Australian lucerne yellows:

pathogen, vector and control

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Abstract

Prior to the work carried out in this thesis, little was known about the causal organism of Australian lucerne yellows (ALuY) and nothing was known of the epidemiology of this disease. Foliar and root symptoms are described for (ALuY), a disease common in Australian lucerne seed crops. A phytoplasma was detected in plants exhibiting symptoms, but not in symptomless lucerne plants. Oligonucleotide primers specific to the phytoplasma 16S-23S rRNA intergenic spacer region (SR) were used in polymerase chain reaction (PCR) assays on DNA extracted from lucerne plants with and without symptoms and detected a novel phytoplasma associated with the presence of symptoms. Identical restriction fragment length polymorphism (RFLP) profiles were obtained for PCR products amplified from ten symptomatic lucerne samples. RFLP profiles obtained for four restriction enzymes were different from those of the tomato big bud (TBB) phytoplasma, which is also present in lucerne seed stands. ALuY phytoplasma PCR products were sequenced to determine phylogeny and were found to fall within the faba bean phytoplasma group and phytoplasma group 16srII. Transmission electron microscopy revealed phytoplasmas in the phloem of ALuY affected plant samples but not in symptomless plant samples. The role of fungal, bacterial and viral agents in the aetiology of ALuY was excluded.

Three newly-sown lucerne stands in the mid Lachlan Valley region of New South Wales, Australia were sampled over 50 weeks for ALuY symptom distribution and severity. Leafhopper populations were also monitored.

Symptoms developed in all three stands within 32 weeks of sowing. There were statistically significant spatial patterns in the density of symptomatic plants on two occasions at two sites. Two possible insect vectors, *Austroagallia torrida* and *Batracomorphus angustatus*, were more numerous in some sections of crop-margins at two sites. These two species and a third possible insect vector, *Orosius argentatus*, each had a statistically significant spatial and temporal correlation with symptomatic plant numbers at one site on one occasion. Two subsequent border treatment experiments evaluated the effect of crop-margin insecticidal and herbicidal treatments on leafhopper movement into and from the stand. The second border treatment experiment examined also the treatment effect on ALuY disease incidence. Treatment with insecticide or herbicide significantly reduced the overall movement of leafhoppers. In addition, the insecticide treatment lowered the incidence of disease expression in adjacent lucerne. Significantly more leafhoppers of *A. torrida* and *O. argentatus* were caught on sticky traps 300 mm from the soil surface than in higher traps, up to 690 mm from the soil surface. This suggests that the insects were migrating into lucerne by short-range, trivial movement rather than long-range, directed flight. Results suggest that there is scope for management of this plant disease by reducing immigration of leafhopper vectors into lucerne from non-crop vegetation.

Leafhopper species *O. argentatus*, *A. torrida* and *B. angustatus* were used in transmission tests to determine their vector status for the phytoplasma associated with ALuY. Caged, seed-grown lucerne plants were monitored for foliar symptom expression after feeding by leafhoppers transferred from ALuY

symptomatic lucerne plants. Twelve of 25 plants developed phytoplasma disease-like symptoms including stunting and yellowing. The most pronounced foliar symptoms were displayed by five plants that had been fed on by *O. argentatus* and four plants that had been fed on by *A. torrida.* One plant, fed on by *O. argentatus*, showed the distinctive root symptoms of ALuY*.* A phytoplasma was identified by electron microscopy in two plants fed on by *O. argentatus* and one by *A. torrida.* For each group of plants that had been fed on by a single leafhopper species, one plant was phytoplasma positive as determined by the PCR using universal primers. The phytoplasma detected by PCR in the plant fed on by *A. torrida* was identified by RFLP analysis as the TBB phytoplasma. The PCR product from two plants fed on by *B. angustatus* and *O. argentatus* were too faint for RFLP analysis. PCR assays were conducted on DNA extracted from the head and thorax of each leafhopper species from transmission tests and from field-collected insects but no phytoplasma DNA was detected. These findings suggest *O. argentatus* as a vector of the ALuY pathogen and *A. torrida* as a vector of the TBB phytoplasma.

A randomised block, split-plot design experiment was established within an established irrigated lucerne stand in the mid Lachlan Valley to assess the effect of several treatments on alleviation of ALuY disease. Treatments included applications of supplementary water, multi-nutrients, potash, tetracycline antibiotic and a nil control. Supplementary water application resulted in a modest but statistically significant increase of seed yield, though effects on other measures of plant health were not significant. No treatment

had any statistically significant effect on other measures of plant health. Results are discussed in relation to scope for effective management of this disease via symptom alleviation or antibiotic treatment as well as by other options such as vector control.

Identifying and characterising the pathogen responsible for ALuY disease has led to a greater understanding of the disease and likely insect vector species have been identified through field and laboratory experimentation. These results, combined with the preliminary results in relation to disease management, suggest avenues for further research to develop an effective management strategy for ALuY disease.

Dedication

To Nicole and I.

We have a partnership that, despite testing it with most of life's trials in the past eight years (such as 12 months overseas living in each other's pockets, coming back and one of us immediately becoming a "MIA" cohort at times due to a PhD project, home buying, home renovating and broken limbs), we are still together and still in love.

We work.

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Even as I write this it doesn't quite feel as if it's really over. At times I wondered why I started the project to begin with but, to be honest, the other 99% (in the end, I guess, a statistically significant portion) of the time I was having the time of my life. I have enjoyed nearly every second, even the seconds that lasted more than an hour when I was laboriously sucking up insects for days on end in a paddock near Forbes.

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At times I also wonder why I chose to undertake some of the most stressful activities in life all at once – returning to steady work after 12 months overseas, doing a PhD, buying a house, renovating (or at least partially) a house and the list goes on.

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Table of contents

List of tables

List of figures

Chapter One – General introduction

Lucerne

Origins of lucerne

Lucerne (*Medicago sativa* L.) is a forage crop of the family Fabaceae. Wild lucerne has been found in south-western parts of the former Soviet Union and southern Europe, its cultivation before recorded history making its origin difficult to ascertain (Bolton 1962). The recorded history of lucerne, as a forage crop, is well over 2,000 years old (Rogers 1967), and is considered to be the oldest crop of this kind (Fautrier 1967). It is now regarded as the most important forage crop in the world (Lolicato & Lattimore 1998). Lucerne is a high value perennial leguminous plant for stock (Lolicato & Lattimore 1998) with 11 million hectares of cultivated lucerne in the USA alone (Summers 1998).

Lucerne in Australia

Lucerne seed was probably introduced to Australia in the late 18th century with its first record being in a report written by Governor King in 1806 (Bolton 1962). Lucerne has been used extensively for grazing, conserved fodder and the production of value-added products such as green feed, baled hay and processed lucerne hay in Australia since around 1925 (Morgan 1955, Fitzgerald *et al.* 1980). Cultivation reached approximately 400,000 hectares in 1954-1955 (Bolton 1962) increasing to 1,133,000 hectares in 1966-67 (Bolton *et al.* 1972). In 2002, it was estimated that Australia had 620,000 ha of

lucerne (McDonald *et al.* 2003) though estimates are difficult to verify because statistics have not been collected since 1996.

Lucerne agronomy

Lucerne is a perennial plant that has a deep root system, making it increasingly important in managing aspects of environmental sustainability, such as rising water tables and soil salinity (Fitzgerald *et al.* 1980). This deep tap-root makes the plant well suited to areas where deep fertile soils are present, such as river plains, helping the plant survive drier conditions (Rogers 1977), and it has been grown in degraded areas to assist in the restoration of soil quality (Rogers 1981). Lucerne is one of the most reliable pasture species providing relatively high energy and protein levels in livestock diets (McDonald *et al.* 2003).

The crop is best suited to well-drained, neutral to slightly alkaline soils (Morgan 1955, Hanson & Kehr 1972, Rogers 1974, Buffier & Green 1975) and cultivars are often developed to suit the area in which they are grown (Leach 1967). The plant is suitable for a wide range of climates, although it responds well to plentiful water and warm conditions during the summer growing season (Buffier & Green 1975). Lucerne makes excellent growth at all times of the year except in the colder months of winter, the inherent seasonality dictated by the degree of a cultivar's inbred dormancy which makes some more adapted to particular climatic regions and conditions (McDonald *et al.* 2002).

Many factors influence the growth of lucerne including soil type, soil pH, intensity of grazing and cutting, rainfall or irrigation creating water stress by over or under supply, nutrient deficiencies, weed competition, and a variety of diseases and arthropod pests (McDonald *et al.* 2003). Sub-optimal environmental conditions may adversely affect the crop and give rise to conditions such as widespread chlorosis, waterlogging-induced root rot, drooping in terminal leaves (frost damage), stunting of plants with curled, small dark green to purple leaves (phosphorus deficiency) and small yellow spots near the margins of lower leaves (manganese toxicity) (Lloyd *et al.* 2002). These conditions are common and easily distinguishable from most pathogenic lucerne diseases (Lloyd *et al.* 2002). Lucerne responds well to careful grazing or cutting, making it easy to maximise the productivity of the stand (Mitchell & Denne 1967, Howarth 1988). Grazing management must not only consider the health of the plant but also the response to grazing livestock. Care must be taken in grazing to avoid problems such as bloat in livestock (Howarth 1988).

Lucerne, like all legumes, obtains nitrogen from symbiotic, nodule-forming *Rhizobium* bacteria and inoculation with an appropriate strain of bacterium prior to sowing ensures this occurs efficiently (Lolicato & Lattimore 1998). A variety of sowing techniques such as banded drilling combine, direct drilling or surface sowing is used when moisture and temperature are optimal, generally during March to May or August to mid September in Australia (McDonald *et al.* 2003). Effective management of weeds and other competitive plant species in the previous rotation is generally preferable prior to sowing as pre-emergent

herbicides are much more efficient and cost effective than are post-emergent herbicides (Lolicato & Lattimore 1998).

Lucerne utilisation ranges from hay production from irrigated stands to stock forage in large semi-arid pastures (Stovold 1983). Lucerne, an important component of many crop rotations, is also grown for seed production, silage, green fodder, pellets, sprouts and protein fractionation (McDonald *et al.* 2002, McDonald *et al.* 2003). The management of lucerne often allows the crop to be used for multiple purposes. Seed and hay crops, for example, are often grazed as an integral part of their management (Lolicato & Lattimore 1998, McDonald 1999).

The first cut of the crop is made when individual plants are at least 20cm tall (Lolicato & Lattimore 1998). Arthropod management is sometimes underpinned by monitoring populations of pests and applying insecticide when numbers reach a threshold level (McDonald *et al.* 1995, McDonald *et al.* 2003), though a more common approach is a prophylactic application of insecticide. Lucerne seed stands are generally “closed up” for seed production, when livestock are excluded from the stand and no cutting takes place, to allow the stand to set seed from early to late December and seed may be harvested from March to late February (Elliott & Smith 1996).

Diseases of lucerne

Diseases have the potential to affect many stages of plant development causing significant damage to seed and hay production (Ryley 1994) with

several reviews of lucerne diseases available, including Stuteville and Erwin's (1990) comprehensive international review of lucerne diseases. Most lucerne diseases can occur in any stand of lucerne, although geographic and individual paddock conditions can influence the severity of some diseases (Stovold 1983). The most common loss to Australian lucerne crops from disease is a result of phytophthora root rot and common leaf spot (Buffier & Green 1975, McDonald *et al.* 2003).

Bacterial diseases - Bacterial wilt of lucerne, caused by *Clavibacter michiganensis* subsp*. insidiosus* (McCulloch), was first recorded in the United States in 1926 (Chand *et al.* 1987) and is recognised as a major disease in many lucerne growing areas in the United States (Fahy 1974, Samac *et al.* 1998). In England bacterial wilt was first recorded in 1965 (Close & Mulcock 1972) and was first identified in Australia in 1966 (Smith & Taylor 1967). Symptoms include a stunting of the entire lucerne plant and a discolouration under the periderm of the tap-root extending into the stele (Fahy 1974, Hill 1981). The last reported incidence of the disease in Australia was in 1986 in the Hunter Valley, New South Wales (Priest, 2002, pers. comm., 28 Nov.). The disease was widely reported in New South Wales prior to 1986 (Stovold 1983) although, based on its reported incidence, its level has dropped dramatically. Although bacterial wilt is a disease that impacts heavily on the lucerne growing industry in other countries, it has had relatively low impact in Australian lucerne crops. Even though bacterial wilt in lucerne has not been reported in Australia since 1986, it remains an economically important bacterial lucerne disease in Australia (McDonald *et al.* 2003).

Two other bacterial diseases that occur internationally are bacterial stem blight, caused by *Pseudomonas medicaginis* Sackett, and bacterial leaf and stem blight, caused by *Xanthomonas alfalfae* Rikar, Jones and Davis. Symptoms include dark lesions on stems and leaves with spots being water soaked in the case of the latter disease. Internationally, neither is considered to be of economic significance (Chand *et al.* 1987) and no literature of their occurrence in Australia is available.

Fungal diseases - The main fungal diseases of lucerne in Australia are phytophthora root rot, caused by *Phytophthora megasperma medicaginis* Hansen & Maxwell, anthracnose, caused by *Colletotrichum trifolii* Bain & Essary and common crown rot, a disease complex of the fungi *Acrocalymma medicaginis* Alcorn & Irwin, *Phomopsis* spp., *Phoma medicaginis* Malbr. & Roum. and several other fungi. Other common fungal diseases include stemphylium leaf spot, caused by *Stemphylium botryosum* Wallroth, leaf rust, caused by *Uromyces trifolii-repentis* Liro, pepper spot, caused by *Leptosphaerulina trifolii* (Rostrup) Petrak and common leaf spot, caused by *Pseudopeziza trifolii* (Bivona-Bernardi) Fückel (Thomson & Ockey 1984, McDonald *et al.* 2002).

Phytophthora root rot, caused by *P. medicaginis*, is considered to be an important disease (Irwin 1974) and continues to have a high impact on the Australian lucerne industry (McDonald *et al.* 2003). Symptoms include wilting, yellowing and death of individual plants or patches of lucerne plants resulting from extensive root rot (Stovold 1983). Phytophthora root rot is considered to

have a high impact on lucerne and causes yellow-brown to dark brown lesions on the tap-root often surrounded by a yellow discolouration (Faris & Sabo 1981). Other fungal disease of lucerne include colletotrichum crown rot, caused by *Colletotrichum trifolii*, stagonospora crown rot, caused by *Stagonospora meliloti* (Lasch) Petr., rhizoctonia root and stem canker, caused by *Rhizoctonia solani* Kühn, fusarium wilt, caused by *Fusarium oxysporum* Schlechtend. and sclerotium crown rot, caused by S*clerotium rolfsii* Sacc. (Irwin 1977, McDonald *et al.* 2003). These are just a few of the numerous fungal pathogens of lucerne. On the other hand, *Pythium* spp. cause few problems and while affected plants may rarely exhibit primary stem rotting (Fitzgerald *et al.* 1980), this pathogen generally causes very little damage in the majority of the Australian lucerne growing areas (Johnstone & Barbetti 1987). Although commonly regarded as fungi, *Phytophthora* spp. and *Pythium* spp. have been classed as Oomycetes, within the Stramenopile Kingdom (Sogin & Patterson 2003).

Virus diseases - There are three viruses reported to cause diseases in Australian lucerne: alfalfa mosaic virus (AMV), lucerne transient streak sobemovirus (LTTV) and lucerne latent nepovirus (LALV) (Blackstock 1978, Johnstone & Barbetti 1987). Symptoms of AMV include mild to severe mosaic, leaf stunting and rolling, chlorotic vein-banding and leaf-reddening (Hajimorad & Francki 1988). Lucerne plants infected with LTTV typically develop chlorotic streaks around the main lateral veins, necrotic and chlorotic lesions of leaflets, none of which are expressed in summer (Blackstock 1978). Strains of LTTV have been found in Australia and symptoms for all variants

are similar (Dall *et al.* 1990). Plants naturally infected with LALV show no symptoms (Blackstock 1978).

Nematode diseases - The stem nematode, *Ditylenchus dipsaci* (Kühn) Filipjev, is a nematode of particular importance in Australian (McDonald *et al.* 2003) and international lucerne crops (Gubiš 1994). Symptoms include dwarfing, distorted and swollen shoots and death of plants in patches causing significant damage (McDonald *et al.* 2003). The root-lesion nematode, *Pratylenchus penetrans* (Cobb) Filipjev & Schuumans Stekhoven, is one of the most important nematode pests internationally (Summers 1998) but, whilst it has been reported in other cropping systems (Riley & Kelly 2002), no literature could be found reporting it's incidence in Australian lucerne crops.

Other nematodes that are known to cause damage to lucerne stands are the northern root-knot nematode, *Meloidogyne hapla* Chitwood, southern root-knot nematode, *M. incognita* (Kofiod & White) Chitwood, the Javanese root-knot nematode, *M. javanica* (Treub.), peanut root-knot nematode, *M. arenaria* Chitwood, the Columbia root-knot nematode, *M. chitwoodi* Golden, O'Bannon, Santo & Finley and *Aphelenchoides ritzemabosi* (Schwartz) Steiner & Buhrer (Summers 1998, Milano de Tomasel & McIntyre 2001).

Phytoplasma diseases - Phytoplasmas cause a variety of symptoms in lucerne including witches' broom (Khan *et al.* 2002a), phyllody (Autonelli & Faccioli 1980) and yellowing (Chapter Two). Several phytoplasmas have been reported in lucerne such as alfalfa witches' broom phytoplasma (AWB)

(Smrz *et al.* 1981, Salehi *et al.* 1995, Marcone *et al.* 1997b, Marcone *et al.* 1999, Khan *et al.* 2002a); the stolbur phytoplasma from lucerne in Italy (Marzachi *et al.* 2000); little leaf phytoplasma in India (Suryanarayana *et al.* 1996); and aster yellows phytoplasma in Wisconsin (Peters *et al.* 1999). Phytoplasmas do not always cause disease in plants, though they may exist as a reservoir for infection of other plants. Lucerne, for example, is suspected to be a reservoir for canola yellows (Wang & Hiruki 2001b).

Witches' broom of lucerne was first reported in America in 1925 (Menzies 1946) and later in Australia in 1935 (Edwards 1935). Bowyer *et al*. (1969) provided evidence that witches' broom was not caused by a virus and showed it was caused by "mycoplasma-like organisms". Subsequent transmission testing of the disease was conducted by Bowyer (1974) and it was found that *Orosius argentatus* (Evans) (Cicadellidae: Deltocephalinae) was not capable of transmitting the disease. This finding was later disputed by Grylls (1979) who showed that the leafhopper was a vector. Witches' broom of lucerne has not had a major impact on production (Stovold 1983, McDonald *et al.* 2003) and management options for this disease are limited to removing the crop and either rotating or re-sowing (Stovold 1983, McDonald *et al.* 2003).

Very little literature exists regarding phyllody symptoms in lucerne. Autonelli and Faccioli (1980) briefly detail the symptoms in lucerne grown in Italy. Peters *et al*. (1999) showed that the aster yellows phytoplasma causes inter-veinal chlorosis and purpling on the stems and leaves of lucerne.

'Yellows' symptoms have been recorded in Australian lucerne since the early 1950s (Anonymous 1953).

Disease management in lucerne

There are several commonly used methods of disease management including manipulation of cultural practices such as crop rotation and crop hygiene, chemical application, host plant resistance, biological control and use of transgenic species. For some diseases, in lucerne in addition to other plant species, a combination of several of these techniques is necessary for the management of disease (Vanneste 2000, McDonald *et al.* 2003).

There are many different disease management strategies available to lucerne growers and early detection and diagnosis of a disease assists in effective management (Mpunami *et al.* 1996). Management strategies range from preventative measures such as chemical seed dressings applied prior to sowing to the drastic destruction of an entire crop to prevent further disease spread into other paddocks (Chand *et al.* 1987). Bacterial diseases are generally avoided by selecting certified seed that is free of bacterial pathogens, sowing resistant varieties of lucerne or changing management practices to avoid damaging the root or foliage, so preventing further spread (Chand *et al.* 1987, McDonald *et al.* 2003). Crop rotation, sanitation and management practices such as timing of sowing, cutting or strategic grazing of crops all aid in the management of fungal diseases of lucerne (Chand *et al.* 1987, McDonald *et al.* 2003).

Cultural - The cultural management of plant diseases includes strategies that do not comprise the use of chemicals, resistance breeding or biological control methods (Termorshuizen 2001). The oldest example of a cultural practice to reduce the level of disease incidence is crop rotation. Other examples are planting many species of crops together or in rows, removing the inoculum by heat treating or sterilisation, using certified seed from disease free crops, timing the sowing of plants to avoid peak infection periods and careful consideration of irrigation practices (Termorshuizen 2001). Practices that avoid damage to the plant are also considered cultural practices (Waller & Lenné 2001), as they limit the pathogen's ability to infect the plant. When damage to the plant tissue is unavoidable, practices such as cutting infected stands last in order to prevent the spread of inoculum to other crops on mower blades are acceptable (Stovold 1983).

Limiting the movement of insect vectors into a crop may lower the incidence of disease (Chancellor *et al.* 1996, Lindblad & Areno 2002) or reduce the effect of a disease (Randles 1986). Schaber *et al*. (1990) demonstrated that physical barriers, such as farm access roads or irrigation channels, limit the movement of insects and so the deliberate introduction of a physical barrier represents a cultural practice. Insect vectors of virus diseases are often dependent on alternative plant hosts and insect reservoirs of the pathogen for survival. Viruses can be managed either by the removal of the insect vector or pathogen reservoir (Holt *et al.* 1999). Organic additives, such as manures, may be used to reduce the effect of many diseases (Lazarovits *et al.* 2001). These amendments have the ability to limit soil borne diseases because they

contain many antagonistic soil micro-organisms (Akhtar & Malik 2000) and add nutritional value to the plant or crop (Trankner 1992).

In Australian lucerne production, cultural practices are the main basis of effective disease management. The effects of phytophthora root rot are lessened by the selection of free-draining soils prior to sowing and the use of efficient irrigation throughout the season (McDonald *et al.* 2003). Avoiding mechanical injury to the plants is advised as a means of disease management for common crown rot, common leaf spot and stemphylium leaf spot, caused by *Stemphylium botryosum*. Damping off, caused by *Pythium* spp., is avoided by sowing in dry, warm weather (McDonald *et al.* 2003); AMV and bacterial wilt are controlled using certified seed from disease free areas (Stovold 1983). Prior to breeding for resistance, anthracnose was controlled by avoiding sowing lucerne into affected areas for three to four years (Stovold 1983).

There are no economically viable management strategies for lucerne diseases such as downy mildew (caused by *Peronospora trifoliorum* de Bary) and therefore the only option is to wait for warm dry weather to clear symptoms in the paddock. Common leaf spot and other foliage diseases of lucerne are managed by removing the infected material to prevent its spread by cutting or grazing and to reduce the inoculum available for reinfection (McDonald *et al.* 2003). Root and stem rots of lucerne, caused by *Sclerotinia* spp. and *Sclerotium* spp., are most effectively controlled by long rotations or deep ploughing of the field (Chand *et al.* 1987) though the pathogen can persist for many years in soil (Agrios 1997).

Chemical - Chemicals are now used to either eliminate or inactivate plant pathogens such as bacteria and fungi, or make the plant surface incompatible with the establishment of new infections (Psallidas & Tsiantos 2000). Chemicals fall broadly into several areas; eradication of a pathogen by sterilising or fumigating soil; establishing a protective chemical barrier covering seeds or growing plants; or applications to seeds or growing crops that systemically protect the plant against disease (Hollomon 2001). They may be applied to seed, soil, or the foliage (Matthews 2001). Tetracycline antibiotics have been applied to plants with phytoplasma infections to reduce their symptoms and are either applied as a foliar spray (Bowyer & Atherton 1972) or as an injection directly into the plant (McCoy 1974). Soil drenches have been ineffective against dwarf disease in mulberry plants (Ishiie *et al.* 1967) as tetracyclines characteristically bind to soil (McCoy 1982).

Host plant resistance - All plants have some level of resistance to disease and cultivar or species selection based on its level of resistance is the most common form of disease management in many plant systems (Waller & Lenné 2001). Plant resistance mechanisms include mechanical barriers and morphological characteristics that help prevent infection from occurring. Chemical barriers like gums, tannins and hypersensitive reactions can aid resistance to both infection and establishment by the pathogen (Waller & Lenné 2001). Breeding for improved nitrogen fixation can be detrimental to resistance against bacterial wilt (Viands *et al.* 1980) highlighting the complexity of developing breeding lines.

Exhaustive screening techniques are used to survey plant lines for resistance against disease caused by phytophthora root rot (Hine *et al.* 1975, Faris & Sabo 1981). The level of resistance in lucerne is commonly low for this disease and this is attributed to traits within the plant rather than to particularly virulent strains of the pathogen (Nygaard & Grau 1989). The selection of resistant varieties lowers the impact of anthracnose and colletotrichum crown rot and is the only advice currently available to lucerne growers for these diseases (McDonald *et al.* 2003). Similarly, the current advice for bacterial wilt of lucerne is to select varieties of lucerne that show high levels of resistance (McDonald *et al.* 2003) although initial resistance against this disease was poor (Close & Mulcock 1972).

Cultural practices are used to manage damping-off disease caused by *Pythium* species and lucerne seedlings have a degree of resistance, though there is minimal difference between varieties (Altier & Thies 1995). Virus diseases of lucerne are also managed by selecting resistant cultivars (McDonald *et al.* 2003) and similar screening techniques were used to select several species resistant to *Fusarium* root and crown rots in New York State (Miller-Garvin & Viands 1994). There are resistance breeding programs for lucerne in Australia for diseases such as phytophthora root-rot (Rogers *et al.* 1978), colletotrichum crown rot (Stovold & Francis 1988) and bacterial wilt (Fahy 1974) and pests such as the potato leafhopper (*Empoasca fabae* L.) (Lefko *et al.* 2000), spotted alfalfa aphid (*Therioaphis trifolii* f. maculata (Monell)) and blue-green aphid (*Acyrthosiphon kondoi* Shinji) (Williams & Young 1996).

Biological control - Farmers' opinions and concerns about the use of pesticides have resulted in a wish for more environmentally-sustainable practices to be utilised in agriculture (Whipps & Lumsden 2001) and biological control agents are a viable alternative to chemical control. Organisms that successfully compete with, antagonise or parasitise insect pests and plant pathogens can be exploited to control these organisms (Navi & Bandyopadhyay 2001). There is increasing interest in their use for controlling pests, weeds and diseases (Butt *et al.* 2001).

Biological control agents are generally targeted against fungal pathogens. The biological control of the bacterial disease fire blight of pome fruit, for example, has focused on the interaction between a bacterial antagonist and the fire blight pathogen (Johnson & Stockwell 2000). Johansson *et al*. (2003) investigated the inhibitive properties of several bacterial isolates when used to suppress the symptoms caused by the fungal disease snow mould in wheat seedlings, caused by *Microdochium nivale* Fries, and seedling blight in wheat seedlings, caused by a complex of *Fusarium* species.

Transgenic species - Often plants have traits and characteristics that are highly prized in their production or their marketability, but suffer high levels of disease. When plant resistance cannot be established and other methods of disease management fail, the resistance genes from other species may be directly introduced into these varieties (Norelli & Aldwinckle 2000). Transgenic methods have been used in disease management for annual

medics, the coat protein gene of a South Australian strain of AMV being introduced to *Medicago truncata* cv. Jemalong 2HA (Jayasena *et al.* 2001).

Phytoplasmas

First report of phytoplasmas

Phytoplasmas were first known as “Mycoplasma like organisms” (MLO) because of their morphological similarity to mycoplasmas, pathogens known to occur in animals (Smart *et al.* 1996, Davis *et al.* 1997, Seemüller *et al.* 1998, Schneider *et al.* 1999a). These organisms were first discovered in 1967 (Namba *et al.* 1993) and are parasitic prokaryotes of the class Mollicutes (Schneider *et al.* 1993), defined as such primarily because they are capable of varying their shape, because they lack cell walls and because they are bound by a single unit membrane (Gundersen *et al.* 1994).

Phytoplasmas are phylogenetically related to gram-positive bacteria (Bove & Garnier 2000) and are sensitive to tetracycline antibiotics (Tsai 1979). Previous studies of phytoplasma disease etiology were based on the assumption that each species of phytoplasma caused one set of symptoms (Davis & Sinclair 1998). Original classifications of phytoplasmas were based on symptom expression, host range and vector relationships (Chiykowski & Sinha 1990, Seemüller *et al.* 1994). This was problematic as the tomato big bud phytoplasma, for example, causes phyllody symptoms in sesame (*Sesamum indicum* L.) (Wilson *et al.* 2001), flower virescence and some stunting in the epiphytic orchid *Sarcochilus hartmanii* F.Muell x *S. falcatus* R. Br. (Gowanlock *et al.* 1998) and big bud symptoms in tomatoes (*Lycopersicon*

spp.) (Osmelak 1984). Serological and DNA hybridisation techniques have been used for detection of phytoplasmas in plants. Polymerase chain reaction (PCR) assays have since proved more versatile and reliable in detecting the pathogen in plants and insect vectors (Smart *et al.* 1996, Webb *et al.* 1999). Use of PCR has confirmed that different diseases in different hosts can be caused by the same phytoplasma.

Phytoplasmas are associated with a large number of plant diseases around the world (Marcone *et al.* 1997a, Davis & Sinclair 1998, Schneider *et al.* 1999b). It is estimated that more than 600 phytoplasma species are in existence (Lee & Davis 1992) and they have been reported from several hundred plant species (Davis *et al.* 1988, Marcone & Ragozzino 1995, Jarausch *et al.* 1996, Hwang *et al.* 1997). Symptoms associated with phytoplasmas in many plant species include excessive branching (witches' broom), reduced leaflet size (little leaf), decreased rate of growth or shortening of the internode (stunting), virescence or petal greening (phyllody) and floral gigantism (big bud) (McCoy 1979).

Initially, phytoplasma diseases were often attributed to viral pathogens (Davis *et al.* 1997) mainly because of common symptoms such as yellowing and stunting and the inability to culture the pathogen within the laboratory (Tsai 1979). In the 1960s it was discovered that the causal agents for these diseases were not viruses (Bowyer *et al.* 1969) and phytoplasmas are now frequently found to be associated with yellows diseases such as grapevine

yellows (Maixner *et al.* 1994, Padovan *et al.* 1995, Liefting *et al.* 1998) and strawberry lethal yellows (Padovan *et al.* 1998).

To satisfy Koch's postulates (Agrios 1997) and identify a given pathogen as the causal agent of a disease, there must be a consistent association of the pathogen with all diseased plants, the pathogen must be isolated from a diseased plant and grown in pure culture and when healthy plants of the same species are inoculated from the pure culture in the laboratory the pathogen must be isolated from these diseased plants, grown in pure culture and all characteristics must be consistent with the original isolate. Phytoplasmas cannot be cultured under axenic conditions (Schneider *et al.* 1997). For this reason, it is impossible to satisfy Koch's postulates and therefore the classification of a phytoplasma as the causal agent of any disease will always be a tentative one in the view of Agrios (1997). Phytoplasma researchers, therefore, have developed alternative norms when seeking to infer causality on the part of phytoplasmas isolated from symptomatic plants. These norms are explored fully in Chapter 4.

Phytoplasmas in Australia

Symptoms typical of phytoplasmas were first reported in Australia in 1902 on tomato (Cobb 1902) and later named tomato big bud (TBB) (Samuel *et al.* 1933). Economic losses have been attributed to various phytoplasma diseases in Australia including lucerne witches' broom and papaya dieback (Davis *et al.* 1997).

It was not until the late 1960's that many of the symptoms we now recognise as being caused by phytoplasmas in tomato (Cobb 1902, Samuel *et al.* 1933) and field crops were attributed to phytoplasmas (Bowyer *et al.* 1969). With the advent of molecular technology improving the detection and characterisation of phytoplasmas (Davis *et al.* 1997), many Australian diseases, for example Australian grapevine yellows (AGY), have been attributed to phytoplasmas (Padovan *et al.* 1996).

Extensive surveys have been conducted in Australia and phytoplasmas have been identified in over 60 host plants. These surveys included 34 species of Fabaceae, five species of Solanaceae and several Poaceae (Davis *et al.* 1997, Schneider *et al.* 1999b). In addition to those host plants identified in the survey, several other host plants have been identified such as *Carica papaya* L. (Gibb *et al.* 1996, Liu *et al.* 1996, Guthrie *et al.* 1998), *Stylosanthes scabra* Vog. (De La Rue *et al.* 2001), *Fragaria* sp. (Greber & Gowanlock 1979, Padovan *et al.* 2000), *Vigna radiata* L. (Wilson *et al.* 2001), *Pyrus communis* L. (Schneider & Gibb 1997), and *Sarcochilus hartmanii* x *S. falcatus* (Gowanlock *et al.* 1998).

Detection and classification of phytoplasmas

Initially the majority of phytoplasma diseases were classified as 'yellows' (Maramorosch *et al.* 1975) and different groups of the pathogen could only be distinguished by examining their latent period (the time between inoculation and symptom expression), the symptoms they expressed and their vectors (Bowyer & Atherton 1972, Sinha & Chiykowski 1984, Schneider *et al.* 1999a).

The latent period of phytoplasma diseases ranges from approximately 40-60 days in the case of the eastern peach X-Mycoplasma like organism (Chiykowski & Sinha 1988) to as low as 14-25 days in many other plant systems (Bowyer 1974, Chiykowski & Sinha 1990, Carraro *et al.* 2001b).

Fluorescence microscopy (Seemüller *et al.* 1976, Osmelak *et al.* 1989) and electron microscopy (Capoor *et al.* 1972) have been used to study phytoplasmas. Though slow and labour-intensive, these techniques became valuable tools in the detection of phytoplasmas (Sinha & Chiykowski 1986). Their use is limited to plants with a relatively high phytoplasma titre (Ahrens & Seemüller 1992) and the technique could not differentiate between phytoplasma species. Other detection techniques have included dot hybridisation (Bonnet *et al.* 1990), enzyme-linked immunosorbent assay (ELISA) (Sinha & Chiykowski 1986), southern blot analysis (Bertaccini *et al.* 1990), cloned DNA probes (Bellardi *et al.* 1992), immunosorbent electron microscopy (ISEM) (Lee & Davis 1992), and serology (Khadhair *et al.* 1997).

The use of oligonucleotide probes in polymerase chain-reaction assays (PCR) that have high sequence homology to the phytoplasma's 16SrRNA genes has increased the sensitivity of detection and was first reported to have been used for phytoplasmas by Deng and Hiruki (1991a). During the PCR process, DNA is extracted from infected plant material and insects, then enriched and amplified (Dellaporta *et al.* 1983, Lee & Davis 1988, Sears & Klomparens 1989).

PCR is not without its faults. Rubbery wood, a disease in apples, was associated with phytoplasmas following the use of traditional electron and fluorescence microscopy (Minoiu *et al.* 1980, Minoiu & Craciun 1983), though Poggi Pollini *et al*. (1995) failed to confirm phytoplasma infection in symptomatic apples with PCR analysis. Bertaccini *et al*. (1998) used nested PCR techniques, where the product from first round PCR is subsequently amplified a second time, to increase the sensitivity of the assay that was previously unsuccessful and were able to amplify and characterise a phytoplasma in rubbery-wood symptomatic apples. The rubbery-wood study continued to be hindered by phytoplasma low titres, common in phytoplasma patho-systems (Gundersen & Lee 1996, Heinrich *et al.* 2001). Molecular assays do not consistently concur with traditional detection techniques. Davis *et al*. (1997) reported unsuccessful amplification of phytoplasma rDNA from symptomatic *Amaranthus* sp., *Phaseolus aureus* Roxb. Non Zucc. and *Vigna unguiculata* ssp. *dikinditanal* (Harms), where traditional detection techniques identified phytoplasma bodies.

The primers used in early PCR methods for phytoplasma research generally suffered a lack of sensitivity and specificity and often amplified non-phytoplasma or non-specific DNA particularly in woody hosts such as fruit and ornamental trees (Gundersen & Lee 1996). A further complication that may adversely affect currently used primers is that some oligonucleotides can create “dimers” or “hair-pins” where they attach themselves to other primers or fold upon themselves (Heinrich *et al.* 2001), thereby reducing the amount of target DNA that is amplified, possibly culminating in false negatives.

False negatives can also be explained by the titres of phytoplasmas in plant tissue often being very low. Cell titres of phytoplasmas in plant tissue are generally low and can account for less than 0.1% of total DNA in extracts, such as in phytoplasma infected *Catharanthus roseus* (L.) G. Don. (Schaff *et al.* 1992). In addition to the inherent low titres of phytoplasmas within the plant, these titres can be influenced by sampling date and the part of the plant from which tissue was taken for extraction of DNA. This characteristic was demonstrated in paulownia trees, *Paulownia tomentosa* (Thunb.) Steud., when PCR assays were conducted on trees showing symptoms of witches' broom (Sahashi *et al.* 1995). Inability to amplify phytoplasma DNA from symptomatic plant tissue, which occurred during studies of phytoplasma infection in *Prunus mahaleb* (L.) cv. *cemany*, may occur as the result of degeneration of tissue and the degeneration of prokaryote DNA as the disease advances and symptoms become more severe (Varga *et al.* 2001). The uneven distribution of phytoplasmas within diseased plants, such as in phytoplasma infected strawberries, can make detection difficult or unreliable, even with the sensitive molecular methods that have been developed (Gundersen & Lee 1996, Bertaccini *et al.* 1997). Other factors that may affect the efficiency of PCR include the presence of polysaccharides. These carbohydrates, which occur in high concentration in diseased New Zealand flax rhizomes, have inhibitory effects when present in PCR assays (Davis *et al.* 1997, Andersen *et al.* 1998). Such factors contribute towards the possibility of false negatives. Because PCR detection can fail to associate the pathogen with symptoms, some workers have used a combination of molecular methods and epidemiological data to provide evidence of a

phytoplasma infection e.g. Gatineau *et al.* (2001) showed that *Beta vulgaris* L. was infected with a phytoplasma by providing evidence of aerial transmission, phytoplasma symptomology and preliminary results of phytoplasma detection.

Amplifying a false positive is also a factor that needs to be considered in PCR assays. Many primers have sequence homology with the 16S-spacer region of plastids and chloroplasts giving a positive band of a size indicative of the presence of phytoplasma DNA, an error easily resolved by further genetic differentiation with restriction fragment length polymorphism (RFLP) analysis (Heinrich *et al.* 2001). False negatives in PCR can be a lot harder to identify and rectify.

Little progress was made initially into the classification of phytoplasmas due to the inability of researchers to detect and compare these pathogens (Deng & Hiruki 1991b). Using sequence information or RFLP analysis of rDNA, a large amount of information has been gathered over the last ten years in relation to the classification and characterisation of phytoplasma pathogens (Seemüller *et al.* 1998). RFLP analysis continues to be used for differentiating and classifying phytoplasmas such as differentiating between the clover phyllody and potato witches' broom phytoplasmas (Deng & Hiruki 1991b), phytoplasmas from the aster yellows group (Davis & Lee 1993), between elm yellows and aster yellows phytoplasma (Lee *et al.* 1993), several unknown phytoplasmas (Namba *et al.* 1993) and differentiating the monarda yellows phytoplasma from clover phyllody and potato witches' broom phytoplasmas (Wang *et al.* 1998). Additionally, RFLP has been used as a tool to classify

phytoplasmas into a series of groups or subgroups (Lee *et al.* 1993, Schneider *et al.* 1993, Gundersen & Lee 1996, Wang *et al.* 1998) for taxonomic purposes.

Sequence analysis has been used to taxonomically characterise a large number of phytoplasmas using the 16S rDNA and 16S/23S spacer region (Schneider *et al.* 1995, Padovan *et al.* 1996, Schneider *et al.* 1997, Davis & Sinclair 1998, Seemüller *et al.* 2002). Using sequence information, phytoplasmas may be differentiated into a greater number of taxonomic groups than those indicated by RFLP characterisation (Lee *et al.* 1998b, Seemüller *et al.* 2002).

Insect Vectors

Though vectors often do not cause economic damage from their feeding, their vector status and the losses suffered from the introduction of pathogens can be very damaging (Moya-Raygoza & Nault 1998). Insect vectors play an important role in the etiology of phytoplasma diseases as they bridge the gap between disease-free plant communities and nearby pathogen reservoirs (Sdoodee 2001, Wilson *et al.* 2001). Phytoplasmas are vectored almost exclusively by homopterous insects (Tsai 1979, Hanboonsong *et al.* 2002) with three insects of the genus *Halyomorpha* (Hemiptera: Pentatomidae) being the only exceptions (Hill & Sinclair 2000). Homopterous vectors include leafhoppers (Cicadelloidea), planthoppers (Fulgoroidea) and psyllids (Psylloidea) (Tsai 1979, Ploaie 1981, Hill & Sinclair 2000).

Not all insects that ingest phytoplasmas are capable of vectoring the organism (Blanche *et al.* 1999). The phytoplasma must circulate and multiply within the insect, passing from the gut into the hemocoel and from there into the salivary glands before the pathogen can be transmitted to plant tissue (Hill & Sinclair 2000, Feeley *et al.* 2001, Klein *et al.* 2001, Tanne *et al.* 2001). There is evidence that some phytoplasmas can pass from one vector generation to the next through transovarial transmission, such as the sugarcane white leaf phytoplasma in the vector *Matsumuratettix hiroglyphicus* (Matsumura) (Hanboonsong *et al.* 2002), the transovarial transmission of phytoplasmas in *Scaphoideus titanus* Ball (Alma *et al.* 1997) and mulberry dwarf phytoplasmas detected in the organs of the insect vector *Hishimonoides sellatiformis* Ishihara (Kawakita *et al.* 2000). This reduces the requirement for the pathogen to have a reservoir in another plant system. This is not the case for all phytoplasma vectors. Dabek (1983), for example, showed that the *Rhynchosia* little leaf phytoplasma is not transovarially transmitted. It has been shown that nymphs are efficient at acquiring infectivity status, as are adults of the species (Chiykowski & Sinha 1988, Chiykowski 1991).

Phytoplasma pathogen/vector/host systems are diverse and complex. Often, the vectoring status of the insect species is not host specific such that they may transmit the pathogen to several different species of plants that are susceptible to phytoplasma infection, demonstrated by the leafhopper vector *Paraphlepsius irroratus* (Say) and its ability to transmit eastern peach X-mycoplasmalike organism to chokecherry, *Prunus virginiana* L. var. *melanocarpa* (Sarg.), and also to periwinkle, *C. roseus* (Chiykowski & Sinha

1988). Similarly, insect vectors may become infective with more than one species of phytoplasma and hence mixed infections may be possible within the plant and insect system as with grapevine flavescence dorée disease and the insect vector *S. titanus* (Alma *et al.* 1996). Further, phytoplasmas are often not vector specific and some phytoplasmas, such as the alfalfa witches' broom phytoplasma and its vectors *Aceratagallia* sp., *Neokolla hieroglyphica* (Say), *Cuerna septentrionalis* (Walker) and *Macrosteles fascifrons* (Stål), are vectored by several insect species (Khadhair *et al.* 1997). The plants that become infected with the phytoplasma may not be the preferred host of the vector (Lee *et al.* 1998a). In addition, phytoplasmas such as California aster yellows phytoplasma is transmitted by 24 leafhopper species, showing a low insect vector specificity, in contrast to phytoplasmas such as American elm yellows phytoplasma that shows high vector specificity and is transmitted by one vector (Lee *et al.* 1998a).

Since phytoplasmas are phloem-restricted (Guthrie *et al.* 2001), insects that feed outside the phloem cells can be disregarded as possible vectors (McCoy 1979). In 1920, only three species of leafhopper vectors and two phytoplasma diseases were known and 60 years later more than 100 vectors were identified (Tsai 1979). Several techniques have been used to identify vectors. One approach is using insect surveys that aim to find dense populations of homopterous insects in or near symptomatic plants. The presence of eligible vectors in high numbers and/or their temporal relationship with the disease symptoms has been used to implicate many insect vectors (Khadhair *et al.* 1997, Grilli & Gorla 1998, Zhang *et al.* 2000, Lindblad & Areno

2002). The presence of an insect responsible for vectoring other phytoplasma diseases, such as the vector *Macrosteles quadrilineatus* Forbes and its association with witches' broom in *Nasturtium officinale* R. Br. and the association of numerous leafhoppers with dieback, yellow crinkle and mosaic in *C. papaya*, has been considered sufficient to implicate that insect in the diseased plant community in which it is found (Feeley *et al.* 2001, Borth *et al.* 2002, Elder *et al.* 2002).

Monitoring of suspected insect vectors and relating this information to the temporal dynamics of disease symptoms can be useful in identifying the vector or developing a management strategy for the disease (Randles 1986). Insect sampling techniques include mercury vapour and ultra-violet light traps (Osmelak 1987, Labonne *et al.* 1998), sweep net sampling (Osmelak 1984, Larsen & Whalon 1988), suction traps (Kersting *et al.* 1997), suction sampling (Hossain *et al.* 1999), malaise traps (Labonne *et al.* 1998), direct collection from plants (Power *et al.* 1992) and sticky traps (Purcell & Elkinton 1980, Mensah 1996). Sticky traps have been used for examining population densities (Smith & Ellis 1983) and have been used extensively for examining the flight characteristics of potato leafhoppers, *Empoasca fabae* Harris (DeGooyer *et al.* 1998), and in particular to examine the directional characteristics of flying insects such as *E. fabae* (Fleischer *et al.* 1983).

Detection of phytoplasmas in insects

Evidence of vector status of insect species include: visualisation of the phytoplasmas within the salivary glands of the insect using electron

microscopy (Ishiie 1970, Grylls 1979), transmission testing by relocating insects from symptomatic plants to a known uninfected plant and subsequent examination of the plant for symptom expression and relocating insects from symptomatic plants to a glucose feeding medium and conducting PCR assays for phytoplasmas on the medium to indicate if the insect is capable of vectoring the phytoplasma (Tanne *et al.* 2001). The development of molecular analysis has allowed fast screening of insects for the presence of phytoplasma DNA, leading to the possible detection of vectors (Vega *et al.* 1993, Blanche *et al.* 1999).

Insect acquisition rates are often quite low and in fact PCR testing for phytoplasmas indicates that efficiency for known vectors can be less than 2%. One of 60 *M. quadripunctulatus* and two of 60 *E. incisus* leafhopper vector tested positive for phytoplasmas in PCR assays (Alma *et al.* 2000), a phytoplasma was detected in one of 87 *Colladonus clitellarius* Say adults (Hill & Sinclair 2000). Often no positives are achieved such as with the 34 leafhopper species assayed for ash yellows phytoplasma (Feeley *et al.* 2001) and 13 leafhopper species collected from papaya dieback, yellow crinkle and mosaic affected areas (Elder *et al.* 2002). Research using transmission testing has revealed greater insect efficiency such as psyllid vectors of pear decline (24% positive in 33 samples) (Avinent *et al.* 1997), several leafhopper species responsible for transmitting strawberry lethal yellows (9% positive in 36 samples) (Charles *et al.* 2002), the planthopper vector of stolbur phytoplasma at 2% to 13% (Gatineau *et al.* 2001) and Cixiid planthoppers

associated with the German grapevine yellows phytoplasma (38% positive in 17 samples) (Maixner *et al.* 1995).

Amplification of phytoplasma DNA from the entire body of an insect does not prove its vector status (Vega *et al.* 1993) but simply implicates the presence of the pathogen, probably in the gut. In the past, insect DNA extraction techniques involved grinding the whole leafhopper, including the gut contents (Khadhair *et al.* 1997, Gatineau *et al.* 2001, Klein *et al.* 2001, Charles *et al.* 2002) such as the two leafhopper species, *M. quadripunctulatus* and *E. incisus*, associated with chrysanthemum yellows (Marzachi *et al.* 1998), the implication of the leafhoppers *Diastrombus* sp. and *Meenoplus* spp. in the transmission of lethal disease of *Cocos nucifera* L. (Mpunami *et al.* 2000) and PCR assays on DNA extracted from *H. obsoletus* for the German grapevine yellows phytoplasma (Maixner *et al.* 1995). This leads to the possibility that the insect has simply fed on an infected plant and therefore contains phytoplasmas in its gut. This does not establish that the insect is capable of vectoring the phytoplasma. In these cases, vector status has to be established through demonstrated disease transmission and recovery of the pathogen from test plants.

Insects species may be used as a transmission tool if it is an efficient and reliable vector (Chiykowski 1988, Ganguly & Mukhopadhyay 1989) though care needs to be taken as the vector often only transmits the disease approximately 60% of the time as is the case with the eastern peach X-mycoplasmalike organism and its leafhopper vector, *P. irroratus* (Chiykowski

& Sinha 1988). The efficiency of transmission is influenced largely by the titre of the pathogen and by the behaviour of the insect (Tanne *et al.* 2001).

Transmission testing

An important aspect of phytoplasma etiology is studying transmission characteristics in the field or under laboratory conditions. There are many transmission methods available including graft transmission tests (Alivizatos 1993, Pastore *et al.* 2001), mechanical inoculation (Clark & Guy 2000), dodder transmission (Cook & Wilton 1985, Chiykowski 1988, Salehi *et al.* 1995), and caged insect transmission (Blanche *et al.* 1999, Gatineau *et al.* 2001, Jarausch *et al.* 2001). Understanding insect transmission is very important in understanding the etiology of phytoplasmas and developing possible management strategies for these diseases. Insect transmission tests can provide information on the pathogen's ability to multiply inside the insect host and be transmitted to the plant.

Insect transmission tests use insects reared on symptomatic plants or alternative hosts such as *C. roseus* (Gatineau *et al.* 2001). Insects for use in transmission tests, for example the pear decline phytoplasma and its vector *C. pyri*, are often reared on symptomatic plants to maximise the chances of the insect acquiring the disease (Carraro *et al.* 2001a), since the stage of its lifecycle during which the insect acquires the disease is often not known (Gatineau *et al.* 2001, Palermo *et al.* 2001). Field-collected insects may also be used in the assumption that many will have been feeding on symptomatic plants and so will be infective (Maeso Tozzi *et al.* 1993, Carraro *et al.* 2001a,

Jarausch *et al.* 2001), though the risk of multiple infections or transmission of an entirely different disease becomes possible.

There are three key time frames in the vectoring of phytoplasma diseases: the acquisition period of the pathogen, the latency period in the insect before it is transmissible and the inoculation period (Carraro *et al.* 2001b). The acquisition time of the insect can vary from very short periods such as five minutes for the insect vector *Circulifer tenellus* (Baker) to acquire the beet leafhopper-transmitted virescence agent phytoplasma (Golino *et al.* 1987), or 1-2 days for the insect vector *C. pruni* to acquire the European stone fruit yellows phytoplasma (Carraro *et al.* 2001b), to as long as 54 days for the eastern peach X-mycoplasmalike organism to be acquired by its vector *P. irroratus* (Chiykowski & Sinha 1988). The latency period varies from 10-18 days for the beet leafhopper transmitted virescence agent phytoplasma and its vector *C. tenellus* (Golino *et al.* 1987) and the chrysanthemum yellows phytoplasma and its associated leafhopper vectors *M. quadripunctulatus* and *E. variegatus* (Palermo *et al.* 2001) to 65 days for *Dalbulus maidis* (Delong & Wolcott), the vector of maize bushy stunt phytoplasma, to become transmissible (Moya-Raygoza & Nault 1998). Inoculation can be as short as the acquisition time of five minutes, for example the vector *C. tenellus* (Golino *et al.* 1987).

Management of Phytoplasma diseases

Management of phytoplasma diseases usually targets the prevention of infection rather than reducing the severity of symptoms (Lee *et al.* 2000).

Strategies in the management of phytoplasma diseases include using clean seed or planting material, exclusion of disease through quarantine, limiting densities of insect vectors and reducing their migration into the crop, breeding for host-plant resistance and the use of chemotherapy, which is usually restricted to tetracycline injections in tree species (Lee & Davis 1992). Controlling the vector in different stages of its lifecycle has been shown to be important in the control of many diseases such as pear decline phytoplasma in the vector *Cacopsylla pyri* (Carraro *et al.* 2001a). Removing the phytoplasma-infected papaya plants from healthy individuals or ratooning the infected material, which involves pruning much of the upper branches and crown of the plant, has been shown to be a successful means of managing phytoplasma associated diseases in papaya (Guthrie *et al.* 1998).

Some of these strategies, for example using clean seed or planting material, are inappropriate for the management of phytoplasma diseases. Tests performed on AWB-symptomatic lucerne plants in Oman provided preliminary evidence of seed transmission of several phytoplasma species found in lucerne (Khan *et al.* 2002b). Support for seed transmission of another phytoplasma disease, witches' broom of lime, has also been presented (Khan *et al.* 2003). All other literature concerning seed transmission of phytoplasmas argues against such a possibility (Kleinhempel *et al.* 1975, Shin 1980).

In some phytoplasma disease systems, such as lethal yellowing in coconut palm where mortality can range from 0% to 94% in plants screened, breeding

for resistance to the disease is the only mechanism available to growers (Been 1997). As in some fungal disease systems such as damping off, caused by *Pythium* spp., and black stem, caused by *P. medicaginis* (Chand *et al.* 1987, McDonald *et al.* 2003), annual crops may be sown at particular times in order to avoid periods of high vector densities so limiting contact between plants at a highly susceptible growth stage and immigrating vectors such as infestations of *D. maidis* in maize, *Zea mays* L. (Hruska & Gomez Peralta 1997).

Australian lucerne yellows

In the 1970s, Australian lucerne yellows (ALuY) levels were low (McGechan 1980). Disease levels increased through the 1980s (McGechan 1980) and it is now a disease that impacts heavily on the Australian lucerne seed industry (Pilkington *et al.* 1999). The history of ALuY is addressed in Appendix One.

ALuY is one of several major lucerne diseases in New South Wales (Stovold 1983, McDonald *et al.* 2003) and is attributed to a phytoplasma (Fletcher 1980, McGechan 1980). In the 1970s, Australian lucerne yellows (ALuY) levels were low (McGechan 1980). Disease levels increased through the 1980s (McGechan 1980) and it is now a disease that impacts heavily on the Australian lucerne seed industry (Pilkington *et al.* 1999). The disease has a severe effect on seed production, frequently causing death of plants and reduced vigour in those that survive (Stovold 1981). The disease also causes a reduction in seed yield and has led to the cutting or ploughing-under

of seed crops, resulting in estimated losses of $7m annually to the Australian lucerne seed industry (Pilkington *et al.* 1999).

Symptoms associated with ALuY include yellowing of foliage (Stovold 1983) and roots that have a characteristic yellow-brown discoloration immediately under the periderm of the tap-root (Stovold 1983, Chapter Two). The association between ALuY and phytoplasmas is addressed in Chapter Two.

No current advice for disease management is available for ALuY other than to plough-in the infected crop and rotate if heavily infested (Pilkington *et al.* 1999, McDonald *et al.* 2003). Management strategies are addressed in Chapters Three and Four.

Overall aims of the study

This project aims to deliver an understanding of, and contribute to a disease management strategy for, “Australian lucerne yellows”. Identification of the pathogen and the vectors responsible for its spread will be key aspects of this work. This will be accomplished using molecular biology DNA techniques in addition to confirming the presence of the phytoplasma using transmission electron microscopy (TEM).

Specific objectives were:

1. Identify and study the pathogen by developing and refining molecular techniques that may be used in the identification of the ALuY

phytoplasma. TEM was used to confirm the presence of a phytoplasma in symptomatic plants. The phytoplasma will be phylogenetically characterised and the level of association between the ALuY phytoplasma and the disease will be explored. Symptomatic plants will be assayed for non-phytoplasma pathogens. This objective is addressed in Chapter Two.

2. Monitor insect field populations in lucerne and nearby non-crop vegetation and monitor ALuY symptomatic plants in order to establish which insects are possible vectors of ALuY. The vector status of insects will be demonstrated with examination of the spatial and temporal relationships between the distributions of insects within the crop and disease incidence. This objective is addressed in Chapter Three.
3. Examine the effect of insecticide treatment, herbicide treatment and a combination of both treatments on non-crop vegetation to the movement of leafhopper species identified in objective 2. Disease severity will be monitored to examine the effect of these treatments to levels of disease in the crop. This objective is addressed in Chapter Three.
4. Determine the vector status of leafhopper species identified in objective 2. This objective is addressed in Chapter Four.
5. Refine DNA extraction techniques for insects by excluding gut content from extraction process. Test field populations of *O. argentatus, B. angustatus* and *A. torrida* for presence of ALuY phytoplasma. This objective is addressed in Chapter Five.

6. Test symptom alleviation techniques in the field as a possible means of disease management. This objective is addressed in Chapter Six.

Raw data for each section of this study is presented in spreadsheets stored on the mini-disk located on the inner back cover of this thesis.

Chapter Two – Detection and identification of a phytoplasma from lucerne with 'Australian lucerne yellows' disease

Introduction

Lucerne (*Medicago sativa*) is a perennial, deep-rooted pasture legume of increasing world-wide significance due to its use in managing aspects of environmental sustainability, such as rising water tables and soil salinity (Fitzgerald *et al.* 1980). The production of lucerne seed is an important sector of Australia's pasture seed industry but is affected by the disease Australian lucerne yellows (ALuY) (Pilkington *et al.* 1999).

Phytoplasmas have been detected in 38 plant species in Australia (Schneider *et al.* 1999b) including lucerne. The TBB and sweet potato little leaf strain V4 (SPLL-V4) phytoplasma have been detected in lucerne (Gibb *et al.* 2000, Wilson *et al.* 2001, Gibb, 2002, pers. comm., 26 August).

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

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

Materials and Methods

Source of material

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

Fungal isolations from roots

A segment of the tap-root, approximately five centimetres long, was cut from each plant and washed thoroughly in tap water then in sterile distilled water and dried with paper towelling. A small section of the root cambium was

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

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

Bacterial isolations from roots, stems and leaves

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

All exterior surfaces of the root section were removed aseptically using standard sterile techniques. Discoloured tissue from ALuY affected plants, and matching tissue from symptomless plants, were sliced into fine pieces

(approximately 1 x 1 x 0.5 mm) and teased out. The slices were placed in 10 ml sterile distilled water for one hour.

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

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

Detection of phytoplasmas

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

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

The primers P1 (Deng & Hiruki 1991a) and P7 (Kirkpatrick *et al.* 1994), fU5 (Lorenz *et al.* 1995) and m23sr (Padovan *et al.* 1995) were used in PCR and nested PCR assays. PCR cycling conditions were as follows: denaturation for one minute (two minutes for first cycle) at 95°C, annealing temperature of 55°C for one minute and an extension time of 1.5 minutes at 72°C for 35 cycles (9.5 minutes on final cycle). TBB phytoplasma DNA and sterile distilled water (SDW) were used for positive and negative controls, respectively. Sixteen nested PCR assays were conducted, each consisting of sixteen ALuY

DNA samples, two symptomless lucerne DNA samples, one TBB sample and one SDW sample using the universal primers P1/P7. One μL of each P1/P7 PCR cocktail was then subjected to re-amplification using the primer pair fU5/m23sr and the same cycling conditions. After each nested PCR assay, 2 μL of PCR product were analysed by electrophoresis on a 1.0% agarose gel and stained with ethidium bromide prior to being visualised with a UV transilluminator.

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

Restriction fragment length polymorphism analysis

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

Sequence analysis

The entire PCR product obtained from a DNA sample extracted from a single ALuY lucerne plant that tested positive for phytoplasma by PCR was purified

using the QIAquick PCR purification kit (Qiagen, Clifton Hill, NSW, Australia). Sequencing of products was performed at the Australian Genome Research Facility (St Lucia, Queensland, Australia). Sequencing primers consisted of P3 (Schneider *et al.* 1995), rP3 (reverse and complement of P3), 16R723f, r723SEQ (the reverse and complement of 16R723f), rU3 (Lorenz *et al.* 1995), fsLYa (5' CAAACCACGAAAGTTGGC 3'), fsLYb (5' AAAAACAGTCCCAGTCCG 3'), fU5 (Lorenz *et al.* 1995) and M23sr (Padovan *et al.* 1995). The ALuY 16S rDNA sequence was compiled using CodonCode Assembler version 0.000918 (CodonCode Corporation, Dedham, MA, USA) available through BioNavigator (Entigen Corporation, Sunnyvale, CA, USA). ALuY phytoplasma 16S rDNA was aligned with other phytoplasmas using ClustalW (Thompson *et al.* 1994) (Table 2. 1). A phylogenetic tree was prepared using DNAdist and Neighbour (Felsenstein 1989) and phylodendron (D. G. Gilbert & BioNavigator, Entigen Corporation). Pairwise comparisons between ALuY phytoplasma and several closely related phytoplasmas (Table 2. 1) were conducted using the programme GAP (Accelrys, San Diego, CA, USA). *Acholeplasma palmae* and *A. laidlawii* were used as outgroups.

Electron microscopy

Leaf midribs from six ALuY affected and two unaffected lucerne plants were dissected into approximately $1mm^3$ pieces containing phloem tissue. Samples were fixed with standard methods (Bozzola & Russell 1992). Specimens were infiltrated with 100% acetone/Spurrs resin (1:1) overnight at room temperature on rotators, transferred to 100% Spurrs resin overnight on rotators and embedded in fresh Spurrs resin and polymerised at 60°C

overnight. Specimens were then cut into ultra-thin (80nm) sections and viewed in a Philips Biofilter CM120 (120kV) electron microscope.

Table 2. 1 Phytoplasma names, abbreviations and EMBL accession numbers

Phytoplasma	Abbreviation	Accession number
Sweet potato witches' broom	SPWB	L33770
Sweet potato little leaf	SPLL	X90591
Tomato big bud	TBB	Y08173
Faba bean phyllody	FBP	X83432
Bonamia little leaf	BoLL	Y15863
Clover phyllody	CPh	L33762
Oenothera aster yellows	OAY	M30970
American aster yellows	AAY	X68373
Australian grapevine yellows	AGY	X95706
Phormium yellow leaf	PYL	U43571
Alfalfa witches' broom	AlfWB	AF438413
Stolbur disease	STOL	X76427
Peanut witches' broom	PnWB	L33765
Sunhemp witches' broom	SUNHP	X76433
Vergilbungskrankheit	VK	X76428
Sugarcane white leaf	SCWL	X76432
Bermuda grass white leaf	BGWL	Y14645
Rice yellow dwarf	RYD	L26997
Pigeon pea witches' broom	PPWB	L33735
Clover yellow edge	CYE	L33766
Coconut lethal yellowing	LY	L27030
Loofah witches' broom	LfWB	L33764
Ash yellows	AshY	L33759
Clover proliferation	CP	LL33761
Elm yellows	EY	L33763
Flavescence dorée	FD	X76560
Spartium witches' broom	SPAR	X92869
Omani alfalfa witches' broom	OaWB	AF438413
Papaya yellow crinkle	PPYC	Y10095
Papaya mosaic	PPMz	Y10096
Pear decline	PD	X76425
Acholeplasma palmae		L33734
Acholeplasma laidlawii		M23932

Results

Fungal isolations

Eighteen distinct taxa of fungi were isolated from symptomatic and asymptomatic plants (Table 2. 2). *Fusarium solani* was isolated from three of the twelve diseased plants examined. Several other fungi e.g. *Phoma medicaginis*; *Colletotrichum trifolii*, were identified less commonly from plants both with and without symptoms. No consistent association between any fungus and ALuY symptoms was apparent.

Bacterial isolations

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

Detection of phytoplasmas in lucerne

No bands were amplified by simple PCR from either ALuY or symptomless plants, but in all assays the TBB phytoplasma control was positive and

Table 2. 2 Fungi isolated from individual ALuY symptomatic and asymptomatic lucerne plants

	Symptomatic	Asymptomatic
No isolations	4/12	3/12
Fusarium solani (Mart.) Sacc.	3/12	0/12
Mucorales undet.	2/12	2/12
Phoma sp.	2/12	2/12
Ascomycete undetermined	2/12	1/12
Verticillium sp.	2/12	1/12
Colletotrichum trifolii Bain. & Essary	2/12	0/12
Penicillium sp.	2/12	0/12
Phoma medicaginis Malbr. & Roum.	1/12	3/12
Alternaria alternata (Fr.) Keissler	1/12	0/12
Fusarium subglutinans (Wollenw. & Reinking) Nelson, Toussoun & Marasas	1/12	0/12
Paecilomyces sp.	1/12	0/12
Cladosporium sp.	0/12	2/12
Fusarium oxysporum Schlecht.	0/12	2/12
Trichoderma sp.	0/12	2/12
Fusarium avenaceum (Corda ex Fr.) Sacc.	0/12	1/12
Gloeosporium sp.	0/12	1/12
Phomopsis sp.	0/12	1/12
Undetermined	1/12	0/12

amplified a 1.6kb band. In nested PCR using primers P1/P7 followed by fu5/m23sr, the TBB phytoplasma positive controls gave a product of 1.1kb while water controls gave no amplified product. Of the 260 ALuY samples tested from 130 individual yellows affected plants, 63 samples gave a product of 1.1kb when amplified in nested PCR assays. No positive signal was observed with DNA extracted from the 30 symptomless plant samples. A 1.6kb PCR product was observed when 18 ALuY DNA samples that had tested negative were spiked with TBB phytoplasma DNA and subjected to single round PCR. A representative PCR result for 16 ALuY affected plants and two symptomless plant samples is shown in Figure 2. 1.

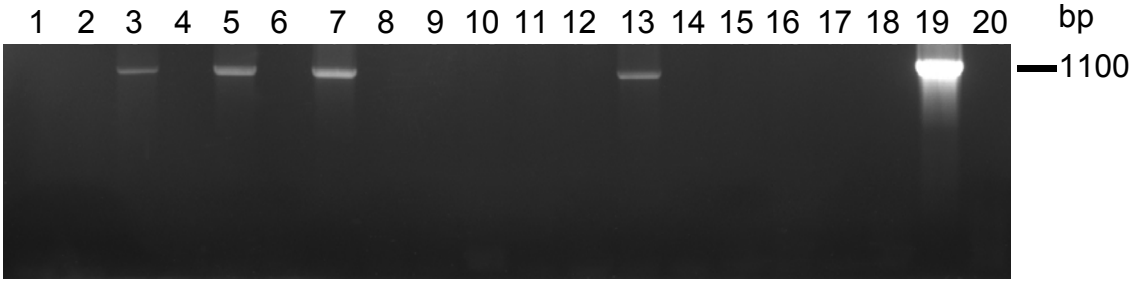

Figure 2. 1 Polymerase chain reaction amplification of phytoplasma DNA from ALuY affected lucerne using the primer pairs P1/P7 and fU5/m23sr. Lanes 1 – 16 ALuY plants; 17 and 18 symptomless lucerne; lane 19 TBB; lane 20 water control. The size marker indicated on the right hand side of the gel was used to determine the size of the PCR products.

RFLP

When 10 PCR products amplified from 10 separate ALuY plant samples were digested with the restriction enzymes *Mse*I, *Alu*I, *Rsa*I and *Hpa*II, all resulting RFLP profiles for each enzyme were identical, but differed from the patterns of the TBB digests. In all ALuY RFLP profiles for *Alu*I and *Hpa*II enzymes, extra

bands were present that were absent from TBB profiles. These extra bands result in a total fragment size larger than 1.1kb. Representative RFLP profiles of ALuY and TBB phytoplasmas are shown in Figure 2. 2.

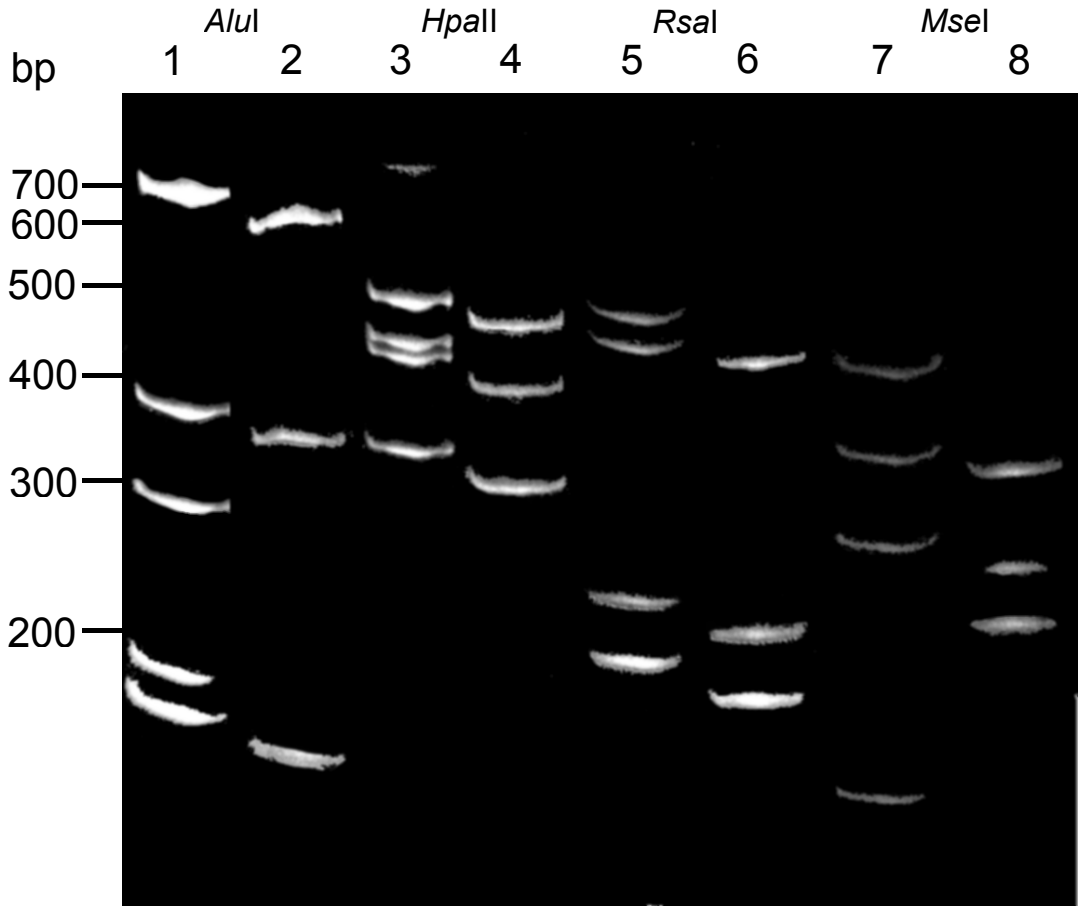

Figure 2. 2 RFLP profiles of 16s rDNA amplified by nested PCR from the phytoplasma associated with ALuY and TBB phytoplasma. Lanes 1, 3, 5 and 7 ALuY DNA digested with *Alu*I, *Hpa*II, *Rsa*I and *Mse*I respectively. Lanes 2, 4, 6 and 8 TBB DNA digested with *Alu*I, *Hpa*II, *Rsa*I and *Mse*I respectively.

Sequence Analysis

The entire PCR product of approximately 1.1kb amplified from a DNA sample extracted from an ALuY diseased lucerne plant was sequenced. The region

sequenced included the 16S rRNA gene and the entire 16S/23S spacer region (SR). The 16S/23S spacer region (accession number AJ315966) was 241 bases long which is consistent in size with other phytoplasmas (Cronje *et al.* 2000, Tran-Nguyen *et al.* 2000). The 16S rRNA region (accession number AJ315965) represents a partial sequence (position 520 to the start of the spacer region at position 1480).

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

Table 2. 3 Sequence similarity (%) matrix of the partial 16s region (approximately 5' 520 to the start of the IGS 5' 1480) of several phytoplasma species from the FBP group rounded to whole.

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

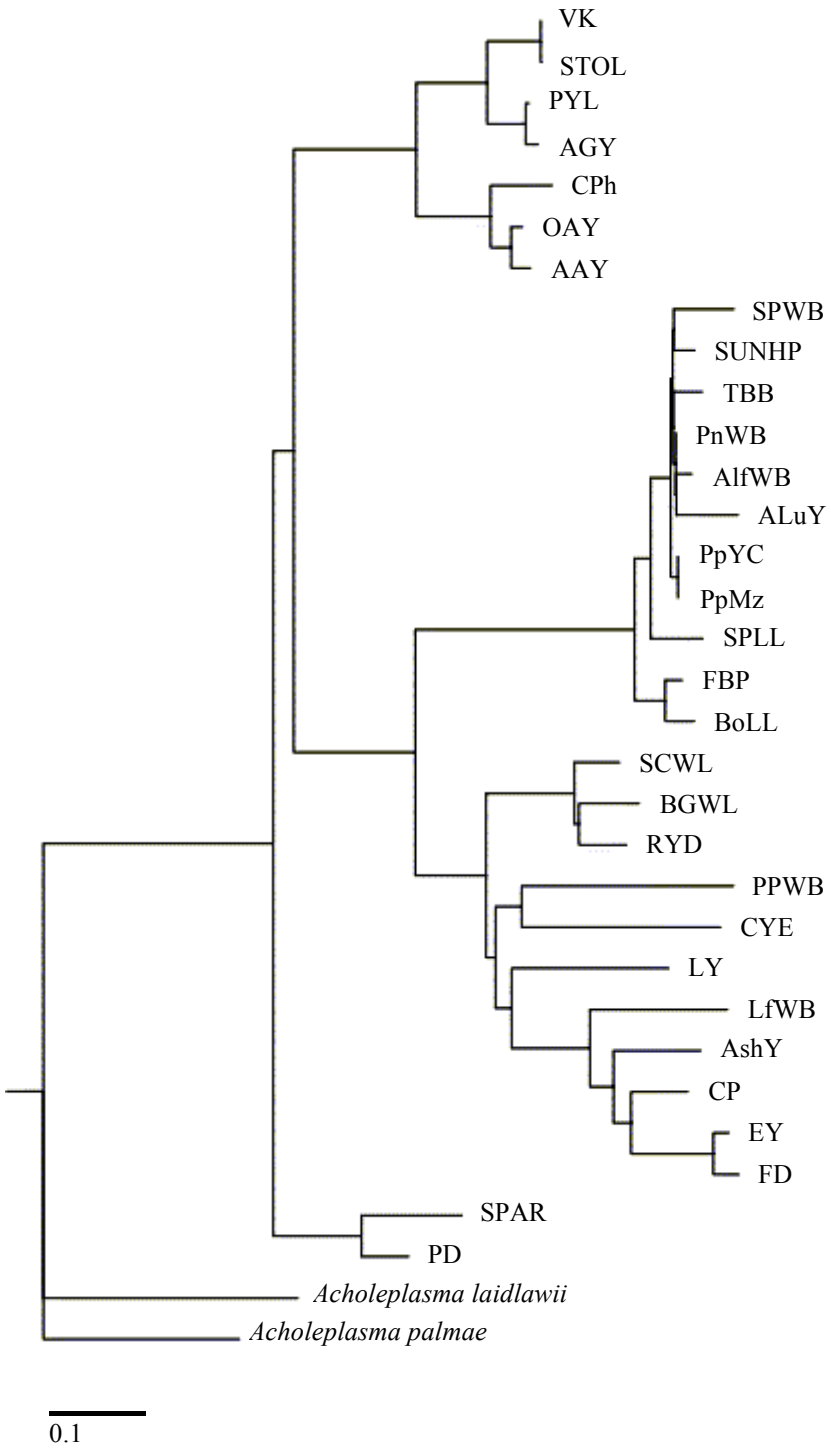

Figure 2. 3 Phylogenetic tree of the 16srRNA gene sequence of the phytoplasma associated with ALuY (partial sequence of 960bp) and other selected phytoplasma 16srRNA sequences. *Acholeplasma laidlawii* and *Acholeplasma palmae* were used as outgroups. The bar represents a phylogenetic distance of 10%. Phytoplasma strains are given in Table 2. 1.

Electron Microscopy

Examination of ultrathin cross-sections of leaf midrib from ALuY affected plants showed numerous phytoplasmas (200-400nm diameter) in the phloem of four of the plants. The structures were spherical to ovoid, were enclosed by a single unit membrane and contained dark structures centrally located that were consistent in appearance to bundles of DNA (Figure 2. 4). Some phloem cells were completely occluded with phytoplasmas. No phytoplasmas were evident in sieve tube sections of two symptomless plants examined.

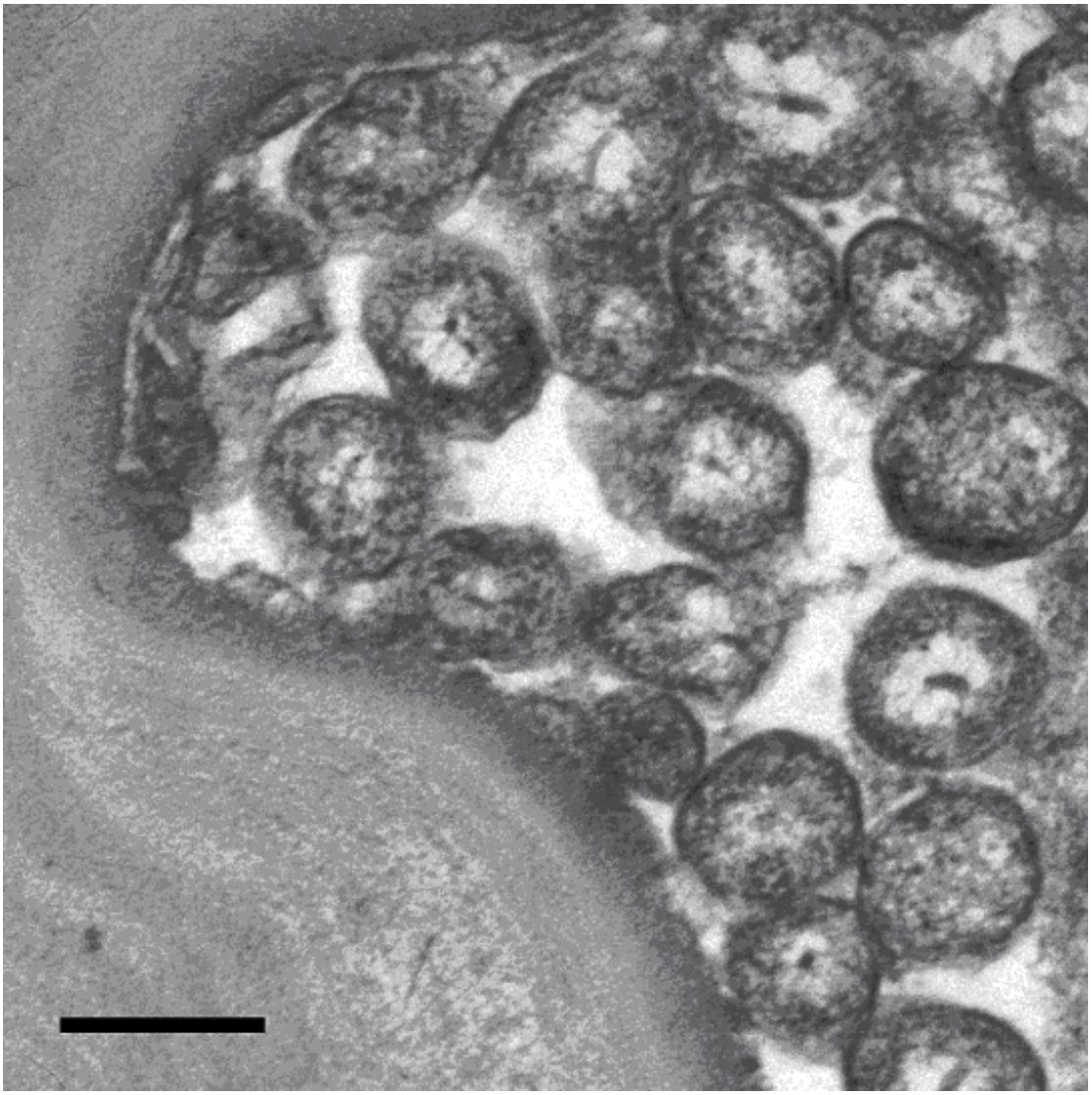

Figure 2. 4 A phloem cell of a lucerne plant affected with ALuY showing phytoplasma bodies (bar = 0.30μm).

Discussion

Lucerne with ALuY symptoms was tested for the presence of potential pathogens including fungi, bacteria and phytoplasmas. No apparent association was found between symptoms and any individual fungus. Five of the 12 fungal species isolated from ALuY affected plants were also isolated from symptomless plants and six other species were recovered solely from symptomless plants (Table 2. 2). *Fusarium solani was* the most frequently isolated fungus from yellows affected plants and previously has been associated with crown and root rots of lucerne (Leath & Kendall 1978, Nikandrow 1990), its symptoms distinct from those of ALuY.

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

Two known bacterial plant pathogens were tentatively identified by fatty acid analysis: *R. fascians* is known to cause fasciation in many plant hosts (Crespi *et al.* 1994, Stange *et al.* 1996) but no evidence indicates that it causes a disease in lucerne, whilst *C. michiganense* subsp. *insiodosum* causes bacterial wilt of lucerne but its tentative identification was not supported by subsequent, more detailed, ELISA studies. Bacterial wilt causes yellowed and stunted leaves in lucerne and symptoms are most apparent immediately after cutting or grazing (Stovold 1983). Root symptoms of bacterial wilt are a

yellow to brown discoloration throughout the stele of the tap-root and thus are distinct from the symptoms seen in plants infected with ALuY (Stovold 1983) in which discolouration occurs directly beneath the cambium layer of the tap-root (Pilkington *et al.* 1999). Aside from differences in symptoms, no obvious association with *C. michiganense* subsp. *insidiosus* could be inferred because like *R. fascians* it was isolated from only one of the 12 ALuY affected plants tested. The involvement of a culturable bacterial pathogen with lucerne yellows has also been ruled out in previous studies (Hellemere 1972).

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

Several phytoplasmas have been reported in lucerne. Alfalfa witches' broom (AWB) is distributed world-wide (Khan *et al.* 2002a). Others include the stolbur phytoplasma from lucerne in Italy (Marzachi *et al.* 2000); little leaf

phytoplasma in India (Suryanarayana *et al.* 1996); and aster yellows phytoplasma in Wisconsin (Peters *et al.* 1999). Lucerne has been implicated as being a reservoir for phytoplasma diseases such as canola yellows (Wang & Hiruki 2001b). The most common phytoplasma, AWB, is associated with several different phytoplasma groups depending on geographical location. AWB has been associated with phytoplasmas from the faba bean phyllody (FBP) group (Marcone *et al.* 1997b, Khan *et al.* 2002a), the clover proliferation (CP) group (Wang & Hiruki 2001a) and the aster yellows group (Valiunas *et al.* 2000).

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

RFLP analysis is useful for differentiating phytoplasmas (Gundersen *et al.* 1996) and has been used to classify phytoplasmas into a series of groups or subgroups (Schneider *et al.* 1993) for taxonomic purposes (Schneider *et al.* 1993). RFLP profiles for ALuY phytoplasma that were digested with the enzymes *Alu*I and *Hpa*II produced extra bands and the total fragment size was therefore greater than the 1.1kb fragment expected. Phytoplasmas contain two 16SrRNA operons (Schneider & Seemüller 1994) and these can sometimes be resolved as double bands in agarose gel electrophoresis of PCR products (De La Rue *et al.* 2001).

It is possible to visualise double banding, if the two bands are several bases differing in size, by running the product in a very low voltage electrophoesis gel. This will exentuate differences in the sizes though this was not successful in this case. Whilst only a single band was consistently amplified from ALuY DNA samples, it cannot be ruled out that the extra bands in the RFLP analysis may have resulted from slight differences in the 16SrRNA gene sequences from each operon. Although these differences may be so slight that the PCR product co-migrates on an agarose gel (Schneider & Seemüller 1994, Liefting *et al.* 1996), any sequence differences that affect restriction enzyme recognition sites will result in different inter-operon banding patterns that can be resolved on an acrylamide gel. An alternative explanation for the additional RFLP bands is that ALuY diseased plants are subject to a mixed phytoplasma infection, though this is unlikely as the RFLP patterns observed were consistent across all samples. PCR products amplified from individual ALuY affected plants gave consistent RFLP patterns

that differed from those of the positive control, the TBB phytoplasma. Such a finding indicates that the phytoplasma detected in ALuY-diseased lucerne is distinct from the widespread TBB phytoplasma (Davis *et al.* 1997, Schneider *et al.* 1999a) and on this basis it is now referred to as the Australian lucerne yellows phytoplasma (ALuY).

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

The work in this chapter presents evidence of the causal agent of ALuY disease. The work does not indicate which leafhopper species may be responsible for the transmission of the disease and this is explored further in Chaper Three.

Chapter Three – Reducing the immigration of suspected leafhopper vectors and severity of Australian lucerne yellows disease

Introduction

Phytoplasmas are associated with over 300 plant diseases around the world (Davis *et al.* 1988) and are transmitted exclusively by insects (Hanboonsong *et al.* 2002); specifically leafhoppers (Cicadelloidea), planthoppers (Fulgoroidea) and psyllids (Psylloidea) (Tsai 1979, Ploaie 1981). More than 30 species from these superfamilies have been identified in Australian (Bishop & Holtkamp 1982, Osmelak *et al.* 1989) and American lucerne stands (Sulc *et al.* 2001).

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

As phytoplasmas are restricted to the phloem of infected plants (Guthrie *et al.* 2001), it follows that their vectors feed on phloem (McCoy 1979). *Balclutha* sp., which feeds exclusively on grasses (Knight 1987), and the known parenchyma feeding species, *Austroasca* sp. and *Zygina* sp. (Carver *et al.* 1991), were discounted as possible vectors of ALuY. *O. argentatus* has been shown to be responsible for transmission of lucerne witches' broom and has been implicated in many other phytoplasma diseases, including Australian

grapevine yellows (AGY) (Padovan *et al.* 1996). *A. torrida* is a known vector of viral and bacterial plant diseases (Grylls 1979) and both *A. torrida* and *B. angustatus* have also been suggested as possible vectors of AGY (Osmelak *et al.* 1989). Their presence in ALuY-symptomatic lucerne stands suggests that they are possible vectors of this disease.

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

To assist studies of disease-vector relationships it is useful to consider information such as the spatial distribution of symptomatic plants (Arnò *et al.*

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

The aims of this study were first to survey three lucerne stands over 12 months to capture, for the first time, information on the etiology of ALuY disease. The second aim was to collect data on the spatial and temporal appearance of disease symptoms and correlate these with equivalent data on incidence of the three most common leafhopper species to provide a

preliminary indication of the vector status of each leafhopper species. The third aim of this study was to utilise pesticide treatments to crop-margin vegetation aimed to measure the extent of disease management that may be achieved by reducing potential vector immigration.

Materials and Methods

Symptom and leafhopper survey

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

Each stand was divided into either 99 or 104 sub-regions using a grid format with width and length divided into intervals such that, on a power transformation, the sub-regions were of equal size (Figure 3. 1). This power transformation was chosen so that, when dimensions used in each stand of lucerne were back-transformed, sub-regions nearer the boundaries (where it was anticipated greater precision in spatial sampling would be required) were smaller than sub-regions closer to the centre of the stand where greater homogeneity was likely.

On each sampling date for insect distribution (fortnightly in summer and monthly in winter for 12 months commencing on the 8 November 2000) evidence of symptom expression was monitored. Plant disease surveys were initiated at each site at the first appearance of ALuY symptoms. For each of the three sites, disease data were recorded from each sub-region on the following occasions. Site one was sampled monthly on three occasions after

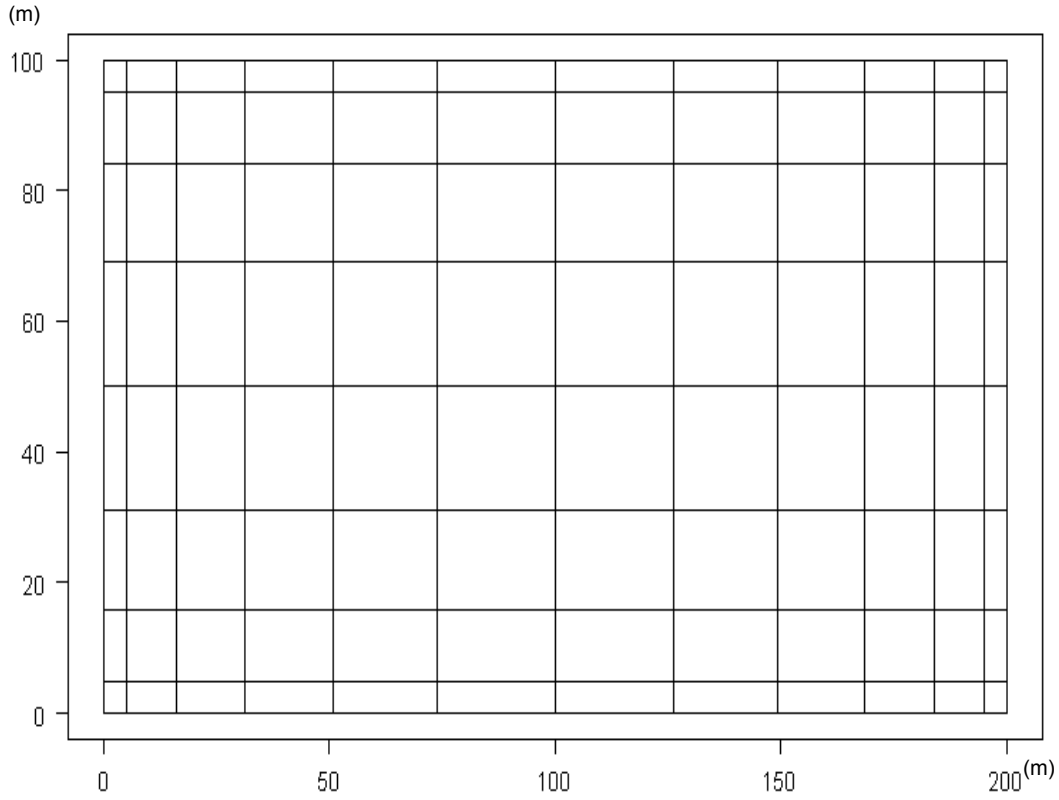

Figure 3. 1 Example of division of a site into sub-regions.

symptoms appeared on 4 January 2001, site two was sampled monthly on four occasions after ALuY symptoms appeared on 22 January 2001 and site three was sampled monthly on five occasions after symptoms appeared on 23 January 2001. The first sample dates for each site were within 32 weeks of the date of sowing for each stand.

On each sampling occasion, a small ball was cast into each sub-region, the nearest 100 lucerne plants identified and the area these plants occupied was measured. The number of plants showing ALuY symptoms was recorded and the severity of the symptoms for each plant rated on a scale of one to five (Table 3. 1). Where there was more than one symptomatic plant, within the sample of 100, the distance from each symptomatic individual to its nearest

symptomatic neighbour (of the sampled 100 plants) was recorded. Numbers of symptomatic plants, symptom severity scores and numbers of symptomatic plants per square metre were initially mapped to identify factors with marked spatial trends that merited further analysis. Regression analysis was subsequently made of numbers of ALuY symptomatic plants versus the two dimensions of the lucerne stand using Genstat 6th edition statistical software package (GenStat Committee). An example output of this style of statistical analysis is presented in Appendix Three.

Insect distribution survey

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

Table 3. 1 ALuY symptom severity assessment scale.

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

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

Regression analysis of numbers of leafhoppers caught versus row distance and column distance was performed using Genstat 6th edition statistical

software package (GenStat Committee) to model the spatial distribution of each species within the stand. An example of the output from this style of statistical analysis is presented in Appendix Three. On two occasions (20 November 2000 and 29 December 2000), a two dimensional model (insect numbers and distance from a stand edge) was used as this maximised the variance accounted for by the regression model. On all other dates, three dimensional models (that included insect numbers and distance from stand edge in two dimensions) were used.

Border treatment experiment

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

The strip on each of the two sites was divided into nine 20m by 10m plots (three blocks, each with three treatments). The allocation of treatments to plots was undertaken using Spades (Coombes & Gilmour 1999) to generate

nearest neighbour designs and was randomly allocated to herbicide (1.5 L/ha 360 g/L glyphosate), insecticide (0.465 L/ha 300 g/L dimethoate) and a control, where no application was made. To ensure sufficient replication to generate neighbour balance, the experiment was designed on the assumption that the strips on each of the two sites were contiguous.

Bi-directional sticky traps were used to measure insect movement from the neighbouring vegetation into the lucerne field and vice versa. Each trap ($0.0637m^2$) was constructed from ten 90mm-diameter Petri dishes mounted on a 1800mm-tall wooden stake. The inner surface of each Petri dish base was coated with a thin layer of Tanglefoot sticky trap glue (Australian Entomological Supplies, Bangalow, Australia). Five Petri dishes (total area of $0.0318m^2$) faced the stand and five on the opposite edge of the stake faced the non-crop vegetation. The Petri dishes on each face of the stake were arranged vertically with their edges touching. The centre of the lowest dish was 300mm from the soil surface and the centre of the top dish was 690mm from the soil surface. Each of these was nested, with its sticky surface outermost, within the lid of the Petri dish that was fixed to the stake by a drawing pin. The Petri dish lids had previously been sprayed with three coats of yellow paint (Carnival Yellow, Dulux, Clayton, Australia). The coloured lids remained attached to the stakes whilst the sticky bases were collected twice weekly between 21st September 2001 and 5th November 2001. This period was the 45 days immediately following the application of treatments to the non-crop vegetation. For each plot, a single trap was placed on the boundary of the lucerne field/non-crop vegetation, equidistant from the plot's edges. For

each collection date, leafhoppers on each trap were identified and counted using a binocular microscope (10X). Catches of each leafhopper species were pooled over all dates. Analysis of variance using Genstat 6th edition statistical software package (GenStat Committee) was used to test for effects of pesticide treatment, direction of flight and trap height following a square root transformation ($\sqrt{(x+0.5)}$) on all data. An example of the output from this style of statistical analysis is presented in Appendix Three.

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

Insect movement into and out of each lucerne stand was monitored using bi-directional yellow sticky traps (total area of 0.0254m^2) placed on the crop-margin. Traps were constructed as described in experiment 1 using two Petri dishes facing in each direction, the centre of the bottom dish being of the top dish being 300mm and 390mm from the soil surface respectively. Two traps were placed in the lucerne stand on the boundary with non-crop vegetation and were five metres either side of the mid point of the plot. The traps were changed weekly (12 November 2002 to 23 December 2002) and, for each collection date, leafhoppers on each trap were identified and counted using a binocular microscope (10X). Catch data were analysed as in experiment 1.

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

Results

Symptom and leafhopper survey

ALuY symptoms developed at site one 29 weeks after sowing and after 31 weeks at sites two and three. Maximum numbers of symptomatic plants on any given date at all sites were relatively low and dates that had a symptomatic plant count maximum of less than five were excluded from analyses. Separate three dimensional maps of symptomatic plant numbers, symptom severity and symptomatic plant density showed no significant differences within sample dates so regression analysis was performed on symptomatic plant numbers only. Regression analysis of data from two dates from site one (4 January 2001 and 8 February 2001) indicated statistically significant spatial effects (Fig. 3. 2, $P<0.001$ and $P=0.042$) with regression models counting for up to 20.6 percent of the variability in 300 symptomatic plants. On both dates, symptom incidence, when length and width were used together as parameters, was significantly higher in some parts of the crop-margin than in other parts of the margin (Figure 3. 2, $P<0.001$ and $P=0.042$).

Distribution of both *A. torrida* and *B. angustatus*, showed a significant edge effect at sites one (length and width for 28 December 2000, $P=0.038$, width for 20 November 2000, $P=0.008$ and length for 29 December 2000, $P=0.020$) and three (length and width for each of 29 December 2000, $P=0.003$, 19 January 2001, $P=0.013$, 31 January 2001, $P=0.003$ and 13 February 2001, $P=0.009$) on at least one date (Figure 3. 3 and Figure 3. 4, respectively). Whenever a significant spatial effect was found, catches of leafhoppers were highest in one or more sections of the crop-margin and lower in the stand interior,

though catches were not consistently high in all margins. At site one, symptomatic plant numbers across the entire site on 8 February 2001 were positively correlated with *O. argentatus* distribution, nine days earlier, on 31 January 2001 (r=0.195, P=0.05). Symptomatic plant numbers, with parameters length and width, at site three showed a correlation on 20 February 2001 with the spatial distribution of *B. angustatus*, 54 days earlier, on 28 December 2000 (r=0.318, P<0.05). Similarly, there was a significant correlation between the symptomatic plant numbers on 4 January 2001 with the spatial distribution of *A. torrida* on 11 April 2001 at site one (r=0.300, P<0.05)

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

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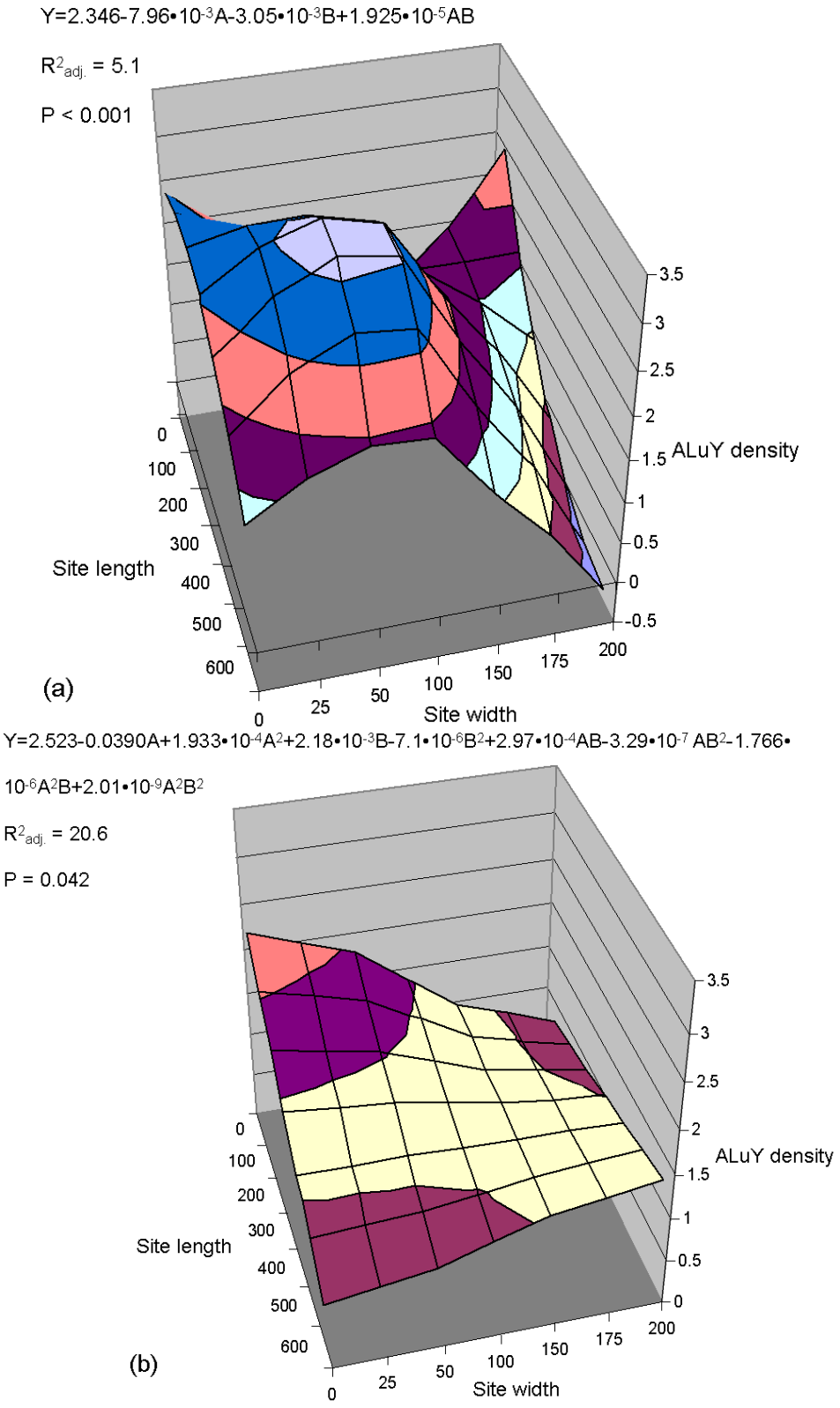

Figure 3. 2 Fitted models representing spatial distribution of ALuY disease severity for site 1 on (a) 4 January 2001 and (b) 8 February 2001.

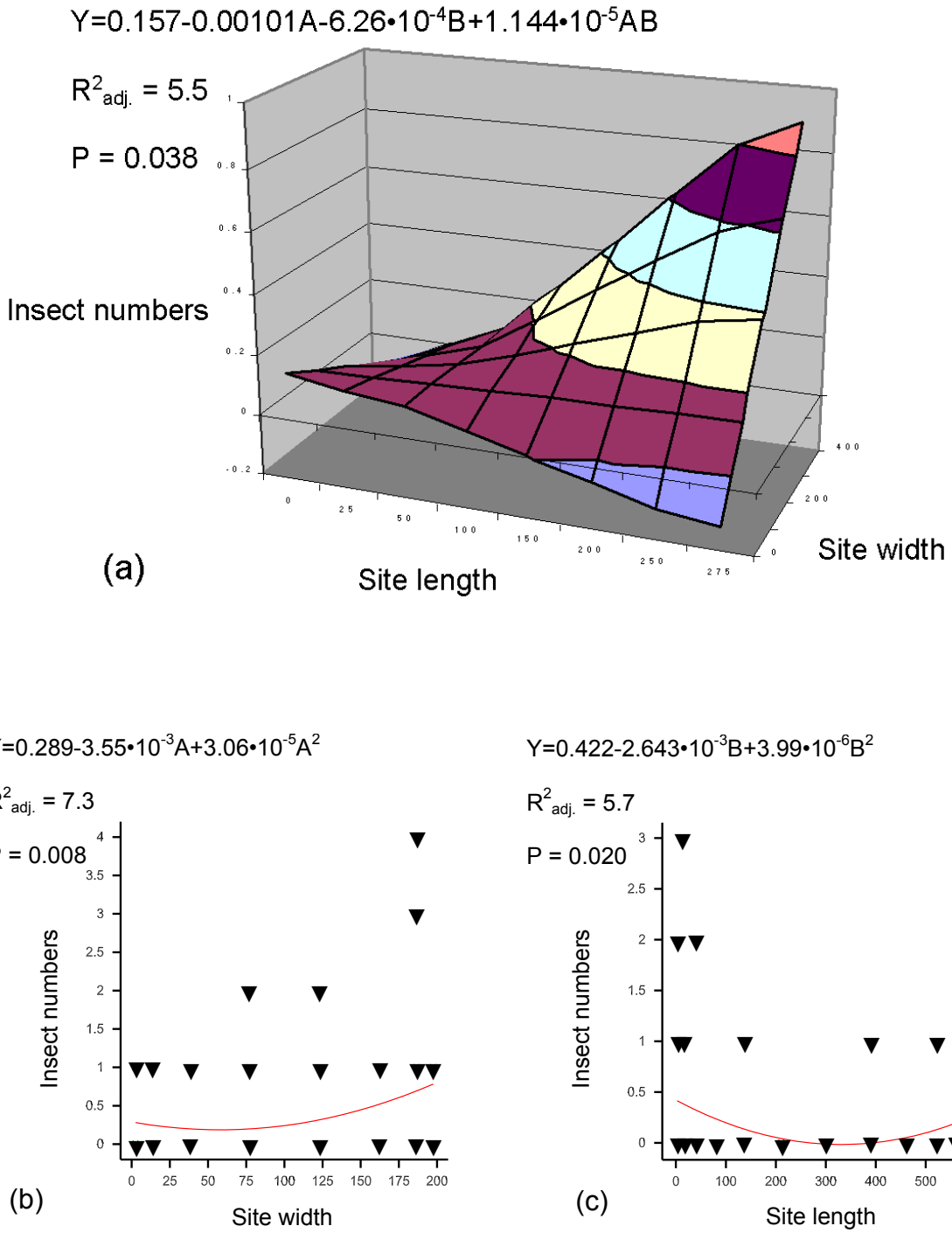

Figure 3. 3 Fitted models representing spatial distribution of *B. angustatus*. (a) three dimensional distribution on 28 December 2000 at site 3; (b) two dimensional distribution on 20 November 2000 at site 1; (c) two dimensional distribution on 29 December 2000 at site 1.

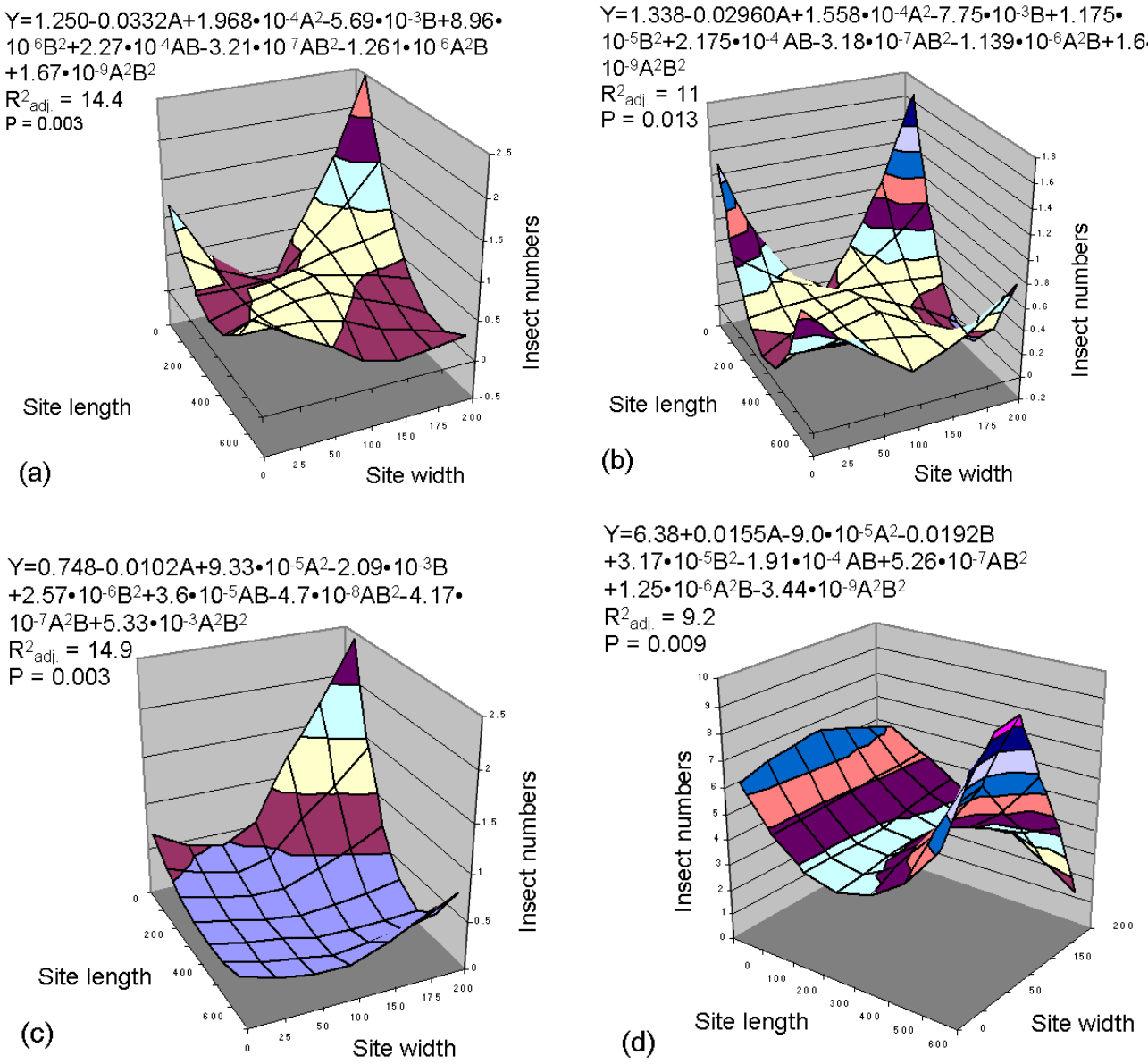

Figure 3. 4 Fitted models representing insect numbers at site 1. (a) *A. torrida* on 29 December 2000; (b) *A. torrida* on 19 January 2001; (c) *A. torrida* on 31 January 2001; and (d) *A. torrida* on 13 February 2001.

P. aviculare, *C. myriocarpus* and *H. europaeum*. Trends in the distribution of leafhoppers in non-crop vegetation were non clear-cut and were not correlated with symptomatic plant numbers within the stand.

Border treatment experiment

Experiment 1 - Only nine *B. angustatus* were caught in all treatments over the course of the experiment so data for this species were excluded from analyses. Border treatments did not significantly affect catches of either *A. torrida* or *O. argentatus* but catches were strongly affected by trap height with catches declining as trap height increased (Fig. 3. 5a, b).

Experiment 2 - No statistically significant spatial effects were found in numbers of *B. angustatus*. Irrigation at one of the sites ceased in early November 2002 due to the grower's reduced water allocations during drought conditions. This site was excluded from analyses due to desiccation of the stand and non-crop vegetation leading to low insect catches for all treatments at that site. For the remaining three sites, pooled counts of *A. torrida* and *O. argentatus* were significantly higher ($P<0.001$) in the lower traps (Fig 3. 5c, d). Herbicide treatment reduced *A. torrida* migration into the lucerne as well as overall (i.e. pooled immigration and emigration) catches to a statistically significant ($P=0.02$ and $P=0.005$, respectively) extent compared with the control treatment (Table 3. 2). Similarly, catches of pooled *O. argentatus* were reduced significantly by herbicide treatment (Table 3. 2, $P=0.029$). Catches of emigrating *O. argentatus* were reduced significantly by insecticide

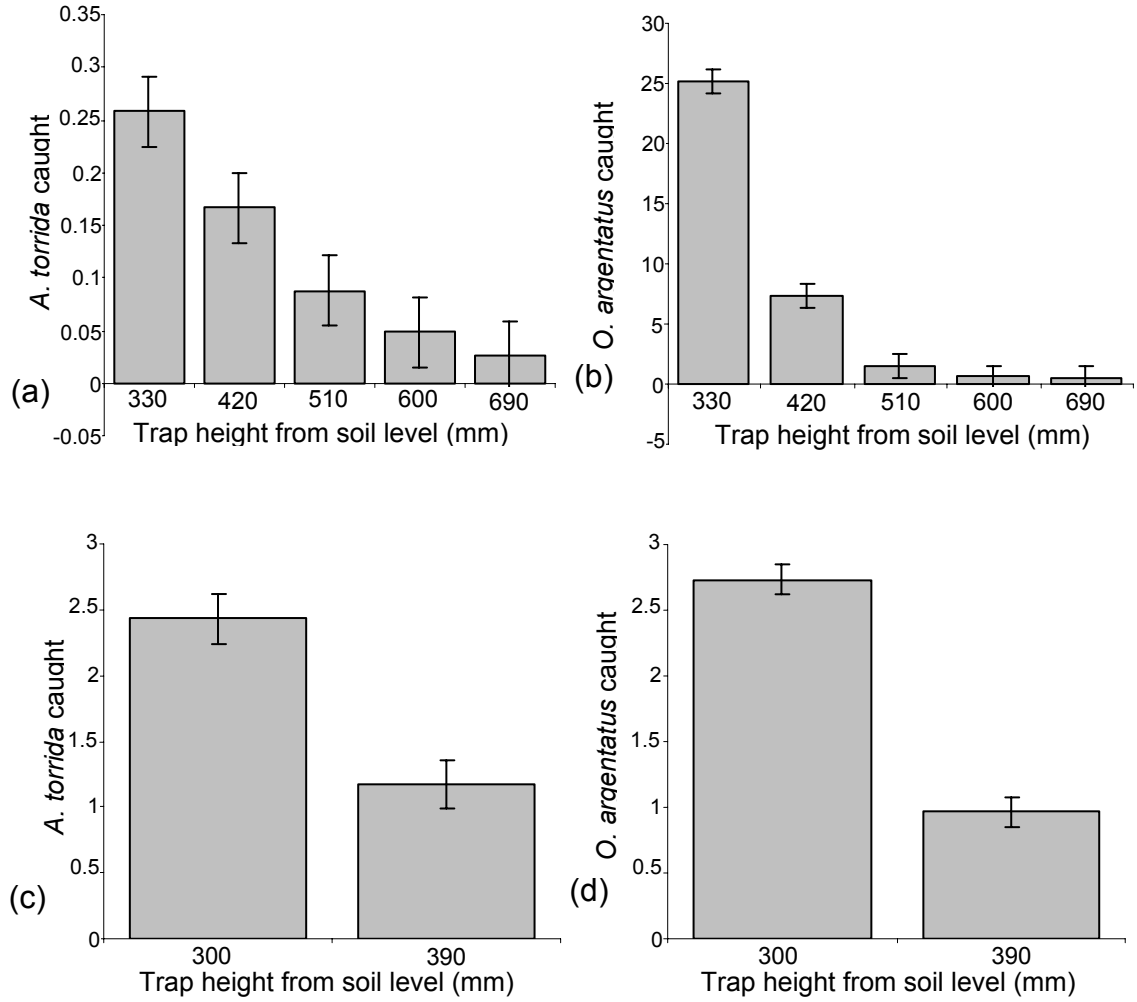

Figure 3. 5 Effect of trap height on catches of *A. torrida* and *O. argentatus* in experiment 1 (a and b) and; experiment 2 (c and d).

Catches of emigrating *O. argentatus* were reduced significantly by insecticide treatment (P=0.024). Throughout all treatments there were higher numbers of *O. argentatus* and *B. angustatus* migrating into the stand than from the stand.

The numbers of plants expressing ALuY symptoms was significantly reduced (P=0.007) in plots treated with insecticide (mean 4.50) when compared with the control (mean 6.33). The reduction in disease levels achieved by

herbicide treatment (mean 5.00) fell just outside of 95% confidence limits of significance (P=0.052) when compared with the control.

Table 3. 2 Effect of border treatments on catches of leafhoppers on lucerne borders when compared with the control.

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

Discussion

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

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

The spatial distribution of leafhoppers on some dates was significantly correlated with symptomatic plant numbers on other dates. On 28 December 2000 the distribution of *B. angustatus* at site three was significantly correlated

($P<0.05$) with the distribution of symptoms 54 days later. The length of this incubation period is consistent with that known for other phytoplasma diseases (Chiykowski & Sinha 1988). Whilst the distribution of *O. argentatus* at site one was correlated ($P=0.05$) with disease incidence only nine days later, the immigration of the vectors into the stand may have happened up to 12 days earlier when the preceding sample was taken, placing the disease incubation time within the range known for other phytoplasma diseases (Chiykowski & Sinha 1990). Catches of *A. torrida* prior to emergence of ALuY symptoms may have been too highly variable to allow the detection of a statistically significant relationship with symptoms, though a significant relationship ($P<0.05$) between symptoms and later densities of *A. torrida* was found.

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

The significant edge effects evident in field surveys for *A. torrida* and *B. angustatus* is consistent with the finding that leafhopper catches were greater

in lower traps than in identical traps placed further from the ground. This suggests that the leafhoppers do not undergo long-range dispersal to reach newly-sown lucerne stands but enter by trivial, short-range movement from adjacent vegetation. There were no significant spatial patterns detected for the leafhopper *O. argentatus*.

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

The use of an ALuY management strategy based on heavy pesticide inputs to large areas of non-crop vegetation is unlikely to be acceptable to farmers, regulatory authorities or the broader community. A narrow strip of treated non-crop vegetation, however, may suffice to give useful levels of disease suppression. A normal application rate would require 4.9kg of dimethoate to treat a 35ha stand of lucerne in comparison with 660g of dimethoate to treat a

20 m wide band of the non-crop vegetation bordering the stand. It would seem that removal of host species with an effective weed control strategy might have the most potential and greatest acceptability to farmers.

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

Results indicate that the successful management of ALuY disease may be achieved by limiting the movement of leafhoppers into lucerne stands, though further studies need to be undertaken. The reduction of disease incidence in relation to the lowered movement of *O. argentatus* and *A. torrida* into the lucerne stand is the best indication to date that one or both of these leafhopper species is a vector of ALuY. These results are not evidence of the vector status of these leafhopper species and further experimentation is required. Transmission testing and testing of field collected leafhoppers was conducted and is presented in Chapter Four.

Chapter Four – Vector status of three leafhopper species for Australian lucerne yellows phytoplasma

Introduction

Insects that have been shown to be responsible for the vectoring of phytoplasmas are leafhoppers (Membracoidea), planthoppers (Fulgoromorpha) and psyllids (Psylloidea) (Chiykowski 1981, Hill & Sinclair 2000). Fletcher (1980) conducted insect trapping studies in order to identify the vectors responsible for the spread of ALuY, and analysis of the occurrences of the 26 cicadellid species collected indicated that 23 were not associated with the disease. The three possible vector species for the disease were to undergo subsequent transmission tests though these tests were not undertaken and no evidence of other similar studies could be found in the current literature.

ALuY disease is associated with a phytoplasma (Chapter Two). Three leafhoppers; *Orosius argentatus* (Evans); *Austroagallia torrida* (Evans); and *Batracomorphus angustatus* (Osborn) have been shown to have a spatio-temporal correlation with disease symptoms and are possible vectors of the ALuY pathogen (Chapter Four). To date, however, there has been no direct experimental evidence to implicate any of the species as ALuY vectors.

The disease named tomato big bud (TBB) by Samuel *et al.* (1933) was first reported in Australia in 1902, being described on tomato plants (Cobb 1902). TBB, originally thought to be caused by a viral pathogen (Bowyer 1974), was

shown to be caused by a mycoplasma-like organism, now known as a phytoplasma, by Bowyer *et al.* (1969) and further work, including the treatment of symptoms with antibiotics, was continued (Bowyer & Atherton 1972, Osmelak 1986). The leafhopper, *O. argentatus,* was shown to be a vector for the pathogen (Hill 1943) which is widespread in a range of plant species throughout Australia (Davis *et al.* 1997). *O. argentatus* is responsible for the transmission of witches' broom in lucerne and has been implicated as a vector of many other phytoplasma pathogens (Tsai 1979, Osmelak *et al.* 1989, Padovan *et al.* 1996).

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

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

transmitting the pathogen (Maeso Tozzi *et al.* 1993, Carraro *et al.* 2001a, Jarausch *et al.* 2001).

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

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

The aims of this study were to identify vectors for the disease ALuY using field-collected and laboratory-reared leafhoppers in caged transmission tests.

Plants were to be assayed using PCR techniques and monitored for symptom expression. A supplementary aim was to identify other possible phytoplasma pathogens that may be transmitted by field-collected insects.

Materials and methods

Source of ALuY symptomatic lucerne

ALuY symptomatic plants were collected from certified lucerne cv. Aurora seed stands growing in the Lachlan Valley region of central New South Wales, Australia in September 2002. Six symptomatic plants were provisionally identified by foliar symptoms and each plant was carefully removed from the ground, removing the soil surrounding the bulk of each plant's root system. A lateral root was exposed and ALuY diagnosis was confirmed based on root symptoms (Chapter Two). Each plant was transferred to a 16.5 L pot that was back filled with an Australian standard premium potting mix (Pot'n'Peat potting mix, Envirogreen, Castlereagh, Australia) and thereafter, watered regularly. The foliage was sprayed with insecticide (active constituents 0.4 g/L pyrethrins, 1.6 g/L piperonyl butoxide) applied as a fine mist over the entire plant, and rinsed thoroughly with water 24 hours later prior to introduction of leafhopper colonies to ensure that all other insect species were removed.

Transmission tests

Source of insects - *A. torrida* and *B. angustatus,* adults and nymphs, were obtained by sweep-netting certified lucerne seed stands, cv. Aurora, growing in the Lachlan Valley region of central New South Wales, Australia in September 2002. Leafhoppers were removed from the sweep net and placed, with some green lucerne foliage, into a container stored in a 12V car refrigerator set at approximately 9°C for transport back to the laboratory. In the laboratory, leafhoppers were anaesthetised with carbon dioxide and sorted to species. Specimens of *O. argentatus* were sourced from a laboratory

culture maintained at Northern Territory University as only very low numbers were present in the field at the time other species were captured. *O. argentatus* suffered high mortality on lucerne plants in previous culturing attempts so were placed on two seed-grown, caged, faba bean (*Vicia faba* L.) plants at 28°C on which they bred readily.

A. torrida feeding trial - Fifty individuals of *A. torrida* were placed on two individually caged ALuY symptomatic plants for an acquisition period of seven days. Insects were then transferred to nine caged, seed-grown lucerne plants for an inoculation period of 50 days.

B. angustatus feeding trial - Fifty *B. angustatus* were placed on two individually caged ALuY symptomatic plants for an acquisition period of seven days. Insects were then transferred to eight caged, seed-grown lucerne plants for an inoculation period of 50 days.

O. argentatus feeding trial - Approximately 50 *O. argentatus* individuals were removed from the colony maintained on beans and transferred to individual leaflets on two ALuY symptomatic plants in batches of two or three insects per clip cage. Clip cages were constructed using metal hairclips, fine mesh material, sponge and two lengths of 15 mm plastic pipe cut to 15 mm. The hairclip tips were heated using a Bunsen burner and each tip was inserted into each length of pipe. The fine mesh was glued onto the outside opening of the tube and sponge was attached to the two pipe ends that contacted with the leaf. The acquisition period was seven days followed by a latency period on

faba bean for 30 days. The insects were then transferred to eight individually caged, healthy, seed-grown lucerne plants and placed on individual leaflets, each plant having three clip cages with batches of two to three leafhoppers for an inoculation period of seven days.

Monitoring plants and insects - At the end of each inoculation period, insects were removed from the test plants and stored at -20°C for phytoplasma screening by PCR. Stored insects were labelled with numbers that corresponded to each test plant. Test plants were removed from the pots and planted in the field under large, insect-proof cages on The University of Sydney, Orange campus farm in November 2002 and monitored weekly for expression of foliar symptoms consistent with phytoplasma infection. After seven months, the plants were removed from the ground, their roots washed thoroughly with water and the periderm exposed to check for expression of ALuY root symptoms on each plant.

PCR assays of test plants - For each test plant, two samples were taken from different parts of the plant. A sample was three leaf mid-ribs and DNA was extracted from each sample (Dellaporta *et al.* 1983). Each 50 µl PCR reaction mixture contained 1 µl of DNA, 1.25 units of Taq polymerase, Taq buffer consisting of 1.4 mM $MgCl_2$, 0.4 µM of each primer and 0.1 mM of each dNTP (all components listed supplied by GeneWorks, Adelaide, Australia). Universal phytoplasma primers P1 (Deng & Hiruki 1991a) and P7 (Kirkpatrick *et al.* 1994) were used in first round PCR and primers fU5 (Lorenz *et al.* 1995) and m23sr (Padovan *et al.* 1995) were used in nested PCR assays. PCR

cycling conditions were as follows: denaturation for one minute (two minutes for first cycle) at 95°C, annealing temperature of 55°C for one minute and an extension time of 1.5 minutes at 72°C for 35 cycles (9.5 minutes on final cycle). TBB phytoplasma DNA, collected from a tomato plant exhibiting symptoms of TBB disease, and sterile distilled water (SDW) were used as positive and negative controls respectively. After each nested PCR assay, 2 μl of PCR product were analysed by electrophoresis on a 1% agarose gel and stained with ethidium bromide prior to being visualised with a UV transilluminator.

PCR assays of leafhoppers

A. torrida and B. angustatus - For each species, 50 leafhoppers that had been stored at –20°C following their use in transmission tests were assayed for phytoplasma in batches of five to ten. To test for phytoplasmas, the head and thorax of each insect was dissected and the batch assayed by PCR using the same technique described for the test plants.

O. argentatus - Individuals of laboratory-reared *O. argentatus* were fed on disease free faba bean for 7 days after the inoculation period to clear the gut of diseased lucerne. They were then screened for phytoplasma in the same manner as the other two insect species, the abdomen also removed for consistency of methods.

Phytoplasma screening of field-collected insects - Leafhoppers were collected using a Weed Eater® motorised vacuum sampler (Model GB^{TM} 30v, 1993,

Poulan: Division of WCI Outdoor Products, Inc., Shreveport, LA 71129, USA) (Hossain *et al.* 1999) from three newly sown certified lucerne (cv. Aurora) seed stands in the mid Lachlan Valley region of New South Wales, Australia between December 2000 and February 2001. The leafhoppers were kept refrigerated at approximately 6°C for transport back to the laboratory and stored at -20°C. One hundred and fifteen *B. angustatus,* 145 *A. torrida* and 155 *O. argentatus* were tested for phytoplasma by PCR using the same protocols as described for the insects used in the feeding trials in bacthes of five to ten.

RFLP of PCR product

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

Microscopy

For transmission electron microscopy (TEM), leaf mid-ribs from all test plants were dissected into approximately 1 mm^3 pieces containing phloem tissue and were fixed using standard methods (Bozzola & Russell 1992). Specimens were infiltrated with 100% acetone/Spurrs resin (1:1) overnight at room temperature on rotators, transferred to 100% Spurrs resin overnight on

rotators and embedded in fresh Spurrs resin and polymerised at 60°C overnight. Specimens were then cut into ultra-thin (80 nm) sections and viewed in a Philips Biofilter CM120 (120 kV) electron microscope.

From plants selected for fluorescence microscopy, leaf mid-ribs from all test plants, a symptomless lucerne plant as negative control and a lucerne plant affected with witches' broom symptoms as positive control were dissected into approximately 1 – 2 mm^3 pieces containing phloem tissue and were chilled and transported at 4°C to Northern Territory University for specimen preparation and fluorescence microscopy. Samples were transported in DAPI (4',6' diamidino-2-phenylindole) fixative made by combining 9.7 mL 0.2 M sodium cacodylate buffer (21.4 g $Na(CH_3)_2AsO_2.3H_2O$ in 500 mL of sterile distilled water) with 9.7 mL sterile distilled water and 600 µL 25% glutaraldehyde.

Three insects of each species were selected from transmission trials and examined with fluorescence microscopy. DAPI stain was mixed by combining 4.05 mL of a 0.2 M solution of dibasic phosphate (Na_2HPO_4) (Sigma Aldrich, Castle Hill, Australia) with 0.95 mL of a 0.1 M monosodium phosphate ($NaH_2PO_4.H_2O$) (Sigma Aldrich, Castle Hill, Australia) buffer with 4 mL of water. This buffer was adjusted to 7.0 pH before two grains of DAPI (Sigma Aldrich, Castle Hill, Australia) and two drops of aniline blue stain (Sigma Aldrich, Castle Hill, Australia) were added. Thick cross-sections of each leafhopper species, three of each, were left in the stain for three hours before

being visualised on a Nikon E800 microscope using ultra-violet filter cubes (EX 380, DM 400, BA 520).

Results

A. torrida feeding trial and phytoplasma assays

Three of the nine lucerne plants fed on by field-collected *A. torrida* showed no symptoms. Of the remainder, one showed leaf chlorotic vein-banding and leaf reddening, symptoms that are consistent with alfalfa mosaic virus. Two plants showed leaf stunting and some yellowing of leaves. The remaining three plants fed on by *A. torrida* showed severe stunting of leaves and stems and in one case die-back of stems. None of the plants fed on by *A. torrida* exhibited any root symptoms. When these plants were tested by PCR, only the plant that showed both severe leaf stunting and die-back of stems was phytoplasma positive by PCR. None of the leafhoppers were phytoplasma positive by PCR. The phytoplasma DNA detected in the plant fed on by *A. torrida* was digested with the restriction enzymes *Alu*I, *Hpa*II and *Rsa*I. The digestion pattern for all three enzymes was indistinguishable from the pattern displayed by the TBB phytoplasma reference sample (Figure 4. 1). The results for symptom expression, PCR assays and microscopy examination are summarised in Table 4. 1.

B. angustatus feeding trial and phytoplasma assays

Six of the eight plants fed on by field-collected *B. angustatus* showed no symptoms of phytoplasma infection. The remaining two plants had mild stunting of leaves on some shoots near the crown of the plant. None of the plants fed on by *B. angustatus* exhibited any root symptoms. No plants were phytoplasma positive by PCR except for one of the asymptomatic plants. No leafhoppers were phytoplasma positive by PCR. The PCR product from the

Table 4. 1 Summary table of evidence from transmission tests with leafhopper species *A. torrida*.

	ALuY Symptoms		Molecular		Microscopy
Plant[a]	Foliar[b]	Roots[c]	PCR[d]	RFLP[e]	EM[f]
1	-	-	-	-	-
2	-	-	-	-	-
3	-	-	-	-	-
4	+ (Leaf stunting and yellowing)	-	-	-	-
5	+ (Leaf stunting and yellowing)	-	-	-	-
6	+ (Severe stunting of leaves)	-	-	-	-
7	+ (Severe stunting of leaves)	-	-	-	-
8	+ (Severe stunting and Die back)	-	+	+ (TBB)	+
9	+ (Inter veinal yellowing)	-	-	-	-

[a] Healthy lucerne plant exposed to feeding by *A. torrida*
[b] Indicates what foliar symptoms were expressed by transmission test plant after being exposed to feeding by *A. torrida*
[c] Indicates what root symptoms were expressed by transmission test plant after being exposed to feeding by *A. torrida*
[d] Indicates whether phytoplasma DNA was amplified from transmission test plant after being exposed to feeding by *A. torrida*
[e] Indicates characterisation of any PCR product produced by PCR assays of DNA extracted from transmission test plant after being exposed to feeding by *A. torrida*
[f] Indicates whether phytoplasma bodies were visualised by TEM examination in tissue from transmission test plant after being exposed to feeding by *A. torrida*

asymptomatic plant was too faint for RFLP analysis. The results for symptom expression, PCR assays and microscopy examination are summarised in Table 4. 2.

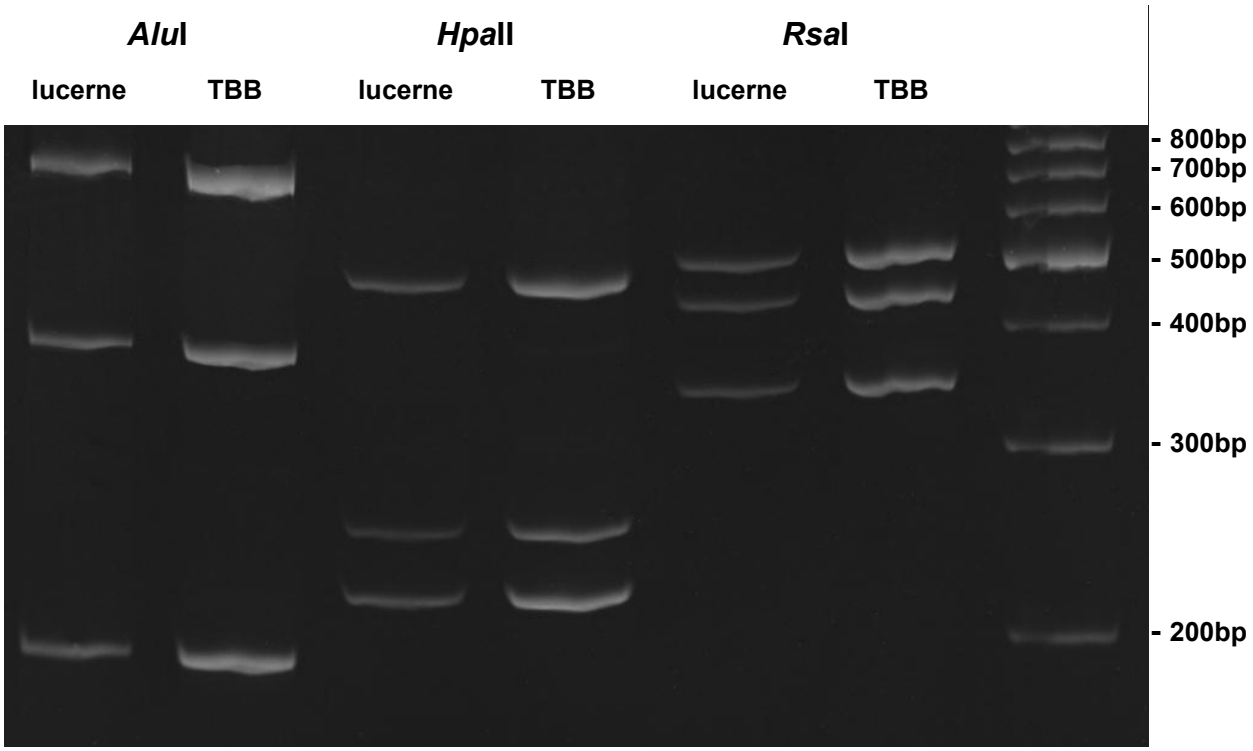

Figure 4. 1 Polyacrylamide gel of RFLP digestions of amplified product from lucerne plant fed on by *Austroagallia torrida* ("lucerne") and tomato big bud positive control ("TBB") using digestions enzymes *Alu*I, *Hpa*II and *Rsa*I.

O. argentatus feeding trial and phytoplasma assays

One of the eight plants fed on by *O. argentatus* showed no symptoms. Of the remainder, two plants showed some stunting of leaves, limited to a few shoots. Three plants showed more extensive stunting of leaves and shoots with a further two plants showing signs of yellowing. One plant, showing severe stunting and yellowing of its foliage, had a dark discolouration immediately under the periderm of its tap-root (Figure 4. 2). This

Table 4. 2 Summary table of evidence from transmission tests with leafhopper species *B. angustatus*.

	ALuY Symptoms		Molecular		Microscopy
Plant[a]	Foliar[b]	Roots[c]	PCR[d]	RFLP[e]	EM[f]
10	- (Root rot)	-	-	-	-
11	-	-	+	Inconc.	-
12	-	-	-	-	-
13	-	-	-	-	-
14	-	-	-	-	-
15	-	-	-	-	-
16	+ (Slight stunting)	-	-	-	-
17	+ (Slight stunting)	-	-	-	-

[a] Healthy lucerne plant exposed to feeding by *B. angustatus*
[b] Indicates what foliar symptoms were expressed by transmission test plant after being exposed to feeding by *B. angustatus*
[c] Indicates what root symptoms were expressed by transmission test plant after being exposed to feeding by *B. angustatus*
[d] Indicates whether phytoplasma DNA was amplified from transmission test plant after being exposed to feeding by *B. angustatus*
[e] Indicates characterisation of any PCR product produced by PCR assays of DNA extracted from transmission test plant after being exposed to feeding by *B. angustatus*
[f] Indicates whether phytoplasma bodies were visualised by TEM examination in tissue from transmission test plant after being exposed to feeding by *B. angustatus*

discolouration did not extend into the stele of the tap-root typical of bacterial wilt, caused by *Clavibacter michiganensis* subsp. *insidiosus* (McCulloch), and was consistent with root symptoms expressed by lucerne plants with ALuY disease (Chapter Two). Another plant, with some minor stunting of leaves, had a light discoloration under the periderm of its tap-root. This discolouration extended slightly into the stele of the tap-root. All plants were phytoplasma negative by PCR except for one of the plants with leaf stunting but without root symptoms. None of the leafhoppers were phytoplasma positive by PCR. The PCR product from the stunted plant was too faint for RFLP analysis. The results for symptom expression, PCR assays and microscopy examination are summarised in Table 4. 3.

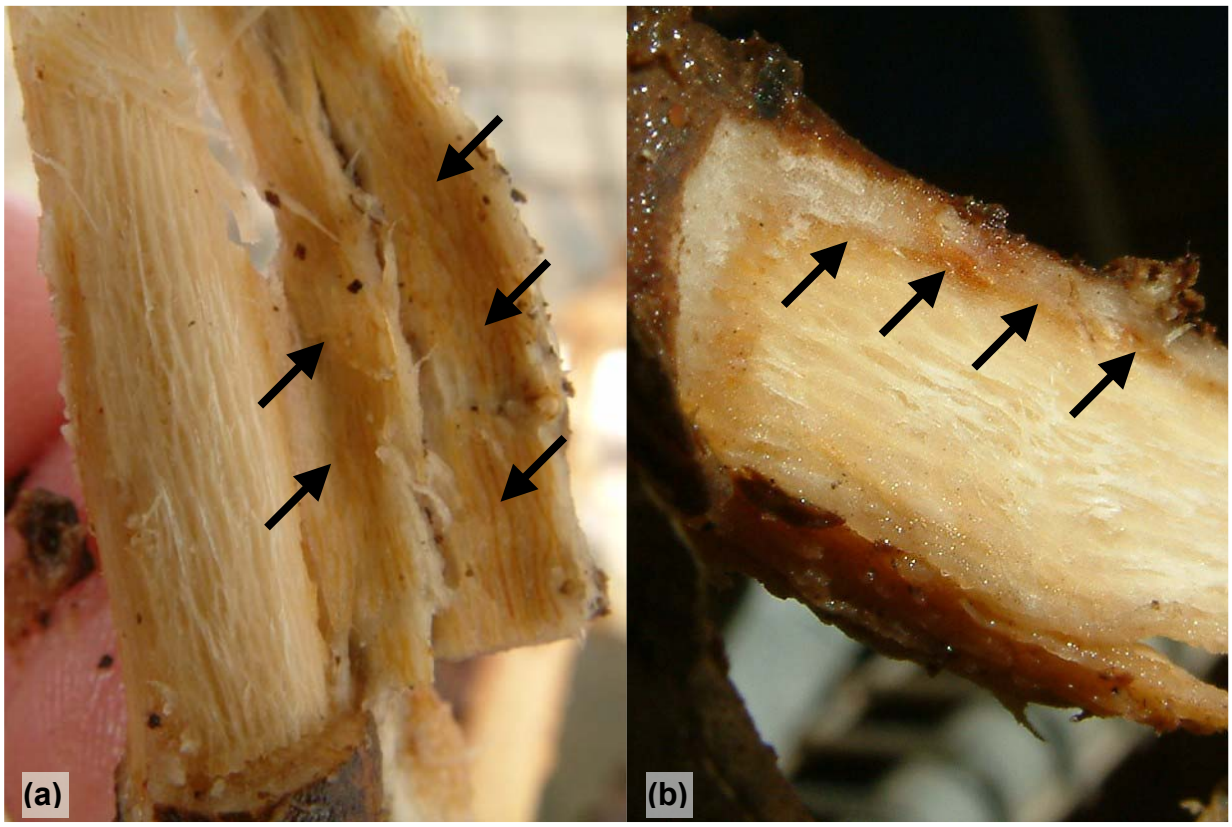

Figure 4. 2 Dark discolouration of the root periderm of a lucerne plant exposed to *O. argentatus*.

Table 4. 3 Summary table of evidence from transmission tests with leafhopper species *O. argentatus*.

	ALuY Symptoms		Molecular		Microscopy
Plant[a]	Foliar[b]	Roots[c]	PCR[d]	RFLP[e]	EM[f]
18	-	-	-	-	-
19	+ (Leaf stunting)	-	+	Inconc.	+
20	+ (Leaf stunting and yellowing)	-	-	-	-
21	+ (Leaf stunting and yellowing)	+ (Discolouration under periderm)	-	-	+
22	+ (Stunting of leaves)	-	-	-	-
23	+ (Stunting of leaves)	-	-	-	-
24	+ (Some stunting of leaves)	-	-	-	-
25	+ (Some stunting of leaves)	+ (Slight discolouration under periderm, extends into stele)	-	-	-

[a] Healthy lucerne plant exposed to feeding by *O. argentatus*
[b] Indicates what foliar symptoms were expressed by transmission test plant after being exposed to feeding by *O. argentatus*
[c] Indicates what root symptoms were expressed by transmission test plant after being exposed to feeding by *O. argentatus*
[d] Indicates whether phytoplasma DNA was amplified from transmission test plant after being exposed to feeding by *O. argentatus*
[e] Indicates characterisation of any PCR product produced by PCR assays of DNA extracted from transmission test plant after being exposed to feeding by *O. argentatus*
[f] Indicates whether phytoplasma bodies were visualised by TEM examination in tissue from transmission test plant after being exposed to feeding by *O. argentatus*

PCR assays of field-collected leafhoppers

No phytoplasmas were detected in leafhoppers collected from the field.

Microscopy

Ultra-thin cross-sections of leaf mid-ribs from each transmission test plant were examined and phytoplasmas (200-400nm diameter) were observed in the phloem of two plants fed on by *O. argentatus.* One of these was phytoplasma positive in PCR assays and the other displayed distinct ALuY root symptoms but which was phytoplasma negative in PCR assays. Phytoplasmas were also observed in the lucerne plant fed on by *A. torrida* that was TBB phytoplasma positive in PCR and RFLP assays (Figure 4. 3). The structures were consistent with those found in lucerne plants affected with ALuY disease (Chapter Two). No phytoplasma bodies were observed in other plants.

No fluorescence associated with phytoplasma structures were visualised by fluorescence microscopy in plant or insect tissue.

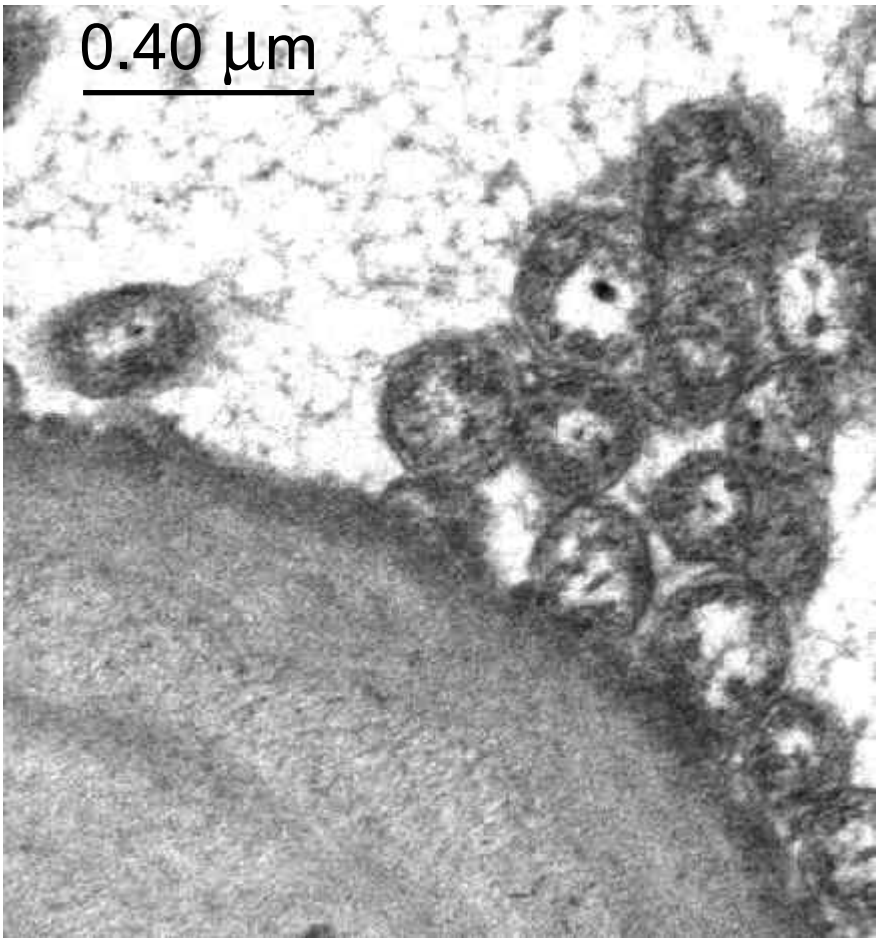

Figure 4. 3 Phytoplasmas observed in a phloem cell from a symptomatic lucerne plant fed on by *O. argentatus* in transmission tests.

Discussion

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

Lucerne plants that had been fed on by *B. angustatus* showed little or no symptoms, six plants being symptomless and the remaining two plants showing very slight stunting on some shoots and leaflets. There were no root symptoms to indicate ALuY disease. Phytoplasma DNA was amplified from one of these symptomless plants by PCR but the product was too faint for RFLP analysis. Attempts to reamplify the product for RFLP failed. *B. angustatus* insects used in the feeding trials had been collected from the field and, therefore, they may have carried a range of phytoplasma pathogens. Without confirming the identity of the phytoplasma amplified in PCR assays using RFLP analysis, and no supporting implication of *B. angustatus* as a vector of any phytoplasma, little information may be inferred from the positive PCR result.

Colony bred *O. argentatus* were given access to an ALuY plant and then to a healthy lucerne plant, the latter of which tested phytoplasma positive by PCR. Although the phytoplasma was not identified, it is likely that the *O. argentatus* acquired the phytoplasma from the ALuY plant because few vectors of phytoplasmas have been shown to be capable of transovarial transmission (Chiykowski 1981) and as a result they are not capable of transmitting to the next generation of the insect vector (Kawakita *et al.* 2000). *O. argentatus* were laboratory-reared for more than 20 generations on plants known not to contain phytoplasmas and, therefore, it is unlikely that they carried pathogens other than those acquired from ALuY symptomatic plants.

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

Fluorescence microscopy on insect tissue failed to identify any bodies that could be associated with phytoplasmas. There was a great deal of

background fluorescence in each insect and it was impossible to differentiate between the insect and any fluorescence that may have come from external bodies. Fluorescence microscopy on plant tissue, similarly, showed a great deal of background fluorescence. In some samples there was fluorescence evident in phloem tissue but not consistent with fluorescence of phytoplasma bodies.

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

As reflected in the present results, rates of symptom expression in test plants are often reported to be low. The vector of pear decline phytoplasma caused symptoms in 17 out of 56 test plants (30%) and was considered to be a ‘highly active vector’ (Carraro *et al.* 2001a), and Jarausch *et al*. (2001) found less

than 1% (one of 50 test plants) transmission success in trials between *C. pruni* (Scopoli) and apricot seedlings infected with European stone fruit yellows phytoplasma. A 14-18% (four to five of 25 test plants) rate of successful transmission of symptoms was demonstrated in trials involving *Nephotettix virescens* (Distant) and rice yellow dwarf transmission (Rajappan *et al.* 1999) and a successful transmission rate of 3.5% (three of 88 test plants) was considered evidence for the leafhopper *Oncopsis alni* (Schrank) to be the vector of grapevine yellows symptoms (Maixner *et al.* 2000).

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

The RFLP assay conducted on the PCR product obtained from an *A. torrida* fed plant was identical to that obtained for the TBB positive controls used in

the assay. That plant exhibited severe stunting and die-back. Though these symptoms are not typical of TBB in lucerne, symptoms for a given phytoplasma can vary widely within, as well as between, host plant species (Wilson *et al.* 2001).

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

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

clearly symptomatic plants not yielding positive results in PCR tests (Bertaccini *et al.* 1997) as may have been the case in the present study.

PCR products were not amplified from DNA extracted from the heads and thoraxes of leafhoppers used in transmission tests or from leafhoppers collected from the field. This is the first time that an attempt has been made to amplify phytoplasma DNA from only the heads and thoraxes of leafhoppers, effectively isolating the area of the leafhopper that contains the salivary glands and excluding the gut. Phytoplasmas are found in all body parts of infective leafhoppers (Chiykowski 1979), though false positives resulting from amplification of residual phytoplasma DNA remaining in the gut after feeding on infected material is possible. Sampling a small section of the individual insect, however, compounds the inherent problem of low concentration of phytoplasma DNA within leafhoppers.

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

The results presented in this study are supported by spatial and temporal survey data of the three leafhopper species examined in the current study that were found to be common in lucerne stands and margins (Chapter Four). Further, herbicide and insecticide treatments of non-crop vegetation adjacent

to lucerne stands resulted in statistically significant reductions in the overall movement of *O. argentatus* and herbicide treatments resulted in statistically significant reductions in the overall movement of *A. torrida* (Chapter Three). Insecticide treatment of the non-crop vegetation successfully lowered ALuY disease incidence adjacent to the treatment (Chapter Three). These findings support the vector status results presented in this study and offer scope for developing disease management strategies in future studies.

This study provides evidence that *O. argentatus* is a vector for the ALuY pathogen and *A. torrida* has the ability to transmit the TBB phytoplasma. This is the first report of the vector status of these two leafhoppers with two important Australian phytoplasma pathogens. Further work was considered necessary to establish and test management strategies for ALuY and this is presented in Chapter Five.

Chapter Five – Management of Australian lucerne yellows disease by water, nutrient and antibiotic

Introduction

Management strategies for plant diseases range from preventative measures prior to sowing to the drastic destruction of an entire crop to prevent further spread into other paddocks (Chand *et al.* 1987). The management options for lucerne diseases vary widely. These range from practices such as the selection of free-draining sites and efficient irrigation throughout the growing season for the reduction of Phytophthora root rot symptoms to the use of resistant varieties of lucerne for Anthracnose crown rot and stem nematode control and cutting or grazing to reduce inoculum in infected material for diseases such as common leaf spot, rust and pepper spot (McDonald *et al.* 2003).

More generally, the application of supplementary water, for example in the management of Leucostoma canker of stone fruit trees caused by either of the two fungal pathogens *Leucostoma persoonii* Hohn or *Leucostoma cincta* (Fr. ex Fr.) Hohn, increases the vigour of the host plant, often decreasing symptom severity (Agrios 1997). In many plant disease systems, infection does not lead to the death of the plant, but to an overall qualitative or quantitative reduction in yield (Jones 1987). Australian lucerne yellows (ALuY) at times tends to lead to reduced vigour in plants rather than death (Stovold 1981, Chapter Two). Alleviation of symptoms would be a highly desirable outcome for ALuY disease management.

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

Tetracycline compounds inhibit the activity of phytoplasmas (Bowyer & Atherton 1972) and was first demonstrated with the suppression of phytoplasma symptoms of mulberry dwarf disease in mulberry (*Mulberry* spp.) plants (Ishiie *et al.* 1967). The use of such compounds inhibits the pathogen and often results in a marked increase of vigour in the host plant (McCoy 1982). Application of tetracycline compounds several times a week, as was the case in the study by McCoy (1982), is likely to be uneconomical and impracticable in lucerne production, but a less frequent application regime may give economic control and so merits experimental evaluation for ALuY.

No current advice for the management of ALuY is available to growers and this has resulted in destructive practices such as ploughing-in the infected crop and using a crop rotation or insecticides on the (previously untested) assumption that insects vector the pathogen (Pilkington *et al.* 1999, McDonald *et al.* 2003). This situation requires studies of symptom alleviation or

suppression of the pathogen to improve plant health and increase seed yield for producers.

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

Materials and methods

Study Site

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

Experimental design

One hundred ALuY-symptomatic plants were selected in December 2001 based on foliar symptoms rather than destructive root inspections. Test plants were selected to fit a randomised plot design with two plants per plot in a split plot design, the split based on subsequent sampling date. Each plant was marked with a 2 m-tall, 5 mm-diameter fibreglass pole driven into the ground within five centimetres to the north of the plant crown. Individual plants were at least 20 m apart and were selected for uniformity of symptom expression with a score of three on a scale of one to five (Chapter Three, Table 3. 1). Soil type, drainage and irrigation patterns were consistent across the study area.

Treatments

Treatments were (i) nil control, (ii) 3 L water applied once a fortnight, six applications in total, and a woven synthetic fabric mulch mat (Mulch Mat Products Pty Ltd, Australia) placed around the base of the lucerne plant to reduce evaporation, (iii) 20 g of prilled sulphate of potash (Incitec Pivot Limited, Melbourne, Australia) applied to 1 m^2 around the plant and watered into the soil with three litres of water on one occasion at the outset of the experiment, (iv) 30 g of prilled MultiGro (Incitec Pivot Limited, Melbourne,

Australia), N:P:K 10:3:6, applied to 1 m^2 around the plant and watered into the soil with three litres of water on one occasion at the outset of the experiment, and (v) 0.1 L of 0.1 g/L tetracycline hydrochloride (Sigma Aldrich, Castle Hill, Australia) antibiotic applied using a hand-held sprayer to deliver a fine mist to all foliar parts of the plant once a fortnight, six applications in total.

The watering regime, which equates to six applications of 0.03 ML/ha, supplemented the grower's irrigation that occurred in late December 2001 and late February 2002 at the rate of 1 ML/ha on each occasion and rainfall that totalled 2 mm (0.02 ML/ha) in December 2001 and 108 mm (1.08 ML/ha) in February 2002. No rainfall or irrigation occurred in January 2002.

Measurements

One plant per plot (50 plants in total) was destructively sampled on 10 February 2002, 12 weeks after initial application of treatments. Remaining plants were sampled on 25 February 2002, two days before the host farmer harvested seed from the entire stand in which the experiment was located. Immediately prior to each destructive sampling, symptom severity of individual plants was visually assessed on a scale of one to five. Shoots from individual plants were then cut just above the crown of the foliage and placed in a labelled brown paper bag for transport to the laboratory. Fresh weight was recorded immediately then plants were dried to constant weight in a dehydrator at 55°C. Seeds from the second batch of plants were extracted using a small threshing machine, plant debris was removed and the seed yield recorded.

The germination rate of the collected seed was measured following protocols developed by the International Seed Testing Association (Anonymous 1993). Thick paper towel was placed on the bases of shallow stainless steel trays. Seeds from each plant (100 seeds in total) were placed on the paper towel and covered with another layer of paper towel. 250ml of distilled water was slowly added to each tray, which was then covered with a glass plate and held at 5°C for 7 days. The trays were then removed to an incubator at 20°C. Germination was assessed after 10 days.

Analysis of variance (ANOVA) using Genstat 6th edition statistical software package (GenStat Committee) was used to test for effects of treatments following a logarithmic transformation on fresh weight ($\log_{10}$ (x+0.5)), dry weight ($\log_{10}$ (x+0.5)) and seed weight ($\log_{10}$ (x+0.01)). Data for parameters assessed on both the first and second collection dates were analysed using a split-plot ANOVA. An example of the output from this style of statistical analysis is presented in Appendix Three. There was no statistical difference evident between dates so treatment means, only, are shown and discussed. Data for parameters collected on only one date were analysed using a one-way ANOVA in randomised blocks. The seed germination rate data was assessed for appropriateness of square root and angular transformations and transformation was found to be unnecessary (Snedecor & Cochran 1989). Because of the ordinal nature of the symptom expression data, a model for ordinal responses was used (McCullagh & Nelder 1989) on untransformed data. For all variates, treatments were individually compared with the control

to test the *a priori* hypothesis that each treatment would increase plant health compared with the control.

Results

There was no statistically significant treatment effect on fresh weights or dry weights, though the effect of the potash treatment on the latter fell close to the threshold for statistical significance (P = 0.098). Other treatments numerically increased yields compared with the nil control with the exception of antibiotic effect on dry weight.

The water application treatment significantly (P = 0.040) increased seed yield compared with the control. No statistically significant differences were found for the remaining treatments, though all numerically increased seed weight compared with the nil control (Figure 5. 1).

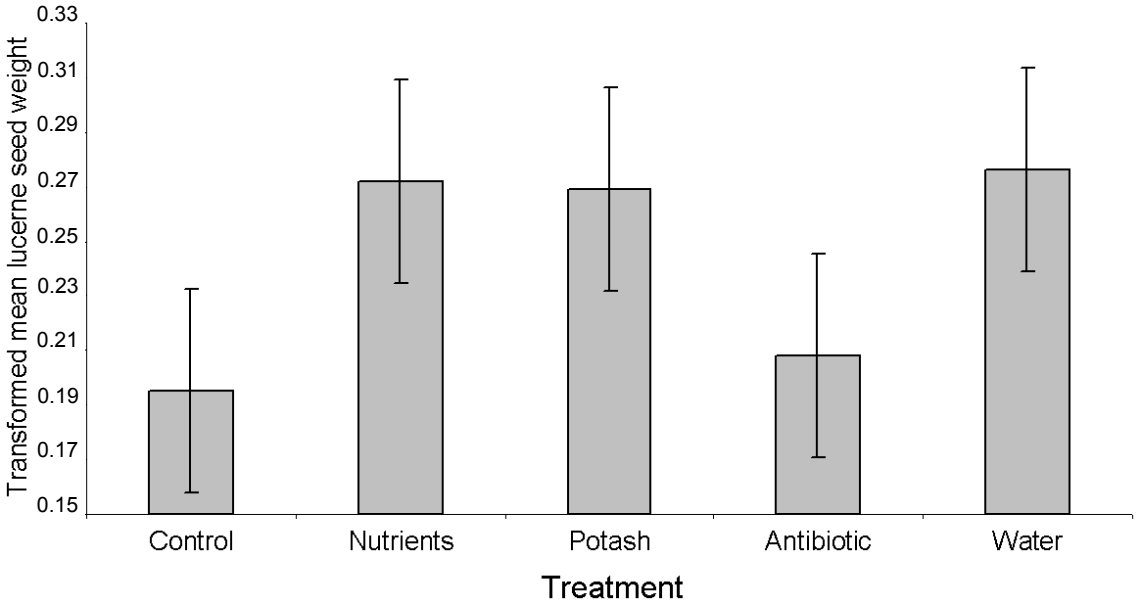

Figure 5. 1 Effect of treatments on seed yield of ALuY infected lucerne. Error bars indicate standard error of means.

There were no statistically significant treatment effects on seed germination rate, though nutrients and antibiotic treatments numerically increased the seed germination rate compared with the nil control.

There were no statistically significant treatment effects on symptom severity, though all treatments numerically reduced the severity of symptoms compared with the nil control.

Discussion

The results obtained in this study suggest sufficient scope to manage ALuY disease via symptom alleviation or antibiotic treatment to merit future studies. Such studies should test treatments on a range of sites and over several years. Dose-response studies are required before recommendations can be made to lucerne growers. The effect of supplementary water on seed yield was the only statistically significant response, though all treatments led to a numerical increase in all or most of the measures of plant health.

Seed yields in lucerne are maximised when soil moisture is adequate to prevent severe moisture stress, but reduced when watering promotes excessive vegetative growth (Rincker *et al.* 1988). Total seed yield is highly dependant on optimal water availability and under or over supply of water can lead to significant seed yield losses (Fick *et al.* 1988), the timing of irrigation having a large impact on seed production (Rincker *et al.* 1988). A statistically significant (P=0.040) increase in the seed weight was achieved with supplementary water treatment that was volumetrically modest compared with the grower's irrigation and rainfall. The increase in seed yield achieved with supplemental water application in this study needs to be interpreted with a degree of caution. An increase in seed yield may have occurred with disease-free plants, so increased seed yield from symptomatic plants may not have been directly associated with alleviation of symptoms though a numerical improvement in symptom severity was observed for the water treatment compared with the nil control. The effect of water treatment does, however, suggest that refined irrigation management may form part of a successful

management strategy for ALuY whereby, irrespective of the mechanism, the grower's yield of seed is improved.

Applications of nutrients gave the numerically highest increase for fresh weight, dry weight and seed germination rate when compared with the nil control. Potash treatment also numerically increased fresh weight, dry weight and numerically lowered symptom severity when compared with the control. The increase of fresh weight was only marginally outside 95% confidence limits. This increase in yield could, like supplementary water applications, be directly related to increased plant health and not in fact a result of an interaction with disease symptoms. Further examination using higher rates of nutrient may reduce foliar symptoms to a useful extent. Future research will need to take account of the nutrient status of the soil because if the elements used in nutrient treatments are plentiful in the soil, the likelihood of a response is diminished. Further work should also test the effect of foliar-applied nutrients as it is not known whether ALuY interferes with nutrient uptake and translocation.

Tetracycline treatment had no effect on growth parameters, though it is known that plant response to tetracycline compounds varies according to plant age, symptom severity and method of treatment (McCoy 1982). Coconut palms (*Cocos nucifera* L.), for example, showed a marked decrease in symptom expression in early stages of lethal yellows infection and failed to improve when the disease was at a more advanced stage (Cha & Tattar 1993). Plants in the present study were selected on the basis of moderate to severe

symptom severity and this factor may explain the lack of effect. Commencing treatment earlier in the season when plants are less severely affected should be tested in future studies.

The type of tetracycline antibiotic used may affect the level of symptom alleviation achieved (McCoy 1982). Tetracycline hydrochloride has been used with varying degrees of success in many studies on phytoplasma symptom alleviation. Bowyer and Atherton (Bowyer & Atherton 1972) showed that applications of tetracycline hydrochloride in a 100 μg/mL foliar spray resulted in eight out of eight *Nicotinia glutinosa* L. plants showing symptoms of infection with legume little (LL) phytoplasma recovered. Hunt *et al*. (Hunt *et al.* 1974) showed that out of 12 coconut palms, *Cocos nucifera* L., showing symptoms of lethal-yellowing, five recovered, five showed delayed symptoms and two had no remission of symptoms at all. Applications of 100 mg/L tetracycline hydrochloride to coconut palms resulted in no remission of symptoms whereas higher rates of application resulted in 32 of 37 palms infected with kainkope disease, a phytoplasma disease similar to lethal yellowing, showed a full recovery (Steiner 1976). Variations in the efficiency of tetracycline hydrochloride in the remission of phytoplasma symptoms vary between and within plant systems and phytoplasma pathogens. Other types of tetracycline antibiotics have been used in other studies such as chlortetracycline hydrochloride (Bowyer & Atherton 1972), oxytetracyline hydrochloride (Giunchedi & Pollini 1986), doxycycline compounds (Ali *et al.* 1987) and demeclocycline hydrochloride (Varma *et al.* 1975). Applications of tetracyclines should have been tested prior to the commencement of this

major study. Further studies of application rates and type of antibiotic are warranted.

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

Tetracyclines are used extensively to retard symptom expression, but these cases are confined to woody plant systems such as lethal yellowing in coconut palm (Steiner 1976, Maramorosch 1999) where application is by trunk injection. Symptom alleviation of ALuY disease by foliar applications of antibiotic is unlikely to be a viable disease management strategy because broad acre spraying of compounds of this type, especially in the lucerne system which includes livestock grazing, will not be acceptable to growers, consumers or regulators.

The fact that this experiment did not include ALuY-free control plants was the result of financial and practical constraints. Future studies of ALuY symptom alleviation should include such controls. This would allow the effects of

treatments on the disease process to be quantified in relation to the direct effects of treatments on plant physiology.

The present study constitutes only a preliminary appraisal of ALuY disease management treatments and suggests that further study using more complex methodologies is warranted. All results discussed in this chapter must be viewed with an extremely high level of caution. Numerical increases in any of the measured factors of plant health are in no way indicative of what may be a successful treatment but they do indicate where future studies, beyond the scope of this PhD, may be concentrated to maximise the chances of success. Though there appears to be some scope for reducing the impact of the disease by cultural practices such as optimising plant nutrition and water availability, especially with respect to the key economic parameter of seed yield, control of infection by reducing vector immigration needs to be considered as part of an integrated disease management strategy.

Chapter Six – General discussion

Australian lucerne yellows (ALuY) is an important disease that has had a large effect on the Australian lucerne seed industry since the middle of last century. Previous work (Pilkington *et al.* 1999, Appendix One) has shown that the disease has a significant economic impact on the Australian lucerne seed industry yet, prior to the present investigation, very little was known about the etiology of ALuY disease. Gaps in knowledge regarding the pathogen, vector and management strategies are evident in the scant literature regarding ALuY disease.

Studies of all aspects of a disease serve the practical purpose of assisting in the development of rational and effective management strategies for a disease (Agrios 1997). Identifying and characterising the pathogen associated with a disease is crucial in identifying potential insect vectors, particularly with phytoplasma pathogens, as they are often highly vector-specific (Davis *et al.* 1997). Taxonomy of phytoplasmas was previously determined using symptomology, host range, geographic area in which they occurred and the vector associated with the pathogen (Schneider *et al.* 1997) and now integrates the use of molecular biology (Seemüller *et al.* 1994). There is no indication in the literature that the ALuY phytoplasma has previously been characterised at any level. This study addresses this knowledge gap in Chapter Two. The spatial and temporal appearance of symptoms and their relationship with vectors is part of this understanding (Lindblad & Areno 2002) as is the disease-vector relationship itself (Arnò *et al.*

1993, Madden *et al.* 1995). Patterns of symptom expression can provide valuable information that underpins effective control strategies. This includes information about the time between inoculation and symptom expression (Guthrie *et al.* 1998) and the therapeutic effects of some compounds such as tetracycline antibiotics (Bindra *et al.* 1972).

The following sections explore the contribution of each piece of work to the overall understanding of the ALuY disease system including the pathogen, the vectors and possible management strategies.

Detection and identification of the ALuY phytoplasma

The first phase of the work, Chapter Two, aimed to identify and characterise the pathogen responsible for ALuY disease. Hellemere (1972) eliminated bacterial wilt and nutrient disorders as possible causes for ALuY and suggested a mycoplasma-like organism, now known as a phytoplasma, as the causal agent. McGechan and Stovold (1976) built on these studies and, with the use of electron microscopy, further implicated mycoplasma-like organisms as the causal agents. Literature on ALuY since the mid 1970s is sparse and only a few reports on disease incidence and severity are available to add to the understanding of this disease (Anonymous 1975). Since then, advice given regarding disease management (Stovold 1981, Stovold 1983, McDonald *et al.* 2003) has often been inappropriate, as is detailed in Chapter One within the *Management of phytoplasma diseases* section.

Association of a phytoplasma pathogen with ALuY disease allowed the study to move onto further work, as reviewed in following sections, to determine the possible vectors of the disease. Identifying the pathogen associated with a disease is important in narrowing the field of possible vectors. Based on the *a priori* understanding that phytoplasmas are transmitted exclusively by leafhoppers, planthoppers and psyllids (Tsai 1979, Ploaie 1981), other vectors such as aphids, often associated with the transmission of virus diseases (Irwin & Goodman 1981), could be excluded from further investigation.

This study has succeeded in identifying the pathogen responsible for ALuY disease, confirming early work suggesting a phytoplasma etiology. The possibility of viral, fungal or bacterial pathogens was ruled out with the use of symptomology and traditional plant pathology techniques and the amplification of phytoplasma DNA in PCR assays was confirmed with TEM. Once the pathogen was successfully detected in plants affected by ALuY, DNA sequencing of the phytoplasma intergenic spacer region was undertaken and showed that the phytoplasma is a novel species, possibly endemic to Australia.

The work detailed in Chapter Two of this thesis identified and addressed a fundamental gap in knowledge for this disease and is a significant contribution to science and lucerne agronomy.

Identifying the vector of ALuY

Fletcher (1980) conducted studies on leafhoppers associated with lucerne in Australia and suggested the leafhopper species *Orosius argentatus*, *Batracomorphus angustatus* and *Austroagallia torrida* as possible vectors of the disease. Further studies such as transmission tests were suggested at the time though not implemented. These three leafhopper species were used in subsequent studies of ALuY disease/vector relationships.

Spatio-temporal distribution of ALuY disease and suspected insect vectors

Chapter Three aimed to identify the leafhopper species responsible for transmitting the ALuY disease by looking for spatio-temporal patterns in insect populations and disease severity in several lucerne seed stands in New South Wales, Australia. Results for this phase of the work are summarised in Table 6. 1.

Statistically significant spatial patterns for ALuY disease symptoms were evident such that symptoms were more severe in the stand margins. This suggested that the disease may be being introduced to the crop system by insects that move into the crop via short-range flight from adjacent non-crop vegetation. This also suggests that the disease is not seed-borne, as might be suggested by uniformity of symptom severity throughout the stand (Jones 1987). A preliminary molecular study was conducted to test the hypothesis that the disease may be seed borne (Appendix Two). Results did not support the hypothesis. This phase of the work was also valuable in that it identified

Table 6. 1 Table of evidence for spatio-temporal and border treatment experiments

	Distribution			Border experiment	
Leafhopper species	Spatial[a]	Margin[b]	Temporal[c]	Catch[d]	ALuY incidence[e]
O. argentatus	X	X	✓	✓	✓
		Just outside 5% confidence limits	Symptom distribution matches insect distribution +9 days	Insecticide and herbicide lowered catch	Insecticide reduced numbers of symptomatic plants
B. angustatus	✓	✓	✓	O	O
	Statistically significant patterns detected on three dates	One 3D edge effect and two 2D edge effects	Symptom distribution matched insect distribution +54 days	Numbers too low to assess effect	Numbers too low to assess effect
A. torrida	✓	✓	X	✓	✓
	Statistically significant patterns detected on four dates	Four 3D edge effects	Symptoms prior to insect pattern	Insecticide and herbicide lowered catch	Insecticide reduced numbers of symptomatic plants

[a] Indicates whether a statistically significant spatial pattern was detected in the leafhopper distribution
[b] Indicates whether leafhopper numbers were more prevalent in sections of the crop margins to a statistically significant level
[c] Indicates whether spatial distribution of leafhoppers correlated to a statistically significant level with spatial distribution of ALuY symptom expression
[d] Indicates whether border treatments had a statistically significant effect on leafhopper catches
[e] Indicates whether border treatments lowed ALuY disease severity to statistically significant levels

that ALuY disease can have a significant impact on a lucerne crop within 32 weeks from sowing, a major contribution to understanding the disease.

The suspected leafhopper vectors, *A. torrida* and *B. angustatus*, were monitored for 12 months and shown to have a statistically significant spatial pattern within the crop. The third suspected leafhopper vector, *O. argentatus*, showed no such spatial pattern within the lucerne stands but demonstrated an edge effect that fell marginally outside 95% confidence limits. In addition to *A. torrida* and *B. angustatus* having a spatial pattern, their densities were concentrated in the sections of the stand margins, suggesting that the leafhoppers were predisposed to short-range dispersal. This was shown in spatial examination of leafhopper populations and supported by the trap data collected in the border treatment phase of this study. Such a short-range dispersal characteristic of the leafhoppers is consistent with patterns demonstrated in ALuY disease incidence.

Significant correlations were demonstrated between spatial patterns observed in insect distribution and ALuY disease incidence patterns. A spatial distribution of *O. argentatus* was correlated, nine days later, with spatial patterns of ALuY disease symptoms at one site. At another site, the spatial distribution of *B. angustatus* was correlated, 54 days later, with spatial patterns of ALuY disease. *A. torrida* had a significant correlation between its spatial distribution and the distribution of ALuY symptoms that occurred prior to the spatial pattern of the leafhopper. The significance of these correlations is discussed in greater depth in Chapter Three.

These findings do not, in isolation, rule out any of the three leafhopper species as the vectors of ALuY disease so all three species were included in subsequent transmission experiments. The results suggested that further work needed to be undertaken to explore the relationship of these insects with the disease and also that, because of their short-range dispersal, further work needed to be concentrated on the boundary of the crop rather than the entire stand.

Border treatment studies

Findings from this phase of the study were not conclusive, though an important step in the rigorous testing of the hypothesis that these insects are possible vectors of ALuY. Results are summarised and presented in Table 6.1. The short-range dispersal of the leafhoppers, suggested from earlier results in the spatial analysis of leafhopper populations, was further examined by the trap-height effect discussed in Chapter Three. Greater numbers of leafhoppers were caught in lower traps than in higher traps suggesting a low-level flight pattern and short movements to the lucerne plants. Flight characteristics of *O. argentatus* have been examined between 0.38 m and 4.12 m (Hosking & Danthanarayana 1988) showing a significantly greater level of leafhopper movement at lower heights. This flight pattern is consistent with the observation that ALuY-symptomatic plants tend to be more common in the margins of lucerne stands. However, some *O. argentatus* individuals were caught at high levels, as well as close to the ground, indicating that movement is not restricted to short flight patterns though nevertheless more common. Such high-level flight evident for *O. argentatus*

could explain the appearance of ALuY symptoms in the interior of a lucerne stand. Alternatively, even short-range dispersal of infective vectors from the crop margin could lead to infection of lucerne plants in the crop interior if these insects moved in a series of short flights.

Herbicidal or insecticidal treatment of non-crop vegetation significantly reduced catches of leafhoppers on the crop margin/border vegetation interface and reduced the numbers of symptomatic lucerne plants immediately adjacent to the treatment plots. Distribution of disease symptoms is often directly related to the insect patterns adjacent to the crop. Weeds and non-crop vegetation often serve as a host for the pathogen and the insect vector (Groves *et al.* 2001). Stopping an insect vector from moving into the crop can reduce the disease severity in the crop. For example, preventing the movement of the lettuce necrotic yellows virus vector *Hyperomyzus lactucae* L. from its preferred host of *Sonchus oleraceus* L. (Boakye & Randles 1974) reduced the disease severity in lettuce (*Lactuca sativa* L.) (Randles 1986). The vector, *H. lactucae*, was found predominantly on sowthistle, *S. oleraceus*, and this understanding of the vector/host relationship has had important implications on the management of the lettuce necrotic yellows disease (Martin 1983) and may be a model that will work in lucerne crops for ALuY. Further work to successfully identify the vector responsible was considered necessary and in order to expand on these findings transmission tests were conducted.

The three leafhopper species were found on non-crop vegetation of *Polygonum aviculare* and *Heliotropium europaeum* with *A. torrida* and *O. argentatus* also being common on *Marrubium vulgare* (Chapter Three). The distribution of these weed species was not, however, found to be statistically correlated with the distribution of ALuY symptoms. Despite this lack of correlation a knowledge of the alternative plant hosts for these insects could lead to a better targeted pest management system in which these weed species are better managed. Lacy *et al*. (1979) described the association between high levels of peach X-disease, the possible insect vectors *Colladonus clitellarius* (Say) and *Scaphytopius acutus* (Say) and the reservoir *Prunus virginiana* Linnaeus. Management of that disease was discussed on the basis of controlling leafhopper populations or by removing non-crop vegetation that was assisting vector movement into the crop (Lacy *et al.* 1979).

The apparent importance of short-range dispersal of *O. argentatus* and *A. torrida*, only a few centimetres above the canopy, suggests that physical barriers such as shade mesh material mounted to existing fences may constitute effective barriers to leafhopper migration into the crop. Schaber *et al*. (1990) conducted studies showing that physical impediments to the movement of short-range insects were successful in limiting their numbers in crop systems. This method of insect population management should be tested as a priority in follow up work.

Transmission testing

The third section, Chapter Four, aimed to test the vector status of the three leafhopper species that were identified in earlier phases of the work to be possible ALuY vectors. PCR assays carried out on field-collected leafhoppers or those from transmission tests failed to amplify phytoplasma DNA. Further development of the technique used for the first time in this study of separating the heads and thoraxes of leafhoppers prior to DNA extraction needs to be explored in future studies.

Many studies have been conducted, such as with the possible vectors *Macrosteles quadripunctulatus* and *Euscelidius variegatus* of the phytoplasma disease chrysanthemum yellows (Palermo *et al.* 2001), examining the transmission efficiency of insect vectors from diseased plants to healthy plants. In controlled transmission testing for ALuY, and the three leafhopper species listed in previous sections, results obtained suggested *O. argentatus* is the most likely vector though *B. angustatus* cannot be ruled out as an additional vector. The third species, *A. torrida*, is considered unlikely to be a vector for ALuY but was shown to be capable of transmitting the TBB phytoplasma - a phytoplasma that causes witches' broom and phyllody in lucerne (Gibb, K. S. 2002, pers. comm., 26 Aug.) (Gibb *et al.* 2000). The results of transmission tests using each leafhopper species, detailed in Chapter Four, are summarised in Table 6. 2.

Table 6. 2 Table of evidence for transmission testing experiments

	Symptoms		Molecular (plant)		Molecular (insect)		Plant Microscopy		Insect microscopy	
Leafhopper species	Foliage[a]	Root[b]	PCR[c]	RFLP[d]	PCR[e]	RFLP[f]	Fluoro[h]	TEM[i]	Fluoro[j]	TEM[k]
O. argentatus	✓ 7/8 plants showed symptoms. 3 plants showed ALuY-type yellowing	✓ 1 plant showed clear symptoms. 1 plant showed faint symptoms	✓ 1/8 plants positive for a phytoplasma	O PCR product too faint for phytoplasma characterisation	X 0/8 batches	X	X 0/8 - no fluorescence typical of phytoplasma was visualised	✓ 2/8 plants had phytoplasma bodies visualised in plant tissue	X 0/3 (back-ground fluoresc.)	X Fixation failed
B. angustatus	X 2/8 showed symptoms atypical of ALuY	X 0/8	✓ 1/8 plants positive for a phytoplasma	O PCR product too faint for phytoplasma characterisation	X 0/9 batches	X	X 0/9 - no fluorescence typical of phytoplasma was visualised	✓ 0/9 No phytoplasma bodies were found in plant tissue	X 0/3 (back-ground fluoresc.)	X Fixation failed
A. torrida	✓ 5/9 showed phytoplasma symptoms, 2 of which showed ALuY-type yellowing.	X 0/9	✓ 1/9 plants positive for a phytoplasma	✓ TBB phytoplasma	X 0/9 batches	X	X 0/9 - no fluorescence typical of phytoplasma was visualised	✓ 1/9 plant had phytoplasma bodies visualised in plant tissue	X 0/3 (back-ground fluoresc.)	X Fixation failed

[a] Indicates the test plant developed foliar symptoms typical of phytoplasma disease after hosting the leafhopper
[b] Indicates the test plant developed root symptoms typical of ALuY disease after hosting the leafhopper
[c] Indicates the successful amplification of phytoplasma DNA from transmission test plants
[d] Indicates the characterisation of DNA amplified from transmission test plants
[e] Indicates the successful amplification of phytoplasma DNA from leafhoppers used in transmission tests
[f] Indicates the characterisation of DNA amplified from leafhoppers used in transmission tests
[h] Indicates the visualisation of phytoplasma bodies in plant tissue using fluorescence microscopy
[i] Indicates the visualisation of phytoplasma bodies in plant tissue using transmission electron microscopy
[j] Indicates the visualisation of phytoplasma bodies in insect tissue using fluorescence microscopy
[k] Indicates the visualisation of phytoplasma bodies in insect tissue using transmission electron microscopy

A. torrida - Tomato big bud (TBB) has been associated with the leafhopper vector *O. argentatus* (Grylls 1979) and literature implicating other leafhopper species was not available. The presence of TBB phytoplasma, combined with the foliar symptoms expressed by the plant, provides the first evidence that *A. torrida* can transmit the TBB phytoplasma. The results do not indicate that this leafhopper species is a vector of ALuY.

B. angustatus - Very few symptoms were expressed by test plants fed on by *B. angustatus*, though a faint positive was amplified during PCR assays from one plant used in this section of the study. This DNA could not be identified by RFLP assays as the PCR product obtained from this plant was too faint. Phytoplasma bodies were visualised within the phloem of a separate plant, confirming that it is possible that this leafhopper species is capable of transmitting a phytoplasma pathogen.

These results do not conclusively establish that *B. angustatus* is a vector for ALuY disease though it cannot be ruled out as a possible vector. The faint positive amplified in PCR assays is inconclusive due to the inability to identify the phytoplasma with RFLP. One could speculate that ALuY was present on the basis of the intensity of the band, as outlined in Chapter Four and the variation inherent in amplification of phytoplasma DNA (Bertaccini *et al.* 1996), being faint when compared to TBB phytoplasma amplifications, but this is impossible to confirm. Whilst its status as a vector for ALuY or TBB is impossible to ascertain from these results, *B. angustatus* should not be ruled out of further work in relation to its vector status.

O. argentatus - During transmission tests two of the plants expressing phytoplasma-like symptoms showed yellowing of the foliage that was consistent with ALuY infection. One of these plants clearly showed the characteristic discolouration of the tap-root that is a definitive symptom of ALuY (Stovold 1983, Chapter Two). One of the eight plants showing stunting of leaves, broadly consistent with phytoplasma infection, also yielded a faint product in PCR assays that was of the expected size for phytoplasma DNA. RFLP assays failed on this sample because of the small amount of amplified DNA and hence identification of the phytoplasma was not possible. During TEM examination, phytoplasma bodies were visualised within the phloem of this plant.

The development of ALuY root symptoms in a plant fed on by *O. argentatus* is a significant finding that implicates this leafhopper species as a vector of ALuY. The low rate of symptom development, detailed in greater depth in Chapter Four, is consistent with similar studies investigating leafhopper transmission such as one in ten test plants in a study by Jarausch *et al*. (2003).

Caution should be exercised when examining the results of this phase of the work. The transmission experiments involving *O. argentatus* differed slightly in design to that used for *A. torrida* and *B. angustatus*. The latter two leafhopper species were field-collected and the possibility exists that these insects may have carried other phytoplasma pathogens from affected plants. This possible infection with alternative phytoplasma pathogens may have

precluded them from acquiring the ALuY phytoplasma from diseased lucerne. This is, however, considered unlikely because multiple phytoplasma infections are common (Lee *et al.* 1995). Any risk of such an effect is minimised by having insect colonies cycle through several generations on healthy plants prior to giving them access to diseased plants. Since reports indicate that the chance of transovarial transmission is low (Dabek 1983), suspected vectors should be bred on healthy lucerne prior to giving them access to ALuY affected caged plants and this method should be used in future studies. Colonies of *O. argentatus* were considered to be clear of alternative phytoplasma pathogen infection, as this species was laboratory-reared on experimental plants that were frequently tested for phytoplasma infection.

Individuals of *O. argentatus* suffer a high rate of mortality and appear not to feed when caged on lucerne plants, suggesting that lucerne is not a preferred host for the leafhopper species. However, Alma *et al*. (2000) showed that *E. incisus* suffered similar mortality rates and yet was still capable of vectoring a phytoplasma pathogen in the apparent dead-end host cyclamen. Further, if *O. argentatus* is responsible for vectoring the disease, the plant system may also be a dead-end host for ALuY, the disease not being vectored from lucerne plant to lucerne plant but rather being introduced from an external plant host. Such a scenario would explain the tendency for ALuY infected plants to occur singly rather than in patches or foci as is typical of diseases where plants can infect a neighbouring conspecific.

The fact that lucerne plants affected by ALuY are found in isolation is consistent with a vector that transmits a phytoplasma while probing a non preferred host and that shows a high rate of movement to find a preferred food source. Finch and Collier (2000) discuss a theory whereby individual insects select appropriate host plants by probing and scrutinising the appropriateness of any green material as a host plant. This study by Finch and Collier (2000) demonstrated that insects are attracted to odorous molecules emitted by host plants that are often mixed with non-host emissions by the wind or flight of the insect. This confusion of these signals often led to insects not landing on their preferred host or not remaining on the plant to feed or oviposit. Such a scenario could apply to *O. argentatus* and lucerne, the individual insects landing on lucerne, probing the plant, finding it unpalatable and resuming flight in search of a more suitable host plant leading to isolated individuals infected with ALuY.

These results, as discussed in Chapter Four, suggest that the leafhopper *O. argentatus* is a vector for ALuY disease. This is a major contribution to the etiology of ALuY disease.

Disease management strategies for ALuY

This phase of the study, Chapter Five, attempted to identify methods by which lucerne growers could, in a tractable and affordable way, manage the ALuY disease through applications of additional water, potash, multi-nutrients or tetracycline antibiotics. The border treatment experiments, outlined in

Chapter Three, also aimed to identify treatments to the non-crop vegetation adjacent to the crop that would reduce ALuY disease incidence.

Field experimentation suggests limited scope for disease management via symptom suppression though supplementary water had some effect on seed yield of diseased plants. The modest volume of water application yielded statistically significant results in symptom alleviation. This suggests that, after further research into the effect of irrigation on ALuY symptom expression, modification of the agronomy of seed lucerne could have a significant impact in reducing the adverse affect of ALuY disease or at least could result in increased yield of seed from ALuY affected plants. Other treatments tested showed a numerical trend of alleviation of ALuY symptoms and further research of these treatments is warranted. Preliminary experiments should have been conducted to evaluate the effect of tertracyclines on ALuY symptoms. Further, true controls should have been used. Time and financial contrainsts limited the use of such controls although they could have been used at the expense of another treatment. The information inferred by these results would have been useful in explaining the failure of many of the treatments. Environmental conditions, soil tests and soil moisture may have all been used to great advantage in this study and should be utilised thoroughly in future examinations of this kind.

With the details on the edge effect apparent in ALuY symptom expression in lucerne stands, there is scope to continue trials of treatments to alleviate symptoms in small blocks of lucerne along the borders of stands rather than

with individual plants. Experiments of this nature could be done in second-season stands where the level of ALuY disease infection is established from the previous season and would allow use of larger plots.

Antibiotics can be used as a powerful diagnostic tool in associating diseases with phytoplasma infection (McCoy 1974). The failure of tetracycline applications to alleviate the symptoms of ALuY is discussed at depth in Chapter Five and considers factors such as plant age, health, type of tetracycline used and frequency of applications. Broad scale application of tetracyclines as a disease management strategy are not realistic because of environmental considerations and cost.

Conclusion

The importance of understanding a disease system is an accepted part of disease management (Agrios 1997). Major gaps have been identified in knowledge of ALuY disease and the formal identification and characterisation of the ALuY pathogen has been the most conclusive outcome of this study and constitutes a major contribution to the understanding of this important disease.

Another important facet of plant pathology is understanding the dynamics of disease transmission. Identifying one vector of ALuY, *O. argentatus*, and a possible additional vector, *B. angustatus*, is an important step towards a successful disease management strategy. The identification of *A. torrida* as a leafhopper with the ability to transmit the TBB phytoplasma is a significant

discovery that may have implications for phytoplasma disease research in lucerne and other cropping systems. This association may assist in the field management of the lucerne diseases witches' broom and phyllody, both associated with the TBB phytoplasma (Gibb *et al.* 2000). This is the first report of *A. torrida* being capable of transmitting the TBB phytoplasma.

The phase of study focusing on the management of ALuY disease was less conclusive but constitutes useful preliminary work. The study has left scope to investigate the benefits of some treatments, most notably water. Further, the reduction of disease severity immediately adjacent to non-crop vegetation that was treated with insecticide (and just outside 95% confidence limits with herbicide) suggests the possibility of significantly reducing the effects of ALuY disease by such border treatments. This reduction may be achieved with minimal use of pesticides by limiting applications to small strips of non-crop vegetation adjacent to lucerne stands. The alternative possibility, suggested by trap height results, of erecting physical barriers such as shade mesh on crop margins to limit the migration of the vectors also merits further work. Whilst the work on disease management reported herein does not offer a proven management strategy, scope for refining the treatments for which preliminary results have been obtained is shown.

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Appendices

Appendix One – Occurrence and severity of lucerne yellows disease in Australian lucerne seed crops

This paper is not part of the PhD study. Because it is a direct prelude to the project and was written by the PhD candidate and the supervisory team it is appropriate to include this work as an appendix to the major work.

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Abstract

Production of lucerne seed is one of the most important seed industries in Australia. Anecdotal reports suggested that the disease lucerne yellows was having a marked impact on the industry, but no firm data existed on its occurrence and severity. An interview-based survey obtained detailed information from eight seedhouse offices and a telephone-based survey equivalent data from 62 individual growers. Only two of the 36 growers surveyed in New South Wales (NSW) and four of the 26 growers from South Australia (SA) had not observed the disease in their crops. In one instance the incidence of lucerne yellows was reported to be as high as 50–75 percent of plants but the majority of interviewees estimated the incidence to be less than five percent. Responses from farmers in NSW did not indicate a significantly greater severity for the disease than was apparent in SA. The results from the seedhouses support those from individual growers in showing that lucerne yellows disease occurs widely in both States and that its severity is such that significant economic losses are likely.

Introduction

Lucerne is one of the most valuable pasture legumes in Australia (Anon. 1998). Because of its deep root system and perenniality, it is increasingly important in managing aspects of environmental degradation such as rising water tables and soil salinity. Lucerne yellows is one of several significant lucerne diseases in Australia (McDonald *et al*. 1995) and severe crop losses have been recorded (Anon. 1974; McGechan 1978). Depending on its incidence, it is likely to have a severe impact on seed production as infected plants rarely recover (Stovold 1983).

Though lucerne yellows has been recorded in Australia for at least 18 years (Stovold 1981), little is known about its biology and etiology. A leafhopper-transmitted phytoplasma (mycoplasma-like organism) is suspected to be the cause (McGechan 1980; Fletcher 1980). However, definitive confirmatory research, which would allow the development of a disease management strategy, has not been undertaken. Such research would be warranted only if the distribution and severity of the disease indicated that a current problem existed. Since such information was entirely lacking, complementary surveys were undertaken to obtain relevant information from seedhouses and individual growers in the two major lucerne seed-growing States.

Methods

Survey of lucerne seed growers Contact details for established lucerne seed growers in South Australia (SA) and New South Wales (NSW) were obtained via certification scheme records for each State, and a total of 83 provisionally agreed to participate. These growers were then sent a 'fact sheet' (Gurr *et al.* 1998) and a brief questionnaire. The 'fact sheet' sought to maximise the reliability of responses by including colour illustrations and providing, in flow chart form (Figure 1), a guide to identify the disease and distinguish it from similar lucerne diseases and disorders.

Growers were subsequently contacted by telephone and their responses to each of the questions noted. During this interview, growers were also asked about the basis for the diagnosis of lucerne yellows in their crops (i.e. foliar symptoms, root examination or confirmation by a plant pathologist) and whether they viewed the disease as a potential, actual or non-problem. Some growers also volunteered additional information, which was noted. Telephone feedback was obtained from 24 growers in SA and 27 in NSW. The remaining 32 growers were sent a letter explaining that difficulty had been experienced in reaching them by telephone and inviting them to return their completed questionnaire in a post-paid envelope. Subsequently, written

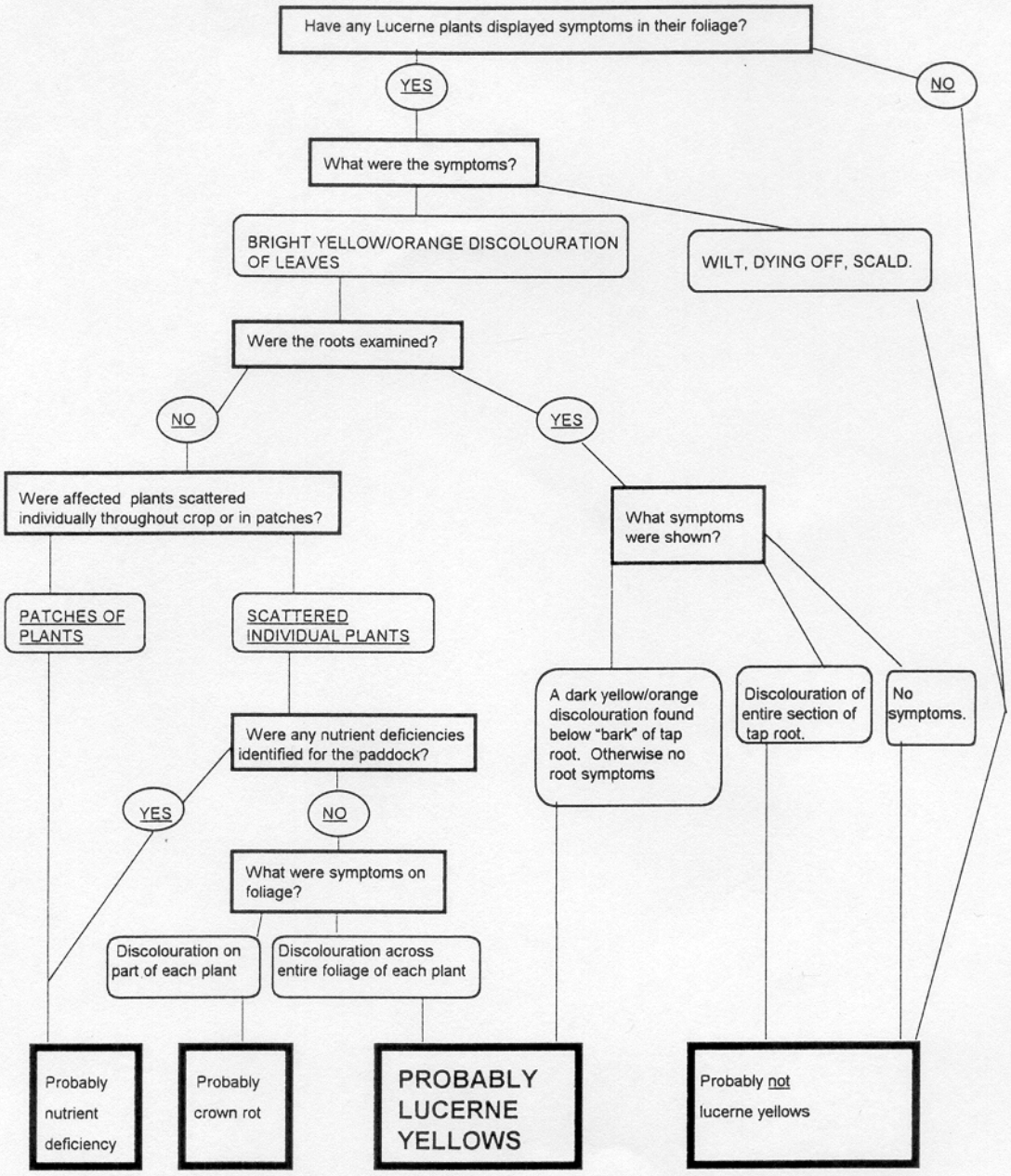

Figure 1 Guide to distinguishing lucerne yellows from similar lucerne diseases and disorders (as used in illustrated 'factsheet' for surveyed growers).

feedback was received from an additional nine NSW growers and two from SA.

Growers in NSW were located as far west as Condobolin, north to Nyngan and south to Cootamundra but the majority were in the commercially important Forbes/Eugowra district of the Lachlan Valley. In SA, growers were predominantly in the Keith-Naracoorte district with a smaller cluster to the north between Kapunda and Jamestown.

Survey of seedhouses Visits were arranged to the offices of five seedhouses involved in lucerne seed production. The most appropriate staff members were made available to provide, during a structured interview, information on the impact of the disease to each company's business. For one of the seedhouses, two separate interviews were held with staff based at different offices within NSW. For a second seedhouse, three separate interviews were held, two at offices in SA and one in NSW. This yielded data on the impact of lucerne yellows for four seedhouse offices in each state. Interviews were based on the questionnaire used for lucerne seed growers. Areas covered by seedhouse offices in NSW were the Lachlan and Macquarie valleys and the Riverina. In SA, offices covered the areas around Bordertown, Padthaway and Clare.

Because of the commercial sensitivity of data, the names of farmers and companies were suppressed, their locations indicated by State only and cultivars identified by code numbers.

Data analysis Results from the survey of lucerne seed growers were subject to Pearson chi-squared tests to compare data from the two States.

Results

Survey of lucerne seed growers Results were obtained from 36 seed growers in NSW and from 26 growers in SA. In NSW, 34 growers reported symptoms within their crops consistent with the descriptions and colour illustration on the 'fact sheet'. The equivalent figure for SA was 22 of the 26 growers (Table 1). Foliar symptoms were the most widely used basis for identifying the presence of the disease. Only two growers had inspected roots for symptom expression and only one had used a plant pathologist to confirm disease identity. In both States the most common response to the question about how frequently symptoms had been observed was 'every year' (Table 1).

In NSW, the majority of growers reported the disease in all or most of their lucerne stands, whereas, in SA, occurrence in very few stands was reported as frequently as the other categories (Table 1). In one instance the incidence of symptoms was

Table 1 Questionnaire responses of lucerne seed growers in New South Wales (NSW) and South Australia (SA) on the incidence of lucerne yellows disease

Question	Response category	NSW (n = 36)	SA (n = 26)	χ^2 analysis
Symptoms observed	Yes	34	22	$\chi^2 = 1.67$
	No	2	4	dof = 1
				$P = 0.196$
Frequency of symptoms	Every year	17	12	$\chi^2 = 0.30$
	Most years	10	6	dof = 3
	Occasionally	6	3	$P = 0.959$
	Rarely	1	1	
Occurrence on property	All stands	10	6	$\chi^2 = 5.35$
	Most stands	15	6	dof = 3
	Few stands	7	4	$P = 0.148$
	V. few stands	2	6	
Incidence within affected stands	>75%	0	0	$\chi^2 = 5.79$
	50–75%	1	0	dof = 4
	25–50%	2	0	$P = 0.215$
	10–25%	5	5	
	5–10%	5	2	
	2–5%	10	3	
	<2%	11	12	

reported to be as high as 50–75 %, but the majority of interviewees indicated 5% or fewer plants were affected (Table 1).

In NSW, three of the 34 growers who reported the disease used insecticides in an attempt to control it, one had ploughed-in affected crops and another had cut seed crops. No growers in SA reported taking action against the disease. Some growers indicated that marked differences were evident in susceptibility of cultivars and collectively commented on the perceived susceptibility/resistance of approximately 30 cultivars. However, responses were not consistent. Only one cultivar in NSW and one in SA was reported to have been unaffected. In NSW, 20 of the 26 growers who expressed an opinion viewed the disease as an actual or potential problem, whereas in SA this view was shared by 13 of 24 growers.

Though the results suggested that the disease was more serious in NSW than in SA, chi-squared analysis showed for each measure of severity that the States did not differ significantly ($P>0.05$).

Survey of seedhouses Seedhouse offices reported overseeing between 15 and in excess of 200 crops per annum. Three of the four offices based in NSW reported that lucerne yellows was evident in their crops every year. The fourth office reported it for most years. In SA, two offices reported the disease every year, and one office reported it for the 'occasional' and the 'rarely' response category. The identification of the disease was most commonly based on foliar symptoms alone. Two offices in NSW and one in SA reported inspecting plant roots for disease symptoms. Only one office (in NSW) had the identity of the disease confirmed by a plant pathologist.

The frequency of lucerne yellows in NSW crops tended to be higher than in SA. In the latter State, all interviewees reported the disease to occur in 'few' or 'very few' stands. Estimated levels of disease incidence within affected crops were in the 10–25% category or greater for NSW respondents. In contrast, all offices in SA reported levels in the 10–25% or lower categories.

Recommendations which seedhouses had made for action against lucerne yellows included high intensity grazing over winter and spring (one), ploughing plus insecticide sprays (one) and insecticide use (two). Half of the offices did not recommended action.

The overall view of seedhouse offices of the importance of lucerne yellows disease varied between States. All four offices in NSW considered it to be a problem but only one in SA shared this view. One office based in SA did not view the disease as a problem, and two regarded it as constituting a potential problem. Estimates of yield losses resulting from lucerne yellows included 25%, 50% (said to be worth $0.5 million (M) to that office in 1997), 10–60% and up to 90% in "bad" years.

Discussion

The results of this survey clearly indicate that lucerne yellows disease is a serious problem within the Australian lucerne seed industry. The impact of the disease is reflected by the actions of growers and staff from seedhouses. One grower had resorted to ploughing-in crops and four had taken other action in response to the disease in the form of cutting or spraying insecticide. A greater proportion of seedhouse offices (one half) had recommended action which included grazing affected seed crops. Such actions were taken despite the fact that the nature of the pathogen and how it is spread are unknown.

Findings suggest that a range of lucerne yellows susceptibility/resistance is exhibited within current cultivars so one avenue for future research is to undertake selective breeding to increase resistance levels. However, this strategy is a long term one and, as a unilateral approach, is unlikely to be effective. Some individual cultivars were considered by surveyed growers to have good resistance, yet other growers reported the same cultivars to have been severely affected. This suggests that resistance to lucerne yellows may be poly/oligogenic rather than vertical (i.e. major gene-based) and/or mediated by interaction with environmental factors. Resistance of this type is more difficult to select for and demands that the environmental factors which contribute to disease development in the field should be understood.

Investment in further research and development is therefore clearly warranted, especially as feedback from interviewees suggested that the disease was becoming more serious over time. This may be related to the more widespread use of cultivars from overseas, particularly from the United States of America; these imported cultivars were thought to be more susceptible by one seedhouse office based in SA and several growers.

The present results do not allow a precise quantitative estimate of the economic impact of lucerne yellows disease on the Australian lucerne seed industry. However, balancing the high number of growers reporting symptoms consistent with lucerne yellows against the relatively low incidence in terms of affected plants per stand (mostly below 25%), an average seed yield loss over the lifetime of a stand of 10% appears conservative. This equates to an economic loss in the order of $2M per annum in NSW alone. A more detailed economic analysis is possible for SA where 1997/98 production figures are available from the certification process. Based on the amount of seed produced and current retail prices, a value of $49.73 M per annum for the crop is indicated and the yield loss average of 10% suggested above equates to a loss of about $5M per annum in SA. Lucerne yellows disease is clearly a serious problem within an important industry. Research is planned for development of an appropriate control strategy.

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Appendix Two - Molecular testing for phytoplasma DNA in seeds from Australian lucerne yellows infected

Introduction

Seed transmission of phytoplasma diseases is not considered possible by many researchers and some argue against the possibility of such transmission being possible (Kleinhempel *et al.* 1975, Shin 1980). Recently, preliminary evidence became available suggesting that some phytoplasma diseases, namely alfalfa witches' broom symptomatic lucerne plants (Khan *et al.* 2002b) and witches' broom of lime (Khan *et al.* 2003), are capable of transmission to plant progeny through seed.

These findings came to light very late in the current project and this constrained the extent to which work could explore the possibility that Australian lucerne yellows (ALuY) could be seed borne. A small-scale molecular study was undertaken on seed harvested from ALuY symptomatic lucerne and subjected to molecular assays to test the hypothesis, in a limited way, that ALuY is a seed-borne pathogen.

Materials and Methods

Source of material

ALuY symptomatic lucerne plants were used in a symptom alleviation study (Chapter Five) in the mid Lachlan Valley, New South Wales, 50 km west of Forbes. The site was an established, irrigated, certified lucerne cv. Aurora seed stand. After harvest and measurement of alleviation parameters including seed yield, seed samples from six ALuY symptomatic lucerne plants was retrieved for use in this study.

Detection of phytoplasmas

DNA Extraction

DNA was extracted as described by Dellaporta, *et al*. (1983) from 0.2g of seed collected from six ALuY symptomatic lucerne plants. Ethanol-precipitated DNA pellets were each re-suspended in 50 µl 1 x TE buffer (10 mM Tris-HCl, 1 mM EDTA) and stored at –20°C until used.

Primers and PCR protocols

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

The primers P1 (Deng & Hiruki 1991a) and P7 (Kirkpatrick *et al.* 1994), fU5 (Lorenz *et al.* 1995) and m23sr (Padovan *et al.* 1995) were used in PCR and

nested PCR assays. PCR cycling conditions were as described in Chapter Two. Tomato big bud phytoplasma DNA and sterile distilled water were used for positive and negative controls, respectively. Fourteen nested PCR assays were conducted, each consisting of DNA extracted from six possible ALuY infected seed batches DNA samples at 1:1 and repeated at 1:10 dilutions, one tomato big bud phytoplasma sample and one sterile distilled water sample using the universal primers P1/P7. One μL of each P1/P7 PCR cocktail was then subjected to re-amplification using the primer pair fU5/m23sr and the same cycling conditions. After each nested PCR assay, 2 μL of PCR product were analysed by electrophoresis on a 1.0% agarose gel and stained with ethidium bromide prior to being visualised with a UV transilluminator.

Results

A PCR product of approximately 1.1 kb was amplified in samples containing tomato big bud positive control DNA. No PCR product was amplified from samples containing sterile distilled water or DNA extracted from suspected ALuY-infected seed batches at either the 1:1 or 1:10 dilutions.

Discussion

The findings from this study do not support the hypothesis that ALuY is a seed-borne disease. The sample size was limited, though chances of phytoplasma DNA amplification was maximised by use of seeds from ALuY symptomatic lucerne plants. This preliminary study does not preclude the possibility of seed transmission of the disease. The results, combined with the field distribution of ALuY symptoms (detailed in Chapter Three) and findings from other studies, such as those undertaken by Kleinhempel *et al*. (1975) and Shin (1980), suggest that it is unlikely that ALuY is a seed-borne disease.

The same method of DNA extraction and PCR protocols were used in this preliminary study as was shown successfully to detect ALuY DNA in molecular studies in ALuY symptomatic plants (Chapter Two). Using a method proven able to detect ALuY phytoplasma DNA in plant material maximised the likelihood for detection in seeds. Khan *et al*. (2002b) used batches of 40 seeds, reported as 2 g, compared with batches of approximately 100 seeds used in this study which weighed only 0.2 g. The amount of seed material used in this study was in accordance with details set out by Dellaporta *et al*. (1983) for extraction of DNA from plant material.

Investigating seed-transmission of witches' broom in lime Khan *et al*. (2003) used seedlings from infected plant material grown under quarantine conditions and extracted DNA from this plant material rather than the seeds themselves. This method is employed to avoid false positives that are possible from

amplification of phytoplasma DNA located in parent plant tissue that is found within the seed structure (Bertaccini A. F. 2003, pers. comm., 20 March). Accordingly, had phytoplasma DNA been amplified in the present study further work would have been required to establish that parent tissue was not the source.

More extensive seed sampling over many sites is needed in future examinations of this hypothesis. Several different techniques would need to be used including propagating plants from suspected ALuY infected seed in cages and examining for foliar or root symptom expression. DNA collected from seed material should also be “spiked” with a tomato big bud positive control to exclude the possibility of inhibitors preventing successful amplification of DNA. Further, PCR should be performed on seedlings grown, under controlled conditions, from seed collected from ALuY symptomatic lucerne.

Appendix Three – Examples of statistical output

Regression analysis of ALuY symptom and leafhopper distribution

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Response variate: A_t
     Fitted terms: Constant + Row + Column + Row.Column
                   (FACTORIAL limit for expansion of formula = 2)
        Submodels: POL(Row; 2)
                   POL(Column; 2)


*** Summary of analysis ***

             d.f.         s.s.         m.s.      v.r.  F pr.
Regression      8        11.02       1.3771      2.58  0.013
Residual       95        50.64       0.5330
Total         103        61.65       0.5986

Percentage variance accounted for 11.0
Standard error of observations is estimated to be 0.730
* MESSAGE: The following units have large standardized residuals:
         Unit     Response    Residual
          712        4.000        4.05
          713        4.000        3.70
          733        2.000        2.62
* MESSAGE: The error variance does not appear to be constant:
           intermediate responses are more variable than small or large
responses


*** Estimates of parameters ***

                              estimate         s.e.     t(95)  t pr.
Constant                         1.338        0.329      4.07  <.001
Row Lin                        -0.02960      0.00906     -3.27  0.002
Row Quad                      0.0001558    0.0000441      3.53  <.001
Column Lin                     -0.00775      0.00306     -2.53  0.013
Column Quad                  0.00001175   0.00000496      2.37  0.020
Row Lin .Column Lin           0.0002175    0.0000841      2.58  0.011
Row Lin .Column Quad      -0.000000318  0.000000137     -2.33  0.022
Row Quad .Column Lin      -0.000001139  0.000000410     -2.78  0.007
Row Quad .Column Quad         1.64E-09     6.64E-10      2.47  0.015


*** Accumulated analysis of variance ***

Change                           d.f.         s.s.         m.s.      v.r.  F
pr.
+ POL(Row; 2)                       2       2.2488       1.1244      2.11
0.127
+ POL(Column; 2)                    2       3.4828       1.7414      3.27
0.042
+ POL(Row; 2).POL(Column; 2)
                                    4       5.2849       1.3212      2.48
0.049
Residual                           95      50.6374       0.5330

Total                             103      61.6538       0.5986
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Analysis of variance of border treatment experiments (1 and 2)

Variate: Atsqrt

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	2	0.005954	0.002977	1.00	
Block.*Units* stratum					
Treatment	2	0.005954	0.002977	1.00	0.372
Residual	85	0.253063	0.002977		
Total	89	0.264972			

***** Tables of means *****

Variate: Atsqrt

Grand mean 0.7129

Treatment	Control	Herbicide	Insecticide
	0.7071	0.7244	0.7071

*** Standard errors of differences of means ***

Table	Treatment
rep.	30
d.f.	85
s.e.d.	0.01409

*** Least significant differences of means (5% level) ***

Table	Treatment
rep.	30
d.f.	85
l.s.d.	0.02801

Analysis of variance for symptom alleviation study

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Variate: Seedwtlog_01
Source of variation     d.f.(m.v.)      s.s.       m.s.    v.r.  F pr.
block stratum              9          21.892      2.432    0.92
block.*Units* stratum
Treatment                  4          24.110      6.027    2.28  0.084
Residual                  30(6)       79.197      2.640
Total                     43(6)      118.879

* MESSAGE: the following units have large residuals.
block 4     *units* 3            2.68   s.e. 1.26
block 7     *units* 3            3.29   s.e. 1.26

***** Tables of means *****
Variate: Seedwtlog_01

Grand mean  -2.56
 Treatment      Control     Multigro       Potash Tetracycline        Water
                  -3.60        -1.88        -2.83        -2.80        -1.70

*** Standard errors of differences of means ***

Table            Treatment
rep.                    10
d.f.                    30
s.e.d.               0.727

(Not adjusted for missing values)

*** Least significant differences of means (5% level) ***

Table            Treatment
rep.                    10
d.f.                    30
l.s.d.               1.484

(Not adjusted for missing values)

***** Stratum standard errors and coefficients of variation *****

Stratum                   d.f.          s.e.         cv%

block                        9         0.697        27.2
block.*Units*               30         1.625        63.4

**** Missing values *****

Unit  estimate
    8     -2.64
   22     -1.17
   45     -3.52
   48     -1.87
   50     -3.88
   87     -1.45

Max. no. iterations 4

                     Mean    Variance
    Treatment
      Control      0.0331      0.0046
     Multigro      0.4827      0.6426
       Potash      0.4845      0.6906
 Tetracycline      0.1234      0.0308
        Water      0.4716      0.6875
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