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

L. J. Pilkington[a*†], K. S. Gibb[b], G. M. Gurr[a], M. J. Fletcher[c], A. Nikandrow[c], E. Elliott[d], R. van de Ven[c] and D. M. Y. Read[a]

[a]*University of Sydney, Orange, PO Box 883, Orange, New South Wales, 2800;* [b]*Northern Territory University, Darwin, Northern Territory, 0909;* [c]*NSW Agriculture, Orange Agricultural Institute, Forest Road, Orange, New South Wales, 2800; and* [d]*NSW Agriculture, PO Box 369, Forbes, New South Wales, 2871, Australia*

Foliar and root symptoms are described for Australian lucerne yellows (ALuY), a disease common in Australian lucerne seed crops. A phytoplasma was detected in plants exhibiting symptoms, but not in symptomless lucerne plants. Oligonucleotide primers specific to the phytoplasma 16S-23S rRNA intergenic spacer region (SR) were used in polymerase chain reaction (PCR) assays on DNA extracted from lucerne plants with and without symptoms. Identical restriction fragment length polymorphism (RFLP) enzyme profiles were obtained for PCR products amplified from 10 yellows-affected lucerne samples. RFLP profiles obtained for four restriction enzymes were different from those of the tomato big bud (TBB) phytoplasma. ALuY phytoplasma PCR products were sequenced to determine phylogeny and were found to fall within the faba bean phyllody phytoplasma group, or phytoplasma group 16srII. Transmission electron microscopy revealed phytoplasmas in the phloem of yellows-affected plant samples, but not in symptomless plant samples. Fungal, bacterial and viral agents in the aetiology of Australian lucerne yellows were ruled out.

Keywords: alfalfa, faba bean phyllody phytoplasma, *Medicago sativa*, yellows disease

Introduction

Lucerne (*Medicago sativa*) is a perennial, deep-rooted pasture legume of increasing worldwide significance as a result of its use in managing aspects of environmental sustainability, such as rising water tables and soil salinity (Fitzgerald & Simmons, 1978). 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) (Pilkington *et al*., 1999).

Australian lucerne yellows 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 $7 million annually to the Australian lucerne seed industry (Pilkington *et al*., 1999).

*To whom correspondence should be addressed.

†E-mail: leigh.pilkington@orange.usyd.edu.au

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Symptoms associated with ALuY include 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 tomato big bud (TBB) and sweet potato little leaf strain V4 (SPLL-V4) phytoplasmas have been detected in lucerne (Gibb *et al*., 2000; Wilson *et al*., 2001; K. S. Gibb, 2002, Northern Territory University, Darwin, personal communication).

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).

The aim of the present study was to analyse plants with and without symptoms for the presence of (i) phytoplasmas and (ii) bacterial and fungal pathogens, as well as to characterize the phytoplasmas detected in

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ALuY-affected lucerne plants using molecular techniques. A preliminary report on the aetiology of Australian lucerne yellows has been published previously (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 1–4, 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 that 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 1 and 2. An additional set of two plants each with and without symptoms were collected from site 3 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 4 in the Lachlan Valley, NSW, for bacterial examination. A tomato plant exhibiting symptoms of TBB disease was 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 taproot, approximately 5 cm 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 $2 \times 2 \times 0{\cdot}5$ 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 (1/4PDA) supplemented with 100 $\mu g\ mL^{-1}$ 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 5 days of incubation and the leading edge of each individual colony was subcultured onto 1/4PDA 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 $1 \times 1 \times 1$ cm) was removed from the taproot leaving the cambium layer intact. This section was surface-sterilized for 2 min in 1% sodium hypochlorite, agitating every 30 s, then rinsed twice in sterile distilled water for 2 min.

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 $1 \times 1 \times 0{\cdot}5$ mm) and teased out. The slices were placed in 10 mL sterile distilled water for 1 h.

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 2 min, the pieces (approximately $1 \times 1 \times 1$ mm) aseptically cut, roughly macerated and placed into 10 mL of sterile distilled water for 1 h. The suspension was streaked out with a 1-mm loop onto each of four plates of sucrose peptone agar (SPA), SPA + 250 p.p.m. glycohexamide and nutrient agar (NA) Oxoid (Oxoid Ltd, Basingstoke, UK). Plates were sealed with Parafilm (American Can Company, Greenwich, CT, USA) and placed in an incubator at 25°C. After 3 days, cultures were examined and individual colonies were subcultured onto the same medium from which they had been isolated.

Eleven colonies were selected and submitted for fatty acid analysis (Agilent Technologies 6890 N Network GC System Machine) at the Orange Agricultural Institute, New South Wales, Australia. Cultures identified as *Clavibacter michiganense* ssp. *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.

Detection of phytoplasmas

DNA extraction

DNA was extracted as described by Dellaporta *et al.* (1983) from 0·5 g combined leaf midribs, stems and roots from lucerne plants with and without symptoms of ALuY within 12 h 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 resuspended in 50 μL $1 \times$ TE buffer (10 mM Tris-HCl, 1 mM EDTA) and stored at −20°C until use.

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 aliquots of each in PCR reactions. 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 primers and 0·1 mM dNTPs (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 1 min (2 min for the first cycle) at 95°C, an annealing temperature of 55°C for 1 min, and an extension time of 1·5 min at 72°C for 35 cycles (9·5 min on the 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 16 ALuY DNA samples, two symptomless lucerne DNA samples, one TBB sample and one SDW sample using the universal primers P1/P7. One microlitre of each P1/P7 PCR cocktail was then subjected to reamplification 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 visualized 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 microlitre of DNA from each ALuY-affected plant was combined with an equal volume of the control (TBB) DNA and subject to PCR using primers P1/P7.

RFLP analysis

Nested PCR products from 10 ALuY-affected lucerne plants and six TBB phytoplasma controls were subjected to RFLP analysis. Following the manufacturer's instructions (New England Biolabs, Inc., Beverley, MA, USA), 5 μL of each PCR product were 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 visualized by UV transillumination.

Sequence analysis

The entire PCR product obtained from a DNA sample extracted from a single ALuY-affected 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 (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). 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 GAP program (Accelrys, San Diego, CA, USA). *Acholeplasma palmae* and *A. laidlawii* were used as outgroups.

Table 1 Phytoplasma names, abbreviations and EMBL accession numbers

Phytoplasma	Abbreviation	Accession number
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

Electron microscopy

Leaf midribs from six ALuY-affected and two unaffected lucerne plants were dissected into approximately 1 mm^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 (22°C) on rotators, transferred to 100% Spurrs resin overnight on rotators and embedded in fresh Spurrs resin and polymerized at 60°C overnight. Specimens were then cut into ultrathin (80 nm) sections and viewed in a Philips Biofilter CM120 (120-kV) electron microscope.

Figure 1 PCR amplification of phytoplasma DNA from ALuY-affected lucerne using the primer pairs P1/P7 and fU5/m23sr. Lanes 1–16, ALuY-affected plants; lanes 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.

Results

Fungal isolations

Eighteen distinct fungal taxa were isolated from plants with and without symptoms. *Fusarium solani* was isolated from three of the 12 diseased plants examined. Several other fungi, e.g. *Phoma medicaginis* and *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* ssp. *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* ssp. *insidiosus* of 0·702 was lower than that for the alternative identification of *Leifsonia aquatica* (0·780 SI), a nonlucerne pathogen. Both isolates tentatively identified as *C. michiganense* ssp. *insidiosus* were, however, negative when tested by ELISA.

Detection of phytoplasmas in lucerne

No bands were amplified by simple PCR of either ALuY-affected or symptomless plants, but in all assays the TBB-phytoplasma control was positive and amplified a 1·6-kb band. In nested PCR using primers P1/P7 followed by fu5/m23sr, the TBB-phytoplasma-positive controls gave a product of 1·1 kb while water controls gave no amplified product. Of the 260 ALuY samples tested from 130 individual yellows-affected plants, 63 gave a product of 1·1 kb when amplified in nested PCR assays. No positive signal was observed with DNA extracted from the 30 symptomless plant samples. A 1·6-kb PCR product was observed when 18 ALuY DNA samples that had tested negative were 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.

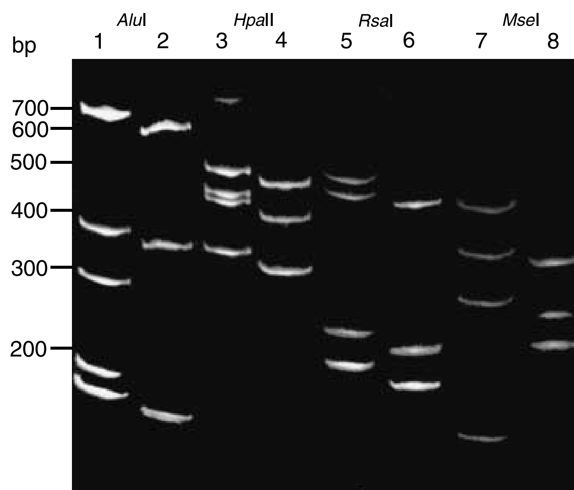

Figure 2 Restriction fragment length polymorphism profiles of 16S rDNA amplified by nested PCR from the phytoplasma associated with ALuY and from the TBB phytoplasma. Lanes 1, 3, 5 and 7: AluY DNA digested with *Alu*I, *Hpa*II, *Rsa*I and *Mse*I, respectively; lanes 2, 4, 6 and 8: TBB DNA digested with *Alu*I, *Hpa*II, *Rsa*I and *Mse*I, respectively.

RFLP

When 10 PCR products amplified from 10 separate ALuY-affected 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 1·1 kb. Representative RFLP profiles of ALuY and TBB phytoplasmas are shown in Fig. 2.

Sequence analysis

The entire PCR product of approximately 1·1 kb amplified from a DNA sample extracted from an ALuY-diseased

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

	ALuY	OaWB	BoLL	FBP	PpMz	PpYC	TBB	PnWB	SUNHP	SPLL	SPWB	
ALuY	0											ALuY
OaWB	99	0·0										OaWB
BoLL	97	98	0·0									BoLL
FBP	97	98	99	0·0								FBP
PpMz	99	100	99	98	0·0							PpMz
PpYC	99	100	99	98	100	0·0						PpYC
TBB	99	99	98	98	99	99	0·0					TBB
PnWB	99	100	98	98	100	100	100	0·0				PnWB
SUNHP	99	99	98	98	100	100	99	100	0·0			SUNHP
SPLL	98	99	98	98	99	99	98	99	98	0·0		SPLL
SPWB	98	99	97	97	99	99	99	99	99	98	0·0	SPWB
	ALuY	OaWB	BoLL	FBP	PpMz	PpYC	TBB	PnWB	SUNHP	SPLL	SPWB	

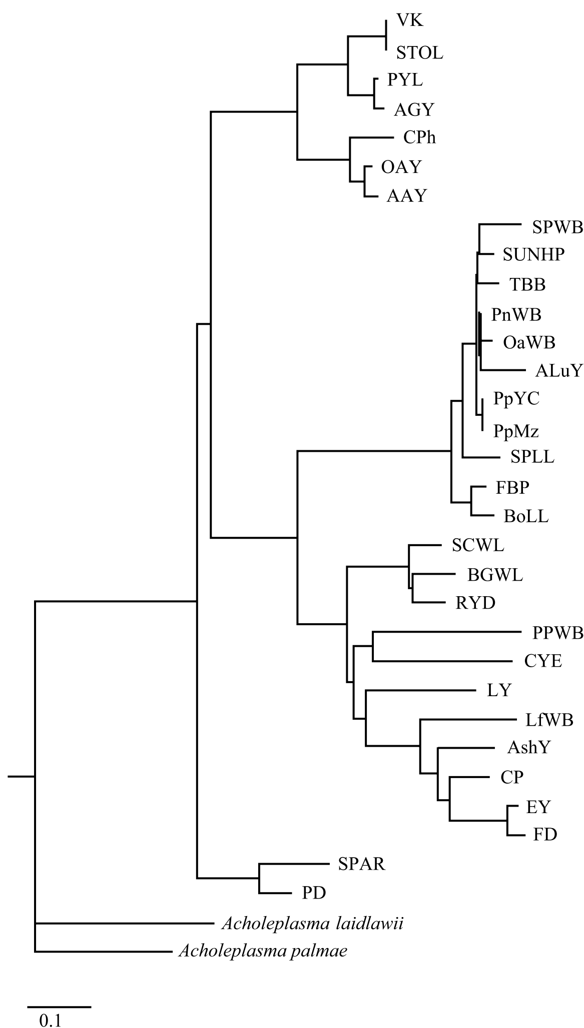

Figure 3 Phylogenetic tree of the 16S rRNA gene sequence of the phytoplasma associated with ALuY (partial sequence of 960 bp) and other selected phytoplasma 16S rRNA sequences. *Acholeplasma laidlawii* and *A. palmae* were used as outgroups. The bar represents a phylogenetic distance of 10%. Phytoplasma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

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 (Cronjé *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 was 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 indicated that the former was associated with the FBP phytoplasma (16srII) group (Lee *et al*., 1998; Seemüller *et al*., 2002).

Electron microscopy

Examination of ultrathin cross-sections of leaf midrib from ALuY-affected plants showed numerous phytoplasmas (200–400 nm in diameter) in the phloem of four of the plants. The structures were spherical to ovoid, enclosed by a single unit membrane and contained dark structures centrally located that were consistent in appearance with 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.

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

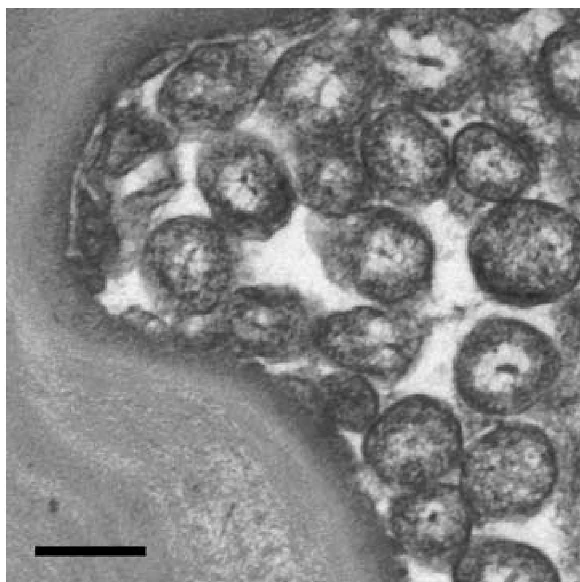

Figure 4 A phloem cell of a lucerne plant affected with ALuY showing phytoplasma bodies (bar = 0.30 μm).

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, was 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* ssp. *insiodosus* 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-brown discoloration throughout the stele of the taproot, and thus are distinct from the symptoms seen in plants infected with ALuY (Stovold, 1983), in which discoloration occurs directly beneath the cambium layer of the taproot (Pilkington *et al*., 1999). Aside from differences in symptoms, no obvious association with *C. michiganense* ssp. *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 Australian latent nepovirus* (LALV) and *Lucerne transient streak sobemovirus* (LTSV) (Blackstock, 1978; Johnstone & Barbetti, 1987). Symptoms of AMV include mild to severe mosaicking, 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 LTSV 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 LTSV 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 worldwide (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-affected 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 digested with the enzymes *Alu*I and *Hpa*II produced extra bands and the total fragment size was therefore greater than the 1·1-kb fragment expected. Phytoplasmas contain two 16S rRNA 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 16S rRNA gene sequences from each operon. Although these differences may be so slight that the PCR product comigrates on an agarose gel (Schneider & Seemüller, 1994; Liefting *et al*., 1996), any sequence differences that affect restriction enzyme recognition sites will result in different interoperon banding patterns that can be resolved on an acrylamide gel. An alternative explanation for the additional RFLP bands in this study is that ALuY-diseased plants were subject to a mixed phytoplasma infection, although this was 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 was 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 characterized 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 South-east 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.

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