Development of qPCR assays to detect and quantify inoculum of grapevine trunk disease pathogens from environmental samples

*R. Billones-Baaijens, J. Urbez-Torres, M. Ayres, M. Sosnowski and S. Savocchia*

rbaaijens@csu.edu.au
Eutypa dieback (ED) and Botryosphaeria dieback (BD)

• Two most important trunk diseases of grapevines in Australia.

• Caused by Diatrypeaceae and Botryosphaeriaceae fungi

• Their ascospores and/or conidia - dispersed by rain splash and wind

• Spores infect pruning wounds leading to cankers, dieback and death of vines.

• Threaten vineyard sustainability – wine industry contributes ~$40 billion to the Australian economy.

• Rank in the top five priority diseases of the Australian wine and grape industry.
Spore trapping in Australian vineyards

• No published data on spore dispersal patterns for Australian vineyards.

• Identify high risk infection periods of ED and BD pathogens in Australian vineyards

Objective:

Develop molecular tools to detect and quantify Diatrypaceae and Botryosphaeriaceae spores from the environment.
DNA extraction protocol

Evaluated 4 protocols
2 commercial kits, 2 published

Inoculate tapes
ED and BD spores

Nested PCR & qPCR
Efficiency, consistency

- **Modified Gentra® Puregene®**
  - commercial buffers + conventional methods
  - efficient; consistent; easy to use
  - Detection - 6 conidia/ascospores per reaction
Diat qPCR using multi-species primers

- *Eutypa lata*
- *Cryptovalsa ampelina*
- *C. rabenhorstii*
- *Diatrypella vulgaris*
- *E. leptoplaça*
- *Eutypella citricola*
- *Eu. microtheca*
- *Eu. cryptovalsoidea*
Photos by M. Sosnowski

18 S  5.8 S  28 S

DIA 16F  DIA 89R

SYBR Green

125 bp
Construction of standard curve using gBlocks® gene fragments

Synthesised

Diat gBlock® (460 bp) 0.5 ag per copy (10^{-18} g)

DIAT 16F  DIAT 89R

\[ y = -3.3449x + 34.031 \]

\[ R^2 = 0.9954 \]

Efficiency = 99%
**gBlocks® Gene Fragments**

10-fold dilutions - $10^1$ to $10^6$

Limit of detection – 10 copies

**Genomic DNA** (4 species)

10-fold dilutions - 2 ng to 20 fg

Limit of detection - 20 fg

---

**How many rDNA copies in ONE ascospore?**

<table>
<thead>
<tr>
<th>Species</th>
<th>rRNA gene/ ng of gDNA</th>
<th>rRNA copies/ haploid genome</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cryptovalsa ampelina</em></td>
<td>1,942,500</td>
<td>105</td>
</tr>
<tr>
<td><em>Eutypa lata</em></td>
<td>2,039,750</td>
<td>110</td>
</tr>
<tr>
<td><em>Eutypa leptoplaca</em></td>
<td>2,119,115</td>
<td>114</td>
</tr>
<tr>
<td><em>Eutypella citricola</em></td>
<td>2,046,464</td>
<td>111</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>2,036,957</strong></td>
<td><strong>110</strong></td>
</tr>
</tbody>
</table>

*Eutypa lata* haploid genome = 54 mbp (Morales-Cruz *et al.* 2015)
Bot qPCR using multi-species primers

- *Diplodia seriata*
- *D. mutila*
- *Neofusicoccum parvum*
- *N. luteum*
- *N. australe*
- *Botryosphaeria dothidea*
- *Lasiodiplodia theobromae*
- *Spencermartinsia viticola*
- *Dothiorella ibérica*
Construction of standard curve using gBlocks® gene fragments

\[ y = -3.5018x + 38.709 \]
\[ R^2 = 0.9978 \]
Efficiency = 94%

Synthesised

\[ \beta\text{-tubulin nuclear gene} \]

Bot BtF1  Bot BtR1

Bot gBlock® (410 bp)  0.45 ag per copy

Quantification cycle (Cq)
Copy number (Log)
gBlocks® Gene Fragments

10-fold dilutions - $10^1$ to $10^6$

Limit of detection – 7 copies

Genomic DNA
(2 species)

10-fold dilutions - 3 ng to 30 fg

Limit of detection - 300 fg

Is β-tubulin gene a single copy gene?

<table>
<thead>
<tr>
<th>Species</th>
<th>copies/ng of gDNA</th>
<th>β- tubulin gene /haploid genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diplodia seriata</td>
<td>32,211</td>
<td>1.20</td>
</tr>
<tr>
<td>N. parvum</td>
<td>32,488</td>
<td>1.36</td>
</tr>
</tbody>
</table>

*Diplodia seriata* genome = 37.1 mbp
*Neofusicoccum parvum* genome = 42.6 mbp (Morales-Cruz, et al. 2015).
<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>Amplification for each primer pair&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5S-16F and DIA-89R</td>
</tr>
<tr>
<td><strong>Diatrypaceae species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryptovalsa ampelina</td>
<td>11</td>
<td>+</td>
</tr>
<tr>
<td>Cryptovalsa rabenhorstii</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>Diatrypella vulgaris</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>Eutypa lata</td>
<td>13</td>
<td>+</td>
</tr>
<tr>
<td>Eutypa leptoplaca</td>
<td>6</td>
<td>+</td>
</tr>
<tr>
<td>Eutypella citricola</td>
<td>8</td>
<td>+</td>
</tr>
<tr>
<td>Eutypella cryptovalsoidea</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>Eutypella microtheca</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td><strong>Botryosphaeriaceae species</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Botryosphaeria dothidea</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Diplodia mutila</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>Diplodia seriata</td>
<td>17</td>
<td>-</td>
</tr>
<tr>
<td>Dothiorella iberica</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Lasiodiplodia. theobromae</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Neofusicoccum australae</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Neofusicoccum parvum</td>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>Neofusicoccum luteum</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Spencermartinsia viticola</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td><strong>Other grapevine pathogens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus sp.</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Botrytis cinerea</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Colletotrichum acutatum</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Colletotrichum gloeosporoides</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Greneria uvicola</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Ilyonectria liriodendri</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Ilyonectria macrodyma</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Phaeoacremonium minimum</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Phaeomoniella chlamydospora</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Phomopsis viticola</td>
<td>7</td>
<td>-</td>
</tr>
<tr>
<td>Erysiphe necator</td>
<td>3</td>
<td>-</td>
</tr>
</tbody>
</table>
SUMMARY

• Two qPCR assays using multi-species primers developed

• Rapid and sensitive in detecting of ED and BD pathogens from environmental samples

• Currently being used to analyse spore trap tape samples collected from Australian vineyards.
Wine Australia for Australian Wine

Practical Management of Grapevine Trunk Diseases
(2013-2016)

National Project Leader:
Mark Sosnowski (SARDI)

Collaborators:
Jose Urbez-Torres (SuRDC)
Eileen Scott (Uni of Adelaide)
Chris Steel, Gavin Ash (NWGIC)
Trevor Wicks (ex. SARDI)

RESEARCH TEAM

Matthew Ayres, Mark Sosnowski (SARDI)
Regina Billones Baaijens Sandra Savocchia (NWGIC)

Institutes and Organizations

SARDI Plant Research Centre
University of Adelaide
NWGIC
Trunk Disease Team

Technical Staff

Jenny Bannister
Pete Carey
Dave Foster

Students

Sandra Savocchia
Reggie Baaijens
Chris Steel

Cyprien Goupil
Intern

Jennifer Niem
PhD student

Pierluigi Reveglia
PhD student

Lindsay Greer
Meifang Liu
Gurli Nielsen
ACKNOWLEDGEMENTS

Wine Australia for Australian Wine