Hort Innovation

Final Report

Investigating Tree Mortality During Early Field Establishment

Project leader:

Dr Elizabeth Dann

Delivery partner:

University of Queensland

Project code:

AV14012

Project:

Investigating Tree Mortality During Early Field Establishment – AV14012

Disclaimer:

Funding statement:

This project has been funded by Hort Innovation, using the avocado research and development levy and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

Publishing details:

ISBN 978 0 7341 4415 7

Published and distributed by: Hort Innovation

Level 8
1 Chifley Square
Sydney NSW 2000

Telephone: (02) 8295 2300

www.horticulture.com.au

Content

Summary	4
Keywords	4
Introduction	5
Methodology	6
Outputs	9
Outcomes	11
Monitoring and evaluation	13
Recommendations	14
Refereed scientific publications	15
References	15
Intellectual property, commercialisation and confidentiality	16
Acknowledgements	16
Appendices	16

Summary

Black root rot, a severe disease of young avocado trees, is caused by soilborne fungal pathogens in the Nectriaceae family, with symptoms including black rotten roots, tree stunting and leaf wilt, often resulting in tree death within a year after transplanting into the orchard. This project has enhanced our knowledge of the fungi associated with black root rot, their relative ability to cause disease, some insights on how this disease may be managed, and has provided a molecular diagnostic test for rapid identification of the key pathogens.

More than 120 fungal isolates were collected from roots of nursery and field avocado trees, and a further 30 from other hosts, such as peanuts, blueberry, papaya and custard apple. Phylogenetic studies of the extracted fungal DNA, as well as traditional microscopy, have identified six genera including *Calonectria*, *Dactylonectria*, *Ilyonectria*, *Cylindrocladiella*, *Mariannaea* and *Gliocladiopsis*. The detailed analyses have assisted with the resolution of species and names of the fungi, which was often confusing in previous literature. Three new species of *Gliocladiopsis* were described, with several more new species or species complexes to be formally resolved and named. Many species were isolated from roots of both nursery and established field trees, however, *Calonectria ilicicola* seems to be specifically distributed in avocado nursery trees. Isolates of *C. ilicicola* from avocado were identical to those from peanut, papaya and custard apple. Pathogenicity studies demonstrated that *C. ilicicola* was the most aggressive of the fungi tested, and the knowledge of alternate hosts is new and important for orchard management, particularly where avocado may be planted into sites where these susceptible crops were previously grown. The four species of *Dactylonectria* tested were all pathogenic to avocado seedlings, however, are less aggressive pathogens than *C. ilicicola*, taking longer to cause extensive root rot and not causing stunting of plants. *Ilyonectria* (including species which cause black foot disease in grapevine), *Gliocladiopsis* and *Cylindrocladiella* species tested were not pathogenic on avocado seedlings. A molecular diagnostic test was developed and optimised which can detect the pathogenic *Calonectria* and *Dactylonectria* in necrotic roots in around half an hour. This is an extremely useful tool for the avocado industry, especially for the ANVAS clean planting scheme, but will also be valuable for other industries impacted by these pathogens. The test has already been successfully employed in a sample of avocado roots sent to a commercial diagnostic laboratory.

Options for managing black root rot were explored in this project. In particular, one fungicide, fludioxonil, was effective at low concentrations at inhibiting growth of the key fungi *in vitro*, and reduced root damage in seedlings, compared with other fungicides previously reported to be effective. Thus, it is likely to be feasible for suppressing, but probably not eliminating, the effects of the pathogen such that with careful post-planting care, the trees can produce new clean roots and establish even in the presence of the pathogen. There were indications from *in vitro* and field trials that incorporation of Brassica biofumigant crops prior to planting could be an effective disinfestation strategy in fields with pre-existing soilborne disease pressure. It is unlikely to be effective as a nursery treatment. Studies evaluating susceptibility of different rootstocks to the disease were inconclusive and further testing of root reaction to inoculation under controlled conditions is required. The efficacy of biofumigant green manure crops as a pre-plant disinfestation treatment will be evaluated more extensively in AV16007, based on information gained in this project.

The outputs from this project have been communicated to the international scientific and avocado communities, ANVAS nursery scheme reviewers as well as diagnostic laboratories in Brisbane, Perth and Mareeba. The pathology team will continue to support industry in its diagnosis and management of black root rot.

Keywords

Avocado, nectriaceous, black root rot, LAMP, biofumigation

Introduction

The nursery is a key element in any avocado orchard establishment program and therefore disease control in the nursery is essential. The fundamental principle of disease control in the nursery is that is preferable to avoid disease than to have to apply control once disease has developed. Many of these diseases are caused by soilborne or waterborne fungi, and these can generally be avoided by strict nursery hygiene procedures. *Phytophthora cinnamomi* has always been central to avocado nursery research, and the means to detect, identify and control this pathogen are well known.

However plant diseases are rarely static and in recent years other pathogens have been found causing black root rot disease in nurseries. Infection and serious destruction of roots of avocado and bay laurel (*Persea nobilis*) trees by *Calonectria ilicicola* (syn. *Cylindrocladium parasiticum*) in Australia and Italy, respectively, have recently been reported (Dann *et al.*, 2012; Polizzi *et al.*, 2012; Vitale *et al.*, 2012). *C. ilicicola* was isolated from roots of nursery material (Dann *et al.*, 2012). Related fungi, *Ilyonectria radicicola* or *I. macrodidyma* (syn. *Cylindrocarpon destructans*) have also been reported to cause severe losses in young avocado trees in South Africa (Darvas, 1979), Chile (Besoain and Piontelli, 1999), Spain (Lopez-Herrera and Melero-Vara, 1992), Israel (Zilberstein *et al.*, 2007), Italy (Vitale *et al.*, 2012) and New Zealand (A.W. Whiley and E.E. Jones, personal communication).

It is difficult to estimate economic losses due to these pathogens in the Australian avocado industry. Several growers have reported to us significant losses of young trees after planting, for example Grower 1 Lost 100/100 of trees planted, Grower 2 lost 450/500 trees planted, Grower 3 lost 80% and Grower 4 lost 30+% of trees planted. *Calonectria* sp. or *Ilyonectria* sp. were isolated from the roots of samples sent by these growers, thus indicating that in Australia a complex of species may be involved in these avocado root diseases. We are aware that other growers have had trees die soon after planting. Conservative estimates of tree loss, repurchase, soil fumigation and lost fruit production would be in excess of $1.5 min the last few years (Guest, 2014).

Our knowledge of these soilborne pathogens was limited prior to this project. The fungal nomenclature (names) were confusing and there was no systematic survey or isolation of these fungi from nurseries or orchards. Similarly, the pathogenicity of a range of species associated with black root rot was unknown. The aim of the project was to increase our understanding of this family of fungi causing black root rot disease responsible for tree deaths after outplanting, and to develop a rapid molecular-based diagnostic tool. Another objective was to investigate management options, so as to provide practical management procedures for nurserymen and growers. This will lead to improved tree establishment and health in avocado orchards.

Benefits to Industry will include:

1. The supply of healthy trees which are free of pathogens to growers
2. High establishment and growth rates of trees after outplanting. This will save replanting, and lead to vigorous growth and rapid onset of fruit production.
3. Recommendations on management of the diseases in the nurseries and also on-farm, which includes nursery hygiene, targeted use of fungicides, and disinfestation prior to planting.

Methodology

Collection of isolates, phylogenetic and pathogenicity studies

This research catalogued the nectriaceous fungi associated with black root rot disease of avocado (*Persea americana*) trees in Australia. Fungal isolates were collected from the roots of diseased and healthy avocado nursery trees, young orchard transplants and established orchard trees from growing regions in Queensland, New South Wales, Victoria, South Australia and Western Australia. Nectriaceous fungi were also collected from other hosts, including grapevine, peanut, pinto peanut, papaya, custard apple, blueberry, tea tree, *Elaeocarpus* sp., *Heliconia* sp., camphor laurel (*Cinnamomum camphora*), *Endiandra* sp. and *Cryptocarya* sp. Tissue around characteristic lesions or black spots were transferred to fungal growth media, and fungi belonging to the Nectriaceae family were selected for molecular characterisation based on the shape and size of the spores after microscopic examination. A total of 91 plants were sampled and 153 fungal isolates were identified or classified using phylogenetic analyses of partial sequences of ITS, β-tubulin and histone H3 gene loci (Appendix 1).

Glasshouse pathogenicity tests of selected *Nectriaceae* isolates collected from avocado roots and other hosts were undertaken. Inoculum of test isolates was prepared by growing on an autoclaved bran:sand media, and this inoculum was incorporated into potting media when seedlings of avocado were re-potted. Plant heights, root and foliar biomass, and root necrosis were all assessed after several weeks. Full details on the method and results have been published (Parkinson et al. 2017b, Appendix 4).

Development of LAMP molecular diagnostic assay

Loop-mediated isothermal amplification (LAMP) molecular diagnostic assays were developed for the rapid detection of *Calonectria ilicicola* (Ci) *Dactylonectria macrodidyma* (Dm), and species within the broader *Dactylonectria* genus in avocado roots. Histone H3 and β-tubulin gene sequence data from the large phylogenetic analysis were used to design species-specific LAMP primers which anneal to target fungal DNA and amplify the target gene sequence for diagnostic detection. The LAMP primers were tested for specificity and sensitivity on 82 fungal isolates, which included the target species, *C. ilicicola* and *D. macrodidyma*, *D. anthuriicola*, *D. novozelandica*, *D. pauciseptata* and *D. vitis*; and isolates of non-target species including *Calonectria* sp., *Cylindrocladiella* sp., *Cy. pseudoinfestans*, *Gliocladiopsis forsbergii*, *G. peggii*, *G. whileyi*, *Ilyonectria* sp., *Mariannaea* sp., *M. humicola*, *Fusarium* sp. (non-target species) and *Phytophthora cinnamomi* (non-target). Validation of the LAMP diagnostic was demonstrated by testing the diagnostic with purified DNA, cultured fungal mycelia mixed cultures containing both target pathogens, and in inoculated avocado roots. The diagnostic assay was modified to include a DNA extraction step for use with fresh plant tissue. Glasshouse cv. Reed seedlings, 3 or 9 months old, were inoculated with each pathogen separately or co-inoculated with both pathogens. Roots were collected after 2 weeks. The assays were modified to accommodate a DNA extraction step and use of avocado roots as DNA templates. Detection in avocado roots ranged between 12 min 30 s – 24 min 45 s for *C. ilicicola*, 12 min – 25 min 30 s for *D. macrodidyma* and 14 min – 29 min 45 s for species in *Dactylonectria*. The specificity of the assays were found to be dependent on time and isothermal amplification temperature, with optimal specificity occurring in reactions under 30 minutes and at temperatures of 67°C for *C. ilicicola* and *D. macrodidyma* assays and at 69°C for *Dactylonectria* genus-wide assays. The species-specific LAMP assays were sensitive and specific at DNA concentrations of 0.001 ng/µl for *C. ilicicola* and 0.01 ng/ µl for *D. macrodidyma*, while the *Dactylonectria* genus-wide assay was sensitive to 0.1 ng/µl.

In vitro, in planta and in-field biofumigation studies

In vitro Petri dish assays were conducted by amending molten agar with 0.5 (equivalent to 50t/Ha fresh), 1 or 2g powdered brassica, Caliente, Mustclean, Biofum, BQ Mulch, Nemat or Tillage, (previously harvested, dried, ground to a fine powder), then pouring plates and placing a plug of Dm, Ci or *Phytophthora cinnamomi* (Pc) in the centre of the plate and measuring colony diameter. The activity of volatile compounds was assessed by placing the powdered brassica in the bottom of a Petri dish, adding water to activate, and then inverting another Petri dish containing agar and a plug of inoculum over the biofumigant and taping both halves of the Petri dish together so that the brassica powder was not in contact with the fungus.

Glasshouse trials *Dactylonectria macrodidyma* (Dm) and *Phytophthora cinnamomi* (Pc) were selected as the soilborne pathogens of interest for the study with glasshouse seedlings. While *Calonectria ilicicola* is highly pathogenic to avocado, it does not have the same widespread distribution as Dm, and was not included in the

present study. The commercially available biofumigants Nemat (*Eruca sativa)* and Caliente (*Brassica juncea*) were obtained from John Duff (DAF, Gatton). Whole plants were harvested at full-flowering stage and oven-dried at 40°C, ground to a fine powder and maintained in the freezer until use. Composted chicken manure was obtained from Anderson Horticulture, Duranbah. There were 12 treatments, with 9 or 10 seedlings per treatment. Biofumigant, 7g DW, or composted chicken manure 20g, was introduced at the same time as fungal pathogen inoculum, during transplanting of seedlings to larger pots. Pots were watered and sealed with a plastic bag for 2 days to retain any volatile compounds produced. Plant heights were measured weekly, and the trial terminated at 6 weeks by unpotting plants, washing roots and evaluating % root necrosis, and measuring fresh and dry weights of root systems and above-ground tissues.

Field testing Gauze pouches containing approximately 10–15 dried wheat grain colonized with *C. ilicicola* or *D. macrodidyma* were prepared for use in the field experiment. Field tests were conducted in collaboration with John Duff, DAF, Gatton. Commercial *Brassica* Biofum *(Raphanus sativus + Sinapsis alba)*, BQ Mulch *(Brassica napus + B. campestris)*, and Caliente, Mustclean and Nemfix (all Indian mustard, *Brassica juncea*) were grown from seed, each in three replicate field plots of approximately 2 × 0.5 m for 59–89 days. The control plots were left fallow. The experimental field plots were rotary hoed shortly after flower development, and the fresh macerated *Brassica* spp. were incorporated into the top soil (Fig. 1). The pouches of wheat grain inoculum were buried adjacently in 10 cm trenches (Fig. 2) immediately after Brassica mulch incorporation, then recovered three and seven days later for plating onto media to determine viability of fungus. The trial was conducted over multiple weeks in September through to November, 2017, with incorporation, burial and recovery of inoculum occurring on different dates, and were subject to varied weather conditions.

Fig. 1 – Incorporation of Brassica biofumigant, BQ Mulch, as fresh mulch via rotary hoe.

Fig. 2 – Inoculum pouches of *Calonectria ilicicola* and *Dactylonectria macrodidyma* buried in 10 cm trenches (left) containing incorporated Brassica biofumigant (right).

In vitro and in planta fungicide studies

Eight fungicides (prochloraz, imazilil, propiconazole, paclobutrazol, azoxystrobin, A19649B, chlorothalonil and fludioxonil) were tested for their *in vitro* efficacy against one isolate each of *C. ilicicola*, *D. macrodidyma* and *D. novozelandica*. A 1:9 serial dilution of each fungicide was prepared and incorporated into molten media. An agar plug containing the test fungus was placed in the centre of the plate and colony diameters measured and compared against non-fungicide amended control plates.

Glasshouse fungicide trials were conducted by inoculating seedlings (Reed or Zutano) with either Dm or Ci, allowing infection to establish for at least 2 weeks, then drenching with test fungicide solution. Assessments of plant and root growth parameters and root necrosis were undertaken several weeks later.

Rootstock evaluation

Two glasshouse experiments were conducted to evaluate development of root rot disease in different avocado cultivars after inoculation with *Calonectria ilicicola* or *Dactylonectria macrodidyma*. There were 6 rootstock varieties tested in the first experiment, Hass seedlings, Reed seedlings, Zutano seedlings, Dusa clones, SHSR04 clones and Velvick as both seedlings and clonally-propagated plants. In the second experiment, Reed seedlings, Dusa clones and Velvick clones were evaluated.

Industry support, extension, training etc.

Presentations have been delivered at several Australian avocado grower field days, R&D updates, and also at scientific conferences, World Avocado Congress 2015, South African Avocado Growers Research Symposium and Avocado Brainstorming. A full list of these is provided in Outputs section. There have also been articles published in grower literature. Industry support activities include interaction with ANVAS scheme coordinators, nursery operators, individual growers and other state government diagnostic pathology labs (eg. WA, NSW, Growhelp and Mareeba).

Outputs

1. Report on the pathogenicity and epidemiology of root pathogens of avocado to be delivered in the project final report.

This research tested the pathogenicity of 19 isolates from *Calonectria*, *Cylindrocladiella*, *Dactylonectria*, *Gliocladiopsis* and *Ilyonectria*, spp. collected from young avocado trees and other hosts. Glasshouse pathogenicity tests with avocado (*Persea americana*) cv. Reed seedlings confirmed that *Calonectria ilicicola* is a severe pathogen of avocado, causing stunting, wilting and seedling death within 5 weeks of inoculation. Isolates of *Calonectria ilicicola* from peanut, papaya and custard apple, were also shown to be aggressive pathogens of avocado, demonstrating a broad host range. An isolate of *Calonectria* sp. from blueberry, and avocado isolates of *Dactylonectria macrodidyma*, *D. novozelandica*, *D. pauciseptata* and *D. anthuriicola* caused significant root rot, but not stunting within 5–9 weeks of inoculation. An isolate of *Ilyonectria* sp. from grapevine closely related to *I. liriodendri*, and avocado isolates of *Cylindrocladiella pseudoinfestans*, *Gliocladiopsis peggii* and *Ilyonectria* sp. were not pathogenic to avocado (Parkinson et al. 2017b, Appendix 4).

Isolates associated with black root rot of avocado were recovered in six genera of fungi in the *Nectriaceae* namely, *Calonectria* (15 isolates), *Cylindrocladiella* (2 isolates), *Dactylonectria* (64 isolates), *Gliocladiopsis* (28 isolates), *Ilyonectria* (41 isolates) and *Mariannaea* (3 isolates). The species identified were *Calonectria ilicicola*, *Cylindrocladiella pseudoinfestans*, *Dactylonectria macrodidyma*, *D. novozelandica*, *D. anthuriicola, D. vitis*, *D. pauciseptata* and *Mariannaea humicola*. Over 20 new species across 6 genera in the *Nectriaceae* have been provisionally identified. There were some unresolved taxa in paraphyletic clades and species complexes in a number of the genera examined. A draft manuscript on this work is in preparation).

2. Report of disinfestation practices including fungicides and biofumigants for the management of soilborne diseases to be delivered in the project final report.

Eight fungicides were tested for their efficacy against one isolate each of *C. ilicicola*, *D. macrodidyma* and *D. novozelandica* (see Appendix). There were large differences in efficacy, including between *Calonectria* and *Dactylonectria* isolates. A new chemistry from Syngenta was highly inhibitory to all isolates. Fludioxonil was also inhibitory at extremely low concentrations of active ingredient (a.i.). Prochloraz and propiconazole were inhibitory at low concentrations to *Dactylonectria* isolates, while chlorothalonil was efficacious against *Calonectria*. Imazilil, paclobutrazol and azoxystrobin were the least inhibitory fungicides to all isolates.

Two fungicide trials with inoculated glasshouse seedlings have demonstrated that fludioxonil (Scholar) is extremely inhibitory to Ci, resulting in reduced root necrosis (both experiments) and increased root biomass (Experiment 2, Appendix). This demonstrates the efficacy of fludioxonil to at least partially inhibit growth of the aggressive pathogen such that roots are able to regenerate. In a nursery situation, and in newly planted trees in orchards, this suppression (it is unlikely there will be complete eradication of the pathogen) will be sufficient to retard disease development allowing for sufficient healthy root regeneration to establish the trees in the field.

Field trials conducted at Gatton demonstrated that burying wheat seeds colonised with *Dactylonectria macrodidyma* in plots at the same time as incorporation of brassica biofumigant species (particularly Caliente, Mustclean and Nemfix), initially suppressed viability of the fungus, but did not completely eradicate it at 7 days after incorporation. There was no significant suppression of viability of *Calonectria ilicicola* by any of the brassica incorporation treatments. The study found that time also has an effect on the efficacy of Brassica sp. on fungal suppression and regular incorporation of Brassica biofumigants into orchard soils may reduce pre-existing *Dactylonectria* inoculum and potentially improve mortality of young orchard transplants.

3. Report on treatments which have potential for management of the diseases in the nurseries and also on-farm to be delivered in the project final report and through relevant industry communication mechanisms such as Talking Avocados, or grower/industry workshops.

The most promising result from the laboratory and glasshouse work demonstrating the efficacy of drench applications with fludioxonil (Scholar) to reduce root necrosis caused by Calonectria. The glasshouse studies are being repeated in AV16007 for *C. ilicicola* and *D. macrodidyma*, and the intention is to prepare this work for peer-review publication. Care must be taken (and advice sought from Hort Innovation's R&D Manager for Chemicals) prior to communicating this information to Industry in any format, as fludioxonil is not registered for soil drench

application in avocado (or other nursery).

In vitro studies investigating effects of biofumigant brassica plant tissue on growth of *Phytophthora cinnamomi*, *C. ilicicola* and *D. macrodidyma* were promising, with a clear demonstration of inhibition of fungal growth. There was a clear inhibition by volatile compounds emanating from moistened brassica tissue, demonstrating the "biofumigation" effect. Effects in glasshouse and field trials were less convincing, however. In the first glasshouse experiment, amendment of potting media with Caliente reduced root necrosis in avocado seedlings by nearly 40% five weeks after inoculation with *Phytophthora cinnamomi*, compared with plants that had been inoculated but received no amendment treatment. However, Caliente did not reduce root necrosis caused by Dm. In the second glasshouse experiment, root necrosis after inoculation with Dm was reduced by Nemat and Caliente amendment, but not significantly. There was no effect of Caliente improving root health after inoculation with Pc.

4. A molecular diagnostic test for two soilborne pathogens, *Calonectria ilicicola* and *Dactylonectria macrodidyma*

Three loop-mediated isothermal amplification (LAMP) diagnostic assays were developed for rapidly identifying *C. ilicicola*, *D. macrodidyma* complex and species in the *Dactylonectria* genus in avocado roots. The species-specific LAMP assays were sensitive and specific at DNA concentrations of 0.001 ng/µl for *C. ilicicola* and 0.01 ng/ µl for *D. macrodidyma*, while the *Dactylonectria* genus-wide assay was sensitive to 0.1 ng/µl. Detection of *C. ilicicola* occurred within 10 mins 5 s – 14 min 54 s or 15 min – 29 min 15 s when the template was pure DNA or fungal mycelia respectively. Detection of *D. macrodidyma* was between 12 min 16 s – 28 min 30 s with pure DNA and 16 min 13 s – 29 min 21 s with fungal mycelia. Detection using the *Dactylonectria* genus-wide assay found *Dactylonectria* spp. detection ranging 6 min – 24 min 5 s with fungal DNA and 7 min 15 s – 23 min with fungal mycelia. The specificity of the assays were found to be dependent on time and isothermal amplification temperature, with optimal specificity occurring in reactions under 30 minutes and at temperatures of 67°C for *C. ilicicola* and *D. macrodidyma* assays and at 69°C for *Dactylonectria* genus-wide assays. The assays were modified to accommodate a DNA extraction step and use of avocado roots as DNA templates. Detection in avocado roots ranged between 12 min 30 s – 24 min 45 s for *C. ilicicola*, 12 min – 25 min 30 s for *D. macrodidyma* and 14 min – 29 min 45 s for species in *Dactylonectria*. *Calonectria ilicicola* was detected in 100% (9 out of 9) of avocado seedlings inoculated or co-inoculated with *C. ilicicola*, with a detection speed ranging ~12 mins – 22 mins. *Dactylonectria macrodidyma* was detected in 87.5% (7 out of 8) of plants inoculated or co-inoculated with *D. macrodidyma*, with a detection speed ranging ~12 – 23 mins. Consistent detection of *D. macrodidyma* in avocado roots may be subject to level of infection. (see draft manuscript sent separately).

The Calonectria LAMP assay has already been successfully used to test suspect roots obtained in a recent ANVAS sample, demonstrating its immediate usefulness to industry.

5. High quality scientific research papers arising from the related PhD project and biofumigation aspects of the study

There has been significant scientific and industry-relevant communication arising from this project.

Student Thesis

Parkinson, L. E. 2017. Investigating soilborne nectriaceous fungi impacting avocado tree establishment in Australia, PhD Thesis, University of Queensland.

Papers already published

- Parkinson, L., Shivas, R., Dann, E. 2018. *Dactylonectria* Lombard and Crous, Pathogen of the Month – April 2018 https://www.appsnet.org/Publications/POTM/PDF/Apr18.pdf
- Parkinson, L. E., Shivas, R. G., Dann, E. K. 2017. Pathogenicity of nectriaceous fungi on avocado in Australia. *Phytopathology* **107**, 1479-1485. http://dx.doi.org/10.1094/PHYTO-03-17-0084-R
- Parkinson, L. E., Shivas, R. G., Dann, E. K. 2017. Novel species of *Gliocladiopsis* (*Nectriaceae, Hypocreales, Ascomycota*) from avocado roots (*Persea americana*) in Australia. *Mycoscience* **58**, 95-102.

Papers in advanced stages of preparation

- Parkinson, L. E., Shivas, R. G., Dann, E. K. (2018?) Development of a LAMP assay for detection of Calonectria ilicicola and Dactylonectria spp., fungi causing black root rot, in preparation.
- Parkinson, L. E., Shivas, R. G., Dann, E. K. (2018?) Phylogeny and diversity of nectriaceous fungi associated with black root rot of avocado (*Persea americana*) in Australia, in preparation.

Seminars and poster presentations at conferences, workshops and field days

- Grower field days in 2014 – NNSW (April), SE QLD (May),
- AAL R&D meeting, June 2014
- QAAFI Annual Research Meeting Nov 2014
- Australasian Soilborne Disease Symposium, Hobart, November 2014
- 8th World Avocado Congress, Lima, Peru, September 2015
- TropAg, Brisbane, September 2015
- Grower field days in 2016 – SE QLD (May and June), Central NSW (Nov)
- TropAg, November 2017
- Avocado R&D Planning Forum 2017, Brisbane. 8–9 November 2017
- Science Protecting Plant Health 2017, Brisbane. 25–29 September 2017
- South African Avocado Growers Association Research Symposium February 2017
- Grower field days in 2017 – WA (June), North QLD (Oct), Central NSW & Bundaberg (Nov)
- Avocado Brainstorming researchers meeting, Tzaneen, South Africa, May 2018
- AVOCO NZ growers conference, Auckland, June 2018
- International Congress of Plant Pathology, Boston, USA, July 2018

Talking Avocados articles, other publications

- L. Parkinson, E. Dann and R. Shivas (2017) Black root rot of avocado – what do we know and how can we manage it? Talking Avocados 28(3):35-39.
- E. Dann, L. Parkinson (2017) Three other diseases impacting avocado productivity in Australia. South African Avocado Growers Association Yearbook, 40: 24-27.
- E. Dann (2015) Investigating black root rot in avocado: A new research project, Talking Avocados, 26(3):30-31.

Outcomes

1. We would expect that 90% of avocado trees received by growers from nurseries for planting will be free of soilborne pathogens within 3 years of project completion.

This will partly be achieved by our input into the revision and support of the ANVAS clean planting scheme. The significant new information concerning pathogenic species and their accurate and rapid diagnosis is critical to ensure an effective clean planting scheme. It is a concern that there are many "pop-up" nurseries now in Australia, given the buoyancy of the industry and demand for trees. The vast majority of nurseries are not ANVAS accredited and are not subjected to routine testing and accountability. Our study demonstrated that black root rot pathogens are easily detected in such nurseries, which inevitably means that thousands of trees may be planted with pre-existing infections by soilborne pathogens (not only *Calonectria* and *Dactylonectria*, but potentially *Phytophthora* as well). Nursery operators (whether part of ANVAS or not), have access to diagnostic labs for routine testing of their stock. It would be advantageous for all nurseries to engage in frequent testing, and depending on the circumstances, destruction of trees deemed to be infected. Trees with a low level of infection may establish well if all care is taken during transportation, planting and post-planting, including appropriate use of prophylactic fungicides or anti-oomycete chemistry, good irrigation (particularly, not over-irrigating which would exacerbate disease problems), correct nutrition and planting during the least environmentally stressful times of the year. This outcome will be realized over the next several years.

2. Improved establishment of orchard blocks with outplanting survival rates in excess of 95%, within 3 years of project completion, through clean planting material and adoption of recommended management practices.

The project has identified pathogens (other than *Phytophthora cinnamomi*) which are responsible for tree mortality during the initial stages (12 months) of establishment. This information is available now and is being extended to the industry via articles in *Talking Avocados*, personal communication with nurseries, and presentations at industry field days. The awareness of these pathogens has thus increased considerably since project commencement. Through AV16007, we will continue to transfer our project outputs to industry with the aim of boosting nursery best practice. As mentioned above, an impediment to full adoption is the craving by growers, both established and new, for planting material, and the explosion of back yard nurseries which are unregulated and do not necessarily adhere to best hygiene practices. This has potentially come at a cost to tree quality, although there is no information available on numbers or cost of tree losses due to inferior (eg. diseased) stock.

Any nursery or orchard management strategy relies on timely and accurate diagnosis of causal organisms. This project has delivered detection and identification tools for the key pathogens causing black root rot. These tools are available now to all nurseries in the industry. Knowledge of presence or absence of pathogens is critical in any clean planting scheme, and may have implications on planting and establishment strategies, such that mortality post planting can be minimized. While frequent application of fungicide drenches in the nursery is never recommended, we have shown in this project that fludioxonil, the active component in Scholar®, effectively reduces severity of black root rot allowing feeder root regeneration and development of a strong root system required for establishment under field conditions. Scholar is not currently registered for this use pattern, and discussions with the Nursery and Garden Industry of Australia, the Hort Innovation R&D Manager for Chemicals, and Syngenta are warranted to investigate registration options.

Other management practices such as resistant rootstocks and pre-plant disinfestation strategies are likely to be important, but trials in this project did not conclusively demonstrate effects which could be immediately translated to commercial production. There are likely to be opportunities within AV16007 to pursue these strategies further.

3. Enhanced capacity for Industry and for the pathology R&D project team via increased knowledge on improved disease management gained through the project.

The project has contributed vital information to the pathology team, industry, and the international plant pathology discipline. Accurate diagnosis is critical for establishing whether a pathogen or disease is present and recommending any management strategies. Not all soilborne plant pathogens are managed the same way. The pathology team has published a key paper in a seminal international plant pathology journal on which of the fungi associated with black root rot are severe pathogens. The same information has been extended to the industry via articles in Talking Avocados, conference and field day presentations, and numerous personal communications with nursery operators, diagnosticians and growers, as appropriate. This capacity is being further strengthened as new team members are trained and become experts on this group of fungi.

4. At least two publications submitted to refereed scientific journals, and two articles in Talking Avocados by the end of the project (May 2018).

Two papers have already been published in international peer-reviewed journals, and another two are in advanced stages of preparation and will be submitted in August-September, 2018. Two articles have been published in *Talking Avocados*, and one in the SAAGA Yearbook, 2017. There is no doubt that this research has already had significant scientific and industry impact. There are at least three more peer-reviewed journal articles to be published from this project within the next 12-18 months. These include 1) the efficacy of different fungicides on reduction of root necrosis and improvement in plant growth parameters in plants infected with *Calonectria ilicicola* or *Dactylonectria macrodidyma* 2) The full characterization and phylogeny of all isolates used in this study, including the molecular description and naming of several new species, taxa or species complexes and 3) details of the loop mediated isothermal amplification (LAMP) assay. Key components of these papers will be distilled for publication and/or dissemination in media more appropriate for industry, enabling adoption and realization of all outcomes.

5. Student expected to graduate with her PhD before the end of the project (May 2018).

Louisa Parkinson fulfilled the requirements for her PhD in September 2017 and graduated in December 2017. The capacity for undertaking pathology research has been boosted, with the training of the student, and her subsequent training of other students, colleagues and staff within the team and eternally, with respect to this group of soilborne pathogens. We are fortunate that Louisa has taken a position as post-doctoral fellow in project AV16010, concerning avocado biosecurity capacity and preparedness.

Monitoring and evaluation

This project was contracted prior to the introduction of the M&E plan requirement, however some discussion on the success and impact of this project is provided below.

This project had a total budget from Hort Innovation of <$200,000, and was conducted over 4.5 years from February 2015 to July 2018. The knowledge generated and impact to be realized is thus substantial for such a relatively small investment. The success of the project relied upon the hard work of an excellent PhD candidate. Such students are few and far between and we were fortunate that most of her stipend was paid by an Australian Postgraduate Award, significantly reducing salary cost to the project budget. Louisa has thus been trained in many aspects of basic and highly molecular plant pathology techniques, and gained knowledge of many aspects of the Australian and international avocado industries. As a post-doctoral fellow, Louisa continues to be employed in the industry, via project AV16010. She remains a valuable resource to the pathology team, and will soon be co-supervising other students. Capacity for plant pathology and biosecurity research within horticulture, and avocados specifically, has thus been enhanced.

There is no doubt that this project has significantly increased the global understanding of black root rot in avocado, and the relatedness of causal fungi to those from other hosts. Thus, the applications from this project reach beyond avocado to other horticultural crops and peanuts, the nursery industry and diagnostic facilities around the country. This is the first study to comprehensively survey and analyse the group of pathogens associated with black root rot of avocado. There were few reports prior to this project, and they reported on species associated with black root rot, often without testing to evidence respective pathogenicity. We now know that there are 6 genera associated with black root rot symptoms, but of those tested so far, only *Calonectria* and *Dactylonectria* were shown to be pathogenic on avocado seedlings. *Calonectria* seems to be restricted to nursery origins, while there are multiple species of *Dactylonectria* in nurseries and mature orchards. We don't believe *Dactylonectria* spp. alone is causing decline in tree health, or productivity in mature orchards, and this would be difficult to determine.

The demonstration of a fungicide drench which can significantly reduce root death in infected seedlings is a key finding of this project. Fludioxonil was more effective than prochloraz, which was previously reported in the literature (well prior to the development and release of the new generation fludioxonil) to be effective. Fungicide drench did not completely eliminate (kill) the fungi, so any further registration and use of this fungicide for use in the nursery or soon after planting will have to be part of an integrated disease management strategy.

The study has highlighted the importance of correct nomenclature (naming) of fungal genera and species, so that pathogenic fungi can be correctly diagnosed and management practices implemented. For example, industry previously used the name *Cylindrocarpon* as one genera of pathogenic fungi. This study showed that *Cylindrocarpon* is now actually split into several new species, and in avocado *Dactylonectria* is pathogenic while the *Ilyonectria* isolates tested were not pathogenic on avocado seedlings. Industry is being encouraged to adopt the new names to avoid confusion and potential misdiagnoses.

Recommendations

1. Discussions should be initiated with the industry and nursery representatives about the value of offering (and advertising) a screening service to all nurseries, not just ANVAS members, to check for presence or absence of these pathogens. The cost will have to be borne by the nurseries who participate and send samples. This service is essentially already available through GrowHelp Australia, who have the capacity for invoicing clients. The avocado pathology team have worked closely with GrowHelp for many years. The ability of GrowHelp to accurately detect and diagnose the black root rot pathogens has been significantly enhanced through the interaction during this project.

2. Growers should be encouraged to source trees from accredited nurseries to ensure they are free of the black root rot pathogens. Avocados should not be planted in ground which may be infested with these pathogens from previous susceptible crops, such as peanut, papaya, custard apple or blueberry. Best practices for site preparation, planting and post-planting care, particularly irrigation management, should be strictly followed. Nursery trees which have a sparse, necrotic root system should not be planted, and a sample sent to a diagnostic lab. These simple procedures are outlined in the YouTube avocado tree planting video.

3. Fludioxonil drench applications have been shown to reduce root necrosis caused by two pathogenic species of black root rot fungi. Final glasshouse trials are currently being completed, but are expected to confirm the efficacy of this fungicide. It is recommended that this information be shared with the Hort Innovation R&D Manager for Chemicals, NGIA and Syngenta to facilitate discussion concerning whether fludioxonil should be registered (emergency or minor use) for nurseries. Although this fungicide does not eradicate the pathogens, it seems to suppress the fungus and limit new infections from occurring such that new feeder roots can emerge and the plant can effectively out-grow the pathogen, leading to successful establishment. If required, the data may be supplied to support application for registration through APVMA.

4. Continue to investigate biofumigation with brassica species for disinfestation of soilborne pathogens prior to planting. This is currently being done as part of project AV16007, however the target pathogen is *Phytophthora cinnamomi*, as there are no sites heavily infested with the nectriaceous pathogens which would be suitable for such a trial. Two brassica species and chicken manure will be compared with untreated plots at a site with high Phytophthora disease pressure. After incorporation of the plant and chicken manure, one half of the plots will be covered with plastic to seal in the fumigants. After several weeks, nursery avocado trees will be planted and their establishment over the ensuing months monitored.

5. *Mariannaea* sp. was one of the genera isolated at low frequencies and identified in the phylogenetic analyses, and has not been tested for its pathogenicity in avocado. Therefore, it is recommended that this be tested to determine its ability to cause root rot and other symptoms in avocado.

6. It is recommended that the project team continue to disseminate information on black root rot to industry and provide support to ANVAS, nursery operators and growers who are impacted by these pathogens. This will include further articles in *Talking Avocados*, information for the online Best Practice Resource and other extension materials, presentations at field days, R&D meetings and other conferences, as well as assisting diagnostic laboratories with correct detection and identification of fungi associated with tree mortality. Any opportunity to investigate the epidemiology of these pathogens, eg. how they enter and spread throughout nurseries, should be utilized as appropriate. These activities will be continued as contracted in project AV16007, and as such no further industry R&D investment is required, at this stage.

7. Although not reported here, Louisa conducted preliminary experiments on the role of fungal toxins from *Calonectria ilicicola* and *Dactylonectria macrodidyma* in the infection and development of disease in avocado, and this has been reported in other crops. It is recommended that a student/s continue to investigate this important interaction, as insights into the underlying mechanisms of pathogenicity may elucidate targets for management of the disease.

Refereed scientific publications

Journal articles

1. Parkinson, L. E., Shivas, R. G., Dann, E. K. (2018?) Development of a LAMP assay for detection of *Calonectria ilicicola* and *Dactylonectria* spp., fungi causing black root rot, in preparation.

2. Parkinson, L. E., Shivas, R. G., Dann, E. K. (2018?) Phylogeny and diversity of nectriaceous fungi associated with black root rot of avocado (*Persea americana*) in Australia, in preparation.

3. Parkinson, L., Shivas, R., Dann, E. 2018. *Dactylonectria* Lombard and Crous, Pathogen of the Month – April 2018 https://www.appsnet.org/Publications/POTM/PDF/Apr18.pdf

4. Parkinson, L. E., Shivas, R. G., Dann, E. K. 2017a. Novel species of *Gliocladiopsis* (*Nectriaceae, Hypocreales, Ascomycota*) from avocado roots (*Persea americana*) in Australia. *Mycoscience* **58**, 95-102.

5. Parkinson, L. E., Shivas, R. G., Dann, E. K. 2017b. Pathogenicity of nectriaceous fungi on avocado in Australia. *Phytopathology* **107**, 1479-1485. http://dx.doi.org/10.1094/PHYTO-03-17-0084-R

Whole book (Thesis)

Parkinson, L. E. 2017. Investigating soilborne nectriaceous fungi impacting avocado tree establishment in Australia, PhD Thesis, University of Queensland.

References

Besoain, X.C. and Piontelli, E.L. (1999) Black root rot in avocado plants (*Persea americana* Mill.) by *Cylindrocarpon destructans*: pathogenicity and epidemiological aspects. *Boletin Micologico* 14, 41-47.

Dann, E.K., Cooke, A.W., Forsberg, L.I., Pegg, K.G., Tan, Y.-P. and Shivas, R.G. (2012) Pathogenicity studies in avocado with three nectriaceous fungi, *Calonectria ilicicola*, *Gliocladiopsis* sp. and *Ilyonectria liriodendri*. *Plant Pathology* 61, 896-902.

Darvas, J.M. (1979) Ecology of avocado root pathogens. *South African Avocado Growers' Association Research Report* 3, 31-32.

Guest, D (2014) HAL Project AV13022 "Review of AV13005", unpublished.

Lopez-Herrera, C.J. and Melero-Vara, J.M. (1992) Diseases of avocado caused by soil fungi in the southern Mediterranean coast of Spain In 'Second World Avocado Congress' pp. 119-121

Polizzi, G., Vitale, A., Aiello, D., Guarnaccia, V., Crous, P. and Lombard, L. (2012) First Report of Calonectria ilicicola Causing a New Disease on Laurus (Laurus nobilis) in Europe. *Journal of Phytopathology* 160, 41-44.

Vitale, A., Aiello, D., Guarnaccia, V., Perrone, G., Stea, G. and Polizzi, G. (2012) First Report of Root Rot Caused by Ilyonectria (=Neonectria) macrodidyma on Avocado (Persea americana) in Italy. *Journal of Phytopathology* 160, 156-159.

Zilberstein, M., Noy, M., Levy, E., Elkind, G., Zeidan, M., Teverovski, E. and Ben Ze'ev, I. (2007) Wilting disease of young avocado trees caused by *Neonectria radicicola* in Israel. In '6th World Avocado Congress'. (Viña Del Mar, Chile)

Intellectual property, commercialisation and confidentiality

No project IP, project outputs, commercialisation or confidentiality issues to report.

Acknowledgements

The majority of this project was undertaken by PhD student Louisa Parkinson. Louisa was the recipient of an Australian Postgraduate Award, for the duration of her PhD studies. She was awarded a University of Queensland Graduate Student International Travel Award in 2015, which allowed her to attend (and present her work) at the World Avocado Congress in Peru, and the associated Study Tour of Chile.

Prof Roger Shivas (Biosecurity Queensland) was a co-supervisor during Louisa's candidature, and is continuing to assist with publications. Ms Yu Pei Tan and Dr Alastair McTaggart (Biosecurity Queensland) provided assistance to Louisa for the molecular and phylogenetic analyses, and interpretation of data.

Dr Paulo de Souza was a visiting scientist with the avocado pathology team from March 2016 to May 2017, and he propagated the clonal plants (Dusa, Velvick and SHSR04) for the rootstock trials and assisted with evaluation. Paulo and Dr Duy Le conducted the *in vitro* assays with the biofumigant species. Dr Akila Devi Prabhakaran has undertaken the glasshouse trials evaluating the effects of fungicides in infected seedlings.

Mr John Duff (Queensland Department of Agriculture and Fisheries) provided Brassica material for testing *in vitro* and *in planta*, and assisted with the field trial evaluations at Gatton.

Anderson Horticulture provided seedlings for the various glasshouse screening tests, and we thank Graham (Snr) and David Anderson for their assistance with our research.

Several plant pathology colleagues provided isolates of fungi from hosts other than avocado, for the study. Thanks to Dr Andrew Manners and Jan Dean (Growhelp), Dr Ken Pegg (QDAF), Mr Peter Trevorrow and Ms Kathy Grice (DAF Mareeba), Dr Len Tesoriero and Dr Rose Daniel (NSW DPI).

Finally, a very big thank you to the many nursery and orchard operators we visited in order to build the collection of isolates. The ongoing support for our research by individual growers and businesses and industry as a whole, is greatly appreciated.

Appendices

1. Summary and results of work which has not yet been published, eg. details of isolate collection, biofumigation, fungicide and rootstock studies

2. Parkinson, L., Shivas, R., Dann, E. 2018. *Dactylonectria* Lombard and Crous, Pathogen of the Month – April 2018 https://www.appsnet.org/Publications/POTM/PDF/Apr18.pdf

3. Parkinson, L. E., Shivas, R. G., Dann, E. K. 2017a. Novel species of *Gliocladiopsis* (*Nectriaceae, Hypocreales, Ascomycota*) from avocado roots (*Persea americana*) in Australia. *Mycoscience* **58**, 95-102.

4. Parkinson, L. E., Shivas, R. G., Dann, E. K. 2017b. Pathogenicity of nectriaceous fungi on avocado in Australia. *Phytopathology* **107**, 1479-1485. http://dx.doi.org/10.1094/PHYTO-03-17-0084-R

Appendix 1

Details of fungal isolate collection

Table 1. Nectriaceous fungal isolate collection identified with sequencing and multigene phylogenetic analyses of ITS, β-tubulin and histone H3.

Herbarium Accession (BRIP) no.	**Fungal Isolate**	**Host**	**Locality**
53653 a	*Calonectria ilicicola*	*Persea americana*	QLD
53933 a	*Calonectria ilicicola*	*Carica papaya*	South Johnstone, QLD
54018 a	*Calonectria ilicicola*	*Persea americana*	QLD
55531 a	*Calonectria ilicicola*	*Persea americana*	Evelyn, QLD
60388	*Calonectria ilicicola*	*Arachis hypogaea*	Atherton, QLD
60389	*Calonectria ilicicola*	*Arachis hypogaea*	Tolga, QLD
60397	*Calonectria ilicicola*	*Arachis hypogaea*	Kairi, QLD
60982	*Calonectria ilicicola*	*Persea americana*	Childers, QLD
60992	*Calonectria ilicicola*	*Carica papaya*	South Johnstone, QLD
61291	*Calonectria ilicicola*	*Annona reticulata*	Woombye, QLD
15920 a	*Calonectria* sp.	*Annona squamosa*	Eumundi, QLD
16747 a	*Calonectria* sp.	*Arachis pintoi*	Eumundi, QLD
60981	*Calonectria* sp.	*Vaccinium* sp.	NSW
61448	*Calonectria* sp.	*Melaleuca alternifolia*	Narellan, NSW
63712	*Calonectria* sp.	*Heliconia bihai x H. caribaea*	Eumundi, QLD
60986	*Cylindrocladiella pseudoinfestans*	*Persea americana*	Woombye, QLD
61292	*Cylindrocladiella* sp.	*Cinnamomum camphora*	Alstonville, NSW
60985	*Dactylonectria anthuriicola*	*Persea americana*	Hampton, QLD
61306 b	*Dactylonectria anthuriicola*	*Persea americana*	Bellthorpe, QLD
61429 b	*Dactylonectria anthuriicola*	*Persea americana*	Duranbah, NSW
61437 b	*Dactylonectria anthuriicola*	*Persea americana*	Childers, QLD
60907 b	*Dactylonectria macrodidyma*	*Persea americana*	Childers, QLD
60979	*Dactylonectria macrodidyma*	*Persea americana*	Hampton, QLD
61090 c	*Dactylonectria macrodidyma*	*Persea americana*	Beechmont, QLD
61090 f	*Dactylonectria macrodidyma*	*Persea americana*	Beechmont, QLD
61195 d	*Dactylonectria macrodidyma*	*Persea americana*	Comboyne, NSW
61259 c	*Dactylonectria macrodidyma*	*Persea americana*	Norfolk Island
61260 c	*Dactylonectria macrodidyma*	*Persea americana*	Norfolk Island
61294 a	*Dactylonectria macrodidyma*	*Persea americana*	Alstonville, NSW

61294 b	*Dactylonectria macrodidyma*	*Persea americana*	Alstonville, NSW
61306 a	*Dactylonectria macrodidyma*	*Persea americana*	Bellthorpe, QLD
61349 e	*Dactylonectria macrodidyma*	*Persea americana*	Mullumbimby, NSW
61349 f	*Dactylonectria macrodidyma*	*Persea americana*	Mullumbimby, NSW
61427 a	*Dactylonectria macrodidyma*	*Persea americana*	Alstonville, NSW
61431 c	*Dactylonectria macrodidyma*	*Persea americana*	Green Pigeon, NSW
61434 a	*Dactylonectria macrodidyma*	*Cryptocarya* sp.	Moggill, QLD
61436 a	*Dactylonectria macrodidyma*	*Persea americana*	Childers, QLD
61440 a	*Dactylonectria macrodidyma*	*Persea americana*	Pemberton, WA
61442	*Dactylonectria macrodidyma*	*Persea americana*	Manjimup, WA
61444 a	*Dactylonectria macrodidyma*	*Persea americana*	Capel, WA
61546 a	*Dactylonectria macrodidyma*	*Persea americana*	Robinvale, VIC
61546 b	*Dactylonectria macrodidyma*	*Persea americana*	Robinvale, VIC
61546 c	*Dactylonectria macrodidyma*	*Persea americana*	Robinvale, VIC
61546 d	*Dactylonectria macrodidyma*	*Persea americana*	Robinvale, VIC
61546 e	*Dactylonectria macrodidyma*	*Persea americana*	Robinvale, VIC
61546 f	*Dactylonectria macrodidyma*	*Persea americana*	Robinvale, VIC
61546 k	*Dactylonectria macrodidyma*	*Persea americana*	Robinvale, VIC
62000 b	*Dactylonectria macrodidyma*	*Persea americana*	Gol Gol, NSW
62000 g	*Dactylonectria macrodidyma*	*Persea americana*	Gol Gol, NSW
62001 a	*Dactylonectria macrodidyma*	*Persea americana*	Robinvale, VIC
62001 b	*Dactylonectria macrodidyma*	*Persea americana*	Robinvale, VIC
62002 a	*Dactylonectria macrodidyma*	*Persea americana*	Robinvale, VIC
62002 b	*Dactylonectria macrodidyma*	*Persea americana*	Robinvale, VIC
62005 a	*Dactylonectria macrodidyma*	*Persea americana*	Waikerie, SA
62005 c	*Dactylonectria macrodidyma*	*Persea americana*	Waikerie, SA
52550 a	*Dactylonectria pauciseptata*	*Persea americana*	Woombye, QLD
60991 a	*Dactylonectria pauciseptata*	*Persea americana*	Mt Binga, QLD
61089	*Dactylonectria pauciseptata*	*Persea americana*	Mareeba, QLD
61295 d	*Dactylonectria pauciseptata*	*Persea americana*	Alstonville, NSW
61428 b	*Dactylonectria pauciseptata*	*Persea americana*	Nimbin, NSW
61428 c	*Dactylonectria pauciseptata*	*Persea americana*	Nimbin, NSW
61428 d	*Dactylonectria pauciseptata*	*Persea americana*	Nimbin, NSW
61432 c	*Dactylonectria pauciseptata*	*Cinnamomum camphora*	Moggill, QLD
61433 a	*Dactylonectria pauciseptata*	*Endiandra* sp.	Moggill, QLD
61436 c	*Dactylonectria pauciseptata*	*Persea americana*	Childers, QLD
62007 a	*Dactylonectria pauciseptata*	*Persea americana*	Hampton, QLD
63707 a	*Dactylonectria pauciseptata*	*Persea americana*	Busselton, WA
63711 b	*Dactylonectria pauciseptata*	*Persea americana*	Waikerie, SA
63713	*Dactylonectria pauciseptata*	*Persea americana*	Duranbah, NSW

61195 b	*Dactylonectria vitis*	*Persea americana*	Comboyne, NSW
61263 e	*Dactylonectria vitis*	*Carica papaya*	Norfolk Island
61263 f	*Dactylonectria vitis*	*Carica papaya*	Norfolk Island
61263 g	*Dactylonectria vitis*	*Carica papaya*	Norfolk Island
61263 h	*Dactylonectria vitis*	*Carica papaya*	Norfolk Island
61263 i	*Dactylonectria vitis*	*Carica papaya*	Norfolk Island
63708 b	*Dactylonectria vitis*	*Elaeocarpus eumundii*	New Farm, QLD
62000 a	*Dactylonectria zovozelandica*	*Persea americana*	Gol Gol, NSW
62000 c	*Dactylonectria zovozelandica*	*Persea americana*	Gol Gol, NSW
62000 d	*Dactylonectria zovozelandica*	*Persea americana*	Gol Gol, NSW
61352 c	*Dactylonectria* sp.	*Persea americana*	Norfolk Island
61354 c	*Dactylonectria* sp.	*Persea americana*	Norfolk Island
60984	*Gliocladiopsis forsbergii*	*Grevillea* sp.	Burbank, QLD
61349 a	*Gliocladiopsis forsbergii*	*Persea americana*	Mullumbimby, NSW
53654	*Gliocladiopsis peggii*	*Persea americana*	QLD
60983	*Gliocladiopsis peggii*	*Persea americana*	Woombye, QLD
60988	*Gliocladiopsis peggii*	*Persea americana*	Walkamin, QLD
62003 a	*Gliocladiopsis peggii*	*Persea americana*	Hampton, QLD
62008 a	*Gliocladiopsis peggii*	*Persea americana*	Hampton, QLD
62843 a	*Gliocladiopsis peggii*	*Persea americana*	Hampton, QLD
62844 a	*Gliocladiopsis peggii*	*Persea americana*	Sunshine Coast, QLD
62844 c	*Gliocladiopsis peggii*	*Persea americana*	Sunshine Coast, QLD
62845 a	*Gliocladiopsis peggii*	*Persea americana*	Duranbah, NSW
62845 e	*Gliocladiopsis peggii*	*Persea americana*	Duranbah, NSW
63709 c	*Gliocladiopsis peggii*	*Persea americana*	Woombye, QLD
63709 d	*Gliocladiopsis peggii*	*Persea americana*	Woombye, QLD
63710 a	*Gliocladiopsis peggii*	*Persea americana*	Woombye, QLD
63711 d	*Gliocladiopsis peggii*	*Persea americana*	Waikerie, SA
61430	*Gliocladiopsis whileyi*	*Persea americana*	Duranbah, NSW
54019	*Gliocladiopsis* sp.	*Persea americana*	QLD
60987	*Gliocladiopsis* sp.	*Persea americana*	Walkamin, QLD
60990	*Gliocladiopsis* sp.	*Persea americana*	Woombye, QLD
61446 b	*Gliocladiopsis* sp.	*Persea americana*	WA
62845 b	*Gliocladiopsis* sp.	*Persea americana*	Duranbah, NSW
62845 c	*Gliocladiopsis* sp.	*Persea americana*	Duranbah, NSW
62845 d	*Gliocladiopsis* sp.	*Persea americana*	Duranbah, NSW
63709 a	*Gliocladiopsis* sp.	*Persea americana*	Woombye, QLD
63709 b	*Gliocladiopsis* sp.	*Persea americana*	Woombye, QLD
63710 c	*Gliocladiopsis* sp.	*Persea americana*	Woombye, QLD
63711 c	*Gliocladiopsis* sp.	*Persea americana*	Waikerie, SA

53498 a	*Ilyonectria* sp.	*Vitis vinifera*	Hunter Valley, NSW
53652 a	*Ilyonectria* sp.	*Persea americana*	QLD
54020 a	*Ilyonectria* sp.	*Persea americana*	QLD
55532 a	*Ilyonectria* sp.	*Persea americana*	Evelyn, QLD
60980	*Ilyonectria* sp.	*Persea americana*	Duranbah, NSW
60989	*Ilyonectria* sp.	*Persea americana*	Duranbah, NSW
61193 a	*Ilyonectria* sp.	*Persea americana*	Comboyne, NSW
61193 b	*Ilyonectria* sp.	*Persea americana*	Comboyne, NSW
61194 a	*Ilyonectria* sp.	*Persea americana*	Comboyne, NSW
61194 b	*Ilyonectria* sp.	*Persea americana*	Comboyne, NSW
61194 c	*Ilyonectria* sp.	*Persea americana*	Comboyne, NSW
61349 d	*Ilyonectria* sp.	*Persea americana*	Mullumbimby, NSW
61293	*Ilyonectria* sp.	*Persea americana*	Duranbah, NSW
61432 b	*Ilyonectria* sp.	*Cinnamomum camphora*	Moggill, QLD
61435 b	*Ilyonectria* sp.	*Persea americana*	Childers, QLD
61435 c	*Ilyonectria* sp.	*Persea americana*	Childers, QLD
61438 b	*Ilyonectria* sp.	*Persea americana*	Mt Binga, QLD
61090 a	*Ilyonectria* sp.	*Persea americana*	Beechmont, QLD
61303 d	*Ilyonectria* sp.	*Persea americana*	Bellthorpe, QLD
61352 f	*Ilyonectria* sp.	*Persea americana*	Norfolk Island
61439 c	*Ilyonectria* sp.	*Persea americana*	Bellthorpe, QLD
61441	*Ilyonectria* sp.	*Persea americana*	Pemberton, WA
61443	*Ilyonectria* sp.	*Persea americana*	Manjimup, WA
61446 a	*Ilyonectria* sp.	*Persea americana*	WA
61546 g	*Ilyonectria* sp.	*Persea americana*	Robinvale, VIC
61546 h	*Ilyonectria* sp.	*Persea americana*	Robinvale, VIC
61546 i	*Ilyonectria* sp.	*Persea americana*	Robinvale, VIC
61546 j	*Ilyonectria* sp.	*Persea americana*	Robinvale, VIC
62000 e	*Ilyonectria* sp.	*Persea americana*	Gol Gol, NSW
62000 f	*Ilyonectria* sp.	*Persea americana*	Gol Gol, NSW
62002 c	*Ilyonectria* sp.	*Persea americana*	Robinvale, VIC
62002 d	*Ilyonectria* sp.	*Persea americana*	Robinvale, VIC
62002 e	*Ilyonectria* sp.	*Persea americana*	Robinvale, VIC
62002 f	*Ilyonectria* sp.	*Persea americana*	Robinvale, VIC
62004 b	*Ilyonectria* sp.	*Persea americana*	Robinvale, VIC
63705 a	*Ilyonectria* sp.	*Persea americana*	Duranbah, NSW
63707 b	*Ilyonectria* sp.	*Persea americana*	Busselton, WA
63711 f	*Ilyonectria* sp.	*Persea americana*	Waikerie, SA
63711 g	*Ilyonectria* sp.	*Persea americana*	Waikerie, SA
63714 a	*Ilyonectria* sp.	*Persea americana*	Duranbah, NSW

63714 b	*Ilyonectria* sp.	*Persea americana*	Duranbah, NSW
63711 e	*Mariannaea humicola*	*Persea americana*	Waikerie, SA
61192 c	*Mariannaea* sp.	*Persea americana*	Comboyne, NSW
61192 f	*Mariannaea* sp.	*Persea americana*	Comboyne, NSW

Results of Biofumgiant and Fungicide Trials

In vitro and in-field biofumigation studies

Several trials were undertaken in the lab, glasshouse and field with a range of brassica biofumigant species. John Duff, DAF, Gatton, assisted with providing fresh and dried brassica material, and by allowing us to test viability of inoculum in the field trials. Table 2. summarises the various species we used, and their active glucosinolate compound.

Table 2. Brassica biofumigant species used for *in vitro*, glasshouse and field studies

Name	Brassica	Glucosinolate
BQ Mulch	*Brassica napus* + *B. campestris*	glucobrassicanapin + sinigrin
Caliente	*B. juncea*	sinigrin
Mustclean	*B. juncea*	sinigrin
Nemat	*Eruca sativa*	glucoerucin
Biofum	*Raphanus sativus* + *Sinapsis alba*	glucoraphanin
Tillage radish	*R. sativus*	glucoraphanin
Nemfix	*B. juncea*	sinigrin

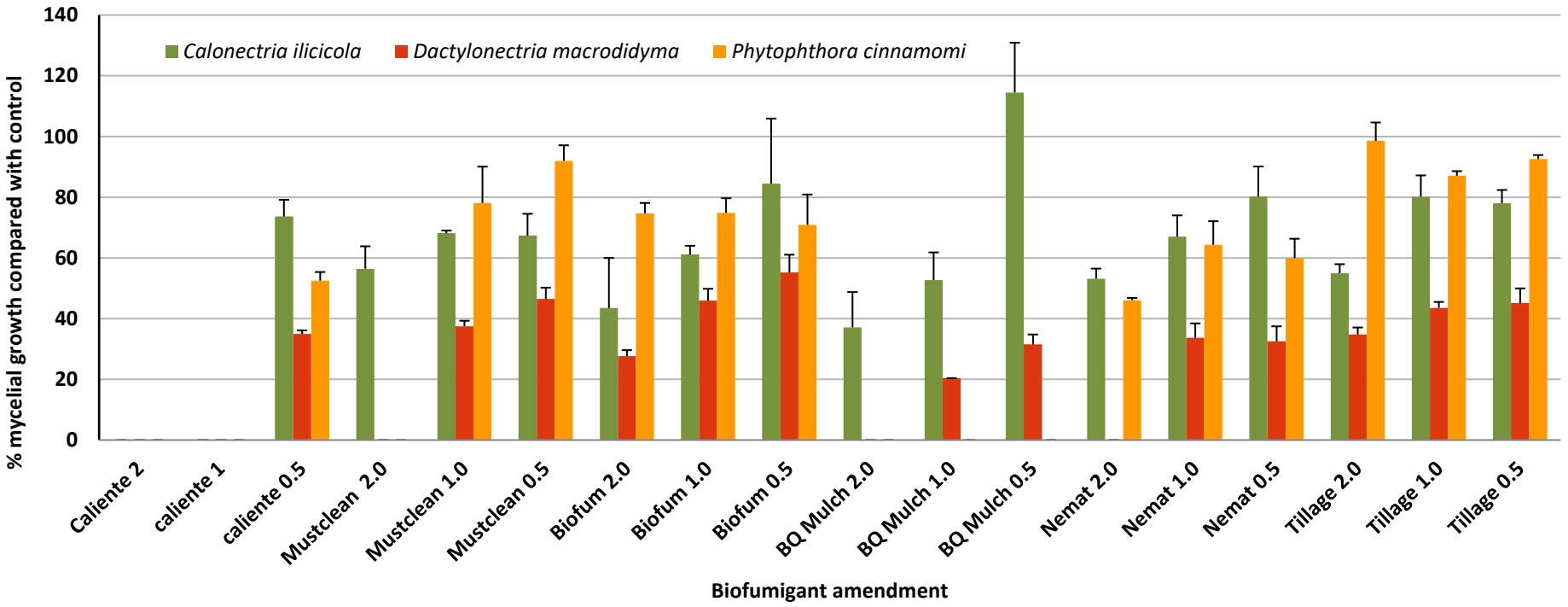


Figure 1. Effect of amending media with brassica biofumigant on growth of three soilborne pathogens of avocado *in vitro*. n=3 replicate Petri dishes for each biofumigant species and concentration for each pathogen tested. Bars represent standard errors of the means.

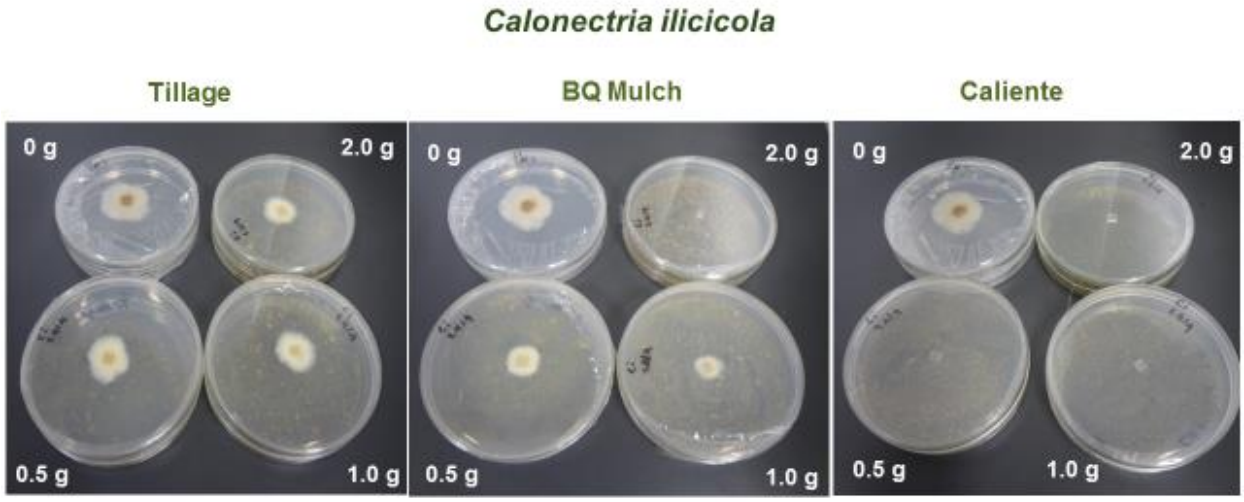


Figure 2. Effect of brassica biofumigant volatile compounds on growth of *Calonectria ilicicola in vitro*

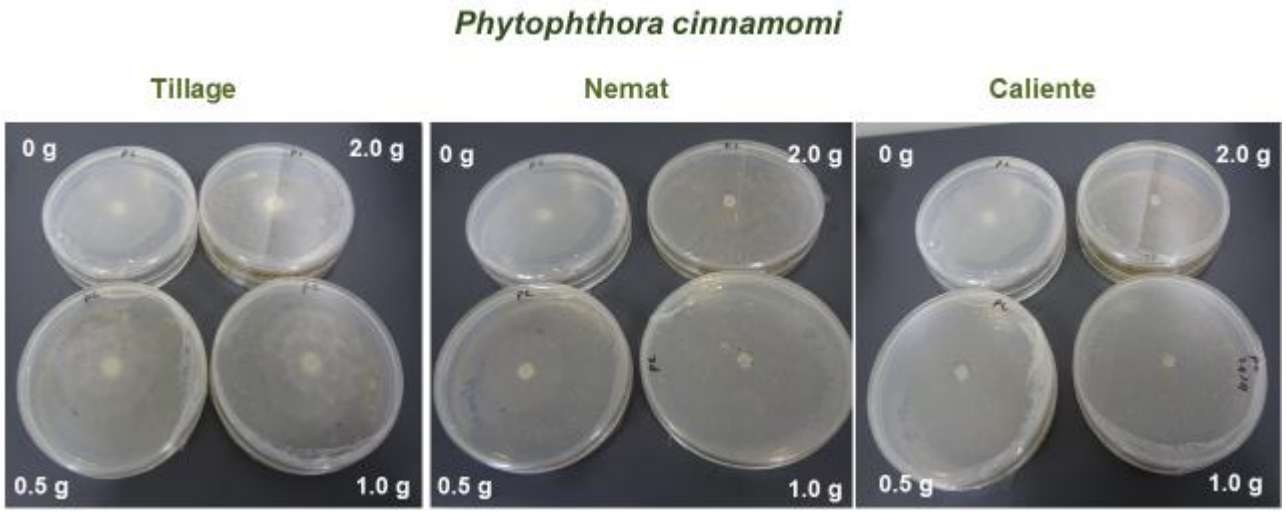


Figure 3. Effect of brassica biofumigant volatile compounds on growth of *Phytophthora cinnamomi in vitro*

Testing the effect of Brassica biofumigant amendment of potting mix for root rot and plant health after inoculation of cv. Hass seedlings with *Phytophthora cinnamomi* and *Dactylonectria macrodidyma* under glasshouse conditions

Table 3. The effect of two biofumigants, Nemat and Caliente, and composted chicken manure on growth, biomass and root necrosis of avocado seedling cv. Hass, after inoculation with soilborne pathogens *Phytophthora cinnamomi* or *Dactylonectria macrodidyma*, Experiment 1

Treatment	n	Change in height (cm)	Leaves+ stems FW (g)	Roots FW (g)	Leaves+ stems DW (g)	Roots DW (g)	% necrotic roots	
No treatment, no pathogen	8	14.8	26.6	16.6	6.69	1.54	18.8	e
No treatment, Pc	8	10.8	37.3	19.9	11.3	2.44	72.5	a
No treatment, Dm	8	18.8	47.9	22.7	12.0	2.01	45.0	bcd
Nemat, no pathogen	8	18.9	30.3	17.1	8.04	1.71	30.6	de
Nemat, Pc	8	16.2	35.8	21.6	9.35	2.11	62.5	ab
Nemat, Dm	8	19.1	39.4	21.4	9.93	2.38	45.0	bcd
Caliente, no pathogen	8	12.5	32.4	17.5	9.36	2.34	35.6	cde
Caliente, Pc	8	14.8	36.4	17.3	10.8	2.31	45.0	bcd
Caliente, Dm	8	13.8	29.7	14.5	7.12	1.53	52.5	abc
Chicken manure, no pathogen	8	14.4	39.2	24.2	10.5	2.62	16.3	e
Chicken manure, Pc	8	11.6	34.7	16.8	9.74	1.85	60.0	ab
Chicken manure, Dm	8	19.2	42.9	17.1	10.9	1.81	63.8	ab
P		**0.695**	**0.061**	**0.815**	**0.161**	**0.920**	**<0.001**	

Growth and root necrosis assessed five weeks after treatment and inoculation. For % necrotic roots, means followed by the same letter(s) are not significantly different at P<0.05. For all other parameters, there were no significant differences among treatments as indicated by P>0.05.

Table 4. The effect of two biofumigants, Nemat and Caliente, and composted chicken manure on growth, biomass and root necrosis of avocado seedling cv. Hass, after inoculation with soilborne pathogens *Phytophthora cinnamomi* (Pc) or *Dactylonectria macrodidyma* (Dm), Experiment 2

Treatment	n	Change in height (cm)		Leaves+ stems FW (g)		Roots FW (g)		Leaves+ stems DW (g)		Roots DW (g)		% necrotic roots	
No treatment, no pathogen	10	3.5	a	25.3	ab	13.4	ab	6.80	a	1.88	b	9.5	e
No treatment, Pc	10	0.7	d	14.8	fg	5.05	de	4.30	cd	0.79	d	70.1	b
No treatment, Dm	10	2.9	ab	15.5	fg	4.04	e	4.01	cd	0.88	d	27.5	cd
Nemat, no pathogen	10	2.4	abc	21.3	bcde	12.1	abc	5.63	ab	1.75	b	13.3	e
Nemat, Pc	9	1.8	bcd	18.0	defg	10.7	bc	5.15	bc	1.53	bc	76.2	ab
Nemat, Dm	10	3.3	a	24.2	abc	9.40	c	5.97	ab	1.94	b	19.1	de
Caliente, no pathogen	10	3.1	ab	17.1	efg	9.93	bc	4.74	bcd	1.59	b	15.8	de
Caliente, Pc	9	1.2	cd	18.5	def	4.41	e	5.59	ab	0.95	cd	67.0	b
Caliente, Dm	10	3.2	a	21.6	bcd	10.2	bc	5.84	ab	1.77	b	20.5	de
Chicken manure, no pathogen	10	2.7	ab	20.3	cde	8.50	cd	4.96	bcd	1.74	b	15.3	e
Chicken manure, Pc	9	0.7	d	13.7	g	2.64	e	3.79	d	0.67	d	86.2	a
Chicken manure, Dm	10	3.3	a	26.4	a	14.7	a	6.86	a	2.67	a	35.2	c

Growth and root necrosis was evaluated six weeks after treatment and inoculation. Within each column, means followed by the same letter are not significantly different at $P<0.05$.

Testing the efficacy of Brassica biofumigants for reducing *Calonectria ilicicola* and *Dactylonectria macrodidyma* inoculum under field conditions

Figure 3 shows how the effects of biofumigant incorporation on fungal inoculum viability was assessed. Wheat grain, which had been colonised with one of the soilborne pathogens, then buried in trenches the same time as the brassica biofumigant was incorporated, was recovered 3 or 7 days after burial and surface sterilised then plated onto selective media. The numbers of grain with target fungal growth was recorded as a percentage of total grains plated.

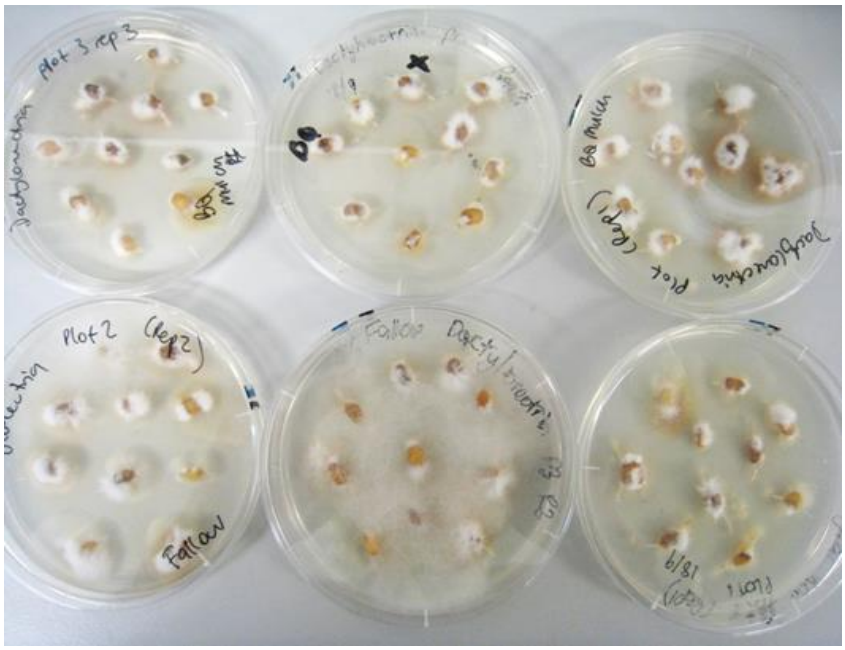


Figure 3. Frequency of *Dactylonectria macrodidyma* isolation in colonized grain buried in plots incorporated with BQ Mulch (top row) or plots left fallow (bottom row).

The frequency of isolation from the colonised wheat grains of target pathogens was initially recorded 3 days after culturing. At three days post incorporation with Caliente, Mustclean or Nemfix the frequency of isolation of *D. macrodidyma* was significantly reduced, compared with BQ mulch, Biofum or fallow plots (Figure 4). There was complete suppression of *D. macrodidyma* growth from colonised grain in plots incorporated with Caliente. There was no significant difference in frequency of isolation of *C. ilicicola* from colonised grain among the different biofumigant or fallow plots (Figure 5).

At 7 days post incorporation, there were no significant differences in frequencies of isolation of either *D. macrodidyma* or *C. ilicicola* among biofumigant or fallow plots (Figure 6 & Figure 7). The pathogens were least frequently isolated from colonised grain buried in BQ Mulch or Fallow 1 plots.

Data were re-analysed to investigate growth on selective media 3, 5 and 7 days after plating colonised grain. This showed that the complete suppression of *D. macrodidyma* by incorporation with Caliente, at 3 days (after plating), was not maintained and the fungus grew from about 75% of colonised grains by 7 days after plating (Figure 8). This frequency of isolation assessed at 7d was still significantly less than all other incorporation treatments, which had attained 100% at 7d after plating. This suggests an initial retardation of fungal growth, but not a complete eradication, by Caliente, Mustclean and Nemfix. There were no significant differences among biofumigant incorporation treatments for 3 or 7d on the recovery of *C. ilicicola* 3, 5 and 7 days after plating colonised grain onto media (Figure 7 & Figure 11). However, there was an indication of suppression by Biofum when colonised grain was recovered 7d after incorporation.

Despite initial reduction of *C. ilicicola* frequency at 3 and 5 days after culturing (Figure 10), there was no significant difference in pathogen incidence in inoculated grains buried in plots containing biofumigants compared to plots left fallow at any of the measurement times. At 7 days post incorporation with Brassica biofumigants, there was no significant difference in *C. ilicicola* incidence in inoculated grains buried in plots containing biofumigants compared to plots left fallow at any of the measurement times (Figure 11).

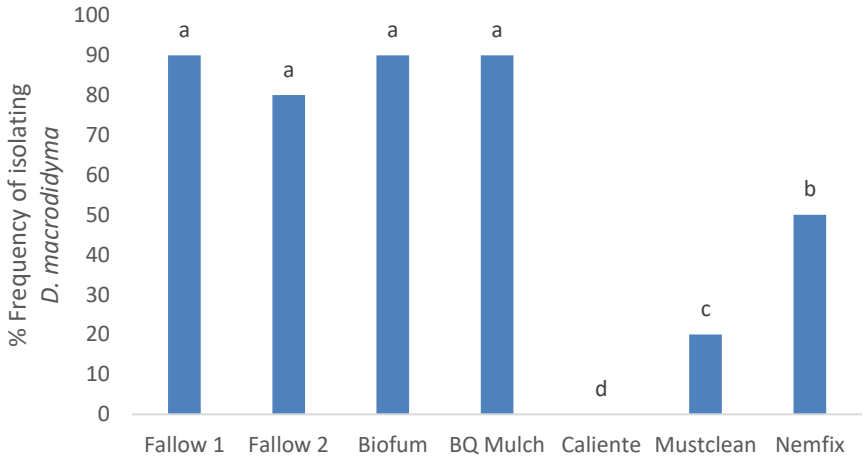


Figure 4. Mean frequency of isolating *Dactylonectria macrodidyma* at 3 days post incorporation with biofumigant, measured 3 days after culturing. Means with the same letter are not significantly different at P<0.05. n=3 replicate plots per biofumigation or control (fallow) treatment.

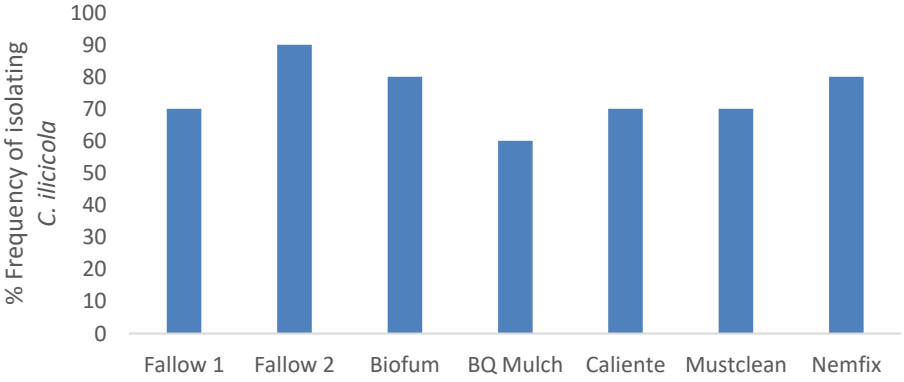


Figure 5. Mean frequency of isolating *Calonectria ilicicola* at 3 days post incorporation with biofumigant, measured 3 days after culturing. Means are not significantly different.

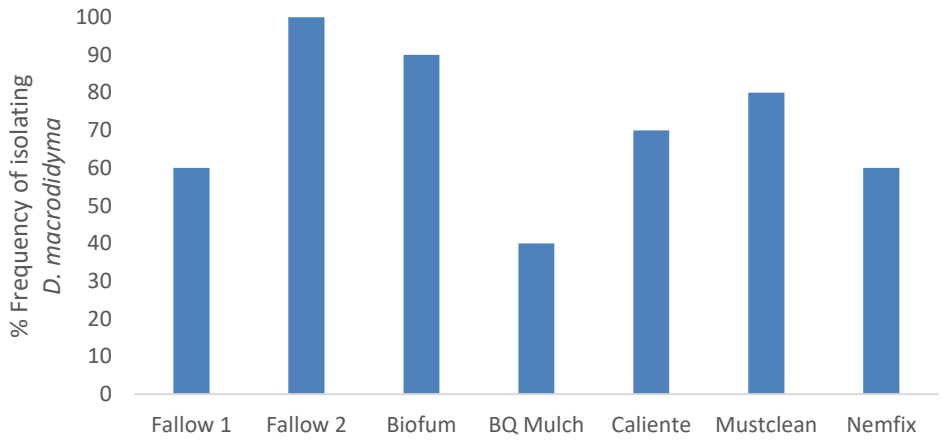


Figure 6. Mean frequency of isolating *Dactylonectria macrodidyma* at 7 days post incorporation with biofumigant, measured 3 days after culturing. Means are not significantly different (P>0.05).

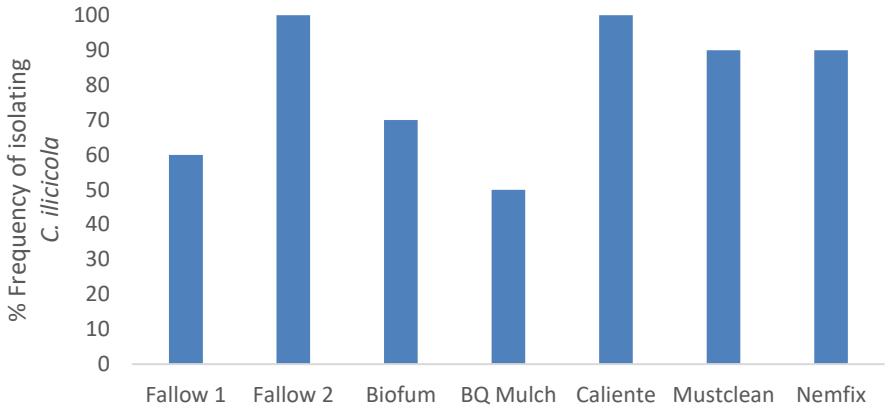


Figure 7. Mean frequency of isolating *Calonectria ilicicola* at 7 days post incorporation with biofumigant, measured 3 days after culturing. Means are not significantly different (P>0.05).

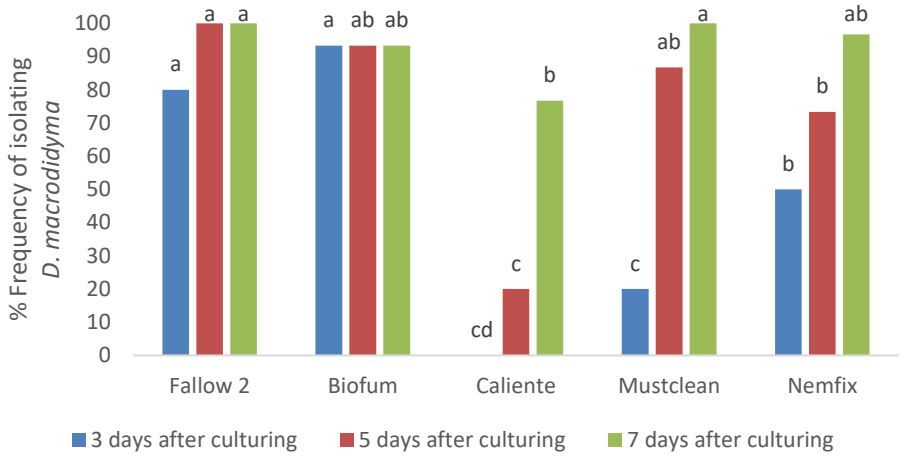


Figure 8. Mean frequency of isolating *Dactylonectria macrodidyma* at 3 days post incorporation with biofumigant, measured at 3, 5 and 7 days after culturing. Means with the same letter are not significantly different at P<0.05.

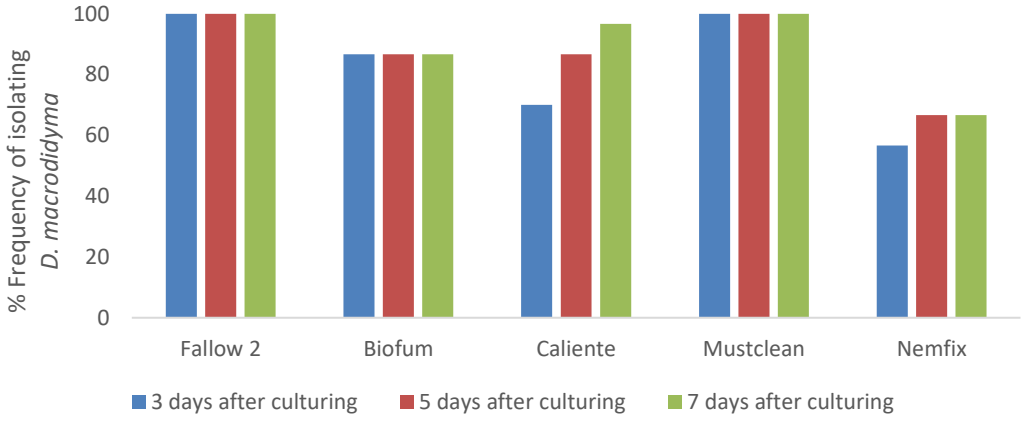


Figure 9. Mean frequency of isolating *Dactylonectria macrodidyma* at 7 days post incorporation with biofumigant, measured at 3, 5 and 7 days after culturing. Means of the same colour are not significantly different (P>0.05).

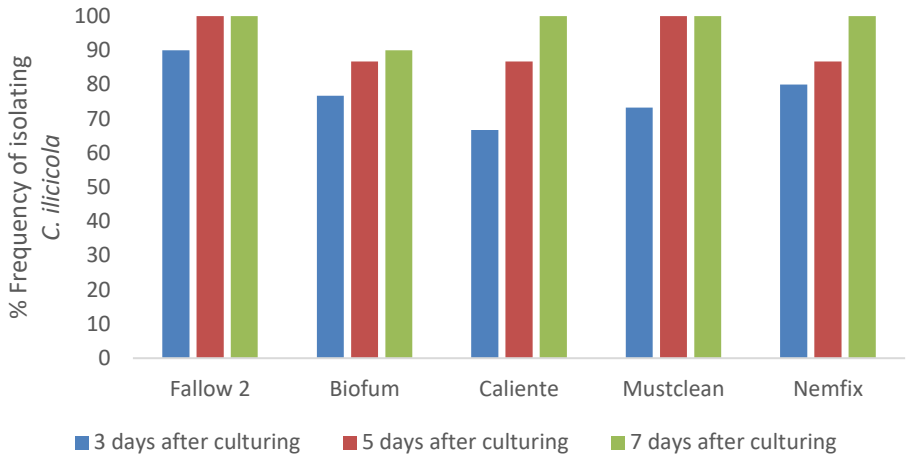


Figure 10. Mean frequency of isolating *Calonectria ilicicola* at 3 days post incorporation, measured at 3, 5 and 7 days after culturing. Means of the same colour are not significantly different.

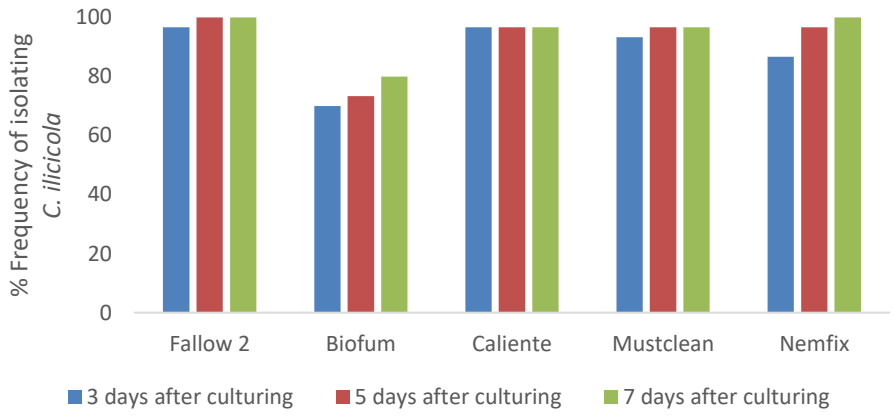


Figure 11. Mean frequency of isolating *Calonectria ilicicola* at 7 days post incorporation, measured at 3, 5 and 7 days after culturing. Means of the same colour are not significantly different.

Table 5. Efficacy of 8 fungicides against three Nectriaceae isolates. IC50 is the concentration of active ingredient at which fungal growth is inhibited by 50%.

	prochloraz	imazilil	propiconazole	paclobutrazol	azoxystrobin	A19649B	chlorothalonil	fludioxonil
C. ilicicola								
IC50 (mg/mL)	0.01819	0.08787	0.3158	8.703	2.128	0.000001199	0.0001933	0.000009343
R^2	0.9808	0.9592	0.976	0.8886	0.7654	0.4775	0.5023	0.4139
D. macrodidyma								
IC50 (mg/mL)	~ 0.0008827	0.005791	0.001537	0.04407	0.03011	interrupted	0.008405	0.0000637
R^2	0.9925	0.9963	0.9694	0.9395	0.9683		0.8014	0.1859
D. novozelandica								
IC50 (mg/mL)	0.0004478	0.01833	0.006183	0.3038	2.078	interrupted	0.04412	0.004475
R^2	0.9228	0.9974	0.9885	0.9282	0.8622		0.9645	0.7678

R^2 is a statistical measure of how close the data are to the fitted regression line, ie.the “goodness of fit” of the data. n=15 (3 replicate plates for each of 5 concentrations of fungicide tested).

In vitro and in planta fungicide studies

Eight fungicides have been tested for their efficacy against one isolate each of *C. ilicicola*, *D. macrodidyma* and *D. novozelandica* (Table 5). There were large differences in efficacy, including between *Calonectria* and *Dactylonectria* isolates. A new chemistry from Syngenta was highly inhibitory to all isolates. Fludioxonil (Scholar) was also inhibitory at extremely low concentrations of active ingredient (a.i.). Prochloraz (Sportak) and propiconazole were inhibitory at low concentrations to *Dactylonectria* isolates, while chlorothalonil was efficacious against *Calonectria*. Imazilil, paclobutrazol and azoxystrobin were the least inhibitory fungicides to all isolates.

Table 6 summarises the mean effect of drench treatments on the health of inoculated avocado cv. Reed seedlings five weeks after inoculation with *Calonectria ilicicola*, in glasshouse Experiment 1. There was no significant difference in plant height, plant biomass and root biomass for inoculated plants treated with Sportak® or Scholar® compared to inoculated and uninoculated plants treated with water. However a significant difference in root necrosis was found. In inoculated plants treated with Scholar®, a ~16.5% improvement in necrotic symptoms was found compared to inoculated plants treated with water. Moreover, the percentage of necrotic roots in inoculated plants treated with Scholar® was statistically similar to uninoculated plants treated with water.

The root necrosis of inoculated plants treated with Sportak® was statistically similar to those treated with Scholar®, although the roots of Sportak®-treated plants had ~11.7% higher root necrosis than Scholar®-treated plants. The percentage of necrotic roots in inoculated plants treated with Sportak® improved symptoms by ~4.8% compared to inoculated plants treated with water, however this finding was not significant. The roots of inoculated plants treated with Sportak® were significantly more necrotic by ~23% than uninoculated plants treated with water.

The frequency of isolating *C. ilicicola* from inoculated avocado roots was not significantly different between any of the treatment groups (Table 6) thus suppression of *C. ilicicola* by fungicide treatment could not be demonstrated.

In the second glasshouse experiment, seedlings drenched with Scholar and Banrot had significantly greater root fresh weight after inoculation with Calonectria than the inoculated controls, and were similar to root weights of seedlings which were not inoculated (Figure 12). There were no significant differences in root fresh weight amongst treatments after inoculation with Dactylonectria (Figure 12).

The fungicide Scholar significantly reduced the percentage of root necrosis after Calonectria inoculation in avocado seedling roots compared to the other fungicides tested and inoculated control (Figure 13). Root necrosis caused by Dactylonectria was low in this experiment, however, results show that drenching with Scholar or Banrot actually increased the percentage of necrotic roots compared with inoculated controls. This experiment will be repeated. Anolyte is a chlorine-based sanitiser, and while it reduced the root necrosis caused by Calonectria, the plants were stunted (data not shown).

Table 6. Effect of soil drenching on health of avocado cv. Reed seedlings 5 weeks after inoculation with *Calonectria ilicicola*, Experiment 1.

Treatment Group	Plant height (cm)	Leaf + stem biomass (g)		Root biomass (g)		Root necrosis (%)		Freq. isolation from roots (%)
		Fresh	Dry	Fresh	Dry			
Uninoculated control + water	34.7	16.0	4.89	8.50	1.23	16.7	c	0
C. ilicicola + water	33.1	13.8	4.19	10.0	1.39	44.6	a	38.9
C. ilicicola + Sportak 110mL/100L	32.8	14.8	4.47	10.1	1.57	39.8	ab	29.2
C. ilicicola + Scholar 120mL/100L	33.6	16.2	4.73	9.53	1.28	28.1	bc	26.4

Letters representing Fisher's least significant difference (LSD) rank the means. Mean values within columns with the same letter or no letter are not significantly different at P<0.05.n=12 replicate plants for uninoculated + water control, and n=24 replicate plants for each of the other treatments.

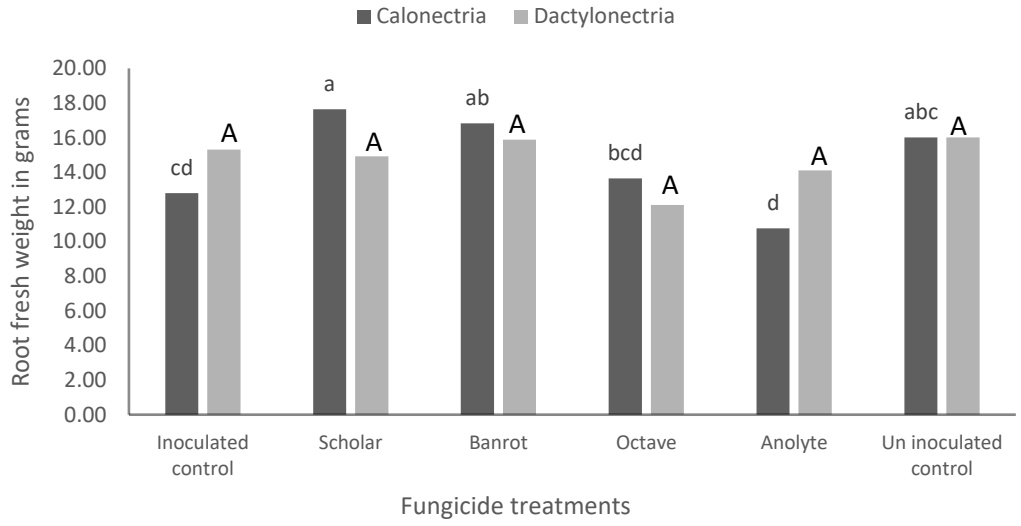


Figure 12. Effect of different fungicide treatments on avocado seedling root fresh weight, inoculated with *Calonectria* or *Dactylonectria* sp., Experiment 2. For each fungus, bars with same letter (upper and lower cases differentiate pathogen) indicate no significant difference among means at P<0.05. n=12 replicate plants per treatment for each pathogen.

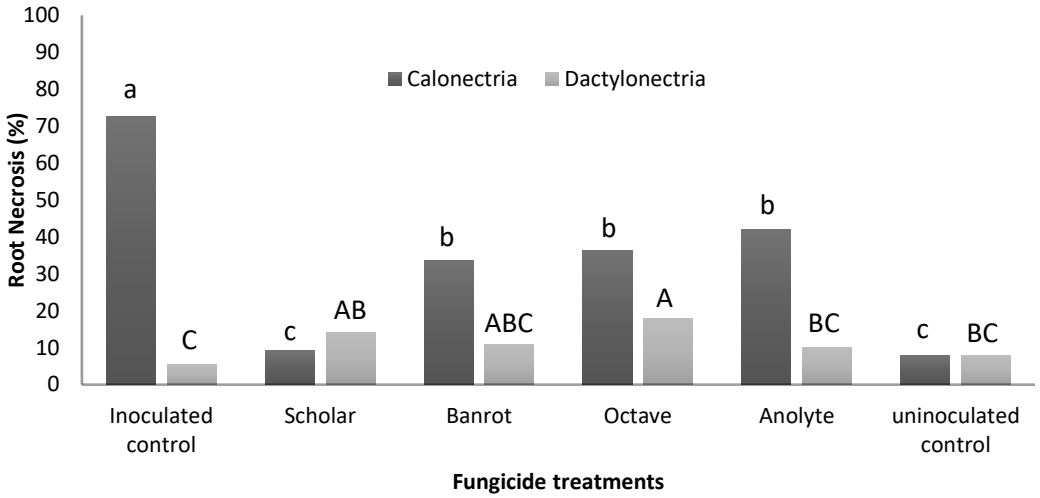


Figure 13. Effect of different fungicide treatments on root necrosis in avocado seedlings previously inoculated *Calonectria* or *Dactylonectria*, Experiment 2. For each fungus bars with same letter (upper and lower cases differentiate pathogen) indicate no significant difference. n=12 replicate plants per treatment for each pathogen.

Effect of rootstock on disease caused by black root rot under glasshouse conditions

Two glasshouse experiments were conducted to evaluate development of root rot disease in different avocado cultivars after inoculation with *Calonectria ilicicola* or *Dactylonectria macrodidyma*. There were 6 rootstock varieties tested in the first experiment, with Velvick included as both seedlings and clonally-propagated plants. Analyses and comparisons have been made separately for each pathogen inoculation. Inoculation with Ci resulted in significantly more severe root rot in each rootstock variety than respective uninoculated controls (Table 7). Clonal Velvick, and SHSR04 had significantly healthier roots than all other varieties (seedling Hass, Reed, Zutano, Velvick and clonal Dusa), after inoculation with Ci. The next healthiest root system after inoculation was clonal Dusa, which had significantly less severe root necrosis than in seedling Hass. Plant heights were significantly reduced by Ci inoculation in seedling Hass and Reed only, compared with respective uninoculated controls.

Inoculation with Dm resulted in significantly more severe root rot in each rootstock variety than respective uninoculated controls (Table 8). Plant heights were not significantly reduced by Dm inoculation and, in fact, were significantly greater in seedling Velvick and clonal SHSR04 than in the uninoculated control. Amongst the Dm inoculated rootstocks, seedling Hass had significantly more severe root necrosis than all other rootstocks, at 74% necrosis. Clonal SHSR04, Zutano seedling and Reed seedling had intermediate levels of root necrosis, while Velvick seedling and Dusa clones had the least root rot (significantly less only than Hass and Zutano). Velvick clones were unavailable for testing against Dm in Experiment 1.

There were fewer rootstocks available for testing in the second experiment. All rootstocks succumbed to root rot, the most severe necrosis after inoculation with Ci, which is consistent with previous reports. While there were no statistically significant differences amongst rootstocks in severity of root

rot caused by Ci, Reed seedling and clonal Dusa had similar levels of disease, but clonal Velvick had much more severe root rot (Table 9). Irrespective of inoculation treatment, Velvick clones grew very little during the experiment (less than 1cm) but their root systems were larger (dry weight) than those of Reed seedlings, which grew 10-15cm over the experiment. Dusa clones had intermediate growth but larger root systems (for uninoculated and Ci-inoculated) than all other rootstock treatments. This demonstrates that despite severe root infection young plants in the glasshouse are able to grow well, and we know that this happens in the commercial nurseries. Plants are watered, fertilised and well tendered in nurseries, such that infections go unnoticed, yet may succumb to the root infection when planted into less ideal conditions in the field.

Table 7. Effects of rootstock on growth and root rot 7 weeks after inoculation with *Calonectria ilicicola* (Ci), Glasshouse Experiment 1

		n	Total growth (cm)	Necrotic roots (%)	Roots DW (g)	Stem+leaf DW (g)
Hass seedling	no pathogen	10	31.6 bcd	16.5 ef	7.49 b	25.1 a
Hass seedling	Ci	10	17.9 ef	83.0 a	5.30 c	24.2 a
Reed seedling	no pathogen	10	39.7 ab	21.5 e	5.48 c	22.9 ab
Reed seedling	Ci	10	30.9 cd	74.0 ab	5.15 c	19.0 bcd
Zutano seedling	no pathogen	10	41.0 a	33.0 d	4.22 cd	17.3 cde
Zutano seedling	Ci	10	35.7 abc	74.0 ab	5.10 c	18.1 bcd
Velvick seedling	no pathogen	10	24.2 de	18.0 ef	9.50 a	24.7 a
Velvick seedling	Ci	10	24.8 de	78.0 ab	7.82 ab	22.1 abc
Velvick clone	no pathogen	6	8.50 fgh	18.3 ef	4.63 cd	13.7 def
Velvick clone	Ci	6	6.67 gh	51.7 c	1.39 e	9.29 fg
Dusa clone	no pathogen	10	17.4 ef	10.0 f	2.99 de	12.7 ef
Dusa clone	Ci	10	13.7 fg	68.0 b	2.82 de	14.0 def
SHSR04 clone	no pathogen	7	4.14 h	27.9de	1.35 e	4.43 g
SHSR04 clone	Ci	8	13.6 fgh	51.3 c	1.54 e	9.60 fg

Within each column, means followed by the same letter are not significantly different at P<0.05.

Table 8. Effects of rootstock on growth and root rot 7 weeks after inoculation with *Dactylonectria macrodidyma* (Dm), Glasshouse Experiment 1

		n	Total growth (cm)	Necrotic roots (%)	Roots DW (g)	Stem+leaf DW (g)
Hass seedling	no pathogen	10	31.6 abc	16.5 ef	7.49 abc	25.1 a
Hass seedling	Dm	9	29.2 bc	74.4 a	6.81 bc	21.4 abc
Reed seedling	no pathogen	10	39.7 ab	21.5 e	5.48 cd	22.9 ab
Reed seedling	Dm	10	41.7 a	46.0 bc	6.88 bc	24.3 a
Zutano seedling	no pathogen	10	41.0 a	33.0 d	4.22 de	17.3 cde
Zutano seedling	Dm	10	35.6 ab	57.0 b	5.91 bcd	18.7 bcd
Velvick seedling	no pathogen	10	24.0 cd	18.5 ef	9.59 a	24.7 a
Velvick seedling	Dm	10	34.8 ab	39.0 cd	8.12 ab	22.5 abc
Velvick clone	no pathogen			Not tested		
Velvick clone	Dm			Not tested		
Dusa clone	no pathogen	10	17.4 de	10.0 f	2.99 ef	12.7 ef
Dusa clone	Dm	10	16.4 de	39.0 cd	3.72 def	13.5 def
SHSR04 clone	no pathogen	7	4.14 f	27.9 de	1.35 f	4.43 g
SHSR04 clone	Dm	8	10.5 ef	48.8 bc	1.57 f	8.55 fg

Within each column, means followed by the same letter are not significantly different at P<0.05.

Table 9. Effects of rootstock on growth and root rot 5 weeks after inoculation with *Dactylonectria macrodidyma* (Dm), or *Calonectria ilicicola* (Ci), Glasshouse Experiment 2

	n	Total growth (cm)	necrotic roots (%)	Roots DW (g)	Stem+leaf DW (g)
Reed seedling no pathogen	10	10.8 ab	15.5 cde	1.45 c	13.0 b
Reed seedling Ci	10	10.0 b	36.0 ab	1.59 bc	14.5 ab
Reed seedling Dm	10	14.6 a	25.0 bcd	1.52 bc	17.2 a
Dusa clone no pathogen	10	6.90 bc	8.00 e	3.99 a	17.6 a
Dusa clone Ci	10	5.10 c	36.0 ab	3.90 a	17.2 a
Dusa clone Dm	10	5.60 c	28.0 bc	2.01 bc	11.9 bc
Velvick clone no pathogen	10	0.50 d	10.5 de	2.13 bc	9.18 c
Velvick clone Ci	10	0.30 d	49.0 a	2.46 b	11.7 bc

Within each column, means followed by the same letter are not significantly different at P<0.05.

April 18 Pathogen of the month – April 2018

Dactylonectria Lombard & Crous

Fig. 1. Black root rot symptoms in young orchard transplants (a), necrotic avocado roots (b, c) *Dactylonectria macrodidyma* on ½ sPDA at 3.75 cm after 10 days growth (d, e), *D. macrodidyma* macroconidia at 40 × magnification (f)

Disease: Black root rot of avocado
Name: *Dactylonectria* spp. including *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata* and *D. anthuriicola*
Classification: K: Fungi, D: Ascomycota, C: Sordariomycetes, O: Hypocreales, F: Nectriaceae

Black root rot caused by nectriaceous fungi is a severe disease of avocado nursery trees and young orchard transplants, causing decline and death within one year of planting. Symptoms include stunting, wilt, leaf chlorosis and browning, leaf drop prior to tree death caused by severe necrosis of the root system. In Australia black root rot of avocado is caused by *Calonectria ilicicola* and several *Dactylonectria spp.*

The Pathogen:

Species of *Dactylonectria* (reported as *Cylindrocarpon* in older literature) have often been isolated from necrotic avocado roots. *Dactylonectria macrodidyma* is the most prevalent of the pathogens found in symptomatic avocado roots. *Dactylonectria novozelandica*, *D. pauciseptata* and *D. anthuriicola* have also been isolated from avocado roots and shown to be pathogenic in glasshouse tests with seedlings. While *Dactylonectria* spp. can be isolated from roots of established orchard trees, there is no evidence that mature trees are severely impacted. Root infection caused by *Dactylonectria* spp. is often undetected as trees may appear symptomless under nursery conditions, although tree decline and death may occur after planting. *Dactylonectria* spp. produce cylindrical, straight to slightly curved 1–4 septate macroconidia (Fig. 1) and ellipsoid to ovoid, straight 0–1 septate microconidia. The disease cycle of *Dactylonectria* spp. in avocado is not known.

Impact:

Black root rot caused by nectriaceous fungi has led to significant commercial loss of new plantings in avocado orchards around Australia over the last 10 years.

Host range and distribution:

Dactylonectria spp. cause root rot diseases in various hosts including avocado (*Persea americana*), grapevine (*Vitis vinifera*), cherimoya (*Annona cherimola*), kiwifruit (*Actinidia deliciosa*) and olive (*Olea europaea*). *Dactylonectria* spp. associated with avocado have been reported in Australia and Italy. However the fungal genus is reported globally across numerous horticultural industries.

Management options:

Hygiene is important for reducing the risk of spread. Recommended management options include removal of diseased or symptomatic plants, use of clean planting material, pasteurisation of soil, care not to over irrigate or over fertilize, adequate space between plants in the nursery and keeping plants off the ground, sourcing plants from accredited nurseries, and taking extreme care, monitoring tree health during planting and post planting establishment. Fungicides and other treatment options are being investigated.

Further Reading:

Parkinson LE, Shivas RG, Dann EK. 2017. Pathogenicity of nectriaceous fungi on avocado in Australia. *Phytopathology*, 107, 1479-1485.

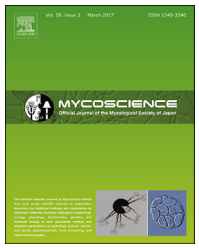
Key Contacts: Dr Louisa Parkinson (l.Parkinson@uq.edu.au) and Dr Elizabeth Dann (e.dann@uq.edu.au)
Authors: Dr Louisa Parkinson, Prof Roger Shivas and Dr Elizabeth Dann

Available online at www.sciencedirect.com

MYCOSCIENCE

ISSN 1340-3540 (print), 1618-2545 (online)

journal homepage: www.elsevier.com/locate/myc



Full paper

Novel species of *Gliocladiopsis* (Nectriaceae, Hypocreales, Ascomycota) from avocado roots (*Persea americana*) in Australia

Louisamarie E. Parkinson [a], *Roger G. Shivas* [b], *Elizabeth K. Dann* [a,*]

[a] *Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Ecosciences Precinct, 41 Boggo Road, Dutton Park, QLD 4102, Australia*

[b] *Plant Pathology Herbarium, Department of Agriculture and Fisheries, Ecosciences Precinct, 41 Boggo Road, Dutton Park, QLD 4102, Australia*

ARTICLE INFO

Article history:
Received 24 June 2016
Received in revised form
27 October 2016
Accepted 30 October 2016
Available online 7 December 2016

Keywords:
Lauraceae
Phylogeny
Rhizosphere
Taxonomy

ABSTRACT

Root rot of avocado (*Persea americana*) is an important disease in seedling nurseries as well as in the field in eastern and southern Australia. During an investigation into the causal organisms of avocado root rot, 19 isolates of *Gliocladiopsis* were obtained from necrotic lesions on avocado roots and examined by morphology and comparison of DNA sequences from three gene loci (the internal transcribed spacer region of the nuclear rDNA, Histone H3 and β-tubulin). Three new species of *Gliocladiopsis* are described as a result of phylogenetic analysis of these data. One of the new species, *G. peggii*, formed a monophyletic group that may represent an unresolved species complex as it contained a polytomy that included a well-supported clade comprising two subclades. *Gliocladiopsis peggii* is sister to *G. mexicana*, which is known from soil in Mexico. The remaining two new species, *G. whileyi* and *G. forsbergii*, formed a clade sister to *G. curvata*, which is known from Ecuador, Indonesia and New Zealand.

1. Introduction

Gliocladiopsis (Hypocreales, Nectriaceae) is a genus of soilborne fungi found mostly in tropical and sub-tropical regions of the world (Lombard and Crous 2012). Crous (2002) regarded *Gliocladiopsis* species as secondary plant pathogens or saprobes. These fungi are often isolated from necrotic roots, although their pathogenicity has rarely been tested. Dann et al. (2012) showed that an isolate of *Gliocladiopsis* sp. from necrotic avocado (*Persea americana* Mill.) roots in Australia was not pathogenic and actually increased the height of avocado seedlings in two of three soil amendment trials.

Species of *Gliocladiopsis*, based on the type *G. sagariensis* Saksena (1954), have densely penicillate and branched conidiophores that resemble those of *Calonectria* (syn. *Cylindrocladium*) and *Cylindrocladiella*, yet lack the stipe extensions found in these genera (Lombard et al. 2015). Species of *Gliocladiopsis* are difficult to distinguish morphologically as the

* *Corresponding author*. Fax: +61 7 3844 4529.
E-mail address: e.dann@uq.edu.au (E.K. Dann).

http://dx.doi.org/10.1016/j.myc.2016.10.004

branching structure of conidiophores as well as the size and shape of conidia are similar between species (Liu and Cai 2013). Lombard and Crous (2012) used DNA sequence data to infer that *Gliocladiopsis* was a phylogenetically distinctive genus and further described four new species. Currently *Gliocladiopsis* contains 10 species, namely, *G. curvata*, *G. elghollii*, *G. guangdongensis*, *G. indonesiensis*, *G. irregularis*, *G. mexicana*, *G. pseudotenuis*, *G. sagariensis*, *G. sumatrensis* and *G. tenuis* (Lombard and Crous 2012; Liu and Cai 2013), none of which have been reported in Australia.

The aim of this study was to identify isolates of *Gliocladiopsis* that had been collected from the necrotic roots of seedlings and mature avocado trees in eastern and southern Australia. Morphology and multigene phylogenetic inference from DNA sequence analysis was used to classify these isolates, resulting in the recognition of three novel species that are formally described here.

2. Materials and methods

2.1. Isolates

Nineteen isolates of *Gliocladiopsis* were obtained from roots of avocado with brown to black, sunken necrotic lesions collected from orchards and production nurseries in eastern Australia between 2013 and 2016 (Table 1). Roots were sampled from mature trees in the field as well as from young trees in nurseries that displayed symptoms of wilt or chlorosis. The isolates were obtained using the method described by Dann et al. (2012).

Prior to examination the cultures were grown on plates of potato dextrose agar amended with streptomycin (sPDA) at room temperature, under black light for 4–5 d. Representative isolates derived from a single germinated conidium were deposited in the Queensland Plant Pathology Herbarium (BRIP), Ecosciences Precinct, Dutton Park, Australia. Cultures were grown on sPDA for 7 d prior to cultural and molecular examination.

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was extracted from mycelia using the Promega Wizard® genomic DNA purification kit (Promega Corporation, Alexandria, Australia). The manufacturer's protocol (Promega Corporation 2010) was modified in that 50–100 mg of mycelium was ground by hand with a pestle in an Eppendorf tube in 600 μL nuclei lysis solution, then incubated at 65 °C for 15 min. The purified DNA was rehydrated with sterile deionised (sd) water instead of DNA rehydration solution. The absorbance of the DNA at A260/A280 nm was measured with a spectrophotometer (BioDrop®, Pacific Laboratory Products, Blackburn, Australia) and used to quantify the DNA concentration (Dhanoya 2012). The DNA was diluted to working solutions of up to 50 ng/μL.

The internal transcribed spacer (ITS) regions 1 and 2 and the 5.8S gene of the ribosomal RNA, β-tubulin and Histone H3 partial gene loci for each isolate were amplified separately in PCR with Invitrogen® Taq PCR reagents (Life Technologies, Mulgrave, Australia) and RNase-free water at master mix concentrations of 1 unit Invitrogen® Taq Polymerase, 0.6 μM forward primer, 0.6 μM reverse primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$ and 1 × concentration of PCR buffer. For each PCR reaction, 2 μL of DNA template at 25–50 ng/μL was used. The partial ITS region (~600 bp) was amplified with the universal primers ITS 4 and ITS 5 (White et al. 1990); the partial β-tubulin gene (~600 bp) was amplified with the primers T1 (O'Donnell and Cigelnik 1997) and CYLTUB1R (Crous et al. 2004); and the partial Histone H3 gene (~500 bp) was amplified with the primers CYLH3F and CYLH3R (Crous et al. 2004). The PCR thermal cycling consisted of initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. The PCR products were sent to Macrogen Inc. (Seoul, Rep. of Korea) for sequencing in both directions with the same primer pairs used for amplification. Contigs were assembled from the forward and reverse sequences using a de novo assembly in Geneious v. 7.1.5 (Biomatters Ltd., Auckland) (Kearse et al. 2012).

2.3. Phylogenetic analysis

DNA sequences were aligned using the MAFFT 7 (Katoh and Standley 2013) Geneious plugin. The multiple alignments for each gene were cured in Gblocks (www.phylogeny.fr) (Talavera and Castresana 2007) and concatenated in Geneious. A maximum likelihood (ML) and Bayesian inference (BI) phylogeny were constructed in a partitioned setup, with an invariable GTRGAMMA model of evolution. ML phylogenetic trees were generated using RAXML v. 7.2.6 (Stamatakis 2006) with a rapid bootstrap analysis and 1000 runs on the distinct random starting tree. The BI phylogenetic trees were generated with MrBayes v. 3.2.5 (Huelsenbeck and Ronquist 2001) with the Markov Chain Monte Carlo (MCMC) consisting of 4 runs. Each run contained 4 chains analysed with 10 million generations, and the trees were sampled and printed every 1000 generations. The cold chain heating temperature was 0.25. The phylogenetic trees were viewed with FigTree v. 4.2 (Rambaut 2007). The ML bootstrap support values of ≥70% or BI posterior probabilities of ≥0.95 were considered to indicate statistical significance.

For the phylogenetic analysis, sequence data generated from the 19 isolates in this study was compared with the gene sequences from all 10 ex-type cultures of *Gliocladiopsis* available on the NCBI GenBank database (Table 1). *Cylindrocladiella parva* (ATCC 28272), the type species of that genus (Lombard et al. 2012), was chosen as the outgroup taxon as *Cylindrocladiella* is sister to *Gliocladiopsis* (Lombard et al. 2015). Novel sequences derived in this study were deposited in GenBank (www.ncbi.nlm.nih.gov/genbank) and MycoBank (www.mycobank.org). The alignments and phylogenetic trees were placed in TreeBASE (www.treebase.org/treebase/index.html).

2.4. Morphological examination

Isolates were grown on carnation leaf agar at 24 °C under 12 h dark/12 h near-ultraviolet light. Approximately 7–10 d after culturing, conidiophores were observed on carnation leaves under a dissecting microscope, collected and then placed on a

Table 1 – Details of the cultures of *Gliocladiopsis* species and the outgroup taxon (*Cylindrocladiella parva*) used in this study.

Species	Culture no.[a]	Origin	Substrate	Collector	NCBI GenBank accession no.[b,c]		
					BT	HIS3	ITS
Cylindrocladiella parva	ATCC 28272^T	New Zealand	*Telopea speciosissima*	H.J. Boesewinkel	AY793486	AY793526	AF220964
Gliocladiopsis curvata	CBS 194.80	Ecuador	*Persea americana*	J.P. Laoh	JQ666120	JQ666010	JQ666044
	CBS 112365^T	New Zealand	*Archontophoenix purpurea*	F. Klassen	JQ666126	JQ666016	JQ666050
	CBS 112935	Indonesia	*Syzygium aromaticum*	M.J. Wingfield	JQ666127	JQ666017	JQ666051
G. elghollii	CBS 206.94	USA	*Chamaedorea elegans*	N.E. El-Gholl	JQ666130	JQ666020	JQ666054
	CBS 116104^T	USA	*Chamaedorea elegans*	N.E. El-Gholl	JQ666131	JQ666021	JQ666055
G. forsbergii	BRIP 60984	Australia	*Grevillea* sp.	K.G. Pegg	**KX274036**	**KX274053**	**KX274070**
	BRIP 61349a^T	Australia	*Persea americana*	L.E. Parkinson	**KX274037**	**KX274054**	**KX274071**
G. guangdongensis	LC 1340^T	China	Submerged wood	F. Liu & L. Cai	KC776124	KC776120	KC776122
	LC 1349	China	Submerged wood	F. Liu & L. Cai	KC776125	KC776121	KC776123
G. indonesiensis	CBS 116090^T	Indonesia	Soil	A.C. Alfenas	JQ666132	JQ666022	JQ666056
G. irregularis	CBS 755.97^T	Indonesia	Soil	A.C. Alfenas	JQ666133	JQ666023	AF220977
G. mexicana	CBS 110938^T	Mexico	Soil	M.J. Wingfield	JQ666137	JQ666027	JQ666060
G. peggii	BRIP 53654	Australia	*Persea americana*	E.K. Dann & A.W. Cooke	JN255247	–	JN255246
	BRIP 54019	Australia	*Persea americana*	E.K. Dann & A.W. Cooke	JN243766	JN243767	JN243765
	BRIP 60983^T	Australia	*Persea americana*	K.G. Pegg	**KX274038**	**KX274065**	**KX274083**
	BRIP 60987	Australia	*Persea americana*	K.G. Pegg	**KX274040**	**KX274062**	**KX274074**
	BRIP 60988	Australia	*Persea americana*	K.G. Pegg	**KX274043**	**KX274063**	**KX274082**
	BRIP 60990	Australia	*Persea americana*	A.G. Manners	**KX274044**	**KX274055**	**KX274077**
	BRIP 62845a	Australia	*Persea americana*	L.E. Parkinson	**KX274039**	**KX274067**	**KX274076**
	BRIP 62845b	Australia	*Persea americana*	L.E. Parkinson	**KX274050**	**KX274058**	**KX274072**
	BRIP 62845d	Australia	*Persea americana*	L.E. Parkinson	**KX274047**	**KX274061**	**KX274079**
	BRIP 63709a	Australia	*Persea americana*	L.E. Parkinson	**KX274041**	**KX274057**	**KX274085**
	BRIP 63709b	Australia	*Persea americana*	L.E. Parkinson	**KX274046**	**KX274056**	**KX274073**
	BRIP 63709c	Australia	*Persea americana*	L.E. Parkinson	**KX274048**	**KX274064**	**KX274081**
	BRIP 63710a	Australia	*Persea americana*	L.E. Parkinson	**KX274051**	**KX274068**	**KX274084**
	BRIP 63710c	Australia	*Persea americana*	L.E. Parkinson	**KX274049**	**KX274060**	**KX274080**
	BRIP 63711c	Australia	*Persea americana*	L.E. Parkinson	**KX274042**	**KX274059**	**KX274078**
	BRIP 63711d	Australia	*Persea americana*	L.E. Parkinson	**KX274045**	**KX274066**	**KX274075**
G. pseudotenuis	CBS 114763	Indonesia	*Vanilla* sp.	M.J. Wingfield	JQ666139	JQ666029	JQ666062
	CBS 116074^T	China	Soil	M.J. Wingfield	JQ666140	JQ666030	AF220981
G. sumatrensis	CBS 754.97^T	Indonesia	Soil	M.J. Wingfield	JQ666142	JQ666032	JQ666064
	CBS 111213	Indonesia	Soil	M.J. Wingfield	JQ666144	JQ666034	JQ666066
G. tenuis	IMI 68205^T	Indonesia	*Indigofera* sp.	F. Bugnicourt	JQ666150	JQ666040	AF220979
	CBS 111964	Vietnam	*Coffea* sp.	P.W. Crous	JQ666147	JQ666037	JQ666068
	CBS 114148	Vietnam	Soil	P.W. Crous	JQ666149	JQ666039	JQ666070
G. sagariensis	CBS 199.55^T	India	Soil	S.B. Saksena	JQ666141	JQ666031	JQ666063
G. whileyi	BRIP 61430^T	Australia	*Persea americana*	E.K. Dann	**KX274052**	**KX274069**	**KX274086**
Gliocladiopsis sp. 1	CBS 111038	Colombia	Soil	M.J. Wingfield	JQ666151	JQ666041	JQ666071
Gliocladiopsis sp. 2	CBS 116086	Indonesia	Soil	A.C. Alfenas	JQ666152	JQ666042	JQ666072

^{T} Ex-type cultures. Taxonomic novelties are in bold print.

[a] BRIP: Biosecurity Queensland Plant Pathology Herbarium, Department of Agriculture and Fisheries, Dutton Park, Australia. CBS: CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands. CPC: Working collection of Pedro Crous housed at CBS. IMI: International Mycological Institute, CABI-Bioscience, Egham, UK. ATCC: American Type Culture Collection, Manassas, USA. IMUR: Institute of Mycology, University of Recife, Recife, Brazil. LC: Herbarium of Microbiology, Academia Sinica, Taipei, Taiwan.

[b] BT = β-tubulin; HIS3 = Histone H3; ITS = Internal transcribed spacer regions 1, 2 and 5.8S ribosomal RNA gene.

[c] Newly deposited sequences are shown in bold.

microscope slide containing clear 100% lactic acid. Fungal structures were viewed and measured at ×1000 magnification by differential interference contrast using a Leica DM2500 microscope (Leica Microsystems, North Ryde, Australia). Conidial sizes were expressed as 95% confidence intervals derived from 20 observations with extremes of conidial measurements given in parentheses. Colonies were described after 7 d incubation on sPDA using the colour charts of Rayner (1970). Novel species were registered in Mycobank (Crous et al. 2004).

3. Results

3.1. Phylogeny

Amplicons of approximately 400–600 bp were obtained for each of ITS, β-tubulin and Histone H3. The phylogenetic analysis included 12 ingroup taxa, with *Cylindrocladiella parva* (ATCC 28272) as the outgroup taxon. The combined dataset

consisted of 1562 characters. ML and BI phylogenies based on the concatenated ITS, β-tubulin and Histone H3 partial gene sequences produced congruent tree topologies (TreeBASE ID: 19374). The BI topology was selected for graphical representation (Fig. 1), which showed the Australian isolates from avocado clustered in two clades, one of 16 and the other with three. The larger clade may represent a species complex as it contained a polytomy that included a well-supported clade comprising two subclades.

3.2. *Taxonomy*

Three novel species are described based on phylogenetic inference and morphology (Supplementary Table S1).

Gliocladiopsis forsbergii L.E. Parkinson, E.K. Dann & R.G. Shivas, sp. nov. Figs. 2, 3.
MycoBank no.: MB 817263.

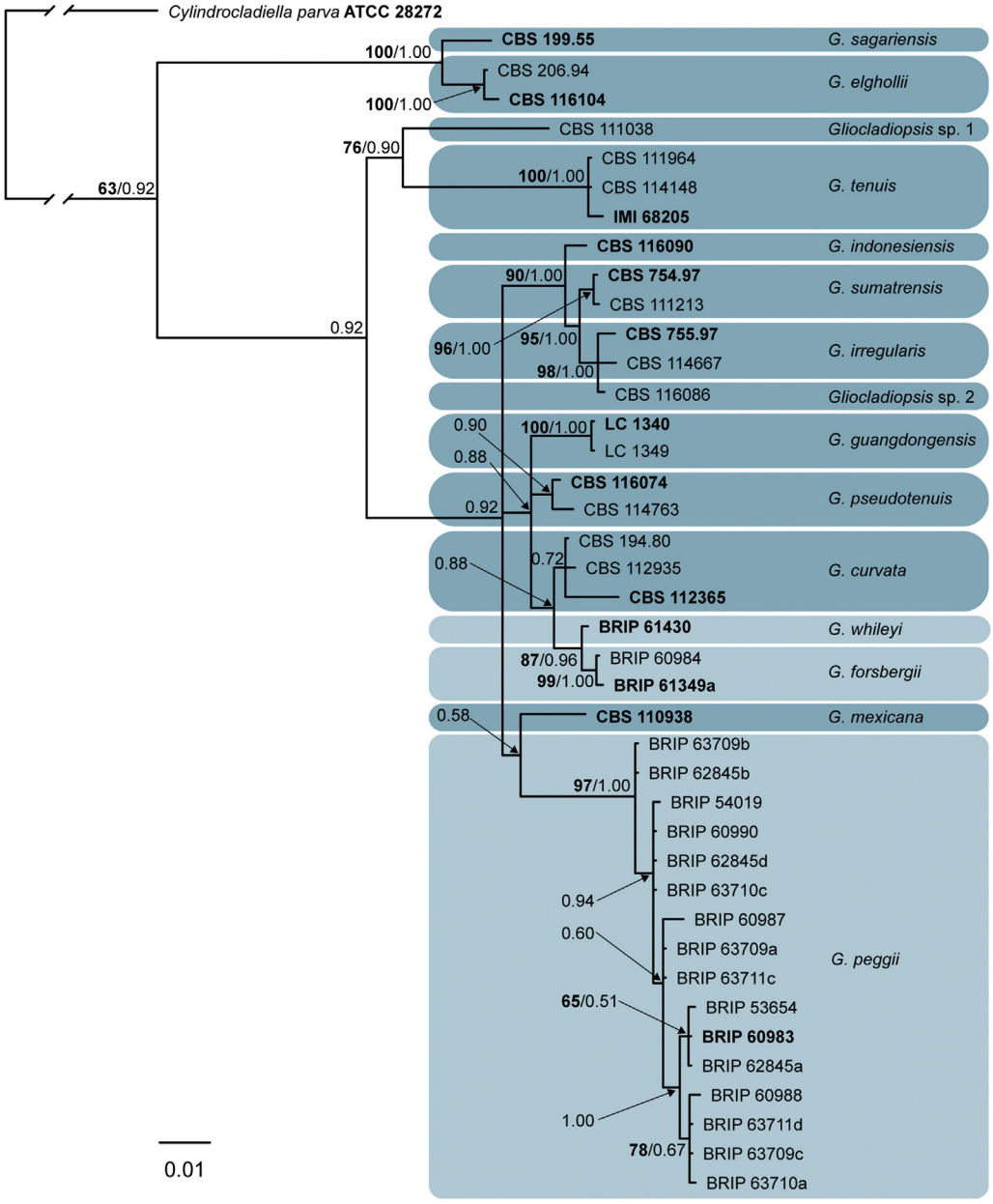


Fig. 1 – Phylogenetic tree generated by maximum likelihood analysis and Bayesian inference of the combined sequences of β-tubulin, ITS and Histone H3 gene loci of the *Gliocladiopsis* isolates. Bootstrap support values (bold) over 60% and posterior probabilities over 0.5 are shown at the nodes. Darker blocks indicate previously reported species; lighter blocks indicate new species. The tree was rooted to *Cylindrocladiella parva* (ATCC 28272). The accession numbers of ex-type cultures are shown in bold. The scale bar represents the expected number of nucleotide changes per site.

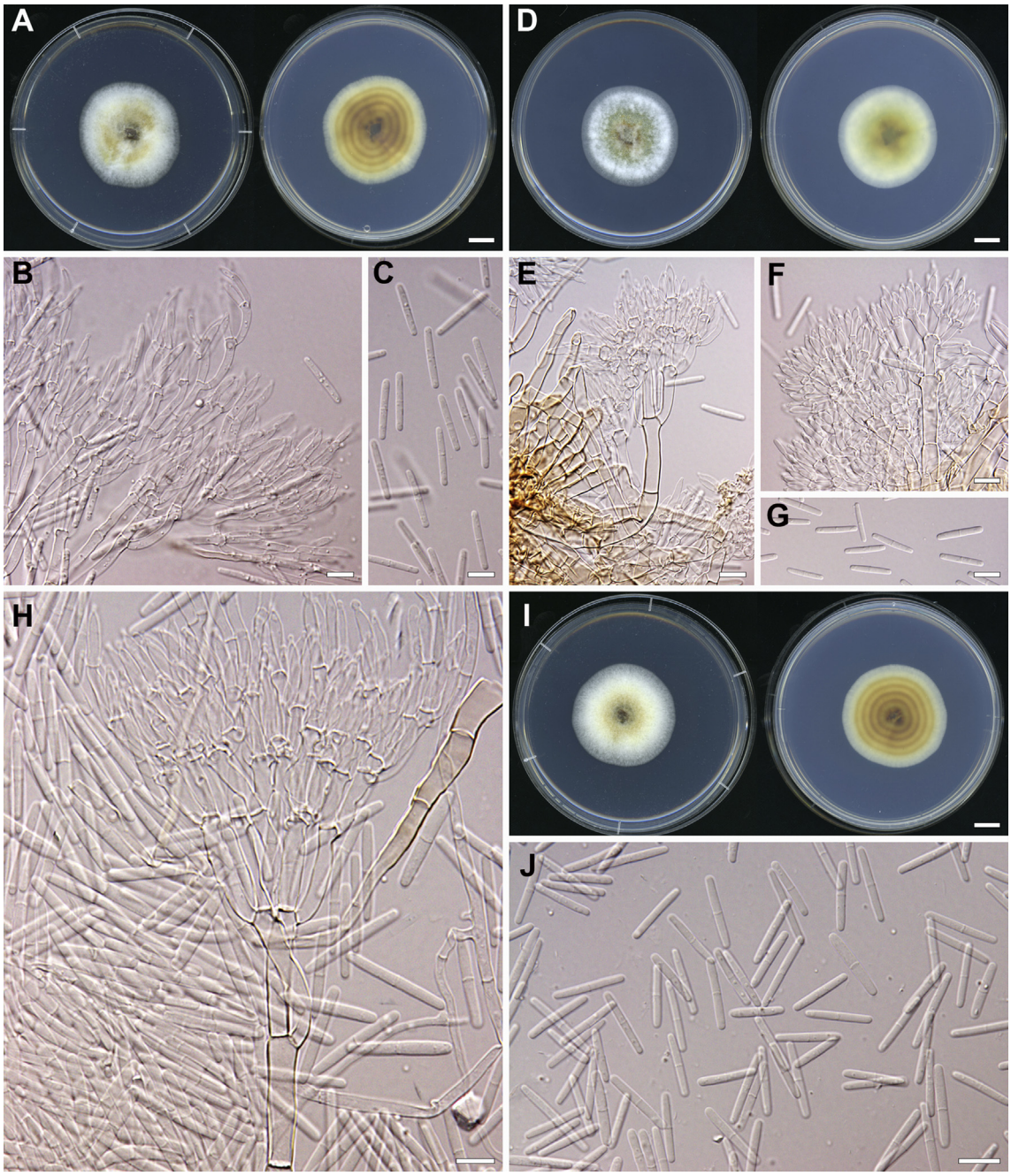


Fig. 2 – *Gliocladiopsis forsbergii* (BRIP 61349a; A–C), *G. peggii* (BRIP 60983; D–G) and *G. whileyi* (BRIP 61430; H–J). A, D, I: Colonies on sPDA at 7 d (left, upper side; right, reverse). B, E, F, H: Conidiophores. C, G, J: Conidia. *Bars*: A, D, I 1 cm; B, C, E–H, J 1 μm.

Diagnosis: *Gliocladiopsis forsbergii* has a conidiogenous apparatus that sometimes produces quinary branches, which differentiates it from all known species (Lombard and Crous 2012), including those described in this paper.

Holotype: AUSTRALIA, New South Wales, Mullumbimby, on necrotic roots of *Persea americana* in a seedling nursery, Mar 2014, leg. L.E. Parkinson (BRIP 61349a, permanently preserved in a metabolically inactive state).

Etymology: Named after the Australian plant pathologist Leif Forsberg, who collected and identified many hundreds of fungal plant pathogens during the time that he ran the plant pest and disease diagnostic service in Queensland from 1996 to 2013. The isolation techniques that he developed were used in this study.

Colonies on sPDA reach 4 cm diam after 7 d under near UV light at 24 °C, floccose, pale ochreous in the central part becoming cream at the margin, margin entire; reverse umber, becoming paler towards the margin, zonate. Conidiophores arise from a yellowish brown sporodochium up to 80 μm diam, penicillate 50–90 × 4–7 μm, with subhyaline to hyaline stipes. Conidiogenous apparatus with several branch series; primary branches 27–35 × 4–6 μm, aseptate; secondary branches

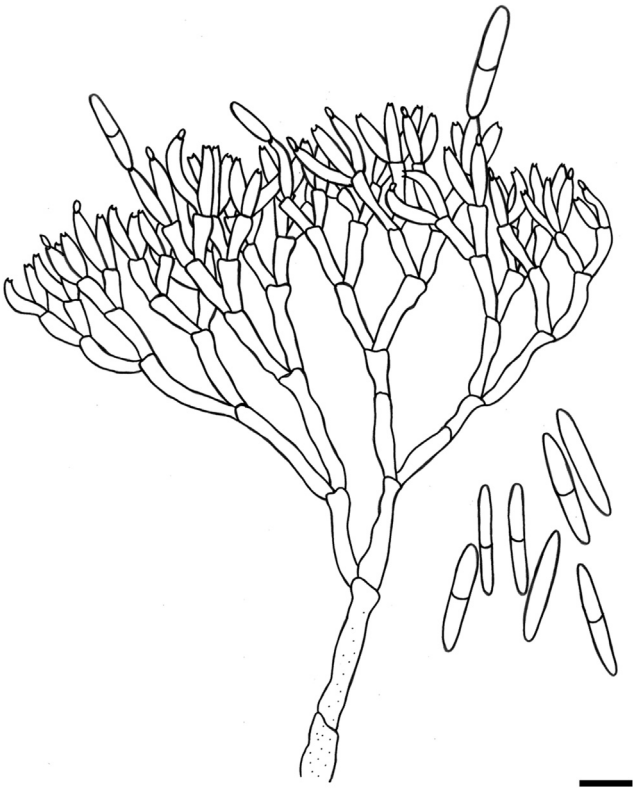


Fig. 3 – *Gliocladiopsis forsbergii* (BRIP 61349a) on carnation leaf agar at 7 d. Penicillate conidiophore, conidiogenous cells and conidia. *Bar*: 10 μm.

30–80 × 4–6.5 μm, aseptate to 1-septate; tertiary branches 16–21.5 × 2.5–3.5 μm, aseptate; quaternary branches 10–13 × 1.5–2 μm, aseptate; quinary branches 10–12 × 1.5–2 μm, aseptate; phialides lageniform to cylindrical, 7.5–12 × 2 μm, straight or curved, with a terminal collarette, single or in whorls of 2–3. Conidia cylindrical (13.5–)15.5–19(–20) × (1–)1.5–2.5(–3) μm (n = 20), hyaline and mostly 1-septate. Sexual morph unknown.

Additional culture examined: AUSTRALIA, Queensland: Burbank, on necrotic stem lesions of *Grevillea* sp., Feb 2014, leg. K.G. Pegg (BRIP 60984).

Substrate and distribution: On roots of *Persea americana* (Lauraceae), *Grevillea* sp. (Proteaceae); Australia.

Notes: Phylogenetic inference is required to identify *G. forsbergii*. Unique fixed nucleotides were identified for *G. forsbergii* at three loci: β-tubulin positions 173 (T), 328 (C), 377 (G) and 407 (A); Histone H3 positions 51 (C), 56 (T), 117 (A) and 439–440 (CTC); ITS at positions 3 (A), 191–199 (-TGGCA-T) and 478 (G).

Gliocladiopsis peggii L.E. Parkinson, E.K. Dann & R.G. Shivas, sp. nov. Figs. 2, 4.
MycoBank no.: MB 817264.

Diagnosis: *Gliocladiopsis peggii* formed a monophyletic group that may represent an unresolved species complex as it contained a polytomy that included a well-supported clade comprising two subclades (Fig. 1). The type of *G. peggii* was selected from one of these subclades.

Holotype: AUSTRALIA, Queensland, Woombye, on necrotic roots of *Persea americana* in a seedling nursery, 18 Jul 2013, leg. L.E. Parkinson (BRIP 60983, permanently preserved in a metabolically inactive state).

Etymology: Named after Ken G. Pegg AM, one of Australia's most respected and inspirational plant pathologists for more than five decades.

Colonies on sPDA reach 4 cm diam after 7 d under near UV light at 24 °C, floccose at the margin, flat and zonate towards the centre, covered in conspicuous small white droplets, cream to pale luteous, margin entire; reverse zonate, ochreous, darker at the centre becoming paler towards the margin. Conidiophores penicillate, 80–110 × 4–7.5 μm, with subhyaline stipes. Conidiogenous apparatus with several series of hyaline branches; primary branches 20–25 × 3–4.5 μm, aseptate; secondary branches 8–15 × 2.5–4 μm, aseptate; phialides lageniform to cylindrical, 10–15 × 2–3 μm, arranged in whorls of 3–4 per branch, straight or curved with a minute collarette. Conidia cylindrical, (10.5–)14–18(–19) × (1.5–)2–3 μm (n = 20), hyaline and medianly 1-septate. Sexual morph unknown.

Additional cultures examined: On *Persea americana* (Lauraceae), AUSTRALIA, Queensland: Woombye, seedling nursery, on necrotic roots, Jun 2010, leg. E.K. Dann & A.W. Cooke (BRIP 53654); Walkamin, seedling nursery, on necrotic roots, 2 Dec 2013, leg. K.G. Pegg (BRIP 60987); Walkamin, seedling nursery, in soil, 2 Dec 2013, leg. K.G. Pegg (BRIP 60988);

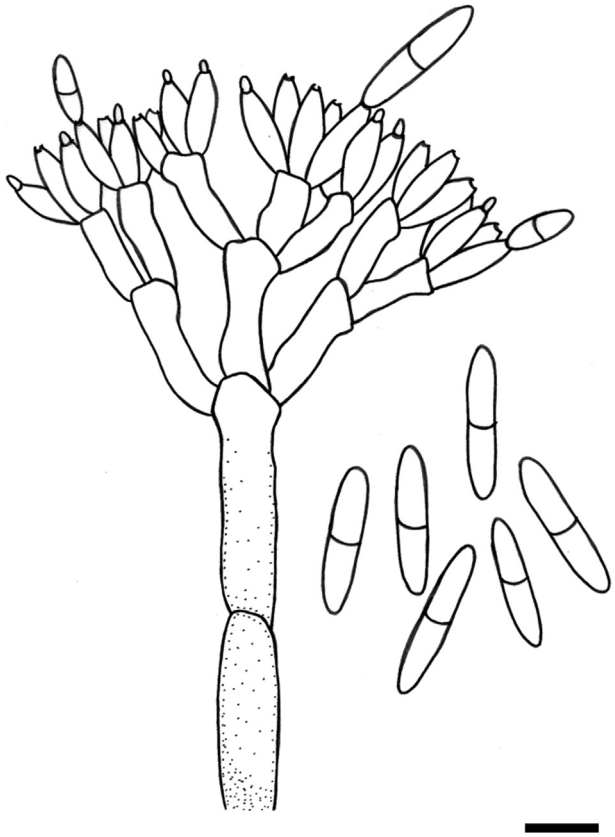


Fig. 4 – *Gliocladiopsis peggii* (BRIP 60983) on carnation leaf agar at 7 d. Penicillate conidiophore, conidiogenous cells and conidia. *Bar*: 10 μm.

Woombye, seedling nursery, on necrotic stem lesions, 12 Dec 2013, leg. A.G. Manners (BRIP 60990); Woombye, seedling nursery, on necrotic roots, Aug 2015, leg. L.E. Parkinson (BRIP 63709a, BRIP 63709b, BRIP 63709c, BRIP 63710a, BRIP 63710c). AUSTRALIA, Queensland: Woombye, seedling nursery, on necrotic roots, Jun 2010, leg. E.K. Dann & A.W. Cooke (BRIP 54019); Duranbah, seedling nursery, on necrotic roots, Jul 2015, leg. L.E. Parkinson (BRIP 62845a, BRIP 62845b, BRIP 62845c). AUSTRALIA, South Australia: Waikerie, in an orchard, on necrotic roots, Nov 2015, leg. L.E. Parkinson (BRIP 63711c, BRIP 63711d).

Substrate and distribution: On *Persea americana* (Lauraceae); Australia.

Notes: *Gliocladiopsis peggii* is sister to *G. mexicana*, which is only known from soil in Mexico. *Gliocladiopsis peggii* was found on necrotic avocado roots, stem lesions or soil near diseased trees in eastern and southern Australia. The geographical distribution may be a reflection of the widespread movement of avocado planting material from major nurseries in the eastern states of Australia to all production areas. An isolate of *G. peggii* (BRIP 53654) was shown to significantly increase the height of avocado seedlings in a soil amendment trial (Dann et al. 2012 as *Gliocladiopsis* sp.). *Gliocladiopsis peggii* and *G. mexicana* both have penicillate conidiophores with secondary branching, or rarely tertiary branching in *G. mexicana* (Lombard and Crous 2012). Unique fixed nucleotides were identified for *G. peggii* at three loci, which differ from *G. mexicana*: β-tubulin at positions 29 (C), 237 (G), 421 (T) and 431 (T); Histone H3 at positions 59 (A), 69 (C), 77–79 (TCG), 283 (C), 291–293 (AAG), 318 (T), 322 (T) and 432 (T); ITS at positions 78 (T), 182–199 (-------TC--TGGCA-T) and 361 (C).

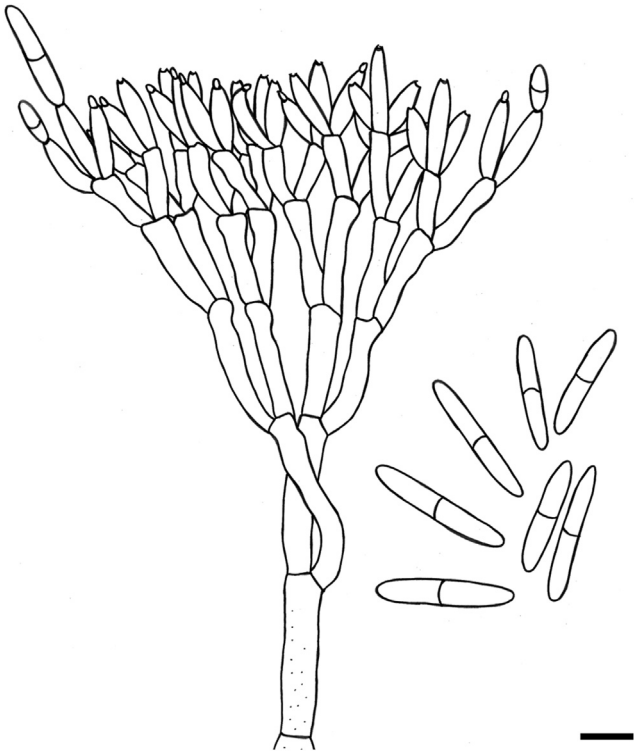


Fig. 5 – *Gliocladiopsis whileyi* (BRIP 61430) on carnation leaf agar at 7 d. Penicillate conidiophore, conidiogenous cells and conidia. *Bar*: 10 μm.

Gliocladiopsis whileyi L.E. Parkinson, E.K. Dann & R.G. Shivas, sp. nov. Figs. 2, 5.
MycoBank no.: MB 817265.

Diagnosis: *Gliocladiopsis whileyi* is most closely related to *G. curvata* (Fig. 1). *Gliocladiopsis whileyi* has a conidiogenous apparatus that contains quaternary branches, with 2–3 phialides per terminal branch, whereas *G. curvata* has mostly tertiary branches and rarely quaternary branches, with phialides in whorls of 2–6 per terminal branch (Lombard and Crous 2012). *Gliocladiopsis whileyi*, differs in morphology to sister species, *G. forsbergii* in conidiophore branching, in which *G. forsbergii* has quinary branches.

Holotype: AUSTRALIA, New South Wales, Duranbah, on necrotic roots of *Persea americana* cultivated in an orchard, May 2014, leg. L.E. Parkinson (BRIP 61430, permanently preserved in a metabolically inactive state).

Etymology: Named after Anthony (Tony) Whiley AM, whose holistic approach to horticultural production and research, and his ability to effectively communicate his research, has benefited both avocado growers and researchers in Australia.

Colonies on sPDA reach 4 cm diam after 7 d under near UV light at 24 °C, floccose, pale ochreous in the central part becoming cream at the margin, margin entire; reverse umber, becoming paler towards the margin, zonate. Conidiophores penicillate, 70–100 × 5–7 μm, with subhyaline to hyaline multi-septate stipes. Conidiogenous apparatus hyaline with several series of branches; primary branch 18–27 × 3–4 μm, aseptate; secondary branches 15–20 × 2.5–3 μm, aseptate; tertiary branches 13–15 × 2–3 μm, aseptate; quaternary branches 7–10 × 2–3 μm, aseptate; phialides lageniform to cylindrical, 11–17 × 2–3 μm, central phialides occasionally extended, straight or curved, with a minute terminal collarette, single or in whorls of 2–3. Conidia cylindrical, (16–)17–21(–22) × 1.5–3 μm (n = 20), straight or slightly curved, hyaline and medianly 1-septate. Sexual morph unknown.

Substrate and distribution: *Persea americana* (Lauraceae); Australia.

Notes: *Gliocladiopsis whileyi* and *G. forsbergii* share identical and unique fixed nucleotides in the ITS at positions 3, 182–188, 191–199 and 478. However in ITS *G. whileyi* and *G. forsbergii* are significantly different to *G. curvata*, demonstrating the separation of these species from *G. curvata*. *Gliocladiopsis whileyi* differs from *G. forsbergii* in the β-tubulin gene at positions 170–173, 178, 209–214, 377–383, whereas *G. whileyi* is identical to *G. curvata* at these positions.

4. Discussion

Multigene phylogenetic analysis and morphological comparisons of fungal structures of Australian cultures of *Gliocladiopsis* isolated from necrotic avocado roots, revealed three new species, *G. forsbergii*, *G. peggii* and *G. whileyi*. This brings the total number of *Gliocladiopsis* species to 13 worldwide (Table 1).

Gliocladiopsis species are generally soilborne (Lombard et al. 2015) with many of the known reported isolates collected from soil (Lombard and Crous 2012). However some *Gliocladiopsis* isolates have been collected from diverse symptomatic terrestrial plant material in various countries including avocado (in Ecuador), *Syzygium aromaticum* (Indonesia), *Archontophoenix purpurea* (New Zealand), *Chamaedorea elegans* (USA), *Araucaria* sp. (Malaysia), *Vanilla* sp. (Indonesia) and *Indigofera* sp. (Indonesia) (Lombard and Crous 2012). Interestingly, *Gliocladiopsis guangdonensis* was collected from decaying wood submerged in forest freshwater in China (Liu and Cai 2013).

All species of *Gliocladiopsis* are associated with the necrotic roots of diseased plants, plant litter or soil (Lombard and Crous 2012; Liu and Cai 2013). Only one species of *Gliocladiopsis* has been tested for pathogenicity, namely *G. peggii*, which actually increased the height of avocado seedlings in two of three soil amendment trials (Dann et al. 2012, as *Gliocladiopsis* sp.). It is noteworthy that all of the isolates of *G. peggii* were collected from young, unhealthy avocado trees, mostly in nurseries and orchards. Isolates of *G. peggii* were morphologically indistinguishable, although the phylogenetic analysis showed that it may represent an unresolved species complex as it contained a polytomy that included a well-supported clade comprising two subclades. The ecological role of *Gliocladiopsis* species in the rhizosphere, including their potential contribution to tree growth and decline, warrants further investigation.

Disclosure

The research undertaken in this study contains no conflicts of interest and complies with the current laws in Australia.

Acknowledgments

This project (AV14012) is funded by Horticulture Innovation Australia Limited using the avocado levy and funds from the Australian Government. This project is jointly supported by the Department of Agriculture and Fisheries (DAF) and the University of Queensland (UQ). The first author receives an Australian Postgraduate Award. We wish to thank the avocado nursery operators and growers who kindly allowed us to collect samples to build our isolate collection; and Dr Alistair McTaggart (UQ) and Ms Yu Pei Tan (BRIP, DAF) for their assistance with the phylogenetic analysis and data submission.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.myc.2016.10.004.

REFERENCES

Crous PW, 2002. *Taxonomy and pathology of* Cylindrocladium *(Calonectria) and allied genera*. The American Phytopathological Society, Minnesota.

Crous PW, Groenewald JZ, Risède JM, Simoneau P, Hywel-Jones NL, 2004. *Calonectria* species and their *Cylindrocladium* anamorphs: species with sphaeropedunculate vesicles. *Studies in Mycology* 50: 415–186.

Dann EK, Cooke AW, Forsberg LI, Pegg KG, Tan YP, Shivas RG, 2012. Pathogenicity studies in avocado with three nectriaceous fungi, *Calonectria ilicicola*, *Gliocladiopsis* sp. and *Ilyonectria liriodendri*. *Plant Pathology* 61: 896–902; http://dx.doi.org/10.1111/j.1365-3059.2011.02579.x.

Dhanoya A, 2012. Quantification of microliter volumes of DNA: an alternative to traditional low volume spectroscopy techniques is now available. *Genetic Engineering and Biotechnology News* 32: 32–33; http://dx.doi.org/10.1089/gen.32.17.14.

Huelsenbeck JP, Ronquist F, 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755; http://dx.doi.org/10.1093/bioinformatics/17.8.754.

Katoh K, Standley DM, 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* 30: 772–780; http://dx.doi.org/10.1093/molbev/mst010.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, 2012. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28: 1647–1649; http://dx.doi.org/10.1093/bioinformatics/bts199.

Liu F, Cai L, 2013. A novel species of *Gliocladiopsis* from freshwater habitat in China. *Cryptogamie, Mycologie* 34: 233–241; http://dx.doi.org/10.7872/crym.v34.iss2.2013.233.

Lombard L, Crous PW, 2012. Phylogeny and taxonomy of the genus *Gliocladiopsis*. *Persoonia* 28: 25–33; http://dx.doi.org/10.3767/003158512X635056.

Lombard L, Shivas RG, To-Anun C, Crous PW, 2012. Phylogeny and taxonomy of the genus *Cylindrocladiella*. *Mycological Progress* 11: 835–868; http://dx.doi.org/10.1007/s11557-011-0799-1.

Lombard L, Van Der Merwe N, Groenewald J, Crous P, 2015. Generic concepts in Nectriaceae. *Studies in Mycology* 80: 189–245; http://dx.doi.org/10.1016/j.simyco.2014.12.002.

O'Donnell K, Cigelnik E, 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* 7: 103–116; http://dx.doi.org/10.1006/mpev.1996.0376.

Promega Corporation, 2010. *Wizard® genomic DNA purification kit technical manual*. Promega Corporation, Madison.

Rambaut A, 2007. FigTree, a graphical viewer of phylogenetic trees. In: *Molecular evolution, phylogenetics and epidemiology*. *Institute of Evolutionary Biology*. University of Edinburgh. http://tree.bio.ed.ac.uk/software/figtree. Accessed 13 Mar 2016.

Rayner RW, 1970. *A mycological colour chart*. Commonwealth Mycological Society, Surrey.

Saksena SB, 1954. A new genus of Moniliaceae. *Mycologia* 46: 660–666.

Stamatakis A, 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2688–2690; http://dx.doi.org/10.1093/bioinformatics/btl446.

Talavera G, Castresana J, 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* 56: 564–577; http://dx.doi.org/10.1080/10635150701472164.

White TJ, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds), *PCR protocols: a guide to methods and applications*. Academic Press, Orlando, pp 315–322.

Phytopathology • 2017 • 107:1479-1485 • https://doi.org/10.1094/PHYTO-03-17-0084-R

Etiology

Pathogenicity of Nectriaceous Fungi on Avocado in Australia

Louisamarie E. Parkinson, Roger G. Shivas, and Elizabeth K. Dann†

First and third authors: Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, and second author: Plant Pathology Herbarium, Biosecurity Queensland, Department of Agriculture and Fisheries, Ecosciences Precinct, 41 Boggo Road, Dutton Park, QLD 4102, Australia.
Accepted for publication 17 July 2017.

ABSTRACT

Black root rot is a severe disease of young avocado trees in Australia causing black necrotic roots, tree stunting, and leaf drop prior to tree death. Nectriaceous fungi (Nectriaceae, Hypocreales), are commonly isolated from symptomatic roots. This research tested the pathogenicity of 19 isolates from *Calonectria*, *Cylindrocladiella*, *Dactylonectria*, *Gliocladiopsis*, and *Ilyonectria*, spp. collected from young avocado trees and other hosts. Glasshouse pathogenicity tests with 'Reed' avocado (*Persea americana*) seedlings confirmed that *Calonectria ilicicola* is a severe pathogen of avocado, causing stunting, wilting, and seedling death within 5 weeks of inoculation. Isolates of *C. ilicicola* from peanut, papaya, and custard apple were also shown to be aggressive pathogens of avocado, demonstrating a broad host range. An isolate of a *Calonectria* sp. from blueberry and avocado isolates of *Dactylonectria macrodidyma*, *D. novozelandica*, *D. pauciseptata*, and *D. anthuriicola* caused significant root rot but not stunting within 5 to 9 weeks of inoculation. An isolate of an *Ilyonectria* sp. from grapevine closely related to *Ilyonectria liriodendri*, and avocado isolates of *Cylindrocladiella pseudoinfestans*, *Gliocladiopsis peggii*, and an *Ilyonectria* sp. were not pathogenic to avocado.

Additional keywords: *Dactylonectria novozelandica*, *Dactylonectria pauciseptata*, *Ilyonectria liriodendri*.

Black root rot is a severely damaging disease of young avocado (*Persea americana*, family Lauraceae) trees caused by soilborne nectriaceous fungi (Nectriaceae, Hypocreales) (Dann et al. 2012; Ramírez-Gil and Morales-Osorio 2013; Vitale et al. 2012). Symptoms of black root rot in young avocado trees include tree stunting, wilt, leaf chlorosis and browning, leaf drop, and rapid decline and death of young orchard transplants (Fig. 1) (Dann et al. 2012). Affected roots have brown to black, sunken lesions which coalesce to destroy the root completely (Fig. 1).

Infected nursery trees have been reported to die within 1 to 5 years of transplantation into orchards, causing significant commercial loss in Australia (Dann et al. 2012), Chile (Besoain and Piontelli 1999), Colombia (Ramírez-Gil and Morales-Osorio 2013), Israel (Zilberstein et al. 2007), Italy (Vitale et al. 2012), and New Zealand (Boesewinkel 1986). Species confirmed by pathogenicity tests as the cause of black root rot in avocado include *Calonectria ilicicola* in Australia (Dann et al. 2012), which also caused severe stunting, and *Dactylonectria macrodidyma* (as *Ilyonectria macrodidyma*) in Italy (Vitale et al. 2012). *I. liriodendri* and an undescribed *Gliocladiopsis* sp. were not pathogenic to avocado seedlings in glasshouse pathogenicity tests (Dann et al. 2012). Other fungi have been reported associated with black root rot of avocado, including *Cylindrocladiella parva* (Crous et al. 1991; Dann et al. 2012), *Gliocladiopsis peggii*, *G. whileyi*, and *G. forsbergii* (Parkinson et al. 2017).

There are reports of *I. destructans* as a pathogen of avocado (as *Cylindrocarpon destructans* in Besoain and Piontelli 1999; Darvas 1978; Ramírez-Gil and Morales-Osorio 2013; and as *Neonectria radicicola* in Zilberstein et al. 2007). The first report of *I. destructans* isolated from avocado was from South Africa (Darvas 1978); later, it was found in Chile, where 22,000 nursery trees were killed between 1994 and 1995 (Besoain and Piontelli 1999). More recently, *I. destructans* has been reported in avocado seedlings in Israel (Zilberstein et al. 2007) and Columbia (Ramírez-Gil and Morales-Osorio 2013). However, conclusive evidence of pathogenicity and accurate identification of the causal agent was not demonstrated in these studies. That is, confirmation of pathogenicity by demonstrating Koch's postulates were not recorded in the studies of Darvas (1978) or Zilberstein et al. (2007). Moreover, the studies in Colombia had carried out pathogenicity tests and Koch's postulates but relied on morphology alone to identify the fungi (Ramírez-Gil and Morales-Osorio 2013), which potentially risks misidentification of cryptic and closely related species. *I. destructans* has had numerous taxonomic nomenclature changes over time (Lombard et al. 2015) and correct identification by phylogenetic methods and gene sequencing is important for accurate identification of this species. Thus far, there are no studies that have shown that *Ilyonectria* spp. are pathogens of avocado.

Dactylonectria is a genus recently separated from *Ilyonectria* (Lombard et al. 2014, 2015) and a number of species have been reported as soilborne pathogens, including *D. macrodidyma* causing black foot disease of grapevines (as *C. macrodidymum* in Halleen et al. 2004; and as *I. macrodidyma* in Agustí-Brisach and Armengol 2013; Cabral et al. 2012; Whitelaw-Weckert et al. 2013) and apple seedling replant disease (Tewoldemedhin et al. 2011b). *D. macrodidyma* caused significant root rot in 100% of potted grapevines (*Vitis vinifera* 'Chardonnay') inoculated with *D. macrodidyma* in Western Australia (Whitelaw-Weckert et al. 2013). However, *D. macrodidyma* has never been associated with avocado disease in Australia. Many previous records of *Cylindrocarpon* spp. on avocado in Australia (Dann et al. 2012) warrant reidentification because these fungi have been recently transferred to other genera, including *Cylindrodendrum*, *Dactylonectria*, *Ilyonectria*, and *Neonectria* (Lombard et al. 2014, 2015).

Six genera in the family Lauraceae have species that are reported as hosts for *Calonectria* spp. (Crous 2002; Lombard et al. 2010b), including *Persea* (Dann et al. 2012) and *Laurus* (laurels) (Polizzi et al. 2012). *Calonectria pauciramosa* is reported as a dominant nursery pathogen in Australia (Lombard et al. 2011) and South Africa (Crous 2002; Lombard et al. 2010a, 2011), while *C. ilicicola* is highly pathogenic to horticultural and field crops, causing several diseases, including red crown rot in soybean (Kuruppu et al. 2004;

†Corresponding author: E. K. Dann; E-mail: e.dann@uq.edu.au

Ochi et al. 2011), Cylindrocladium black rot of peanut (Wright et al. 2010), collar rot of papaya (Male et al. 2012), crown and root rot of bay laurel (Polizzi et al. 2012), and leaf spot in holly (*Ilex aquifolium*) (Lechat et al. 2010). Damage caused by *C. ilicicola* is reported to be as high as 50% yield loss in both peanut (Wright et al. 2010) and soybean (Kuruppu et al. 2004). The ability of nectriaceous pathogens of other crops to infect and cause disease in avocado is yet to be investigated.

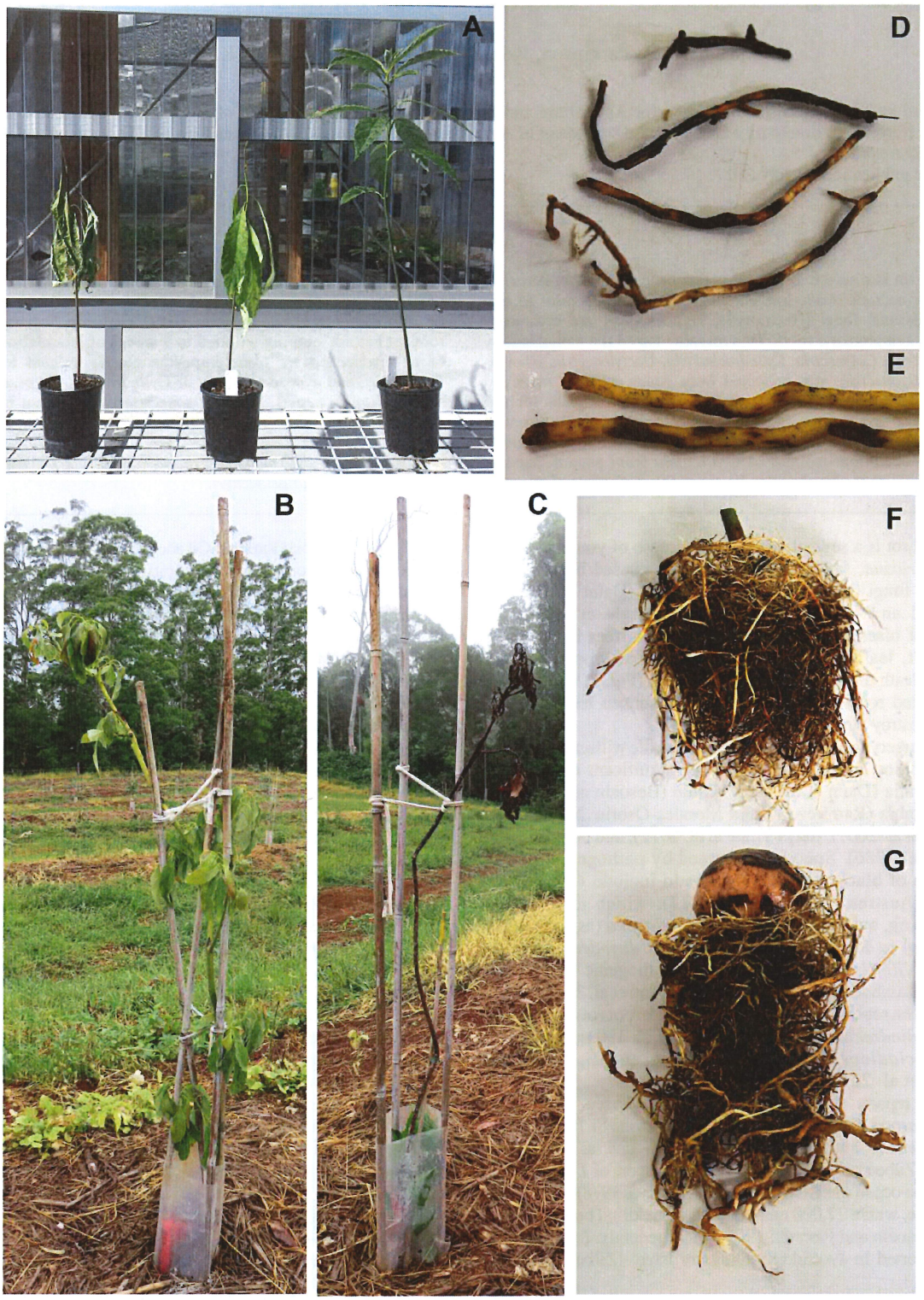


Fig. 1. Symptoms of black root rot disease in avocado seedlings and orchard transplants. **A,** Leaf wilt and tree stunting in Reed avocado seedlings 5 weeks after inoculation with *Calonectria ilicicola* compared with uninoculated seedling. Black root rot symptoms in 1-year-old avocado orchard transplants: **B,** leaf wilt followed by **C,** rapid death. **D** and **E,** Characteristic black, necrotic root lesions caused by nectriaceous pathogens, which coalesce to rot the entire root. Avocado black root rot symptoms after 9 weeks of inoculation with **F,** *C. ilicicola* and **G,** *Dactylonectria macrodidyma*.

This investigation tested several Australian isolates from the nectriaceous genera *Calonectria*, *Dactylonectria*, *Ilyonectria*, *Gliocladiopsis*, and *Cylindrocladiella* in experiments for pathogenicity in avocado roots.

MATERIALS AND METHODS

Isolate identification and inoculum preparation. Nineteen fungal isolates from the genera *Calonectria*, *Cylindrocladiella*, *Dactylonectria*, *Gliocladiopsis*, and *Ilyonectria* (Table 1) were chosen for pathogenicity tests. The isolates included species from diseased roots of nursery and field plants of avocado, blueberry, custard apple, grapevine, papaya, and peanut. The isolates were cultured on half-strength potato dextrose agar amended with streptomycin (sPDA) and kept at room temperature under black light (12 h of black light and 12 h of darkness) for 7 days prior to preparation of inoculum for glasshouse experiments and DNA extractions.

The fungal inoculum contained four 1-cm^2 sPDA cubes of the respective fungal isolate, which were added to separate 2-liter flasks containing autoclaved media consisting of 200 g of sand, 20 g of bran, and 80 ml of water (ratio 10:1:4 [wt/wt]) (Dann et al. 2012), maintained at room temperature on a laboratory bench, and shaken daily for 7 to 10 days to distribute the inoculum evenly.

Isolates were identified by morphology; partial gene sequencing of β-tubulin, histone H3, and internal transcribed spacer (ITS) regions 1 and 2 and the 5.8S gene of the ribosomal RNA; and phylogenetic analyses.

DNA was extracted from fungal mycelia from cultures grown on sPDA for 7 to 10 days using the Promega Wizard genomic DNA purification kit (Promega Corp. 2010), with modifications to the protocol, including 50 to 100 mg of hyphae ground in 600 µl of nuclei lysis solution by tissue lysis (Tissue Lyser; Qiagen) at 30 shakes/s for 3 to 6 min or by hand with a microfuge tube pestle. DNA extracts of 50 ng/µl, measured with a BioDrop spectrophotometer (Dhanoya 2012), were selected as DNA templates for polymerase chain reaction (PCR).

The ITS, β-tubulin, and histone H3 gene loci for each isolate were amplified in PCR with 1 U of Invitrogen *Taq* polymerase, 0.6 µM forward primer, 0.6 µM reverse primer, 0.2 mM each DNTP, 1.5 mM $MgCl_2$, and 1× PCR buffer. The PCR primers used included ITS5 and ITS4 (White et al. 1990) for amplifying 600 bp of the ITS region; T1 (O'Donnell and Cigelnik 1997) and CYLTUB1R (Crous et al. 2004) for amplifying 600 bp of the partial β-tubulin gene; and CYLH3F and CYLH3R (Crous et al. 2004) for amplifying 500 bp of the partial histone H3 gene.

Thermal cycling consisted of initial denaturation at 95°C for 2 min; followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min; and terminated at 72°C for 5 min. Amplicons were sent to Macrogen Inc. (Republic of Korea) for sequencing.

Approximate genus identities were determined from consensus sequences using a Basic Local Alignment Search Tool (National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov/). Multiple alignments of three gene loci were performed on the sequences of interest and sequences from type species were

TABLE 1. List of fungal isolates tested in glasshouse pathogenicity experiments

Fungal species	BRIP[y]	Host	Locality, state[z]	Approximate age of plant, health	Place of collection	Substrate collector
Calonectria sp.	60981	*Vaccinium* sp.	NSW	Unknown, diseased	Field	E. K. Dann
Calonectria ilicicola	53933a	*Carica papaya*	South Johnstone, QLD	Unknown, diseased	Field	P. Ibell
	54018a	*Persea americana*	QLD	Young potted nursery tree, <1 year old, diseased	Nursery	E. K. Dann, A. W. Cooke, L. I. Forsberg (Dann et al. 2012)
	60389	*Arachis hypogaea*	Tolga, QLD	Unknown, diseased	Field	L. Owens
	60982	*P. americana*	Woombye, QLD	Young potted nursery tree, <1 year old, diseased	Nursery	A. W. Cooke, A. G. Manners
	60992	*C. papaya*	South Johnstone, QLD	Unknown, diseased	Nursery	L.L. Vawdrey
	61291	*Annona reticulata*	Woombye, QLD	Young potted nursery tree, <1 year old, diseased	Nursery	A. G. Manners
Cylindrocladiella pseudoinfestans	60986	*P. americana*	Woombye, QLD	Young potted nursery tree, <1 year old, diseased	Nursery	L. McDonald
Dactylonectria macrodidyma	61294a	*P. americana*	Alstonville, NSW	Established orchard tree, >20 years old, diseased	Orchard	L. E. Parkinson
	61294b	*P. americana*	Alstonville, NSW	Established orchard tree, >20 years old, diseased	Orchard	L. E. Parkinson
	61349e	*P. americana*	Mullumbimby, NSW	Young potted nursery tree, <1 year old, diseased	Nursery	L. E. Parkinson
	62001b	*P. americana*	Robinvale, VIC	Established orchard tree, <1 year old, diseased	Orchard	L. E. Parkinson
Dactylonectria novozelandica	62000d	*P. americana*	Gol Gol, NSW	Young potted nursery tree, <1 year old, diseased	Nursery	L. E. Parkinson
Dactylonectria pauciseptata	61428d	*P. americana*	Nimbin, NSW	Established orchard tree, <1 year old, diseased	Orchard	L. E. Parkinson
Dactylonectria anthuriicola	60985	*P. americana*	Hampton, QLD	Established orchard tree, >8 years old, healthy	Orchard	K. G. Pegg, L. E. Parkinson
Gliocladiopsis peggii	60987	*P. americana*	Walkamin, QLD	Young potted nursery tree, <1 year old, diseased	Nursery	K. G. Pegg
	60990	*P. americana*	Woombye, QLD	Young potted nursery tree, <1 year old, diseased	Nursery	A. G. Manners
Ilyonectria sp.	53498a	*Vitis vinifera*	Hunter Valley, NSW	Unknown, diseased	Vineyard	Unknown
Ilyonectria sp.	61349d	*P. americana*	Mullumbimby, NSW	Young potted nursery tree, <1 year old, diseased	Nursery	L. E. Parkinson

[y] Culture accession number.
[z] States in Australia: QLD = Queensland, NSW = New South Wales, and VIC = Victoria.

downloaded from GenBank. Fungal species identities were confirmed with congruent maximum-likelihood and Bayesian inference phylogenetic trees of partitioned gene loci (data not shown in this study).

Pathogenicity tests. There were three separate pathogenicity experiments, with two replicate trials per experiment, each with 10 (experiments 1 and 3) or 12 (experiment 2) plants per treatment (isolate tested). 'Reed' avocado test plants were grown from seed in the glasshouse (approximately 22 to 24°C [day] and 18°C [night]) for 3 to 6 months, until seedlings were approximately 30 to 40 cm high.

Potting soil (Searles Premium Potting Mix) was added to the bottom 3 cm of each plant pot (12.5 cm in diameter). Inoculum from flasks was mixed with vermiculite (grade 3) at a 3:1 ratio (percent vol/vol) of vermiculite/inoculum, filling each 2-liter flask; then, approximately 165 to 200 ml of the mixture was distributed into the pots for each treatment group or isolate. A single avocado seedling was transplanted into each pot, with the roots touching the inoculum, and the pots were filled with potting soil. Plant height (top of the seed to the plant apex) was measured immediately after transplantation, then weekly, and any visible disease symptoms were recorded. At 5 weeks (experiments 1 and 3) or 9 weeks (experiment 2) postinoculation, the seedlings were uprooted, the roots were washed to remove potting mixture, and the percentage of necrotic or discolored roots relative to total roots was assessed for each plant. Plant (leaves and stems) and roots were weighed, dried at 55°C for 3 days, and reweighed. The causal agents were confirmed by surface sterilizing fresh root samples in 50% ethanol and plating on sPDA from three to four representative plants of each tested isolate, and observing the fungal morphological structures under a light microscope after 4 to 6 days of growth under black light (12 h of black light and 12 h of darkness) at room temperature.

Experiment 1: Pathogenicity testing of *Calonectria* and *Ilyonectria* spp. The isolates included *C. ilicicola* (BRIP 54018a), collected from symptomatic nursery avocado (*P. americana*), which was used as a positive control for disease symptom development in all experiments, and nectriaceous species from other hosts on which the isolates caused disease; for example, *C. ilicicola* from custard apple (*Annona reticulata*) (BRIP 61291), peanut (*Arachis hypogaea*) (BRIP 60389), and papaya (*Carica papaya*) (BRIP 60992 and BRIP 53933a); an undescribed *Calonectria* sp. from blueberry (*Vaccinium* sp.) (BRIP 60981); and an undescribed *Ilyonectria* sp. from grapevine (*V. vinifera*) (BRIP 53498a) (Table 1).

Experiment 2: Pathogenicity testing of *Calonectria*, *Dactylonectria*, and *Ilyonectria* spp. The isolates tested were collected from symptomatic avocado (1 year old) nursery trees and young orchard transplants (mostly <1 year old; two isolates >20 years old) and one established healthy orchard tree (>8 years old) (Table 1), including four *D. macrodidyma* isolates (BRIP 61294a, BRIP 61294b, BRIP 61349e, and BRIP 62001b), *D. novozelandica* (BRIP 62000d), *D. pauciseptata* (BRIP 61428d), *D. anthuriicola* (BRIP 60985), *Ilyonectria* sp. (BRIP 61349d), and *C. ilicicola* (BRIP 54018a) (Table 1).

Experiment 3: Pathogenicity testing of *Calonectria*, *Cylindrocladiella*, and *Gliocladiopsis* spp. The isolates tested in this experiment were collected from symptomatic nursery avocado trees and included one *Cylindrocladiella pseudoinfestans* isolate (BRIP 60986), two *G. peggii* isolates (BRIP 60987 and BRIP 60990), and *C. ilicicola* (BRIP 54018a) (Table 1).

Statistical analyses. The data from each experimental trial were pooled because there was no significant treatment × trial interaction. The statistical analysis of pooled means was performed using the software GenStat (16th edition; VSN International Ltd.). Plant heights over time, plant and root biomasses, and percentage of necrotic roots were each analyzed by analysis of variance. Fisher's least significant difference was used to rank the means.

RESULTS

Experiment 1: Pathogenicity testing of *Calonectria* and *Ilyonectria* spp. The effects of inoculation with *Calonectria* and *Ilyonectria* isolates on avocado were tested in the glasshouse over 5 weeks. A significant difference between the mean plant heights was observed at 4 weeks postinoculation ($P < 0.001$) (data not shown). At 5 weeks postinoculation, all *C. ilicicola* isolates caused significant ($P < 0.001$) stunting in seedlings (Fig. 1; Table 2). Plants inoculated with a *Calonectria* sp. (BRIP 60981) from blueberry and an *Ilyonectria* sp. (BRIP 53498a) from grapevine were not significantly different in height from the uninoculated controls (Table 2).

At 5 weeks postinoculation, all *C. ilicicola* isolates significantly reduced leaf and stem biomass (Table 2) compared with uninoculated plants. Leaf and stem biomass of plants inoculated with a *Calonectria* sp. from blueberry and an *Ilyonectria* sp. from grapevine were not significantly different from that of plants grown in uninoculated media (Table 2).

All *C. ilicicola* isolates significantly reduced root biomass compared with uncolonized control media, with an approximately 74% average reduction in fresh weight (Table 2). The largest reductions in root biomass was caused by inoculation with *C. ilicicola* isolated from avocado (BRIP 54018a), custard apple (BRIP 61291), peanut (BRIP 60389), and papaya (BRIP 53933a). There was significant variation in root biomass and necrosis between the two *C. ilicicola* isolates from papaya, despite both papaya isolates significantly reducing root biomass compared with uninoculated controls (Table 2). The average root biomass of plants inoculated with a *Calonectria* sp. (BRIP 60981) or an *Ilyonectria* sp. (BRIP 53498a) was not statistically different from those from uninoculated plants (Table 2).

All *Calonectria* isolates caused significantly greater avocado root necrosis than uncolonized media, with *C. ilicicola* isolated

TABLE 2. Effect of inoculation with *Calonectria* and *Ilyonectria* spp. isolated from other hosts on growth of Reed avocado seedlings and percentage of root necrosis at 5 weeks after inoculation[y]

Inoculum	BRIP[z]	Host	Plant height (cm)	Leaf + stem biomass (g)		Root biomass (g)		Root necrosis (%)
				Fresh	Dry	Fresh	Dry	
Uncolonized media	...	...	27.5 a	17.0 a	4.75 a	16.6 ab	2.18 a	14.6 d
Calonectria ilicicola	54018a	*Persea americana*	21.1 c	8.17 d	2.72 c	4.33 d	0.57 c	81.3 a
Calonectria sp.	60981	*Vaccinium* sp.	26.0 ab	15.9 a	4.56 a	14.8 bc	1.94 ab	27.6 bc
C. ilicicola	61291	*Annona reticulata*	21.3 c	8.78 cd	2.88 c	6.61 d	0.80 c	70.5 a
C. ilicicola	60389	*Arachis hypogaea*	22.4 bc	8.46 d	2.98 c	4.09 d	0.54 c	79.1 a
C. ilicicola	53933a	*Carica papaya*	21.4 c	9.64 cd	3.10 bc	4.72 d	0.58 c	79.1 a
C. ilicicola	60992	*C. papaya*	23.1 bc	12.0 bc	3.44 bc	11.5 c	1.56 b	31.4 b
Ilyonectria sp.	53498a	*Vitis vinifera*	23.6 abc	14.0 ab	3.97 ab	18.6 a	2.35 a	17.2 cd

[y] Mean values within columns with the same letter are not significantly different ($P < 0.001$).
[z] BRIP accession of fungal isolate.

from peanut (BRIP 60389), papaya (BRIP 53933a), custard apple (BRIP 61291), and avocado (BRIP 54018a) causing the most severe necrosis. Although the biomass of roots inoculated with the blueberry *Calonectria* isolate were similar to uninoculated roots, the percentage of necrotic roots was significantly higher (Table 2). The percentage of symptomatic avocado roots in plants inoculated with an *Ilyonectria* sp. isolated from grapevine (BRIP 53498a) was not significantly different from that of uninoculated avocado plants (Table 2).

Experiment 2: Pathogenicity testing of *Calonectria*, *Dactylonectria*, and *Ilyonectria* spp. The effects of inoculation with *C. ilicicola*, *Dactylonectria* spp., and *Ilyonectria* isolates on avocado were tested in the glasshouse over 9 weeks. A significant difference between the mean plant heights was observed from 6 weeks postinoculation ($P < 0.001$) (data not shown), where plants inoculated with *C. ilicicola* were significantly shorter than uninoculated plants or those inoculated with an *Ilyonectria* sp. and all isolates of *Dactylonectria* spp., and remained significantly shorter for the rest of the trial period. By 9 weeks postinoculation, *C. ilicicola*-inoculated plants were 24% shorter than uninoculated plants (Table 3). Plants inoculated with an *Ilyonectria* sp. or *Dactylonectria* spp. were not significantly different from uninoculated plants across all time periods. However, at 9 weeks, wilting was observed in some plants inoculated with *D. macrodidyma* (BRIP 61349e and BRIP 61294a), *D. pauciseptata* (BRIP 61428d), and *C. ilicicola* (BRIP 54018a) (data not shown).

Plants inoculated with *C. ilicicola* had significantly reduced fresh weight and dry weight leaf and stem biomass compared with uninoculated plants, with a 33.5 to 33.6% biomass reduction (Table 3). Plants inoculated with *D. macrodidyma* (BRIP 61349e) were statistically similar in leaf and stem biomass to *C. ilicicola*, with a 16.6 to 18.7% reduction in fresh weight and dry weight, respectively. However, *Dactylonectria* spp. and the *Ilyonectria* sp. did not cause significant stunting or a reduction in biomass compared with uninoculated controls (Table 3). Root biomass of plants inoculated with *C. ilicicola*, an *Ilyonectria* sp., and *Dactylonectria* spp. were not significantly different from uninoculated plants ($P = 0.071$).

Inoculation with *C. ilicicola* and *Dactylonectria* spp. resulted in reduced avocado root health (Fig. 1), where the percentage of necrotic roots was significantly greater compared with uninoculated plants (Table 3). The percentage of necrotic roots after *C. ilicicola* inoculation was significantly greater than any other treatment: 2.8× higher than uninoculated controls and 1.3 to 2.2× higher than plants inoculated with the *Ilyonectria* sp. and *Dactylonectria* spp. Plants inoculated with any of the *Dactylonectria* isolates had significantly more symptomatic roots than uninoculated controls; while those inoculated with the *Ilyonectria* sp. had root symptoms similar to those of uninoculated controls (Table 3).

Experiment 3: Pathogenicity testing of *Calonectria*, *Cylindrocladiella*, and *Gliocladiopsis* spp. Glasshouse experiments tested the effects of inoculation with *C. ilicicola*, *Cylindrocladiella pseudoinfestans*, and *G. peggii* isolates on avocado seedlings over 5 weeks (Table 4). A significant difference between the mean plant heights was observed at 5 weeks postinoculation, where plants inoculated with *C. ilicicola* (BRIP 54018a) were significantly shorter than the uninoculated group and those inoculated with all other isolates ($P < 0.001$). The height of plants inoculated with *C. ilicicola* (BRIP 60982), *Cylindrocladiella pseudoinfestans* (BRIP 60986), and *G. peggii* (BRIP 60987 and BRIP 60990) was not significantly different from uninoculated plants or each other. However, plants inoculated with *G. peggii* (BRIP 60987) were significantly taller than the plants inoculated with *C. ilicicola* (BRIP60982).

Plants inoculated with *C. ilicicola* (BRIP 54018a and BRIP 60982) had significantly lower biomass compared with uninoculated controls (Table 4), with a 52% reduction in fresh leaf and stem

TABLE 3. Effect of inoculation with *Dactylonectria* and *Ilyonectria* spp. on growth of Reed avocado seedlings and percentage of root necrosis at 9 weeks after inoculation[y]

Inoculum	BRIP[z]	Plant height (cm)	Leaf + stem biomass (g)		Root biomass (g)		Root necrosis (%)
			Fresh	Dry	Fresh	Dry	
Uncolonized media	...	38.6 ab	35.1 ab	11.3 ab	26.9	2.93	20.6 d
Calonectria ilicicola	54018a	29.3 c	23.3 c	7.55 c	18.2	2.31	58.5 a
Ilyonectria sp.	61349d	36.8 ab	35.5 ab	11.1 ab	25.6	2.96	26.5 cd
Dactylonectria anthuriicola	60985	38.0 ab	36.9 a	12.0 a	27.5	3.31	34.5 bc
D. macrodidyma	61294a	36.4 ab	32.8 ab	10.3 ab	23.8	2.73	39.9 b
D. macrodidyma	61294b	40.0 a	35.3 ab	11.2 ab	25.1	2.90	33.3 bc
D. macrodidyma	61349e	35.5 ab	29.3 bc	9.23 bc	20.9	2.42	42.8 b
D. macrodidyma	62001b	38.6 ab	35.7 a	11.3 ab	25.9	2.80	39.2 b
D. novozelandica	62000d	34.5 b	32.4 ab	10.3 ab	25.0	2.84	42.8 b
D. pauciseptata	61428d	37.9 ab	33.8 ab	10.2 ab	25.2	2.82	40.5 b

[y] Mean values within columns with the same letter are not significantly different ($P < 0.001$).
[z] BRIP accession of fungal isolate.

TABLE 4. Effect of inoculation with *Calonectria*, *Cylindrocladiella*, and *Gliocladiopsis* spp. on growth of Reed avocado seedlings and percentage of root necrosis at 5 weeks after inoculation[y]

Inoculum	BRIP[z]	Plant height (cm)	Leaf + stem biomass (g)		Root biomass (g)		Root necrosis (%)
			Fresh	Dry	Fresh	Dry	
Uncolonized media	...	46.9 ab	21.3 a	5.84 a	12.9 b	1.31 b	22.2 b
Calonectria ilicicola	54018a	38.1 c	11.1 b	3.58 b	3.12 c	0.42 c	78.1 a
C. ilicicola	60982	41.3 abc	10.2 b	3.88 b	2.93 c	0.46 c	70.6 a
Cylindrocladiella pseudoinfestans	60986	45.2 abc	20.2 a	5.52 a	16.3 a	1.72 a	30.0 b
Gliocladiopsis peggii	60987	48.2 a	21.9 a	5.99 a	14.8 ab	1.62 ab	34.0 b
G. peggii	60990	44.5 abc	20.0 a	5.66 a	14.1 ab	1.53 ab	24.9 b

[y] Mean values within columns with the same letter are not significantly different ($P < 0.001$).
[z] BRIP accession of fungal isolate.

biomass and a 77% reduction in fresh weight root biomass (Table 4). The leaf and stem biomass and root biomass of plants inoculated with *G. peggii* were not significantly different from those from uninoculated plants. The leaf and stem biomass of plants inoculated with *Cylindrocladiella pseudoinfestans* was not significantly different from uninoculated plants; however, the root biomass of *C. pseudoinfestans*-inoculated plants was significantly higher than uninoculated plants by approximately 21%. Plants inoculated with *Calonectria ilicicola* had the highest percentage of necrotic roots compared with uninoculated plants, averaging 70 to 78% necrosis. Severity of root necrosis of *Cylindrocladiella* or *Gliocladiopsis*-inoculated plants was not significantly different from that of uninoculated plants (Table 4).

All of the isolates were successfully reisolated from the roots in selected plant specimens, fulfilling Koch's postulates. In all three trials, the uninoculated controls showed some measure of root discoloration which contributed to the percentage of necrotic roots in the root assessment. However, no pathogens were isolated from selected uninoculated plant root samples. The root discoloration in uninoculated controls was likely due to suberization rather than necrosis caused by disease.

DISCUSSION

C. ilicicola was shown to be an aggressive pathogen of avocado seedlings, causing significant root rot, reduced plant and root biomass, stunting, wilt, and death within 5 weeks of inoculation in glasshouse experiments. This confirmed a previous study by Dann et al. (2012), who found significant stunting caused by *C. ilicicola* in 'Velvick' avocado seedlings from 3 to 14 weeks postinoculation and in 'Hass' from 10 to 19 weeks. Contrasting with this study, the Reed seedlings tested in the study by Dann et al. (2012) were not significantly different in height to the uninoculated control group. However, similar to this study, in the Reed plants inoculated with *C. ilicicola*, significant root rot was found (Dann et al. 2012). *C. ilicicola* originally isolated from custard apple, papaya, and peanut and a *Calonectria* sp. from blueberry, caused black root rot disease in avocado seedlings, which demonstrates that these *Calonectria* spp. are potentially pathogenic to more than one host. The unidentified *Calonectria* sp. isolated from blueberry was closely related to *C. pauciramosa*, which Lombard et al. (2011) reported as a dominant nursery pathogen in Australia. Accurate identification of the fungal species responsible for avocado root rot has significant implications for multi-crop production nurseries and orchard disease management strategies.

In this study, *D. macrodidyma*, *D. novozealandica*, *D. pauciseptata*, and *D. anthuriicola* caused significant root rot but did not cause significant stunting in seedlings. A previous study demonstrated that *D. macrodidyma* was pathogenic to avocado, causing wilting, root rot, and tree death 2 months after inoculation (Vitale et al. 2012); however, plant height was not measured. This is the first report confirming the pathogenicity of *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata*, and *D. anthuriicola* on avocado trees in Australia.

A number of the pathogenic *Calonectria* and *Dactylonectria* isolates were collected directly from roots of nursery trees or from young trees that had declined within a year after being transplanted into the orchard. Once nursery stock is contaminated with nectriaceous pathogens, the spread of disease is exacerbated by frequent irrigation (and over-irrigation), crowded seedling arrangements, and poor nursery hygiene practices (Crous 2002). Desiccation and unfavorable environmental conditions for fungal growth have little effect on the primary survival of propagules, microsclerotia, and chlamydospores (Crous 2002; Sinclair and Backman 1989), because these highly resistant resting structures can survive for several years, infesting soil and host debris, and will germinate or sporulate when conditions become favorable (Crous 2002). Clean planting material is critical for preventing young tree deaths after transplanting (Dann et al. 2012, 2013). Australian avocado growers are able to source trees from several nurseries registered and frequently tested under the Australian Avocado Nursery Voluntary Accreditation Scheme and Nursery Industry Accreditation Scheme Australia.

Cylindrocladiella pseudoinfestans and *G. peggii* isolates from avocado were not pathogenic. *C. pseudoinfestans* increased root biomass but did not produce taller trees, whereas *G. peggii* did not cause any significant difference from the uninoculated controls. The isolates tested in this study are likely saprobic rhizosphere inhabitants (Lombard and Crous 2012) or root endophytes (Liu and Cai 2013). *Cylindrocladiella* spp. are generally not regarded as important plant pathogens (Lombard et al. 2012). However, *C. parva* was associated with avocado roots and cuttings in South Africa (Crous et al. 1991; Darvas 1978; van Coller et al. 2005), and the death of 3-year-old 'Wurtz' trees in Woombye, Australia in the 1980s (Dann et al. 2012). *C. parva* is reported as a common soil saprobe (Brown et al. 2013) associated with a number of hosts in genera such as *Acacia*, *Eucalyptus*, *Pinus* (Crous et al. 1991), and *Vitis* (Brown et al. 2013). The pathogenicity of *C. parva* to avocado remains unknown because pathogenicity experiments have never been reported, although based on our findings, it is unlikely to be responsible for severe root disease and tree death.

The undescribed *Ilyonectria* sp. isolate (BRIP 53498a) from grapevine was closely related to *I. liriodendri*, a pathogen of grapevine (Cabral et al. 2012), whereas the reported *I. liriodendri* isolate tested in Dann et al. (2012) has subsequently been found to be a potentially novel species, phylogenetically distinct from *I. liriodendri* (L. E. Parkinson, R. G. Shivas, and E. K. Dann, *unpublished data*). The other undescribed *Ilyonectria* sp. (BRIP 61349d) in this study is also a potentially novel species (L. E. Parkinson, R. G. Shivas, and E. K. Dann, *unpublished data*). Closely related to *I. capensis*, which causes Ilyonectria black foot rot in members of the family Proteaceae (Lombard et al. 2013). However, both *Ilyonectria* isolates in this study had no effect on avocado seedlings, consistent with the findings of Dann et al. (2012). Although the tested *Ilyonectria* isolates were not directly pathogenic, *Ilyonectria* spp. are also reported as soil saprobes (Agustí-Brisach and Armengol 2013) and their isolation from symptomatic avocado roots may be incidental.

However, there is a possibility that the pathogenic and other nectriaceous fungi found in association with young avocado orchard transplants predispose their hosts to infection by more aggressive pathogens (e.g., *Phytophthora cinnamomi*). Co-infection studies on grapevine cultivars with Botryosphaeriaceae spp. and *I. liriodendri* or *D. macrodidyma* significantly increased black foot disease severity compared with inoculation with *I. liriodendri* or *D. macrodidyma* alone (Whitelaw-Weckert et al. 2013). Similarly, synergistic pathogenicity between *D. macrodidyma* and *Pythium irregulare* was reported in apple seedlings; co-inoculated plants were significantly reduced in plant weight and height compared with inoculation with these species individually (Tewoldemedhin et al. 2011a). The prevalence of rapid death and decline of young avocado orchard transplants may be explained by previous infection with nectriaceous species in the nursery, followed by secondary infection by aggressive soilborne pathogens in the orchard.

This study found further causal agents of black root rot disease of avocado trees in Australia. Further studies should investigate the facilitation of disease by co-infection of a number of nectriaceous species and investigate other disease management methods to improve current management practices.

ACKNOWLEDGMENTS

AV14012 was funded by Horticulture Innovation Australia Limited using the avocado levy and funds from the Australian Government. This project is jointly supported by the Queensland Department of Agriculture and Fisheries (DAF) and the University of Queensland. L. E. Parkinson is a recipient of an Australian Postgraduate Award. We thank R. Daniel

(New South Wales Department of Primary Industries) and P. Trevorrow (DAF) for providing fungal isolates and the avocado nursery operators and growers who kindly allowed us to collect samples to build our isolate collection.

LITERATURE CITED

Agustí-Brisach, C., and Armengol, J. 2013. Black-foot disease of grapevine: An update on taxonomy, epidemiology and management strategies. Phytopathol. Mediterr. 52:245-261.

Besoain, X., and Piontelli, E. 1999. Black root rot in avocado plants (*Persea americana* Mill.) by *Cylindrocarpon destructans*: Pathogenicity and epidemiological aspects. Bol. Micol. 14:41-47.

Boesewinkel, H. J. 1986. New plant disease records from New Zealand. Australas. Plant Pathol. 15:18-21.

Brown, D., Jaspers, M., Ridgway, H., Barclay, C., Jones, E., and Zydenbos, S. 2013. Susceptibility of four grapevine rootstocks to *Cylindrocladiella parva*. N. Z. Plant Prot. 66:249-253.

Cabral, A., Cecília, R., Crous, P. W., and Oliveira, H. 2012. Virulence and cross-infection potential of *Ilyonectria* spp. to grapevine. Phytopathol. Mediterr. 51:340-354.

Crous, P. W. 2002. Taxonomy and Pathology of Cylindrocladium (*Calonectria*) and Allied Genera. The American Phytopathological Society, St. Paul, MN.

Crous, P. W., Groenewald, J. Z., Risède, J.-M., Simoneau, P., and Hywel-Jones, N. L. 2004. *Calonectria* species and their *Cylindrocladium* anamorphs: Species with sphaeropedunculate vesicles. Stud. Mycol. 50: 415-430.

Crous, P. W., Phillips, A. J. L., and Wingfield, M. J. 1991. The genera *Cylindrocladium* and *Cylindrocladiella* in South Africa, with special reference to forest nurseries. S. Afr. For. J. 157:69-85.

Dann, E. K., Cooke, A. W., Forsberg, L. I., Pegg, K. G., Tan, Y. P., and Shivas, R. G. 2012. Pathogenicity studies in avocado with three nectriaceous fungi, *Calonectria ilicicola, Gliocladiopsis* sp. and *Ilyonectria liriodendri*. Plant Pathol. 61:896-902.

Dann, E. K., Ploetz, R. C., Coates, L. M., and Pegg, K. G. 2013. Foliar, fruit and soilborne diseases. Pages 380-422 in: The Avocado: Botany, Production and Uses. B. Schaffer, B. N. Wolstenholme, and A. W. Whiley, eds. CABI, Homestead, FL.

Darvas, J. 1978. Common root pathogens from avocados. *South African Avocado Growers'*. Assoc. Res. Rep. 2:3-4.

Dhanoya, A. 2012. Quantification of microliter volumes of DNA. Genet. Eng. Biotechnol. News 32:32-33.

Halleen, F., Schroers, H., Groenwald, J. Z., and Crous, P. W. 2004. Novel species of *Cylindrocarpon* (*Neonectria*) and *Campylocarpon* gen. nov. associated with black foot disease of grapevines (*Vitis* spp.). Stud. Mycol. 50: 431-455.

Kuruppu, P. U., Schneider, R. W., and Russin, J. S. 2004. Factors affecting soybean root colonization by *Calonectria ilicicola* and development of red crown rot following delayed planting. Plant Dis. 88:613-619.

Lechat, C., Groenewald, J. Z., and Crous, P. W. 2010. The enigma of *Calonectria* species occurring on leaves of *Ilex aquifolium* in Europe. IMA Fungus 1:101-108.

Liu, F., and Cai, L. 2013. A novel species of *Gliocladiopsis* from freshwater habitat in China. Cryptogam.: Mycol. 34:233-241.

Lombard, L., Bezuidenhout, C. M., and Crous, P. W. 2013. Ilyonectria black foot rot associated with *Proteaceae*. Australas. Plant Pathol. 42:337-349.

Lombard, L., and Crous, P. W. 2012. Phylogeny and taxonomy of the genus *Gliocladiopsis*. Persoonia 28:25-33.

Lombard, L., Crous, P. W., Wingfield, B. D., and Wingfield, M. J. 2010a. Phylogeny and systematics of the genus *Calonectria*. Stud. Mycol. 66: 31-69.

Lombard, L., Crous, P. W., Wingfield, B. D., and Wingfield, M. J. 2010b. Species concepts in *Calonectria* (*Cylindrocladium*). Stud. Mycol. 66: 1-13.

Lombard, L., Polizzi, G., Guarnaccia, V., Vitale, A., and Crous, P. W. 2011. *Calonectria* spp. causing leaf spot, crown and root rot of ornamental plants in Tunisia. Persoonia 27:73-79.

Lombard, L., Shivas, R. G., To-Anun, C., and Crous, P. W. 2012. Phylogeny and taxonomy of the genus *Cylindrocladiella*. Mycol. Prog. 11:835-868.

Lombard, L., Van Der Merwe, N. A., Groenewald, J. Z., and Crous, P. W. 2014. Lineages in Nectriaceae: Re-evaluating the generic status of *Ilyonectria* and allied genera. Phytopathol. Mediterr. 53:515-532.

Lombard, L., Van Der Merwe, N. A., Groenewald, J. Z., and Crous, P. W. 2015. Generic concepts in Nectriaceae. Stud. Mycol. 80:189-245.

Male, M. F., Tan, Y. P., Vawdrey, L. L., and Shivas, R. G. 2012. Recovery, pathogenicity and molecular sequencing of *Calonectria ilicicola* which causes collar rot on Carica papaya in Australia. Australas. Plant Dis. Notes 7:137-138.

Ochi, S., Yoshida, M., Nakagawa, A., and Natsume, M. 2011. Identification and activity of a phytotoxin produced by *Calonectria ilicicola*, the causal agent of soybean red crown rot. Can. J. Plant Pathol. 33:347-354.

O'Donnell, K., and Cigelnik, E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Mol. Phylogenet. Evol. 7:103-116.

Parkinson, L. E., Shivas, R. G., and Dann, E. K. 2017. Novel species of *Gliocladiopsis* (Nectriaceae, Hypocreales, Ascomycota) from avocado roots (*Persea americana*) in Australia. Mycoscience 58:95-102.

Polizzi, G., Vitale, A., Aiello, D., Guarnaccia, V., Crous, P., and Lombard, L. 2012. First report of *Calonectria ilicicola* causing a new disease on Laurus (*Laurus nobilis*) in Europe. J. Phytopathol. 160:41-44.

Promega Corp. 2010. Wizard Genomic DNA Purification Kit Technical Manual. Promega Corp., Madison, WI.

Ramírez-Gil, G., and Morales-Osorio, J. G. 2013. First report of *Cylindrocarpon destructans* (Zinss) Scholten affecting avocado (*Persea americana* Mill) seedling in Colombia. Rev. Prot. Veg. 28:27-35.

Sinclair, J., and Backman, P. 1989. Compendium of Soybean Diseases. American Phytopathological Society Press, St. Paul, MN.

Tewoldemedhin, Y. T., Mazzola, M., Labuschagne, I., and McLeod, A. 2011a. A multi-phasic approach reveals that apple replant disease is caused by multiple biological agents, with some agents acting synergistically. Soil Biol. Biochem. 43:1917-1927.

Tewoldemedhin, Y. T., Mazzola, M., Mostert, L., and McLeod, A. 2011b. *Cylindrocarpon* species associated with apple tree roots in South Africa and their quantification using real-time PCR. Eur. J. Plant Pathol. 129:637-651.

van Coller, G. J., Denman, S., Groenewald, J. Z., Lamprecht, S. C., and Crous, P. W. 2005. Characterisation and pathogenicity of *Cylindrocladiella* spp. associated with root and cutting rot symptoms of grapevines in nurseries. Australas. Plant Pathol. 34:489-498.

Vitale, A., Aiello, D., Guarnaccia, V., Perrone, G., Stea, G., and Polizzi, G. 2012. First report of root rot caused by *Ilyonectria* (=*Neonectria*) *macrodidyma* on avocado (*Persea americana*) in Italy. J. Phytopathol. 160: 156-159.

White, T. J., Bruns, T., Lee, S., and Taylor, J. W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, Inc., New York.

Whitelaw-Weckert, M. A., Rahman, L., Appleby, L. M., Hall, A., Clark, A. C., Waite, H., and Hardie, W. J. 2013. Co-infection by *Botryosphaeriaceae* and *Ilyonectria* spp. fungi during propagation causes decline of young grafted grapevines. Plant Pathol. 62:1226-1237.

Wright, L., Davis, A., Wingfield, B., Crous, P., Brenneman, T., and Wingfield, M. 2010. Population structure of *Cylindrocladium parasiticum* infecting peanuts (*Arachis hypogaea*) in Georgia, USA. Eur. J. Plant Pathol. 127: 199-206.

Zilberstein, M., Noy, M., Levy, E., Elkind, G., Zeidan, M., Teverovski, E., and Ben Ze'ev, I. 2007. Wilting disease of young avocado trees caused by *Neonectria radicicola* in Israel. Pages 25-30 in: 6th World Avocado Congr. Proc. Conf. Viña Del Mar, Chile.