Pistachio Bushy Top Syndrome:
Disease Etiology and diagnosis procedure

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April 7, 2015

The Problem:

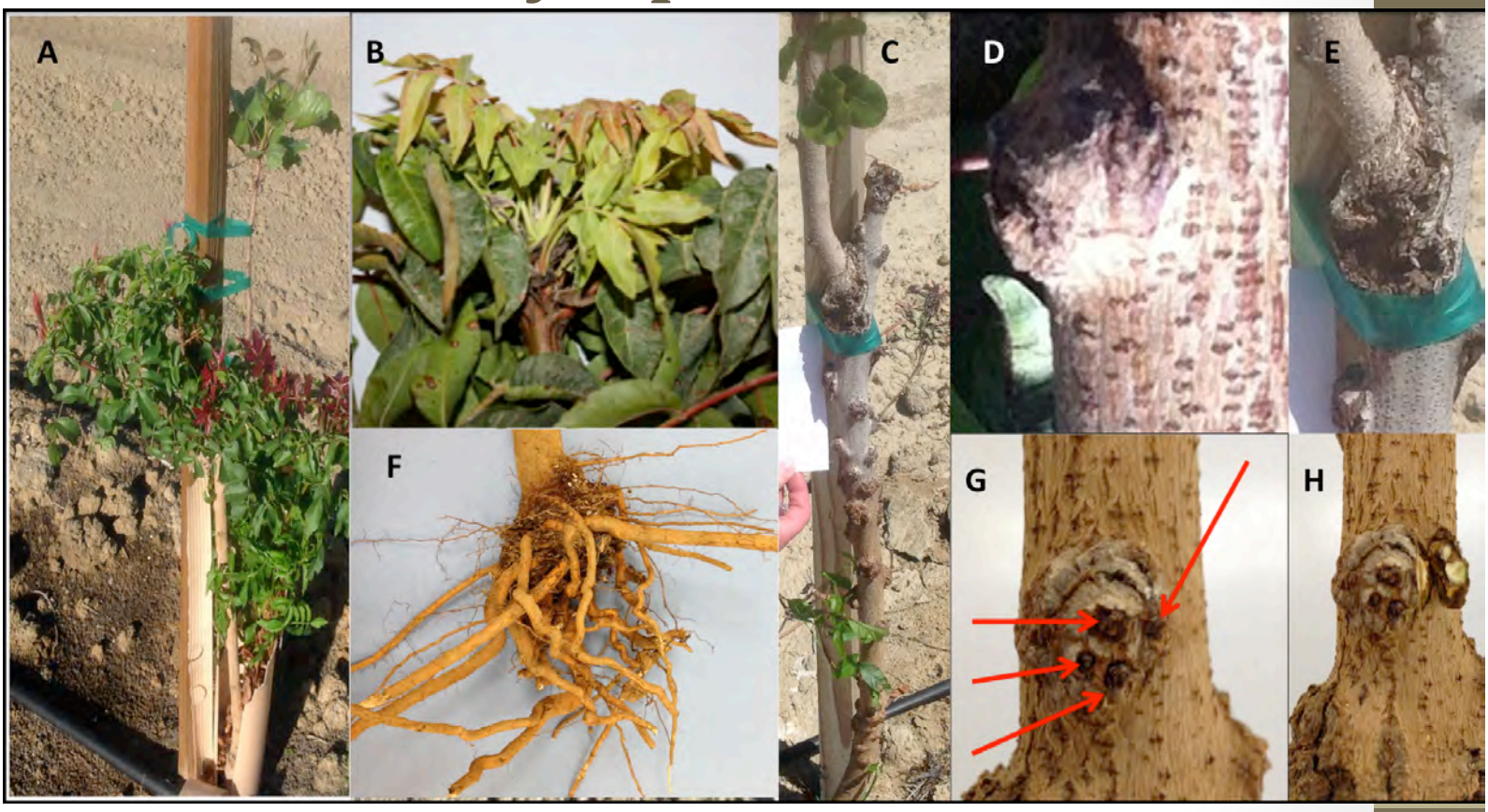
Approximately 2 million UCB-1 rootstock planted from 2011-2014 in California and Arizona demonstrated stunted growth, shortened internodes, swollen lateral buds, and atypical bushy growth.

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

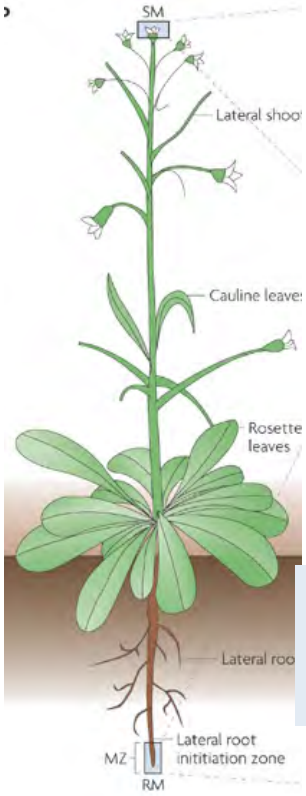
STUNTED TREES: Trees planted in 2013 pictures taken in May 2014

Tree 12

Tree 25

RHODOCOCCUS FASCIANS MODULATES PHYTOHORMONES!

Cytokinins act antagonistically to auxins



CK

Promote branching in the shoot

Auxin

Inhibit branching in the shoot

CK

Inhibit branching in the root

Promote branching in the root

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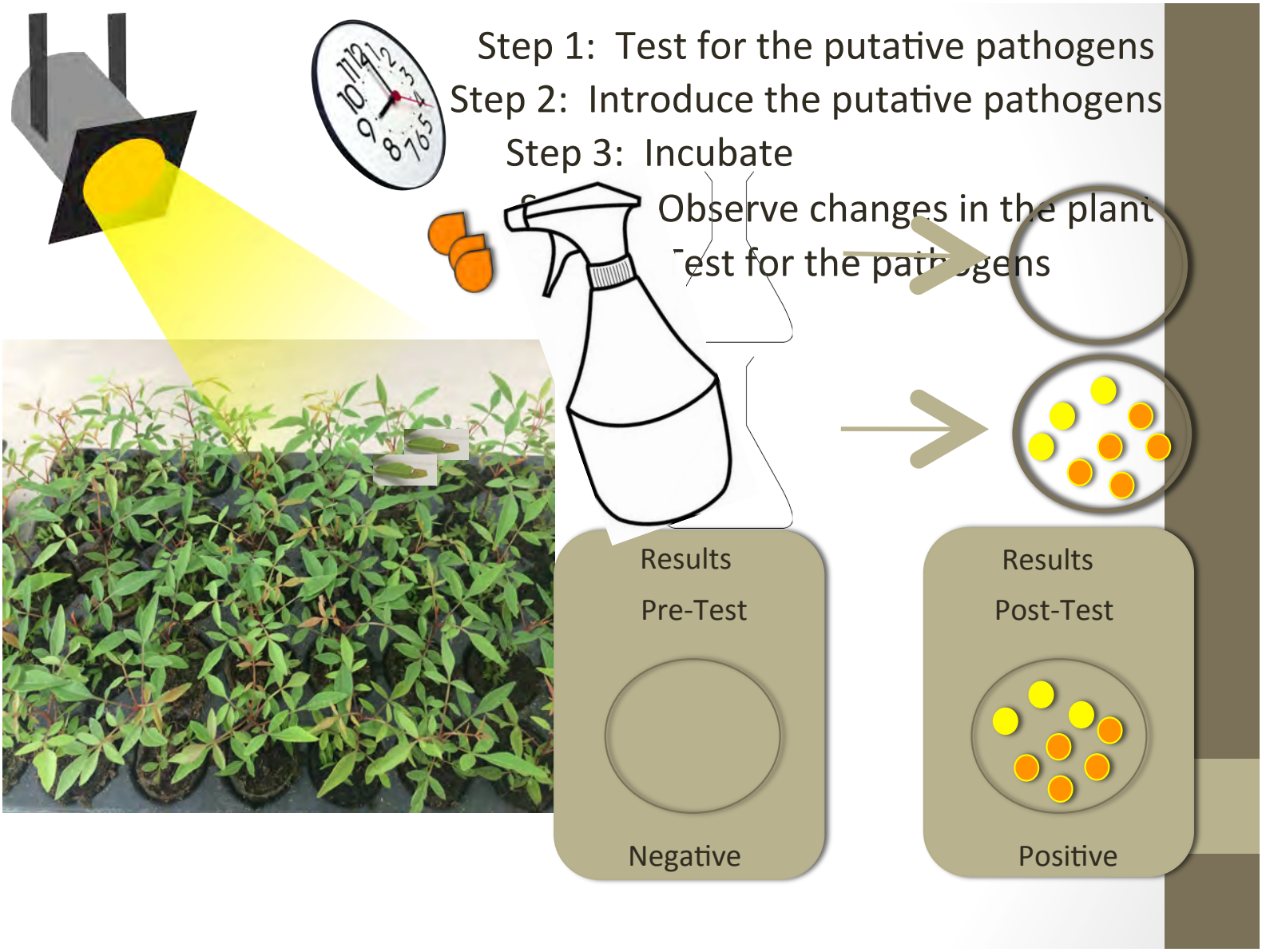
Slide from ASPB 2010 Teaching Tools

KOCH'S POSTULATES


Step 1: Test for the putative pathogens
Step 2: Introduce the putative pathogens
Step 3: Incubate
Observe changes in the plant
Test for the pathogens
Results
Pre-Test
Negative
Results
Post-Test
Positive

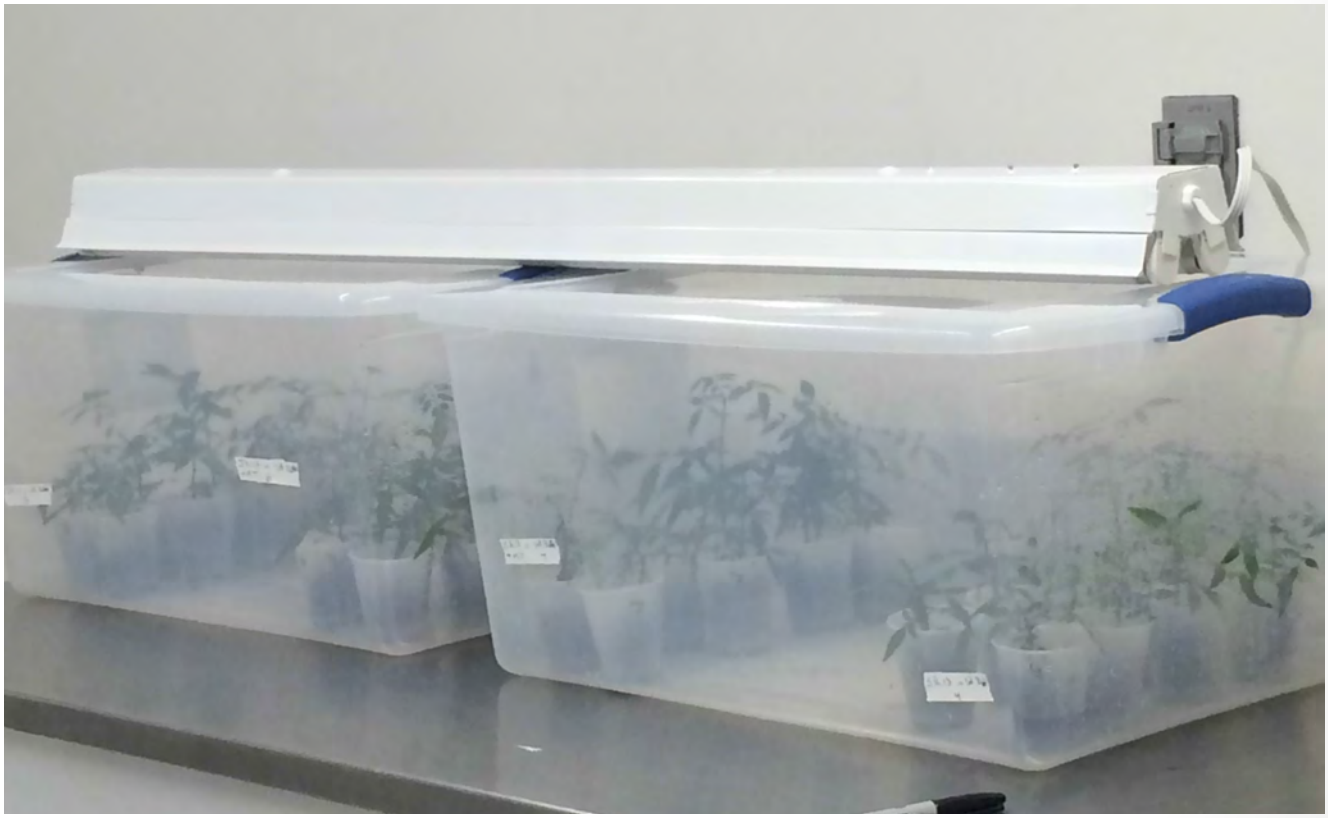
Testing Pistachio UCB-1 with *Rhodococcus* isolates

- 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

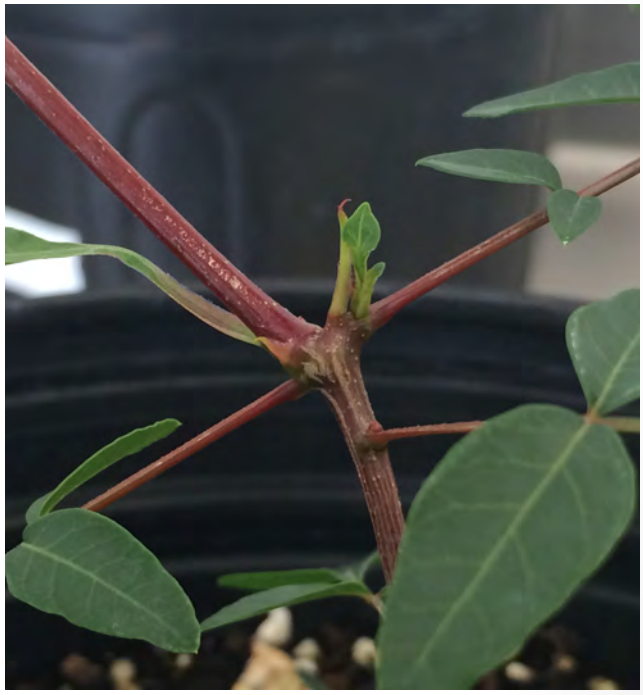
Treatments for Koch's

1. Control plants (no bacteria).
2. Inoculated Rhodococcus isolate 1.
3. Inoculated Rhodococcus isolate 2.
4. Rhodococcus isolates 1 + 2.

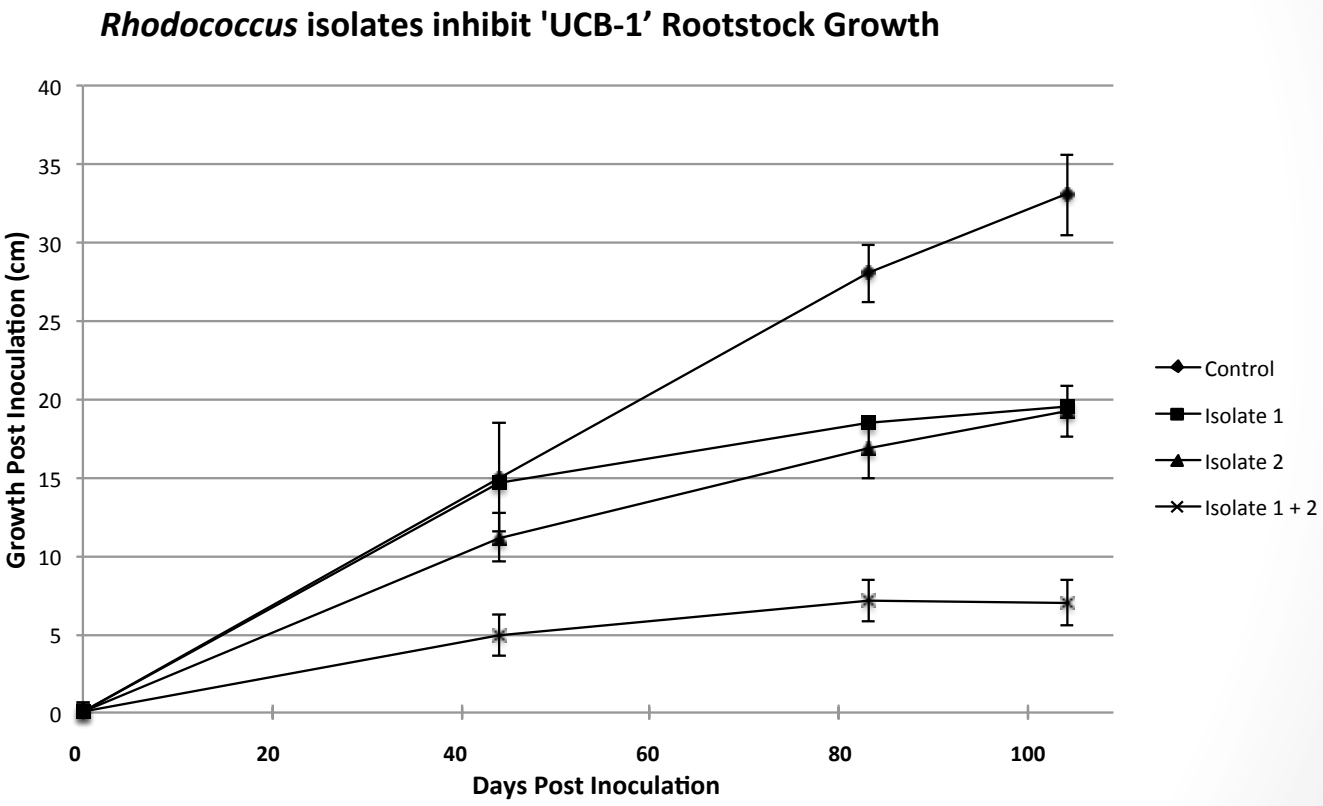
Rhodococcus isolates sprayed onto plants



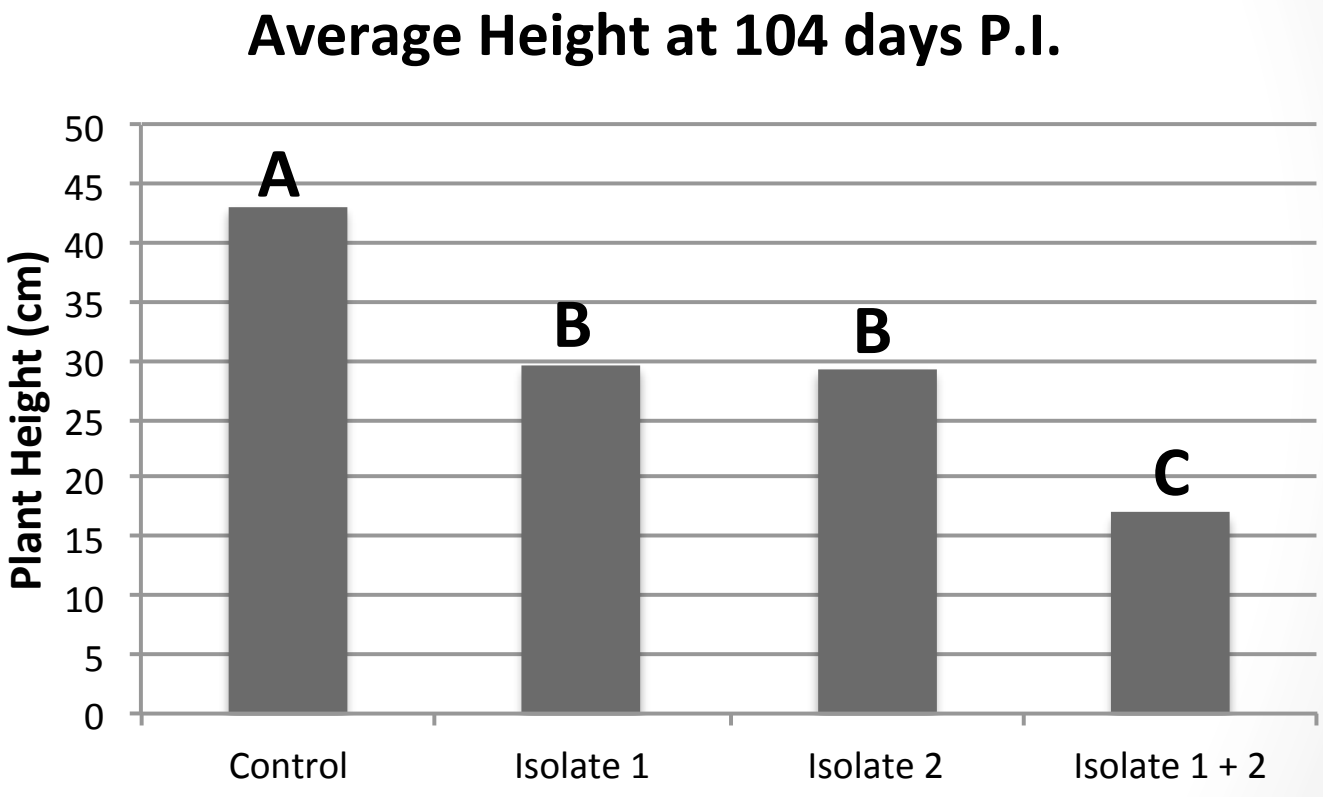
Initial Symptoms
Trial 1: *Rhodococcus* isolate 2



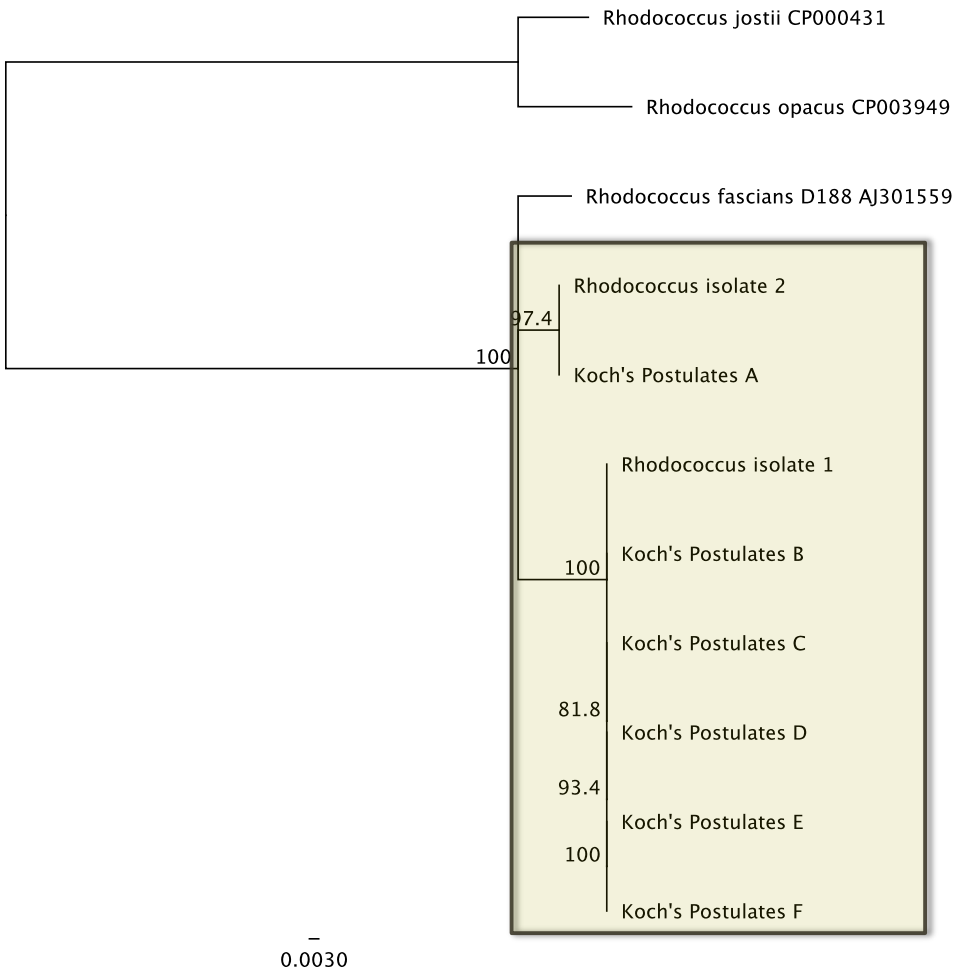
Growth Rates of Treatments



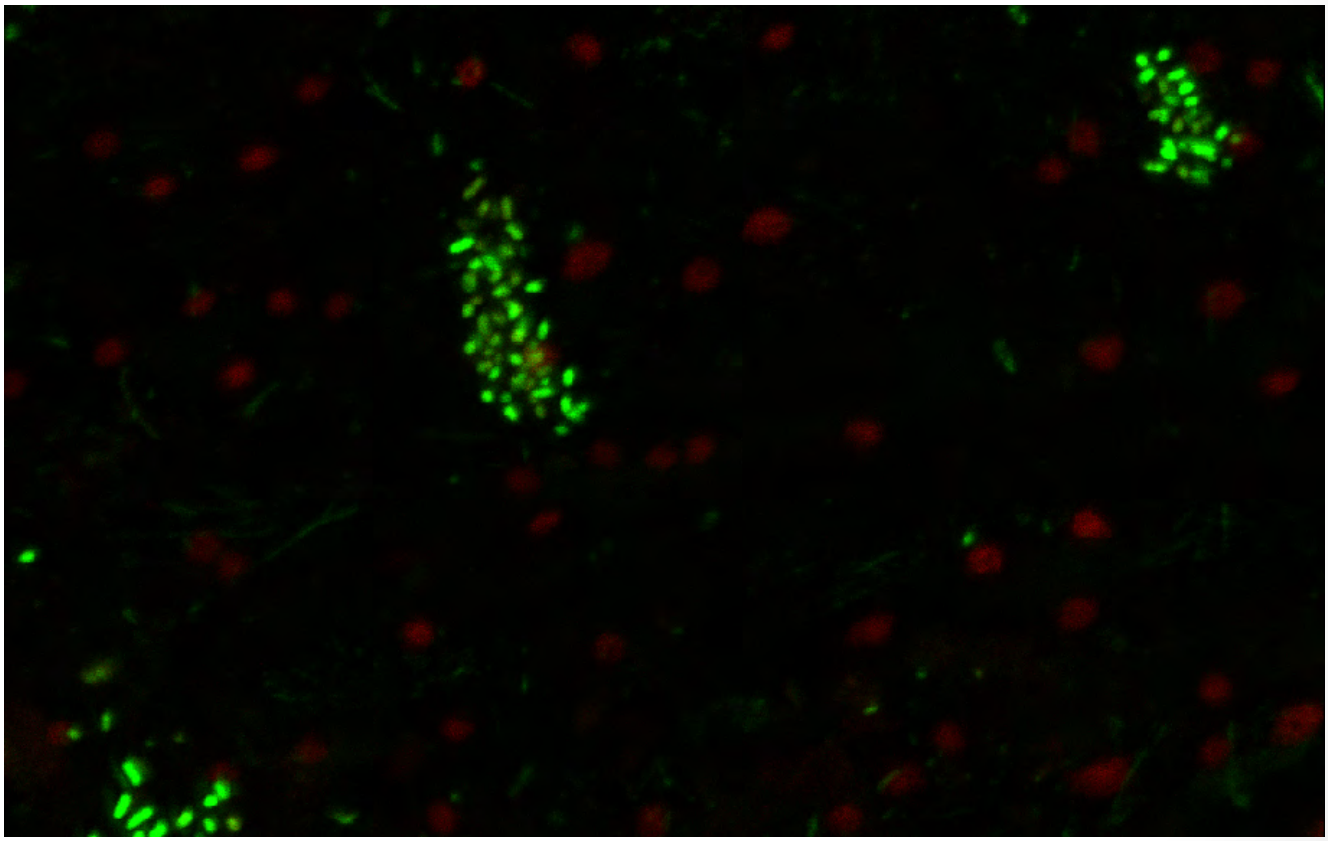
Average Height of Treatments

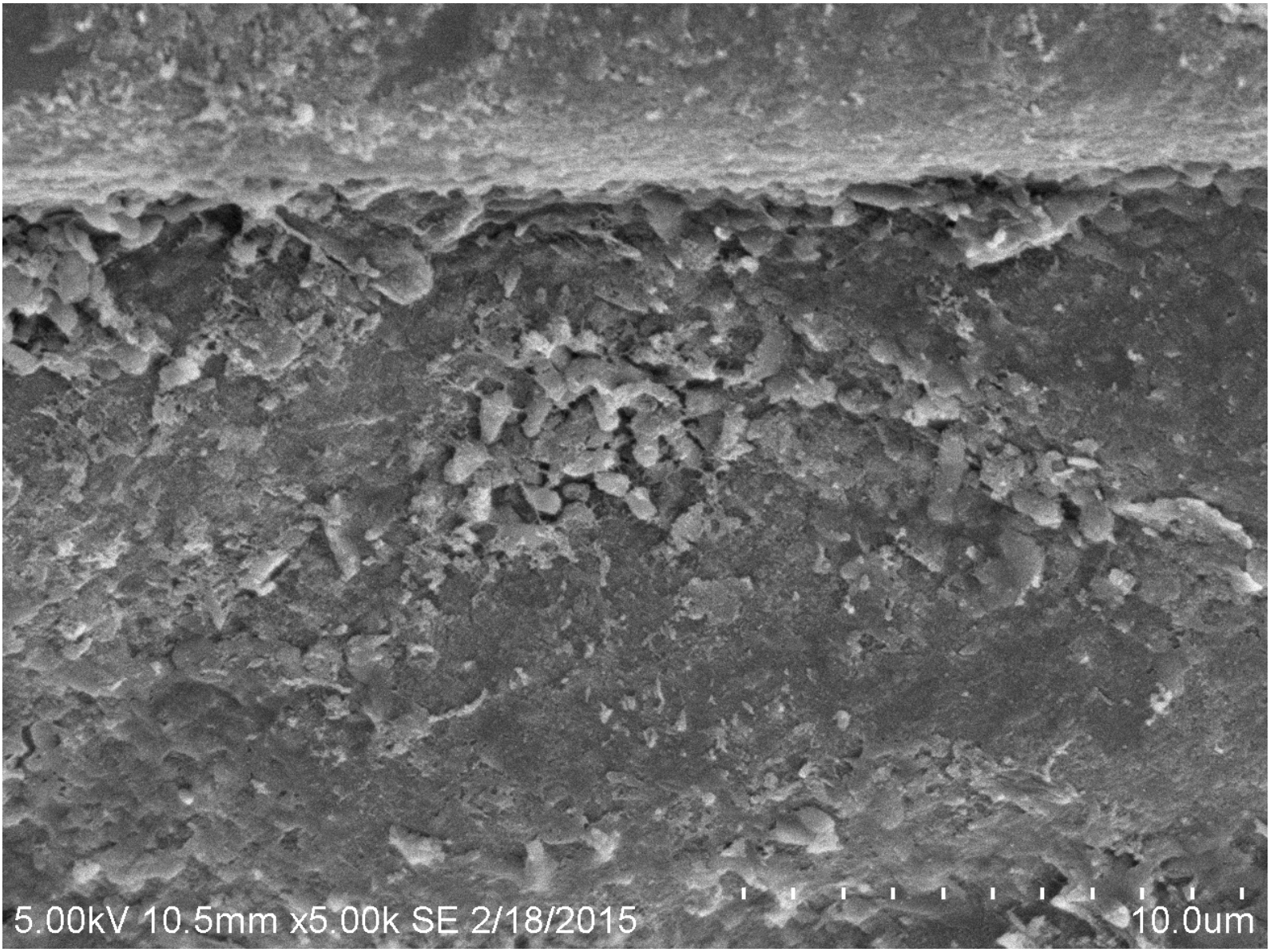


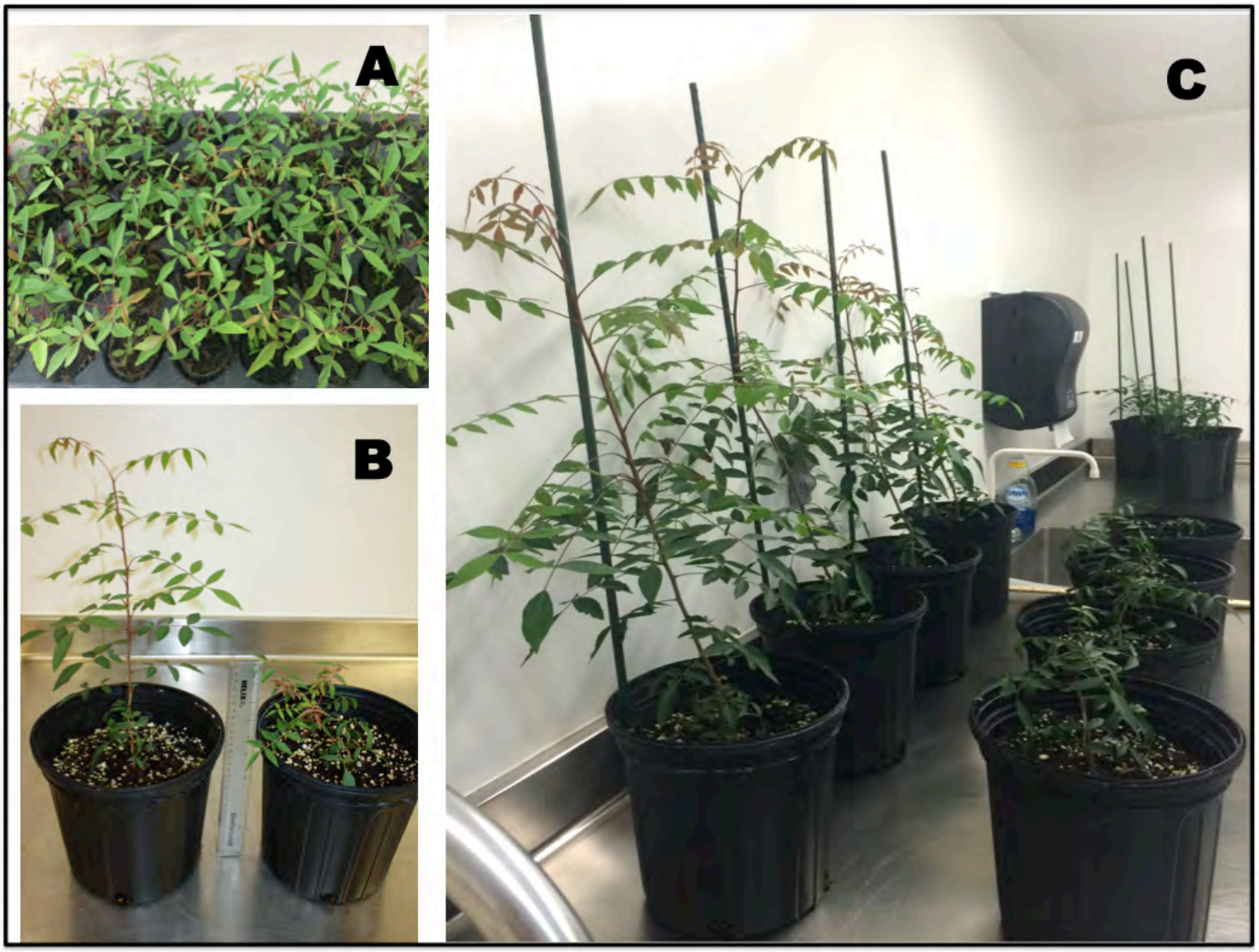
Genetic Verification of Bacteria on symptomatic plants from Koch's postulates



Bacteria still alive on surface 104 days post inoculation




5.00kV 10.5mm x5.00k SE 2/18/2015
10.0um



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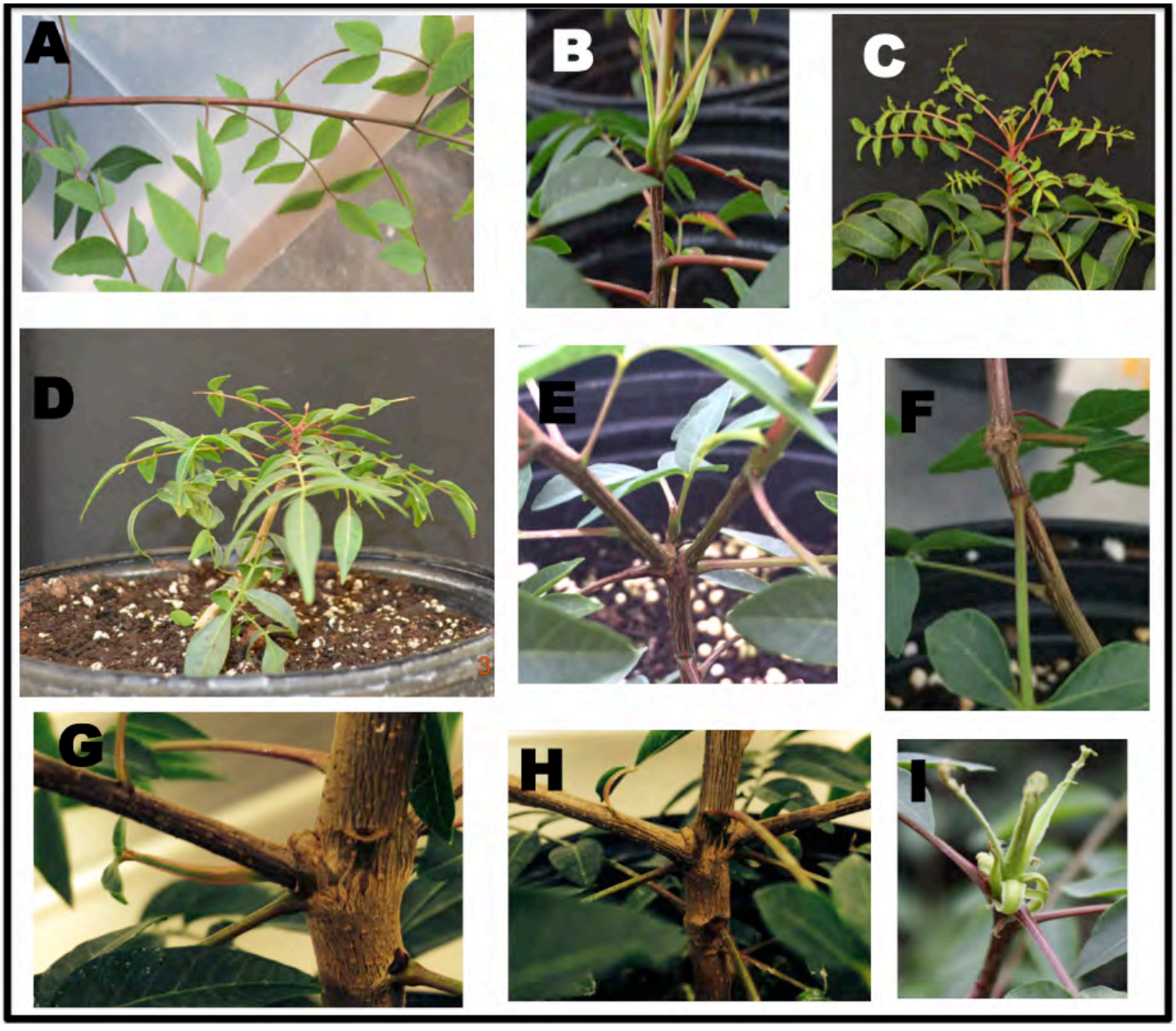
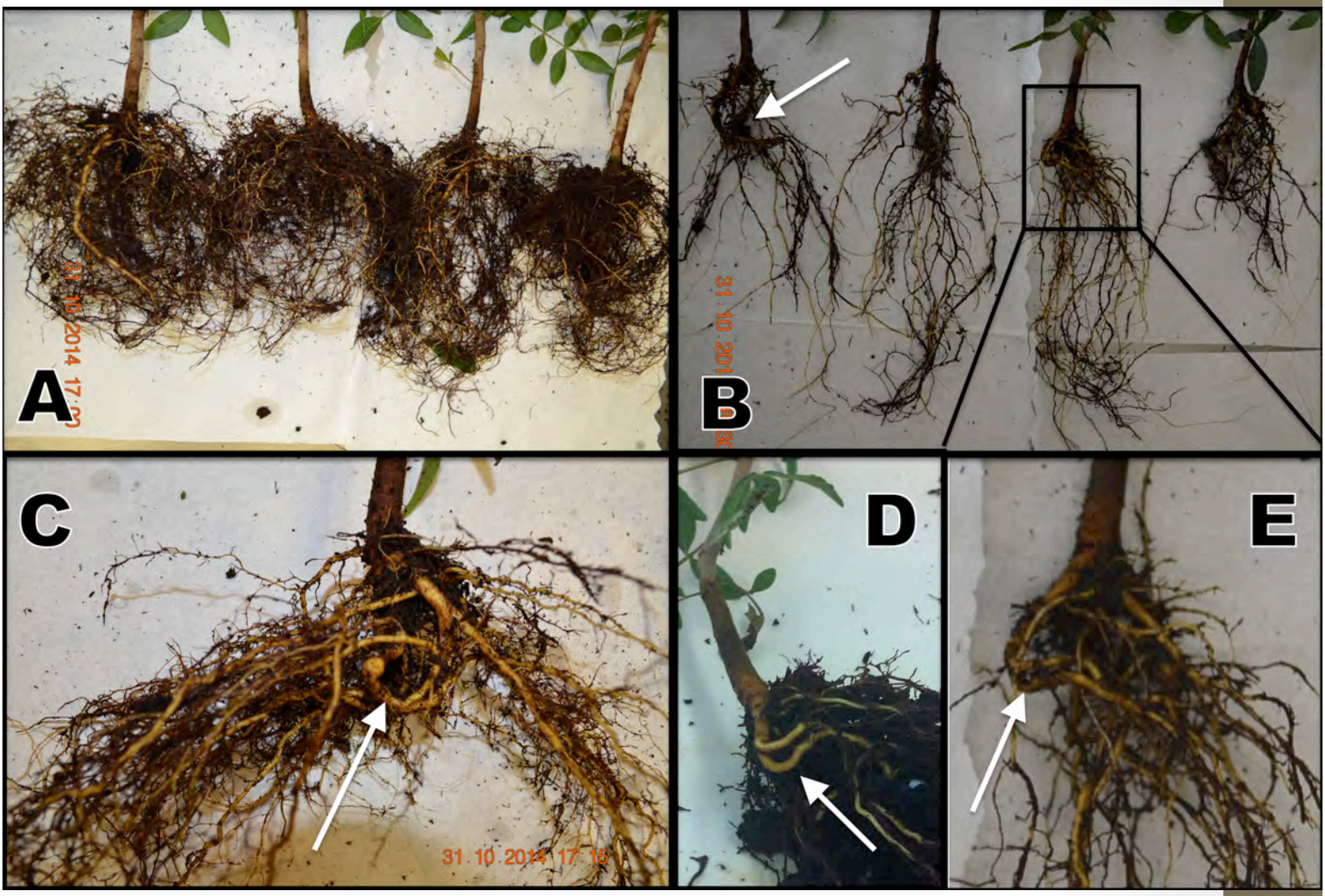
Leaf phenotype


A
B
C
D
E
F
G
H
I



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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

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.

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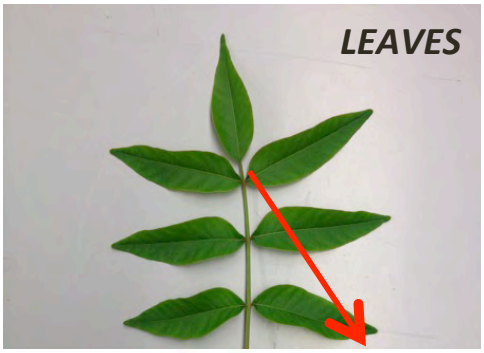

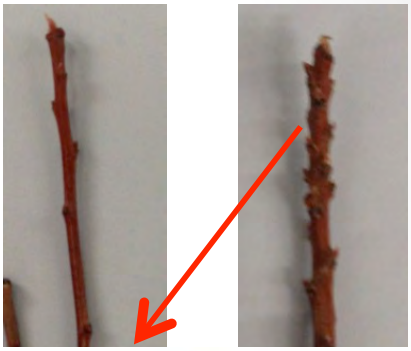

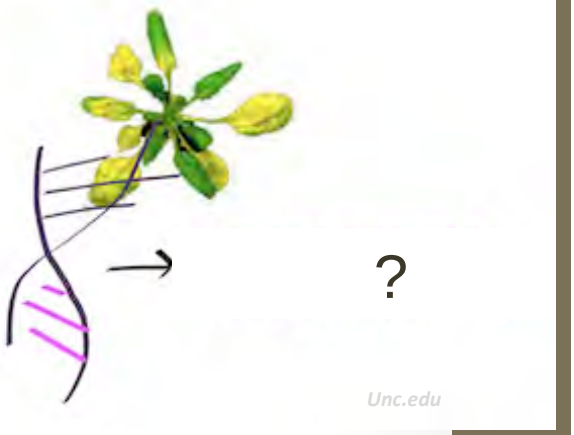

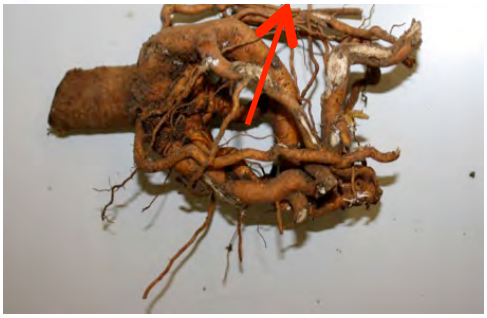

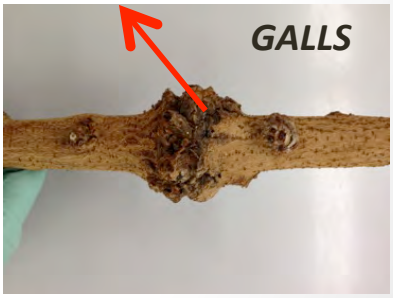

Total DNA can be isolated from tissues and screened for *Rhodococcus*

- 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

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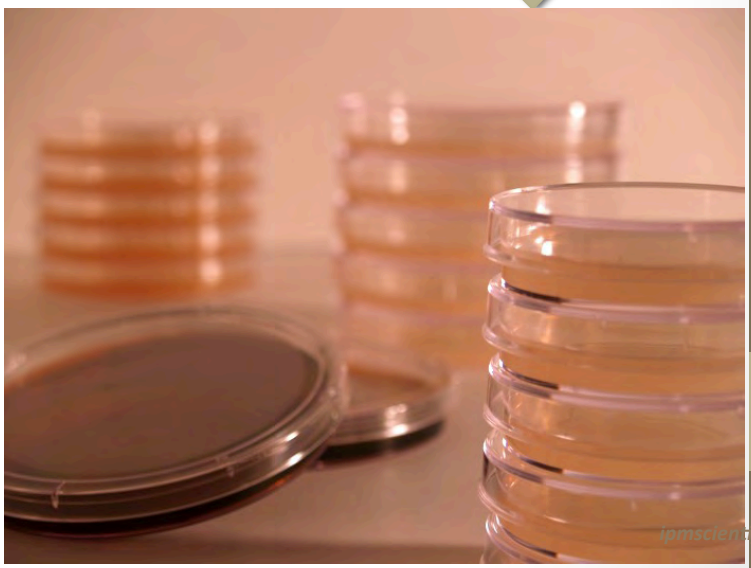
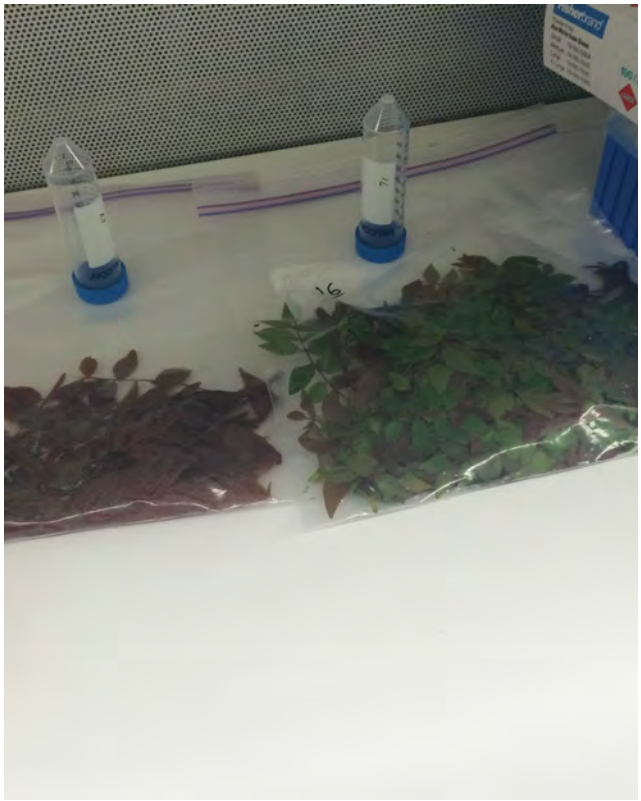
Pathogenicity Tests

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



INCUBATION FOR 7-10 DAYS

TISSUE PRINT



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.

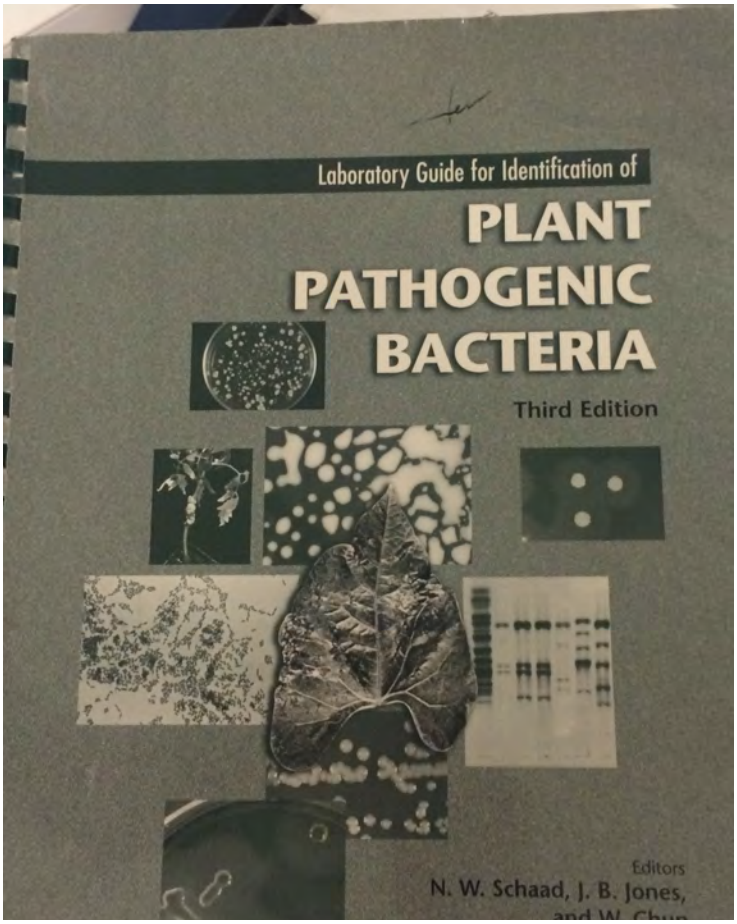
Tissue used for grinding

- 5 ml of sDH_2O 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



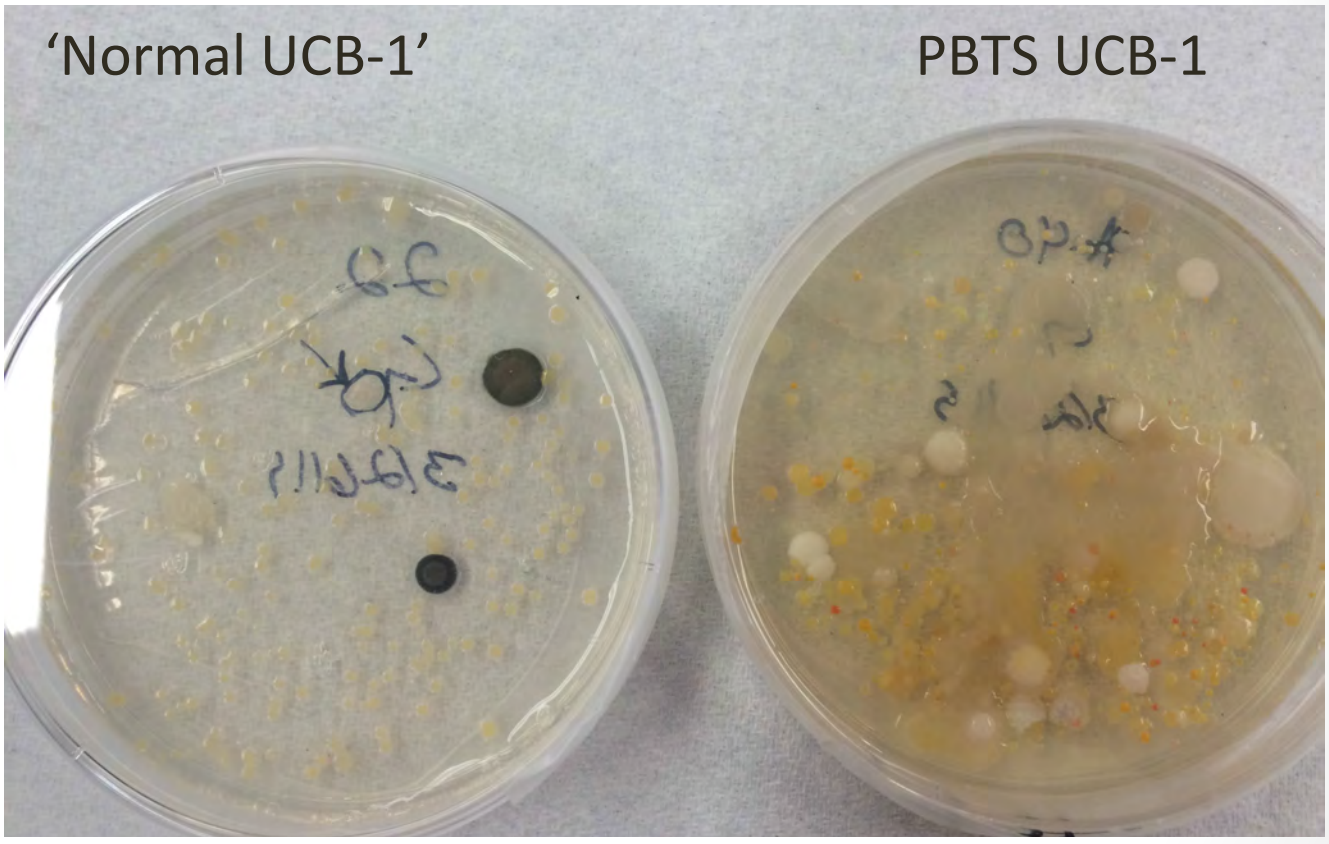
D2 Media and MD2
Kado and Heskett 1970

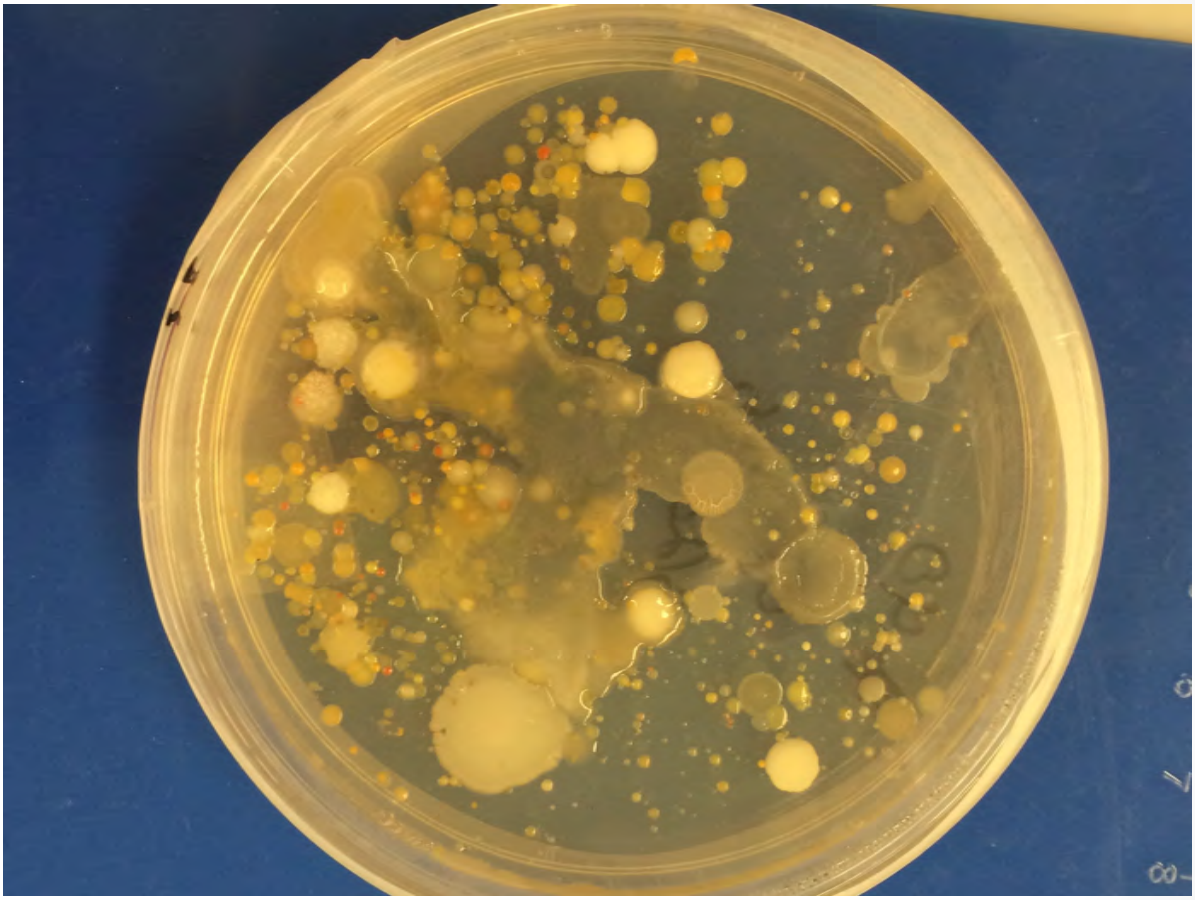
	per L	mD2
Glucose	10.0 g	
Casein hydrolysate	4.0 g	
Yeast extract	2.0 g	
NH_4Cl	1.0 g	
$MgSO_4 \cdot 7H_2O$	0.3 g	
LiCl	5.0 g	
Tris [tris (hydroxymethyl) amino methane]	1.2 g	
Agar	15.0 g	

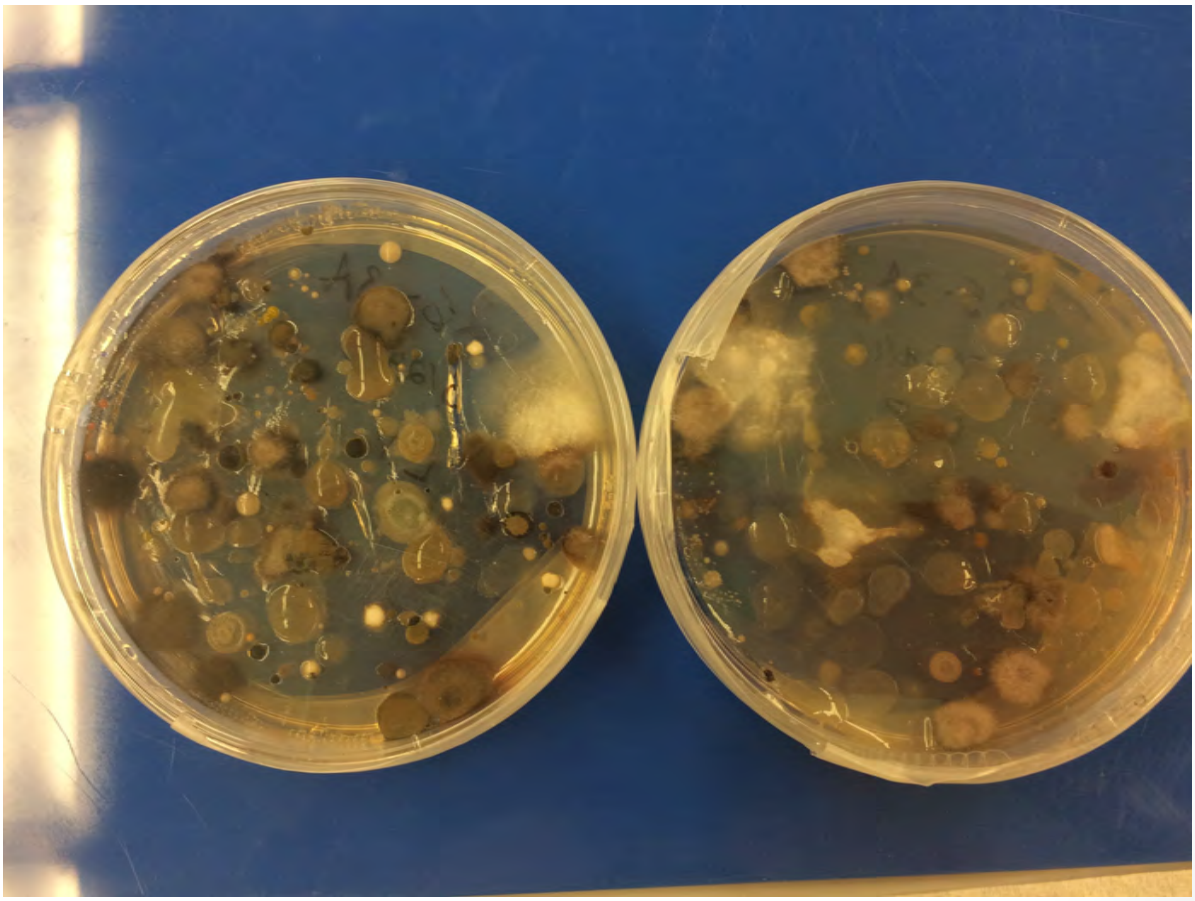
Adjust to 7.8 with HCl and autoclave. The medium is cooled to about 50° C and polymyxin sulfate and sodium azide are added.

Polymyxin B sulfate*	0.4 mg	40mg
Sodium azide	2.0 mg	0.5mg

Plates with Colonies: Internal







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

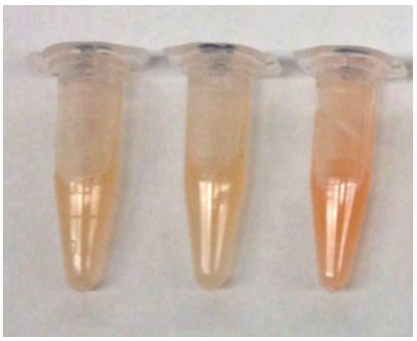
DNA Extraction

Bacterial Isolation

PCR & Sequencing

Microscopy

Pathogenicity Tests



PLANT DNA
BACTERIAL DNA

16S PCR & Sequencing
VicA PCR & Sequencing
Plasmid PCR & Sequencing

Genomic DNA Isolation

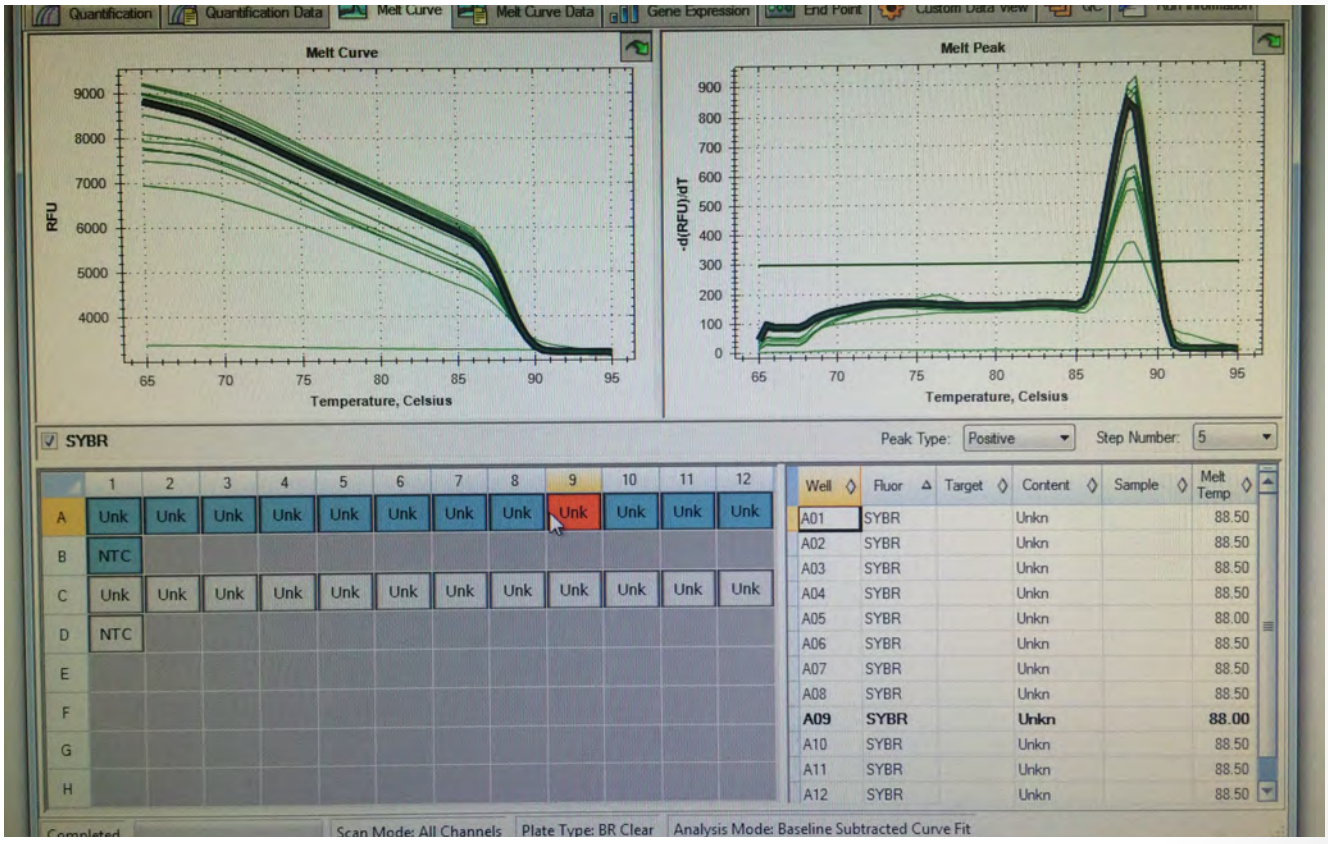
DNA

Amplification Conditions

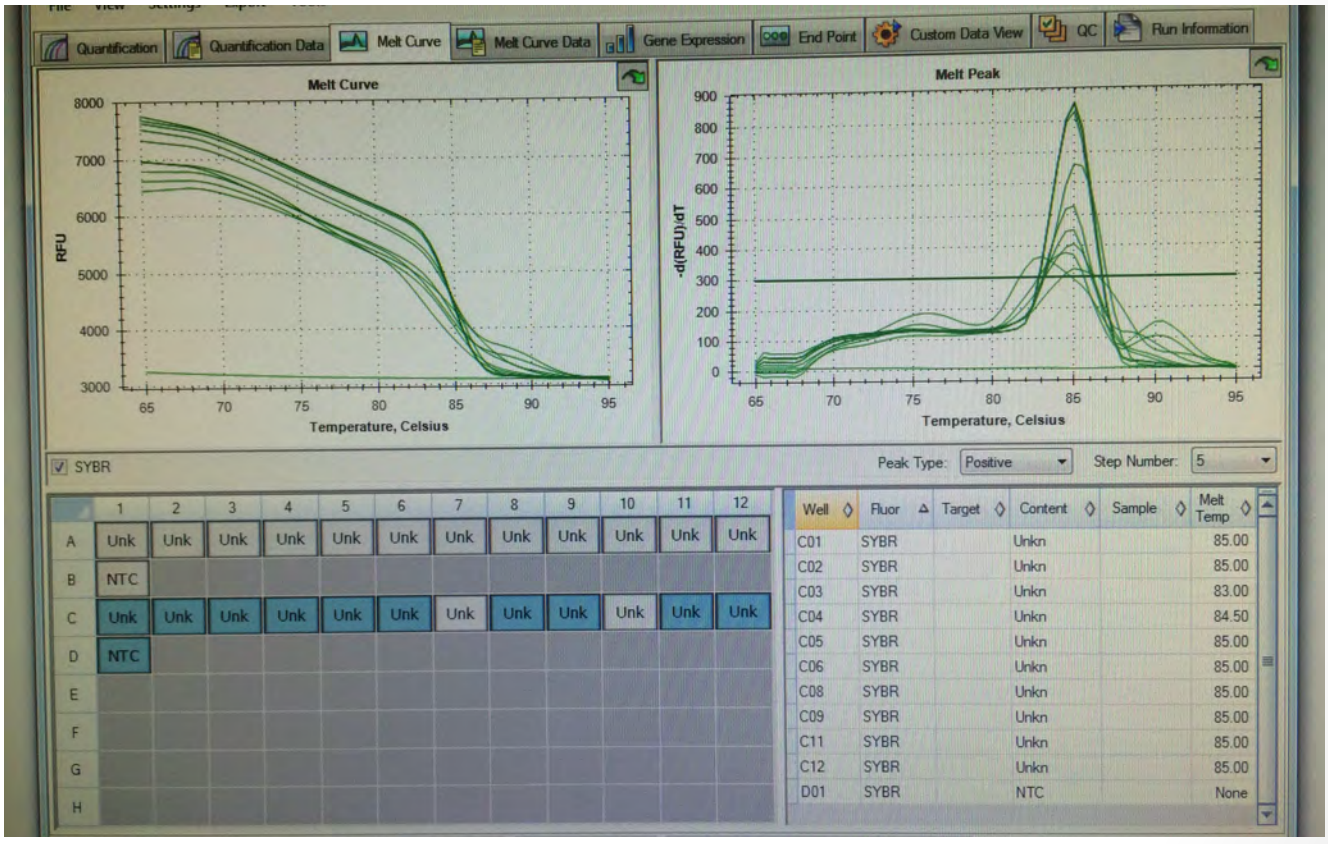
- 1. 95 C for 3 min.
- 2. 95 C for 15 sec
- 3. 64 C for 30 sec (+plate read)
- 4. Go To step 2 39X
- Melt Curve from 65 - 95 C with 0.5 degree increments

PRIMER NAME	PRIMER SEQUENCE	Reference
vicA 5' forward	5'TCTGGATCTCGAAGTGCAAACCGT3'	Nikolaeva et al. 2012
vicA 3' reverse	5'AGCGTACAAGGCCTTCCTGAAAGA3'	Nikolaeva et al. 2012
Rf *fasD* internal 5' forward	5'GCATTGAGTCACTCGGCTCC3'	Randall Lab (Stamler et al. 2015)
Rf *fasD* internal 3' reverse	5'CGCACTGCTGGGCAAAGTAG3'	Randall Lab (Stamler et al. 2015)
Rf *attA* internal 5' forward	5'CGTGTAGCCGGGTAGAACTG3'	Randall Lab (Stamler et al. 2015)
Rf *attA* internal 3' reverse	5'CTGCTCGACATGGAGAGCAG3'	Randall Lab (Stamler et al. 2015)
Rf -229F	5' ATGGCACAGACGCAAGCAA3'	Nikolaeva et al. 2012
Rf-408R	5' GATACGGTCGGCAACAACAATC3'	Nikolaeva et al. 2012
Rf-366P	/6FAM/AATTGGAAAGAGCGCCGAAGCGAGCAAGTT/BHQ_1/	Nikolaeva et al. 2012

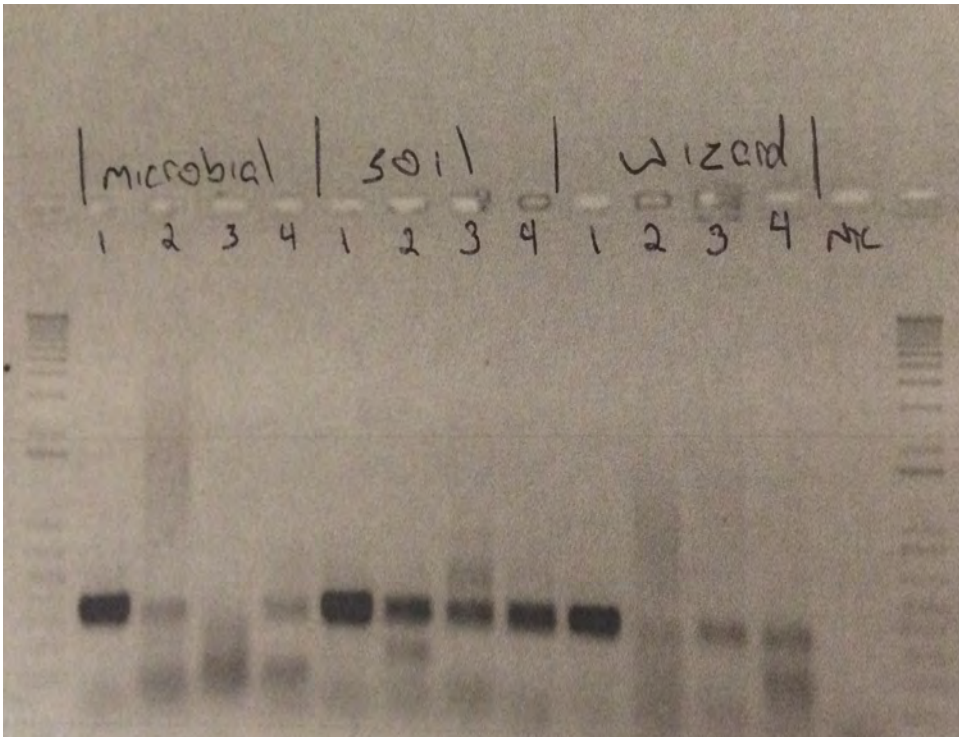
vicA amplification Tm:88.5



fasD internal Tm 85.0



fasD amplification with kits



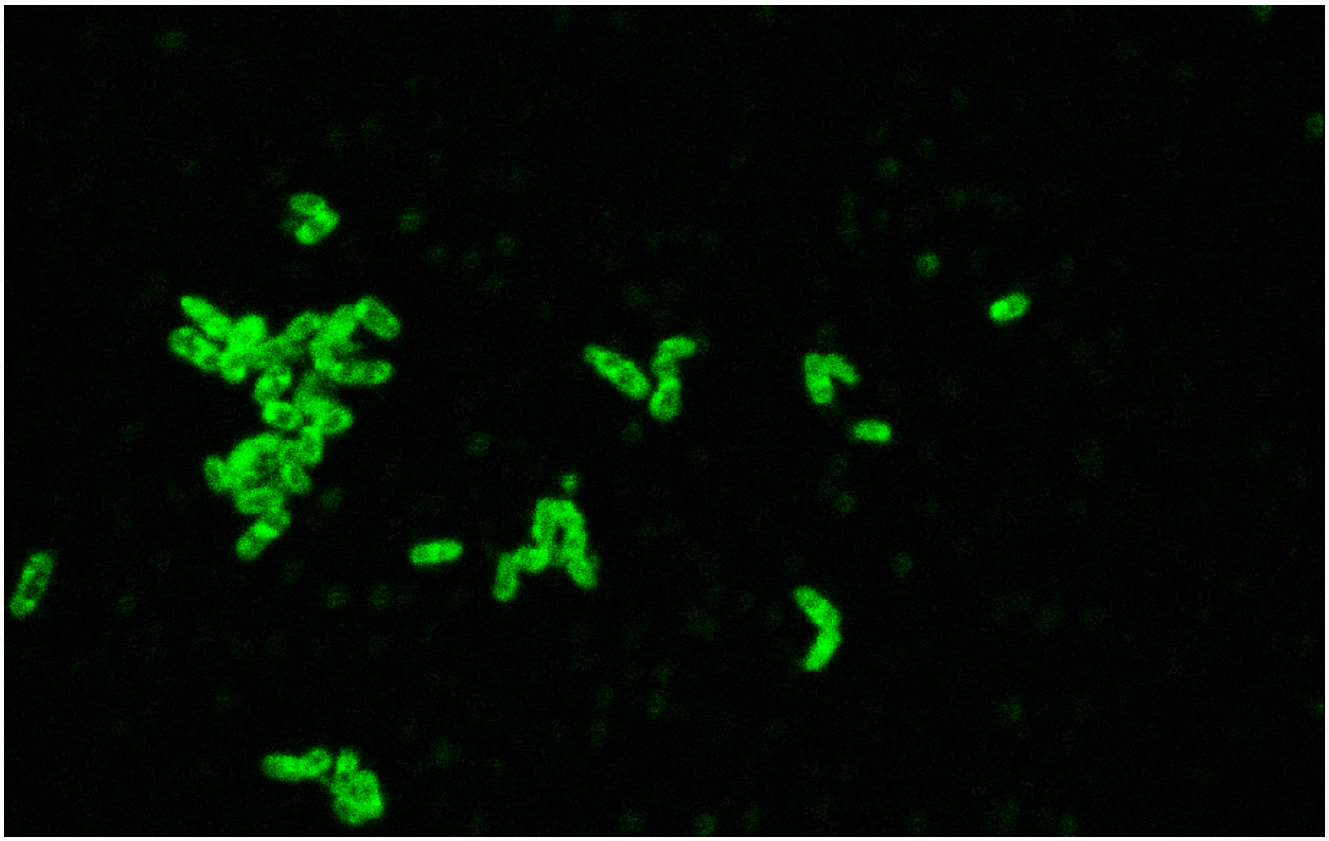
Sequence Analysis of Amplicons

- 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

- Pistachio Growers in California and Arizona; **Thank you for allowing us to tour your orchards and take samples for testing.**

- New Mexico State University Experimental Station

- California Pistachio Research Board

Collaborators on Project

- **Jennifer J. Randall,** New Mexico State University
- **Elizabeth Fichtner**, UCCE Tulare County
- **Florent Trouillas,** University of California, Davis
- **Craig Kallsen,** UCCE Kern County
- **Isolde Francis,** California State University Bakersfield
- **Melodie Putnam**, Oregon State University
- **Richard Heerema,** New Mexico State University

RANDALL LABORATORY