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L. E. Parkinson, D. P. Le, R. G. Shivas and E. K. Dann

Dr Louisamarie Parkinson BBiotech(Hons), PhD

Centre for Horticultural Science
Queensland Alliance for Agriculture and Food Innovation (QAAFI)
The University of Queensland, Brisbane Qld 4072 Australia
l.parkinson@uq.edu.au | https://www.qaafi.uq.edu.au

THE UNIVERSITY OF QUEENSLAND
St Lucia, Brisbane, Queensland Australia

PATHOGENICITY AND MOLECULAR DETECTION OF NECTRIACEOUS FUNGI ASSOCIATED WITH BLACK ROOT ROT OF AVOCADO

L. E. Parkinson[1], D. P. Le[1], R. G. Shivas[2], E. K. Dann[1]

[1] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Australia

[2]Centre for Crop Health, The University of Southern Queensland, Australia

KEY WORDS

Calonectria, *Calonectria ilicicola*, *Dactylonectria*, *Dactylonectria macrodidyma*, diagnostic test, diversity, loop-mediated isothermal amplification (LAMP)

SUMMARY

Black root rot of avocado associated with soilborne nectriaceous fungi is an aggressive disease of nursery trees and young orchards transplants, causing tree stunting, wilt, severe root necrosis, rapid decline and death within a year after planting. This study aimed to identify the fungal genera associated with the disease, determine the causal agents of black root rot, and develop a rapid molecular test for detection of key pathogens in avocado roots. A disease survey in all Australian growing regions collected 153 nectriaceous fungal isolates from roots of 91 symptomatic and healthy avocado trees and other hosts including peanut, papaya, blueberry, custard apple and grapevine. The fungal isolates were identified with phylogenetic analyses of ITS, β-tubulin and Histone H3 sequenced genes. Six genera were found associated with black root rot; *Calonectria*, *Cylindrocladiella*, *Dactylonectria*, *Gliocladiopsis*, *Ilyonectria* and *Mariannaea*. Glasshouse pathogenicity tests on 3–6 month-old avocado cv. Reed seedlings determined *Calonectria ilicicola* to be an aggressive pathogen, causing stunting and death within 5 weeks of inoculation. *C. ilicicola* isolated from peanut, papaya and custard apple also caused black root rot in avocado, demonstrating a broad host range. *Calonectria* sp. from blueberry and *Dactylonectria macrodidyma*, *D. novozelandica*, *D. pauciseptata* and *D. anthuriicola* from avocado caused significant root rot but not stunting within 5–9 weeks. *Ilyonectria* sp. from grapevine, and *Cylindrocladiella pseudoinfestans*, *Gliocladiopsis peggii* and *Ilyonectria* sp. isolates from avocado were determined to be non-pathogenic. Three loop-mediated isothermal amplification (LAMP) assays were developed for the detection of *C. ilicicola*, *D. macrodidyma* and the *Dactylonectria* genus. The assays were sensitive and specific at DNA concentrations of 1 pg/µl, 0.01 ng/µl and 0.1ng/µl for *C. ilicicola*, *D. macrodidyma*, and *Dactylonectria* spp. respectively. Detection in avocado roots averaged from 12–26 min for *C. ilicicola* and *D. macrodidyma* and 14–30 min for *Dactylonectria* spp.

INTRODUCTION

Black root rot of avocado, caused by soilborne fungal pathogens in the Nectriaceae family, is a severely damaging disease of nursery avocado trees and young orchard transplants, causing tree stunting, wilt, black, rotten and necrotic roots and rapid tree decline and death within a year after planting (Parkinson et al., 2017b). Typical early symptoms include sunken, brown or black necrotic lesions throughout the root which coalesce to rot the entire root (Parkinson et al., 2017b). Nectriaceous pathogens are a threat to new plantings; black root rot can potentially be undetected in young nursery trees as above ground symptoms of stunting and wilt may not develop until after planting in the field (Parkinson et al., 2017b).

A number of nectriaceous genera are associated with the disease, however until recently the known causal agents of black root rot were *Calonectria ilicicola* in Australia (Dann et al., 2012) and *Dactylonectria macrodidyma* in Italy (as *Ilyonectria macrodidyma* in Vitale et al., 2012). Other reported genera associated with black root rot of avocado include *Gliocladiopsis* (Dann et al., 2012; Parkinson et al., 2017a), *Ilyonectria* (Dann et al., 2012; Parkinson et al., 2017b) and *Cylindrocladiella* (Parkinson et al., 2017b). These genera share similar fungal spore morphology and identification to genus and species level with molecular methods, such as PCR and gene sequencing, is sometimes necessary for an accurate diagnosis.

Some fungi causing black root rot are commonly known by their historical genus names as '*Cylindrocarpon*' or '*Cylindrocladium*', causing confusion, as modern taxonomic studies have reclassified and separated these genera. *Cylindrocarpon* is now four separate genera known as *Neonectria*, *Ilyonectria*, *Cylindrodendrum* and *Dactylonectria* (Lombard et al., 2015) while *Cylindrocladium* is now known as *Calonectria* (Crous, 2002). Care must be considered when reporting the diagnostic identities of fungal isolates as use of the old names may impact perceptions on pathogenicity of the isolates.

Standard diagnostic practices for identifying black root rot include observing symptoms, isolating and culturing the causal agents from symptomatic root tissue on selective media, and identification of microbial species by microscopy or DNA sequencing. However these procedures can take several days to weeks to achieve a diagnosis (Niessen, 2015; Notomi et al., 2015). A rapid, sensitive and specific molecular method, such as loop-mediated isothermal amplification (LAMP), for early detection of black root rot can help enable faster implementation of disease management strategies and reduce commercial loss of young trees. LAMP is a nucleic acid amplification technique which can detect pathogens from diseased tissue in under 60 minutes (Fu et al., 2011; Notomi et al., 2000). LAMP uses 4–6 primers which target and anneal to 6–8 distinct regions of target DNA or nucleic acid sequence (Parkinson et al., 2019). A strand-displacing DNA polymerase initiates synthesis of the target nucleic acid sequence for amplification, and two of the primers form loop structures to facilitate subsequent rounds of amplification (Parkinson et al., 2019).

This investigation identified the nectriaceous fungal genera associated with black root rot in avocado in Australia, determined which fungi caused disease in glasshouse pathogenicity experiments, and developed a rapid molecular LAMP test for detection of the important pathogens.

MATERIALS AND METHODS

Diversity of nectriaceous fungi associated with black root rot of avocado in Australia

A national black root rot survey of orchards and nurseries in all avocado growing regions in Australia was conducted in 2013–2016 and collected nectriaceous fungal isolates from symptomatic roots of avocado and other horticultural crop hosts including peanut, papaya, blueberry, custard apple, grapevine and various forestry and ornamental trees including tea tree, *Elaeocarpus* sp., *Heliconia* sp., camphor laurel, *Endiandra* sp. and *Cryptocarya* sp. Ninety-one trees were surveyed which include 72 healthy and symptomatic avocado trees from nurseries and orchards and 19 symptomatic trees of other hosts. The fungal collection contained 153 nectriaceous fungal isolates, 129 of which came from avocado and 24 from other hosts. The avocado growing regions surveyed included Queensland (Far North, Central and South East Queensland), New South Wales (Northern New South Wales and along the Tri State), the Tri State (New South Wales, Victoria and South Australia along the Murray River), Western Australia and Norfolk Island.

The nectriaceous fungal isolates were initially identified to genus-level by microscopic study of fungal spores, then subsequently identified to species level using PCR, DNA sequencing and multigene phylogenetic analyses, as described in Parkinson et al. (2017b).

Pathogenicity of nectriaceous fungi

Nineteen fungal isolates from the genera *Calonectria*, *Cylindrocladiella*, *Dactylonectria*, *Ilyonectria* and *Gliocladiopsis* were selected for glasshouse pathogenicity testing on 3–6 month-old avocado cv. Reed seedlings. The fungal isolates tested included *Calonectria ilicicola* isolated from avocado, custard apple, peanut and papaya; an undescribed *Calonectria* sp. isolated from blueberry; two undescribed *Ilyonectria* spp. isolated from grapevine and avocado; four isolates of *Dactylonectria macrodidyma* and an isolate of *D. novozelandica*, *D. pauciseptata* and *D. anthuriicola* from avocado; an isolate of *Cylindrocladiella pseudoinfestans* and two isolates of *Gliocladiopsis peggii* from avocado (Parkinson et al., 2017b).

In three glasshouse pathogenicity experiments, avocado cv. Reed seedlings were inoculated with a single fungal isolate following the methods in Parkinson et al. (2017b) and maintained in the glasshouse for 5–9 weeks. Plant heights were measured weekly and at the end of each trial, seedlings were uprooted, the roots assessed for disease severity and the fresh and dry weight of plant roots, stems and leaves were recorded. The causal agents of black root rot were confirmed with Koch's Postulates by re-isolation from symptomatic roots and identification by microscopy. Two independent trials of the glasshouse pathogenicity experiments were conducted. Detail of this study is available in Parkinson et al. (2017b).

LAMP diagnostic test for rapid detection of Calonectria ilicicola, Dactylonectria macrodidyma and Dactylonectria spp. in avocado roots

Species and genus-specific LAMP primers were designed from β-tubulin gene sequence data of *C. ilicicola*, and from Histone H3 of *D. macrodidyma* and the *Dactylonectria* genus (Parkinson et al., 2019).

The LAMP primers were tested for specificity and sensitivity with 82 fungal isolates, which included the target species, *C. ilicicola* and *D. macrodidyma*; species within the target

Dactylonectria genus namely *D. macrodidyma*, *D. anthuriicola*, *D. novozelandica*, *D. pauciseptata* and *D. vitis*; and isolates of non-target species including *Calonectria* sp., *Cylindrocladiella* sp., *Gliocladiopsis forsbergii*, *G. peggii*, *G. whileyi*, *Ilyonectria* sp., *Mariannaea* sp., *Fusarium* sp. and *Phytophthora cinnamomi*. The LAMP protocol was optimised for use with avocado roots and includes fungal DNA extraction from avocado root tissue for use as DNA templates in the LAMP assay. The assay was tested, optimised and validated using pure fungal DNA of 82 fungal isolates, fungal cultures suspended in sterile distilled water, and necrotic avocado root tissue from inoculated glasshouse cv. Reed seedlings (Parkinson et al., 2019). The development of three LAMP assays for the detection of *C. ilicicola*, *D. macrodidyma* and *Dactylonectria* spp. is detailed in Parkinson et al. (2019).

RESULTS AND DISCUSSION

Diversity of nectriaceous fungi associated with black root rot of avocado in Australia

This study recorded the nectriaceous fungi associated with black root rot disease of avocado trees in Australia. Nectriaceous fungi were also collected from other hosts across avocado growing regions. The isolates associated with black root rot of avocado were identified in six genera of fungi in the *Nectriaceae*; *Calonectria*, *Cylindrocladiella*, *Dactylonectria*, *Gliocladiopsis*, *Ilyonectria* and *Mariannaea*. The species identified were *Calonectria ilicicola*, *Cylindrocladiella pseudoinfestans*, *Dactylonectria macrodidyma*, *D. novozelandica*, *D. anthuriicola*, *D. vitis*, *D. pauciseptata* and *Mariannaea humicola*. Likely new species across each genera have been provisionally identified, along with unresolved species, but remain undescribed (data not shown).

The geographical diversity of *Calonectria* spanned Far North, Central, Sunshine Coast and South East Queensland (QLD) and New South Wales (NSW), with *C. ilicicola* only reported in Queensland (Fig. 1). Fifteen isolates of *Calonectria* were collected. Isolates of *Calonectria ilicicola* from peanut, papaya and avocado were from peanut and avocado country in Far North QLD, from avocados and custard apple in the Sunshine Coast region of QLD and Central QLD; undescribed *Calonectria* spp. were from *Heliconia* sp., sugar apple and pinto peanut in Sunshine Coast; and undescribed *Calonectria* spp. were from blueberry and tea tree in NSW (Fig. 1). The *Calonectria* isolates came from nursery plants, young orchard transplants or small field crops and *Calonectria* associated with avocado only appears to be a problem in young trees.

Two *Cylindrocladiella* isolates were collected. *Cylindrocladiella pseudoinfestans* was isolated from symptomatic nursery avocado roots in the Sunshine Coast region of QLD, while an undescribed *Cylindrocladiella* sp. isolate was found in Northern NSW from camphor laurel roots (Fig. 1).

Sixty-four isolates of *Dactylonectria* were collected with over 50% of isolates identified as *D. macrodidyma*. Other isolates were identified as *D. novozelandica*, *D. anthuriicola*, *D. vitis* and *D. pauciseptata*. The geographical diversity of *Dactylonectria* isolates covered Eastern and Southern Australia (Queensland, New South Wales, Victoria, South Australia) to Western Australia, with hosts including avocado, *Endiandra*, *Cryptocarya* and *Cinnamomum* (camphor laurel) in the *Lauraceae* and also *Carica* (papaya) and *Elaeocarpus* (blueberry ash) (Fig. 1).

Twenty-eight isolates were identified as *Gliocladiopsis* and included species *G. peggii*, *G. whileyi*, *G. forsbergii* and undescribed *Gliocladiopsis* sp. from a broad environmental and geographical range in Australia (Fig. 1) (Parkinson et al., 2017a).

Forty-one isolates of *Ilyonectria* were collected and 39 were from avocado across a broad Australian geographic range (Queensland, New South Wales, Victoria and Western Australia) (Fig. 1). All of the *Ilyonectria* isolates are undescribed species.

Three isolates of *Mariannaea* were collected from young orchard avocado trees; undescribed *Mariannaea* sp. from NSW and *M. humicola* from South Australia. *Mariannaea* spp. are generally known as colonizers of wood, bark, pine needle, with some reports from diseased roots and soil (Cai et al., 2010). The pathogenicity of *Mariannaea* remains unknown.

The ages and health of trees from which *Dactylonectria*, *Ilyonectria* and *Gliocladiopsis* were isolated were diverse, with isolates collected from young and mature trees as well as symptomatic and healthy-looking trees.

Fig. 1. Map of nectriaceous fungal species found in various hosts across Australia. Australian avocado growing states include Queensland (QLD), New South Wales (NSW), Victoria (VIC), South Australia (SA) and Western Australia (WA).

Pathogenicity of nectriaceous fungi

Calonectria ilicicola was shown to be an aggressive pathogen of avocado seedlings, causing significant root rot (Fig. 2, 3), stunting (Fig. 2, 3), reduced plant and root biomass, wilt and death, within five to nine weeks after inoculation (Parkinson et al., 2017b). *Calonectria ilicicola* originally isolated from custard apple, papaya and peanut and a *Calonectria* sp. from blueberry, also caused black root rot disease in avocado seedlings (Parkinson et al., 2017b).

Calonectria sp. isolated from blueberry and *Ilyonectria* sp. isolated from grapevine were not able to cause stunting (Fig. 2), while *Ilyonectria* isolates in all pathogenicity experiments were not able to cause disease; the plant heights and root necrosis (Fig. 2, 3), and plant and root weights were statistically similar to uninoculated plants. The *Ilyonectria* sp. isolate from grapevine is a close relative of *I. liriodendri*, a black foot pathogen of grapevine (Cabral et al., 2012). This isolate had no effect on avocado seedlings and although *Ilyonectria* spp. are known soil inhabitants and cause disease on other hosts (Agustí-Brisach & Armengol, 2013), isolation from symptomatic avocado roots may be incidental.

Fig. 2. Average plant height (cm) and percentage of necrotic roots of avocado cv. Reed seedlings at 5 weeks post-inoculation. The fungal isolates tested on avocado were from multiple hosts including avocado, custard apple, peanut, papaya, blueberry and grapevine. Bars with the same letter and case are not significantly different ($P<0.001$).

Multiple *Dactylonectria* spp. isolated from avocado, including isolates of *D. macrodidyma*, *D. novozelandica*, *D. pauciseptata* and *D. anthuriicola*, were demonstrated to cause significant black root rot, but did not cause significant stunting in seedlings within 9 weeks of inoculation

(Fig. 3). *Calonectria ilicicola* isolated from avocado was shown to cause significantly more severe root necrosis and stunting compared to *Dactylonectria* spp. (Fig. 3). *Ilyonectria* sp. isolated from avocado was not able to cause disease, with symptoms statistically similar to uninoculated plants (Fig. 3). *Ilyonectria* and *Dactylonectria* are both *Cylindrocarpon*-like genera (Lombard et al., 2015) and in diagnostic reporting, it is important not to use "*Cylindrocarpon"* in naming, as pathogenicity on avocado appears to only be associated with *Dactylonectria*.

Fig. 3. Average plant height (cm) and percentage of necrotic roots of avocado cv. Reed seedlings at 9 weeks post-inoculation. The fungal isolates tested were from avocado. Bars with the same letter and case are not significantly different (P<0.001).

Cylindrocladiella pseudoinfestans and *Gliocladiopsis peggii* isolates from avocado were not pathogenic; plant height, root health and plant biomass of plants inoculated with *Cylindrocladiella* or *Gliocladiopsis* were not significantly different to uninoculated plants (Fig. 4). However, *Cy. pseudoinfestans* significantly increased root biomass (Fig. 4). These isolates are likely to be soil inhabitants (Lombard et al., 2012) or root inhabitants (Liu & Cai, 2013), however *Cylindrocladiella* spp. are generally not regarded as important plant pathogens (Lombard et al., 2012).

One *C. ilicicola* isolate caused statistically similar plant heights to uninoculated plants (Fig. 4) although in the other parameters tested, disease severity was similar to plants inoculated with the other *C. ilicicola* isolate (Fig 4). There can be variation in severity, above ground, between different isolates of the same species and this could make it difficult to identify obvious signs of black root rot in nursery conditions.

Fig. 4. Average plant height (cm), percentage of necrotic roots and fresh root biomass (g) of avocado cv. Reed seedlings at 5 weeks post-inoculation. The fungal isolates tested were from avocado. Bars with the same letter and case or presence of underlines are not significantly different (P<0.001)

LAMP diagnostic test for rapid detection of Calonectria ilicicola, Dactylonectria macrodidyma and Dactylonectria spp. in avocado roots

The LAMP assays were sensitive and specific at DNA concentrations of 1 pg/µl, 0.01 ng/µl and 0.1ng/µl for *C. ilicicola*, *D. macrodidyma*, and *Dactylonectria* spp. respectively (Table 1). Across all assays and types of templates used, detection was demonstrated to occur in under 30 minutes (Table 1). Detection in avocado roots averaged from 12–26 min for *C. ilicicola* and *D. macrodidyma* and 14–30 min for *Dactylonectria* spp., although the success of detection with avocado roots was subject to the quantity and dilution of necrotic roots used in each test sample, and repetition of the assay to ensure detection (Parkinson et al., 2019).

The specificity of the diagnostic for detecting the target species or genus was also found to be subject to time and isothermal amplification temperature, with specificity being most reliable in amplifications under 30 minutes and non-detection was assumed at time points after 30 minutes (Parkinson et al., 2019). The species-species assays were 100% specific to the targets.

However the *Dactylonectria* spp. genus-wide assay detected two undescribed *Ilyonectria* sp. out of 15 *Ilyonectria* sp. isolates in tests with pure fungal DNA, and one isolate of *C. ilicicola* in tests with crude fungal culture extracts or inoculated root tissue (Parkinson et al., 2019); that is, the *Dactylonectria* genus-wide assay falsely detected three non-target genus isolates out of 82 isolates tested, which is a specificity rate of 96.34%. *Ilyonectria* is a close relative of *Dactylonectria*, in which the latter genus was recently separated from the former (Lombard et al., 2014). *Calonectria* is also a close relative of *Ilyonectria* and *Dactylonectria*, with 3 phylogenetic clades of separation between these genera (Lombard et al., 2015). It can be difficult to design genus-specific primers as conserved gene sequences within species of one genus, on which the primer design is based on, can also be conserved across species from other closely related genera, which can increase the likelihood of false detection. Nevertheless the genus-wide assay is still a useful test for initial identification of potential pathogens in suspect plants.

Table 1. Summary of LAMP test optimisation and validation.

The LAMP assays have been adopted for diagnostic testing of symptomatic nursery trees submitted through the Australian Nursery Voluntary Accreditation Scheme (ANVAS) and *C. ilicicola* and *D. macrodidyma* presence in separate nurseries was confirmed with LAMP and supported with identification by fungal isolation. Use of the LAMP assays for testing nursery trees routinely and prior to dispatching to orchards could help to prevent industry-wide loss of new plantings. The simplicity of the testing method and visualisation of detection on the machine (Fig. 5) enables plant pathology service providers to use the tool as an initial test for confirming presence of important pathogens in nurseries or orchards.

Fig. 5. Image of target *C. ilicicola* DNA detection on a LAMP machine within 10–12 min.

The LAMP primer sequences are available in Parkinson et al. (2019) and there is flexibility of the procedure to be used with a range of reagents and equipment to enable global accessibility. The LAMP assay is applicable to multiple agricultural industries around the world as the target pathogens are also important pathogens of various crops and ornamental plants including soybean, peanuts, grapevine and apple.

How to manage black root rot

Black root rot is typically a problem in young avocado trees (nursery trees and young orchard transplants) and this study identified *Calonectria ilicicola* and *Dactylonectria macrodidyma* as important pathogens. Once nursery stock is contaminated with nectriaceous pathogens, the spread of disease can be exacerbated by frequent irrigation (and over-irrigation), crowded seedling arrangements and poor nursery hygiene practices (Crous, 2002). Sometimes above-ground symptoms of black root rot can be difficult to identify in the nursery; and the environmental stresses associated with orchard transplanting and other aggressive pathogens

such as *P. cinnamomi*, can exacerbate rapid decline and death of infected young trees (Parkinson et al., 2017b). It is believed that mature, established orchard trees are able to overcome root infection by nectriaceous fungi.

The general advice for nursery trees is to:

- pasteurise potting mix
- use clean planting material and sanitised seed, budwood and grafting tools
- have adequate space between plants and keep plants away from the ground
- refrain from over-irrigating
- promptly remove symptomatic plants and dispose old nursery stock
- check root health prior to dispatch to orchards.

The advice for young orchard transplants is to:

- have a careful selection of planting sites, avoiding planting in ground where previous crops have historically had problems with nectriaceous pathogens (eg. ex-peanut fields affected by *C. ilicicola* or ex-vineyards affected by *Dactylonectria* spp.)
- source plants from accredited nurseries
- refrain from over-irrigation or over-fertilization
- closely monitor transplants in the first year of establishment
- plant replacement trees approximately 30–50 cm away from the site of young trees which died due to suspected black root rot.
- Some fungicide drench treatments may suppress the fungi sufficiently to allow establishment in the field.

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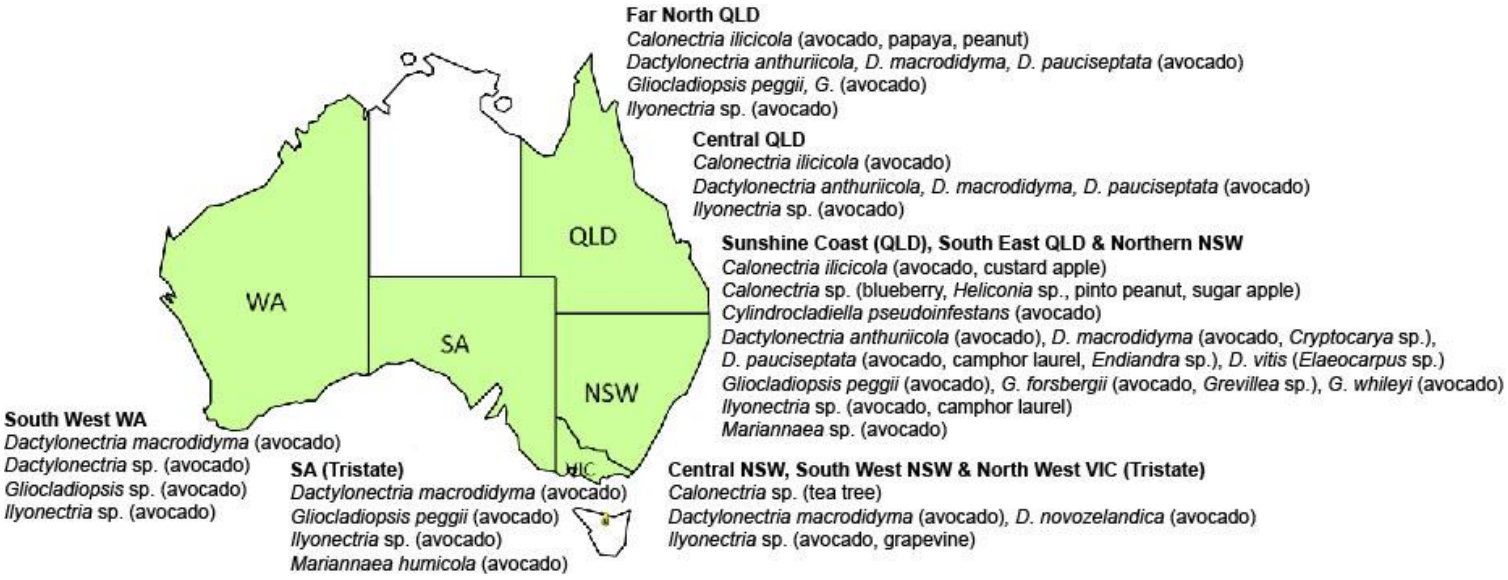
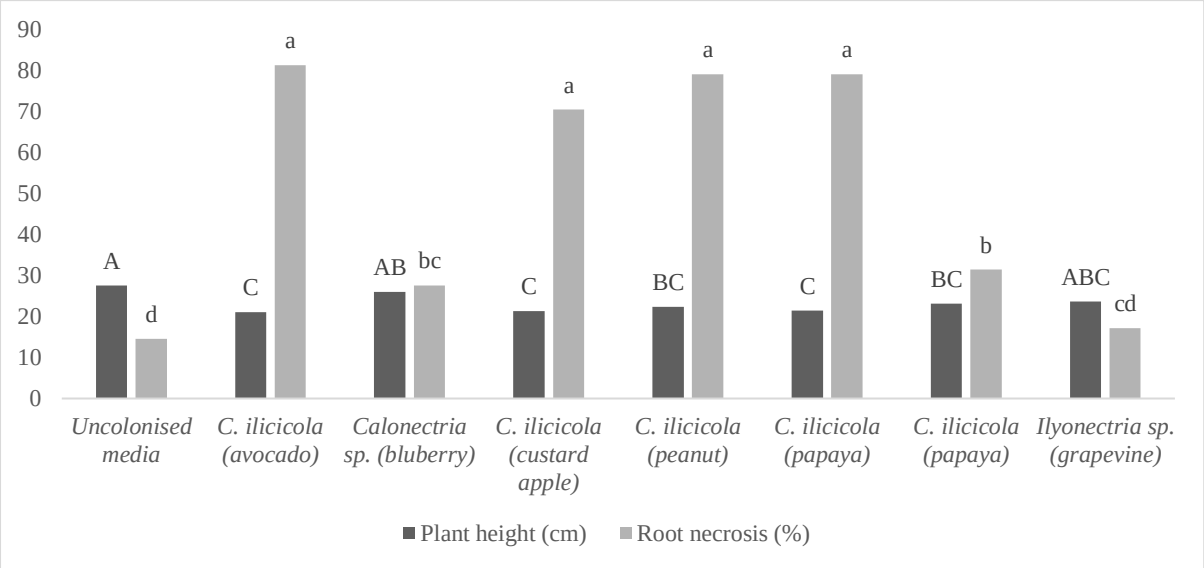


Fig. 1. Map of nectriaceous fungal species found in various hosts across Australia. Australian avocado growing states include Queensland (QLD), New South Wales (NSW), Victoria (VIC), South Australia (SA) and Western Australia (WA).



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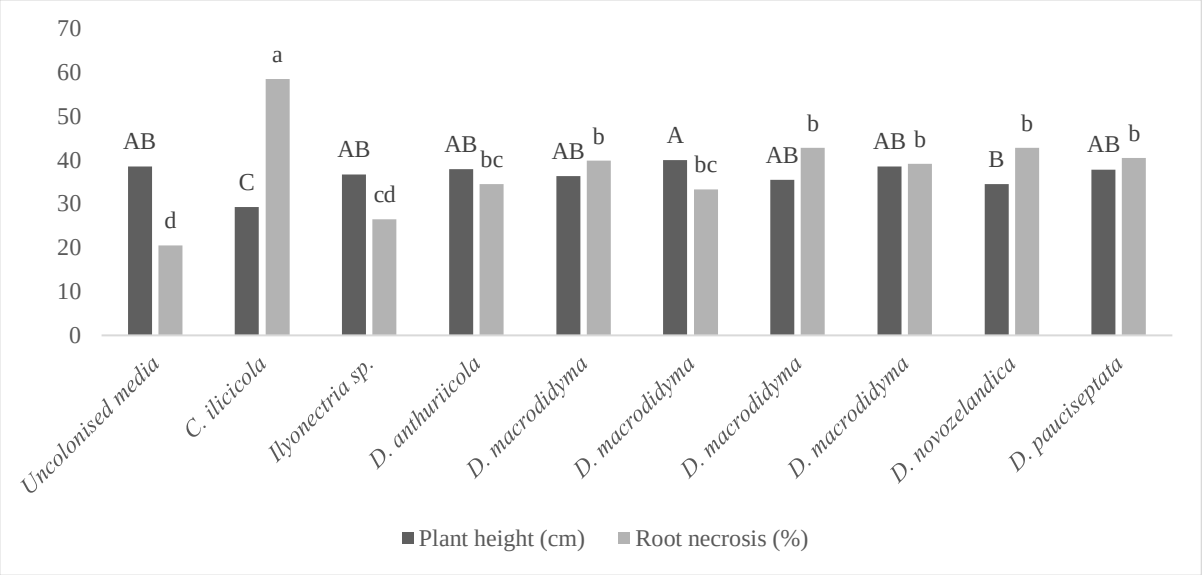

Fig. 3. Average plant height (cm) and percentage of necrotic roots of avocado cv. Reed seedlings at 9 weeks post-inoculation. The fungal isolates tested were from avocado. Bars with the same letter and case are not significantly different (P<0.001).

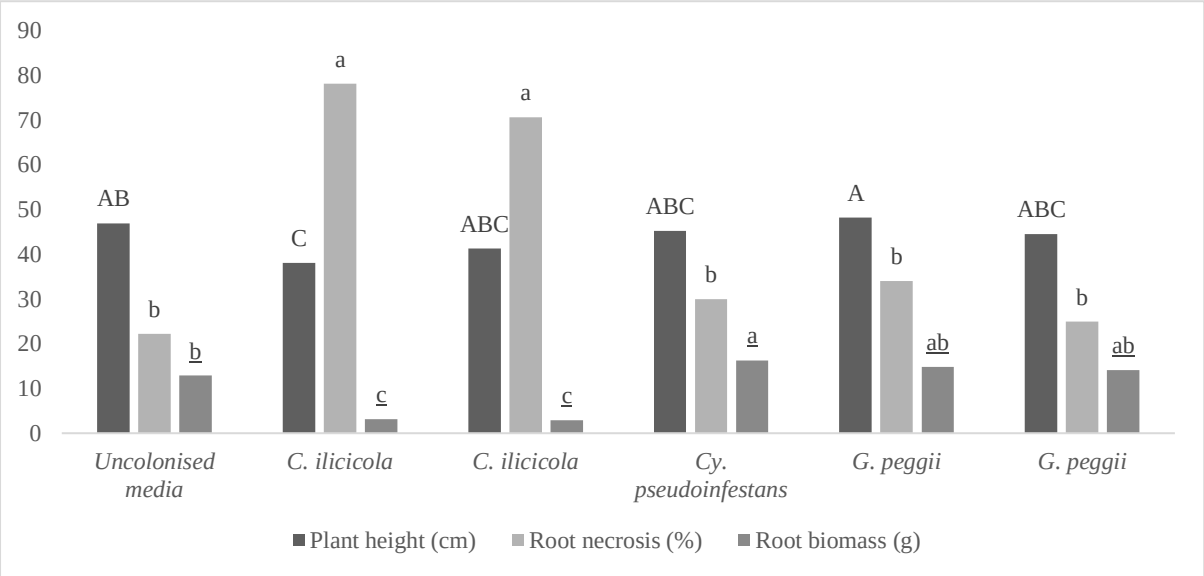

Fig. 4. Average plant height (cm), percentage of necrotic roots and fresh root biomass (g) of avocado cv. Reed seedlings at 5 weeks post-inoculation. The fungal isolates tested were from avocado. Bars with the same letter and case or presence of underlines are not significantly different (P<0.001)

Table 1. Summary of LAMP test optimisation and validation.

	LAMP Assay		
Test	***C. ilicicola***	***D. macrodidyma***	***Dactylonectria* spp.**
Demonstrating sensitivity (ng/µl or pg/µl)	1 pg/µl	0.01 ng/µl	0.1 ng/µl
Demonstrating specificity (%)	100%	100%	96.34%
Detection time with pure DNA	10 – 15 min	12 – 29 min	6 – 25 min
Detection time with fungal cultures	15 – 30 min	16 – 30 min	7 – 23 min
Detection time with avocado roots	12 – 25 min	12 – 26 min	14 – 30 min

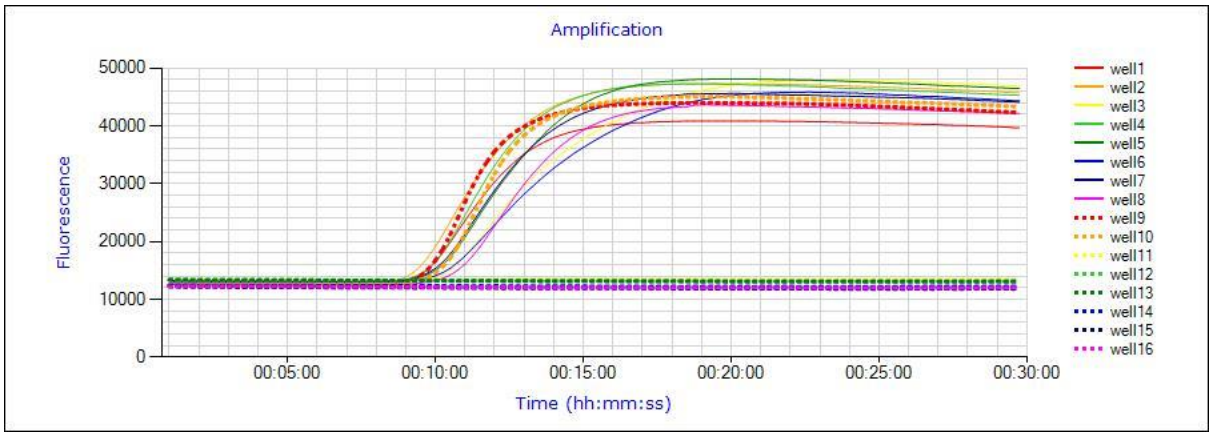

Fig. 5. Image of target *C. ilicicola* DNA detection on a LAMP machine within 10–12 min.