Discrimination between viable and dead *Xanthomonas fragariae* on strawberry host tissue by PCR

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Growing and Protecting New Zealand

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Xanthomonas fragariae (ALS)

- Angular Leaf Spot on strawberry
- Disease affects foliage, calyx and developing daughter plants (vascular)
- Lower yields and/or causes plant death
- Secondary infections caused by exuding bacteria from lesions and water splash
- Infection of fruit calyx reduces quality and fruit marketability
Xanthomonas fragariae (ALS)

- Systemically infected plantlets are almost exclusively the primary source of inoculum in newly planted fields

http://strawberryplants.org/
EPPO lists *X. fragariae* as an A2 quarantine pathogen

- Pathogen absent from majority of strawberry growing countries in Europe, but has the potential to become established there.
X. fragariae (ALS) in New Zealand

- ALS first detected in NZ (1971) from strawberry plants in Auckland
  - progeny of plantlets imported from USA
- 1972, ALS detected from 3 more growers who had received progeny from the original 1971 import.
- Successful eradication program carried out in 1971/72

3. https://gd.eppo.int/taxon/XANTFR/distribution/NZ
More recently....

- In 2011, 2012, 2014 consignments of strawberry fruit showing disease symptoms (calyx) were intercepted by MPI inspectors.
- Samples sent to PHEL for identification.
- *X. fragariae* was detected by PCR at PHEL.
- PCR confirmed by isolation (7 days).
- Consignments were lost due to wait for isolation results.
Viability PCR for detection of live pathogens in a quarantine setting

- PCR techniques are routinely used for the detection of regulated pest in quarantine, biosecurity and food safety.
- One disadvantage is the inability of PCR to differentiate between positive results originating from live or dead microbial cells.
- Validation of test results and interpretation of biological risks can be difficult.
- Risk of “false positive” test results, especially for products that have already undergone treatment or other phytosanitary measures.
Viability PCR for detection of live pathogens in a quarantine setting

• Diagnosis of regulated pests must balance the needs of sensitivity, speed and biological relevance.

• Ambiguity in interpretation of PCR test results without live/dead cell determination can result in unnecessary biosecurity interventions and response type work, and imposition of unnecessary compliance costs.

• DNA intercalating dyes can reduce PCR signals from dead cells
Viability PCR concepts

• Cell membrane integrity is generally accepted as the differential between live and dead cells
  – some disinfection treatments can kill cells without disrupting cell membranes

• VIABILITY is the capacity to form progeny
  – a non-viable microbe is not a quarantine threat
  – a viable microbe is
- PMA penetrates dead cells with compromised membranes
- exposure to light cross links the dye to DNA
- inhibits PCR amplification.

Source – Biotium Inc
PMA pre-treatment of cells

- Add PMA dye to your sample and incubate in the dark (intercalation)
- Cross link PMA dye to DNA by exposing to blue light
- Lyse intact cells and extract DNA
- Conventional or real-time PCR
- Note: PMA is light and temperature sensitive and toxic. Adds approx. 1-2 hours to the PCR detection process.
• Some disinfection treatments can induce cell death without compromising membrane integrity.

Optimize the variables!

- Choice of DNA-intercalating dye (EMA, PMA or PEMAX)
- Dye concentration, incubation time and temperature
- Salt concentration and pH in the reaction
- Different kill treatments
- Photo-activation times
- Effect of different PCR amplicon length and target
- Bacterial cell concentration
- Sample complexity

PCR amplicon length

- The length of the target amplicon was one of the most important parameters.
- Works better with conventional PCR than real-time PCR.
- The amplification of longer sequences correlates with a higher probability of encountering a dye-DNA binding event.

Lanes 1-4  Psa dead cells;
5-6       Psa viable cells;
7-10      Xcc dead cells;
11-12     Xcc viable cells;
13        Xcc dead cells – no PMA;
14        Psa dead cells – no PMA;
15        water
Interpretation of weak positive test results can be a challenge

• The ideal situation is that no DNA is amplified from dead cells. However, this does not always occur.
• The dye or light can not penetrate all dead micro-organisms.
• Potential for false positives i.e. PMA does not completely suppress the amplification of DNA from dead cells.
• Presence of a low number of viable cells mixed with a population of dead cells.
A combination of qPCR tests to improve live/dead cell interpretation

<table>
<thead>
<tr>
<th>Samples</th>
<th>Viable colonies (KBC)</th>
<th>Test 1. qPCR Ct results (no PMA)</th>
<th>Test 2. qPCR Ct results after PMA treatment</th>
<th>Test 3. qPCR Ct results + kill treatment + PMA treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viable cells</td>
<td>+</td>
<td>20.83</td>
<td>21.43</td>
<td>35.64</td>
</tr>
<tr>
<td>Dead cells</td>
<td>-</td>
<td>20.39</td>
<td>35.86</td>
<td>36.85</td>
</tr>
<tr>
<td>Mixed cells</td>
<td>+</td>
<td>21.85</td>
<td>22.17</td>
<td>35.55</td>
</tr>
</tbody>
</table>

**Test 1.** detects all target microbial cells  
**Test 2.** theoretically detects only viable cells  
**Test 3.** differentiates between incomplete suppression of dead cells and mixed populations of viable and dead cells
Detection of viable *Xanthomonas fragariae* on strawberries

- Conventional PCR (amplicon 615bp) does not amplify PMA treated killed cells.

- qPCR (amplicon 129bp) suppresses amplification up to 9 cycles (5µM).

**Legend:**
- **Red** = viable cells with no PMA
- **Green** = viable cells with PMA
- **Dark blue** = killed cells with no PMA
- **Light blue** = killed cells with PMA
Detection of viable *Xanthomonas fragariae* on strawberries

- Conventional vPCR worked
  - amplicon 360bp
  - able to confirm detection of viable cells same day
- Viable qPCR did not work with the PMA pre-treatment
  - amplicon size 120bp
- *X. fragariae* confirmed by isolation, 7 days later on selective media
Conclusions

• This technique shows promise to differentiate between viable and nonviable microbial cells in quarantine samples.

• Interpretation of test results from environmental samples can be challenging but this can be overcome by including additional test controls.

• Potential applications include pathogen detection in import, export, post entry quarantine and response samples.

• Future work to investigate other applications for example, fungal targets
Acknowledgements

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Questions?

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