline compounds have applied the antibiotic treatments every two to three days as was the case with *Nicotiana glutinosa* L., aster (*Symphyotrichum* sp.) and tomato (*Lycopersicon* sp.) plants infected with legume little leaf disease (Bowyer and Atherton 1972). Applications of tetracyclines in this study were applied once every fortnight and future studies on ALuY symptom alleviation should include increased frequency of these applications.

The present study constitutes only a preliminary appraisal of ALuY disease management treatments and suggests that further study is warranted. Though there appears to be some scope for reducing the impact of the disease by cultural practices such as optimising plant nutrition and water availability, especially on the key economic parameter of seed yield, control of infection by reducing vector immigration needs also to be considered as part of an integrated disease management strategy.

5. Implications and Recommendations

Implications

This study has succeeded in identifying the pathogen responsible for Australian lucerne yellows disease. This is a phytoplasma and DNA sequencing has shown this to be a species new to science. A molecular test has been developed for detection of the pathogen in plant tissue and scope exists to refine and commercialise the technique for rapid diagnostics.

Field experimentation suggests limited scope for disease management via symptom suppression though supplementary water had some effect on seed yield of diseased plants. Management of the vectors responsible for introducing the pathogen into newly sown crops and for its subsequent spread, is likely to be important. Work to identify the insect species responsible for vectoring has included field surveys and transmission tests in New South Wales. These suggest that common brown leafhopper (*Orosius argentatus*) is the most likely vector though the large green jassid (*Batracomorphus angustatus*) cannot be ruled out as an additional vector. A third species, the spotted leafhopper (*Austroagallia torrida*) is considered unlikely to be a vector for Australian lucerne yellows but appears to be capable of vectoring tomato big bud – a separate phytoplasma that causes witches' broom and phyllody in lucerne. All three leafhopper species were also present in samples from South Australia.

Newly sown lucerne seed crops in New South Wales are likely to suffer yield loss even in the first year after establishment, for all three closely monitored sites exhibited symptoms within 32 weeks of establishment. Field data suggest strongly that leafhoppers migrate into crops by short-range movement from non-crop vegetation in field margins rather than long-range aerial dispersal.

Herbicidal or insecticidal treatment of this vegetation significantly reduced catches of leafhoppers on the crop: border vegetation interface and the numbers of symptomatic plants within the crop. The apparent importance of short range dispersal only a few centimetres above the canopy suggests that physical barriers such as shade mesh material mounted to existing fences, could constitute effective barriers and this should be tested as a priority in follow up work though individual farmers could also experiment with this approach in an informal manner.

Recommendations

Findings from this study should be disseminated to lucerne growers (especially seed producers) and other relevant industry sectors (eg seed houses) in a concise, well-illustrated format. This will allow better-informed management of lucerne yellows disease. It is suggested that a 'hip pocket guide' and colour poster be produced by the RIRDC in collaboration with the authors.

The results of this work should be published in scientific journals as a major contribution to the understanding of the pathogen, vectors and management for lucerne yellows disease. This will allow follow-up work to refine disease management.

Consideration should be given to allocating a modest level of funding to the present project team in order to conduct field experiments on barriers to the movement of vector insects into lucerne.

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