The fungus, *Lecanicillium muscarium*, is an entomopathogen of passionvine hopper (*Scolypopa australis*)

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Abstract A range of fungi were isolated from cadavers of passionvine hopper (*Scolypopa australis*) (Hemiptera: Ricaniidae) obtained in or close to kiwifruit (*Actinidia deliciosa*) orchards. Several isolates of *Lecanicillium muscarium* (Hyphomycetes), identified by morphological characters, were obtained which proved to be highly pathogenic to passionvine hopper nymphs. Cladograms based on internal transcribed spacer (ITS) and 5.8S rRNA genomic nucleotide sequences, with a single exception, supported the assignment of these isolates as *L. muscarium*, by comparison with other known isolates.

Keywords passionvine hopper; *Scolypopa australis*; *Lecanicillium muscarium*; entomopathogen; ribosomal DNA; *Verticillium lecanii*; *Cephalosporium aphidicola*

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H02035
Received 22 May 2002; accepted 27 November 2002

INTRODUCTION

Passionvine hopper (*Scolypopa australis* Walker) (Hemiptera: Ricaniidae) is widespread in New Zealand with an extensive host range including fruits, vegetables, ornamentals, and ferns. The insect can cause debility of host plants from both feeding activity and from black sooty mould growth on its honeydew (Scott 1984). It is an important economic pest on kiwifruit (*Actinidia deliciosa* (A. Chev) C.F. Liang et A.R. Ferguson) (Ferguson 1976).

Passionvine hopper is a native of Australia where it rarely reaches pest status because of the presence of a wide range of predators and parasitoids. In New Zealand, two egg parasites are present, *Centrodora scolypopae* (Hymenoptera: Aphelinidae) (Cumber 1966) and *Ablerus* sp. (Hymenoptera: Aphelinidae) (J. G. Charles unpubl. data), although these do not exert sufficient control over passionvine hopper as parasitism in New Zealand is very low (Scott 1984). There is a range of broad-spectrum pesticides which have excellent activity against passionvine hopper (Smith et al. 1980; Tomkins 1992); however, many of these pesticides are no longer registered for use on food crops. As a step to potentially reducing insecticide inputs, we investigated the possibility of entomopathogenic fungi associated with the passionvine hopper.

Verticillium section *Prostrata* has recently undergone revision (Zare et al. 2000; Zare & Gams 2001). The new combination *Lecanicillium muscarium* (Petch) Zare & Gams includes such synonyms as *Cephalosporium muscarium* Petch and *Cephalosporium aphidicola* Petch. Zare & Gams (2001) report that *L. muscarium* is distinct from *L. lecanii* on the basis of morphology, internal transcribed spacer (ITS) sequences, and restriction fragment length polymorphism (RFLP) patterns. Zare & Gams (2001) further indicate that many isolates used as biocontrol products as *L. lecanii* are probably *L. muscarium*. Here we report that isolates of *L. muscarium* from passionvine hopper are highly pathogenic to passionvine hopper nymphs.

METHODS

Isolation

Dead, diseased adult and nymph passionvine hoppers were obtained from or near kiwifruit orchards at Te Puke, New Zealand. The insects were covered with fungal mycelia. Fungi were isolated by two methods (Goettel & Inglis 1997). In the first method, fungal fragments were shaken briefly in 100 µl of sterile 0.25% Tween 20. Supernatant aliquots of 50 µl were then spread onto Petri plates of Sabouraud's Dextrose Agar (SDA) (Difco Laboratories, Detroit, MI, United States) and Potato Dextrose Agar (PDA) (Difco Laboratories) and incubated at room temperature until colonies appeared (2–3 days). In the second method, fungal or insect fragments were placed directly on SDA and PDA plates and incubated as described. Fungi were subcultured 3–4 times until 44 pure isolates were obtained. Actively growing cultures on SDA or PDA were chopped into small pieces and stored at –80°C in 10% glycerol. This stored material was used for all subsequent experiments.

Identification

Isolates were transferred to water agar, incubated at room temperature for 3–4 days to facilitate conidial formation, and examined by light microscopy. Isolate numbers were assigned to fungi from individual insects.

To construct a cladogram, the nuclear ITS1-5.8S rRNA-ITS2 region was sequenced. To obtain DNA sequences, selected isolates were grown at room temperature with shaking (100 rpm) for 5 days in 50 ml of YPDY (0.1% yeast extract, 1% peptone, 2% glucose, 0.1% yeast nitrogen base) (Bidochka et al. 1999). A modified method for plant DNA extraction (Willoughby et al. 1998) was used to isolate DNA from each of the fungal isolates. PDA plates, which had been covered with circles of sterile cellophane, were inoculated from the YPDY media (300 µl) and grown at 28°C for 4 days. Hyphae were ground into a fine powder in liquid nitrogen and 0.5 ml of powder was suspended in 0.75 ml of extraction buffer (50 m*M* Tris-HCl, pH 8.0, 50 m*M* EDTA, 0.15*M* NaCl, 2% Sarcosyl, pH 8.0). After addition of 0.75 ml of phenol/chloroform the mixture was incubated (4°C, 30 min), spun (5 min, 15 000*g*), and the upper phase transferred to a clean tube. DNA was precipitated with the addition of 0.6 volume isopropanol, centrifuged (15 min,15 000*g*), and resuspended in 300 µl TE buffer. RNA was digested with the addition of 10 µl of RNAse (10 mg/ml) at 37°C for 10 min before the DNA was re-extracted with 300 µl phenol/chloroform. DNA was isolated with the addition of cold 100% ethanol, centrifuged, air dried, and resuspended in 100 µl TE buffer. The ITS1-5.8S-ITS2 region was amplified using the primers TW81 and AB28 (Chambers et. al. 1986; Curran et al. 1994). PCR amplification was carried out in 25 µl reaction volumes containing 20–100 ng genomic DNA, 1 × PCR buffer, 1.5 m*M* $MgCl_2$, 50 µ*M* of each dNTP, 0.4 µ*M* of each primer and 1 U of High-Fidelity Platinum Taq polymerase (Roche Diagnostics, Auckland, New Zealand). The PCR reactions were denatured for 2 min at 94°C followed by 30 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C. The amplified PCR products were analysed on 1% agarose gels. PCR products of the expected size (c. 600 bp, similar to those of other *Verticillium* section *Prostrata* isolates) were purified (QIAquick PCR purification kit, Qiagen, Valencia, CA, United States). Sequencing was performed (Big Dye Terminator chemistry, Perkin-Elmer, Auckland, New Zealand) in both directions using the PCR primers TW81 and AB28. Sequence was verified from both strands using Sequencer™ version 2.06. Sequence data were aligned using CLUSTAL W (Thompson et al. 1994).

Cladogram construction and phylogenetic determination

The cladogram was generated using the Neighbor-Joining (distance matrix) method (Saitou & Nei 1987) using the program DNAman (Lynnon BioSoft, Canada). We utilised the data set of Zare et al. (2000) that contains clearly identified sequences from an extensive range of *Verticillium* isolates. Initial comparisons showed clearly that our isolates fell within Clade B (Zare et al. 2000). Thus Fig. 2 used largely sequences from within Clade B with representative sequences from other clades as outgroups. Bootstrapping represents 1000 samplings.

Bioassays

Bioassays were carried out at 20°C in the laboratory. A subset of isolates was tested, focused on isolates belonging to genera for which entomopathogenic species were previously known, i.e., *Lecanicillium* and the non-conidiating fungi. Other fungi isolated were assumed to be saprobes and only two, selected at random, were bioassayed.

These fungi were grown overnight in Sabouraud's Dextrose Broth (SDB) (Difco Laboratories), the fungal mass removed, washed twice in sterile

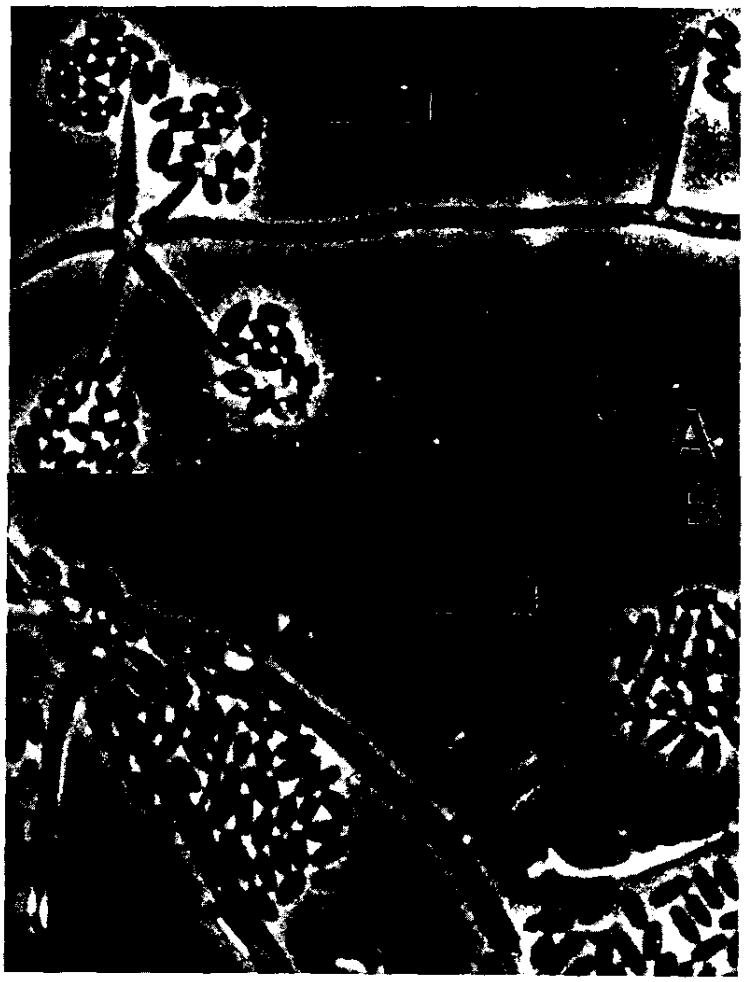

Fig. 1 *Lecanicillium muscarium*. **A**, Isolate F421 at 400× magnification. **B**, Isolate F427 at 400× magnification. Bars represent 10 μm.

distilled water, and ground briefly in a mortar with sterile distilled water. We used this procedure because not all of the isolates we wished to test produced conidia. None of the isolates produced conidia during the short period of submerged growth. Insects were immobilised by exposure to CO_2 then dipped into the medium for 1 s and allowed to dry before placing in 1.2 litre glass jars (Agee Glass Co., Auckland, New Zealand). Controls consisted of insects dipped into sterile water or SDB. Bioassays were carried out using 1–3 jars per isolate with 25–50 insects per jar between January and March 2000.

Passionvine hopper were collected from plants growing in gardens in Palmerston North. The vine *Pandorea pandorana* 'Snowbells' was used as the bioassay plant. The bases of three freshly harvested stems, to which were attached several leaves, were wrapped in wet cotton wool (held in place with aluminium foil and placed in the glass jars). Insects were introduced into each jar, which was loosely capped to prevent their escape. Mortality and disease progression was monitored for 2 weeks. Any dead insects were removed after 24 h and were not used in subsequent analyses. Dead insects were also removed from the jars after 1 week, but these were included in the analysis. Infection was determined at the end of the experiments by placing the dead insects on water agar for 2 days. The presence of *Lecanicillium* was verified by microscopical inspection of the fungi on the cadavers for the presence of diagnostic verticils of conidiogenous cells.

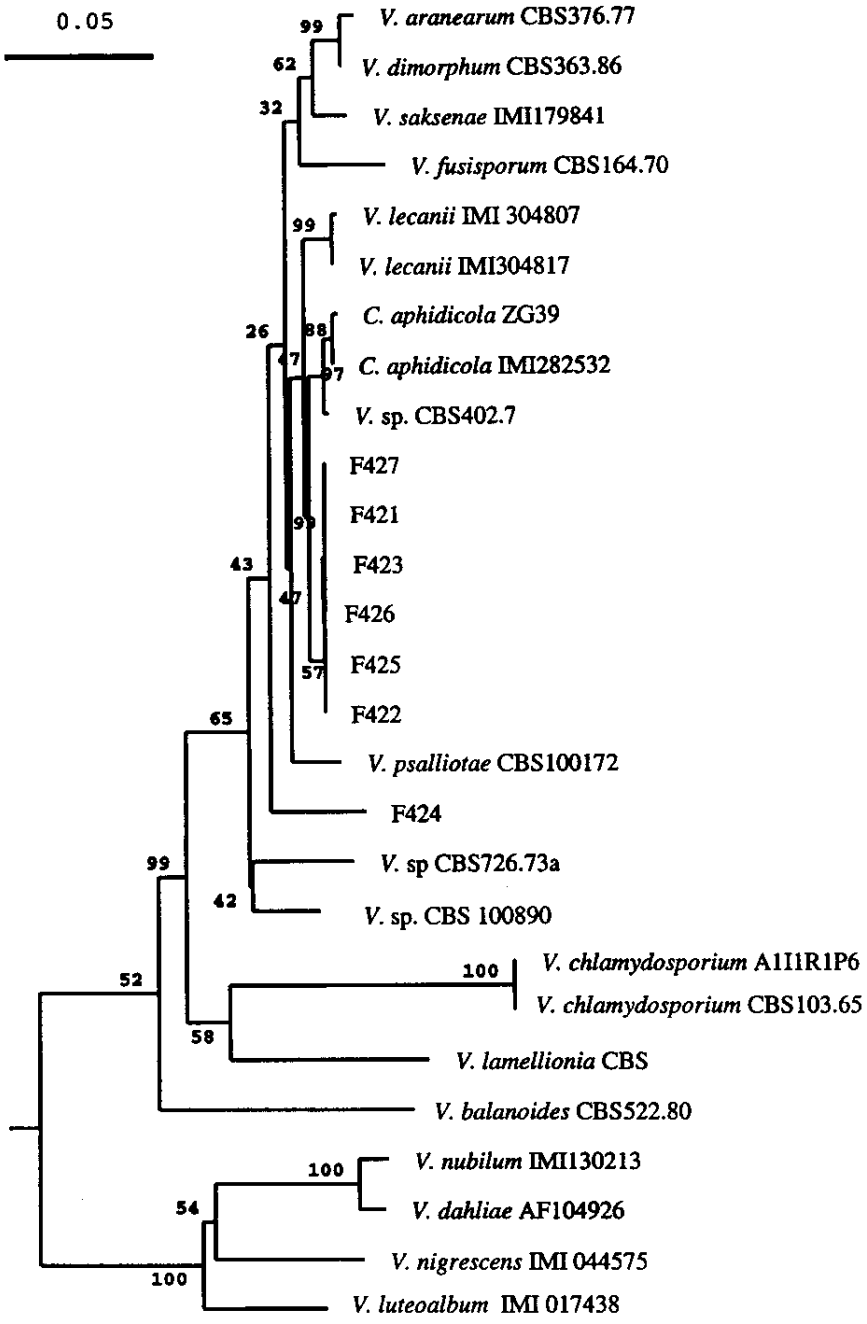

Fig. 2 Phylogenetic analysis of selected *Lecanicillium* spp. and the passionvine hopper pathogenic isolates. Bootstrap values as a percentage, obtained with 1000 resamplings, are shown at the nodes. Bar represents a change of 5% of the bases in the sequences of the isolates along the horizontal axis of the figure. Comparative isolates (except *Verticillium chlamydosporium*: AJ292397 (Morton, C. O.; Mauchline, T. H.; Kerry, B. R.; Hirsch, P.R. 2000 GenBank submission) and *V. dahliae*: AF104926 (Pramateftaki et al. 2000), were described by Zare et al. (2000) whose nomenclature in that paper is retained. Fungal genera are abbreviated: *C.* = *Cephalosporium*, *V.* = *Verticillium*. Representative isolates from beyond the *Lecanicillium* clade B were chosen as outgroups.

Deposition

The pathogenic isolates (F421–F427) have been deposited in the AgResearch Insect Pathogens Culture Collection (Glare et al. 1993), held in cryopreservation at the Canterbury Agriculture Research Centre, P.O. Box 60, Lincoln, New Zealand. Isolates not deposited retain their original numeric designations. ITS sequences for isolates F421–F427 have been entered into Genbank as accessions AF317536–AF317542.

RESULTS

Identification

A range of fungi was identified at 63× magnification that included *Lecanicillium*, *Trichoderma*, *Pithomyces*, *Fusarium*, *Pestalotia*, and *Cladosporium* spp. Several isolates produced a white mycelium but did not produce conidia and these remain unidentified.

To further characterise the *Lecanicillium* isolates, the morphology of seven pathogenic isolates (F421, F422, F423, F424, F425, F426, F427) was examined in the light microscope at 400× magnification (Fig. 1). The key in a recent revision of the genus (Zare & Gams 2001) was used to make a positive identification of these isolates as *L. muscarium*. All key diagnostic characters (Zare & Gams 2001) for the species *L. muscarium* were present in the isolates. Visually, all grew as white colonies on agar, with colourless mycelia and obvious verticils. Using light microscopy at a magnification of 63×, isolates had small water droplets on the tips of typically 2–4 phialides per whorl. At 400× magnification, a distinct size difference was observed, based on c. 25 measurements per isolate. Isolates F421, F422, F423, and F424 produced profuse, colourless, ellipsoidal to cylindrical conidia, 3.5–6 µm in length. Phialides were variable in length and tapering, with a maximum length of 25 µm (Fig. 1A). On the other hand, the other three isolates (F425, F426, and F427) produced profuse, colourless, ellipsoidal to cylindrical conidia, mostly 5–8.5 µm in length. Phialides were tapering, with a maximum length of 25 µm (Fig. 1B). There was no sign of the presence of chlamydospores.

rDNA sequencing and cladogram analysis

To confirm the morphology-based assignment, ribosomal DNA from the seven isolates that were examined morphologically was sequenced and a cladogram constructed (Fig. 2) as described. Over 532 continuous base pairs of sequence, five isolates were identical and another (F423) varied at a single position. The seventh isolate (F424) varied at 12 positions. All isolates grouped closely together as expected from the high degree of homology in their sequences, although isolate F424 was clearly separated from the other isolates (Fig. 2). The separation did not correlate with the differences in size of conidia. The analysis indicated that these fungal isolates are genetically closely related to *L. muscarium* although the identity of isolate F424 is less clear. The isolates other than F424 formed a clear subgroup almost equi-distant *from L. muscarium* (*C. aphidicola*) and *L. lecanii*, suggesting a variety level grouping of *L. muscarium*.

Bioassay

Isolates were bioassayed on up to three separate occasions (Table 1). In the first trial, 2–6 adults were included in each jar and by the end of the experiment no live adults were found. For this reason we restricted subsequent studies to nymphs, the primary target for future biocontrol measures. The results

Table 1 Bioassays to determine pathogenic isolates. Assays were carried out as described in Methods. Replicates (*R*) represent the number of individual jars into which were placed 25–50 insect nymphs. In some instances, 2–6 adults were also present.

Isolate	*R*	Total insects	% dead and infected
Control	10	365	10‖
F421*	3	79	89
F422*	2	46	87
F423*	1	25	84
F424*	1	25	68
F425*	3	64	88
F426*	1	27	96
66-11*	1	34	3
F427*	3	70	59
64-11†	1	20	25
64-13†	1	26	8
64-18†	1	27	26
66-4†	1	25	24
68-4†	2	51	31
68-5†	1	29	7
68-12†	1	35	11
68-14†	2	52	37
64-7y‡	1	23	26
66-7§	1	23	13

**Lecanicillium* isolates.
†Unidentified, non-conidiogenous isolates.
‡*Pestalotia* isolate.
§*Trichoderma* isolate.
‖Range 0–41%, median 8.6%, standard deviation 12.8%.

(Table 1) show that the isolates fell into two groups; those with >58% infection and those with <38% infection. Seven of the eight *Lecanicillium* isolates were in the highly pathogenic group and all of the non-conidiating and putatively saprobic isolates were in the weakly pathogenic-nonpathogenic group. A further single trial (data not shown) indicated that the isolates were pathogenic when presented as conidia.

DISCUSSION

By culturing fungus-infected passionvine hopper we have been able to isolate a range of fungi. Many of these were species not known to be entomopathogenic and were assumed to be saprobes. This assumption was substantiated by inclusion of two putative saprobes in preliminary screens, neither of which appeared to be highly pathogenic to living insects. The only fungus known to be entomopathogenic was *L. muscarium*, although several isolates never produced conidia in culture and remained unidentified. The results indicated that the majority of the *L. muscarium* isolates were highly pathogenic when inoculated onto passionvine hopper nymphs both as mycelial fragments (Table 1) and as conidia (data not shown).

The nymphal populations used in the bioassays were collected from the wild and were not selected for instar. The high mortality observed might be because of the high doses used, which could mask variation between instars in susceptibility. The appearance of adults in the bioassay may not indicate lower susceptibility of later instars since the final moult may have occurred before infection was established. The presence of a high load of *Lecanicillium* in only one of five collections in Palmerston North (data not shown) suggests that, although the fungus is widespread within New Zealand, its role in the population ecology may be variable.

Passionvine hopper has no close relatives in the native fauna. The most closely related species in New Zealand are also Australian, the green planthopper (*Siphanta acuta*, Flatidae) and the grey planthopper (*Sephena cinerea*, Flatidae) (J. Charles pers. comm.). The host range and origin of these *L. muscarium* isolates remain unidentified. The potential for use of these isolates as a biopesticide in kiwifruit orchards is being assessed.

Recent revision of *Verticillium* section *Prostrata* (Zare et al. 2000; Zare & Gams 2001) place *L. muscarium* in a clade with other insect pathogens, including *L. lecanii* and *L. longisporium*, based on morphology, ITS cladogram relationships, and RFLP analyses. Previously, *Verticillium* was a diverse genus of polyphyletic origin (Bidochka et al. 1999; Heale 2000; Zare et al. 2000), classified solely by morphological characteristics (Humber 1997). The nuclear ITS1-5.8S-ITS2 region has been identified as useful for providing molecular sequence data suitable for establishing fungal phylogenies (White et al. 1990; Curran et al. 1994; Bidochka et al. 1999; Zare et al. 2000; Zare & Gams 2001). Our ITS data form a cluster with known isolates of *L. muscarium* when analysed in association with the data set of Zare et al. (2000) thus supporting the morphological assignment of the isolates of the current study to *L. muscarium*.

This is the first report of which we are aware of a hemipteran species being a host to *L. muscarium*. Morphologically, the isolates we found are typical of the genus but appear to fall into two distinct morphotypes with large and small conidia. This is not unusual, for example, in a species of *Metarhizium* a similar situation has been reported with isolates having large or small conidia (Shin et al. 1997).

ACKNOWLEDGMENTS

We thank Bridget Maher (HortResearch, Te Puke) for collecting the dead passionvine hoppers and all members of our lab for providing the live ones; Mike Christensen (AgResearch, Palmerston North) and Adrian Spiers (HortResearch, Palmerston North) for assistance with microscopy and fungus identification; and Nicola Richard (AgResearch, Lincoln) for assistance with PCR. The research was supported by a grant (C06812) from the Foundation for Research, Science and Technology, New Zealand.

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