Pistachio Bushy Top Syndrome: Disease Etiology and diagnosis procedure

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The Problem:

Approximately 2 million UCB-1 rootstock planted from 2011-2014 in California and Arizona demonstrated stunted growth, shortened internodes, swollen lateral buds, and atypical bushy growth.

Many of the trees did not reach sufficient size for budding, and if budded, the buds would not grow well, if at all. Many of the graft unions displayed unusual bark cracking.
Orchards with stunted UCB1 rootstock

Orchards in San Joaquin Valley with abnormal UCB1 rootstocks.
Observed Symptom overview

Stamler et al., 2015  Accepted; In Press.
FIRST LEAF TREE OBSERVATIONS:

STUNTED TREES
SWOLLEN LATERAL BUDS
ROOT MORPHOLOGY ABNORMALITIES
STUNTED TREES: Trees planted in 2013 pictures taken in May 2014
RHODOCOCCUS FASCIANS MODULATES PHYTOHORMONES!
Cytokinins act antagonistically to auxins


Slide from ASPB 2010 Teaching Tools
Koch’s Postulates
Step 1: Test for the putative pathogens
Step 2: Introduce the putative pathogens
Step 3: Incubate
Step 4: Observe changes in the plant
Step 5: Test for the pathogens

Results
Pre-Test
Negative

Results
Post-Test
Positive
Testing Pistachio UCB-1 with *Rhodococcus* isolates

- 340 clonal UCB-1 trees were obtained from a nursery.

- Trees were tested for the presence of *Rhodococcus* by DNA testing and culturing. Trees were negative for the presence of *Rhodococcus*.

- Trials began summer of 2014 in a quarantine greenhouse at New Mexico State University.
Treatments for Koch’s

1. Control plants (no bacteria).
2. Inoculated Rhodococcus isolate 1.
3. Inoculated Rhodococcus isolate 2.
4. Rhodococcus isolates 1 + 2.
Rhodococcus isolates sprayed onto plants
Initial Symptoms
Trial 1: *Rhodococcus* isolate 2
Growth Rates of Treatments

Rhodococcus isolates inhibit 'UCB-1' Rootstock Growth

Days Post Inoculation

Growth Post Inoculation (cm)

- Control
- Isolate 1
- Isolate 2
- Isolate 1 + 2
Average Height of Treatments

Average Height at 104 days P.I.

- **Control**: Height not measured
- **Isolate 1**: Average height not specified
- **Isolate 2**: Average height not specified
- **Isolate 1 + 2**: Average height not specified

The graph shows a comparison of plant heights for different treatments. The control group (A) has the highest average height, while the isolate 1 + 2 group (C) has the lowest.
Genetic Verification of Bacteria on symptomatic plants from Koch’s postulates

- Rhodococcus jostii CP000431
- Rhodococcus opacus CP003949
- Rhodococcus fascians D188 AJ301559
  - Rhodococcus isolate 2
    - Koch’s Postulates A
      - Rhodococcus isolate 1
        - Koch’s Postulates B
          - Koch’s Postulates C
            - Koch’s Postulates D
              - Koch’s Postulates E
                - Koch’s Postulates F

0.0030
Bacteria still alive on surface 104 days post inoculation
Leaf phenotype
DIAGNOSIS OF PISTACHIO BUSHY TOP
Symptom Observations

• Stunted growth
• Reduced Internode length
• ‘Bushy’ appearance
• Inability/reduced grafting efficiency
• Swollen nodes
• Gall formation at nodes
TISSUE COLLECTION FOR RHODOCOCCUS TESTING
Tissue Collection

Analysis & Imaging

DNA Extraction

Bacterial Isolation

PCR & Sequencing

Microscopy

Pathogenicity Tests

LEAVES

STEMS

ROOTS

GALLS
It’s All about that Sample...

• **Efficient testing is best when good quality tissue is submitted.**

• No autopsy samples!
Tissues for Testing

- Leaves and petioles (Root stock and Scion)
- Leaves should be taken at different areas of the tree.
- Stem galls that appear to have multiple shoots
- Root tissues
- Large Sections of the trunk not recommended.
How to collect samples:

• Samples should be removed from the trees using pruning sheers.
• The pruning sheers should be **sanitized between trees with either a bleach solution, or a quaternary ammonia chloride product** (such as Lysol or PW2 etc.).
• The sample should be placed in a large sized ziplock bag and labeled. Sample bag placed in cooler.
Sample Collection

• Hands should also be washed/rinsed between trees or gloves changed between trees.
Quantity of Pistachio tissue (Randall Lab)

- Samples submitted for testing can be bulked to include several trees (5-10 trees). Three leaves from each tree including petioles are sufficient. If including gall tissue, one gall from each tree is sufficient.
Shipping samples

• Once samples are collected it is imperative that the samples are sent directly to the lab for testing. Testing is best when the plant tissues are still fresh. It is recommended that samples remain cold during transport.

• Overnight delivery best
• Appropriate permits should be in place prior to testing!
TESTING SAMPLES
Testing Samples:

1. Total DNA can be isolated from samples and screened using primers specific for *Rhodococcus*.

2. Culture for *Rhodococcus* bacteria from plant samples.
Total DNA can be isolated from tissues and screened for *Rhodococcus*

- DNA isolated from leaves, petioles, stems, roots, and galls.

- Screened with *Rhodococcus* specific primers
Screening total DNA for *Rhodococcus*

- DNA ISOLATED USING PLANT DNA-EASY QIAGEN KITS
- DNA SCREENED USING
  - Primers for *vicA* (located on Chromosome).
  - Primers for bacterial 16S-ITS regions.
  - Primers for *fasD*. 
Screening Total DNA for Rhodococcus using PCR

- Primers
  - *vicA*: amplifies Rhodococcus preferentially
  - *fasD*: *Not recommended.*
  - 16S: Chloroplast amplification!
    
      All microbes associated.
Tissue Collection

Analysis & Imaging

DNA Extraction

Bacterial Isolation

PCR & Sequencing

Microscopy

Pathogenicity Tests

LEAF PRINTS AND GROUND LEAF, STEM, GALLS, ON SELECTIVE BACTERIAL MEDIA

INCUBATION FOR 7-10 DAYS
SURFACE STERILIZATION

• Tissue rinsed with 0.6% Sodium hypochlorite or Sodium dichloroisocynauric acid for 20 seconds with shaking.

**Sodium hypochlorite solution made fresh.**
Surface Sterilization continued.

• Tissue washed with 70% EtOH for 1 minute.
• Tissue washed with sterile DI water three times.
Tissue used for grinding

• 5 ml of sDH₂O or autoclaved D2 media added to the tissue.

• Tissue ground with sterile glass rod.
Plating

- 200 ul of the ‘grindate’ is plated onto D2 or MD2 media. Plates are incubated at 28°C for approximately 4-10 days.
Media Composition—D2 or MD2
D2 Media and MD2
Kado and Heskett 1970

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per L</th>
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<tbody>
<tr>
<td>Glucose</td>
<td>10.0 g</td>
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<tr>
<td>Casein hydrolysate</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0 g</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>1.0 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.3 g</td>
</tr>
<tr>
<td>LiCl</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Tris [tris (hydroxymethyl) amino methane]</td>
<td>1.2 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
</tbody>
</table>

Adjust to 7.8 with HCl and autoclave. The medium is cooled to about 50°C and polymyxin sulfate and sodium azide are added.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (mg)</th>
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<tr>
<td>Polymyxin B sulfate*</td>
<td>40 mg</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>0.5 mg</td>
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Plates with Colonies: Internal

‘Normal UCB-1’ | PBTS UCB-1
Association of Rhodococcus

- *Rhodococcus fascians* cultured and genetically confirmed in >15 PBTS orchards.

- *Rhodococcus fascians* isolates NOT found in non bushy top orchards.
Tissue Collection

DNA Extraction

Bacterial Isolation

**PCR & Sequencing**

Microscopy

Pathogenicity Tests

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**PLANT DNA**

**BACTERIAL DNA**

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**16S PCR & Sequencing**

**VicA PCR & Sequencing**

**Plasmid PCR & Sequencing**
Genomic DNA Isolation
DNA

Amplification Conditions

• 1. 95 C for 3 min.
• 2. 95 C for 15 sec
• 3. 64 C for 30 sec (+plate read)
• 4. Go To step 2 39X
• Melt Curve from 65 - 95 C with 0.5 degree increments
<table>
<thead>
<tr>
<th>PRIMER NAME</th>
<th>PRIMER SEQUENCE</th>
<th>Reference</th>
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<tbody>
<tr>
<td>vicA forward 5’</td>
<td>5’TCTGGATCTCGAAGTGCAAAACCGT3’</td>
<td>Nikolaeva et al. 2012</td>
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<tr>
<td>vicA reverse 3’</td>
<td>5’AGCGTACAAGGCCTTCTGAAAGA3’</td>
<td>Nikolaeva et al. 2012</td>
</tr>
<tr>
<td>Rf fasD internal 5’ forward</td>
<td>5’GCATTGAGTCATCGGCTCC3’</td>
<td>Randall Lab (Stamler et al. 2015)</td>
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<tr>
<td>Rf fasD internal 3’ reverse</td>
<td>5’CGCACTGCTGGGCAAAAGTAG3’</td>
<td>Randall Lab (Stamler et al. 2015)</td>
</tr>
<tr>
<td>Rf attA internal 5’ forward</td>
<td>5’CGTGTAGCCGGTGAAGAAGT3’</td>
<td>Randall Lab (Stamler et al. 2015)</td>
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<tr>
<td>Rf attA internal 3’ reverse</td>
<td>5’CTGCTCGACATGGAGAGCAG3’</td>
<td>Randall Lab (Stamler et al. 2015)</td>
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<tr>
<td>Rf -229F</td>
<td>5’ ATGGCACAGACGCAAGCAA3’</td>
<td>Nikolaeva et al. 2012</td>
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<tr>
<td>Rf-408R</td>
<td>5’ GATACGGTGCGGCAACAACAAATC3’</td>
<td>Nikolaeva et al. 2012</td>
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<tr>
<td>Rf-366P</td>
<td>/6FAM/AATTGGAAAGAGCGCCGAAGCGGAGCAAGT/BHQ_1/</td>
<td>Nikolaeva et al. 2012</td>
</tr>
</tbody>
</table>
vicA amplification  Tm:88.5
fasD internal  Tm 85.0
fasD amplification with kits
Sequence Analysis of Amplicons

• Amplicons treated with Exo-SapIT (Affymetrix).
  • Exonuclease I and shrimp alkaline phosphatase.

• Sequence reactions performed using Big Dye Terminator (Life technologies).
• Reactions loaded onto ABI 3130 (NMSU).
Sequence

• Geneious (software by BioMatters) utilized to analyze sequences, make alignments, and to create phylogenetic trees.
Rhodococcus fascians antibody
Acknowledgements

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