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Vector status of three leafhopper species for Australian lucerne yellows phytoplasma

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Abstract The leafhoppers *Orosius argentatus* (Evans), *Austroagallia torrida* (Evans) and *Batracomorphus angustatus* (Osborn) were used in transmission tests to determine their vector status for the phytoplasma associated with Australian lucerne yellows (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 polymerase chain reaction (PCR) using universal primers. The phytoplasma detected by PCR in the plant fed on by *A. torrida* was identified by restriction fragment length polymorphism (RFLP) analysis as the tomato big bud (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* is a vector of the ALuY pathogen and *A. torrida* is a vector of the TBB phytoplasma.

Key words *Austroagallia torrida*, *Batracomorphus angustatus*, *Orosius argentatus*, PCR, RFLP, transmission.

INTRODUCTION

Phytoplasmas are associated with a large number of plant diseases around the world (Marcone *et al*. 1997; Davis & Sinclair 1998; Khadhair *et al*. 1999; Schneider *et al*. 1999). Symptoms broadly associated with phytoplasmas include witches' broom, little leaf, stunting, phyllody and big bud (McCoy 1979). Insects that have been shown to be responsible for the vectoring of phytoplasmas are leafhoppers (Membracoidea), planthoppers (Fulgoroidea) and psyllids (Psylloidea) (Ploaie 1981; Hill & Sinclair 2000).

Australian lucerne yellows (ALuY) is a serious disease causing an estimated annual loss of $7 m to the lucerne seed industry (Pilkington *et al*. 1999) and has been reported in Australian lucerne stands since the early 1950s (Anonymous 1953). In 1979, Australian lucerne yellows (ALuY) levels were very low amongst crops re-sown after damage from exotic aphids (McGechan 1980). Levels of damage increased in 1980 (McGechan 1980) and since then has increased in intensity to current levels (Pilkington *et al*. 1999). The planthopper family Delphacidae and the cicadellid subfamily Deltocephalinae contain the greatest number of documented phytoplasma vector species (Fletcher 1984). 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 in that study 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 we could find no evidence of other similar studies in the current literature.

Symptoms of ALuY include yellow discolouration of foliage that is typically consistent over the entire plant (Stovold 1983; Pilkington *et al*. 1999, 2002). The plant also expresses a distinctive yellow to dark brown discolouration immediately under the periderm of the tap-root (Stovold 1983; Pilkington *et al*. 2002).

ALuY disease is associated with a phytoplasma (Pilkington *et al*. 2002, 2003a). Three leafhoppers, *Orosius argentatus* (Evans), *Austroagallia torrida* (Evans) and and *Batracomorphus angustatus* (Osborn), have a spatio-temporal correlation with disease symptoms and are possible vectors of the ALuY pathogen (Pilkington *et al*. 2003b). To date, however, there has been no direct experimental evidence to implicate any of the species as ALuY vectors.

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The disease named tomato big bud (TBB) by Samuel *et al*. 1983) 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).

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

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

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

In the past, insect DNA extraction techniques involved grinding the whole leafhopper, including the gut contents (Maixner *et al*. 1995; Charles *et al*. 2002) and subjecting this DNA to 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, contains phytoplasmas in its gut, and does not establish that the insect is capable of vectoring the phytoplasma. Despite this, we could find no reports 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 & Maixner (1998) who cut the leafhopper longitudinally to facilitate PCR assay on one half and enzyme linked immunosorbent assay (ELISA) assays for the other half.

The aims of our study were to identify vectors for the disease ALuY using field-collected and laboratory-reared leafhoppers in caged transmission tests. Plants were 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 seed crops of lucerne cv. Aurora 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 (Pilkington *et al*. 2002). 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^{-1} pyrethrins, 1.6 g L^{-1} piperonyl butoxide), applied as a fine mist over the entire plant, and rinsed thoroughly with water 24 h later, prior to introduction of leafhopper colonies, to ensure that all other insect species were removed.

Transmission tests

Source of insects

Adults and nymphs of *A. torrida* and *B. angustatus* adults and nymphs were obtained by sweep-netting certified lucerne seed crops, cv. Aurora, growing in the Lachlan Valley in September 2002. Leafhoppers were removed from the sweep net and placed, with some green lucerne foliage, into a container stored in a 12-V car refrigerator set at approximately 9°C for transport back to the laboratory. In the laboratory, leafhoppers were anaesthetised with carbon dioxide and sorted into species. Specimens of *O. argentatus* were sourced from a laboratory culture maintained at Charles Darwin University, as only very low numbers were present in the field at the time other species were captured. *Orosius 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.

Austroagallia torrida *feeding trial*

Fifty individuals of *A. torrida* were placed on two individually caged ALuY symptomatic plants for an acquisition period of 7 d. Insects were then transferred to nine caged, seed-grown lucerne plants for an inoculation period of 50 d.

Batracomorphus angustatus *feeding trial*

Fifty *B. angustatus* were placed on two individually caged ALuY symptomatic plants for an acquisition period of 7 d. Insects were then transferred to eight caged, seed-grown lucerne plants for an inoculation period of 50 d.

Orosius 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 2–3 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 7 d followed by a latency period on faba bean for 30 d. 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 2–3 leafhoppers for an inoculation period of 7 d.

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 on The University of Sydney, Orange campus farm in November 2002 and monitored weekly for expression of foliar symptoms consistent with phytoplasma infection. After 7 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 1991) and P7 (Kirkpatrick *et al*. 1994) were used in first round PCR and primers fU5 (Lorenz *et al*. 1995) and m23sr (Padovan *et al*. 1995) were used in nested PCR assays. PCR cycling conditions were as follows: denaturation for 1 min (2 min for first cycle) at 95°C, annealing temperature of 55°C for 1 min and an extension time of 1.5 min at 72°C for 35 cycles (9.5 min on 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

Austroagallia 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 5–10. 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.

Orosius argentatus

Individuals of laboratory reared *O. argentatus* were fed on disease free faba bean for 7 d after the inoculation period to clear the gut of diseased lucerne. They were then screened for phytoplasma as above, 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 30v, 1993, Poulan: Division of WCI Outdoor Products, Shreveport, USA) (Hossain *et al*. 1999) from three newly sown certified lucerne (cv. Aurora) seed stands in the mid Lachlan Valley region 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, and 115 *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.

RFLP of PCR product

Amplified products from PCR assays, including the TBB control, were subjected to restriction fragment length polymorphism (RFLP) analysis. We digested 5 µL of each PCR product separately, following the manufacturer's instructions (New England BioLabs, Beverley, 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 Charles Darwin University for specimen preparation and fluorescence microscopy. Samples were transported in DAPI fixative made by combining 9.7 mL 0.2 M sodium cacodylate buffer (21.4 g $Na(CH3)_2AsO_2{\cdot}3H_2O$ 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 (4′,6′ diamidino-2-phenylindole) 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 ($NaH_2PO_4{\cdot}H_2O$) (Sigma Aldrich) buffer with 4 mL of water. This buffer was made to 7.0 pH before two grains of DAPI (Sigma Aldrich) and two drops of aniline blue stain (Sigma Aldrich) were added. Thick cross-sections of each leafhopper species, three of each, were left in the stain for 3 h before being visualised on a Nikon E800 microscope using ultra-violet filter cubes.

RESULTS

Austroagallia 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 showing severe leaf stunting and die-back of stems was phytoplasma positive by PCR. None of the leafhoppers were phytoplasma positive by PCR. The phytoplasma 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 (Fig. 1).

Batracomorphus 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 asymptomatic plant was too faint for RFLP analysis.

Orosius 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 (Fig. 2). This discolouration did not extend into the stele of the tap-root typical of bacterial wilt, caused by *Clavibacter michiganensis* ssp. *insidiosus* (McCulloch), and was consistent with root symptoms expressed by lucerne plants with ALuY disease

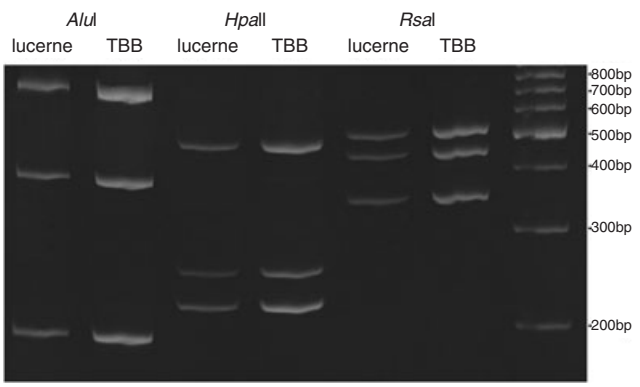

Fig. 1. Polyacrylamide gel of RFLP digestions of amplified product from lucerne plant fed on by *Austroagallia torrida* ('lucerne') and tomato big bud positive control ('TBB') using digestions enzymes *Alu*I, *Hpa*II and *Rsa*I.

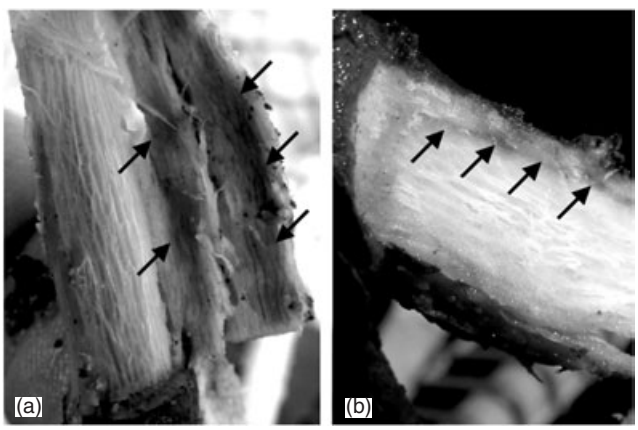

Fig. 2. Dark discolouration of the root periderm of a lucerne plant exposed to *Orosius argentatus*.

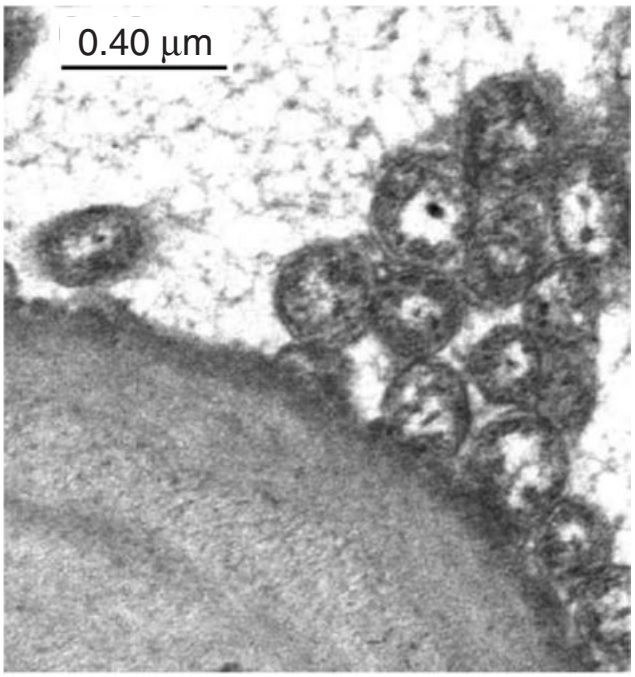

Fig. 3. Phytoplasmas observed in a phloem cell from a symptomatic lucerne plant fed on by *Orosius argentatus* in transmission tests.

(Pilkington *et al*. 2002). 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.

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 (200–400 nm diameter) were observed in the phloem of one of the two phytoplasma positive plants fed on by *O. argentatus*. Phytoplasmas were also observed in the lucerne plant fed on by *A. torrida* that was TBB phytoplasma positive, and in the plant displaying distinct ALuY root symptoms that had been fed on by *O. argentatus* but which was phytoplasma negative by PCR (Fig. 3). The structures were consistent with those found in lucerne plants affected with ALuY disease (Pilkington *et al*. 2003a). No phytoplasma bodies were observed in other plants.

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

DISCUSSION

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. The *B. angustatus* 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 can be inferred from the positive PCR result.

Colony-bred *O. argentatus* were given access to ALuY plants and then to healthy lucerne plants, and one of the latter 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, are not capable of transmitting to the next generation of the insect vector (Kawakita *et al*. 2000). The *O. argentatus* that we used was laboratory reared for more than 20 generations on plants known not to contain phytoplasmas and therefore it is unlikely that they carried pathogens other than those acquired from ALuY symptomatic plants.

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

Five lucerne plants fed on by *A. torrida* showed significant stunting of leaves and some stems, and three lucerne plants fed on by *A. torrida* showed no symptoms. One plant showed symptoms consistent with alfalfa mosaic virus, a seed-borne disease common in Australian lucerne crops (Hajimorad & Francki 1988). The TBB phytoplasma amplified from one plant, identified 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).

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 usually, transmission is less frequent. Jarausch *et al*. (2003) reported 10% (1 of 10 test plants) and 18% (7 of 40 test plants) transmission rates in PCR assays conducted with apple proliferation phytoplasma and its suspected vector *Cacopsylla picta* (Foerster). Results from our 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 a 'highly active vector' (Carraro *et al*. 2001a), and Jarausch *et al*. (2001) demonstrated a less than 1% (1 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% (4–5 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% (3 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 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 (Pilkington, unpubl. data 2001). 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 to infer a phytoplasma infection (Kaminska *et al*. 1999). However, the lack of RFLP characterisation makes it impossible to confirm its precise 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) and may have been the case in our study.

Polymerase chain reaction 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 to amplify phytoplasma DNA from only the heads and thoraxes of leafhoppers has been made, 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.

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.

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