Australian Government

Rural Industries Research and Development Corporation

Australian Lucerne Yellows Disease: *Testing and extension of disease management strategies*

by GM Gurr, MA Getachew, MJ Fletcher, A Mitchell, A Nikandrow and LJ Pilkington

August 2007

RIRDC Publication No 07/120
RIRDC Project No US-131A

ISBN 1 174151 517 3
ISSN 1440-6845

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Publication No. 07/120
Project No.US-131A

Researcher Contact Details
Prof. Geoff M Gurr
Charles Sturt University, PO Box 883, Orange, NSW
2800, Australia.
Voice: +61 2 6365 7551
Fax: +61 2 6365 7885
Mobile: +61 417 480 375
Email: ggurr@csu.edu.au

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

RIRDC Contact Details
Rural Industries Research and Development Corporation
Level 2, 15 National Circuit
BARTON ACT 2600
PO Box 4776
KINGSTON ACT 2604

Phone: 02 6271 4100
Fax: 02 6271 4199
Email: rirdc@rirdc.gov.au.
Web: http://www.rirdc.gov.au

Published in August 2007
Printed on environmentally friendly paper by Canprint

Foreword

Australian lucerne yellows (ALuY) disease affects lucerne (*Medicago sativa* L.) seed production in Australia and causes an estimated $7 million annual loss to the New South Wales (NSW) and South Australian (SA) lucerne seed industries. This project aimed to establish additional information on this disease, including its cause and means of spread and to test possible disease management methods that can be then communicated to growers.

In this project, research in NSW and SA established an association between ALuY symptoms in lucerne plants and two genetically distinct phytoplasmas: *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense. *Candidatus* Phytoplasma aurantifolia was also detected in the bodies of leafhopper insects suspected to be vectors of the pathogen and in seven plant species (mostly weeds).

DNA of *Candidatus* Phytoplasma australiense was amplified from lucerne seeds harvested from a seed crop that had high ALuY disease incidence and from seedlings grown from that material. Incidence was greatest in 'second grade' seed. This constitutes evidence that the pathogen can be seed borne and that phytosanitary arrangements could be important for the management of this disease.

Control of ALuY will need to focus on the identified non-crop plants that may harbour the pathogen, as well as the insects that may carry it into the crop. However, field experiments conducted in this project showed that pesticidal treatments of field margin vegetation and insect exclusion barriers were not effective in restricting the migration of suspected ALuY vector leafhoppers or reducing incidence of ALuY disease in the crop.

An opportunity exists to conduct further research on seed transmission and to develop a commercial diagnostic test for the presence of phytoplasma pathogens. Producers and seed houses will benefit from this new information on an important lucerne disease which will allow them to better target control efforts against the leafhopper and relevant weeds.

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

This report, an addition to RIRDC's diverse range of over 1600 research publications, forms part of our Pasture Seeds R&D Program, which aims to facilitate the growth of a profitable and sustainable pasture seeds industry based on a reputation for the reliable supply, domestically and internationally, of a range of pasture species.

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Peter O'Brien
Managing Director
Rural Industries Research and Development Corporation

Acknowledgments

We thank Mr Eric Elliott for invaluable assistance with early stages of this research. Mr Raymond Christinat based at Keith, South Australia (SA) assisted with the establishment and monitoring of field experiments in SA. Mr Luke Kirkby, Auswest Seed Company, Forbes, NSW, Australia provided lucerne seed samples for molecular tests and assisted liaison with lucerne growers at Griffith. Ms Lucy Trans-Nguyen, Charles Darwin University, provided DNA samples of Australian tomato big bud (TBB) and Phormium yellow leaf (PYL) phytoplasmas. Many thanks also to lucerne growers at Griffith, the Lachlan Valley and Orange for permitting work on their lucerne fields.

At the University of Sydney, Orange, Ms Helen Nicol helped with data analysis; Ms Christine Andrews and Ms Karen Gogala helped with lab work and Ms Marja Simpson supported field work. Staff members of the PCR lab at the Orange Agricultural Institute (OAI) also assisted.

Abbreviations

AAP	Acquisition access period
AGY	Australian grapevine yellows phytoplasma
AlloY	Allocasuarina yellows phytoplasma
ALuY	Australian lucerne yellows
AMV	Alfalfa mosaic virus
AP	Apple proliferation phytoplasma
AY	Aster yellows phytoplasma
AWB	Alfalfa witches' broom
BLOs	Bacteria-like organisms
BWB	Buckthorn witches’ broom phytoplasma
Ca.	*Candidatus*
CP	Clover proliferation phytoplasma
CTAB	cetyltrimethylammonium bromide
cv.	cultivar
CWL	Cynodon white leaf phytoplasma
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBP	Faba bean phyllody phytoplasma
IDM	Integrated disease management
LTTV	lucerne transient streak sobemovirus
OAI	Orange Agricultural Institute
PCR	Polymerase chain reaction
PDB	Papaya dieback phytoplasma
PoiBI	Poinsettia with free branching characteristics
PPT	Potato purple top phytoplasma
PWB	Paulownia witches’ broom phytoplasma
PYL	Phormium yellow leaf phytoplasma
PM	Papaya mosaic phytoplasma
PYC	Papaya yellow crinkle phytoplasma
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
SCGS	Sugarcane grassy shoot phytoplasma
SCWL	Sugarcane white leaf phytoplasma
SDW	Sterile distilled water
SGP	Strawberry green petal phytoplasma
SLY	Strawberry lethal yellows
SPLL	Sweet potato little leaf phytoplasma
SPLL-V4	Sweet potato little leaf variant 4 phytoplasma
SUNHP	Sunhemp witches’ broom phytoplasma
STOL	Stolbur phytoplasma
TBB	Tomato big bud phytoplasma
ViLL	Vigna little leaf phytoplasma
WA	Western Australia
WWB	Walnut witches'-broom phytoplasma

Contents

Executive Summary

What the report is about?
Australian lucerne yellows (ALuY) disease affects lucerne (*Medicago sativa* L.) seed production causing an estimated $7 million in annual loss to the New South Wales (NSW) and South Australian (SA) lucerne seed industries. This report presents additional information on the ALuY disease including its cause and spread and outlines possible management strategies that can be used by growers to control the disease.

Who is the report targeted at?
This report is targeted at lucerne seed growers, seed production agronomists and consultants who may be involved in ALuY disease management. The report (as well as an associated paper[1]) is also targeted towards other researchers who are likely to be able to build further on knowledge of this disease.

Background
This project built on previous RIRDC-funded work that established the impact of ALuY disease and generated information on its cause and possible disease management approaches.

Aims/Objectives
This project aimed to generate additional information on the ALuY disease, its cause and means of spread and to test possible disease management methods that can be communicated to growers.

Methods used
PCR assays were used to test for the DNA of phytoplasmas in lucerne plants collected from stands in NSW and SA. Insects suspected to be the vectors of the phytoplasmas were also tested using PCR. Field surveys in NSW and SA identified plants that harboured potential vector species and these plants were also tested for presence of the phytoplasmas using PCR. Seeds, and the seedlings grown from them, were likewise assayed for phytoplasma using PCR. Field experiments evaluated the efficacy of pesticidal treatment of field margin vegetation and of barriers on the perimeter of lucerne crops for preventing immigration of leafhoppers.

Results/Key findings
Polymerase chain reaction (PCR) assays established an association between ALuY symptoms and two genetically distinct phytoplasmas: *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense, which were detected in 31% and 24% of ALuY symptomatic, field-collected plants, respectively. Mixed infection by these phytoplasmas was not found in ALuY symptomatic plants except in one sample collected from Griffith, suggesting that these phytoplasmas could independently cause ALuY disease. Because phytoplasmas cannot be cultured and inoculated into plants, causality has not been proven and it is not possible to rule out the possibility that another pathogen is involved.

PCR also detected *Candidatus* Phytoplasma aurantifolia in field collected, whole body samples of the leafhopper *Orosius orientalis* (Matsumura) (=*Orosius argentatus* (Evans)) (Deltocephalinae: Opsiini) and the plants yanga bush (*Maeriana brevifolia* (R. Br.) P.G. Wilson), nettleleaf goosefoot (*Chenopodium murale* L.), night shade (*Solanum nigrum* L.), nitre goosefoot (*Chenopodium nitrariacium* (F. Muell) F. Muell ex Benth, ruby salt bush (*Enchylaena tomentose* R. Br.), *Trifolium pratense* L. (red clover) and cotton bush (*Maeriana microphylla* (Moq.) P.G. Wilson). Several plant species in the Chenopodiaceae (*M. brevifolia, E. tomentose, M. microphylla, C. nitrariacium* and *C. murale*) were found to be breeding and winter survival hosts

[1] Getachew, M.A., Mitchell, A., Gurr, G.M., Fletcher, M.J., Pilkington, L.J., and Nikandrow, A. 2007. First report of a "Candidatus phytoplasma australiense"-related strain in lucerne (Medicago sativa) in Australia. *Plant Disease* 91(1): 111

of *O. orientalis*. Large numbers of *O. orientalis* adults and nymphs were sampled from *M. brevifolia*, *E. tomentose*, *M. microphylla* and *C. nitrariacium* in summer, autumn, winter and spring and from *C. murale* in summer. The results suggest that, if *Ca.* Phytoplasma aurantifolia is confirmed to be the etiological agent responsible for ALuY disease, targeted management of chenopod weeds in and around new lucerne fields could reduce ALuY disease incidence. Where there are legislative restrictions on the control of native plant species that host the phytoplasma, growers will need to consider siting new lucerne stands remotely. The leafhoppers *O. orientalis* and *Batracomorphus angustatus* (Osborn) were successfully cultured and maintained on *C. murale* L. and *T. pratense* L. plants, respectively, under controlled conditions. Such information is useful for future disease studies.

Seeds harvested in March 2005 from a seed lucerne crop (cv. CW 5558) that had high ALuY disease incidence during the 2004/2005 crop season were used in DNA studies. DNA of *Candidatus* Phytoplasma australiense was amplified from lucerne seeds and seedlings grown in vector free environments suggesting that seed transmission of this phytoplasma is possible in lucerne. *Candidatus* Phytoplasma australiense was detected in 7/40 and 23/40 seed samples from the 'first' and the 'second' grade seeds, respectively.

Vector management field experiments showed that insecticide or herbicide treatment of a 2-10m wide and 20m long strip of field margin vegetation or 60cm high insect exclusion fence on one boundary of a new lucerne crop field were not effective in restricting the migration of suspected ALuY vector leafhoppers into the crop or to reducing ALuY disease incidence in the crop.

Future ALuY disease studies may consider the use of resistant/tolerant varieties as part of an integrated disease management (IDM) strategy for ALuY disease. If *Candidatus* Phytoplasma australiense is confirmed to be the causal agent for ALuY disease and, if seed transmission of this phytoplasma is confirmed, phytosanitary arrangements at both the local and larger scale also could be part of an integrated disease management strategy for ALuY disease.

Implications for relevant stakeholders for industry:

- Pesticidal treatment of non-crop vegetation around lucerne crops and barriers mounted on existing fences appear to be ineffective in preventing vector insects migrating into lucerne stands.

- Control of Australian lucerne yellows disease will need to focus on the identified non-crop plants that may harbour the pathogen, as well as the insects that may carry it into the crop.

- Consideration should be given to the removal of weeds or siting new lucerne stands well away from specified perennial, native plants.

- Insecticide application should be considered if large numbers of the leafhopper *O. orientalis* are found in lucerne, especially in newly-sown stands.

- The phytoplasmas associated with Australian lucerne yellows disease may be seed borne, necessitating more careful phytosanitary arrangements.

Recommendations

ALuY disease is a challenging problem for growers and researchers alike because the pathogen cannot be cultured and inoculated onto test plants. Detection of the phytoplasma requires electron microscopy or PCR techniques as used in this project. The scientific community needs to develop increasingly powerful methods for the study of phytoplasma diseases which will aid future ALuY disease researchers aiming to definitively resolve causality.

For growers, consultants and seed production technicians aiming to manage ALuY disease more effectively, the results contained in this report provide significant new information that will allow new disease management options to be implemented. These include avoidance of non-crop plants that may harbour the pathogen, control of the likely leafhopper vector, inspection of seed crops for ALuY disease symptoms and more careful choice of seed source to avoid use of seeds that may carry phytoplasmas associated with ALuY disease. This objective may be supported by PCR testing of seed batches and scope exists to develop and offer this as a fee-for-service activity.

An illustrated extension pamphlet has been drafted (see Appendix) and, subject to Corporation approval, this can be mailed to growers. It may also be placed on the Charles Sturt University and/or Corporation web site with a link to the present document for fuller information.

1. Introduction

Lucerne, or alfalfa (*Medicago sativa* L.), is the most important forage legume grown in many parts of the world (Saindon *et al.* 1991) and is sometimes referred to as "Queen of Forages" (Summers 1998) in recognition of its superior qualities as livestock feed. It is highly digestible, rich in protein and cell solutes, and has low levels of indigestible cell wall and neutral detergent fibre (Conrad and Klopfenstein 1988).

Lucerne improves soil structure and, as a legume, fixes atmospheric nitrogen. It is an extremely adaptable crop with considerable tolerance to stresses including drought (Summers 1998). More recently, it has assumed significance due to its use in managing aspects of environmental sustainability, such as rising water tables and soil salinity (Halvorson and Reule 1980; Cocks 2001; Humphries and Auricht 2001; Latta *et al.* 2001). Lucerne is a deep rooted perennial plant and, as such, keeps salt-saturated ground water levels below the root zone of most cultivated crops.

Lucerne seed production is a valuable industry. The Australian lucerne seed industry is worth over $100 million to the Australian economy and in the three years from 2002-2004, the value of lucerne seed exports rose by 55% (Anonymous 2006).

Row or broad paddock cropping systems can be used in lucerne seed production (Kirkby 2006). Row cropping facilitates farm operations such as cultivation and pesticide application. In the USA, lucerne seed growers use the row crop production system with only seed production in mind. In Australia, lucerne seed production generally uses a broad paddock cropping system to allow utilisation of lucerne fields for other purposes such as grazing and hay production (Kirkby 2006). Pests and diseases as well as poor irrigation planning are important factors that reduce the quality and quantity of lucerne seed yield. Flower drop occurs when irrigation is too late whilst too early irrigation leads to excessive vegetative growth.

1.1 Diseases of lucerne

Lucerne is affected by a wide range of fungal, bacterial, viral and phytoplasma diseases. Significant forage yield loss caused by foliar fungal pathogens of lucerne has been reported in Canada (Semeniuk and Rumbaugh 1976), New Zealand (Hart and Close 1976) and Australia (Morgan and Parbery 1980). Common crown rot, a disease complex consisting primarily of the fungi *Phomopsis* sp. and *Acrocalymma medicaginis* Alcorn and Irwin is the most common cause of premature death and thinning of lucerne stands in Australia. Foliar fungal diseases of significance in Australia include black stem caused by *Phoma medicaginis* Mal. *et* Roum v. *pinodella* (L.K. Jones) Boer., leaf spot caused by *Pseudopeziza medicaginis* (Lib.) Sacc., leaf blotch caused by *Leptotrochila medicaginis* (Fuckel) H. Schuepp, common leaf spot or burn caused by *Leptosphaerulina trifolii* (Rostr.) and stemphylium leaf spot caused by *Stemphylium* spp. Other important fungal diseases of lucerne worldwide (though not all present in Australia) include phytophthora root rot caused by *Phytophthora medicaginis* Hansen and Maxwell, fusarium wilt caused by *Fusarium oxysporum* f.sp. *medicaginis* E.F. Sm. W.C. Snyder and H.N. Hans., verticillium wilt caused by *Verticillium albo-atrum* Reinke and Berthold and anthracnose and crown rot caused by *Colletotrichum trifolii* Bain and Essary (Barbetti 1989; Lowe *et al.* 1991; Irwin *et al.* 1997; Liew and Irwin 1997; Mackie and Irwin 1998; Mackie *et al.* 2003; Viands *et al.* 2005). In Australia, phytophthora root rot and anthracnose are important fungal disease of lucerne (Irwin *et al.* 2006). Symptoms of phytophthora root rot include wilting, yellowing and death of individual plants or patches (Krnjaja and Levic 2005).

Bacterial wilt caused by *Clavibacter michiganensis* Spieckermann and Kotthoff, is the most important bacterial disease of lucerne worldwide (Viands *et al.* 2005), although its impact on Australian lucerne crops is minimal (Irwin *et al.* 2006). The most common viral pathogens of lucerne are Alfalfa mosaic virus (AMV) and lucerne transient streak sobemovirus (LTTV). AMV has a world wide distribution and a wide range of plant hosts (CAB International 2002). Lucerne plants infected with AMV develop

mild to severe mosaic, leaf stunting and rolling, chlorotic vein clearing and leaf reddening (Jasnic 2005). Symptoms of LTTV include chlorotic streaks around the main lateral veins and necrotic and chlorotic lesions of leaflets (Krnjaja and Levic 2005).

Several phytoplasma diseases have been reported in lucerne. Alfalfa witches' broom (AWB) disease is distributed worldwide but has been associated with different phytoplasma groups in different parts of the world: with the ash yellows group in Argentina (Conci *et al.* 2005); with the faba bean phyllody in Oman (Khan *et al.* 2002) and Italy (Marcone *et al.* 1997) with the clover proliferation group in Canada (Wang and Hiruki 2001) and with the aster yellows group in Lithuania (Valuinas *et al.* 2000). Other phytoplasma diseases in lucerne include Australian lucerne yellows (Stovold 1983; Pilkington *et al.* 2002; Pilkington *et al*. 2003) and little leaf (Suryanayana *et al.* 1996).

1.2 Yellows type plant diseases

Yellows type plant diseases can be caused by phytoplasmas (Franova *et al*. 2004; Streten *et al*. 2005a), viruses (Keshavarz and Izadpanah 2005; Boubourakas *et al.* 2006) or bacteria-like organisms (BLOs) (Hirumi et al. 1974; Hopkins 1977). Before 1967, when phytoplasmas were discovered, yellows type plant diseases had been presumed to be caused by viruses, although viruses could not consistently be visualised in diseased tissues or isolated from infected plants. Phytoplasmas are now known to be associated with hundreds of yellows type plant disorders.

BLOs have been demonstrated in both the phloem and xylem tissues. Phloem-restricted BLOs are involved in more than 20 plant diseases worldwide (Bove and Garnier 2002). Clover club leaf (Black 1948), yellow vine disease of cucurbits (Bruton *et al*. 1998), citrus greening (Jagoueix *et al*, 1994), strawberry marginal chlorosis (Nourrisseau *et al*. 1993) and papaya bunchy top (Davis *et al*. 1998) are such diseases. In Australia, BLOs have been found associated with strawberry lethal yellows disease (Greber and Gowanlock 1979; Streten *et al*. 2005). Plant disease symptoms caused by BLOs include leaf yellowing/chlorosis (Hopkins 1977), phloem discoloration (yellowing/browning) (Bruton *et al*. 1998) and reduced leaf size and yellowing (Nourrisseau *et al*. 1993).

Australian lucerne yellows disease

Australian lucerne yellows (ALuY) disease has been reported in lucerne since the 1950s in Australia (Stovold 1981). ALuY disease is responsible for losses of approximately $7M per annum to the NSW and South Australian lucerne seed industry (Pilkington *et al.* 1999). Discoloration of leaves ranging from yellow to red that affects the entire foliage and yellowish/honey-brown discoloration of the phloem of the taproot are typical symptoms of ALuY affected plants (Pilkington *et al.* 2002; Pilkington *et al*. 2003). Root rot is not a symptom of ALuY but secondary invaders may cause tissue breakdown and yellowing of the leaves as plants decline.

Association of a new type of phytoplasma with ALuY symptoms has been reported (Pilkington *et al.* 2002; Pilkington 2003), the evidence of which derives from polymerase chain reaction (PCR) and electron microscopy analyses of ALuY symptomatic and asymptomatic lucerne plants. In that study, 24.2% of ALuY symptomatic lucerne plant samples were phytoplasma positive by PCR assays and typical phytoplasma bodies were observed in the phloem of ALuY symptomatic but not asymptomatic plant samples (Pilkington *et al.* 2002; Pilkington 2003). The new type of phytoplasma reportedly associated with ALuY symptoms showed 99% 16s rDNA sequence similarity with Alfalfa witches' broom phytoplasma (Pilkington *et al.* 2002; Pilkington 2003), which is the most widely distributed phytoplasma disease of lucerne (Lee *et al* 2000). According to IPRCM rules, phytoplasmas sharing 97.5% or more 16s rDNA sequence similarity are considered taxonomically highly related/the same (IPRCM 2004). This suggests that further analyses are required to determine whether the ALuY phytoplasma reported by Pilkington *et al*. (2002) was new or a previously described type.

1.3 General properties of phytoplasmas

Association between cell wall-less prokaryotes and yellows type plant diseases was first reported in 1967. Cell wall-less prokaryotes resembling mycoplasmas were discovered in the phloem of plants with mulberry dwarf, potato witches-broom, aster yellows and paulownia witches-broom by electron microscopy (Doi *et al*. 1967).

Phytoplasmas are cell wall-less prokaryotes bounded by a unit membrane. In ultrathin sections, they appear as a complex of multibranched, beaded, filamentous or spheroidal polymorphic bodies ranging from 175-400nm in diameter for the spherical and oblong cells and up to 1700nm long for the filamentous forms (Lee *et al*. 2000; IPRCM 2004). Phytoplasmas are generally present in phloem tissues of plants and in the salivary glands and hemolymph of vector insects (Davis *et al.* 1997b). While phytoplasmas are assumed to multiply in the phloem, little is known about the mechanism. They also multiply in the hemolymph and salivary glands of vector insects (Guthrie *et al.* 1998; Padovan and Gibb 2001). Plants infected with phytoplasmas exhibit a range of symptoms including virescence (the development of green flowers and the loss of normal flower pigments), phyllody (the development of floral parts into leafy structures), witches' broom (proliferation of axillary and auxillary shoots), big bud (bunchy appearance of growth at the ends of the stems) and generalised decline (stunting, dieback of twigs and stems, unseasonal yellowing and reddening/purpling of leaves) (Lee *et al.* 2000).

1.4 Detection and identification of phytoplasmas

Accurate identification of pathogens is a prerequisite for pathogen characterisation and successful management of diseases. Since phytoplasmas are unculturable in vitro, Koch’s postulates are only sometimes fulfilled by using alternative tools, such as insect or dodder inoculation. Isolation of phytoplasmas can be accomplished using a living plant host such as periwinkle from which a healthy test plant can be inoculated using vectors, grafting or dodder. Molecular tests or symptomatology can be used to confirm identity of the phytoplasma. However, this method has limited applications because it is time consuming. This makes diagnosis of phytoplasma-associated plant diseases one of the most difficult in plant pathology research. Until the mid 1980s, diagnosis of phytoplasma-associated plant diseases and classification of phytoplasmas relied primarily on electron microscopy, DNA staining of phloem elements of diseased plants and biological characteristics including phytoplasma-vector relationships, host ranges, and symptomatology (Lim and Sears 1989; Lee *et al.* 1993; Gundersen *et al.* 1994). However, this system is time consuming, laborious and complicated, and often results in misleading conclusions.

Electron microscopy

Observation of phytoplasma bodies in plant phloem by transmission electron microscopy provides direct verification of phytoplasma involvement with any plant disease. In most infected herbaceous plants, phytoplasmas may be observed in the phloem of roots, shoots, flowers and leaves (Jiang *et al.* 2004; Christensen *et al.* 2005). However, phytoplasmas are usually difficult to find in woody plants (Jiang *et al.* 2004). Since some walled bacterial pathogens including BLOs share the phloem habitat of plants, careful examination of electron micrographs is required. Although electron microscopy provides a direct method for detection of phytoplasmas, the characteristic pleomorphic morphology is of little help in the specific identification of these organisms, since there are numerous similar membranous bodies in crude extracts (Christensen *et al.* 2005).

Immunology

Mono and polyclonal antibodies and cloned DNA probes developed in the 1970s, 1980s and 1990s have proven to be useful in analysing phytoplasmas and for rapid disease diagnosis (Wolfe *et al.* 1983; Blomquist *et al.* 2001; Barbara *et al.* 2002; Kakizawa *et al.* 2004). Using partially purified phytoplasma preparations (intact organisms or membrane fractions) as immunogens, monoclonal antibodies have been raised against several phytoplasmas including aster yellows (Sinha and

Benhamou 1983; Lin and Chen 1985; 1986; Vera and Milne 1994), clover phyllody (Sinha 1974; Clark *et al.* 1983), X-disease (Sinha and Chiykowski 1984) and primula yellows (Clark *et al*. 1989).

Polyclonal antisera often have shown low specificity and reacted with antigens from healthy plants or insect vectors, although antisera prepared from phytoplasmas purified from insects have less background reaction with healthy plant antigens (Barbara *et al.* 2002; Herrera M and Madariaga V 2003). Polyclonal antisera are not, therefore, useful for differentiating phytoplasmas.

Monoclonal antibodies react with only one epitope (antigenic site) of the selected antigen (Blomquist *et al*. 2001; Wei *et al.* 2004). This has improved the reliability of immuno-identification of phytoplasmas. Phytoplasma antigens were detected in preparations from diseased lettuce and periwinkle plants using monoclonal antibodies (Guesdon *et al*. 1979; Loi *et al.* 2002). These monoclonal antibodies did not react with phytoplasmas from ash yellows, elm yellows, maize bushy stunt and paulownia witches' broom infected plants (Guesdon *et al.* 1979; Loi *et al.* 2002). Monoclonal antibodies are particularly useful for differentiating closely related strains of phytoplasmas (Guesdon *et al.* 1979). Monoclonal antibodies raised against primula yellows cross-reacted with the European strain of aster yellows and differentiated aster yellows from clover phyllody phytoplasmas (Wei *et al.* 2004). The limitation of monoclonal antibodies is their specific recognition of a single epitope, i.e. homogeneous monoclonal antibody recognises only a single antigenic site on complex immunogens. Therefore, if a particular antigenic site is shared among different immunogens, it is unable to differentiate the immunogens. Thus, certain monoclonal antibodies may sometimes fail to identify the specific disease causing agent in plants with symptoms associated with phytoplasmas.

Enzyme-linked immunosorbent assay (ELISA) has been employed to analyse phytoplasmas (Clark *et al.* 1986; Brzin *et al.* 2003). Earlier applications using polyclonal antisera prepared from partially purified phytoplasmas in ELISA for identification of phytoplasmas in infected plants indicated that polyclonal antisera could not readily differentiate some related phytoplasma strains (Batlle *et al.* 2004; Rowhani *et al.* 2005). However, with proper controls such as cross-absorbing antisera with healthy plant extracts, polyclonal antisera clearly detected disease-specific antigens in infected plants and, in most cases, effectively differentiated distantly related phytoplasmas (Garcia-Chapa *et al.* 2004b). The combination of ELISA and immuno-fluoresence microscopy using monoclonal antibodies greatly improved both the specificity and sensitivity of ELISA for detection and differentiation of phytoplasmas. ELISA using phytoplasma-specific monoclonal antibodies has also been applied to phytoplasma detection in individual insect vectors (Brzin *et al.* 2003; Bertin *et al*. 2004).

Dot blot immuno-assays using tomato big bud phytoplasma-specific monoclonal antibodies were used to differentiate phytoplasma strains in the aster yellows (AY) phytoplasma strain cluster (Bertin *et al.* 2004). The antibodies specifically reacted with the tomato big bud phytoplasma and all other strains in the aster yellows phytoplasma group but phytoplasmas in other groups didn’t react. The dot blot immuno-assay was also applied for detecting phytoplasmas in vector insects (Bertin *et al.* 2004).

Immuno-fluorescence, usually with ELISA, has been used to detect phytoplasmas in tissues (Bertin *et al.* 2004). Although polyclonal antisera could be used in this approach for detection of phytoplasmas in infected tissues for identification and differentiation of phytoplasmas, they did not show high specificity (Garcia-Chapa *et al.* 2004b). In contrast, monoclonal antibodies bound specifically to phytoplasmas in sections of phloem tissue of diseased plants (Bertin *et al*. 2004). The advantages of immuno-fluorescence microscopy are high sensitivity, specificity and simplicity. Immuno-fluorescence microscopy may be useful for revealing the intracellular distribution of phytoplasmas at the light microscopy level (Bertin *et al*. 2004).

Molecular biology

The development of molecular techniques has allowed significant progress to be made in detection and identification of phytoplasmas. Following the first report on DNA probes cloned from the insect vector *Colladonas montanus* (Van Duzee) (Hemiptera: Cicadellidae) (Kirkpatrick *et al*. 1987), other DNA fragments have been cloned from many phytoplasma strains including X-disease (Jiang *et al.* 1989), aster yellows (Kirkpatrick *et al.* 1987; Kuske and Kirkpatrick 1992), elm yellows (Kirkpatrick *et al.* 1987), walnut witches'-broom, pigeon pea witches'-broom (Harrison et al. 1991) and lethal yellowing of coconut palm (Harrison et al. 1992).

Nucleic acid hybridisation

Cloned phytoplasma DNA used as probes have been applied in dot-blot and Southern-blot hybridisation assays for detection and identification of phytoplasmas in plants and insect hosts (Garcia-Chapa *et al*. 2004). DNA probe of walnut witches'-broom (WWB) phytoplasma hybridised to DNA from WWB and pecan bunch tissues, but not to DNA from other unrelated phytoplasmas such as westem X, aster yellows, beet leafhopper-transmitted virescence agent, and *Spiroplasma citri* Saglio (Spiroplasmataceae) (Bosco *et al*. 2002; Marzachi 2004). A number of probes developed from other phytoplasmas were used to investigate their genetic inter-relatedness. Clover proliferation and potato witches'-broom phytoplasmas were shown to be closely related to each other, but distinct from aster yellows and clover phyllody phytoplasmas on the basis of dot-blot and Southern-blot hybridisation (Seemüller and Schneider 2004). Hybridisation studies have shown that phytoplasmas from diverse geographic origins share high nucleotide sequence homology with one another but show relatively little homology with other phytoplasmas or culturable mollicutes (Seemüller and Schneider 2004). Dot-blot and Southern-blot hybridisation have been used to establish distinct phytoplasma groups such as aster yellows, elm yellows, peach X and virescence (Garcia-Chapa *et al*. 2004a).

Polymerase chain reaction

The discovery of PCR assays in the mid 1980s greatly improved both the speed of phytoplasma disease diagnosis and accuracy of test results. PCR provides a highly sensitive method for detection of DNA. It enables the copying of a single DNA molecule over a billion times in a few hours. PCR assays using universal primer pairs designed on the basis of 16s rDNA sequence have been employed effectively to detect and identify a broad array of known and unknown phytoplasmas from various host plants and vector insects. However, universal primer pairs alone are not applicable for epidemiological studies where several phytoplasmas are associated with a given disease. Group specific primers have been applied to detect mixed-phytoplasma infections in a single host plant (Lee *et al.* 2000; Galetto *et al.* 2005). Single PCR amplification occasionally fails to detect phytoplasmas as a result of uneven distribution or low titre of phytoplasmas in host tissues. Nested-PCR assay provides increased detection sensitivity although there is a greater chance of obtaining false positives in this method due to contamination (Marzachi 2004).

Restriction fragment length polymorphism (rflp)

In earlier applications, RFLP together with dot or Southern blot hybridisation assays had been used for differentiation and characterisation of phytoplasmas. Paulownia witches' broom (PWB) and clover proliferation (CP) phytoplasmas showed similar genomic organisation by dot blot hybridisation and RFLP analyses. RFLP and hybridisation analyses revealed that grapevine flavescence doree and southern European grapevine yellows shared some regions of DNA sequence homology but were distinct from each other (Bosco *et al.* 2002). Currently, RFLP and sequence analyses of PCR-amplified 16s rRNA gene are widely applied for identification and characterisation of phytoplasmas. More than 16 phytoplasma groups and over twenty-five subgroups have been identified by RFLP and 16s rDNA sequence analyses (IRPCM 2004). RFLP analysis of 16s rRNA gene sometimes fails to differentiate closely related phytoplasmas since this gene is highly conserved in phytoplasmas (Lee *et al*. 2000).

1.5 Epidemiology of phytoplasma diseases

Phytoplasmas are spread by phloem feeding insects, mainly leafhoppers, planthoppers and psyllids (Kirkpatrick 1989; Fletcher *et al.* 1998; Lee *et al.* 2000), and by vegetative propagation methods such as cuttings and grafting (Hibben and Wolanski 1970; Chiykowski 1988; Lee *et al.* 2000). This section analyses information on vectors and alternative hosts of phytoplasmas with particular emphasis on Australian conditions.

Phytoplasmas are believed to cause yellows type diseases in hundreds of plant species. Worldwide, important crop diseases caused by phytoplasmas are aster yellows, apple proliferation, European stone fruit yellows, coconut lethal yellows, elm yellows, ash yellows, grapevine yellows, peach X disease and pear decline (Agrios 2005). In Australia, phytoplasmas are associated with many crop diseases and some of them cause significant yield loss. Outbreak of tomato big bud (TBB) (Hill and Mandryk 1954), lucerne witches' broom (Helms 1957), little leaf disease of subtropical pasture legumes (Bowyer *et al.* 1969), Australian grapevine yellows (AGY) (Magarey and Wachtel 1986, 1986), papaya dieback (PDB) (Glennie and Chapman 1976; Gibb *et al*. 1996a; Liu *et al*. 1996) and Australian lucerne yellows (ALuY) (Pilkington *et al*. 1999) caused significant financial and economic losses.

1.6 Epidemiology of phytoplasma diseases of crops in Australia

Methods of dissemination

There are several factors that influence the dissemination of phytoplasmas, both locally and geographically, and set this group of plant pathogens apart to some extent from the other types of plant disease agents. A degree of vector specificity, limitations in vector efficiency and distribution in some cases, absence or rare occurrence of seed transmission and difficulty of mechanical transmission limit phytoplasma spread. On the other hand, the presence of alternative host plants for survival and overwintering of the phytoplasma and its vector(s) as well as long retention of phytoplasmas by vectors are factors that may favour phytoplasma disease spread. The susceptibility of a number of vegetatively propagated plant species to infection by phytoplasmas, and introduction of crop plants into new areas are factors that have contributed to the spread of phytoplasmas.

Several phytoplasma diseases in Australia occur in vegetatively propagated plants. Among them are phytoplasmas causing SPLL, AGY, potato purple top (PPT), strawberry lethal yellows/green petal (SLY) and stone fruit disease. Despite the relatively large number of phytoplasma diseases that occur in vegetative propagated plants, evidence for extensive spread of these diseases in nursery stock or other material of this nature is limited. There is no evidence of transmission of AGY by cuttings, instead vector transmission is likely (Constable *et al.* 2003) but no insect vector has been identified. A phytoplasma in stone fruit in Australia is primarily disseminated by nursery stock and provision of testing services for the nursery industry in states where the nursery stock is produced has been suggested (Anonymous 1998).

Although evidence is limited in Australia on dissemination of phytoplasma diseases by vegetative propagation, such information is essential in economic crops (eg. grapevine) because phytoplasma diseases can readily be transmitted from plant to plant by commonly used vegetative propagation methods such as cuttings or grafting (Hibben and Wolanski 1970; Chiykowski 1988; Lee *et al.* 2000). AGY phytoplasma is occasionally detected in symptom-free vines (Constable *et al.* 2003) suggesting that cuttings from apparently healthy plant material could spread the pathogen.

One of the most important factors in the dissemination of plant diseases has been the introduction of crop plants into areas where they had not previously been grown (Agrios 2005). The fact that many economically important crops widely grown in Australia are introductions from the Old or New Worlds leaves open the possibility that these crops could be highly susceptible to plant diseases native to Australia. A recent report (Byliss *et al.* 2005) of *Candidatus* Phytoplasma australiense in Paulownia trees introduced from China as a new plantation timber species illustrates this risk.

The possible introduction of plant diseases by the movement of plants and plant products across geographically isolated locations could result in occurrence of diseases in native plants in epidemic proportions. According to Padovan *et al.* (2000), worldwide distribution of cultivated strawberry might be the key to the original distribution of PYL phytoplasma, which now causes a destructive disease of *P. tenax*, a plant native to New Zealand. Infected runners introduced from overseas could be the original source of inoculum, and later the disease might have been spread by infected runners during propagation or by vectors. A phytoplasma identified to be closely related to the western X group, which includes serious pathogens of fruit trees in Europe and North America (Schneider *et al.* 1999), was detected in poinsettia with free branching characteristics (PoiBI) (Schneider *et al.* 1999). It is likely that PoiBI phytoplasma was introduced to Australia with infected nursery material (Schneider *et al.* 1999).

Insect vectors play a major role not only in the spread of many phytoplasmas from infected to healthy plants but also in determining phytoplasma host plants as phytoplasmas show a high degree of vector specificity (Lee *et al.* 1998).

Phytoplasmas are transmitted in a circulative, reproductive and persistent manner by their vectors (Purcell 1982; Hacket and Clark 1989; Fletcher *et al.* 1998). To acquire the phytoplasma, the vector must feed on a source of inoculum for a certain period of time called acquisition access period (AAP) (Purcell 1982). Once ingested by the vector, phytoplasmas cross the gut barrier and reach the hemolymph (Hacket and Clark, 1989, Fletcher *et al.* 1998). From the hemolymph, the phytoplasma infects other organs and tissues of the vector including the salivary glands (Fletcher *et al.* 1998). Once the phytoplasma reaches the salivary glands, the vector can transmit it during feeding on healthy susceptible plants. The time between vector acquisition of the phytoplasma and the completion of its transmission is called incubation/latent period (Purcell 1982). After acquiring the phytoplasma, the vector remains infective for life (Fletcher *et al.* 1998).

Several insect species, all of them in the Cicadellidae, are known to transmit phytoplasma diseases in Australia. The common brown leafhopper *Orosius orientalis* (Matsumura) (=*Orosius argentatus* (Evans)) (Deltocephalinae: Opsiini) is a vector of the TBB phytoplasma in tomato (Hill, 1943), several legume species (Hill 1943; Hutton and Grylls 1956), and lucerne (Hill and Helson 1949). *Orosius lotophagorum* (Kirkaldy) is a vector of little leaf disease (Behncken 1984). Other suspected phytoplasma vector leafhopper species include *Austroagallia torrida* (Evans) (Agalliinae) in rugose leaf curl (Grylls 1954) and TBB (Pilkington *et al.* 2004), and *Batracomorphus angustatus* (Osborn) (Iassinae: Iassini) in TBB (Grylls 1979). Insects in the Cixiidae, Fulgoridae and Psyllidae are also potential vectors of phytoplasmas in Australia but so far none has been identified from these families.

Padovan *et al.* (2001) detected, using PCR, sweet potato little leaf (SPLL-V4) and stylosanthus little leaf (StLL) phytoplasmas in field collected *Orosius* sp., and vigna little leaf (ViLL) phytoplasma in *Batracomorphus* sp. and *A. torrida.* Blanche *et al*. (2003a) suggested that *Chiasmus varicolor* (Kirkaldy) (Deltocephalinae: Chiasmusini), collected from cynodon white leaf (CWL) phytoplasma infected lawn and tested positive for the phytoplasma, may be a vector of cynodon white leaf (CWL) phytoplasma in Australia. However, data from a follow up study (Blanche *et al.* 2003b) to determine vector status of this insect for CWL disease failed to support that hypothesis. In field experiments, no transmission occurred either between individual *Cynodon dactylon* Author (Poaceae) plants or between *C. dactylon* and sugarcane (*Saccharum* spp.). *C. varicolor* individuals might have failed to transmit the phytoplasma or there may be a requirement for the plants to be stressed (for example water stress) before succumbing to infection by CWL phytoplasma (Blanche *et al.* 2003b). In caged experiments, *C. varicolor* individuals tested positive for CWL phytoplasma after a four day acquisition access period and a three week incubation period to *C. dactylon* with CWL phytoplasma (Blanche *et al.* 2003b). However, following transfer from infected cynodon to healthy *Zea mays* L. (Poaceae), *C. dactylon* or *Saccharum* spp., all individual *C. varicolor* survivors and test plants were negative. *C. varicolor* individuals that tested positive for CWL phytoplasma following acquisition and incubation periods might have ingested the phytoplasma without the phytoplasma multiplying in the

hemolymph and concentrating in the salivary gland (Blanche *et al.* 2003b), a key requirement for a phytoplasma to be transmitted by the vector (Purcell 1982; Fletcher *et al.* 1998). It is important to note in this connection that the overwhelming majority of phytoplasmas associated with crop and non-crop plants in Australia belong to the faba bean phyllody (FBP) group (Davis *et al.* 1997a; Schneider *et al.* 1999), and most of them have been detected in plants belonging to the Fabaceae. This suggests that one or a few widespread species of polyphagous insect vectors, such as *O. orientalis*, may be responsible for the successful spread of this group of phytoplasma throughout Australia.

The presence of wild host plants is potentially important for the epidemiology of a phytoplasma disease because the pathogen could survive and spread without the presence of susceptible crop plants. As would be expected, however, the role of non-crop reservoirs in disease epidemiology varies considerably with different phytoplasma diseases. Padovan *et al.* (2001) stated that the role of non-crop plant species in the epidemiology of phytoplasma diseases of papaya in Northern Australia is minimal. They emphasised that TBB and SPLL phytoplasmas, which occurred in 94% of infected papaya, were present in only a few individual plants of some weed species. However, because TBB and SPLL are the most widespread phytoplasmas in Australia (Davis *et al.* 1997a), the role of non-crop plants as sources of inoculum in papaya disease could be important. Furthermore, *O. orientalis*, which is also widespread throughout Australia, is the most likely vector of these phytoplasmas. It is highly likely, therefore, that non-crop plants particularly those in the Fabaceae family could be sources of inoculum for the vector and papaya. According to Davis *et al.* (1997a), these phytoplasmas were associated mainly with plants in the Fabaceae family in Australia.

In other cases, non-crop hosts of phytoplasmas play important roles in disease epidemiology (Streten *et al.* 2005b; Carraro *et al.* 2002). The weed *Gomphocarpus physocarpus* E.Mey (Apocynaceae) (balloon cottonbush), which tested positive for *Candidatus* Phytoplasma australiense, TBB and SPLL phytoplasmas, is a reservoir to the major phytoplasma strain clusters in Australia (Streten *et al.* 2005b). It is not known, however, if this plant is also a host to the known or potential phytoplasma vector leafhoppers, for example *O. orientalis*. Wild *Prunus* spp. (Rosaceae) play fundamental role in the epidemiology of European stone fruit phytoplasma (Carraro *et al.* 2002). Uncultivated trees of the species *P. spinosa, P. ceracifera* and *P. domestica* not only were found to be infected by stone fruit yellows phytoplasma but also were colonised by *Cacopsylla pruni* (Scopoli) (Hemiptera: Psyllidae), vector of the phytoplasma (Carraro *et al.* 2002).

It is of interest that *G. physocarpus* has been a host for genetically distinct phytoplasmas in Australia. Natural infection of a plant species by more than one type of phytoplasma strain at a time may be a result of either inoculation by different vectors, each capable of transmitting only one type of phytoplasma, or one or two vector species capable of transmitting genetically unrelated phytoplasmas. According to Kusunoki *et al*. (2002) the leafhopper *Hishimonus sellatus* (Uhler) (Deltocephalinae: Opsiini) transmitted rhus yellows and hovenia witches' broom phytoplasmas in Japan.

Sugarcane white leaf (SCWL), sugarcane grassy shoot (SCGS) and cynodon white leaf (CWL) phytoplasmas, detected in a number of native and introduced grasses other than sugarcane in northern Australia, could be a potential threat to the sugarcane industry in the region (Blanche *et al.* 2003a). The epidemiological role of these alternative hosts at the present time is limited and insect vectors are unknown.

It is generally believed that phytoplasmas are not seed borne. Sieve elements of the plant phloem, to which phytoplasmas are thought to be confined (McCoy 1979), lack direct connection to the developing embryo (Cordova *et al.* 2003) and this makes seed transmission unlikely. Systemic infection of plants occurs when phytoplasmas are moved with photosynthates (products of photosynthesis) (Cordova *et al.* 2003). Phytoplasmas have been found in floral tissues (Clark *et al.* 1986) and sterility in phytoplasma-infected plants (Lee *et al.* 2000) could be the direct effect of phytoplasma presence in floral tissues.

The report on the presence of phytoplasma DNA in embryos of coconut palm infected with lethal yellowing disease (Cordova *et al.* 2003) constitutes the first peer-reviewed study that lends broad support for earlier (non-refereed) reports of seeds and seedlings of *Medicago sativa* testing positive for witches' broom phytoplasma (Khan *et al.* 2002). Collectively, these recent reports indicate the possibility of seed transmission in certain plant host-phytoplasma pathosystems, a phenomenon that clearly needs further attention because of the major implications for disease management at both the local scale and international phytosanitary arrangements.

Origins of Australian phytoplasmas

The origins of Australian phytoplasmas are unclear, but it is of interest that Davis *et al.* (1997a) suggested that Australian phytoplasmas might have Australasian/Asian origin. TBB and SPLL phytoplasmas showed a close genetic relationship, based on restriction fragment length polymorphism (RFLP) and sequence analyses, to the sunhemp witches' broom (SUNHP), sesame phyllody and phyllody of *Cloeme viscose* phytoplasmas from Thailand (Davis *et al.* 1997a). TBB/SPLL and AGY are the major phytoplasma strain clusters prevalent in Australia (Davis *et al.* 1997a). Australian TBB and AGY are phylogenetically distant groups of phytoplasmas (Davis *et al.* 1997a; Schneider *et al.* 1997) and were described under two taxa: *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense (IRPCM 2004). *Candidatus* Phytoplasma aurantifolia, first described in Zreik *et al.* (1995) to denote the phytoplasma associated with witches' broom disease of small-fruited acid lime, now included Australian TBB, papaya yellow crinkle (PYC), papaya mosaic (PM), SPLL and others because the 16S rRNA gene of these phytoplasmas shares 99.5% sequence similarity (IRPCM 2004). *Candidatus* Phytoplasma australiense is associated with the plant diseases AGY, PDB, strawberry lethal yellows (SLY), and strawberry green petal (SGP) in Australia and to *Phormium* yellow leaf (PYL) disease in New Zealand (Streten *et al.* 2005a; 2005b). Australian TBB is by far the most widespread phytoplasma on the continent (Gibb *et al.* 1996b).

In further support for the theory of an Australasian/Asian origin of Australian phytoplasmas, Davis *et al.* (1997a) assert that PYL, which has close genetic relationship with PDB and AGY, is endemic to New Zealand because its host *Phormium tenax* and its vector *Oliarus atkinsoni* Myers (Cixiidae) are native to New Zealand (Davies *et al.* 1997a). If both the host and the pathogen were native to New Zealand and *P. tenax* was the primary host to PYL phytoplasma, they could be expected to have reached a state of equilibrium, through long association, in which the host plant could support phytoplasma increase without showing appreciable injury or even without showing symptoms. In the *Phormium* yellows phytoplasma pathosystem, however, the disease invariably causes symptoms in infected plants and sometimes death (Liefting *et al.* 1996). This may indicate that PYL in New Zealand, as now constituted, has not had long association with the plant (*P. tenax*) and that the phytoplasma had its origin in some other type of plant or was introduced to the country via infected plant material or other means.

According to Davis *et al.* (1997a), Australian phytoplasmas may not have European or North American origin. However, there is much evidence in support of the origin of at least some Australian phytoplasmas being in Europe. The AGY, which, in its symptomatology, resembles flavescence doree (known to occur only in France, Italy and Spain), bois noir (occurs only in France and Germany) and other grapevine yellows diseases, might have evolved from the same gene pool as Stolbur (STOL) of *Capsicum annum* L. (Solanaceae) from Serbia and grapevine yellows from Germany (Davis *et al.* 1997b). Though potentially originating that gene pool, the geographical location of Australia might have provided the ecological isolation that favoured its unique evolution of AGY (Davis *et al.* 1997b). Two sequences (at positions 5'191-215 3'and 5'999-10133') in the AGY 16S rDNA were not present in 16S rDNA of either STOL or VK (Davis *et al.* 1997b). 16S rDNA amplified from AGY had a different RFLP profile with *Alu*I and *Mse*I from that of either STOL or VK (Davis *et al.* 1997b) probably because of the variation in base identities in their 16S rDNA.

Allocasuarina muelleriana (Miq.) L. A. S. Johnson (Casuarinaceae) (a native Australian tree) yellows (AlloY) disease is caused by *Candidatus* Phytoplasma allocasuarinae (Gibb *et al.* 2003; Marconi *et al.*

2004) that is most closely related to the buckthorn witches' broom (BWB) phytoplasma (*Candidatus* Phytoplasma rhamni) associated with the lethal witches' broom disease in *Rhamnus catharticus* L. (Rhamnaceae) in south-west Germany (Gibb *et al.* 2003), sharing 96% 16S rDNA sequence similarity (Gibb *et al.* 2003; Marconi *et al.* 2004). Both *Candidatus* Phytoplasma allocasuarina and *Candidatus* Phytoplasma rhamni belong to the apple proliferation (AP) (16Sr X) subclade (IRPCM 2004).

In Australia, *Rhamnus alaternus* L. (Rhamnaceae) is also called buckthorn and is common in Western Australia (WA) and South Australia (SA) (Gibb *et al.* 2003). It is not known, however, if *R. alaternus* (Australian buckthorn) is also a host to *Candidatus* Phytoplasma allocasuarinae. Gibb *et al.* (2003) suggested that if the Australian buckthorn is found to be infected in nature by *Candidatus* Phytoplasma allocasuarinae, it could provide an explanation for the close relationship between the Australian and European phytoplasmas within the apple proliferation (AP) phytoplasma group (16Sr X).

Padovan *et al.* (2000) cast doubt on the idea that PYL/*Candidatus* Phytoplasma australiense is endemic to New Zealand. They indicated that a phytoplasma from strawberry in Florida, USA, was placed in the same taxonomic group (16SrRNA I-J) as AGY (Jomantiene *et al.* 1998) suggesting genetic similarities between *Candidatus* Phytoplasma australiense and the phytoplasma isolated from strawberry in Florida, USA. Neither the origin nor the course or means of dissemination of *Candidatus* Phytoplasma australiense is clear. The association of the monophagous planthopper *O. atkinsoni*, the only known vector of Phytoplasma australiense in New Zealand (Liefting *et al.* 1997), and *P. tenax* could provide an explanation for the ability of this insect to transmit PYL disease in New Zealand. The vector(s) of *Candidatus* Phytoplasma australiense is unknown in Australia. Absence of *O. atkinsoni* in Australia and, more important, association of the insect only with *P. tenax* suggest that other, unidentified, vector(s) of *Candidatus* Phytoplasma australiense might exist in Australia; or that the disease is spread by other means, possibly through propagation materials. Under current circumstances, preventing introduction of *O. atkinsoni* from New Zealand into Australia and use of clean propagation material is important.

It seems probable that Australian phytoplasmas have their origin both in Australasia/Asia and Europe/North America. The *Candidatus* phytoplasma aurantifolia strain cluster might have its origin in Australasia/Asia and the *Candidatus* phytoplasma australiense strain cluster might have originated in Europe or North America. The *Candidatus* phytoplasma australiense is not only genetically distinct from the *Candidatus* Phytoplasma aurantifolia but also has genetic relationship to the stolbur phytoplasma, which is distantly related genetically to the aster yellows phytoplasma (Schneider *et al.* 1997). 16S rRNA gene sequences of 27 previously described phytoplasma '*Candidatus'* species were subjected to pairwise similarity analysis using Neighbor-Joining (NJ) method for clustering. The resulting phylogenetic tree (Fig. 1) shows that *Candidatus* Phytoplasma australiense has genetic relationship with the stolbur phytoplasma (16Sr XII) that predominantly exists in Europe (Lee *et al.* 2000). The widespread occurrence and success of *Candidatus* Phytoplasma aurantifolia in Australia might have been the result of its arrival to the continent from Asia much earlier, as a result of geographical proximity, or had its origin in the Australia. The *Candidatus* phytoplasma australiense strain cluster, in contrast, has limited distribution and host range in Australia and probably arrived on the continent more recently.

1.7 Management of phytoplasma diseases

The goal of studying plant diseases is to prevent or control the diseases. Since phytoplasmas were discovered as agents of yellows-type plant diseases, different control measures have been used with varying degrees of success. These include exclusion of insect vectors and inoculum sources, chemotherapy, disease-free stocks and breeding of disease-resistant varieties.

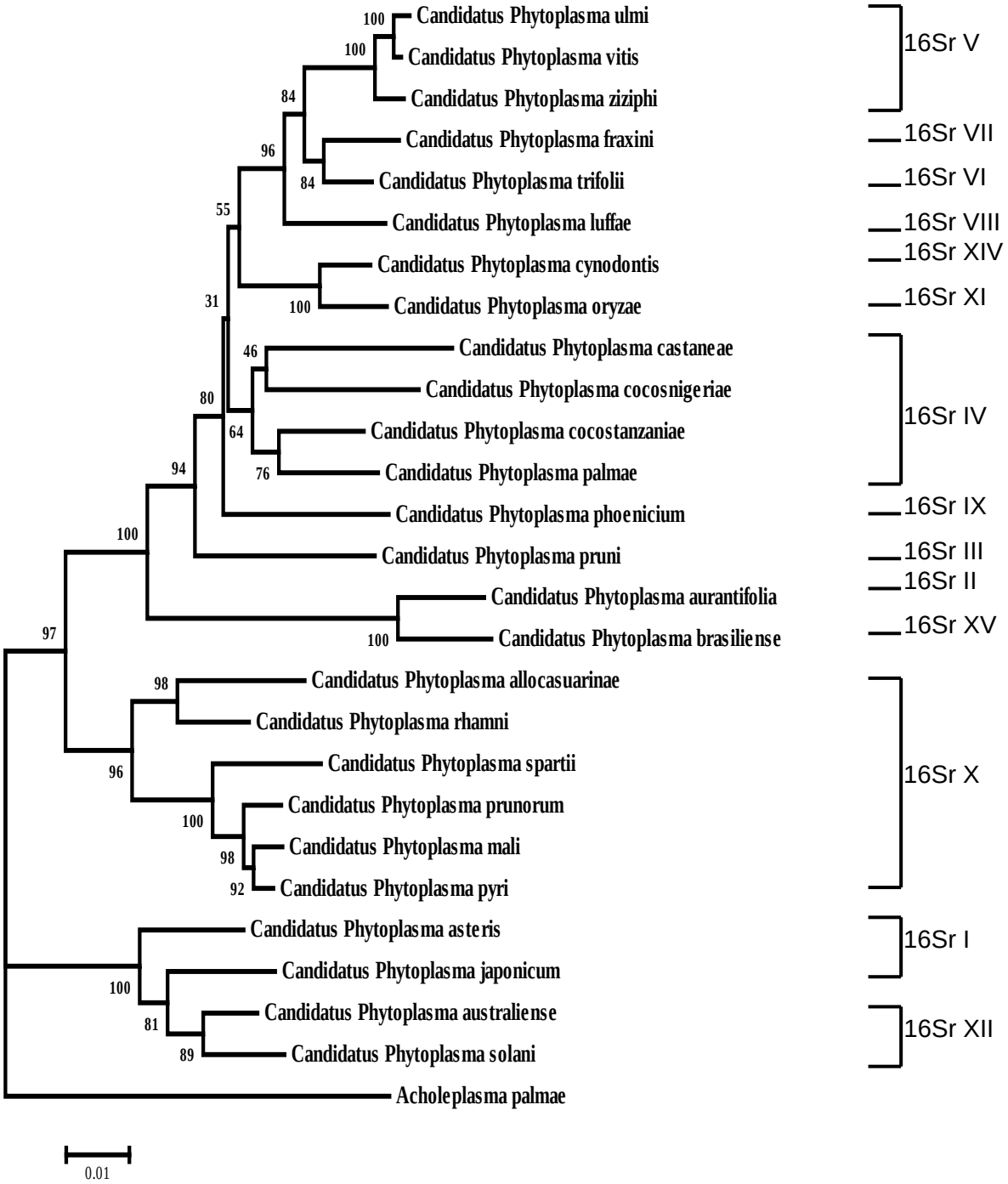

Fig1.1 Phylogenetic tree constructed by the neighbour-joining method of 16S rRNA gene sequences from previously described phytoplasmas, employing *Acholeplasma palmae* as an out-group. Bar, 1 substitution in 100 nucleotides. Numbers on the nodes are bootstrap (confidence) values.

Management of vector insects and non crop hosts has been successfully used against certain fungal, bacterial and virus diseases. Insecticide application is the most commonly used method of controlling vector insects, but this approach is not sustainable. Pesticide residues pollute ground water systems and few insecticides discriminate between harmful and beneficial insects. Other methods such as physical barriers exclude vector insects from protected plants and consequently reduce the impact of vector-borne plant diseases. Squash plants covered with fine-mesh fabric secured in such a way that aerial insects were excluded, were PCR negative for cucurbit yellows bacterium and had no foliar symptoms (Bextine *et al*. 2001). On the other hand, 3% disease incidence and 25% PCR positive plants were observed in non-covered plants (Bextine *et al*. 2001). Physical barriers also reduce insect damage to plants as a result of direct feeding. In radish plants, 33% reduction in feeding damage by *Delia radicum* L. (Anthomyiidae) was achieved in plots fenced with fine nylon fabric (135cm high with 25cm overhangs) compared with unfenced control plots (Michael *et al*. 2001). Although not commercially viable, exclusion of insects with insect proof nets was effective in managing dieback disease of papaya caused by *Candidatus* Phytoplasma australiense (Walsh *et al*. 2006). In general, other than on experimental scale, literature is scanty on successful control of phytoplasma diseases through vector management. Investigation of migration patterns of potential and known phytoplasma vector insects may be useful to predict new disease outbreaks and disease spread, thus contributing to effective management of these diseases.

It is believed that ALuY phytoplasma is vectored by one or all of the leafhoppers *O. orientalis*, *A. torrida* and *B. angustatus* (Pilkington *et al*. 2004a). There was correlation in spatial and temporal distribution between ALuY disease symptomatic plants and all of these leafhoppers in the field (Pilkington 2003). Transmission experiments showed *O. orientalis* but not *A. torrida* or *B. angustatus* was capable of transmitting ALuY phytoplasma. Plants fed on by *O. orientalis* developed typical ALuY disease symptoms and tested positive for phytoplasma (Pilkington *et al*. 2003; 2004b) but the identity of the phytoplasma was not determined. Insecticide or herbicide treatment of field margin vegetation reduced ALuY disease incidence and catches of *O. orientalis*, *A. torrida* and *B. angustatus* within the crop (Pilkington *et al*. 2004b).

Non-crop plants or wild relatives of crops have the potential to function as reservoirs of phytoplasmas (Wilson *et al*. 2001). For example, the role of wild *Prunus* species is believed to be fundamental in the epidemic cycle of European stone fruit yellows disease (Carraro *et al*. 2002). Identification and targeted control of alternative hosts of phytoplasmas is therefore important to reduce phytoplasma disease incidence.

Tissue culture in combination with either heat or antibiotic (tetracycline) treatment has been used for eliminating phytoplasmas from plants (Converse and George 1987; Dai *et al*. 1997; Wongkaew and Fletcher 2004; Chalak *et al*. 2005). Phytoplasmas are generally heat labile at temperatures as low as 37^{O}C and are not present in plant meristems after extended heat treatment (Converse and George 1987). The critical step using this approach is to ensure the absence of phytoplasmas in the treated plant materials. If phytoplasma-free plants can be established, control of phytoplasma diseases may be expected by combining with insect vector control. Thus, establishment of clean stocks is the first and essential step toward controlling phytoplasma diseases. Large scale use of chemotherapy in phytoplasma disease control is not economically feasible and, in most cases, there is disease reoccurrence (McCoy 1975; 1982).

Breeding of phytoplasma-resistant or tolerant varieties may be the most cost effective way to protect plants from phytoplasma infection. However, there are only a few success stories so far, such as coconut resistant to lethal yellowing (Been 1981; Cardena *et al*. 2003) and paulownia resistant to paulownia witches' broom (Du *et al*. 2005). Traditional plant breeding approaches to develop disease resistant/tolerant cultivars are time consuming and sometimes difficult. Genetic engineering has made it possible to introduce certain pathogen/pest resistant genes into plants (Chen and Chen 1998) and the method is time efficient. These approaches may be preferred alternatives to manage phytoplasma diseases in the future.

2. Association of *Candidatus* Phytoplasma australiense and *Candidatus* Phytoplasma aurantifolia with Australian lucerne yellows (ALuY) disease

2.1 Introduction

Lucerne (*Medicago sativa* L.) is an important forage crop in Australia (MacDonald *et al.* 2004) and the production of lucerne seed is an important sector of Australia's pasture seed industry with annual production in excess of 10,000 tonnes (RIRDC 2004). Australian lucerne yellows disease reduces seed yield of affected plants resulting in an estimated losses of $7 million annually to the Australian lucerne seed industry (Pilkington *et al.* 1999). Affected plants exhibit leaf yellowing or purpling and honey brown/yellow discolouration of the phloem tissue of the tap root (Pilkington *et al.* 1999; 2002). The disease is believed to be caused by phytoplasma (Hellemer 1972; McGechan 1980; Pilkington *et al.* 2003).

Phytoplasmas are plant pathogenic bacteria that inhabit plant phloem and insects, mainly leafhoppers and planthoppers (Lee *et al.* 2000). They are unculturable organisms, and thus cannot be characterised by the traditional techniques used for bacteria classification (Bove and Garnier, 1998). Molecular methods, particularly PCR, have proved to be useful for detection and identification of a wide array of phytoplasmas believed to cause diseases in hundreds of plant species (Lee *et al.* 2000). Sixteen phytoplasma groups have been identified based on variations in their 16S rDNA sequences (IRPCM 2004).

Two major groups of phytoplasmas are known to exist in Australia: 16SrII (Peanut witches' broom) and 16SrXII (stolbur) (Streten and Gibb, 2006). *Candidatus* Phytoplasma australiense, which belongs to the stolbur group (IRPCM, Phytoplasma Taxonomy Group, 2004), is associated with diseases of the most economically important crops: Australian grapevine yellows (AGY), strawberry lethal yellows (SLY), papaya dieback (PDB) and pumpkin yellow leaf curl diseases in Australia, and strawberry lethal yellows and Phormium yellow leaf (PYL) diseases in New Zealand (Streten *et al.* 2005). Australian tomato big bud (TBB), PYC, sweet potato little leaf (SPLL) and PM phytoplasmas belong to peanut witches' broom (16SrII) (IRPCM 2004). Recently, TBB, PYC, SPLL and PM phytoplasmas have been identified under one species name *Candidatus* Phytoplasma aurantifolia because their 16S rRNA gene shows >99.5% sequence similarity (IRPCM 2004).

Phytoplasma bodies have been observed by electron microscopy in the phloem tissues of ALuY symptomatic but not in asymptomatic lucerne plants and a phytoplasma that shared 99% 16S rDNA sequence similarity with TBB, PM and PYC was detected in symptomatic plants (Pilkington *et al.* 2003). Fungal, bacterial and viral pathogens as possible causes of the disease were ruled out (Pilkington *et al.* 2003).

The aims of the present study were to: (1) further evaluate the hypothesis that phytoplasmas are associated with ALuY disease and lucerne plants with and without ALuY disease symptoms were analysed for the presence of phytoplasmas using molecular methods.

2.2 Materials and methods

Plant collection

Plant samples were collected from three lucerne fields at Griffith, Forbes and Orange between November 2005 and February 2006 (Table 2.1). The stands were between 1 and 4 years of age when samples were collected, and had low to high incidence of ALuY disease. Cultivar CW-5558 at Griffith had the highest ALuY disease incidence whilst cultivar PL90 at Orange and cultivar Aurora at the Lachlan Valley had low to moderate ALuY disease incidence. Each plant with foliar ALuY symptoms was dug up and its tap root checked for phloem discoloration (yellowing), which is a typical symptom of ALuY disease (Pilkington *et al.* 1999, 2003).

Table 2.1. Dates and locations of sample collections

Date	Location	Cultivar	No of samples	
			Symptomatic	**Asymptomatic**
Nov. 2005	Griffith	CW 5558	15	5
Jan. 2006	Griffith	CW 5558	25	-
Feb. 2006	Lachlan Valley	Aurora	45	15
Feb. 2006	Orange	PL90	10	-
Total			**95**	**20**

Extraction of DNA

Approximately 0.3g of fresh leaf midribs and petioles from each plant sample was used for total DNA extraction according to Zhang *et al.* (1998). Plant tissue was crushed in cetyltrimethylammonium bromide (CTAB) buffer (2% CTAB, 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl, pH 8.0 and 0.2% mercaptoethanol) at 60^{o}C using an autoclaved and pre-chilled mortar and pestle. After incubation at 60^{o}C for 30 min in a water bath, 600-800µl of chloroform was added to the mixture, vortexed and centrifuged for ten minutes at 13,200rpm in a micro centrifuge. An equal volume of ice-cold isopropanol was added to the supernatant, mixed well and centrifuged for ten minutes. The nucleic acid pellet was re-suspended in 50-100µl TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0) and left at 4^{o}C overnight to dissolve the pellet. Quality and quantity of nucleic acids was estimated by agarose gel electrophoresis, and the extract was kept at -20^{o}C until used in PCR. The mortar and pestle were left overnight in water diluted (1:10) chlorox, rinsed with tap water and autoclaved (121°C and 15psi) between samples to avoid contamination.

Amplification of phytoplasma DNA using generic primers

Fifteen symptomatic and five asymptomatic plants collected from Griffith in November 2005 were tested. Nested polymerase chain reaction (PCR) was carried out for amplification of the 16S rDNA with the phytoplasma generic primer pairs P1 (Deng and Hiruki 1991) / P7 (Kirkpatrik *et al.* 1994) and fU5 (Lorenz *et al.* 1995) / rM23Sr (Padovan *et al.* 1995). Undiluted DNA was used for the first amplification whilst PCR products from the first amplification were diluted (1:30) in sterile distilled water (SDW) before the final amplification. DNA TBB phytoplasma and SDW were used as positive and negative controls, respectively. PCR was carried out using Perkin-Elmer 2400 thermocycler (PE Applied Biosystems, Foster City, California, USA). The PCR mix contained 1X reaction buffer, 1.5mM $MgCl_2$, 0.2mM each dNTPs, 1.25 unit heat activated Immolase DNA polymerase and 0.2µM

of each primer in 25µl reaction volume. All PCR components were supplied by Bioline Ltd. (Bioline, Sydney, Australia). PCR conditions were as follows: 95°C for seven minutes in the first cycle followed by 40 cycles of denaturation at 94°C for 60s, annealing at 55°C for 60s and extension at 72°C for 90s. Final extension was for 9.5 minutes at 72°C.

Restriction fragment length polymorphism (RFLP)

Five microlitres of PCR products from six positive testing symptomatic plant samples were separately digested with four units of the endonuclease *Hpa*II (FisherBiotech, Perth, Australia). Following an overnight restriction digestion at 37°C, products were separated on a 2.5% agarose gel, stained with ethidium bromide and visualized by transillumination in ultraviolet light.

DNA sequencing, primer design and PCR protocols

The 1.46kb products of nested PCR from three plant samples were purified using a Qiaquick PCR purification kit (Qiagen, Melbourne, Australia) and cycle-sequenced using the end primers fU5/m23sr. Sequence chromatograms were assembled using the Staden software package (Staden, 1996) and the sequences were used in BLAST searches of the GenBank database. The most similar sequences from GenBank were downloaded and used to design internal primers for bidirectional sequencing of the entire fU5/m23sr PCR fragments. Consensus sequences of the entire fU5/m23sr fragments were aligned to other phytoplasma sequences downloaded from the GenBank database and used to design internal PCR primers for specific amplification of either *Ca.* Phytoplasma australiense or *Ca.* Phytoplasma aurantifolia, and for bidirectional DNA sequencing of the entire M23sr/fU5 PCR fragments.

On the basis of the alignment, five primers were designed. Primers, synthesized by Sigma-Genosys Ltd. Australia, were: PF (5'-GCATGTCGCGGTGAATAC-3'), WB-rev (5'-CTTATTTCCTTTAATGTTCCGG-3') and YR (5'-TGAGCTATAGG CCCTTAATC-3'), PPseq-1F (5'-TAAAGGAATTGACGGGAC TC-3'), and PPs-eq-1R(5'- TCGAATTAAACAACATGATCC-3').

Primers PF and WB-rev amplify a 400bp fragment from the 16Sr DNA sequences of *Ca.* Phytoplasma aurantifolia and related strains between base positions 990 and 1464. Primers PF and YR amplify a 250bp fragment from the 16S rDNA sequences of *Ca.* Phytoplasma australiense and related strains between base positions 990 and 1313. Sequence numbering was based on a consensus sequence of the entire fU5/m23sr fragment and other 16S rDNA sequences of phytoplasmas that showed high sequence homology with the query sequences (Table 2.2). The primers PPseq-1F and PPseq-1R were designed for bidirectional DNA sequencing of the entire M23sr/fU5 PCR fragments.

PCR optimisation

The optimum PCR conditions for specific amplification of *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense were examined. The variables examined were: magnesium concentration (1.5mM, 1.625mM, 1.75mM and 2mM), primer concentration (0.1µM, 0.2µM, 0.3µM and 0.4µM), Immolase DNA polymerase concentration (1u/µl, 1.25u/µl and 1.5u/µl) and thermal cycling parameters (temperatures and times). Test concentrations of magnesium, primers and Immolase DNA polymerase were the same for both phytoplasmas. The following thermal cycling conditions were tested: annealing temperatures (52°C, 53°C, 54°C, 55°C, 56°C and 57°C for *Candidatus* Phytoplasma australiense specific primers and 53°C, 54°C, 55°C, 56°C, 57°C, 58°C and 59°C for *Candidatus* Phytoplasma aurantifolia specific primers) and extension or annealing time (15s, 20s, 30s, 45s, 60s and 90s for both phytoplasmas).

One hundred and fifteen plant samples including those tested with the generic primers were tested for both phytoplasmas using the optimised PCR conditions. The PCR mix contained 1X reaction buffer,

1.625mM $MgCl_2$, 0.2mM each dNTPs, 1.25 unit heat activated immolase DNA polymerase, 0.2µM each primer and 1µl undiluted template DNA in 25µl reaction volume. All PCR components were supplied by Bioline Ltd. (Bioline, Sydney, Australia). The following PCR amplification programme was used: seven minutes at 95°C; 40 cycles of denaturation at 94°C for 30s, annealing at 54°C (56°C for *Candidatus* Phytoplasma aurantifolia) for 30s and extension at 72°C for 20s. Final extension was for five minutes at 72 °C.

Table 2.2. Strains of *Candidatus* Phytoplasma australiense and *Candidatus* Phytoplasma aurantifolia that show high 16S rDNA sequence similarity with the query sequences

Accession Number	Phytoplasma species	Phytoplasma strain	Acronym	Origin
U43570	*Ca.* Phytoplasma australiense	*Phormium* yellow leaf	PYL	New Zealand
AJ243044	*Ca.* Phytoplasma australiense	Strawberry green petal	SGP	Australia
AJ243045	*Ca.* Phytoplasma australiense	Strawberry lethal yellows	SLY	Australia
L76865	*Ca.* Phytoplasma australiense	Aust. grapevine yellows	AGY	Australia
AY377868	*Ca.* Phytoplasma australiense	Strawberry virescence	SV	Australia
Y10095	*Ca.* Phytoplasma australiense	Papaya dieback	PDB	Australia
Y08173	*Ca.* Phytoplasma aurantifolia	Australian tomato big bud	TBB	Australia
Y10096	*Ca.* Phytoplasma aurantifolia	Papaya mosaic disease	PM	Australia
Y10097	*Ca.* Phytoplasma aurantifolia	Papaya yellow crinkle	PYC	Australia
AF438413	*Ca.* Phytoplasma aurantifolia	Omani alfalfa witches' broom	OAlfWB	Oman

Template concentrations of *Candidatus* Phytoplasma australiense and *Candidatus* Phytoplasma *aurantifolia* in the same DNA samples:

Effects on detection

Effects of DNA concentrations of *Candidatus* Phytoplasma australiense and *Candidatus* Phytoplasma aurantifolia in the same DNA samples on PCR detection of one or the other phytoplasma was investigated. First, DNA of Phormium yellow leaf (PYL) (a strain in the species *Candidatus* Phytoplasma australiense) and Australian TBB (a strain in the species *Candidatus* Phytoplasma aurantifolia) was amplified separately using the phytoplasma generic primers P1 (Deng and Hiruki 1991) and P7 (Kirkpatrick *et al.* 1994). Concentration of PCR products was determined by Qubit fluorometer (Invitrogen, Melbourne, Australia) and standardised to a concentration of 1500ng/ml for each phytoplasma. This concentration was then serially diluted (1:10, 1:100, 1:1000 and 1:1000) using sterile distilled water to achieve four different template concentrations (150, 15, 1.5 and 0.15ng/ml) for each phytoplasma. The different DNA concentrations of the two phytoplasmas were mixed in 16 different combinations (Table 2.3) and used in PCR assays with primers that differentially amplify DNA of PYL or Australian TBB.

Table 2.3 Mixtures of different DNA concentrations of PYL and TBB phytoplasmas**.**

		PYL			
TBB		**150ng/ml**	**15ng/ml**	**1.5ng/ml**	**0.15ng/ml**
	150ng/ml	Combination 1	Combination 2	Combination 3	Combination 4
	15ng/ml	Combination 5	Combination6	Combination 7	Combination 8
	1.5ng/ml	Combination 9	Combination 10	Combination 11	Combination 12
	0.15ng/ml	Combination 13	Combination 14	Combination 15	Combination 16

2.3 Results

PCR optimisation

Table 2.4 summarizes the optimised conditions for specific amplification of *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense.

Table 2.4. PCR optimisation for specific amplification of *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense

Variable	**Range tested**	**Optimised conditions**	
		P. aurantifolia	**P. australiense**
Mg	1.5 – 2mM	1.625mM	1.625mM
Primer	0.1 – 0.4µM	0.2µM	0.2µM
Annealing temperature	52 – 57 °C	56°C	54°C
Immolase DNA polymerase	1 – 1.5u/µl	1.25u/µl	1.25u/µl
Annealing time	15 – 90s	30s	30s
Extension time	15 – 90s	20s	20s

Amplification of phytoplasma DNA using generic primers and RFLP analysis

A 1.46-kb fragment was amplified in nested PCR of 10 symptomatic lucerne plants using the primer pairs P1/P7 and fU5/m23sr that amplify the 16S rDNA of all known phyotplasmas (Padovan *et al.* 2000). No phytoplasma DNA was amplified from asymptomatic plants. Restriction digestion profiles of nested PCR amplicons from five separate symptomatic plants differed from the control (Australian TBB) but were identical to each other. One nested PCR product from ALuY symptomatic plant and the Australian TBB had identical RFLP profiles. RFLP profiles of ALuY and TBB phytoplasmas are shown in Fig. 2.1.

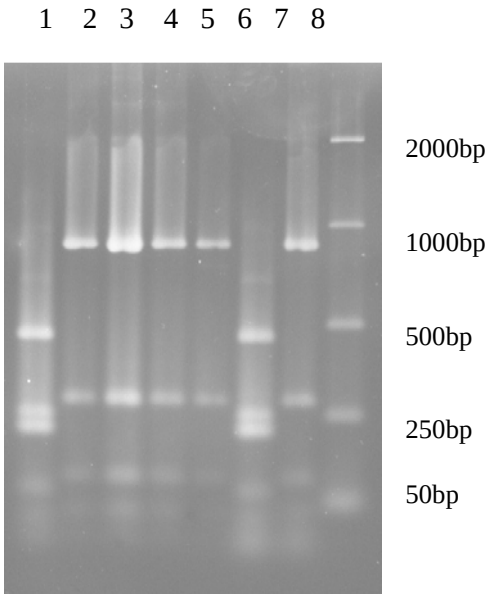

Fig. 2.1 RFLP profiles of phytoplasma 16S rDNA amplified by nested PCR and digested by *Hpa*II. Lane 1 = TBB. Lanes 2-7 = DNA from symptomatic plants. Lane 8 = size marker.

Two PCR products that showed different RFLP profiles from the control (TBB) had identical sequences and the longer sequence has been deposited in the GenBank database (accession number DQ786394). A BLAST search with this sequence showed >99.6% sequence similarity with *Candidatus* Phytoplasma australiense (L76865) and related strains papaya dieback (Y10095), Phormium yellow leaf (U43570), strawberry green petal (AJ243044), strawberry lethal yellows (AJ243045) and strawberry virescence (AY377868) (Table 2.5). The third query sequence that showed the same RFLP profile as the control (Australian TBB) had identical 16S rDNA sequence to papaya yellow crinkle (PYC) and papaya mosaic (PM) phytoplasmas.

Table 2.5 16S rDNA sequence similarities among strains of *Candidatus* Phytoplasma australiense and ALuY phytoplasma

	ALuY	PYL	SLY	SGP	AGY	SV	PDB
ALuY	***	99.73	99.67	99.67	99.75	99.74	99.75
PYL	…	***	99.00	99.00	99.00	99.00	99.00
SLY	…	…	***	99.00	99.00	99.00	99.00
SGP	…	…	…	***	99.00	99.00	99.00
AGY	…	…	…	…	***	99.00	99.00
SV	…	…	…	…	…	***	99.00
PDB	…	…	…	…	…	…	***

Amplification of *Candidatus* Phytoplasma australiense and *Candidatus* Phytoplasma aurantifolia using specific primers

Figure 2.2 shows an example of PCR amplification of *Candidatus* Phytoplasma aurantifolia from ALuY symptomatic plants using specific primers. Fifty two ALuY symptomatic plant samples collected from the Lachlan Valley and Griffith tested positive for phytoplasma (Table 2.6). *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense were detected in 29 and 23 samples, respectively. Of 45 symptomatic plant samples collected from the Lachlan Valley, 26 and two plant samples tested positive for *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense, respectively. Twenty-one and two plant samples from Griffith tested positive for *Candidatus* Phytoplasma australiense and *Candidatus* Phytoplasma aurantifolia, respectively. One sample (from Griffith) tested positive for both phytoplasmas.

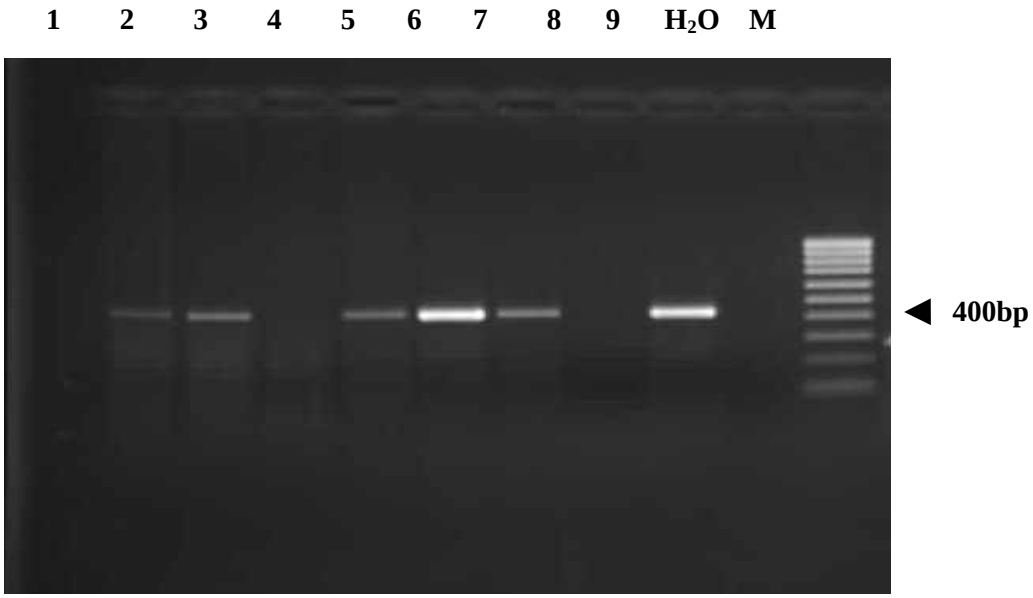

Fig. 2.2 PCR amplification of *Candidatus* Phytoplasma aurantifolia from ALuY symptomatic lucerne plants (Lanes 1-8 are ALuY symptomatic plants; Lane 9 is positive control)

Table 2.6 PCR detection of *Candidatus* Phytoplasma australiense and *Candidatus* Phytoplasma aurantifolia in ALuY symptomatic and asymptomatic plant samples collected from different locations

Sample origin		Symptomatic plant				Asysmptomatic plant			
		Positive			Negative	Positive			Negative
		P. australiense	P. aurantifolia	Both		P. australiense	P. aurantifolia	Both	
Griffith	Nov. 05	10	0	0	5	0	0	0	5
	Jan. 06	11	3	1	10	0	0	0	-
Forbes		2	26	0	18	0	0	0	15
Orange		0	0	0	10	0	0	0	-
Total		**23**	**29**	**1**	**43**	**0**	**0**	**0**	**20**

No phytoplasma DNA was amplified from plant samples collected from the lucerne field near Orange. Both phytoplasmas were significantly associated with ALuY symptoms (P = 0.025, X^2 = 6.05 for *Candidatus* Phytoplasma australiense and P = 0.010, X^2 = 8.16 for *Candidatus* Phytoplasma aurantifolia) (Table 2.7).

Effects of template concentrations on detection of *Candidatus* Phytoplasma australiense and *Candidatus* Phytoplasma aurantifolia from mixed DNA

Different DNA concentrations of *Candidatus* Phytoplasma australiense and *Candidatus* Phytoplasma aurantifolia in the same DNA samples did not affect PCR detection of one or the other phytoplasma. The specific primers detected each respective phytoplasma when the template mixture ratio was both 1:1000 and 1000:1.

Table 2.7 Detection of *Candidatus* Phytoplasma australiense and *Candidatus* Phytoplasma aurantifolia in lucerne

	ALuY symptomatic plants			Asymptomatic plants			X^2	Degree of freedom	P value
	+	-	Total tested	+	-	Total tested			
P. australiense	**23**	**72**	**95**	**0**	**20**	**20**	**6.05**	**1**	**0.025**
P. aurantifolia	**29**	**66**	**95**	**0**	**20**	**20**	**8.16**	**1**	**0.010**

2.4 Discussion

In a previous study to determine the aetiology of ALuY, phytoplasma bodies were consistently observed in sieve elements of Australian lucerne yellows symptomatic lucerne plants but not in asymptomatic plants (Pilkington *et al.* 2003). In the present study, 16S rDNA of two phytoplasmas indistinguishable from *Candidatus* Phytoplasma australiense and *Candidatus* Phytoplasma aurantifolia was detected in tissues of ALuY symptomatic but not in asymptomatic plants. The association suggests that the 16S rDNA amplified from tissues of ALuY symptomatic plants is from phytoplasma bodies observed by electron microscopy (Pilkington *et al.* 2003). Repeated attempts in the present study failed to detect the phytoplasma reported by Pilkington *et al.* (2003) from ALuY symptomatic plants.

In Australia, *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense are associated with the diseases Australian grapevine yellows (Gibb *et al.* 1999; Constable *et al.* 2003), strawberry lethal yellows (Padovan *et al.* 2000) and papaya dieback (Gibb *et al.* 1998) but in all these diseases, *Candidatus* Phytoplasma aurantifolia is detected less frequently. Quite rarely, both phytoplasmas are detected from the same DNA samples. In the present study *Candidatus* Phytoplasma aurantifolia was detected in more ALuY symptomatic lucerne plant samples than was *Candidatus* Phytoplasma australiense and both phytoplasmas were detected from the same DNA sample in only one case.

Phloem discoloration in the tap root is the characteristic symptom of ALuY (Pilkington *et al.* 1999) and was the main sampling criterion in this study. *Candidatus* Phytoplasma australiense causes phloem discoloration in New Zealand cabbage (*Cordyline australis*) (Andersen *et al.* 2001) and occasionally in papaya plants affected by dieback disease (Gibb *et al.* 1998). *Candidatus* Phytoplasma aurantifolia has been reported to cause witches' broom and leaf yellowing in lucerne (Khan *et al.* 2002b), and yellowing in papaya (Gibb *et al.* 1998). It is unclear whether witches' broom (shoot proliferation and small, rounded leaves) and ALuY diseases in lucerne are caused by the same or related organisms. Phytoplasmas that share high 16S rRNA gene sequence similarity can cause different diseases in different plants (Lee *et al.* 1998; IRPCM 2004). It is not known, however, if phytoplasmas that share high 16S rDNA sequence similarity cause different diseases in the same host. More work is required to establish whether there are two different strains of *Candidatus* Phytoplasma aurantifolia that differ in their genes other than the 16S rDNA each causing different disease symptoms in lucerne.

The reason that samples collected in November had more positives for *Candidatus* Phytoplasma australiense than samples collected in January or February was not determined. It may be that the phytoplasma moved from the foliage to other tissues, such as the crown and root where assimilates are stored toward the end of the growing season. Researchers have noted that phytoplasmas are often concentrated in root tissues of trees and perennial weeds (Seemüller 1988). Because of the perennial nature of lucerne, it is probable that *Candidatus* Phytoplasma australiense moves, along with photosynthates, to root and crown tissues toward the end of the growing season. Also, samples collected from older stands (>1 year old) had more positives for *Candidatus* Phytoplasma australiense than samples collected from younger stands (6 months old or less). The perennial nature of the crop may predispose it to phytoplasma infection by allowing a longer time-frame for transmission by vectors and the build-up of inoculum in host tissue.

Not all symptomatic plants were phytoplasma-positive in the present study. Template quality and low phytoplasma titre in plant tissues are possible reasons. However, involvement of other pathogens such as unculturable, phloem limited bacterium like-organisms (BLOs) should not be ruled out. A recent report indicated that BLOs, in addition to the two phytoplasmas detected in lucerne in this study, were associated with SLY disease in Australia (Streten *et al.* 2005). Further, the absence of masking effects on the detection of *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense in an event of mixed infection in the present study suggests that the two phytoplasmas can independently cause ALuY disease or the disease is caused by other pathogen(s) with or without the presence of either or both of these phytoplasmas. The present study showed that *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense are associated with ALuY disease though further work is required to establish the relative importance of each.

3. Aspects of the epidemiology of Australian lucerne yellows disease

3.1 Introduction

Successful management of diseases requires a sound knowledge of disease spread, sources of inocula as well as the environmental factors that lead to disease outbreak. Vectors and alternative host plants play an important role in the epidemic cycle of phytoplasma diseases (Bertaccini 2007). Australian lucerne yellows (ALuY), a phytoplasma associated disease (Pilkington et al. 2003), is believed to be vectored by one or all of the leafhoppers *Orosius orientalis* (Matsumura) (=*Orosius argentatus* (Evans)) (Deltocephalinae: Opsiini), *Austroagallia torrida* (Evans) (Agalliinae) and *Batracomorphus angustatus* (Osborn) (Iassinae: Iassini) (Pilkington 2003; Pilkington *et al.* 2004a). There was correlation between the spatial and temporal distribution between ALuY disease symptomatic plants and all of these leafhoppers in the field (Pilkington 2003; Pilkington *et al.* 2004a). Transmission experiments showed *O. orientalis* but not *A. torrida* or *B. angustatus* was capable of transmitting ALuY phytoplasma. Plants fed on by *O. orientalis* developed typical ALuY disease symptoms and tested positive for phytoplasma (Pilkington *et al.* 2003; 2004b).

O. orientalis is an important vector of phytoplasma diseases in many horticultural and field crops in Australia. As early as the 1940s and 1950s, reports indicated that the insect was capable of transmitting tomato big bud, legume little leaf and witch's broom diseases, which were thought to be caused by viruses at that time (Helson 1951). As a result, the biology of *O. orientalis* has been studied in some detail. *O. orientalis* is a polyphagous insect and completes three generations per year in the field (Grylls 1979).

O. orientalis, *A. torrida* and *B. angustatus* were successfully cultured on *Malva parviflora*, *Datura stramonium* and *Trifolium pratense* for plant disease transmission studies (Grylls 1979). *A. torrida and B. angustatus* fed and bred easily on lucerne (*Medicago sativa*) (Pilkington *et al*. 2003). However, attempts to culture *O. orientalis* on lucerne (*Medicago sativa*) were not successful (Pilkington *et al*. 2003). Information on seasonal abundance and phenology of vector insects helps to predict disease outbreaks and field spread. Little is known about the biology of *A. torrida* and *B. angustatus*.

Non-crop plants or wild relatives of crops have the potential to function as reservoirs of phytoplasmas (Wilson *et al*. 2001). For example, wild *Prunus* species are believed to be fundamental in the epidemic cycle of European stone fruit yellows disease (Carraro *et al.* 2002). Identification and targeted control of alternative hosts of phytoplasmas are therefore important to reduce phytoplasma disease incidence.

Molecular methods such as PCR assays enable sensitive detection of phytoplasma DNA in insects (Weber and Maixner 1998) and, as such, can be applied in the screening of field collected insects. Molecular tools have been used to search for new, unknown vectors and to study seasonal or geographical distribution of infective vectors.

The aims of this work were to: (1) sample lucerne and non-lucerne plants in and around ALuY disease affected lucerne fields for *O. orientalis, A. torrida* or *B. angustatus* throughout the year; (2) test the suitability of lucerne (*Medicago sativa*), red clover (*Trifolium pratense*) and nettleleaf goosefoot (*Chenopodium murale*) plants as ovipositional hosts for *O. orientalis, A. torrida* and *B. angustatus*; and (3) test the presence of ALuY phytoplasma in field collected *O. orientalis, A. torrida, B. angustatus* and other insects using PCR assays.

3.2 Materials and Methods

Vacuum and sweep net samples

ALuY disease affected lucerne fields and adjacent areas suspected of harbouring vectors, including weedy fields in the Lachlan Valley, central NSW, at Griffith, south western NSW and Keith, SA, were sampled from February 2005 using 30cm diameter sweep net or vacuum sampler (Rincon-Vitova, Ventura, CA) with a 0.093m^2 sampling cone. Sweep nets were only used when trees or shrubs were sampled. From year round sampling at various times in the Lachlan Valley and Griffith, and from a single visit to Keith, a total of 38 plant species were sampled (Table 3.1). In vacuum sampling, the vacuum sampler was run for one minute for each sample whilst twenty sweeps per sample were used in sweep net sampling. For each plant species, three individual plants were sampled and collections of *O. orientalis, A. torrida* and *B. angustatus* recorded. Sample bags were returned to the laboratory in a portable 12-volt car refrigerator (~7^{o}C) (Norcord, Houston, Texas) and the number of *O. orientalis, A. torrida and B. angustatus* adults and nymphs were counted using a binocular microscope (Olympus optical, Tokyo, Japan).

Ovipositional preferences

Twenty field collected adults of each of the leafhopper species *O. orientalis, A. torrida* and *B. angustatus* were separately caged with lucerne (*Medicago sativa*), red clover (*Trifolium pratense*) or nettleleaf goosefoot (*Chenopodium murale*) plants in a glasshouse. Plants were started from seeds in 0.5-litre pot, four plants per pot, containing potting mix (Quality Gardening Australia, Tooronga, Victoria), watered to saturation every day and fertilized with liquid fertilizer (Maxicrop Pty. Ltd., Australia) on the day of sowing and every week thereafter. Treatments were arranged in a split plot design with three replications. Plant species (lucerne, nettleleaf goosefoot and red clover) were used as main plots and leafhopper species and insect free control plants as subplots. The numbers of nymphs and adults on caged plants were counted one month after the beginning of the experiment.

The ovipositional host status of *C. murale* to *O. orientalis* was also studied using naturally infested *C. murale* plants. Ten *C. murale* plants naturally infested by adults and nymphs of *O. orientalis* in the field were transplanted to caged pots in a glasshouse. Transplanted plants were sprayed with 0.2% solution of Pyrethrum (a light sensitive contact insecticide) (Andermatt Biocontrol AG, conc. 4% with pyrethrine as active ingredient) to kill existing adults, nymphs and other insects. One day after Pyrethrum treatment, plants were rinsed with tap water then exposed to sun light for two days to remove residual insecticide. Caged plants were maintained in a glasshouse until nymphs and adults emerged.

Detection of phytoplasma in plants and leafhoppers

The presence or absence of phytoplasma in plants and leafhoppers was tested using polymerase chain reaction (PCR) assays. When testing plant samples, portions of leaf midribs, believed to contain high titres of phytoplasma in infected plants, were removed from leaves. Where leaf midribs were difficult to remove, young stems or petioles were used. DNA from samples was extracted and prepared using the method described by Zhang *et al.* (1998). Approximately 0.3g of plant tissue was macerated in hot CTAB (pH 8) DNA extraction buffer using mortar and pestle. The mixture was incubated for 30 minutes at 65^{o}C in hot water bath and DNA was extracted using equal volume of chloroform followed by centrifugation at 13,200 rpm for ten minutes. Two volumes of isopropanol were added to the supernatant and centrifuged as above. The resulting DNA pellet was washed twice in 70% ethanol and once in 100% ethanol and stored, after drying, in 50-100µl TE at -20^{o}C. Plant samples were processed within 24-48h of collection and were stored at 4^{o}C before processing and insects were stored at -20^{o}C before processing.

The same DNA extraction method as for plants was used for insects and 2-5 insects were used per sample. Phytoplasma generic primers were used in the first few months of the PCR tests. Primers specific to *Candidatus* Phytoplasma australiense or *Candidatus* Phytoplasma aurantifolia were used in subsequent PCR tests. The phytoplasma generic primers P1/P7 nested fU5/rU3 that amplify 850-bp

DNA fragment from the 16S rRNA gene of phytoplasmas were used. PCR was carried out using Perkin-Elmer thermocycler (PE Applied Biosystems, Foster City, California, USA). The PCR mix contained 1X reaction buffer, 1.5mM $MgCl_2$, 0.2mM each dNTPs, 1.25 unit heat activated Immolase DNA polymerase and 0.2µM of each primer in 25µl reaction volume. All PCR components were supplied by Bioline Ltd. (Bioline, Sydney, Australia). PCR conditions were as follows: 95^{o}C for seven minutes in the first cycle followed by 40 cycles of denaturation at 94^{o}C for 45s, annealing at 55^{o}C for 45s and extension at 72^{o}C for 90s. Final extension was for 9.5 minutes at 72^{o}C.

PCR conditions for the detection of *Candidatus* Phytoplasma australiense and *Candidatus* Phytoplasma aurantifolia in insects and plants using specific primers were the same as described in Chapter two. The PCR products were run on a 1.5% agarose gel for 50 min at 90 volts and stained with ethidium bromide to visualise the positive 850-bp (all phytoplasmas), 400-bp (*Candidatus* Phytoplasma aurantifolia) and 250-bp (*Candidatus* Phytoplasma australiense) bands. Tomato big bud (TBB) or Phormium yellow leaf (PYL) and sterile distilled water (SDW) were included as positive and negative controls, respectively.

Restriction fragment length polymorphism (RFLP) analysis

Five microlitres of the 850-bp PCR products of five plant and two leafhopper samples were separately digested with four units of the endonuclease *Alu*I (FisherBiotech, Perth, Australia). Following an overnight restriction digestion at 37^{o}C, products were separated on a 3% agarose gel, stained with ethidium bromide and visualized by transillumination in ultraviolet light.

3.3 Results

Vacuum and sweep net samples

Insects were collected from a wide range of plant species using vacuum sampler or sweep net. Large numbers of adults and nymphs of *O. orientalis* (Fig. 3.1) were collected from *Enchylaena tomentosa* at Griffith during spring and *C. murale* (Fig. 3.3) in the Lachlan Valley during summer 2005 (Fig. 3.1 B and D). In autumn and winter, *O. orientalis* adults and nymphs were collected from *C. nitrariacium* and *M. microphylla* in the Lachlan Valley and from *M. brevifolia* (Fig. 3.4) at Griffith. *O. orientalis* was also abundant on *M. brevifolia* during spring and summer. Very few adults and nymphs of *A. torrida* and *B. angustatus* were collected from all plants sampled.

Ovipositional preference studies

Nymphs of *O. orientalis* were observed on *C. murale* plants 22 days after the beginning of the experiment. The numbers of nymphs and adults of *O. orientalis* on *C. murale* were significantly ($P < 0.001$) higher than on lucerne or red clover. The numbers of adults and nymphs of *B. angustatus* were significantly ($P < 0.001$) higher on red clover than on lucerne or *C. murale* plants. Nymphs of *B. angustatus* were observed on red clover plants 30 days after the beginning of the experiment. Five and three adults of *A. torrida* were found on red clover and lucerne plants, respectively but none was found on *C. murale* (Table 3.2). Small numbers of nymphs of *A. torrida* also were found on red clover and lucerne plants. Control plants had no nymphs or adults of *O. orientalis, A. torrida* or *B. angustatus*.

Table 3.2 Mean numbers of adults and nymphs of *O. orientalis, A. torrida and B. angustatus* caged with red clover, goosefoot and lucerne plants

Plant species	**Leafhopper**					
	O. orientalis		***A. torrida***		***B. angustatus***	
	Adult	**Nymph**	**Adult**	**Nymph**	**Adult**	**Nymph**
Red clover	0.67	0.00	3.67	2.00	12.00	10.67
Goosefoot	14.67	8.67	0.00	0.00	0.00	0.00
Lucerne	0.67	0.00	2.33	3.00	3.00	3.00

Detection of phytoplasma in plants and leafhoppers

Phytoplasma DNA was amplified from *O. orientalis* and plant samples (Tables 3.1and 3.3) using phytoplasma generic primers as well as primers specific to *Candidatus* Phytoplasma aurantifolia and related strains. However, both the phytoplasma generic primers as well as primers specific to *Candidatus* Phytoplasma aurantifolia and related strains failed to amplify phytoplasma DNA in *A. torrida*, *B. angustatus* and other insects included in the test. None of the plant samples or insects was positive for *Candidatus* Phytoplasma australiense.

Table 3.3 Results of PCR tests of insects for *Ca.* phytoplasma aurantifolia and *Ca.* Phytoplasma australiense.

Insect	Location	No. positives	
		P. aurantifolia	**P. australiense**
Orosius orientalis	Lachlan Valley and Griffith	18/30 (5 in each group)	0/30 (5 in each group)
Austroagallia torrida	Lachlan Valley	0/15 (3 in each group)	0/15 (3 in each group)
Batracomorphus angustatus	Lachlan Valley	0/10 (2 in each)	0/10 (2 in each)
Austroasca sp.	Lachlan Valley	0/10 (3 in each group)	0/10 (3 in each group)
Balclutha sp.	Lachlan Valley	0/15 (4 in each group)	0/15 (4 in each group)
Delphacidae	Lachlan Valley	0/5 (3 in each group)	0/5 (3 in each group)
Xestocephalus tasmaniensis	Lachlan Valley	0/8 (3 in each group)	0/8 (3 in each group)
Sogatella kolophon	Lachlan Valley	0/10 (2 in each group)	0/10 (2 in each group)
Austroasca merredinensis	Lachlan Valley	0/10 (4 in each group)	0/10 (4 in each group)

Fig. 3.1 *Orosius orientalis* (Matsumura) (=*Orosius argentatus* (Evans)) (Deltocephalinae: Opsiini) (Source: http://www.agric.nsw.gov.au/Hort/ascu/leafhop/cicaspp/orosius.pic1.htm)

RFLP analysis

Restriction digestion profiles of nested PCR amplicons from plants and *O. orientalis* were similar to each other and the control (TBB) but differed from the RFLP profiles of the PYL phytoplasma.

Date	Scientific name	Common name	Location	No. positives		No. leafhopper catches		
				P. aurantifolia	**P. australiense**	***O. orientalis***	***A. torrida***	***B. angustatus***
9 Feb. 05	*Brassica rapa*	Bird rape	Lachlan Valley	0/3	0/3	5	0	0
9 Feb. 05	*Maeriana microphylla*	Eastern cotton bush	Lachlan Valley	5/10	0/10	9	1	2
9 Feb. 05	*Chenopodium nitrariacium*	Nitre goosefoot	Lachlan Valley	1/5	0/5	11	3	2
9 Feb. 05	*Chenopodium album*	Fat hen	Lachlan Valley	0/7	0/7	4	0	0
9 Feb. 05	*Chenopodium murale*	Nettleleaf goosefoot	Lachlan Valley	4/15	0/15	16	0	0
9 Mar. 05	*Malva parviflora*	Mallow	Lachlan Valley	0/2	0/2	0	1	0
9 Mar. 05	*Cucumis myriocarpus*	Paddy melon	Lachlan Valley	0/1	0/1	0	0	0
9 Mar. 05	*Trifolium pratense*	Red clover	Lachlan Valley	1/3	0/3	2	1	5
9 Mar. 05	*Chenopodium pseudomycophyllum*	Small leaf goosefoot	Lachlan Valley	0/10	0/10	3	1	0
9 Mar. 05	*Acacia baileyana*	Cootamundra wattle	Lachlan Valley	0/3	0/3	0	1	2
5 Apr. 05	*Chenopodium pumilio*	Small crumbweed	Lachlan Valley	0/4	0/4	7	1	0
5 Apr. 05	*Portulaca oleracea*	Common pig weed	Lachlan Valley	0/5	0/5	0	0	0
5 Apr. 05	*Glaucium corniculatum*	Red horned-poppy	Lachlan Valley	0/2	0/2	0	0	0
7 Jul 05	*Atriplex semibaccata*	Creeping saltbush	Keith	0/1	0/1	0	0	1
7 Jul 05	*Cressa cretica*	Rosinweed	Keith	0/2	0/2	0	0	0
7 Jul 05	*Trichodesma zeylanicum*	Cattle bush	Keith	0/2	0/2	1	0	0
7 Jul 05	*Polygonum* sp.	Slender lignum	Keith	0/3	0/3	0	0	0

Table 3.1 Collections of *O. orientalis, A. torrida* and *B. angustatus* and PCR tests of plants

Continued

Date	Scientific name	Common name	Location	No. positives		No. leafhopper catches		
				P. aurantifolia	**P. australiense**	***O. orientalis***	***A. torrida***	***B. angustatus***
7 Jul 05	*Chenopodium anidiophyllum*	Mallee goosefoot	Keith	0/9	0/9	2	1	0
7 Jul 05	*Geococcus pusillus*	Earth cress	Keith	0/2	0/2	0	0	0
7 Jul 05	*Acacia delbata*	Silver wattle	Keith	0/1	0/1	1	2	3
9 Jul 05	*Foeniculum vulgare*	Fennel	Griffith	0/2	0/2	0	0	0
23 Aug 05	*Centaurium tenuiflorum*	Branched centaury	Griffith	0/3	0/3	0	0	0
23 Aug 05	*Convolvulus erubescens*	Bind weed	Griffith	0/1	0/1	1	0	1
23 Aug 05	*Echium plantagineum*	Paterson's curse	Griffith	0/1	0/1	2	1	1
23 Aug 05	*Maeriana brevifolia*	Yanga bush	Griffith	5/12	0/12	10	0	0
13 Oct 05	*Enchylaena tomentosa*	Ruby salt bush	Griffith	3/5	0/5	9	1	3
13 Oct 05	*Atriplex holocarpa*	Salt bush	Griffith	0/2	0/2	2	1	3
9 Feb 05	*Solanum nigrum*	Nightshade	Lachlan Valley	0/8	0/8	3	1	0
4 Nov 05	*Ranunculus undosus*	Swamp buttercup	Griffith	0/4	0/4	0	0	0
4 Nov 05	*Urtica urens*	Small nettle	Griffith	0/1	0/1	0	0	0
9 Feb. 05	*Medicago sativa*	Lucerne	Lachlan Valley	Not tested	Not tested	3	5	2
4 Nov 05	*Medicago sativa*	Lucerne	Griffith	Not tested	Not tested	2	7	4
4 Nov 05	*Polygonum arenastrum*	Wire weed	Griffith	0/2	0/2	0	0	0
4 Nov 05	*Solanum nigrum*	Night shade	Griffith	8/10	0/10	3	1	0

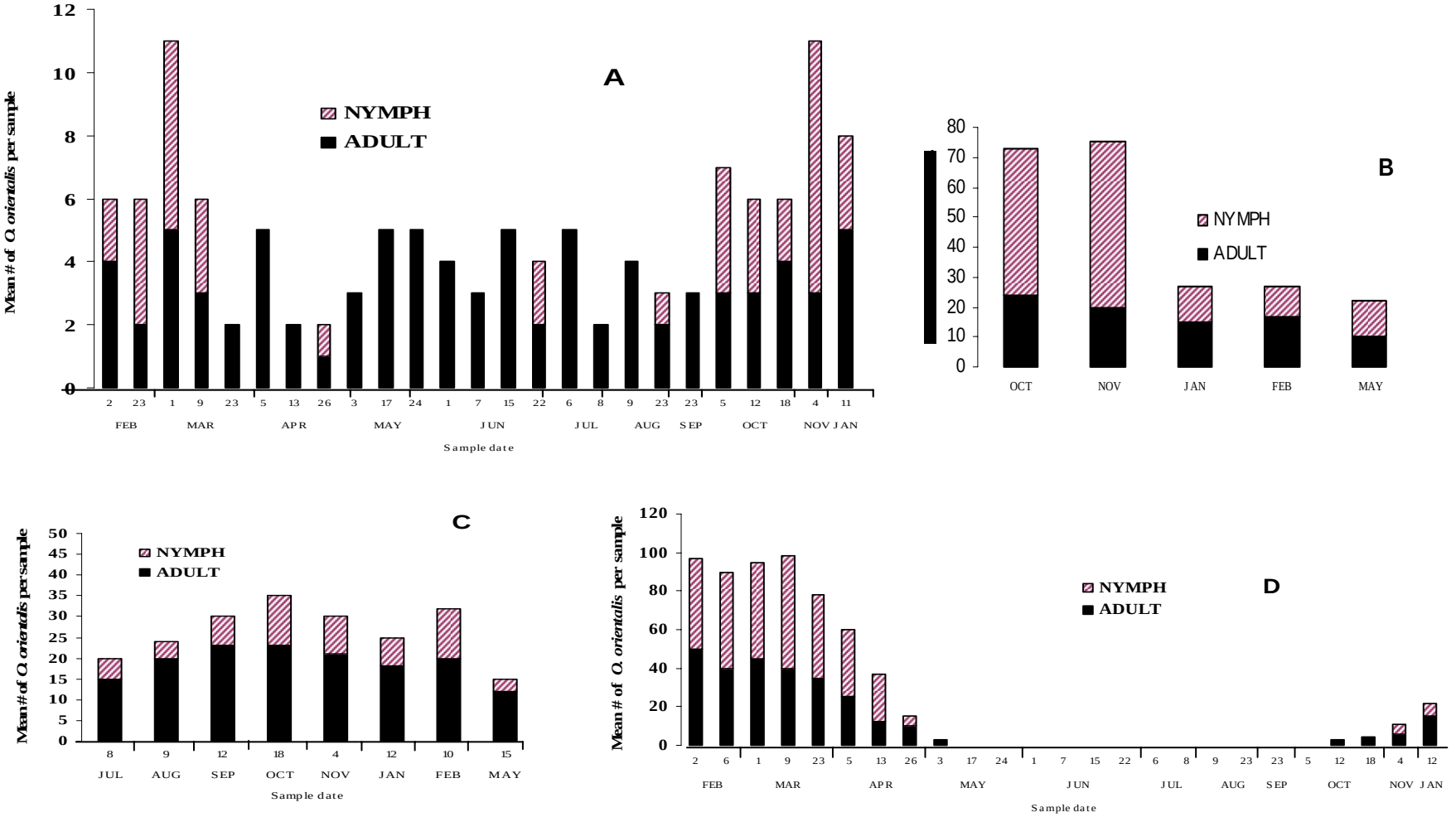

Fig. 3.2 Collections of *O. orientalis* from chenopod weeds: (A) *Maeriana microphylla*, (B) *Enchylaena tomentosa*, (C) *Maireana brevifolia* and (D) *Chenopodium murale*.

Fig. 3.3 *Chenopodium murale*, a plant species testing positive for Phytoplasma aurantifolia and harbouring large numbers of the likely ALuY vector, *Orosius orientalis*.

Fig. 3.4 *Maireana brevifolia*, a plant species testing positive for Phytoplasma aurantifolia and harbouring large numbers of the likely ALuY vector, *Orosius orientalis*.

3.4 Discussion

O. orientalis has been reported to be a polyphagous insect (Grylls 1979). In the present work, although small numbers of this insect were collected from a wide range of plant species, collections in large numbers per sample were only from plants in the Chenopodiaceae. Earlier, a conference paper has been presented on this issue (Getachew *et al.* 2005). Not a single plant species in other plant families sampled had nymphs of the insect, suggesting a possible link between this insect and chenopod plants. Results also show that *O. orientalis* overwinters predominantly as adults on perennial chenopods. Nymphs and adults were collected throughout the year though catches were highest in spring and summer. Temperatures in winter (June to August), spring (September to November), summer (December to February) and autumn (March to May) of 2005 and early 2006 were above average for the locations, and warmer conditions particularly in winter might have contributed to an extended period of reproduction of the insect. Reports indicated that *O. orientalis* reproduced in captivity at temperatures as low as 4^{o}C (Grylls 1979).

Knowledge of maintenance and culture of plant disease vectors is desirable for disease transmission studies under controlled conditions. Reports indicate that *Malva parviflora*, *Datura stramonium* and *Trifolium pretense* can serve as feeding and breeding hosts for *O. orientalis*, *A. torrida* and *B. angustatus* (Grylls 1979). Results of the successful maintenance of *B. angustatus* on *T. pratense* in the present study support a previous report (Grylls 1979). *O. orientalis*, on the other hand, fed and bred easily on *C. murale*. Results of vacuum samples in the field also showed that *O. orientalis* feeds and breeds on *C. murale* in summer. *C. murale* is an annual forb that grows about 30cm high and can be started from seeds or transplanted from field to pots in controlled conditions.

Phytoplasmas are circulative and reproductive within their vectors (Purcell 1982; Hacket and Clark 1989; Fletcher *et al*. 1998). As such, successful overwintering of infected vectors results in the successful overwintering of the pathogen. Findings of *Candidatus* phytoplasma aurantifolia infected *O. orientalis* collected in winter combined with infected non-lucerne plants during the winter months indicate that *Candidatus* phytoplasma aurantifolia can overwinter within *O. orientalis* and plants. Studies showed that in new lucerne seed crops, ALuY symptoms developed in late summer (February) 29-30 weeks after sowing (Pilkington 2003). The results of the present study are consistent with the idea that overwintering *O. orientalis* adults can acquire the pathogen from infected non-lucerne plants during the spring and transmit it to newly sown lucerne crops. Alternatively, infective *O. orientalis* adults that hosted the pathogen throughout the winter months may transmit the pathogen to new lucerne crops in spring without the need for alternative plant hosts for the pathogen.

Few adults and nymphs of *O. orientalis* were collected from *C. murale* in autumn, winter and early spring in the Lachlan Valley. *C. murale* is an annual forb and may not be a reliable feeding and breeding host for *O. orientalis* throughout the winter. Instead the insect may normally retreat to perennial chenopods during the winter months. Large numbers of adults and nymphs of this insect were collected from perennial chenopods during winter. Whilst large numbers of both adults and nymphs were sampled from *M. brevifolia* throughout the year, large numbers of adults and nymphs of *O. orientalis* per sample from *E. tomentosa* were only collected in spring and winter. Large collections of *O. orientalis* from *M. brevifolia* (yanga bush) have been reported (Magarey *et al.* 2002).

The question of *O. orientalis* as a vector of ALuY phytoplasma remains open. The results of PCR detection of *Ca*. Phytoplasma aurantifolia in whole body of *O. orientalis* in the present study suggests this leafhopper is the likely vector of *Ca*. Phytoplasma aurantifolia but this needs to be confirmed by transmission tests. If *Candidatus* Phytoplasma is confirmed to be the etiological agent responsible for ALuY disease, control of chenopod weeds in and around lucerne fields might reduce disease incidence in new lucerne crops. *C. murale* and *T. pratense* can be ideal host plants on which to culture *O. orientalis* and *B. angustatus*, respectively, under controlled conditions.

4. *Candidatus* Phytoplasma australiense in seeds and seedlings of lucerne (*Medicago sativa* L.): evidence of seed transmission.

4.1 Introduction

Candidatus Phytoplasma australiense is associated with Australian grapevine yellows (AGY) (Davis *et al*. 1997a), strawberry lethal yellows (SLY) (Padovan *et al.* 2000; Streten *et al.* 2005c), papaya dieback (PDB) (Gibb *et al.* 1996) and pumpkin yellow leaf curl (Streten *et al*. 2005b) diseases in Australia and with strawberry lethal yellows disease in New Zealand (Andersen *et al.* 1998). Recently, *Candidatus* Phytoplasma australiense has been reported in lucerne (Getachew *et al.* 2007). This phytoplasma and *Candidatus* Phytoplasma aurantifolia have been found associated with Australian lucerne yellows (ALuY) disease (Chapter two), which affects seed yield of lucerne causing millions of dollars in annual loss to the Australian pasture seed industry (Pilkington *et al.* 1999).

To minimize the risk of seed-borne pathogens and pests, as well as to achieve optimal crop establishment, Australian seed companies grade lucerne seed into 'first' and 'second' categories using a gravity table. For seed production, lucerne crops are established using the first grade seed only whilst the second grade seed is used to establish lucerne stands for pasture or hay production (L.M. Kirkby, personal communication, April 2005). First grade seeds typically have yellow or golden seed coat colour and are larger than the second grade seeds that often have a discoloured (brown, green or black) seed coat.

Seed transmission of phytoplasmas is generally dismissed by plant pathologists because it is believed that phytoplasmas are confined to the phloem tissue of plants and there is no direct connection between the phloem tissue of the mother plant and the developing embryo in the seed (McCoy 1979; Cordova *et al.* 2003). However, recent reports of PCR detection of lethal yellows phytoplasma in the embryos of lethal yellows disease affected coconut palm (Cordova *et al.* 2003) and alfalfa witches' broom phytoplasma in seeds and seedling progeny of alfalfa plants infected with witches' broom disease (Khan *et al.* 2002a) raise the possibility of seed transmission in at least some phytoplasma diseases. Prompted by these reports, we tested the hypothesis that *Ca*. Phytoplasma australiense could be seed-borne in lucerne.

4.2 Materials and methods

Seed samples

Two batches of lucerne seed samples one batch from the first grade seed and the other from the second grade seed were obtained from Auswest Seed Company, Forbes, NSW, Australia. The seeds were harvested in March 2005 from a seed lucerne crop (cv. CW 5558) that had high ALuY disease incidence during the 2004/2005 crop season (L.M. Kirkby, personal communication, April 2005). Seed samples were kept at 5^{o}C from acquisition to analysis.

Emergence

Experiment 1. Emergence was tested in a growth chamber (Thermoline, Sydney, Australia) at a temperature of 21^{o}C and RH of 67%. Potting mix (Quality Gardening Australia, Tooronga, Victoria, Australia) and seed trays were steam sterilized (ACA Engineering and Co. Victoria, Australia) and seeds inoculated with rhizobium bacteria (Nodulade, Biocare Technology Pty. Ltd., NSW, Australia) before sowing. From each seed grade, 784 replicates of randomly selected individual seeds were sown in seed trays by hand at a depth of 0.5cm, watered to saturation every three to four days and fertilized with liquid fertilizer (Maxicrop Pty. Ltd., Australia) on the day of sowing and once every week

thereafter. Treatments were laid out in completely randomized design (CRD). Emergence was scored fifteen days after sowing when the most advanced plants were at first true leaf stage. Only plants that grew through the surface of the soil were considered emerged.

Experiment 2. The second grade seed batch was sorted into four categories (treatments) based on size and discolouration of the seed coat. First, seeds were separated into two size groups: small (those that passed through a 1mm mesh sieve) and large (those seeds that did not pass through a 1mm mesh sieve). Then seeds in each size category were separated by hand into two groups based on discolouration of the seed coat. Seeds with a golden/yellow seed coat colour were considered normal whilst seeds with dark brown, green or black seed coat were considered discoloured. Emergence of each of the four categories was assessed under environmental conditions described above. Seeds were sown individually in 'plots' consisting of seventy seeds per treatment. Treatments were laid out in Latin square design with four replications. To avoid the possibility of disease inoculation by infective vector insects entering the growth chamber, each tray was enclosed in a fabric with 200µm holes. Emergence was scored as for experiment one, above.

DNA isolation from seeds and seedlings

On the day that emergence was scored, 40 randomly-selected seedlings of each of the two treatments described in 'experiment 1' and 10 seedlings selected at random from each of the four treatments described in 'experiment 2' were subjected to DNA extraction according to Zhang *et al.* (1998). The entire shoot of each seedling was crushed in hot (60^{o}C) cetyltrimethylammonium bromide (CTAB) buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100mM Tris-HCl, pH 8.0 and 0.2% mercaptoethanol) using a pre-chilled mortar and pestle. After incubation at 65^{o}C for 30 min in a water bath, 600-800 µl of chloroform was added to the mixture, vortexed and centrifuged for ten minutes at maximum speed in an Eppendorf Centrifuge (Eppendorf North America, Westbury, NY). An equal volume of ice-cold isopropanol was added to the supernatant, mixed well and centrifuged for ten minutes. After washing twice in 70% ethanol and drying, the DNA pellet was re-suspended in 50µl TE buffer (10 mM Tris HCl, 1 mM EDTA pH 8.0). Resuspended DNA was left at 4^{o}C overnight to dissolve the pellet then stored at -20^{o}C. Quality and quantity of the nucleic acid was estimated by running 5µl of the sample and a standard marker on a 1% agarose gel using 1X TBE as running buffer.

The method described by Kang *et al.* (1998) was used for DNA isolation from seeds. Forty samples of batches of 10-15 seeds were selected randomly from each seed grade using spatula. Samples were soaked in 400µl extraction buffer (200 mM Tris-HCl (pH 8), 200mM NaCl, 25mM EDTA, 0.5% SDS and 50µg proteinase K) in 1.5ml microcentrifuge tube for three to four hours then crushed using microcentrifuge tubes and micro pestles. When seeds were resistant to crushing, a mortar and pestle were used to ensure uniform homogenisation was obtained. Four hundred microlitre CTAB buffer was added to the crushed seeds and the solution was incubated at 65^{o}C for 30 minutes before extraction with equal volume of chloroform. After this step, the same DNA extraction protocol as described for seedlings was used.

Detection of phytoplasmas in seeds and seedlings using generic primers

Nested polymerase chain reaction (PCR) was carried out for amplification of the 16S rDNA with the phytoplasma generic primer pairs P1 (Deng and Hiruki, 1991)/P7 (Kirkpatrik *et al.* 1994) and fU5 (Lorenz *et al.* 1995) / M23Sr (Padovan *et al.* 1995). Undiluted DNA was used for the first amplification whilst PCR products from the first amplification were diluted (1:30) in sterile distilled water (SDW) before the final amplification. DNA of Australian tomato big bud (TBB) phytoplasma and SDW were used as positive and negative controls, respectively. PCR was carried out using the thermocycler (PE Applied Biosystems, Foster City, California, USA). The PCR mix contained 1X reaction buffer, 1.625mM $MgCl_2$, 0.2mM each dNTPs, 1.25 unit heat activated Immolase DNA polymerase and 0.2µM of each primer in 25µl reaction volume. All PCR ingredients were supplied by Bioline Ltd. (Bioline, Sydney, Australia). PCR conditions were as follows: 95^{o}C for seven minutes in the first cycle followed by 35 cycles of denaturation at 94^{o}C for 60s, annealing at 55^{o}C for 60s and extension at 72^{o}C for 90s. Final extension was for 9.5 minutes at 72^{o}C.

Primer design and PCR protocol for specific detection of *Ca.* Phytoplasma australiense

Primers and the PCR conditions for specific detection of *Candidatus* Phytoplasma in seeds and seedlings were the same as described in Chapter two.

Statistical analysis

Genstat (2002) was used to perform analysis of variance to test differences in emergence between seed size and discolouration categories and a Chi-squared test was used to test differences between seed categories for PCR positives.

4.3 Results

Emergence tests

Experiment 1. The second grade seed had significantly ($P < 0.001$, t = 7.02) lower emergence rate (53.2%) than the first grade seed (80%).

Experiment 2. The seed colour and size groups from the second grade seed differed significantly ($P<0.001$) in rate of emergence. Larger, discoloured seeds had the highest emergence rate (57%) followed by small/discoloured seeds (44.3%) and larger seeds of apparently normal colour (yellow) (41%). Small seeds of apparently normal colour (yellow) had the lowest emergence rate (10%).

Amplification of phytoplasma DNA in lucerne seeds and seedlings using generic primers

Phytoplasma DNA was amplified from the first and the second grade seeds, as well as from seedlings grown from the second grade seed using phytoplasma generic primers (Table 4.1). Phytoplasma DNA was amplified from 3 and 10 seed samples from the first and the second grade seeds, respectively and from 4 seedlings grown from the second grade seed. There were no phytoplasma positive seedling samples from the first grade seed. The second grade seed had a significantly ($P = 0.034$, $X^2 = 4.50$) higher number of phytoplasma positive seed samples than the first grade seed.

Table 4.1 Detection of phytoplasma DNA in seed and seedling samples from the first and the second grade seeds using phytoplasma generic primers.

DNA source	First grade seed		Second grade seed		X^2	df	P value
	+	-	+	-			
Seeds	**3**	**37**	**10**	**30**	**4.50**	**1**	**0.034**
Seedlings	**0**	**40**	**3**	**36**	**3.11**	**1**	**0.100**

PCR amplification of *Ca*. Phytoplasma australiense DNA in seeds and seedlings using specific primers

All seed samples tested using the generic primers were re-tested using primers specific for *Ca*. Phytoplasma australiense and related strains. As a result, a 250bp DNA fragment was amplified from phytoplasma positive seed samples (Fig. 4.1). The specific primers yielded a higher number of positives than the generic primers. Significantly ($P < 0.001$, $X^2 = 13.65$) more seed samples were positive for *Ca* Phytoplasma australiense from the second than the first grade seed (Table 4.2).

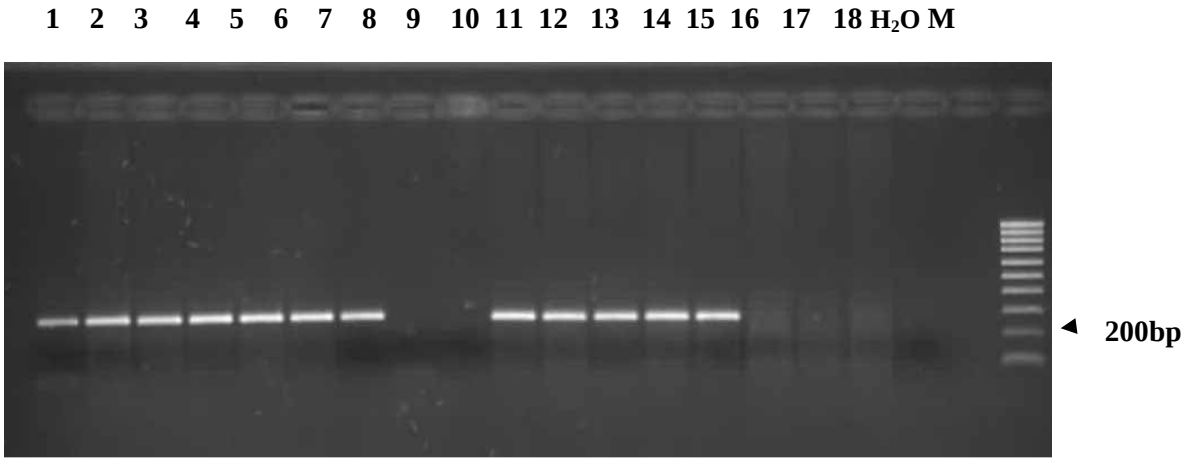

Fig. 4.1 PCR amplification of *Ca.* Phytoplasma australiense from second grade seeds using specific primers. Lane 1 is positive control, lanes 2-18 are seed samples.

Table 4.2 Detection of *Ca.* Phytoplasma australiense in seed and seedling samples from the first and second grade seeds using specific primers

DNA source	First grade seed		Second grade seed		X^2	df	P value
	+	-	+	-			
Seed	**7**	**33**	**23**	**17**	**13.65**	**1**	**<0.001**
Seedling	**2**	**38**	**6**	**34**	**2.22**	**1**	**0.200**

Further grading of the second grade seed showed significant differences in the number of phytoplasma positive seedlings started from the different seed size and colour categories. Seedlings started from discoloured or small seeds had significantly ($P = 0.022$, $X^2 = 9.6$) higher number of positives for *Ca*. Phytoplasma australiense than seedlings started from seeds apparently of normal colour and larger size (Table 4.3).

Table 4.3 Detection of *Ca.* P. australiense in seedlings from different seed categories of second grade seed.

Seed category								X^2	df	P value
Yellow, large		**Yellow, small**		**Discoloured, large**		**Discoloured, small**				
+	-	+	-	+	-	+	-			
0	**10**	**1**	**9**	**1**	**9**	**3**	**7**	**4.3**	**3**	**1.00**

4.4 Discussion

PCR detection of *Ca.* Phytoplasma australiense in seeds originating from a seed lucerne crop that had a high incidence of ALuY, and in seedlings grown from these seeds supports the hypothesis that this phytoplasma could pass from seed to seedling. Amplification of DNA of this phytoplasma both in seeds and seedlings using PCR primers specific to *Ca*. Phytoplasma australiense further supports the fidelity of the results. Furthermore, PCR assays of ALuY symptomatic lucerne plants sourced from the same paddock as the seeds showed the presence of *Ca*. Phytoplasma australiense (Chapter two; Getachew *et al*. 2007)

PCR detection of DNA of *Ca.* Phytoplasma australiense in lucerne/alfalfa seeds and seedlings in the present work is the first evidence on the ability of this phytoplasma to pass from seed to seedling. PCR detection *Ca.* Phytoplasma aurantifolia (witches' broom phytoplasma) in lucerne seeds and seedlings has been reported (Khan *et al.* 2002a). Witches' broom phytoplasma is also believed to be able to pass from seeds to seedlings in small-fruited acid lime (Khan *et al.* 2003) suggesting that this phytoplasma is capable of passing from seed to seedling in different plant species.

The evidence from this and previous studies on the possibility of seed transmission of phytoplasmas (Khan *et al.* 2002, 2003; Cordova *et al.* 2003) challenges the common view that seed transmission of phytoplasmas cannot occur. It is generally believed that phytoplasmas only inhabit the phloem tissue and colonization of the host plant occurs passively when phytoplasmas are moved by photosynthate flows (Kuske and Kirkpatrick 1992). Therefore, absence of a continuous pathway between the phloem tissue of the mother plant and the developing embryo in seeds means that phytoplasma cells cannot reach the developing embryo from the phloem tissue of the mother plant. However, evidence for the confinement of phytoplasmas to the phloem tissue is not conclusive. Amici and Favali (1972) reported evidence of phytoplasma presence in parenchyma cells though McCoy (1979) dismissed this as misidentification of immature phloem as parenchyma cells or as erroneous identification of cytoplasmic vesicles of host origin as phytoplasma bodies. Phytoplasmas are considered too large to move through parenchyma plasmodesmata (McCoy 1979).

Phytoplasmas colonize most tissues and organs of their vectors (Lee *et al.* 2000) probably using the same mechanism as pathogens such as begomoviruses. Recently, similarities between phytoplasma and begomovirus coat proteins have been reported (Marzachi *et al.* 2000). In begomoviruses, cell-to-cell and systemic movement in the plant as well as in the vector is achieved by the presence of both nuclear shuttle and movement proteins (Brown, 2001). In some begomoviruses, for example the phloem-limited Abutilon mosaic virus, the loss of one or both coat proteins resulted in the loss of the virus's ability to actively move from cell to cell and transmissibility by vectors (Avner and Czosnek 2003). If the same mechanism operates in the cellular and systemic infection of phytoplasmas, it could indicate that not all phytoplasmas are phloem-limited.

The high number of *Ca.* Phytoplasma australiense positive seed samples shown by PCR tests in the second grade seed suggests that this phytoplasma reduces seed quality of infected plants. Further grading of the second grade seed based on size and discolouration showed that small or discoloured seeds tended to have high numbers of positives and significantly lower emergence rate. An earlier study (Getachew *et al.* unpublished data) showed that ALuY symptomatic lucerne plants had significantly lower seed yield than their asymptomatic nearest neighbours. Further, seeds collected from ALuY symptomatic plants showed a significant reduction in 1000 seed weight, germination percent and emergence rate. Death of embryos of lucerne seeds from plants infected with this phytoplasma may be the reason for low germination/emergence rate in such seeds.

PCR detection of *Ca.* Phytoplasma australiense in lucerne seeds and seedlings in the present work and of *Ca.* Phytoplasma aurantifolia in seeds and seedlings in a previous study (Khan *et al.* 2002a) suggests seed transmission of phytoplasmas is possible in lucerne. ALuY symptomatic plants usually have scattered distribution and low incidence in lucerne fields. The phytoplasma may kill most infected seeds although disease spread in the field by vectors is possible. In one particular lucerne seed crop (cv. 5558) at Griffith, south western NSW, ALuY disease incidence in the first year of growth of the crop was higher than it was in the second year. Had the disease been spread by vectors only, disease incidence would be expected to increase in successive seasons. Infected seeds could be responsible for high disease incidence in the first year, and, possibly, the disease killed some or most of infected plants in the first year of their growth.

In the present study, symptoms characteristic of ALuY disease (Pilkington *et al.* 1999) did not develop in caged lucerne plants maintained until flowering in growth chamber. However, some plants developed witches' broom type symptoms (shoot proliferation and small, rounded leaves). In future transmission tests, plants could be started in caged plots in the field to provide environmental conditions that may be necessary for typical symptom development.

The presence of phytoplasma in the second grade seeds showed a degree of association with reduction in emergence suggesting this phytoplasma is responsible for reducing lucerne seed viability. However, because lucerne is a host to many pathogens and pests (Summers 1998), the role of other pests and diseases, as well as abiotic factors in reducing seed viability should be investigated.

Although more research is required, the potential for seed transmission of phytoplasmas demonstrated in this work would have significant epidemiological and agronomic consequences. Possible seed transmission of phytoplasmas conflicts with current understanding of phytoplasma distribution within plant tissues. The results from this work warrant further research into the role of seed transmission in the epidemiology of lucerne diseases associated with phytoplasmas.

5. Management of suspected vectors of Australian lucerne yellows disease

5.1 Introduction

Australian lucerne yellows (ALuY) disease, a phytoplasma-associated disease (Pilkington *et al.* 2002), is believed to be vectored by one or all of the leafhoppers *O. orientalis, A. torrida* and *B. angustatus* (Pilkington *et al.* 2004a). *O. orientalis* is the vector of witches' broom disease in lucerne and has been implicated as a vector of many other phytoplasma pathogens (Tsai 1979, Osmelak *et al.* 1989; Padovan *et al.* 1996; Pilkington *et al.* 2004a). Reports also indicated that *O. orientalis* transmits Australian tomato big bud (Hill 1943), which is widespread in a range of plant species throughout Australia (Gibb 1996; Davis *et al.* 1997). *A. torrida* is a vector of viral and bacterial plant diseases (Grylls 1979) and TBB (Pilkington *et al.* 2004a). *A. torrida* and *B. angustatus* have been suggested as possible vectors of Australian grapevine yellows (AGY) (Osmelak *et al.* 1989).

Vector management is one potential strategy to reduce the impact of phytoplasma diseases. As an alternative to heavy reliance on synthetic pesticides, which has environmental costs, reducing the pest population may have a favourable effect. Exclusion of pests is one of the tactics considered to reduce pest populations. Insect exclusion barriers reduce the number of insecticide applications and ingress of insect transmitted pathogens (Vincent *et al.* 2003). Squash plants covered with fine-mesh fabric secured in such a way that insects were excluded had no foliar symptoms nor were PCR positive for cucurbit yellow vine bacterium (Bextine *et al.* 2001). Non covered plants, on the other hand, had 3% disease incidence and 25% PCR positive plants (Bextine *et al.* 2001). It has been reported that physical barriers could be effective in managing *Homalodisca vitripennis* (Germar) [= *H. coagulata* (Say)] (vector of Pierce's disease of grapevine) because 97% of the leafhopper adults trapped between 1 and 7m were caught at an height of 5m or lower (Blua and Morgan 2003). Insect exclusion barriers also can be used to manage insect pests that damage crops by direct feeding. 90cm high fences signficantly reduced the movement of *Delia radicum* (L.) into small plantings of rutabagas (Vernon and Mackenzie 1998). The two-year experiment showed that the number of first flight females inside the enclosures was 80.6% and 82.8% less than in the open check plots in the first and second years, respectively (Vernon and Mackenzie 1998).

A previous study showed that *O. orientalis, A. torrida* and *B. angustatus* migrated from lucerne field margin vegetation into the crop by trivial movement at canopy height rather than via long range dispersal (Pilkington *et al.* 2004a). This suggests the possibility of using physical barriers such as fences to restrict their migrations. Limiting the movement of suspected ALuY vectors into the crop from field margin vegetation may lower ALuY disease incidence.

Outward overhangs increase the efficiency of vertically orientated barriers to exclude the target insect pest (Bomford *et al.* 2000). The vertically orientated barrier intercepts low-flying leafhoppers moving into the crop whilst the overhangs prevent intercepted leafhoppers from crawling up and over the vertically orientated barrier. A fence consisting of vertical panels of nylon window screen, with a downward-sloping outward overhang significantly reduced the immigration of *D. radicum* into plantings of rutabaga (Bomford *et al.* 2000).

The aims of this study were to: 1) evaluate the efficacy of several configurations of a physical barrier constructed of commercial, woven plastic weed mat mounted on existing fences on the edge of a lucerne crop field to reduce immigration of *O. orientalis, A. torrida* and *B. angustatus* into the crop and reduce ALuY disease incidence in the crop; and 2) determine the efficacy of pesticide (insecticide, herbicide or both) applications on a small strip of field margin vegetation (2-10m wide and 20m long) to reduce immigration of *O. orientalis, A. torrida* and *B. angustatus* into the crop from field margin vegetation and reduce ALuY disease incidence in the crop.

5.2 Materials and Methods

Experimental sites

In the Lachlan Valley, central west NSW, more than 75% of seed lucerne crops are irrigated between December and February and lucerne seed production is intensive. The average lucerne seed yield is 600kg/ha. The Lachlan Valley area is one of the "hot spots" for Australian lucerne yellows disease (Eric Elliot, personal communication, February 2005). The other experimental sites were in Griffith and Keith. Griffith is located in the Riverina Plain south west of NSW. About 80% of seed lucerne production in Griffith is irrigated and the average seed yield is 700kg/ha. Keith is located in south eastern SA where almost all lucerne seed production is under irrigation.

Leafhopper exclusion fences

The experiment was conducted during the 2005/2006 crop season in the Lachlan Valley area. The soil is a sandy clay loam. The experimental site was five hectares in area with shelterbelt trees at the northeast border and surrounded by arable land with typical agricultural crops such as wheat, soybean and canola. Lucerne (Aurora) was sown at the experimental site on 29 August 2005.

An experiment was laid out linearly along the margin of the crop using the pre-existing, 60cm high post and wire fence. Four blocks, each containing five treatment plots of 15m long each were set up next to each other (Fig. 5.1). Treatments were: (1) barrier with overhang, (2) barrier only, (3) barrier with overhang and gap, (4) barrier with gap and (5) control plot without barrier. The purpose of the gap was to allow movement of epigeal predatory arthropods, floodwater and wildlife. The lucerne stand had adjacent non-lucerne vegetation consisting of *Trifolium pratense* L. (red clover), *Malva parviflora* L. (Mallow), *Eucalyptus globulus* L., *Silybum marianum* L. (variegated thistle), *Onopordum acanthium* L. (Scotch thistle) and *Solanum nigrum* L. (night shade).

Leafhopper movements into and out of the lucerne field were monitored using bi-directional sticky traps. Sticky traps were constructed using the method used by Pilkington (2003). Each trap ($0.16593m^2$) was constructed from 24, 90mm-diameter Petri dishes mounted on two 65cm-tall wooden stakes posted next to each other at the mid point of each plot. The inner surface of each Petri dish base was coated with a thin layer of Tanglefoot sticky trap glue (Australian Entomological Supplies, Bangalow, Australia). Twelve Petri dishes (total area of $0.08296m^2$) faced the stand and 12 on the opposite edge of the stakes 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 20cm from the soil surface and the centre of the top Petri dish was 60cm 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). Two traps were placed in the lucerne stand on the boundary with non-crop vegetation, at the midpoint of each plot. The traps were changed fortnightly (between 9 September 2005 and 2 February 2006). For each collection date, catches of *O. orientalis*, *A. torrida* and *B. angustatus* on each trap were identified and counted using a binocular microscope (10x) (Olympia Optical, Tokyo, Japan). Leafhopper catch data were pooled across all collection dates following a square root transformation ($\sqrt{(x+0.5)}$). Genstat (Genstat 2002) was used for analysis of variance to test the effects of leafhopper exclusion fences on leafhopper movements and ALuY disease incidence. An example of the output from this analysis is presented in appendix two.

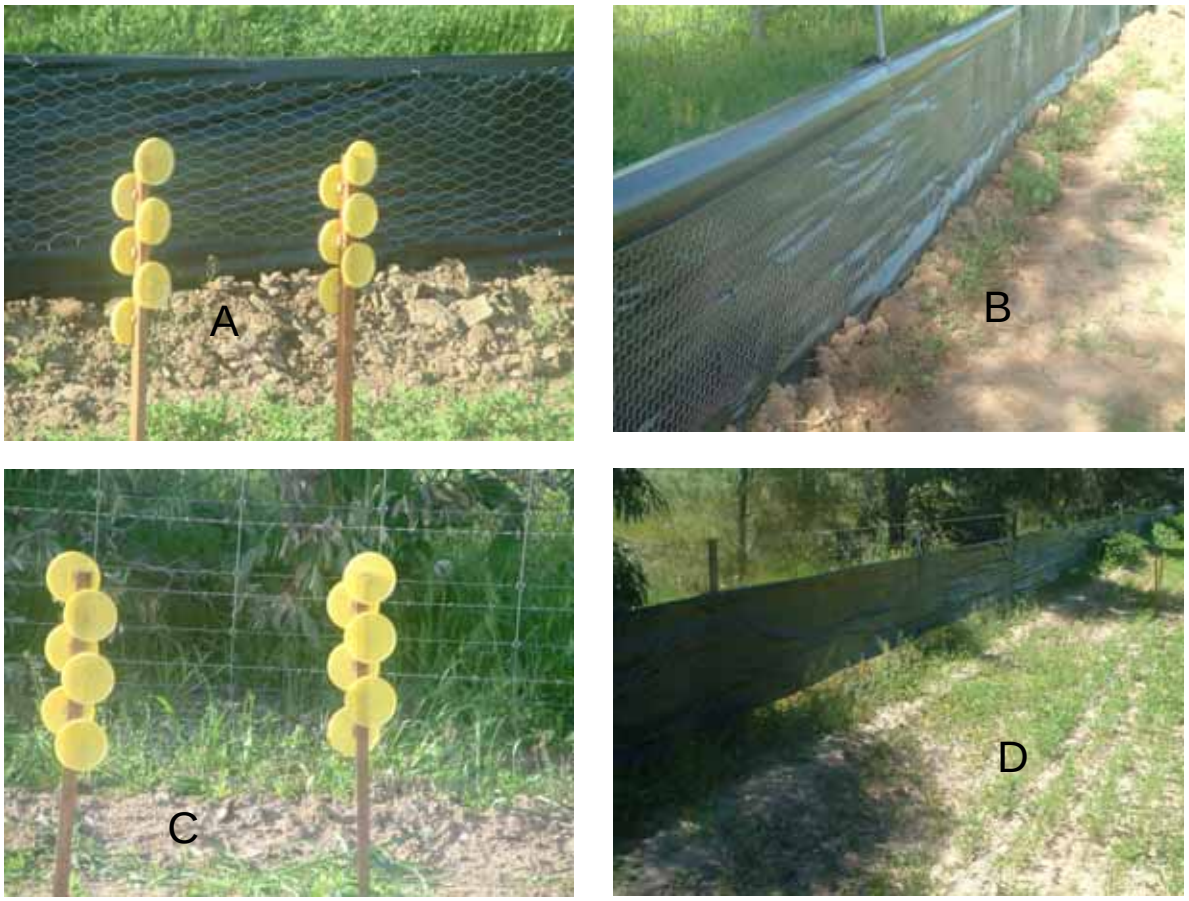

Fig. 5.1 Different treatments of the leafhopper exclusion fence. (A) barrier only; (B) Barrier with outward overhang; (C) control, without barrier and (D) Barrier with gap. **Note:** one of the treatments – barrier with gap and outward overhang is not shown in the pictures.

ALuY disease incidence was assessed on 25 January 2006 using the method used by Pilkington (2003). Assessment was made by delineating an arc with a radius of 30m in the lucerne adjacent to each barrier treatment plot using string attached to the midpoint of each plot's edge. All ALuY symptomatic plants within each arc were counted.

Insecticide or herbicide treatment of field margin vegetation

The border treatment experiment was carried out on sites at Griffith and Keith. Two certified lucerne seed stands, cv Aurora in Griffith and cv Supersriver in Keith, were established. The lucerne stand at Griffith was sown in early August 2005 and had adjacent non-lucerne vegetation consisting of *Trifolium pratense* L. (red clover), *Geococcus pusillus* L. (earth cress), *Foeniculum vulgare* L. (fennel) and *Polygonum arenastrum* L. (wireweed). A 400m long by 10m wide strip at Griffith and a 400m long by 2m wide strip at Keith were marked along one boundary of the lucerne fields. The strip on each of the two sites was divided into 20 plots each 20m long (five blocks, each with four treatments). Figure 5.2 shows an example of herbicide and insecticide treated plot at Griffith. Treatments were: (1) insecticide (0.465L/ha 300g/L dimethoate), (2) herbicide (1.5L/ha 360g/L glyphosate), (3) insecticide and herbicide and (4) control, where no herbicide or insecticide application was made. Leafhopper movements into and out of the lucerne field were monitored using the method described earlier for the fence barrier experiment. The traps were changed fortnightly (between 15 September 2005 and 2 February 2006).

ALuY disease incidence was assessed on 12 January 2006 at Griffith using the method used by Pilkington (2003). An arc with a radius of 30m was delineated in the lucerne adjacent to each Checklist for Final Reports by Pilkington (2003). An arc with a radius of 30m was delineated in the lucerne adjacent to each pesticide treatment plot using string attached to the midpoint of each plot's edge and all ALuY symptomatic plants within each arc were counted.

Fig. 5.2 Herbicide and insecticide treated plot on the edge of a lucerne field at Griffith.

5.3 Results

Leafhopper exclusion fences

When trap data for immigrating *O. orientalis* were pooled at the end of the experimental period, overall catches of immigration of *O. orientalis* were significantly ($P < 0.05$) lower in the control treatment than in other treatments. Few *A. torrida* and *B. angustatus* were caught in sticky traps so data for these leafhopper species were not analysed. *O. orientalis* catches fluctuated substantially over the period of the trial but catches generally increased from spring to summer (Table 5.1 and Figure 5.3). Catches generally increased between September and October, and again between December and January. There was a drop in the number of catches in November.

Table 5.1. Effect of leafhopper exclusion fence on catches of immigrating *O. orientalis*

Treatment	Mean number of catches					
	9/9/05	**12/10/05**	**28/10/05**	**17/11/05**	**30/12/05**	**2/02/06**
No barrier	1.75	8	23.25	11.75	35	40
Barrier with overhang	3.25	12.5	29.5	17	27.25	38.75
Barrier with overhang and gap	2.5	12.25	35.5	27	49.6	56.5
Barrier only	0.25	12	30.75	13.75	40	47.5
Barrier with gap	2.75	11.25	36.5	15	49.75	73

Overall catches of immigrating *O. orientalis* in the control treatment were not significantly different from those in treatments with barriers without gap and with or without an outward overhang. On the other hand, overall catches of immigrating *O. orientalis* in the control treatment were significantly ($P < 0.05$) lower than catches in treatments with barriers and gaps (with or without an outward overhang).

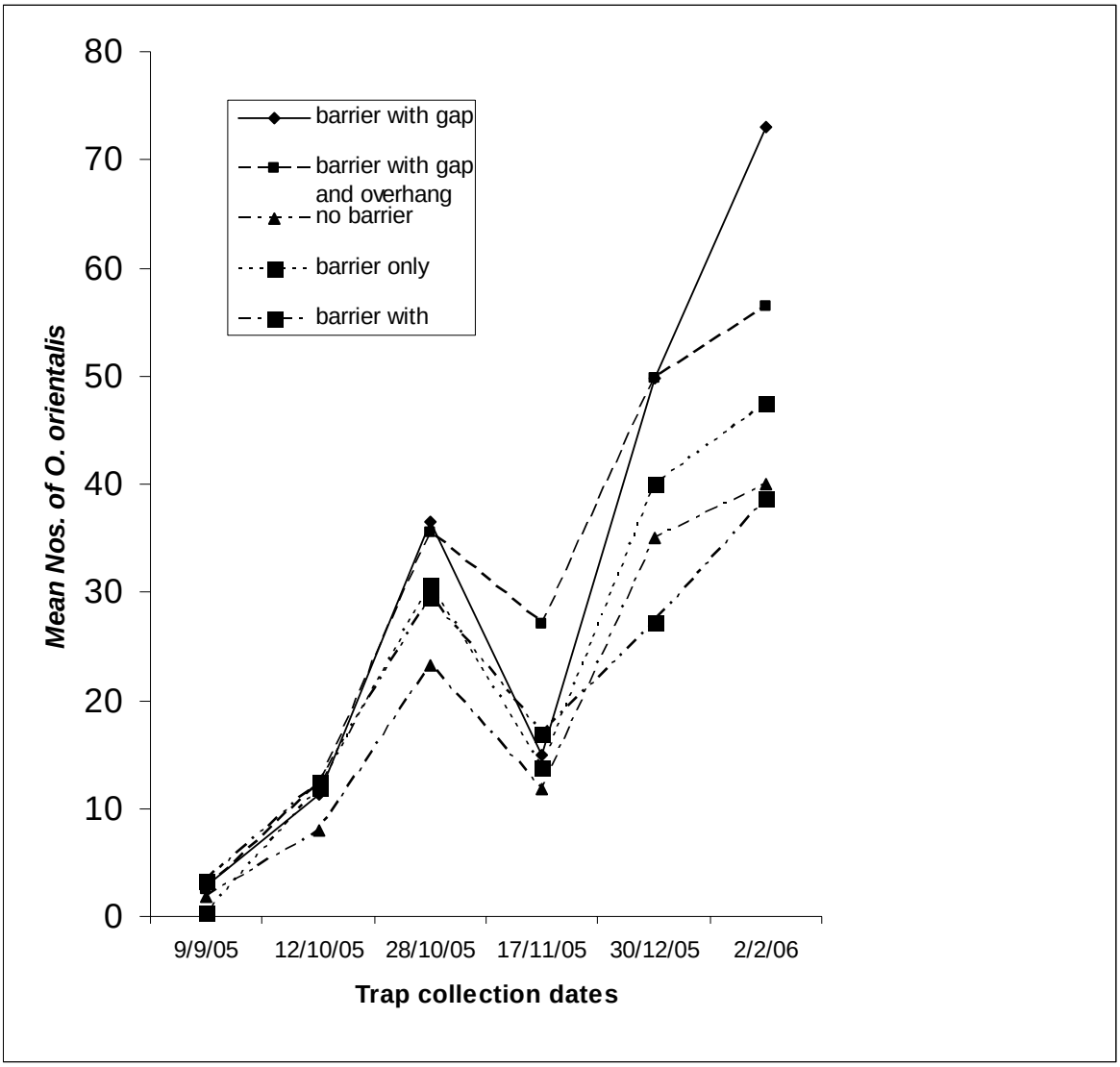

Fig. 5.3 Mean number of immigrating *O. orientalis* caught on yellow sticky traps in plots with differing field-margin barrier treatments (LSD over all dates 8.9)

Pesticidal treatment of field margin vegetation

Few *O. orientalis* and *A. torrida* were caught over the course of the experiment at Griffith so data were not analysed. Only low numbers of leafhoppers were present in sticky traps from the experiment at Keith in September and October so the experiment was discontinued.

Only herbicide or insecticide plus herbicide treatments of field margin vegetation significantly ($P = 0.001$) affected catches of *B. angustatus.* Catches of immigrant *B. angustatus* were highest in the herbicide plus insecticide as well as herbicide only treatments (Fig. 5.4) and lowest in the untreated control. Catches differed significantly between dates, the highest catches being in December (Table 5.2).

Table 5.2. Effect of pesticide treatment of lucerne field margin vegetation on catches of immigrating *B. angustatus*

Treatment	Mean number of catches				
	6/9/05	13/10/05	18/11/05	31/12/05	3/02/06
No pesticide	0.00	1.79	0.18	10.29	3.56
Insecticide	0.00	0.00	0.06	15.93	3.32
Herbicide	0.00	7.77	5.05	20.78	5.36
Herbicide and insecticide	0.01	3.65	3.60	41.39	4.55

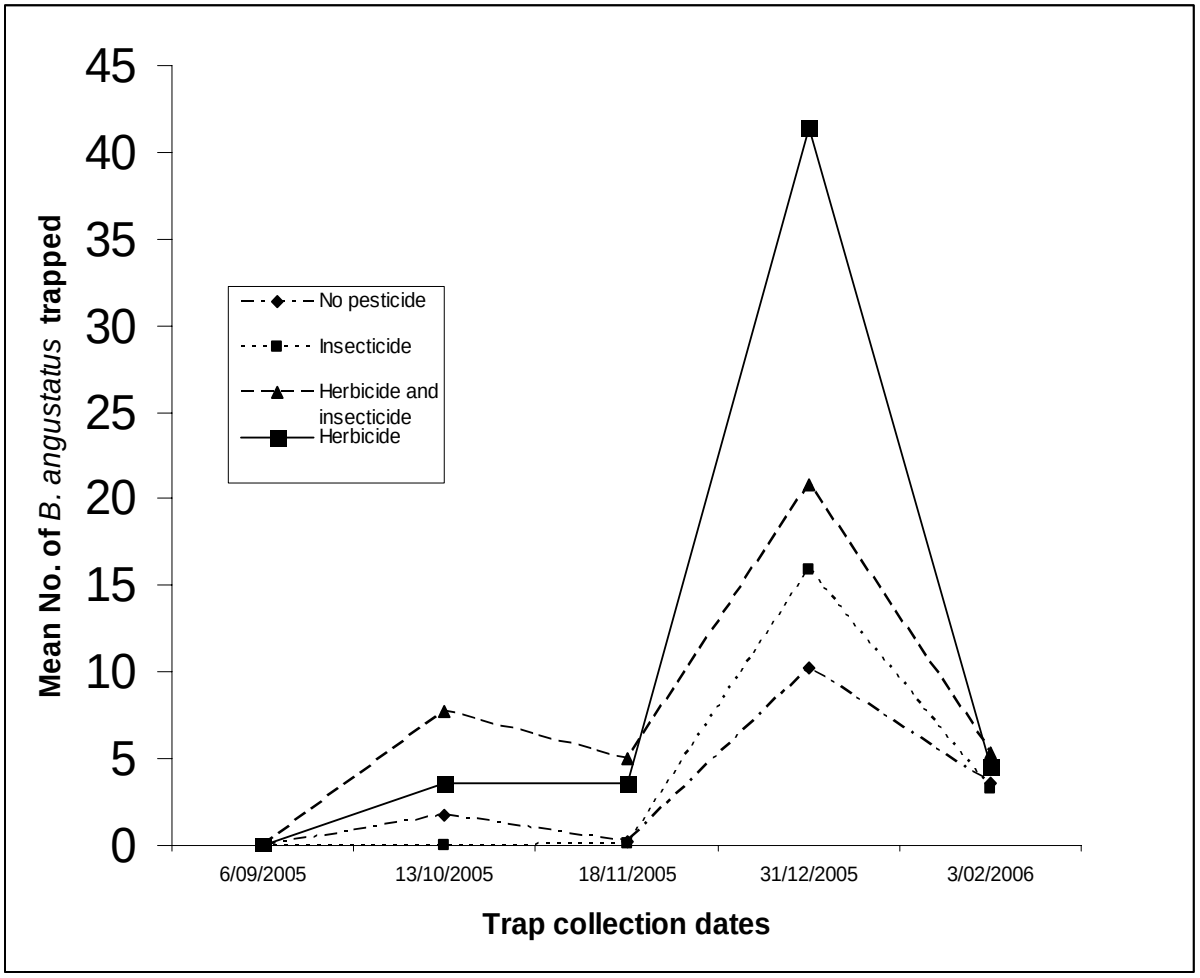

Fig. 5.4. Mean number of *B. angustatus* caught on yellow sticky traps in plots with differing treatments applied to field margin vegetation (LSD over all dates 5.05).

Neither weed mat barrier nor pesticide treatment of field margin vegetation significantly reduced the number of ALuY symptomatic lucerne plants.

5.4 Discussion

Results indicate that insecticide or herbicide treatment of a 2-10m wide and 20m long strip of field margin vegetation or 60cm high insect exclusion fence on one boundary of a new lucerne crop field were not effective in restricting the migration of suspected ALuY vector leafhoppers into the crop or in reducing ALuY disease incidence in the crop. Overall *O. orientalis* catches were lower in control plots than in treatments with a barrier. This may reflect the permeability of the control boundaries that would allow leafhoppers to exit the crop readily without being deflected by barriers to be caught on sticky traps. Such a situation could arise if the crop were well colonized by insects by mechanisms other than trivial movement from the vegetation in the immediate boundary. Either the leafhoppers reached the stand by long range dispersal flying well above the height of the barriers or through the other sides of the crop field where there were no barriers.

While catches of *O. orientalis* immigrating into the crop from field margin vegetation were highest in treatments with barriers and gaps with or without overhang, catches were lowest in the control and in treatments with barrier but without gap. This suggests that enclosing the entire field with a barrier may reduce the number of *O. orientalis* immigrating into a new lucerne crop from bordering vegetation. The use of insect exclusion barriers around the entire lucerne crop field, however, increases the labour and capital costs so it may not a viable option for ALuY disease management. Further, a few individuals of vector insects could overcome the barrier and reach the enclosed areas. However, this approach could be considered as an alternative to heavy reliance on synthetic pesticide to manage insect pests in high value crops. High number of catches of *O. orientalis* in treatments where there were gaps suggests that most leafhoppers moved into the crop by low flight, trivial movement. Reports indicated different dispersal behaviour of this leafhopper. While Grylls (1979) reported migratory long range dispersal of *O. orientalis* during summer nights, Pilkington *et al.* (2004a) observed short range dispersal of this leafhopper.

Catches of *B. angustatus* were significantly higher in herbicide treated plots than in the control. This suggests that the leafhopper moves into the crop by long range dispersal. In the present study, catches of large numbers of *B. angustatus* in sticky traps were observed in summer nights on a couple of occasions suggesting that mass dispersal of this leafhopper may occur in summer nights. Traps placed after 5pm were found to be flooded with this leafhopper when checked at 7am the next morning. There is little information in the literature regarding dispersal of *B. angustatus.* Mass migration of *O. orientalis* in summer nights has been reported (Grylls 1979). According to Grylls (1979), it seems that, in broad outline, the biology of most leafhoppers is similar suggesting the possibility of similar migratory behaviour of *O. orientalis* and *B. angustatus*.

The reason that catches of leafhoppers were low in November was not determined. Sometime between mid October and early November, livestock were allowed to graze in the lucerne stands where the barrier and border treatment experiments were established. It may be that the crop was less suitable and attractive to the leafhoppers during this time. In Australia, lucerne seed production generally uses a broad paddock cropping system to allow utilisation of lucerne fields for other purposes such as grazing and hay production (Kirkby 2006).

Although the experiment at Keith (SA) was established in early July 2005, few leafhoppers were caught in sticky traps replaced at fortnightly intervals between July and early October 2005. Low densities of leafhoppers were present in sticky traps from this experiment and this observation is in agreement with low densities recorded from other sites in the Keith district. Unusually dry conditions during the 2005/2006 crop season may be responsible for this low density of possible ALuY vectors.

The results of the present study suggest that a physical barrier or pesticide treatment of field margin vegetation on one boundary of the crop field is not effective in ALuY disease management. Future ALuY disease studies may consider the use of resistant/tolerant varieties as part an integrated disease

management (IDM) strategy for ALuY disease in addition to managing vectors and alternative host plants of the disease.

6. Concluding discussion

The present study was designed to follow-up the first detailed study on Australian lucerne yellows (ALuY) disease (Pilkington *et al.* 2003). In that study, an association between a novel phytoplasma and ALuY symptoms was reported based on PCR and electron microscopy analyses of ALuY symptomatic and asymptomatic lucerne plants. Fungi, bacteria and viruses were not considered important in the etiology of ALuY disease (Pilkington *et al.* 2003).

Based on transmission studies, *O. orientalis* was implicated as a vector of ALuY phytoplasma (Pilkington *et al.* 2004a). Furthermore, statistically significant correlations between the spatial and temporal distribution patterns of ALuY symptomatic lucerne plants and the leafhoppers *O. orientalis, A. torrida* and *B. angustatus* were found in the field (Pilkington *et al.* 2003). The leafhoppers *O. orientalis, A. torrida* and *B. angustatus* migrated into a new seed lucerne crop from field margin vegetation by short range dispersal (Pilkington *et al.* 2004b). Greater numbers of these leafhoppers were caught in lower traps (up to 30cm high from the ground) than on higher traps (70cm or higher from the ground).

Accurate identification and characterisation of a pathogen is fundamental to the development of successful disease management strategies. Accordingly, the present study initially aimed to repeat and confirm the identity of the supposed causal agent of ALuY disease. Applying the PCR assay protocols of Pilkington *et al.* (2002) to amplify DNA of the ALuY phytoplasma from ALuY disease symptomatic lucerne plants showed an association between other known phytoplasmas and ALuY symptoms (Chapter two).

The novel phytoplasma reported to be associated with ALuY by Pilkington *et al.* (2002) showed 99% 16Sr DNA sequence similarity with Omani alfalfa witches' broom, papaya yellow crinkle and papaya mosaic phytoplasmas (Pilkington *et al.* 2003). According to the IPRCM rules, phytoplasmas that share 97.5% or more 16Sr DNA sequence similarity belong to the same '*Candidatus* Phytoplasma' species unless they represent clearly separate ecological populations; for example unless the two phytoplasmas have different vectors or natural plant hosts (IPRCM 2004). Omani alfalfa witches' broom, papaya yellow crinkle and papaya mosaic phytoplasmas have been described under the same '*Candidatus* Phytoplasma' species named *Candidatus* Phytoplasma aurantifolia because they share 99.5% 16Sr DNA sequence similarity (IPRCM 2004). Papaya yellow crinkle and papaya mosaic phytoplasmas are genetically indistinguishable from Australian tomato big bud (TBB) phytoplasma (Khan *et al.* 2002b), which is the most widespread phytoplasma in Australia (Gibb 1996b; Davis *et al.* 1997a).

Re-examination in the present study of the ALuY sequence reported by Pilkington *et al.* (2001) found it to have many more single base pair insertions than other phytoplasmas and that these insertions are almost always identical to the preceding base. Accordingly, these single base insertions were considered to be spurious base calls by the software so were manually corrected. Re-running the phylogenetic analysis after deleting all potentially spurious base calls showed that ALuY is almost identical to Omani alfalfa witches' broom phytoplasma. This suggests that the ALuY phytoplasma reported by Pilkington *et al.* (2003) was not a novel phytoplasma.

In the present work, *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense were found associated with ALuY symptoms by PCR assays. However, the question of causality and the relative importance of *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense in ALuY disease remain open. Furthermore, many ALuY disease symptomatic lucerne plants were PCR negative for both phytoplasmas although template DNA extraction and preparation methods were the same for all samples. Involvement of other pathogens in ALuY disease cannot be ruled out. Future ALuY disease studies should analyse ALuY symptomatic plants for bacteria like

organisms (BLOs), phytoplasmas and viruses, particularly alfalfa mosaic virus (AMV). BLOs can infect the phloem tissue and cause phloem discolouration (Garnier et al. 2003). BLOs, together with phytoplasmas have been found associated with strawberry lethal yellows (SLY) disease in Australia (Streten *et al.* 2005). Both AMV and ALuY induce leaf yellowing in infected lucerne plants. Three ALuY symptomatic plants were tested for AMV by the enzyme linked immunosorbent assay (ELISA) method and one plant tested positive (data not shown). Whilst this result suggests strongly that further tests are required for a larger number of samples, such work was beyond the scope of the present study. This is an important direction for future research in this system.

It was not unexpected these two phytoplasmas were detected in lucerne as lucerne is a host to several phytoplasma pathogens worldwide such as alfalfa witches' broom (AWB) (Smrz *et al.* 1981, Saleh *et al.* 1995, Marcone *et al*. 1997, Khan *et al.* 2002b), the stolbur phytoplasma in Italy (Marzachi *et al.* 2000), little leaf phytoplasma in India (Surynarayana *et al.* 1996) and the aster yellows phytoplasma in Wisconsin (Peters *et al.* 1999). Furthermore, *Ca.* Phytoplasma australiense and TBB (type strain of *Ca.* Phytoplasma aurantifolia in Australia) are the dominant phytoplasma strains present in Australia (Davis *et al*. 1997).

Most ALuY disease symptomatic lucerne plant samples collected from Griffith were PCR positive for *Candidatus* Phytoplasma australiense whilst most samples collected from the Lachlan Valley area were PCR positive for *Candidatus* Phytoplasma aurantifolia. A mixed infection of *Ca.* Phytoplasma aurantifolia and *Ca.* Phytoplasma australiense was only found in one ALuY symptomatic plant collected from Griffith. PCR tests showed that there was no masking effect of *Candidatus* Phytoplasma aurantifolia or *Candidatus* Phytoplasma australiense in an event of mixed infection. *Candidatus* phytoplasma aurantifolia was detected when its titre concentration was as low as 0.015ng/µl in the presence of *Candidatus* Phytoplasma australiense and vice versa. This suggests that the two phytoplasmas can independently cause symptoms in lucerne plants of ALuY disease. The differing incidence of the two phytoplasmas in these regions may be related to the presence of different species of vectors in the Lachlan Valley and Griffith areas.

Monitoring of leafhopper activity using sticky traps showed that *B. angustatus* and *O. orientalis* were the most active leafhopper species at Griffith and in the Lachlan Valley, respectively. The search for vectors of ALuY phytoplasma in future studies may consider testing the vector status of *B. angustatus* and *O. orientalis* for *Ca.* Phytoplasma australiense and *Ca.* Phytoplasma aurantifolia, respectively. Future work also may consider using a series of nested PCR assays with universal primers followed by group specific or disease specific primers if mixed infection of single plants by multiple phytoplasmas is suspected (Lee *et al.* 2000).

We have reported advances in knowledge of the non-crop phytoplasma host plants in Australia. Of 38 plant species tested for *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense using the PCR assays, *Candidatus* Phytoplasma aurantifolia was detected in six plant species, most of them in the Chenopodiaceae. None of the test plants was PCR positive for *Candidatus* Phytoplasma australiense. PCR also detected *Candidatus* Phytoplasma aurantifolia in whole body samples of field collected *O. orientalis* adults. *O. orientalis* is a suspected vector of Australian TBB, which is one of the strains in the species *Candidatus* Phytoplasma aurantifolia (IPRCM 2004), and the results from the present study support this theory. The vectors of *Candidatus* Phytoplasma australiense (if any) are unknown.

Plants in the Chenopodiaceae also have been found to be feeding, breeding and winter survival hosts for *O. orientalis.* Large numbers of adults and nymphs of *O. orientalis* were collected from *C. murale* in the Lachlan Valley in summer and from *Chenopodium nitrariacium* and *Maeriana microphylla* in winter. Adults and nymphs of *O. orientalis* were collected from *Maeriana brevifolia* in Griffith in summer, autumn, winter and spring. Reports suggest that *O. orientalis* is a polyphagous insect (Helson 1942). In the present study, although small numbers of adults were collected from diverse plant species, collection in large numbers was only from chenopod plants. This suggests that, if *Ca.* Phytoplasma aurantifolia is confirmed to be the etiological agent responsible for ALuY disease,

targeted management of chenopod weeds in and around new lucerne fields could minimise ALuY disease incidence.

We have also reported a simple method to maintain and culture *O. orientalis* and *B. angustatus* under controlled conditions. *O. orientalis* fed and bred readily on *C. murale*, an annual forb easy to start from seed and maintain under controlled conditions. Vacuum samples also showed natural infestations of *C. murale* by *O. orientalis* adults and nymphs in the field during summer. *B. angustatus* was successfully maintained and cultured on *Trifolium pratense* (red clover) under controlled conditions. Such information is useful for future disease transmission studies.

We have reported findings of seed to seedling transmission of a phytoplasma: the most contentious issue in phytoplasmology. Lucerne seeds sourced from a seed lucerne crop that had high incidence of ALuY disease tested positive for *Candidatus* Phytoplasma australiense. Seedlings grown from such seeds in vector-free environments also tested positive for the phytoplasma. Most ALuY disease symptomatic lucerne plants sourced from the same paddock as the seeds also were PCR positive for *Candidatus* Phytoplasma australiense.

The results highlighted the potential importance of seed grading to minimise the effects of seed transmission of *Candidatus* Phytoplasma australiense. The first grade seed had significantly lower numbers of phytoplasma positive samples than the second grade seed. Australian lucerne seed companies usually grade lucerne seed as ‘first’ and ‘second’ based on size using a gravity table. Usually, only first grade seed is sold to growers to establish new lucerne seed crops because the second grade seed has very low germination/emergence rate (Kirkby, personal communication, April 2005). Because of its cheaper price than the first grade seed, the second grade seed is mainly used to establish lucerne crops for pasture or hay production (Kirkby, personal communication, April 2005). A high seed rate of the second grade seed is used to offset its low germination/emergence rate. Lucerne crops started from the second grade seed may serve as sources of inoculum for vectors to transmit the pathogen to seed lucerne crops. Results of the present study also suggest that *Ca.* Phytoplasma australiense may be responsible for reduced viability of the second grade seed though the role of other pathogens and pests in reducing lucerne seed viability cannot be ruled out as lucerne is a host to many pathogens and pests (Summers 1998). Significantly lower numbers of samples of the second grade seed were PCR positive for *Ca.* Phytoplasma australiense than samples of the first grade seed.

Literature is scant regarding seed transmission of phytoplasmas. The first refereed publication on this issue was that of Cordova *et al.* (2003); they reported PCR amplification of DNA of lethal yellowing phytoplasma in embryos of yellowing affected coconut palm trees. An unrefereed publication (Khan *et al.* 2002a) reported PCR detection of Omani alfalfa witches broom phytoplasma in lucerne seeds and seedlings. It is argued that phytoplasmas are confined to the phloem of host plants and that plant host tissue colonization by phytoplasmas only occurs passively when phytoplasmas are moved by photosynthate flows (Kuske and Kirkpatrick 1992). It is also believed that there is no continuous pathway between the phloem tissue of the mother plant and the developing embryo in the seeds implying that phytoplasmas cannot reach the developing embryo from the phloem of the mother plant. However, evidence of PCR detection of lethal yellowing phytoplasma in embryos of yellowing affected coconut palm plants (Cordova *et al.* 2003) contradicts this theory. There is no conclusive evidence, however, regarding the confinement of phytoplasmas in the phloem tissue or the mechanisms involved in the colonization of plant tissues by phytoplasmas.

The reason that symptoms characteristic of ALuY disease (Pilkington *et al.* 2000) did not develop in caged lucerne plants grown from the second grade seed and maintained until flowering in an insect free environment was not determined. It is possible that environmental conditions were not conducive in the growth chamber for typical ALuY symptom development. Future transmission tests may consider starting plants in insect free plots in the field. If *Candidatus* Phytoplasma australiense is confirmed to be the causal agent for ALuY disease and if seed to seedling transmission of this phytoplasma is confirmed, it will have major epidemiological implications for ALuY disease. It will also open new avenues for future epidemiological studies in other phytoplasma diseases.

There are few management options proven to be effective for phytoplasma diseases. The use of tetracycline is not economical except in some high value horticultural or ornamental crops, such as poinsettia, *Euphorbia pulcherrima* Willd. ex Klotzsch (Euphorbiaceae). Thus, alternative control measures such as clean stocks, controlling insect vectors, eliminating alternative host plants and integrated pest and disease management (IPDM) are becoming increasingly important in controlling phytoplasma diseases.

IPDM relies on a number of tactics such as physical barriers, biocontrols and limited use of pesticides (Vincent *et al*. 2003). Physical barriers such as fencing can exclude low-flying insects from enclosed areas (Vincent *et al.* 2003). In chapter five, results of experiments on using a physical barrier or pesticide treatment of field margin vegetation as vector management strategies to reduce ALuY disease incidence in new lucerne seed crops were reported. Insecticide or herbicide treatment of a 5-10m wide and 20m long strip of field margin vegetation or 60cm high insect exclusion physical barriers on one side of a new lucerne crop field failed to restrict the migration of suspected ALuY vector leafhoppers into the crop or to reduce ALuY disease incidence in the crop. Overall *O. orientalis* catches were lower in control plots without a barrier than in plots with a barrier. This may reflect the permeability of the control boundaries that would allow leafhoppers to exit the crop readily without being deflected by barriers to be caught on sticky traps. Such a situation could arise if the crop were well colonized by insects by mechanisms other than trivial movement from the vegetation in the immediate boundary. Either the leafhoppers reached the stand by long range dispersal flying well above the height of the barriers or through the other sides of the crop field where there were no barriers. Migratory long range dispersal of *O. orientalis* has been observed during summer nights (Grylls 1979). On the other hand, short range dispersal of *O. orientalis, A. torrida and B. angustatus* has been reported (Pilkington *et al.* 2004a).

The results from the present study suggest that a physical barrier on one side of the crop field is not effective in ALuY disease management. Documented success stories of the use of exclusion barriers in pest and disease management involved the enclosure of the target crop from all sides of the field/plot. The use of insect exclusion barriers around the entire lucerne crop field, however, increases the labour and capital costs. Further, a few individuals of vector insects could overcome the barrier and reach the enclosed areas. Herbicide or insecticide treatment of non-crop vegetation on the edge of a newly established lucerne stand was also ineffective in restricting the migration of *B. angustatus*, one of the suspect vectors of ALuY disease, into the stand. Future studies may test the effectiveness of this approach by applying insecticides or herbicides around the entire lucerne crop field, not just on one boundary of the crop field.

Conclusions

A series of studies on ALuY disease was carried out. PCR detected phytoplasmas in ALuY symptomatic lucerne plants, non-lucerne plants and insects. Analysis of the original sequences of ALuY phytoplasma reported by Pilkington *et al.* (2001) strongly indicated that ALuY phytoplasma is not a novel phytoplasma. In the present study *Candidatus* Phytoplasma aurantifolia and *Candidatus* Phytoplasma australiense were found to be associated with ALuY symptoms. Although the possibility remains open that other pathogens may be involved in causing symptoms recognised as ALuY.

PCR detected *Candidatus* Phytoplasma aurantifolia in previously undescribed phytoplasma host plant species and in *O. orientalis*. Such information is desirable for future epidemiological studies of phytoplasma diseases. However, none of the test plant species or insects were positive for *Candidatus* Phytoplasma australiense. Vector status of *O. orientalis* for *Candidatus* Phytoplasma aurantifolia needs to be confirmed by transmission tests and the vectors and non-crop host plants of *Candidatus* Phytoplasma australiense should be identified.

PCR evidence of seed to seedling transmission of *Candidatus* Phytoplasma australiense in lucerne warrants further research to resolve the issue. Lucerne is an amenable herbaceous plant and produces appreciable amount of seeds per plant for PCR analyses of seeds and progeny plants.

In general, there are no proven effective phytoplasma disease control measures available at present. In many cases, even after vector insects or alternative host plants have been successfully identified and managed, phytoplasma diseases still cause considerable yield losses (Lee *et al.* 2000). In the case of ALuY, physical barriers or pesticide treatment of field margin vegetation on one boundary of lucerne fields in the present study did not reduce ALuY disease incidence. Future studies should consider breeding/selection of ALuY disease tolerant/resistant lucerne varieties in addition to managing vectors or alternative hosts of ALuY phytoplasma.

Overall, this study has identified several plant species other than lucerne that harbour a phytoplasma that is associated with ALuY. These plants could constitute a significant reservoir for the pathogen. Lucerne growers can now trial the use of control methods (such as tillage, grazing or herbicide) to suppress these species. In the case of the perennial native species (for which control may be constrained as a result of native vegetation legislation), growers will need to site newly-sown lucerne stands as far away as possible. The rationale for this is strengthened by the finding of large numbers of the likely leafhopper vector on these plants and the fact that some of the field collected individuals tested positive for phytoplasma. Insecticidal control of this leafhopper could be warranted when it is present in large numbers within or adjacent to newly sown lucerne seed crops.

The most exciting finding from the present work is strong evidence for seed transmission of one of the phytoplasmas found to be associated with ALuY. Though this is contrary to generally held views on how phytoplasmas are transmitted, it suggests that inspection of seed crops, more careful seed certification requirements (possibly involving diagnostic tests for phytoplasmas) and judicious use of ‘home saved’ seed may play an important role in future ALuY management.

References

Agrios, G.N. 2005. Plant pathology. Academic Press, San Diego.

Ahrens, U. and Seemüller, E. 1992. Detection of DNA of plan pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. *Phytopathology* 82: 828-832.

Amici, A. and Favali, M.A. 1972. Thymidine-3H labeling of mycoplasma-like bodies in roots of rice plants affected by a yellows-type disease. *Protoplasma* 75: 37-43.

Andersen, M.T., Longmore, J., Liefting, L.W., Wood, G.A., Sutherland, P.W., Beck, D.L., and Forster, R.L.S. 1998. Phormium yellow leaf phytoplasma is associated with strawberry lethal yellows disease in New Zealand. *Plant Disease* 82: 606-609.

Andersen, M.T., Beever, R.E., Sutherland, P.W. and Forster, R.L.S. 2001. Association of "*Candidatus* Phytoplasma australiense" with Sudden Decline of Cabbage Tree in New Zealand. *Plant Disease* 85: 462-469.

Anonymous 2006. Pasture seeds research and development update. News letter issue 3. http://www.rirdc.gov.au/programs/PastureSeedsNewsletter.pdf. Accessed 20 December 2006.

Avner, L. and Czosnek, H. 2003. The DNA-B of the non-phloem limited bean dwarf mosaic virus (BDMV) is able to move the phloem-limited Abutilon mosaic virus (AbMV) out of the phloem, but DNA-B of AbMV is unable to confine BDMV to the phloem. *Plant Molecular Biology* 53: 789-803.

Barbara, D.J., Morton, A., Clark, M.F., and Davies, D.L. 2002. Immunodominant membrane proteins from two phytoplasmas in the aster yellows clade (chlorante aster yellows and clover phyllody) are highly divergent in the major hydrophilic region. *Microbiology-Sgm* 148: 157-167.

Barbetti, M.J. 1989. Fungal diseases of pasture legumes in Western Australia. *Journal of Agriculture Western Australia* 24: 10-12.

Batlle, A., Lavina, A., Garcia-Chapa, M., Sabate, J., Folch, C., and Asin, L. 2004. Comparative results between different detection methods of virus and phytoplasmas for a pear and apple certification program. *Acta Horticulturae.* 45: 71-77.

Bayliss, K.L., Saqib, M., Dell, B., Jones, MGK, Hardy, GE 2005. First record of '*Candidatus* Phytoplasma australiense' in Paulownia trees. *Australasian Plant Pathology* 34: 123-24.

Been, B.O. 1981. Observations on Field-Resistance to Lethal Yellowing in Coconut Varieties and Hybrids in Jamaica. *Oleagineux* 36: 9-11.

Behncken, G.M. 1984. *Orosius lotophagorum* subsp. *ryunkyuensis* (Hemiptera: Cicadellidae), a new vector of little leaf disease in Australia. *Australasian Plant Pathology* 13:35-36.

Bertaccini, A. 2007. Phytoplasmas: diversity, taxonomy, and epidemiology. *Frontiers in Bioscience* 12: 673-689.

Bertin, S., Palermo, S., Marzachi, C., and Bosco, D. 2004. Note: A comparison of molecular diagnostic procedures for the detection of aster yellows phytoplasmas (16Sr-I) in leafhopper vectors. *Phytoparasitica* 32: 141-145.

Bextine, B., Wayadande, A., Bruton, B.D., Pair, S.D., Mitchell, F., and Fletcher, J. 2001. Effect of insect exclusion on the incidence of yellow vine disease and of the associated bacterium in squash. *Plant Disease* 85:8795-8878.

Blanche, K.R., Tran-Nguyen, L.T.T., Gibb, KS 2003a. Detection, identification and significance of phytoplasmas in grasses in northern Australia. *Plant Pathology* 52: 505-512.

Blanche, K.R., Tran-Nguyen, L.T.T., Gibb, K.S. 2003b. Tests of transmission of cynodon white leaf phytoplasma to sugarcane and maize in northern Australia. *Australian Journal of Agricultural Research* 54: 423-427.

Blomquist, C.L., Barbara, D.J., Davies, D.L., Clark, M.F., and Kirkpatrick, B.C. 2001. An immunodominant membrane protein gene from the Western X-disease phytoplasma is distinct from those of other phytoplasmas. *Microbiology-Sgm* 147: 571-580.

Blua, M.J. and Morgan, W. 2003. Dispersion of Homalodisca coagulata (Hemiptera : Cicadellidae), a vector of Xylella fastidiosa, into vineyards in southern California. *Journal of Economic Entomology* 96: 1369-1374.

Bomford, M.K., Vernon, R.S. and Pats, P. 2000. Importance of Collection Overhangs on the Efficacy of Exclusion Fences for Managing Cabbage Flies (Diptera: Antomyiidae). *Environmental Entomology* 29:795-799.

Bosco, D., Palermo, S., Mason, G., Tedeschi, R., Marzachi, C., and Boccardo, G. 2002. DNA-based methods for the detection and the identification of phytoplasmas in insect vector extracts. *Molecular Biotechnology* 22: 9-18.

Boubourakas, I.N., Avgelis, A.D., Kyriakopoulou, P.E., and Katis, N.I. 2006. Occurrence of yellowing viruses (Beet pseudo-yellows virus, Cucurbit yellow stunting disorder virus and Cucurbit aphid-borne yellows virus) affecting cucurbits in Greece. *Plant Pathology* 55: 276-283.

Bove J.M. and Garnier, M. 1998. Walled and wall-less eubacteria from plants; Sieve-tube-restricted plant pathogens and plant surface contaminants. *Plant Cell, Tissue and Organ Culture* 52: 7-16.

Bove, J.M. and Garnier, M. 2002. Phloem-and xylem-restricted plant pathogenic bacteria. *Plant Science* 163: 1083-1098.

Bowyer, J.W., Atherton J.G., Teakle D.S., and Ahern G.A. 1969. Mycoplasma-like bodies in plants affected by legume little leaf, tomato big bud, and lucerne witches' broom diseases. *Australian Journal of Biological Science* 22: 271-274.

Brown, J.K. 2001. The molecular epidemiology of begomoviruses. In: *Trends in Plant Virology* Eds.JA Khan and J. Dykstra, Haworth Press, New York, pp. 279-316.

Brzin, J., Ermacora, P., Osler, R., Loi, N., Ravnikar, M., and Petrovic, N. 2003. Detection of apple proliferation phytoplasma by ELISA and PCR in growing and dormant apple trees. *Zeitschrift Fur Pflanzenkrankheiten Und Pflanzenschutz-Journal of Plant Diseases and Protection* 110: 476-483.

CAB International. 2002. Alfalfa mosaic virus. [Distribution map]. In *Distribution Maps of Plant Diseases*, pp. Map 388.

Cardena, R., Ashburner, G.R., and Oropeza, C. 2003. Identification of RAPDs associated with resistance to lethal yellowing of the coconut (*Cocos nucifera* L.) palm. *Scientia Horticulturae* 98: 257-263.

Carraro, L., Ferrini, F., Ermacora, P., and Loi, N. 2002. Role of wild Prunus species in the epidemiology of European stone fruit yellows. *Plant Pathology* 51: 513-517.

Chalak, L., Elbitar, A., Rizk, R., Choueiri, E., Salar, P., and Bove, J.M. 2005. Attempts to eliminate *Candidatus* phytoplasma phoenicium from infected Lebanese almond varieties by tissue culture techniques combined or not with thermotherapy. *European Journal of Plant Pathology* 112: 85-89.

Chen, Y.D., and Chen, T.A. 1998. Expression of engineered antibodies in plants: A possible tool for spiroplasma and phytoplasma disease control. *Phytopathology* 88: 1367-1371.

Chiykowski, L.N. 1988. Maintenance of yellows-type mycoplasmalike organisms. In *Tree mycoplasma diseases*. Ed. C Hiruki. The University of Alberta Press: Alberta, Canada. pp. 123-134.

Christensen, N.M., Axelsen, K.B., Nicolaisen, M., and Schulz, A. 2005. Phytoplasmas and their interactions with hosts. *Trends in Plant Science* 10: 526-535.

Clark, M.F., Barbara, D.J., and Davies, D.L. 1983. Production and Characteristics of Antisera to Spiroplasma-Citri and Clover Phyllody-Associated Antigens Derived from Plants. *Annals of Applied Biology* 103: 251-259.

Clark, M.F., Barbara, D.J., Davies, D.L. 1986. Production and characteristics of antisera to *Spiroplasma citri* and clover phyllody associated antigens derived from plants. *Annals of Applied Biology* 103: 251-259.

Clark, M.F., Lister, R.M., and Barjoseph, M. 1986. Elisa Techniques. *Methods in Enzymology* 118: 742-766.

Clark, M.F., Morton, A., and Buss, S.L. 1989. Preparation of Mycoplasma Immunogens from Plants and a Comparison of Polyclonal and Monoclonal-Antibodies Made against Primula Yellows Mlo-Associated Antigens. *Annals of Applied Biology* 114: 111-124.

Cocks, P.S. 2001. Ecology of herbaceous perennial legumes: a review of characteristics that may provide management options for the control of salinity and waterlogging in dryland cropping systems. *Australian Journal of Agricultural Research* 52: 137-151.

Conci, L., Meneguzzi, N., Galdeano, E., Torres, L., Nome, C., and Nome, S. 2005. Detection and molecular characterisation of an alfalfa phytoplasma in Argentina that represents a new subgroup in the 16S rDNA Ash Yellows group('Candidatus Phytoplasma fraxini'). *European Journal of Plant Pathology* 113: 255-265.

Constable, F.E., Gibb, K.S., Symons, R.H. 2003. Seasonal distribution of phytoplasmas in Australian grapevines. *Plant Pathology* 52: 267-276.

Conrad, H.R., and Klopfenstein, T.J. 1988. Role in livestock feeding - greenchop, silage, hay and dehy. In *Alfalfa and alfalfa improvement*, pp. 539-551. Ed. AA Hanson American Society of Agronomy

Converse, R.H., and George, R.A. 1987. Elimination of Mycoplasmalike Organisms in Cabot Highbush Blueberry with High-Carbon Dioxide Thermotherapy. *Plant Disease* 71: 36-38.

Cordova, I., Jones, P., Harrison, N.A., Oropeza, C. 2003. *In situ* PCR detection of phytoplasma DNA in embryos from coconut palms with lethal yellowing disease. *Molecular Plant Pathology* 4(2): 99-108.

Dai, Q., Hem, F.T., and Liu, P.Y. 1997. Elimination of phytoplasma by stem culture from mulberry plants (*Morus alba*) with dwarf disease. *Plant Pathology* 46: 56-61.

Davis, R.E., Dally, E.L., Gundersen, D.E., Lee, I.M., and Habili, N. 1997a. "*Candidatus* phytoplasma australiense," a new phytoplasma taxon associated with Australian grapevine yellows. *International Journal of Systematic Bacteriology* 47: 262-269.

Davis, R.I., Schneider, B., and Gibb, K.S. 1997b. Detection and differentiation of phytoplasmas in Australia. *Australian Journal of Agricultural Research* 48: 535- 544.

Deng, S., Hiruki, C. 1991. Amplification of 16S rRNA genes from culturable and non-culturable mollicutes. *Journal of Microbiological Methods* 14: 53-61.

Doi, Y., Teranaka, M., K, Y., and H, A. 1967. Mycoplasma or P.L.T group-like micro-organisms found in the phloem elements of plants infected with mulbery dwarf, potato witches' broom, aster yellows or Paulownia witches'-broom. *Annals of Phytopathology Society Japan* 33: 259-266.

Du, T., Wang, Y., Hu, Q.X., Chen, J., Liu, S., Huang, W.J., and Lin, M.L. 2005. Transgenic Paulownia expressing shiva-1 gene has increased resistance to Paulownia witches' broom disease. *Journal of Integrative Plant Biology* 47: 1500-1506.

Fletcher, J., Wayandande A., Melcher U., Ye F. 1998. The phytopathogenic mollicute insect vecor interface: a closer look. *Phytopathology* 88: 1351-1358.

Franova, J., Paltrinieri, S., Botti, S., Simkova, M., and Bertaccini, A. 2004. Association of phytoplasmas and viruses with malformed clovers. *Folia Microbiologica* 49: 617-624.

Galetto, L., Bosco, D., and Marzachi, C. 2005. Universal and group-specific real-time PCR diagnosis of flavescence doree (16Sr-V), bois noir (16Sr-XII) and apple proliferation (16Sr-X) phytoplasmas from field-collected plant hosts and insect vectors. *Annals of Applied Biology* 147: 191-201.

Garcia-Chapa, M., Batlle, A., Lavina, A., Galipienso, L., and Firrao, G. 2004a. Pear decline phytoplasma detection in pear trees and insect vectors by dot-blot hybridization and nested-PCR. *Acta Horticulturae* pp431-436.

Garcia-Chapa, M., Batlle, A., Rekab, D., Rosquete, M.R., and Firrao, G. 2004b. PCR-mediated whole genome amplification of phytoplasmas. *Journal of Microbiological Methods* 56: 231-242.

Garnier, M., Jagoueix-Eveillard, S., and Foissac, X. 2003. Walled bacteria inhabiting the phloem sieve tubes. *Recent Research Developments in Microbiology* 7: 209-223.

Genstat 2002. The guide to GenStat. Oxford, UK, VSN- International Ltd.

Getachew, M.A, Gurr, G.M., Fletcher, M.J., Pilkington, L.J., and Nikandrow, A. Mitchell, A. 2005. Association of Orosius argentatus (Hemiptera: Cicadellidae) with chenopod weeds: a breakthrough in vector-born plant diseases? Australian Entomological Society's 36th AGM and Sceintific Conference. 4-9 December 2005. Australian National Universtiy, Canberra, Australia.

Getachew, M.A., Mitchell, A., Gurr, G.M., Fletcher, M.J., Pilkington, L.J., and Nikandrow, A. 2007. First report of a "Candidatus phytoplasma australiense"-related strain in lucerne (Medicago sativa) in Australia. *Plant Disease* 91(1): 111.

Gibb, K.S., Davis, R.E. and Constable, F.E. 1996b. Tomato big bud-a widespread and 'successful' phytoplasma in Australia. *International Organization for Mycoplasmology Letters* 4: 280.

Gibb K.S., Moran J., Constable, F., and Padovan, A. 1999. Phytoplasmas in Australian grapevines - detection, differentiation and associated diseases. *Vitis* 38 (3): 107-114.

Gibb, K.S., Persley, D.M., Schneider, B., and Thomas, J.E. 1996a. Phytoplasmas associated with papaya diseases in Australia. *Plant Disease* 80: 174-178.

Gibb, K.S., Schneider B., Padovan A.C. 1998. Identification and specific detection of phytoplasmas in papaya. *Plant Pathology* 47: 325-32.

Gibb, K.S., Trans-Nguyen, L.T.T., Randles, J.W. 2003. A new phytoplasma detected in South Australian native perennial shrub, *Allocasuarina muelleriana. Annals of Applied Biology* 142,357-364.

Glennie, J.D. and Chapman, K.R. 1976. A review of dieback- a disorder of pawpaw (*Carica papaya* L.) in Queensland. *Journal of Agricultural and Animal Sciences* 33: 177-188.

Greber, R.S. and Gowanlock, D.H. 1979. Rickettsia-like and Mycoplasma-like organisms associated with two yellow-type diseases of strawberries in Queensland. *Australian Journal of Agricultural Research* 30: 1101-9.

Grylls, N.E. 1954. Rugose leaf curl – a new virus disease transovarially transmitted by the leafhopper *Austroagallia torrida. Australian Journal of Biological Sciences* 7: 47-58.

Grylls, N.E. 1979. Leafhopper vectors and the plan disease agents they transmit in Australia. In: *Leafhopper vectors and plant disease agents* Eds. K Maramorosch and KF Harris Academic Press, London. pp. 179-214.

Guesdon, J.L., Ternynck, T., and Avrameas, S. 1979. Use of Avidin-Biotin Interaction in Immunoenzymatic Techniques. *Journal of Histochemistry and Cytochemistry* 27: 1131-1139.

Gundersen, D.E., Lee, I.M., Rehner, S.A., Davis, R.E., and Kingsbury, D.T. 1994. Phylogeny of Mycoplasmalike Organisms (Phytoplasmas) - a Basis for Their Classification. *Journal of Bacteriology* 176: 5244-5254.

Guthrie, J.N., White, D.T., Walsh, K.B., and Scott, P.T. 1998. Epidemiology of phytoplasma-associated papaya diseases in Queensland, Australia. *Plant Disease* 82: 1107-1111.

Hacket, K.J. and Clark, T.B. (1989). Spiroplasma ecology. In: *The mycoplasmas*, Vol. 5. Eds RF Whitcomb JG Tully,. Academic Press, New York. pp. 113-200.

Halvorson, A.D., and Reule, C.A. 1980. Alfalfa for hydrologic control of saline seeps. *Soil Science Society of America Journal* 44: 370-374.

Harding, R.M. and Teakle, D.S. 1985. Mycoplasma-like organisms as causal agents of potato purple top wilt in Queensland. *Australian Journal of Agricultural Research* 36: 443-449.

Harrison, N.A., Bourne, C.M., Cox, R.L., Tsai, J.H., and Richardson, P.A. 1992. DNA Probes for Detection of Mycoplasmalike Organisms Associated with Lethal Yellowing Disease of Palms in Florida. *Phytopathology* 82: 216-224.

Harrison, N.A., Tsai, J.H., Bourne, C.M., and Richardson, P.A. 1991. Molecular-Cloning and Detection of Chromosomal and Extrachromosomal DNA of Mycoplasmalike Organisms Associated with Witches-Broom Disease of Pigeon Pea in Florida. *Molecular Plant-Microbe Interactions* 4: 300-307.

Hart, P.I.K., and Close, R.C. 1976. Control of leaf diseases of lucerne with benomyl In *Proceedings of the 29th New Zealand Weed Pest Control Conference.* Christchurch, NZ. pp.42-45

Hellemer, J.L. 1972. Lucerne yellows. BCRI (New South Wales Department of Agriculture, Biological and Chemical Research Institute) *News* 6-7.

Helms, K. 1957. Witches' broom disease of Lucerne-I. The occurrence of two strains of the disease and their relation to big bud of tomato. *Australian Journal of Agricultural Research* 8: 135-147.

Helson, G.A.H. 1951. The Transmission of Witches Broom Virus Disease of Lucerne by the Common Brown Leafhopper, Orosius-Argentatus (Evans). *Australian Journal of Scientific Research Series B-Biological Sciences* 4: 115-117.

Herrera, M.G., and Madariaga, V.M. 2003. Immunological, microscopical and molecular evidence of phytoplasma in grapes. *Agricultura Tecnica* 63: 15-22.

Hibben, C.R., Wolanski, B. 1970. Dodder transmission of a Mycoplasma from ash witches'-broom. *Phytopathology* 61: 151-156.

Hill, A.V. 1943. Insect transmission and host plants of big bud of tomato. *Journal of the Council for Scientific and Industrial Research Australia* 16: 85-90.

Hill, A.V. and Helson, G.A. 1949. Distribution in Australia of three virus diseases and of their common vector *Orosius argentatus* (Evans). *Journal of the Australian Institute of Agricultural Science* 15: 160-161.

Hill, A.V. and Mandryk, M. 1954. A study of the virus diseases 'big bud' of tomato and 'yellow dwarf' of tobacco. *Australian Journal of Agricultural Research* 6: 17-625.

Hirumi, H., Kimura, M., Maramoro.K, Bird, J., and Woodbury, R. 1974. Rickettsia-Like Organisms in Phloem of Little Leaf-Diseased Sida Cordifolia. *Phytopathology* 64: 581-582.

Hopkins, D.L. 1977. Diseases Caused by Leafhopper-Borne, Rickettsia-Like Bacteria. *Annual Review of Phytopathology* 15: 277-294.

Humphries, A.W., and Auricht, G.C. 2001. Breeding lucerne for Australia's southern dryland cropping environments. *Australian Journal of Agricultural Research* 52: 153-169.

Hutton E.M., and Grylls N.E., 1956. Legume 'little leaf', a virus disease of subtropical pasture species. *Australian Journal of Agricultural Research* 7, 85-97.

IRPCM, Phytoplasma Taxonomy Group 2004. '*Candidatus* Phytoplasma', a taxon for the wall-less, non-helical prokaryotes that colonize plant phloem and insects. *International Journal of Systematic and Evolutionary Microbiology* 54: 1243-1255.

Irwin, J.A., Aitken, K.S., Mackie, J.M., and Musial, J.M. 2006. Genetic improvement of lucerne for anthracnose (*Colletotrichum trifolii*) resistance. *Australasian Plant Pathology* 35: 573-579.

Irwin, J.A., Maxwell, D.P., and Smith, R.R. 1997. Expression of resistance by tetraploid and diploid lucerne genotypes to Phytophthora medicaginis is not influenced by inoculum level or by temperature. *Australian Journal of Agricultural Research* 48: 181- 190.

Jasnic, S. 2005. Viruses of alfalfa and clover. *Biljni Lekar, Plant Doctor* 33: 576-579.

Jiang, H., Wei, Saiki, T., Kawakita, H., Watanabe, K., and Sato, M. 2004. Distribution patterns of mulberry dwarf phytoplasma in reproductive organs, winter buds, and roots of mulberry trees. *Journal of General Plant Pathology* 70: 168-173.

Jiang, Y.P., Chen, T.A., Chiykowski, L.N., and Sinha, R.C. 1989. Production of Monoclonal-Antibodies to Peach Eastern X-Disease Agent and Their Use in Disease Detection. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* 11: 325-331.

Jomantiene, R., Davis, R.E., Maas, J., Dally, E.L. 1998. Classification of new phytoplasmas associated with diseases of strawberry in Florida, based on analysis of 16S rRNA and ribosomal protein gene operon sequences. *International Journal of Systematic Bacteriology* 48: 269-77.

Kakizawa, S., Oshima, K., Nighigawa, H., Jung, H.Y., Wei, W., Suzuki, S., Tanaka, M., Miyata, S., Ugaki, M., and Namba, S. 2004. Secretion of immunodominant membrane protein from onion yellows phytoplasma through the Sec protein-translocation system in Escherichia coli. *Microbiology-Sgm* 150: 135-142.

Kang, H.W., Cho, Y.G., Yoon, U.H., Eun, M.Y. 1998. A rapid DNA extraction method for RFLP and PCR analysis from a single dry seed. *Plant Molecular Pathology Reporter* 16: 1-9.

Keshavarz, T., and Izadpanah, K. 2005. Etiology of cucurbit yellows in the Boushehr Province, Iran. *Iranian Journal of Plant Pathology* 41: En107-121, pp 291-292.

Khan, A.J., Botti, S, Paltrinieri, S., Al-Subhi, A.M., Bertaccini, A.F. 2002a. Phytoplasmas in Alfalfa seedlings: infected or contaminated seeds? In *Abstracts, 14th International Organization of Mycoplasmology Conference*. Vienna, Austria p 148.

Khan, A.J., Botti, S., A.l. Subhi, A.M., Gunderson-Rindal, D.E., Bertaccini, A.F. 2002b. Molecular identification of a new phytoplasma associated with alfalfa witches'-broom in Oman. *Phytopathology* 92: 1038-47.

Khan, A.J., Lee, R.F., Hartung, J., 2003. Confirming seed transmission of witches' broom disease of lime. *8th International Congress of Plant Pathology* 2: 281.

Kirkby, L. 2006. A travel report presented to RIRDC Pasture Seeds Committee. http://www.rirdc.gov.au/reports/PSE/06-115.pdf. Accessed 1 January 2007.

Kirkpatrick, B.C. 1989. Strategies for charactersing plant pathogenic mycoplasma-like organisms and their effects on plants. In *Plant-microbe interactions. Molecular and Genetic perspectives.* Eds T Kosuge, EW Nester. McGraw Hill Publishing: New York. pp. 241-293.

Kirkpatrick, B.C., Stenger, D.C., Morris, T.J., and Purcell, A.H. 1987. Cloning and Detection of DNA from a Nonculturable Plant Pathogenic Mycoplasma-Like Organism. *Science* 238: 197-200.

Kirkpatrick, B.C., Smart, C.D., Gardner, S., Gao, J.L., Ahrens, U., Maurer, R., Schneider, B., Lorenz, K.H., Seemüller, E., Harrison, N.A., Namba, S., Daire, X. 1994. Phylogenetic relationships of plant pathogenic MLOs established by 16S/23S spacer sequences. *IOM Letters* 3: 228-229.

Krnjaja, V., and Levic, J. 2005. Pathogens of alfalfa and clover seed and seedlings and their control. *Biljni Lekar Plant Doctor* 33: 583-589.

Kuske, C.R., and Kirkpatrick, B.C. 1992. Distribution and Multiplication of Western Aster Yellows Mycoplasmalike Organisms in Catharanthus-Roseus as Determined by DNA Hybridization Analysis. *Phytopathology* 82: 457-462.

Kusunoki, M., Shiomi, T., Kobayashi, M., Okudaira, T., Ohashi, A., Nohira, T. 2002. A leafhopper (*Hishimonus sellatus*) transmits phyllgenetically distant phytoplasmas: Rhus Yellows and Hovenia Witches' broom phytoplasma. *Journal of Genetics and Plant Pathology* 68: 147-154.

Latta, R.A., Blacklow, L.J., and Cocks, P.S. 2001. Comparative soil water, pasture production, and crop yields in phase farming systems with lucerne and annual pasture in Western Australia. *Australian Journal of Agricultural Research* 52: 295-303.

Lee, I.M., Gunderson-Rindal, D.E., Beraccini, A. 1998. Phytoplasma: Ecology and genomic diversity. *Phytopathology* 88: 1359-1366.

Lee, I.M., Davis, R.E., and Gundersen-Rindal, D.E. 2000. Phytoplasma: Phytopathogenic mollicutes. *Annual Review of Microbiology* 54: 221-255.

Lee, I.M., Davis, R.E., and Hsu, H.T. 1993. Differentiation of Strains in the Aster Yellows Mycoplasmalike Organism Strain Cluster by Serological Assay with Monoclonal-Antibodies. *Plant Disease* 77: 815-817.

Liew, E.C., and Irwin, J.A. 1997. Differential disease reactions on lucerne genotypes inoculated with Phytophthora medicaginis isolates from lucerne and chickpea. *Australian Journal of Agricultural Research* 48: 545- 551.

Liefting, L.W., Andersen, M.T., Beever, R.E., Gardner, R.C., Forster, R.L.S. 1996. Sequence hetrogeneity in the two 16S rRNA genes of *Phormium* yellow leaf phytoplasma. *Applied and Environmental Microbiology* 62: 3133-3139.

Liefting, L.W., Beever, R.E., Winks, C.J., Pearson, M.N., Forster, R.L.S. 1997. Planthopper transmission of Phormium yellow leaf phytoplasma. *Australasian Plant Pathology* 26:148-154.

Lim, P.O., and Sears, B.B. 1989. 16s Ribosomal-Rna Sequence Indicates That Plant-Pathogenic Mycoplasmalike Organisms Are Evolutionarily Distinct from Animal Mycoplasmas. *Journal of Bacteriology* 171: 5901-5906.

Lin, C.P., and Chen, T.A. 1985. Monoclonal-Antibodies against the Aster Yellows Agent. *Science* 227: 1233-1235.

Lin, C.P., and Chen, T.A. 1986. Comparison of Monoclonal-Antibodies and Polyclonal Antibodies in Detection of the Aster Yellows Mycoplasmalike Organism. *Phytopathology* 76: 45-50.

Loi, N., Ermacora, P., Carraro, L., Osler, R., and Chen, T.A. 2002. Production of monoclonal antibodies against apple proliferation phytoplasma and their use in serological detection. *European Journal of Plant Pathology* 108: 81-86.

Lorenz, K.H., Schneider, B., Ahrens, U., Seemüller, E. 1995. Detection of the apple proliferation and pear decline phytoplasmas by PCR amplification of ribosomal and non ribosomal DNA. *Phytopathology* 85: 771-776.

Lowe, K.F., Bowdler, T.M., and Ryley, M.J. 1991. Lack of control of phytophthora root rot in lucerne (*Medicago sativa*) by foliar applications of phosphonate. *Australian Journal of Experimental Agriculture* 31: 503 - 507.

McCoy, R.E. 1979. Mycoplasmas and yellows diseases. In *The mycoplasmas*, Vol III, *Plant and Insect Mycoplasmas* Eds Whitcomb, RF and Tully, IG, New York; Academic Press pp. 229-264.

McDonald, W.J., Lattimore, M.A., Williams, R. 2004. *Lucerne: Medicago sativa*. Fact sheet 4th edition. NSW Agriculture.

Mackie, J.M., and Irwin, J.A. 1998. The proportion of individual lucerne plants resistant to *Phytophthora medicaginis* and *Colletotrichum trifolii* in Australian cultivars. *Australian Journal of Experimental Agriculture* 38: 41- 44.

Mackie, J.M., Musial, J.M., O'Neill, N.R., and Irwin, J.A. 2003. Pathogenic specialisation within *Colletotrichum trifolii* in Australia, and lucerne cultivar reactions to all known Australian pathotypes. *Australian Journal of Agricultural Research* 54: 829-836.

Magarey, P.A., Fletcher, M.J., Pilkington, L.J. and Gurr, G.M. 2002. *Orosius argentatus* and yanga bush: The missing links in Australian grapevine yellows? Australian Entomological Society 33rd AGM and Scientific Conference, Fremantle, Australia.

Magarey, P.A., Wachatel, M.F. 1986a. A review of the present status of Australian grapevine yellows. *Agricultural Record* 12: 12-18.

Magarey, P.A., Wachatel, M.F. 1986b. Australian grapevine yellows. *International Journal of Tropical Plant Disease* 4: 1-14.

Marcone, C., Ragozzino, A., and Seemüller, E. 1997. Detection and identification of phytoplasmas in yellows-diseased weeds in Italy. *Plant Pathology* 46: 530-537.

Marcone, C., Gibb, K.S., Streten S., Schneider B. 2004. '*Candidatus* Phytoplasma spartii', '*Candidatus* Phytoplasma rhamni' and '*Candidatus* Phytoplasma allocasuarinae', respectively associated with spartium witches' broom, buckthorn witches' broom and allocasuarina yellows diseases. *International Journal of Systematic Bacteriology* 54: 1025-1029.

McCoy, R.E. 1979. Mycoplasmas and yellows diseases. In *The mycoplasmas*, Vol III, *Plant and Insect Mycoplasmas* (Whitcomb, RF and Tully, IG, eds.), pp. 229-264. New York; Academic Press.

Marzachi, C. 2004. Molecular diagnosis of phytoplasmas. *Phytopathologia Mediterranea* 43: 228-231.

Marzachi, C., Veratti F., d' Aquilio M, Vischi A., Conti M., Boccardo G. 2000. Molecular hybridisation and PCR amplification on non-ribosomal DNA to detect and differentiate stolbur phytoplasma isolates from Italy. *Journal of Plant Pathology* 82: 201-212.

McCoy, R.E. 1975. Effect of Oxytetracycline Dose and Stage of Disease Development on Remission of Lethal Yellowing in Coconut Palm. *Plant Disease Reporter* 59: 717-720.

McCoy, R.E. 1982. Use of Tetracycline Antibiotics to Control Yellows Diseases. *Plant Disease* 66: 539-542.

McGechan, J. 1980. Yellows. BCRI (New South Wales Department of Agriculture, Biological and Chemical Research Institute). Research Report p 11.

Morgan, W.C., and Parbery, D.G. 1980. Depressed Fodder Quality and Increased Estrogenic Activity of Lucerne Infected with Pseudopeziza-Medicaginis. *Australian Journal of Agricultural Research* 31: 1103-1110.

Osmelak, J.A., Emmett, R.W. and Pywell, M. 1989. Monitoring for potential leafhopper vectors (Hemiptera: Cicadellidae and Fulgoroidea) of the causal agent of Australian grapevine yellows. *Plant Protection Quarterly* 4: 8-10

Padovan, A.C., and Gibb, K.S. 2001. Epidemiology of phytoplasma diseases in papaya in northern Australia. *Journal of Phytopathology* 149: 649-658.

Padovan, A.C., Gibb, K.S., Bertaccini, A., Vibio, M., Bonfiflioli, R.E., Magarey, P.A., Sears, B.B. 1995. Molecular detection of the Australian grapevine yellows phytoplasma and comparison with grapevine yellows phytoplasmas from Italy. *Australian Journal of Grape and Wine research* 1: 25-31.

Padovan, A., Gibb, K., and Persley, D. 2000. Association of 'Candidatus Phytoplasma australiense' with green petal and lethal yellows diseases in strawberry. *Plant Pathology* 49: 362-369.

Peters, R.D., Lee, M.E., Grau, C.R., Driscoll, S.J., Winberg, R.M., Kurtzweil, N.C., Lukaesko, L.A., and Lee, I.M. 1999. First report of aster yellows phytoplasma in alfalfa. *Plant Disease* 83: 488.

Pilkington, L.J. 2003. Australian lucerne yellows: pathogen, vector and control. PhD Thesis. University of Sydney, Australia.

Pilkington, L.J., Gibb, K.S., Gurr, G.M., Fletcher, M.J., Nikandrow, A., Elliott, E., van de Ven, R. and Read, D.M.Y.2001. Australian lucerne yellows phytoplasma partial 16S rRNA gene. http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotideandval=16415779. Accessed 1 May 2005.

Pilkington, L.J., Gurr, G.M., Fletcher, M.J., Nikandrow, A., Elliot E. 1999. Occurrence and severity of lucerne yellows disease in Australian lucerne seed crops. *Australasian Plant Pathology* 28: 253-259.

Pilkington, L.J., Gurr, G.M., Fletcher, M.J., Elliott, E., Nikandrow, A., Nicol, H.I. 2004a. Vector status of three leafhopper species for Australian lucerne yellows phytoplasma. *Australasian Journal of Entomology* 43: 366-373.

Pilkington, L.J., Gurr, G.M., Fletcher, M.J., Elliott, E., Nikandrow, A., Nicol, H.I. 2004b. Reducing the immigration of suspected leafhopper vectors and severity of Australian lucerne yellows disease. *Australian Journal of Experimental Agriculture* 44 (10): 983-992.

Pilkington, L.J., Gibb, K.S., Gurr, G.M., Fletcher, M.J., Nikandrow, A., Elliott, E., Van de Ven, R., and Read, D.M.Y. 2002. First report of a phytoplasma associated with Australian lucerne yellows disease. *Plant Pathology* 51: 390-390.

Pilkington, L.J., Gibb, K.S., Gurr, G.M., Fletcher, M.J., Nikandrow, A., Elliott, E., ven de Ven, R., and Read, D.M.Y. 2003. Detection and identification of a phytoplasma from lucerne with Australian lucerne yellows disease. *Plant Pathology* 52: 754-762.

Purcell, A.H. 1982. Insect vector relationships with prokaryotic plant pathogens. *Annual Review of Phytopathology* 20: 397-417.

Rowhani, A., Uyemoto, J.K., Golino, D.A., and Martelli, G.P. 2005. Pathogen testing and certification of Vitis and Prunus species. *Annual Review of Phytopathology* 43: 261-278.

(RIRDC) Rural Industries Research and Development Corporation 2004. Pasture seeds *Annual Report*.

Saindon, G., Michaud, R., and Stpierre, C.A. 1991. Breeding for Root Yield in Alfalfa. *Canadian Journal of Plant Science* 71: 727-735.

Schneider, B., Gibb, K.S., Seemüller, E. 1997. Sequence and RFLP analysis of the elongation factor Tuf gene used in differentiation and classification of phytoplasmas. *Microbiology* 143:3381-3389.

Schneider, B., Gibb, K.S. 1997. Detection of phytoplasmas in declining pears in southern Australia. *Plant Disease* 81: 254-258.

Schneider, B., Padovan, A.C., De La Rue, S., Eichner, R, Davis, R, Bernuetz, A., Gibb, K., 1999. Detection and differentiation of phytoplasmas in Australia: an update. *Australian Journal of Agricultural Research* 50: 333-342.

Sears, B.B., Klomparens K.L. 1989. Leaf tip cultures of the evening primrose allow stable, aseptic culture of mycoplasma-like organism. *Canadian Journal of Plant Pathology* 11: 343-348.

Seemüller, E. 1988. Colonization patterns of mycoplasmalike organisms in trees affected by apple proliferation and pear decline. Pages 179-192 in *Tree Mycoplasmas and Mycoplasma Diseases*, C. Hiruki, ed., The University of Alberta Press, Edmonton, AB. 245 pp.

Seemüller, E., and Schneider, B. 2004. 'Candidatus Phytoplasma mali', 'Candidatus Phytoplasma pyri' and 'Candidatus phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. *International Journal of Systematic and Evolutionary Microbiology* 54: 1217-1226.

Semeniuk, G., and Rumbaugh, M.D. 1976. Reaction of Some Perennial and Annual Medicago Species and Cultivars to Yellow Leafblotch Disease Caused by Leptotrochila-Medicaginis. *Plant Disease Reporter* 60: 596-599.

Siller, W., Kuhbandner, B., Marwitz, R., Petzold, H., Seemüller, E. 1987. Occurrence of mycoplasma-like organisms in parenchyma cells of *Cuscuta odorata* (Ruiz et Pav) *J. Phytopathology* 119: 147-159.

Sinha, R.C. 1974. Purification of Mycoplasma-Like Organisms from China Aster Plants Affected with Clover Phyllody. *Phytopathology* 64: 1156-1158.

Sinha, R.C., and Benhamou, N. 1983. Detection of Mycoplasmalike Organism Antigens from Aster Yellows-Diseased Plants by 2 Serological Procedures. *Phytopathology* 73: 1199-1202.

Sinha, R.C., and Chiykowski, L.N. 1984. Purification and Serological Detection of Mycoplasmalike Organisms from Plants Affected by Peach Eastern X-Disease. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* 6: 200-205.

Smrz, J., Ulrychova M., and Jokes M. 1981. Alfalfa witches' broom in Czechoslovakia and demonstration of mycoplasma-like organisms in the affected plants. *Biologia Plantarum* 23: 381-383.

Staden, R. 1996. The Staden Sequence Analysis Package. *Molecular Biotechnology* 5: 233-241.

Stovold, G.E. 1981. Some crown and root diseases of lucerne. *The Agricultural Gazette of New South. Wales*. 92: 17- 18.

Streten, C. and Gibb, K.S. 2006. Phytoplasma diseases in tropical and subtropical Australia. *Australasian Plant Pathology* 35(2): 129-146.

Streten, C., Conde, B., Herrington, M., Moulden, J., and Gibb, K. 2005a. Candidatus Phytoplasma australiense is associated with pumpkin yellow leaf curl disease in Queensland, Western Australia and the Northern Territory. *Australasian Plant Pathology* 34: 103-105.

Streten, C., Herrington, M.E., Hutton, D.G., Persley, D.M., Waite, G.K., and Gibb, K.S. 2005b. Plant hosts of the phytoplasmas and rickettsia-like-organisms associated with strawberry lethal yellows and green petal diseases. *Australasian Plant Pathology* 34: 165-173.

Streten, C., Waite, G.K., Herrington, M.E., Hutton, D.G., Persley, D.M., and Gibb, K.S. 2005c. Rickettsia-like-organisms and phytoplasmas associated with diseases in Australian strawberries. *Australasian Plant Pathology* 34: 157-164.

Summers, C.G. 1998. Integrated pest management in forage alfalfa. *Integrated Pest Management Reviews* 3: 127-154.

Suryanayana, V., Singh, S., Muniyappa, V., and Redy, H. 1996. Little leaf of Medicago sativa L. - A new phytoplasma disease in India. *International Journal of Tropical Plant Diseases* 14: 167-171.

Tsai, J.H. 1979. Vector transmission of mycoplasmal agents of plant diseases. In: *Plant and insect mycoplasmas* Eds RF whitcomb and JG Tully Academic Press, Inc., New York. pp. 265-307.

Valiunas, D., Jomantiene R., Davis R.E., Sindaraviciene I., Alminaite A., Staniulis J., 2000. Molecular detection and characterisation of phytoplasmas infecting vegetables, legumes, and ornamental plants in Lithuania. *Transaction of the Estonian Agricultural University (Agronomy)* 209: 220-3.

Vera, C., and Milne, R.G. 1994. Immunosorbent Electron-Microscopy and Gold Label Antibody Decoration of MLOs from Crude Preparations of Infected Plants and Vector Insects. *Plant Pathology* 43: 190-199.

Vernon, R.S. and Mackenzie, J.R. 1998. The effect of exclusion fences on the colonization of rutabagas by cabbage flies. *Canadian Entomologist* 130: 153-162.

Viands, D.R., Hansen, J.L., Thomas, E.M., and Neally, J.L. 2005. Registration of 'Guardsman II' alfalfa. *Crop Science* 45: 2644.

Vincent, C., Hallman, G., Panneton, B., and Fleurat-Lessard, F. 2003. Management of agricultural insects with physical control methods. *Annual Review of Entomology* 48: 261-281.

Walsh, K.B., Guthrie, J.N. and White, D.T. 2006. Control of phytoplasma diseases of papaya in Australia using netting. *Australasian Plant Pathology* 35: 49-54.

Wang, K. and Hiruki, C. 2001. Use of heteroduplex mobility assay for identification and differentiation of phytoplasmas in the aster yellows group and the clover proliferation group. *Phytopathology* 91: 546-552.

Wang, K. and Hiruki, C. 2001. Molecular characterisation and classification of phytoplasmas associated with canola yellows and a new phytoplasma strain associated with dandelions. *Plant Disease* 85: 76-9.

Weber, A. and Maixner, M. 1998. Survey of populations of the planthopper *Hyalesthes obsoletus* Sign. (Auchenorrhyncha, Cixiidae) for infection with the phytoplasma causing grapevine yellows in Germany. *Journal of Applied Entomology-Zeitschrift Fur Angewandte Entomologie* 122: 375-381.

Wilson, D., Blanche K.R. and Gibb K.S. 2001. Phytoplasmas and disease symptoms of crops and weeds in the semi-arid tropics of the Northern Territory, Australia. *Australasian Plant Pathology* 30: 159-163.

Wei, W., Kakizawa, S., Jung, H.Y., Suzuki, S., Tanaka, M., Nishigawa, H., Miyata, S., Oshima, K., Ugaki, M., Hibi, T., et al. 2004. An antibody against the SecA membrane protein of one phytoplasma reacts with those of phylogenetically different phytoplasmas. *Phytopathology* 94: 683-686.

Wolfe, P.B., Wickner, W. and Goodman, J.M. 1983. Membrane Assembly from Purified Components. 6. Sequence of the Leader Peptidase Gene of Escherichia-Coli and the Orientation of Leader Peptidase in the Bacterial Envelope. *Journal of Biological Chemistry* 258: 2073-2080.

Wongkaew, P. and Fletcher, J. 2004. Sugarcane white leaf phytoplasma in tissue culture: long-term maintenance, transmission, and oxytetracycline remission. *Plant Cell Reports* 23: 426-434.

Zhang, Y.P., Uyemoto, Z.K. and Kirkpatrick, B.C. 1998. A small scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. *Journal of Virological Methods* 71: 45-50.

Zreik, L., Carle, P., Bove, J.M. and Garrnier, M. 1995. Characterization of the mycoplasmalike organism associated with witches' broom disease of lime and proposition of a "*Candidatus*" taxon for the organism, "*Candidatus* Phytoplasma aurantifolia". *International Journal of Systematic Bacteriology* 45: 449-453.

Appendix – An illustrated extension pamphlet