

# Pathogenicity and molecular detection of nectriaceous fungi associated with black root rot of avocado

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# The Australian avocado industry

**2018: Consumer retail value of AUD \$958 million**

(2015: AUD \$686 million)

**2018: Annual fruit production: ~77,000 tonnes**

(2015: ~50,000 tonnes)

Production and consumption has almost doubled in the last 10 years

- Consumption 3.5kg per person per annum



Hass 78%



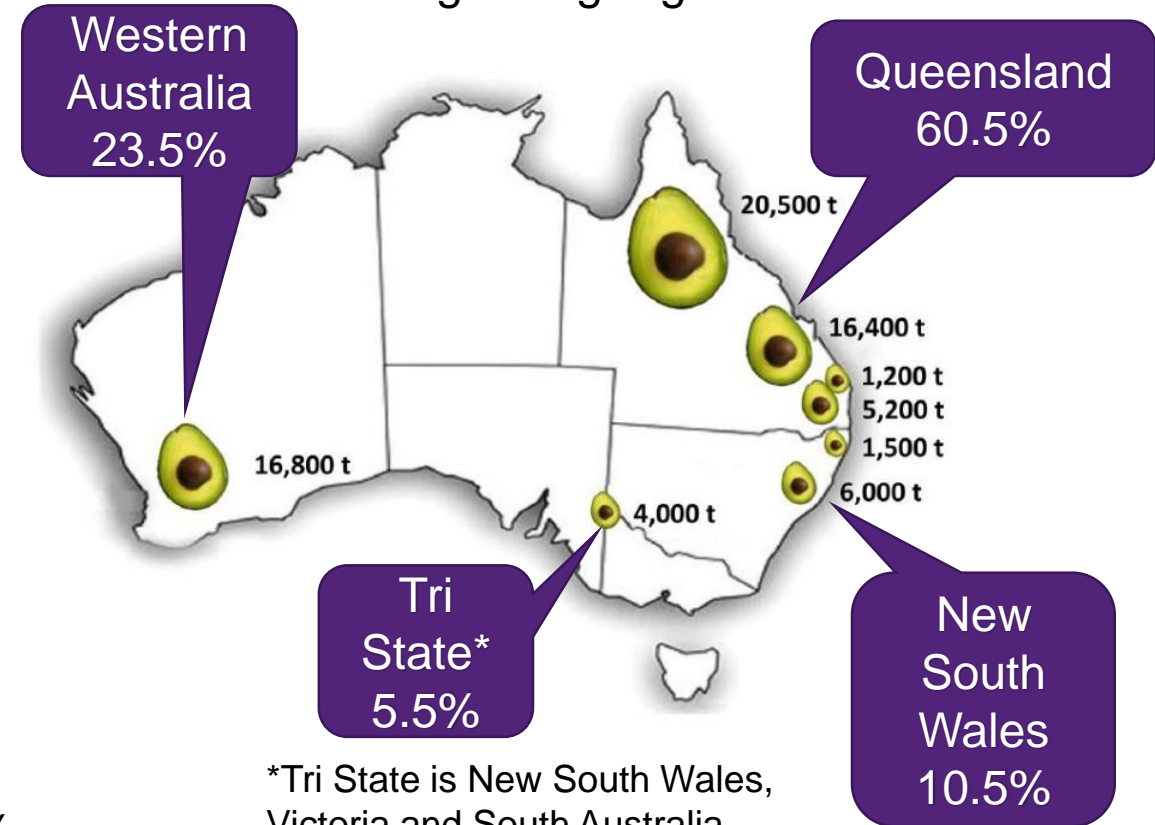
Shepard 19%



Other varieties 3%

Reed, Lamb Hass, Wurtz, Gwen, Sharwil, Fuerte, Pinkerton, Gem, Bacon & Edrinol

## Main growing regions



\*Tri State is New South Wales, Victoria and South Australia along the Murray River

# Black root rot of avocado

- Severe soilborne disease of nursery trees and young orchard transplants.
- Black root rot is caused by fungal pathogens in the Nectriaceae family.
- Rapid death within one year of planting.



# Identifying black root rot symptoms

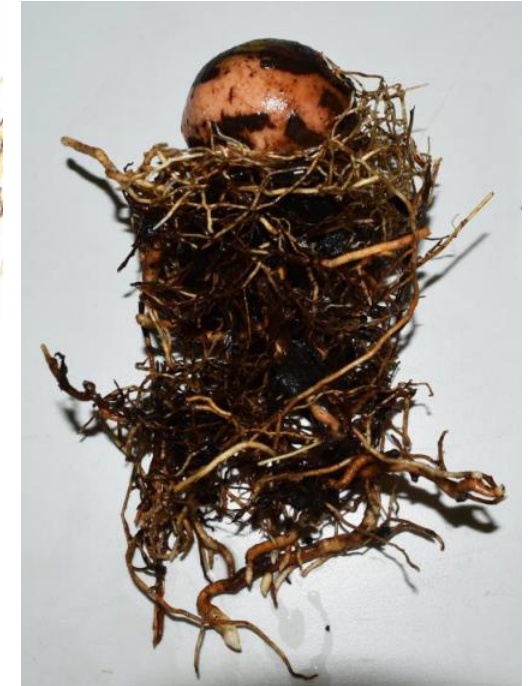


Necrotic lesions “leopard spots” on roots



- Tree stunting
- Wilted and chlorotic leaves

Images: black root rot in nursery seedlings



- Black, rotten & necrotic roots
- Reduced roots

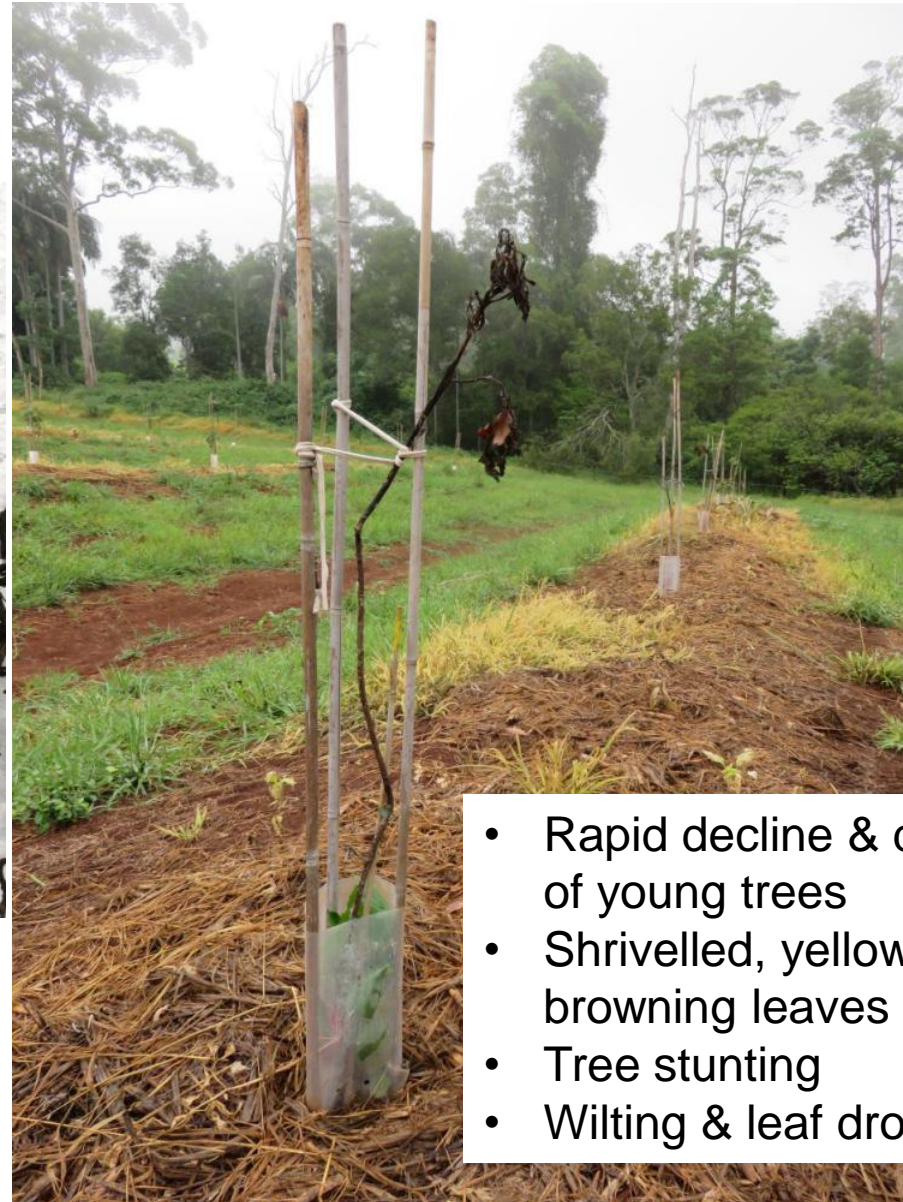
Images: black root rot in young orchard transplants (< 1 year old)

# Identifying black root rot symptoms



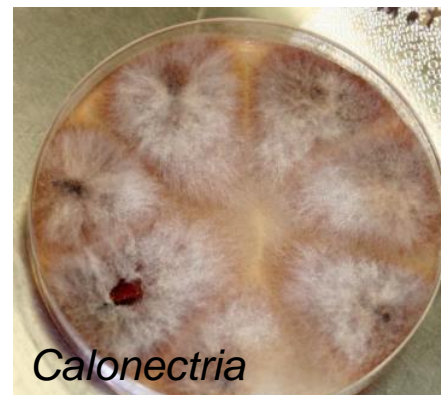
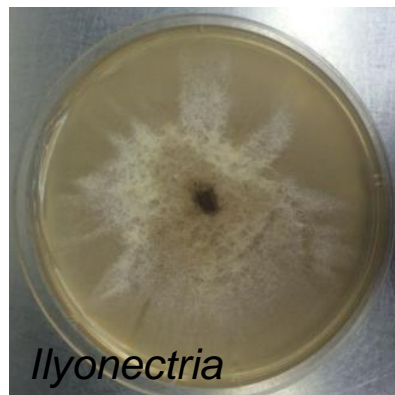
Black/brown  
rotten & reduced  
roots

Orange blobs of perithecia  
(spore producing structures)



- Rapid decline & death of young trees
- Shrivelled, yellowing/ browning leaves
- Tree stunting
- Wilting & leaf drop

# Fungi associated with black root rot of avocado



*Gliocladiopsis*  
*Cylindrocladiella*  
*Mariannaea*  
*Calonectria*  
*Ilyonectria*  
*Dactylonectria*

What about *Cylindrocarpon*?

# Confusion with multiple names for one fungus

All of these fungal genera are “*Cylindrocarpon*”



# Research questions

1. Which fungal genera are associated with black root rot?
2. Which fungal species are pathogens?
3. Can we rapidly test for the pathogens present in avocado roots?



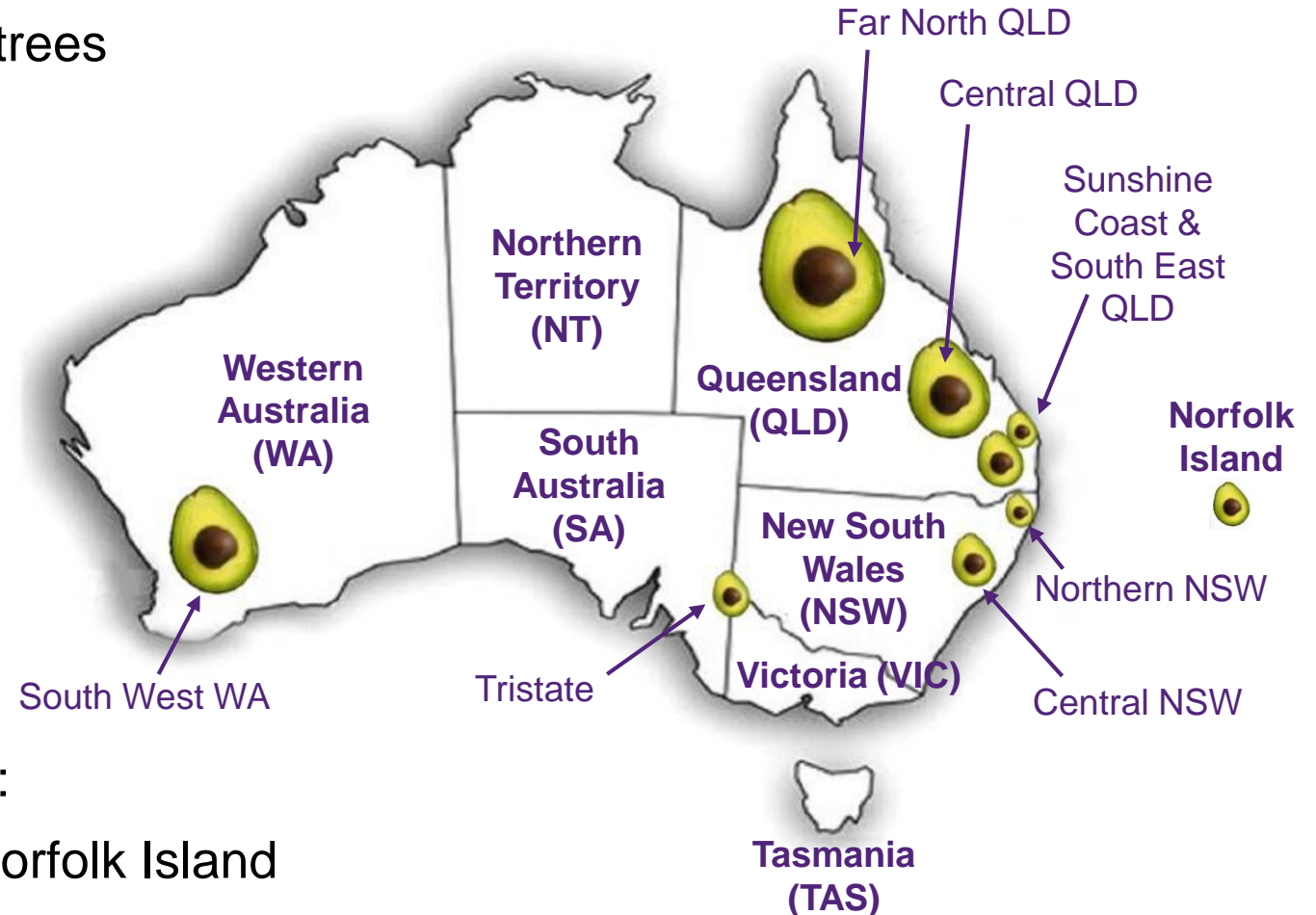
# 1. Which fungal genera are associated with black root rot?

153 fungal isolates collected from 93 trees

- 74 avocado trees
- 19 other hosts
- 129 isolates from avocado
- 24 isolates from other hosts

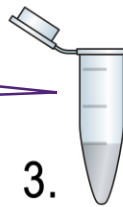
Collected fungi were isolated from:

- Sick and healthy trees
- Young and mature trees
- Nurseries, orchards & fields
- All growing regions in Australia:
  - QLD, NSW, VIC, SA, WA & Norfolk Island

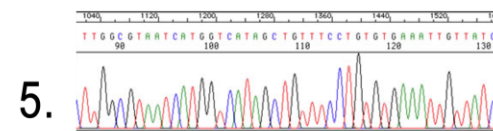
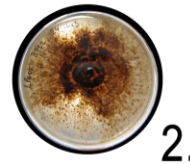


# Methods: Identifying fungal species

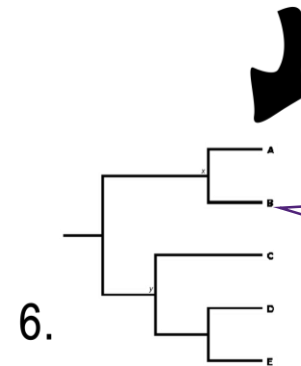
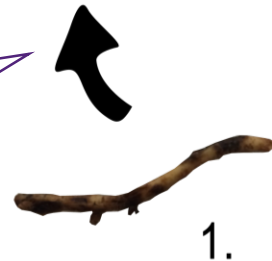
Extracting fungal DNA



Amplifying and sequencing fungal genes: ITS,  $\beta$ -tubulin & Histone H3

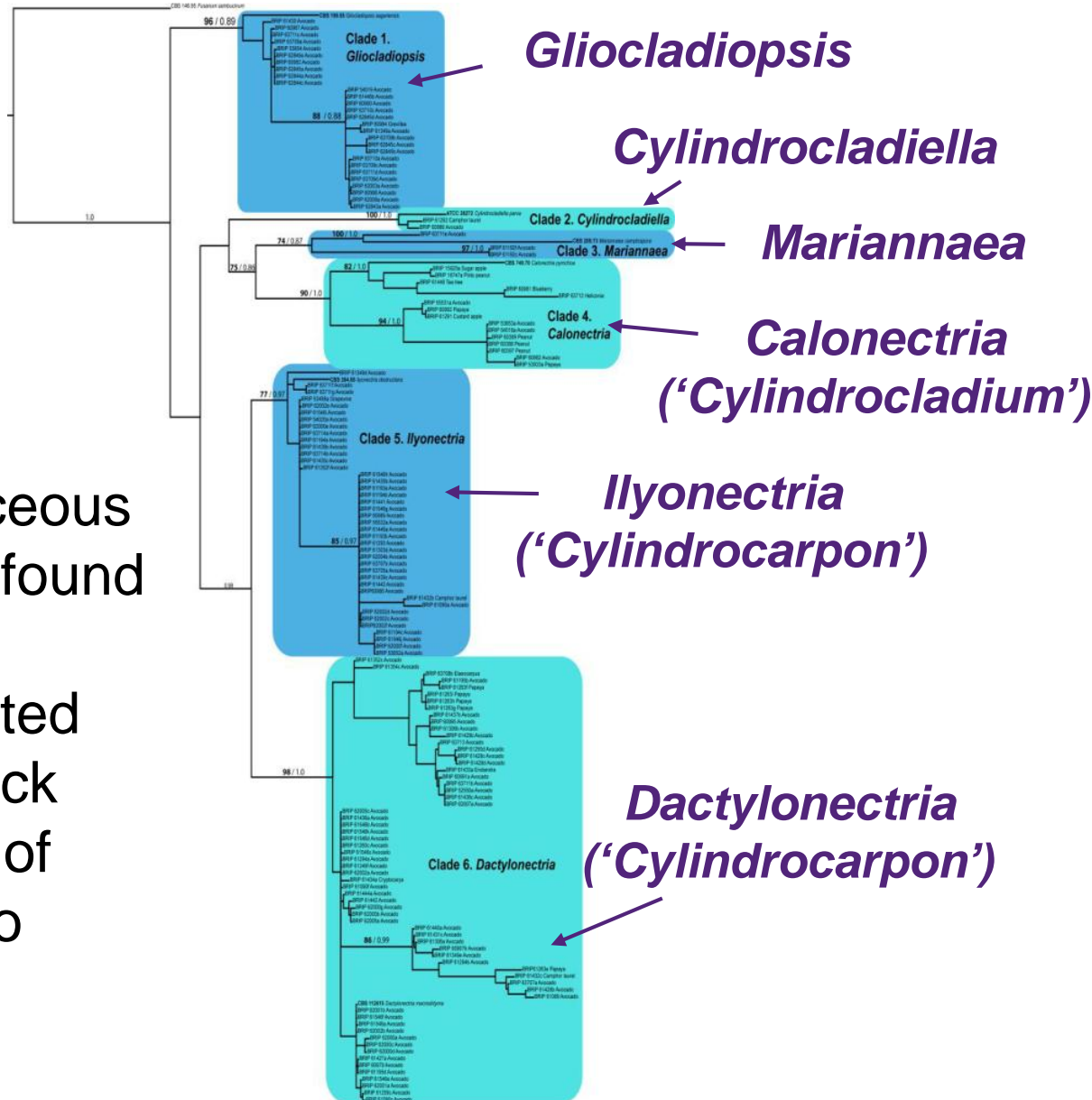


Isolating from necrotic roots and culturing the fungi



Using the sequenced genes & analyses to fully confirm the species

Six nectriaceous genera found to be associated with black root rot of avocado



### Mariannaea

- Not previously reported in avocados.
- Not likely to be a pathogen.

### Calonectria

- Mostly found in nursery trees, young orchard transplants or small field crops on the east coast of Australia.
- *Calonectria* associated with avocado found in young trees.

### Dactylonectria

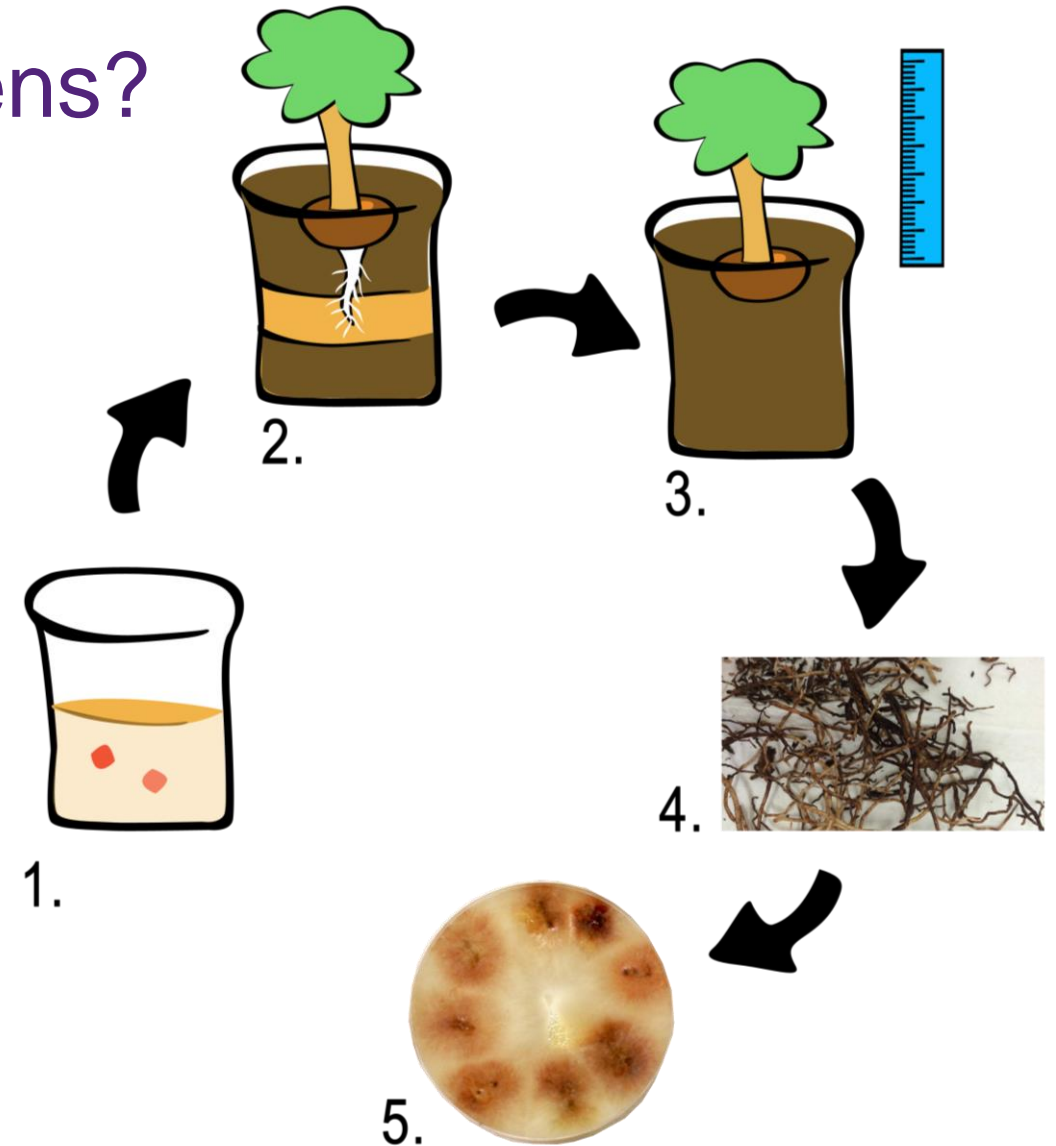
- Has a broad geographic range (found all over Australia).
- Found in both nurseries and orchards.

## 2. Which species are pathogens?

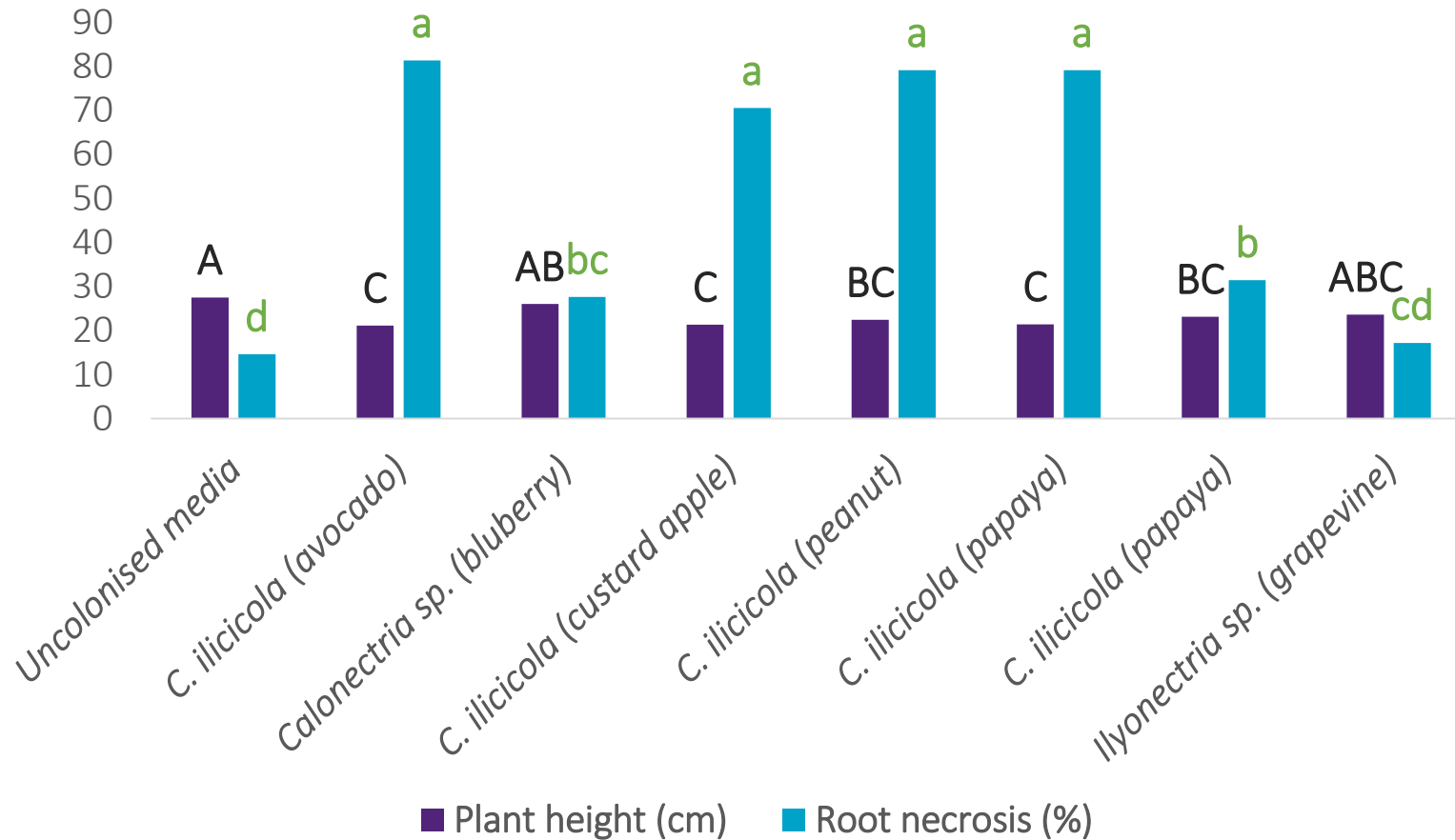
**Glasshouse pathogenicity testing of nectriaceous fungi for ability to cause black root rot in avocado cv. Reed seedlings:**

*Calonectria* & *Ilyonectria* isolates from avocado, peanut, papaya, custard apple, blueberry & grapevine

*Calonectria*, *Dactylonectria*, *Ilyonectria*, *Cylindrocladiella* & *Gliocladiopsis* isolates from avocado



# Which species are pathogens?



Average plant height (cm) and percentage of necrotic roots of avocado cv. Reed seedlings at 5 weeks post-inoculation. Fungal isolates tested on avocado were from multiple hosts.  $P < 0.001$

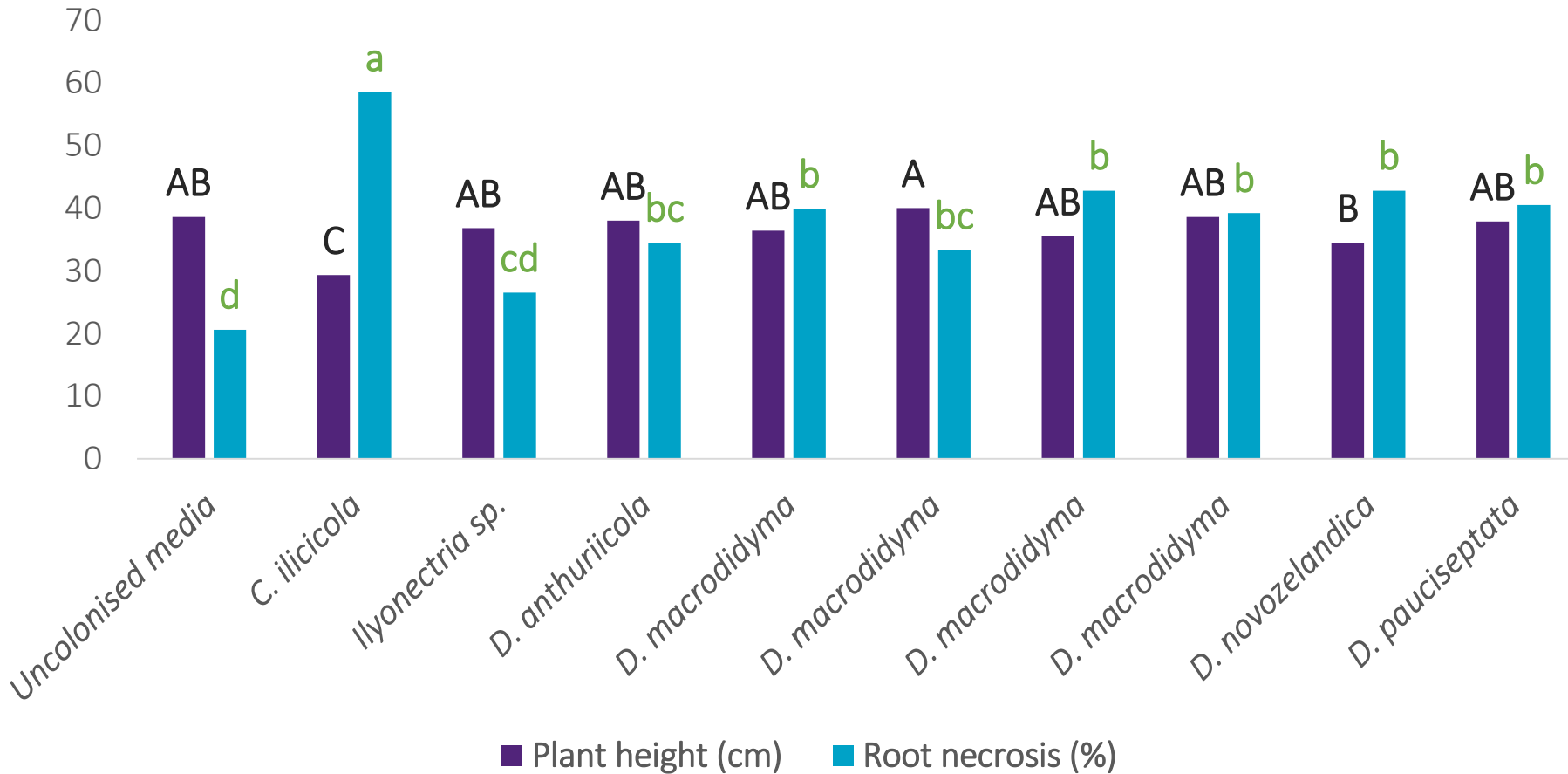
***Calonectria illicicola*** from avocado, papaya, peanut and custard apple extremely pathogenic causing stunting and death.

***Calonectria* sp.** from blueberry also pathogenic.

***Ilyonectria* sp.** from grapevine not pathogenic.



# Which species are pathogens?



***Dactylonectria* spp. from avocado are pathogens**

*D. macrodidyma*  
*D. anthuriicola*  
*D. pauciseptata* and  
*D. novozelandica* caused significant root rot but not stunting.

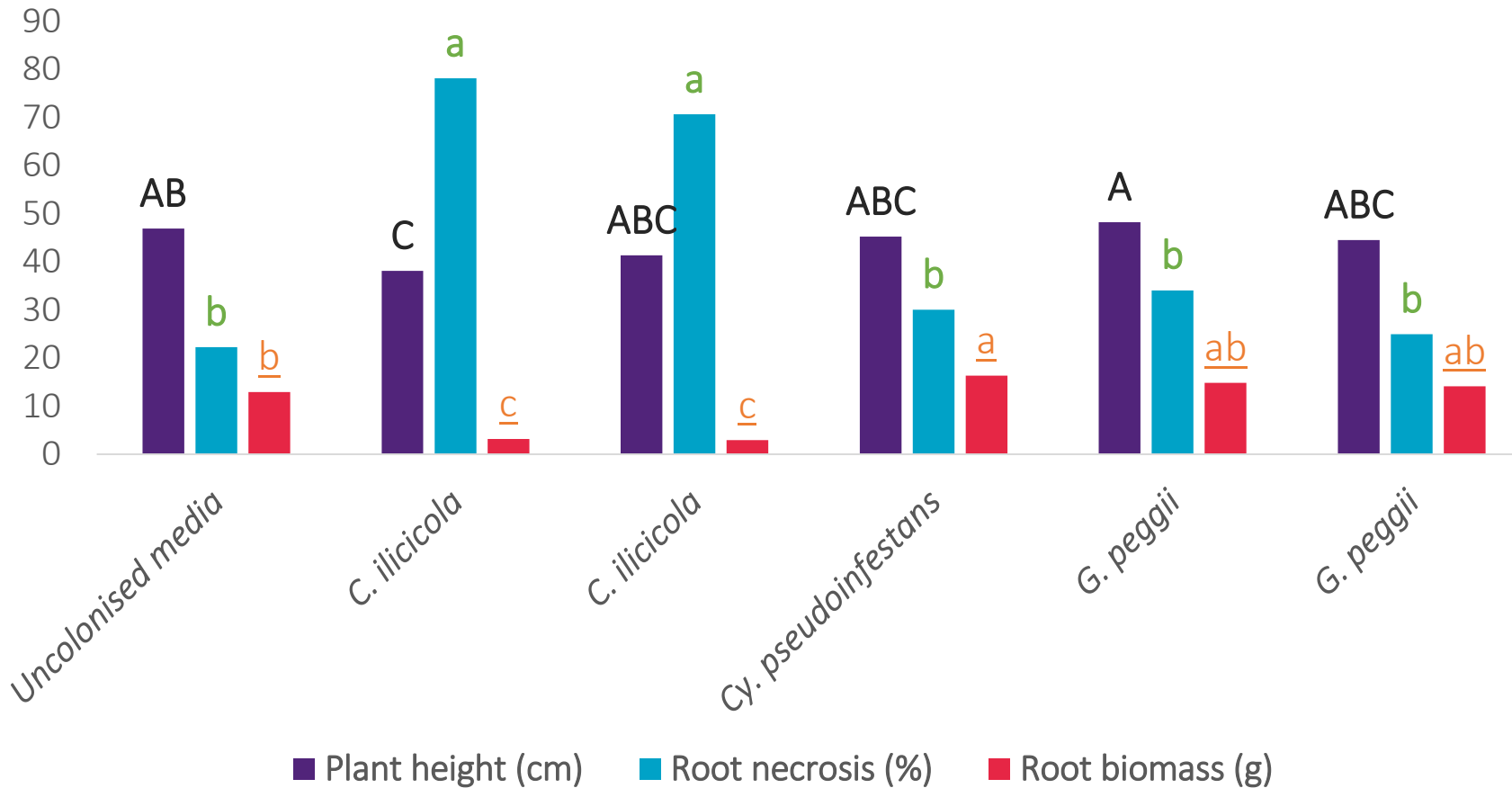
***D. macrodidyma*** most commonly isolated.

***Ilyonectria* sp.** from avocado not pathogenic.

Caution about *Cylindrocarpon*

Average plant height (cm) and percentage of necrotic roots of avocado cv. Reed seedlings at 9 weeks post-inoculation. Fungal isolates tested on avocado were from avocado. P<0.001

# Which species are pathogens?



*Cylindrocladiella pseudoinfestans* and *Gliocladiopsis peggii* from avocado not pathogenic.

*Gliocladiopsis* and *Cylindrocladiella* species are likely soil or root inhabitants.

*Cy. Pseudoinfestans* increased root biomass.

One *Calonectria illicicola* isolate was pathogenic but did not cause stunting. (Can be difficult to identify obvious signs of black root rot in the nursery).

Average plant height (cm) and percentage of necrotic roots and fresh root biomass (g) of avocado cv. Reed seedlings at 5 weeks post-inoculation. Fungal isolates tested on avocado were from avocado. P<0.001

### 3. Developing molecular tests for black root rot pathogens

**Aim:** Develop species and genus-specific molecular tests for detecting black root rot pathogens:

*Calonectria ilicicola*

*Dactylonectria macrodidyma*

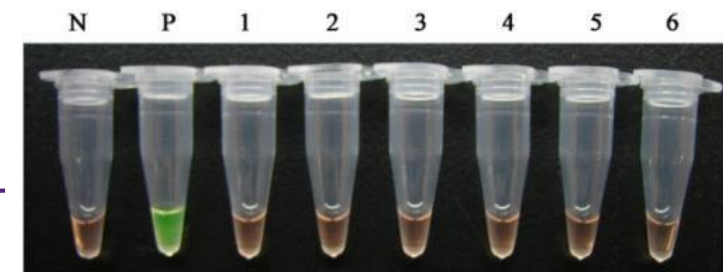
*Dactylonectria* species (a test to detect the entire genus)

#### Molecular test criteria

- Rapid detection in plant tissue
- Sensitive & specific
- Accessible (eg. via publication of primers and ability to be modified for use with cheaper or alternative equipment).
- Fewest steps possible



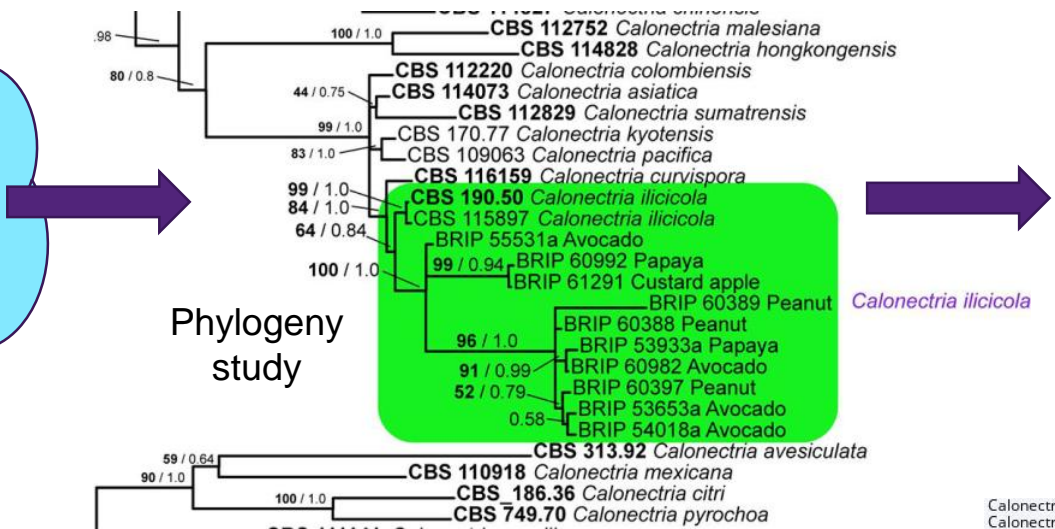
e.g. by machine detection or colorimetric assay





# Using fungal DNA sequence data to develop molecular tests

Choose a target species or genus eg. *Calonectria ilicicola*



Summary - 20 per page - Sort by Default order - Send to: Filters: Manage Fi

See Gene information for beta tubulin cbs  
beta tubulin in *Drosophila melanogaster* 1 Gene record  
cbs in *Homo sapiens* *Mus musculus* *Rattus norvegicus* All 175 Gene records

Choose Destination  
● Complete Record  
○ Coding Sequences  
○ Gene Features

Choose Destination  
● File ○ Clipboard  
○ Collections ○ Analysis Tool

Download 4 items.  
Format: **FASTA**  
Sort by: Default order  
Show GI   
Create File

Download gene sequence data

Amplification positions in the partial β-tubulin gene of *Calonectria ilicicola*

```
TAACATGCGGTAGATTGTAAGTTTCTGCCCTCGCACAGCATCATGCCAGAGTTCTTGGAGAGACC  
GTGCTTGTGTTGCTGGCCCTGAGCGTACCCGCGCCCGGGTTCGACCGCTTGACAACAACAAAGC  
TCGACGACCCCAAGCAGCATGTGATATCGGAGGACAAGGTTGCTGACTATTTCCTCAATTTAGG  
TTCTCCCTCCAGACCGGTCAGTGGCTAAGTGATCATTCCAGCTTCCAAAAACGGCCCTGGGGATTCACT  
AACATTTCGCGATCAGGGTAAACAAATTGGTGCTGCTTCTGGCAGACCATCTCTGGCGAGCACGGCCT  
TGACAGCAATGGTGTCTACAACGGTACCTCCGACCTCCAGTTGGAGCGCATGAACGTCTACTTCAACG  
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```

Design specific primers for detecting the pathogen

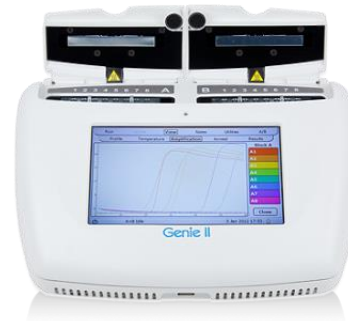
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Calonectria_hongkongensis_CBS_114828_TYPE A A A A T C T C C G T A A A G A T T G A C T G A C A C T T A T T G G C T A G G C  
Calonectria_lateralis_CBS_136629_TYPE A A A A T C T A C C G T G A A G A T T G A C T G A C A C T T A T T G G C T A G G C  
Calonectria_pseudoturangelicola_CERC_7126_TYPE A A A A T C T A C C G T G A A G A T T G A C T G A C A C T T A T T G G C T A G G C  
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Calonectria_malesiana_CBS_112752_TYPE A A A A T T C T G C C A T T G A A G A T T G A C T G A C A C T T A T T G G C T A G G C  
Calonectria_chinensis_CBS_114827_TYPE - G A A T C T G C C A T T C A A G A T T C A C T G A C A C T T G T T G A C T A G G C  
Calonectria_indonesiae_CBS_112823_TYPE A A A A T C T G C C A T T G A A G A T T C A C T G A C A C T T G T G G T T A G G C  
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Calonectria_montana_CERC_8952_TYPE A A A A T C T G C C C G A G T A T T C A C T G A C A C T C G T G G T T A G G C  
Calonectria_penicilloides_CBS_174.55_TYPE A T T C T C T C C A G A G A G A T T C G C T A C A C A T C A C A A A C A G G C  
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BRIP63712_Calonectria_sp A C A T C T C A C - G A C G A T T C A C T G A C A G T T A T C G A C A G G C
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Identify unique gene sequences

# Loop-mediated isothermal amplification (LAMP)

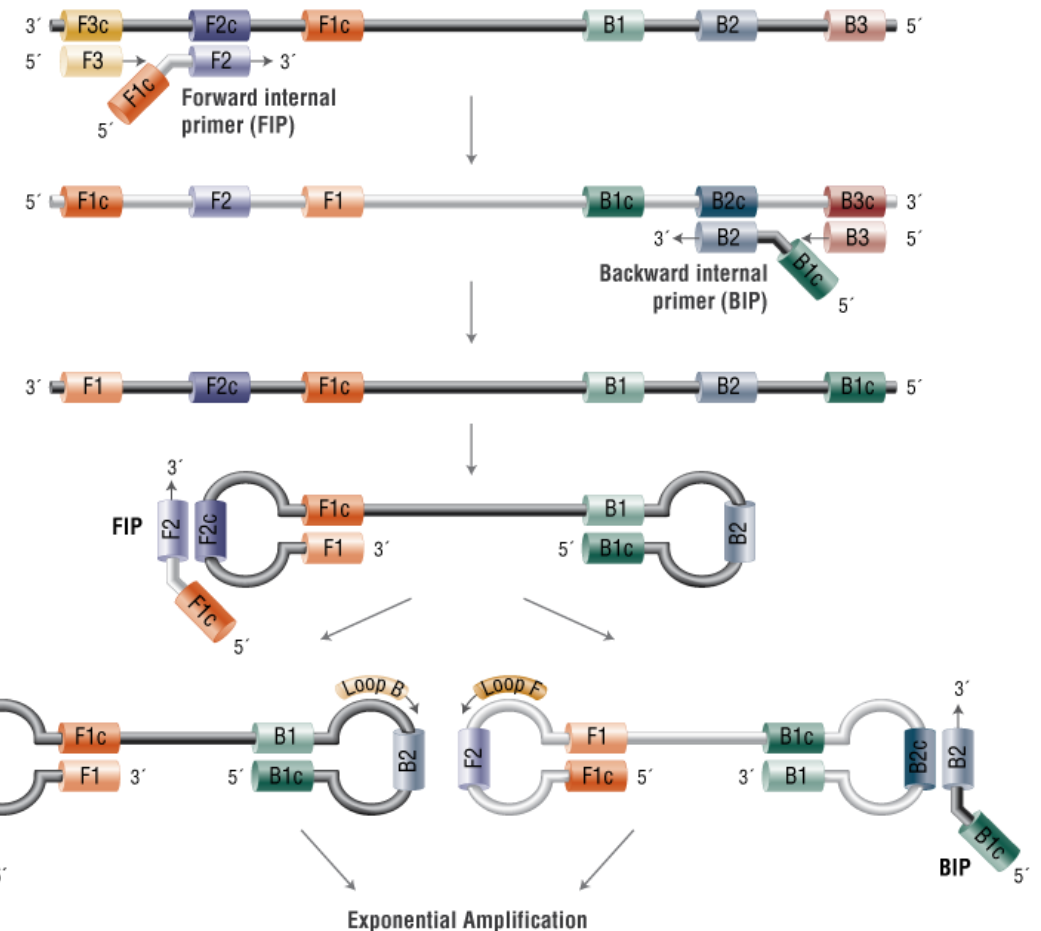
For detection of black root rot pathogens in avocado roots

- *Calonectria ilicicola*
- *Dactylonectria macrodidyma*
- *Dactylonectria* spp.



## Benefits of LAMP

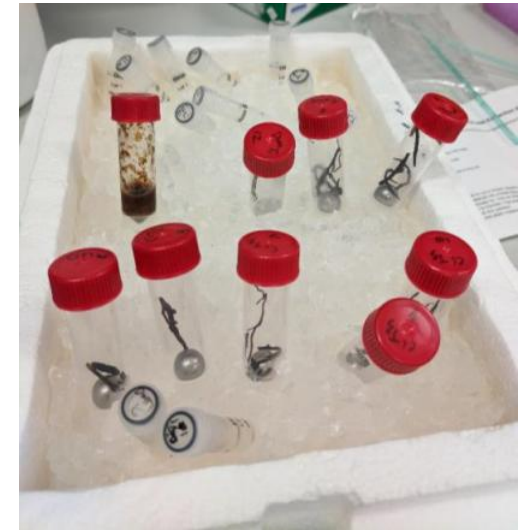
- Alternative reagents for accessibility (eg. Genie II machine, colorimetric assays, water bath & salt precipitation)
- Portable & thermostable (field detection)
- Results in minutes
- Highly specific



# LAMP detection of black root rot pathogens

Test	<i>C. ilicicola</i>	<i>D. macrodidyma</i>	<i>Dactylonectria</i> spp.
Sensitivity	1 pg/μl	0.01 ng/μl	0.1 ng/μl
Specificity	100%	100%	97.6%
DNA	10 – 15 min	12 – 29 min	6 – 25 min
Fungal cultures	15 – 30 min	16 – 30 min	7 – 23 min
Avocado roots	12 – 25 min	12 – 26 min	14 – 30 min

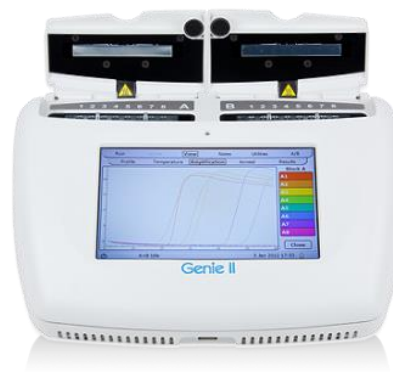
2 *Ilyonectria* isolates out of 82 Nectriaceae isolates falsely detected



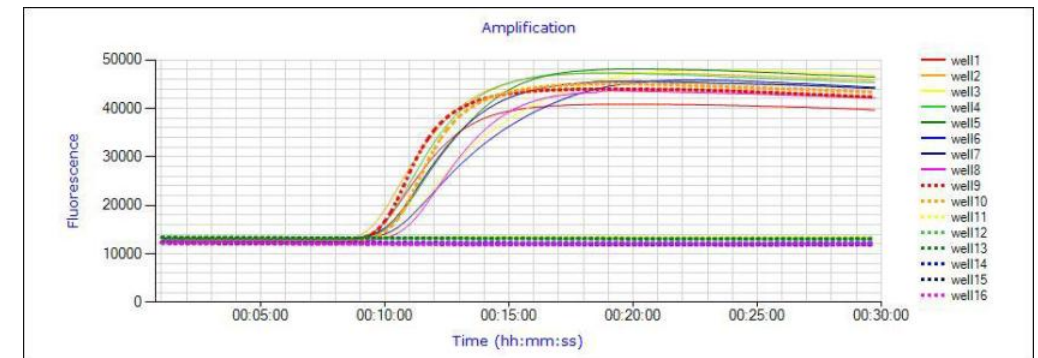
Portable DNA extraction method

## Some shortfalls with genus-wide LAMP assays

- Closely related genera can be hard to separate (eg. 'Cylindrocarpon' fungi *Ilyonectria* and *Dactylonectria*)



Target *C. ilicicola* DNA detected within 10 – 15 min



# How to manage black root rot

## In the nursery

- Promptly remove diseased or sick-looking plants.
- Dispose old nursery stock.
- Always use clean planting material and sanitised seed, budwood and grafting tools.
- Pasteurise potting mix.
- Don't over irrigate.
- Adequate space between plants in the nursery & keep plants off the ground.
- Check & test for disease prior to dispatch.

## In the orchard

- Source plants from accredited nurseries.
- Don't over irrigate or over fertilise.
- Closely monitor transplants in the first year of establishment.
- Be careful about planting sites:
  - Avoid planting in ground which has had previous problems with necrotrophic pathogens (eg. ex-peanut fields or ex-vineyards).
  - Avoid placing replants directly on top of the site of the previous dead tree; plant at least 30–50 cm away from the site.

# Acknowledgements

- 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. For more information on the fund and strategic levy investment visit [www.horticulture.com.au](http://www.horticulture.com.au)

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- Participating avocado growers and operators in Australia
- Participating QLD & NSW Ag. Plant Pathologists



Project members:

A/Prof Elizabeth Dann  
(Project Leader)

Prof Roger Shivas  
(Phylogeny study)

Dr Duy Le  
(LAMP diagnostic)

# Thank you

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