Assays for detection of *Acidovorax citrulli* in matrix seeds

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July 2017

Overview

- Introduction *Acidovorax citrulli* (Acit)
- Detection of Acit using grow-out
- Detection of Acit with seed extract PCR
- Genetic diversity of Acit and lookalikes
- Comparison of different Acit Taqmans
- Validation of seed extract PCR

Bacterial Fruit Blotch



- Acit is causal organism of bacterial fruit blotch (BFB)
- Several hosts amongst the cucurbits
- Relatively "new" disease
  - In the USA in the 90s major problems in commercial productions
- Seed transmittable and therefore liability issue for seed companies
- No reliable selective media for seed matrix: Classic dilution plating is impossible

Symptoms on watermelon

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NSHS and NAL methods

- METHOD Cb1.1 Seedling Grow-out.
  - Class A 10,000-30,000 seeds
- METHOD Cb1.2 Seminis Inc. PCR-Wash method
  - Temporary standard B 30,000 seeds
- METHOD Cb1.3 Syngenta SYBR Green PCR method
  - Temporary standard B 10,000-30,000 seeds
- METHOD Cb1.4 Monsanto Improved PCR method
  - Temporary standard B 10,000-30,000 seeds
- METHOD Cb1.5 CSPL PCR (proprietary)
  - Temporary standard B

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NSHS and NAL methods

- Sweat box method*
  - No NSHS status 10,000-30,000 seeds
- "Syngenta improved PCR method"*
  - No NSHS status 10,000-30,000 seeds
  - Several laboratories in NAL
  - Proficiency testing in 2014
- Sweat bag method (Patented for US/Japan)

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Grow-out in sweat box

- 10,000 or 30,000 seeds per sample
- Sow about 800 fungicide treated seeds per box
- Incubate at high humidity at 28°C
- Evaluate seedlings after 2-3 weeks for symptoms on cotyledons
- Verify presence of Acit in symptomatic plants

Version 1
- Classic PCR 1x
- IF or ELISA
- Pathogenicity assays melon and watermelon

Version 2
- Real time PCR 2x
- Pathogenicity assays melon and watermelon

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Grow-out

Advantages sweat box/greenhouse grow-out
- Relatively low tech for grow-out part
- Compatible with treated seeds
- Detection of viable cells
- Isolation of Acit is possible

Disadvantages
- False negatives after non-symptomatic infections when humidity or temperature are too low
  - Secondary spread Acit necessary
  - Growing conditions should be conducive for Acit
- Labour and space intensive (expensive for 30,000 seeds)
- Takes about 3 weeks
- Fungal growth in sweat box could disguise presence Acit
- Cross-contamination issues

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Seed extract Taqman PCR

1

2

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1. Count 2 x 5,000 or 6 x 5,000 seeds per sample and add extraction buffer
2. Incubate on shaker in BFB buffer for 20 hrs at RT
3. Take 10 ml extract
4. Add low number of *Acidovorax cattleyae* as spike (internal amplification control)

3

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Seed extract Taqman PCR

5. 6

5. Perform slow spin to remove debris
6. Perform fast spin to concentrate bacteria
7. Purify DNA with DNA extraction kit (f.i. A. citrulli Sbeadex kit using a KingFisher)



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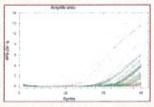
Seed extract Taqman PCR


8. Perform multiplex Taqman PCR
   1. FAM label for *Acit*
   2. TR label for *A. cattleyae* (Internal Amplification Control)
   3. 0.5 and 5 µl in multiplex Taqman PCR to monitor inhibition

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Challenges with seed extract Taqman PCR

- Inhibition of Taqman through PCR inhibitors
  - Spike samples of with small number of *A. cattleyae* cells as internal amplification control (IAC)
  - IAC should be detected to validate test result
  - Use of 10-fold dilutions for each subsample
- Sensitivity of assay
  - Long incubation to multiply Acit
  - Use large volume then centrifugation
  - Use multicopy target in Acit (IS1002 Taqman)
- Specificity of Taqman (NO FALSE NEGATIVES)
  - Use second target

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Characterisation of Acit strains and lookalikes

- Collection of 222 Acit strains and lookalikes from all over the world
  - Seed companies and Naktuinbouw ("Syngenta collection")
  - Turkey (Sumer Horuz)
  - New isolates from Syngenta and Far East (Mitsuru Igarashi)

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Characterisation tools

- AFLP fingerprinting
  - Investigate genetic diversity within species and between related species
- Acit specific Taqmans
  - IS1002 Taqman
  - Contig 21 Taqman
  - Contig 22 Taqman
  - QBX Taqman
- Pathogenicity assays (watermelon and melon)

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AFLP Acit and lookalikes

168 strains group as Acit
54 strains group as lookalike

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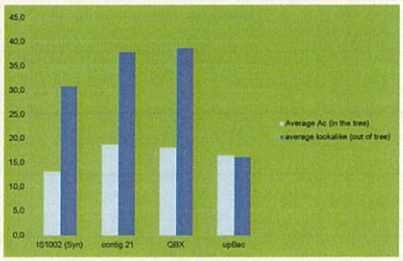
Comparison of 222 strains with 4 Taqmans



168 Acit strains:
average Δ Ct 5 between IS1002 and Contig 21/QBX

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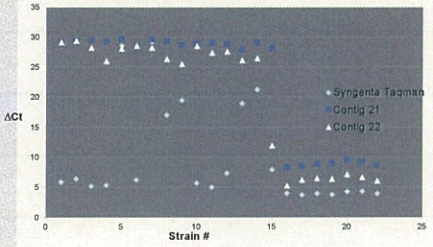
Isolates from Syngenta and Mitsuru Igarashi

Relative Ct values for 3 Acit PCRs



Data Bert Woudt Syngenta

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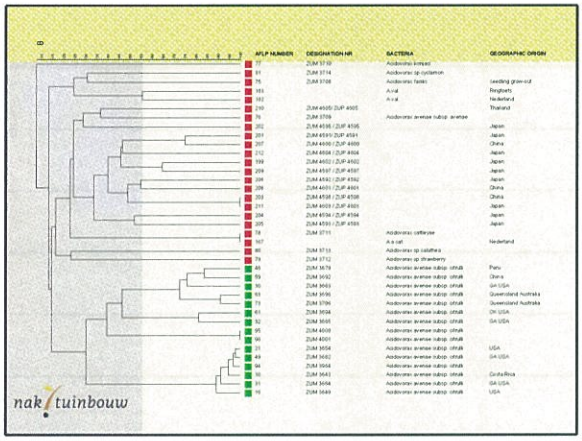

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IS1002 Taqman

	Real Acit	Lookalike
IS1002 Taqman positive	Real Positive 168	False positive 10
IS1002 Taqman negative	False negative 0	Real negative 43

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Validation

1. Trueness
   - Compare with grow-out
2. Analytical sensitivity
   - Dilution series
3. Analytical specificity of primer sets
   - IS1002/contig 22/IAC (done for 222 isolates)
4. Selectivity
   - inhibition in watermelon or melon seed matrix
5. Repeatability and reproducibility
   - within lab
   - between labs

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Advantages of new molecular method

1. Primers and sprobe target a multi-copy element (FAM signal: IS1002) of the Acit genome (higher sensitivity) and specific target (VIC signal: Contig 22)
2. Use of *Acidovorax cattleyae* as IAC (Texas Red signal)
3. DNA isolation using a commercial kit available in single column or 96-well format
4. Data within 48 hrs.
5. Quantitative: lower Ct means presence of more Acit DNA

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Disadvantages of new molecular method

1. No discrimination between dead cells and viable cells
2. Using purified DNA some strongly related lookalike strains were picked up with IS1002 Taqman PCR (false positives)
3. No isolation of Acit and or lookalikes
   - Was noise generated by closely related lookalike or was signal caused by real Acit?
4. Not yet known what Ct threshold has biological relevance. "Conservative" threshold of 35 is now used.

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Application of direct Taqman

- SEED EXTRACT TAQMAN PCR IS A PRESCREENING METHOD!
- Should instruct customers clearly that direct PCR method is prescreening and does not discriminate between dead and viable cells
- Retest direct PCR positive samples with grow-out (SBX or GGO)
  - Positive grow out is smoking gun but in general high Ct levels are negative in grow out
- For direct PCR negative samples it is highly unlikely that disease will occur in practice
- Seed processing could interfere with viability
  - Short soaking time in combination with low volume is a risk

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Running and future research

- Discrimination between viable and non-viable cells
  - PMA PCR
  - Enrichment PCR
- Understand biological relevance of closely related lookalikes
- Determine reliable threshold by comparing data from complementary assays
- Variation in extraction volume and soaking time (Monsanto USA protocol)

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Conclusions

- Several Acit seed and seedlings assays are available
- Complementary molecular and grow out assays
  - Both assay types have been improved
    - Better DNA extraction
    - Improved PCR's
- Some assays have been used widely and have been validated or are under validation

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Thanks

watermelon (*Citrullus lanatus*)

melon (*Cucumis melo*)

pumpkin (*Cucurbita pepo*)

cucumber (*Cucumis sativus*)

squash (*C. maxima, C. moschata*)

citron melon

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Dia 23

hms2 bert welk percentage is ongeveer negatief?
hko; 5-11-2013