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

Western Australia 23.5%
Queensland 60.5%
Tri State* 5.5%
New South Wales 10.5%

*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

Images: black root rot in nursery seedlings

- Tree stunting
- Wilted and chlorotic leaves

- Black, rotten & necrotic roots
- Reduced roots
Identifying black root rot symptoms

Orange blobs of perithecia (spore producing structures)

Black/brown rotten & reduced roots

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

- 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”

<table>
<thead>
<tr>
<th>Neocentria</th>
<th>Ilyonectria</th>
<th>Cylindrochondrum</th>
<th>Dactylonecctria</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBS 198.62 Neocentria neomacrospora</td>
<td>CBS 264.65 Ilyonectria destructans</td>
<td>CBS 129.97 Cylindrochondrum hubeiense</td>
<td>CBS 129085 Dactylonecctria estremocensis</td>
</tr>
<tr>
<td>CBS 324.61 Neocentria neomacrospora</td>
<td>CBS 117527 Ilyonectria liriodendri</td>
<td></td>
<td>CBS 113552 Dactylonecctria novozealandica</td>
</tr>
<tr>
<td>CBS 125435 Neocentria lugdunensis</td>
<td>CBS 132815 Ilyonectria capensis</td>
<td></td>
<td>CBS 129087 Dactylonecctria alcacerensis</td>
</tr>
<tr>
<td>CBS 151.29 Neocentria candida</td>
<td>CBS 132809 Ilyonectria leucospermi</td>
<td></td>
<td>CBS 112615 Dactylonecctria macroidyma</td>
</tr>
<tr>
<td>CBS 788.69 Neocentria isugae</td>
<td>CBS 119606 Ilyonectria coprosmae</td>
<td></td>
<td>CBS 129086 Dactylonecctria torresensis</td>
</tr>
</tbody>
</table>

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

1. Isolating from necrotic roots and culturing the fungi
2. Extracting fungal DNA
3. Amplifying and sequencing fungal genes: ITS, β-tubulin & Histone H3
4. 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.

**Dactyloneectria**
- 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?

**Calonectria ilicicola** 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.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plant Height (cm)</th>
<th>Root Necrosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncolonised media</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. ilicicola (avocado)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calonectria sp. (blueberry)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. ilicicola (custard apple)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. ilicicola (peanut)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. ilicicola (papaya)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ilyonectria sp. (grapevine)</td>
<td></td>
<td></td>
</tr>
</tbody>
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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
The Queensland Alliance for Agriculture and Food Innovation (QAAFI) is a research institute of The University of Queensland (UQ), supported by the Queensland Government.

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.

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**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

**Caution about Cylindrocarpon**
Cylindrocladiella pseudoinfestans and Gliocladiopsis peggi are likely soil or root inhabitants.

Cy. Pseudoinfestans isolate was pathogenic but did not cause stunting. (Can be difficult to identify obvious signs of black root rot in the nursery).
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 (e.g. 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*

**Phylogeny study**

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

<table>
<thead>
<tr>
<th>Amplification Position</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>TAACATCGGATGAGTGAAGTTGTCCGGCTTCGCCACACGTATGCCCCAGAGTTCTTCGAGAGGCC</td>
</tr>
<tr>
<td>F2</td>
<td>GTGCCTTGTAGGGCTGGGCAAGGCCGCGACGCTTCGAGGAGGGCAAGAAGGAAAGG</td>
</tr>
<tr>
<td>F3</td>
<td>TCGAGCCACGCGACGAGTTGATAAGGGAGGACAGGTGCTGGCATCTCGTCTAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>F1 LOOP</td>
<td>TACCCGCAGCGACGCTTCGAGGAGGGCAAGAAGGAAAGG</td>
</tr>
<tr>
<td>F2 LOOP</td>
<td>GACACAAAGAAAGG</td>
</tr>
<tr>
<td>B LOOP</td>
<td>ACTCCAGGCTGGGACAGGACGTCCAGGAGGTGACAGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>B1 LOOP</td>
<td>GTCGAGGAGGAGGTGACAGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>B2 LOOP</td>
<td>AGACTCTGAGGAGGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>B3 LOOP</td>
<td>AAACCTGAGGAGGAGGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>TGACAGCAATGGTGGTCACTACAGGTCATCCTCCGACTGCCAGGTGGAGGCATGACGGTACTCTACAGG</td>
<td></td>
</tr>
<tr>
<td>AAGGTATGGTGGTTAACAACACACCGCTGGCTGTGCAAGAAAACCTTGTGGCACAACATACAGGATACAGG</td>
<td></td>
</tr>
</tbody>
</table>

**Design specific primers for detecting the pathogen**

**Identify unique gene sequences**

Download gene sequence data
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 (e.g., Genie II machine, colorimetric assays, water bath & salt precipitation)
- Portable & thermostable (field detection)
- Results in minutes
- Highly specific
LAMP detection of black root rot pathogen

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

Some shortfalls with genus-wide LAMP assays

- Closely related genera can be hard to separate (e.g., ‘Cylindrocarpon’ fungi Ilyonectria and Dactylonectria)

Portable DNA extraction method

2 Ilyonectria isolates out of 82 Nectriaceae isolates falsely detected
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 nectriaceous 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

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Thank you

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