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Planthopper transmission of *Phormium* yellow leaf phytoplasma

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Abstract

Phormium yellow leaf (PYL) phytoplasma was transmitted from diseased to healthy New Zealand flax (*Phormium tenax*) by the native planthopper, *Oliarus atkinsoni* (Homoptera: Cixiidae). By contrast, transmission was not effected by the introduced passionvine hopper, *Scolypopa australis* (Homoptera: Ricaniidae). Successful transmission of PYL phytoplasma from New Zealand flax to New Zealand flax by *O. atkinsoni* was demonstrated by symptomatology and by polymerase chain reaction (PCR) of the test plants using phytoplasma-specific primers to the 16S rRNA genes. When the salivary glands and the remaining body of the planthoppers used in the transmission studies were tested separately by PCR for the presence of phytoplasma, PYL phytoplasma was detected in 100% of both the salivary glands and the bodies of pre-transmission *O. atkinsoni*, and in 44% and 67% of the salivary glands and the bodies of post-transmission planthoppers, respectively. The phytoplasma was not detected by PCR in the whole bodies of hoppers of *S. australis*.

Additional keywords: *Cordyline australis*, vector

Introduction

Phormium yellow leaf (PYL) is a lethal disease of the large tufted lilioid monocotyledons, New Zealand flax (*Phormium tenax* J. R. et G. Forst.) and mountain flax (*P. cookianum* Le Jolis). PYL was a contributor to the eventual demise of a locally important natural fibre industry based on *P. tenax* (Boyce and Newhook 1953). The disease was first recognised over 80 years ago, but it was not until 1969 when Ushiyama *et al.* (1969) observed phytoplasmas in the phloem of diseased plants and in the proposed planthopper vector (*Oliarus atkinsoni* Myers (Homoptera: Cixiidae)) by electron microscopy, that the etiology of the disease was clarified. More recently, phylogenetic analysis of the 16S rRNA genes of PYL phytoplasma determined that it belongs to a subgroup of the aster yellows group (Liefting *et al.* 1996), and is very closely related to Australian grapevine yellows phytoplasma, sharing 99.5% of the 16S rRNA gene sequence (Padovan *et al.* 1996).

The planthopper, *O. atkinsoni* is endemic to New Zealand where it is confined to *Phormium* spp. Large numbers of *O. atkinsoni* were present on New Zealand flax in badly affected yellow-leaf areas (Cumber 1953). Further, Cumber (1953) found that 16 of the 20 New Zealand flax seedlings caged with adult *O. atkinsoni* developed typical yellow-leaf symptoms, while none of 20 control plants showed any symptoms. A number of further transmission experiments, including an experiment using planthoppers collected from an area free of yellow-leaf, all implicated *O. atkinsoni* as the vector of PYL (Boyce *et al.* 1953). A search for alternative insect vectors of PYL was carried out by Cumber (1954*b*). The mealybug *Trionymus diminutus* (Leonardi), leafhopper *Erythroneura zealandica* Myers, and planthopper *Oliarus oppositus* (Walker) all failed to transmit the disease. However, most of these later transmission experiments were complicated by test plants dying from 'sudden die-back', a disease of unknown etiology (Cumber 1954*a* and *b*). Furthermore, Boyce *et al.* (1953) reported mechanical and seed transmission of yellow-leaf: in view of our current knowledge of phytoplasmas, these observations are almost certainly in error. These later experiments, carried out before the discovery of phytoplasmas (Doi *et al.* 1967), raise doubts as to the validity of the transmission experiments in general. Thus, there was a real need to clarify disease transmission taking into account the proposed etiology.

Since the late 1980s, a syndrome known as 'sudden decline' has caused widespread death of cabbage trees (*Cordyline australis* (Forst. f.) Endl.) in New Zealand (Beever *et al.* 1996). This tufted, woody, lilioid monocotyledon tree is also native to New Zealand and frequently grows in association with *Phormium tenax*. It has been proposed that sudden decline is caused by a phytoplasma, perhaps identical or very closely related to PYL phytoplasma, although this has yet to be proven (Beever *et al.* 1996).

The passionvine hopper, *Scolypopa australis* Walker (Homoptera: Ricaniidae), introduced from Australia, is found in large numbers on a wide variety of native and exotic plants, including *Phormium* spp. (Cumber 1966). The distribution of *S. australis* within New Zealand closely parallels that of sudden decline, leading to the suggestion that it could be a vector of this disease (Beever *et al.* 1996).

The main aim of this research was to carry out transmission experiments to determine if *O. atkinsoni* is a vector of PYL phytoplasma. Also, *S. australis* was examined as a possible additional vector of PYL phytoplasma. Further, attempts were made to transmit PYL phytoplasma to cabbage trees by *O. atkinsoni* and *S. australis* to determine if PYL phytoplasma is involved in the etiology of sudden decline.

Methods

Plant material Healthy *P. tenax* was grown from seed collected from a vegetatively propagated descendant of selection 56 shown by Boyce *et al.* (1953) to be susceptible to PYL phytoplasma. Healthy seedlings of *C. australis* were grown from seed collected from a cutting from a tree that subsequently died from sudden decline. Seedlings of both species were 1 year-old at the time of caging with insects.

Insect samples Adults of *O. atkinsoni* and *S. australis* which were feeding on PYL-affected New Zealand flax were collected during February 1995 from a site near Kawakawa (Northland) where an area of about 1 ha of affected plants occurred naturally. Most of the plants showed yellow-leaf symptoms and many were dying.

Insect transmission experiments The day following collection, groups of ten adult *O. atkinsoni* or *S. australis* were placed on individual test plants in insect-proof cages for a period of 21 days. Six New Zealand flax seedlings and six cabbage trees were used with each insect species. As a control, six New Zealand flax plants and six cabbage trees were placed in mesh bags (three plants per bag) with no insects. An additional 30 insects of each species which were not used in the transmission experiments were stored at –20°C and are referred to as the pre-transmission insects.

At the end of each experiment, the insects that remained alive were removed from the plants and stored at –20°C and are referred to as the post-transmission insects. The plants were maintained in an insect-proof shadehouse and regularly inspected for symptoms.

Plant DNA extraction A modification of the cetyltrimethylammonium bromide (CTAB) method was used (Ahrens and Seemüller 1992). Frozen New Zealand flax leaf bases (2 g) were ground in liquid nitrogen with a mortar and pestle. The powder was transferred to a 30 mL tube and mixed with 10 mL of isolation medium (100 mM Na_2HPO_4, 10% sucrose, 2% polyvinylpyrrolidone [PVP-40], pH 7.6 to which 0.15% bovine serum albumin and ascorbic acid to a final concentration of 1 mM were added just before use). The homogenate was centrifuged at 4°C for 5 min at 1500 *g*. The supernatant was decanted and recentrifuged at 4°C for 25 min at 18 000 *g*. The pellet was resuspended in 10 mL hot (60°C) CTAB buffer (5% CTAB, 100 mM Tris-HCl [pH 8.0], 20 mM EDTA, 1.4 M NaCl, 1% PVP-40) and was incubated at 60°C for 30 min. The lysate was extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v). After centrifugation, the aqueous layer was precipitated with a two-third volume of ice-cold isopropanol, incubated at –20°C for 1 h, and then centrifuged at 10 000 *g* for 15 min. The pellet was washed with ice-cold 70% ethanol, dried under vacuum, and resuspended in 50 µL TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

Preparation of insects for PCR

DNA extraction from insect bodies DNA was extracted from insect bodies by the method of Goodwin *et al.* (1994). Individual insects were macerated in 125 µL extraction buffer (2% CTAB, 1.4 M NaCl, 1% PVP-40, 0.02 M EDTA, 0.1 M Tris-HCl, pH 8.0) in a microfuge tube using a micropestle, made by melting the tip of a large size disposable pipette tip. The

remains of the insects were removed, the tube was briefly vortexed and then incubated at 65°C for 5 min. The suspension was extracted once with an equal volume of chloroform:isoamyl alcohol (24:1, v/v), and the nucleic acids were precipitated by adding 1/10 vol. 3 M sodium acetate, pH 5.2, and 2.5 vol. ice-cold ethanol and incubating at –20°C for at least 1 h. After centrifuging for 15 min at 13 000 *g*, the pellet was washed with 70% ice-cold ethanol, dried under vacuum, and resuspended in 15 µL TE buffer.

Dissection of the salivary glands The salivary gland system of the planthoppers lies in the head and in the fore-part of the thorax and is bilaterally symmetrical. The insects were first briefly dipped in 70% ethanol and were then placed on wax and covered with insect saline solution (1.8 mM $CaCl_2$, 120 mM NaCl). Dissections were performed under a binocular microscope. With the ventral side uppermost, the posterior end of the abdomen was pinned down with entomological pins. The head and the first segment of the thorax were gently separated by pulling with fine forceps and the head was also pinned down while the salivary glands were removed with fine forceps and transferred into a microfuge tube (0.5 mL) containing 5 µL STE (100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

Preparation of the salivary glands for PCR Salivary glands were prepared for PCR by a modification of the method described by O'Neill *et al.* (1992). The dissected salivary glands were homogenised in STE by repetitive pipetting with a micropipette, and incubated with 1 µL of proteinase K (5 mg/mL) for 30 min at 37°C, followed by boiling for 5 min. Samples were centrifuged for 30 sec and 1 µL of the supernatant was used as the template in PCR.

PCR The quality of both the insect and plant samples was tested with the primer pairs ITS5/AB28 and Gd1/Berg54, respectively. ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') (White *et al.* 1990) and AB28 (5'-ATA TGC TTA AGT TCA GCG GGT-3') (Howlett *et al.* 1992) are homologous to conserved regions of the 18S and 28S rRNA genes, respectively, and amplify the intervening ITS1-5.8S-ITS2 sequences of all eukaryotes. Gd1 (5'-ACG GAG AGT TTG ATC CTG-3') and Berg54 (5'-AAA GGA GGT GAT CCA GCC GCA CCT TC-3') were designed by Peter Bergquist, University of Auckland to amplify the entire 1.6 kb 16S rRNA gene from all prokaryotes and some plant organelles such as chloroplasts and mitochondria. The primers NGF (5'-AGG CGG CTT GCT GGG TCT T-3') and NGR (5'-AGC CAT TGT ATC ACG TT-3') were designed to amplify a 500 bp fragment of the 16S rRNA gene of all phytoplasmas (M.T. Andersen, personal communication) and were used to test for the presence of PYL phytoplasma in both plant and insect samples.

PCR amplification was performed with a 20 µL reaction mixture containing 4 µL (10 to 50 ng) of template DNA, 20 µM of each primer, 200 µM dNTPs, 1.25 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase with recommended buffer (Amplitaq, Perkin-Elmer) or 1.75 U of Expand™ High Fidelity PCR System with recommended buffer containing 1.5 mM $MgCl_2$ (Boehringer Mannheim). Amplification involved an initial denaturation at 94°C for 2 min and then 30 cycles of 94°C for 30 sec, 55°C for 15 sec, and 72°C for 1 min in a Corbett FTS-1S Capillary Thermal Sequencer.

PCR products were analysed by electrophoresis through 1% agarose gels for ITS5/AB28 and Gd1/Berg54 products or 1.5% agarose gels for products from NGF/NGR in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) buffer. Gels were stained with ethidium bromide according to standard protocols (Sambrook *et al.* 1989).

Results

Symptoms Five out of six New Zealand flax seedlings developed the typical leaf yellowing and stunting symptoms of PYL 7 months after exposure to planthoppers of *O. atkinsoni* which had previously fed on PYL-affected plants (Table 1). The six New Zealand flax plants exposed to *S. australis* and the six control plants remained symptomless (Table 1). There was a significant reduction in the length of the leaves on the five infected plants which had been exposed to *O. atkinsoni* compared to the control plants, whereas the growth of the plants exposed to *S. australis* was not affected (Figure 1). The five New Zealand flax plants which developed disease symptoms died within 18 months of the start of the transmission experiment.

The cabbage trees remained symptomless approximately 2 years after exposure to the insects (Table 1).

PCR detection of PYL phytoplasma in *P. tenax* Approximately one year after the plants were exposed to the insects, each New Zealand flax plant

from the insect transmission experiment was tested for the presence of PYL phytoplasma by PCR using primers which amplify the 16S rRNA genes. The DNA preparations from each of the test plants were suitable for PCR amplification, as a PCR product was observed from all the plants using the universal 16S rRNA primer pair, Gd1/Berg54 (Table 1). Five out of six New Zealand flax plants which were exposed to *O. atkinsoni* tested positive with the phytoplasma specific primer pair, NGF/NGR (Table 1). These five plants were the same plants as those which developed symptoms of PYL phytoplasma described above. PYL phytoplasma was not detected by PCR in the six plants exposed to *S. australis* and the six control plants (Table 1). These results correlated well with those based on symptomatology and indicate that there were no symptomless infections.

PCR detection of PYL phytoplasma in insects

The pre-transmission insects of both species were tested by PCR for the presence of PYL phytoplasma to determine the potential infectivity of the insects. The salivary glands and the body of the same insect were tested for six adults of *O. atkinsoni*. PYL phytoplasma was detected in both the salivary glands and the remainder of the body of all six insects (Table 2).

The insects used in the transmission experiments were allowed to feed on the test plants for a period of 21 days. After this time, most of the insects had died and decomposed. The nine *O. atkinsoni* and 12 *S. australis* that survived the transmission period were tested for the presence of PYL phytoplasma by PCR. Both the salivary glands and the body of all nine *O. atkinsoni* were tested, that is, the insects were extracted after removal of the salivary glands. Six of these nine insects were infected with PYL phytoplasma, but the infection was present in the salivary glands in only four of them (Table 2).

Phytoplasma 16S rRNA sequences were not detected in DNA prepared from the whole body of *S. australis* (Table 2), even when the Expand™ High Fidelity PCR system was used; this system involves a mixture of *Taq* DNA and *Pwo* DNA polymerases designed to give PCR products with high yield (Boehringer Mannheim technical bulletin).

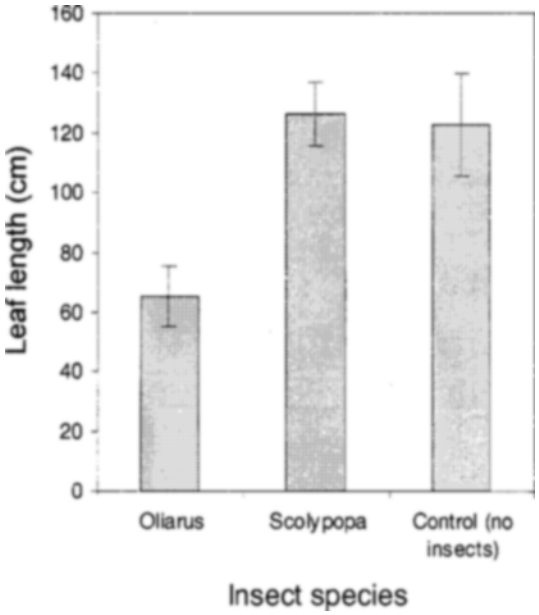

Figure 1 Effect of PYL phytoplasma on the growth of *Phormium tenax*, measured approximately 1 year after exposure to insects. Each bar represents the mean of the three longest leaves of each of the six plants in each treatment ± standard error except that only the five symptomatic plants were included for the treatment with *Oliarus atkinsoni*.

Table 1 Symptomatology and PCR detection of PYL phytoplasma following insect transmission tests

Treatment	Symptoms[A]		PCR of flax using	
	Flax	Cabbage tree	Gd1/Berg54[B]	NGF/NGR[C]
Oliarus atkinsoni	5/6	0/6	6/6	5/6
Scolypopa australis	0/6	0/6	6/6	0/6
Control (no insects)	0/6	0/6	6/6	0/6

[A]Number of plants showing yellow leaf (New Zealand flax) or sudden decline (cabbage tree) symptoms over the total number of test plants.

[B]Number of plants positive over the total number of plants tested (these primers were expected to amplify a 1.6 kb product from both healthy and diseased plants).

[C]Number of plants positive using the phytoplasma-specific primer pair over the total number of plants tested.

Table 2 PCR detection of PYL phytoplasma in *Oliarus atkinsoni* and *Scolypopa australis* used for transmission experiments

	Oliarus atkinsoni				*Scolypopa australis*	
	Pre-transmission[A]		Post-transmission[B]		Pre-transmission[A]	Post-transmission[B]
PCR primers	Salivary glands	Body[C]	Salivary glands	Body[C]	Whole body	Whole body
ITS5/AB28[D]	6/6	6/6	9/9	9/9	30/30	12/12
NGF/NGR[E]	6/6	6/6	4/9	6/9	0/30	0/12

[A]Insects were placed at –20°C at the same time that the post-transmission insects were placed on the recipient plants.
[B]Insects used in transmission experiments.
[C]Body represents the insect remains after dissection of the salivary glands.
[D]Number of insects positive in PCR using the universal ITS5/AB28 primer pair over the total number of insects tested.
[E]Number of insects positive in PCR using the phytoplasma specific primer pair NGF/NGR over the total number of insects tested.

Discussion

The transmission studies and associated PCR tests reported here provide strong evidence to support the earlier transmission experiments by Boyce *et al.* (1953) and Cumber (1953) which relied on symptomatology alone to determine successful transmission of the disease agent. The transmission experiments presented here involved PCR testing of the insects used in the transmission trials. In addition, the presence of the phytoplasma in plants showing typical PYL symptoms was confirmed by PCR, thereby strengthening the correlation between the presence of the phytoplasma and the disease symptoms.

Transmission experiments proved that *O. atkinsoni* is a vector of PYL phytoplasma. Both symptomatology and PCR determined that five of the six New Zealand flax plants (83%) became infected after being fed on by these insects. This closely matches with the transmission experiments by Cumber (1953) where 16 out of 20 New Zealand flax plants (80%) developed symptoms of PYL phytoplasma infection after exposure to *O. atkinsoni*.

PYL phytoplasma was detected in *O. atkinsoni* by PCR in both the total body and in the dissected salivary glands. The NGF/NGR primer pair was designed to amplify a region of the 16S rRNA genes from all phytoplasmas. Therefore, the identity of PYL phytoplasma was confirmed by sequencing the PCR products from some insects. These sequences were identical to the corresponding region of the 16S rRNA gene of PYL phytoplasma sequenced by Liefting *et al.* (1996) (data not shown). All the insects which were immediately frozen (pre-transmission insects) contained phytoplasma as determined by PCR, while the incidence of phytoplasma in the salivary glands and the body of the insects remaining from the transmission experiments (post-transmission insects) was 44% and 67%, respectively. Adults of *O. atkinsoni* begin emerging from fifth instar nymphs around mid-November and have been observed to live for up to 4 weeks (Cumber 1952). The insects used in the transmission experiments were collected from the field in February and only a few survived the 21 day transmission period probably because the planthoppers were near the end of their adult life. The lower number of PCR-positive individuals in the post-transmission compared to the pre-transmission insects suggests that these insects were younger and at an earlier stage of infection, as in some cases phytoplasmas may be pathogenic to their insect vectors and cause premature death (Jensen *et al.* 1967; Nault *et al.* 1984). Ideally, reared insects should be used for transmission experiments. However, attempts to rear *O. atkinsoni* in captivity have failed.

Compared to other insect vectors of phytoplasmas, a higher proportion of *O. atkinsoni* was found to be infected with PYL phytoplasma. Although our results in Table 2 were obtained from only a small number of insects, PCR tests on *O. atkinsoni* collected from other sites also showed consistently high levels of infection (data not shown). By PCR, the proportion of pear psyllids carrying pear decline phytoplasma ranged from less than 1% to approximately 40% (Davies and Eyre 1996), 40% of *Metcalfa pruinosa* were positive for several different

phytoplasmas (Danielli *et al.* 1996), and 38% of *Hyalesthes obsoletus* were positive for German grapevine yellows phytoplasma (Maixner *et al.* 1995). These infectivity values were taken from the total insect and to obtain a more accurate level of potential infectivity, only the salivary glands should be tested, as the phytoplasma can presumably be transmitted only after it has entered and multiplied in the salivary glands. The high level of infectivity of *O. atkinsoni* can be attributed to their 2 year life cycle, during which they probably acquire the phytoplasma as nymphs while living amongst the decayed leaf bases and roots of the rhizome, and to the fact that they feed almost exclusively on New Zealand flax. Consequently *O. atkinsoni* is a very efficient vector of PYL phytoplasma as shown by a high percentage of New Zealand flax plants becoming infected in the transmission experiments.

The passionvine hopper, *S. australis*, which was considered a possible additional vector of PYL phytoplasma due to its occurrence in large numbers on New Zealand flax, failed to transmit the disease. This correlates with the failure to detect phytoplasma in these insects. Detection of the pathogen in an insect species does not necessarily indicate transmissibility of the disease by this insect. For example, Vega *et al.* (1993) detected phytoplasmas in a leafhopper species that does not transmit aster yellows phytoplasma. Once ingested into the insect gut during feeding, some phytoplasmas can apparently multiply in gut cells of non-vectors, but a number of tissue-specific barriers may prevent spread of the phytoplasma to the salivary glands (Kirkpatrick 1992). The failure to detect infection in *S. australis* by PCR indicates that only a very low concentration of phytoplasma is ingested during feeding and that the phytoplasma does not multiply in the gut of these insects. This may be due in part to the different feeding behaviour of *S. australis*. Unlike *O. atkinsoni, S. australis* feeds only on the leaves of the plant and although PYL phytoplasma has been found in the leaves of New Zealand flax, it occurs at a much lower concentration than in the rhizome (Ushiyama *et al.* 1969).

The finding that *S. australis* did not transmit PYL phytoplasma or cabbage tree sudden decline and that phytoplasma was not detected in this insect by PCR, does not completely rule out the possibility that this species is a vector of one or both diseases. *S. australis* may be an inefficient vector such that only a few individuals in the population may be infective.

It is apparent that PCR will become a major tool in the search for potential insect vectors of plant pathogens. Insects which test positive in PCR must still be tested by transmission experiments due to the fact that phytoplasmas are able to multiply in non-vector species (Vega *et al.* 1993). However, the number of potential vectors to be tested by transmission experiments is reduced. For example, Maixner *et al.* (1995) selected four possible vectors of German grapevine yellows phytoplasma frequently collected in infected vineyards. PCR on these insects detected phytoplasma in only one of the species and transmission trials confirmed that this insect is a vector of German grapevine yellows phytoplasma. A further reduction in the number of potential vector species can probably be achieved by dissecting and testing the salivary glands of the insect species which tested positive from the total body extracts.

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