

California Pistachio Research Board
Report

Investigating the association of *Rhodococcus* spp. with Pistachio Bushy Top Syndrome
Funded: July 1, 2014-June 30, 2015

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SUMMARY

In the last three years, a significant percentage of clonal UCB-1 rootstock planted in orchards both in California and in Arizona are exhibiting symptoms not commonly observed with this rootstock. These UCB-1 rootstock trees are stunted, have closely spaced internodes, swollen lateral buds, a light green color, and exhibit “bushy” growth that resembles witches broom. The roots of these plants are twisted and have minimal lateral branching. The percentage of these abnormal rootstocks within affected fields in Arizona and California has varied anywhere from 10% to 90%. Symptomatic rootstocks exhibited only 30% budding success in the field. Many of the trees that were successfully t-budded with *P. vera* developed bark cracking around the bud-union. Many of these trees also have large galls around the stem arising from swollen lateral buds. The cause of these symptoms is unknown and we have termed suite of symptoms in these affected trees ‘Pistachio Bushy Top Syndrome’ (PBTS).

We identified an association of *Rhodococcus* spp. with PBTS clonally propagated UCB-1 rootstock through genetic screening and selective culture techniques. *R. fascians* is a known plant pathogen with a broad host range. Symptoms from this bacterium are often

confused with those caused by phytoplasmas, viruses, *Agrobacterium tumefaciens*, and latent hormone effects in nursery settings (Putnam and Miller, 2007). *R. fascians* can be present on plants as an asymptomatic epiphyte or may gain entry inside plant tissues where it modulates phytohormone activity, resulting in stunted growth, shortened internodes, bushy or bunched top appearance, leafy galls, and modified root development (Cornelis et al., 2001; Stes et al., 2011). While the genetic organization of *R. fascians* genotypes remains unclear, the presence of virulence factors is essential for phytopathogenicity (Crespi et al., 1992). Two *Rhodococcus* isolates were recovered from trees exhibiting PBTS. One isolate is a yellow-orange, pleomorphic, gram-positive culture that is 99% and 97% identical to *R. fascians* D188 at the 16S rDNA and VicA malate synthase chromosomal loci, respectively. The second isolate is a dark orange, pleomorphic and gram-positive culture that is 96% identical to *R. fascians* at the VicA malate synthase locus but 99% identical to *R. corynebacterioides* at the 16S rDNA locus. The purpose of this study is to test whether the *Rhodococcus* spp. isolates from UCB-1 rootstock are responsible for any/all of the observed PBTS symptoms. Pathogenicity testing was performed on *Pisum sativum* (garden pea), an indicator plant that is routinely used to determine pathogenicity of *R. fascians*. The pea assays were inconsistent and additional pathogenicity studies are in progress. Three trials were conducted to test whether the *Rhodococcus* isolates are pathogenic on small clonally propagated UCB-1 plants. Both isolates consistently caused moderate stunting after 80 days post-inoculation, shortened internodes and leaf deformation during pathogenicity tests on clonal UCB-1 rootstock. UCB-1 inoculated with both isolates simultaneously were severely stunted at less than 40 days post-inoculation. These results suggest that the two

Rhodococcus isolates have a synergistic interaction that causes severe symptom development and increased disease progression when both isolates are co-inoculated. Both isolates were recovered and identified from inoculated plants, completing Koch's postulates for characterizing a new plant disease.

PROCEDURES

Pathogenicity of *Rhodococcus* spp. isolates on *Pisum sativum* and *Nicotiana*

***benthamiana*.** Garden pea (*P. sativum*) has been used for decades as an indicator host for virulent *R. fascians* since it shows symptoms within two weeks of inoculation. Pea seeds were inoculated according to the protocol described in Serdani, et al, 2013. Briefly, 'Oregon Sugar Pod II' pea seeds were surface disinfected by prolonged washing followed by 15 min in 0.6% NaOCl, rinsed with sterile DI water, germinated, and soaked in a saline bacterial suspension of 10^8 CFU/ml for 2 h. Individual seeds were then placed into tubes containing Hoagland's solution in a matrix of 5% agar and grown under lights in a growth chamber for 2 weeks, after which shoot length and number of shoots per seed were measured. This procedure was performed twice. *Nicotiana benthamiana* seedlings were also used in a root growth assay with *Rhodococcus* isolates 1 and 2 separately and together. Germinated seeds were inoculated with a drop of inoculum, then placed onto water agar in square plastic dishes, set upright, and root growth was measured after one week. Results were compared with known pathogen D188 and an avirulent isolate.

Koch's postulates with UCB-1 pistachio rootstocks. Clonal UCB-1 trees were purchased from a nursery in plugs. Three trials were performed with 16 plants in each treatment. In the first trial, *Rhodococcus* isolate 2 grown on MD2 plates was suspended

in a 0.01M phosphate buffer pH 7.0 at an OD of 0.7 Small UCB-1 pistachio plants were sprayed with 20 ml of the *Rhodococcus* isolate 2 suspension per tree the first day and each tree was subsequently sprayed for two additional days with 10 ml of the *Rhodococcus* isolate 2 suspension. The ‘mock’ inoculated controls were treated by spraying each UCB-1 tree with 20 ml of 0.01M phosphate buffer pH 7.0 the first day and subsequently sprayed with 10 ml of 0.01M phosphate buffer for two days. All trees were maintained in a humidity chamber under fluorescent lights for two weeks. Rootstocks were transplanted into 8-inch pots two weeks after inoculation and plants were maintained in a quarantine greenhouse for the duration of the experiment. The second and third trials included four treatments: (1) Mock-inoculated control, (2) *Rhodococcus* isolate 1, (3) *Rhodococcus* isolate 2, (4) *Rhodococcus* isolates 1 and 2. Mock-inoculated plants were each sprayed with 10ml of inoculation buffer (10mM MgCl₂ and 10mM MES). For each of the single inoculation treatments *Rhodococcus isolate 1* or *Rhodococcus isolate 2* was scraped from D2 media plates, suspended in inoculation buffer to an OD₆₀₀ of 0.7 and 10 ml of bacterial suspension was sprayed on each clonal UCB-1 tree. Inoculation with both *Rhodococcus* isolates was accomplished by mixing an equal volume of the suspended *Rhodococcus isolate 1* and *Rhodococcus isolate 2* at a final OD₆₀₀ of 0.7 and each clonal UCB-1 tree was sprayed with 10 mL of the combined bacterial suspension. Trees from all treatments remained in humidity chambers under fluorescent lights for two weeks prior to being transplanted into 8-inch pots and moved to a quarantine greenhouse. Plants were monitored and measurements and photos were taken periodically. Statistical significance of $P < 0.05$ was determined using Tukey-Kramer test with Satterthwaite adjustment (SAS 9.2; SAS Institute Inc, Cary, NC) mixed

linear model procedure.

Bacterial Culturing. Fresh leaf tissue was printed directly on D2 (Kado and Heskett, 1970) or mD2 media (D2 media with 40 mg/L of Polymyxin B sulfate and 0.4mg/L of Sodium Azide). Plant leaves were also surface sterilized in 0.6% sodium hypochlorite for 20 seconds followed by 1 minute in 70% Ethanol and three rinses with deionized water. Following surface sterilization the tissue was ground and diluted in water or D2 broth prior to plating. Plates were incubated at 27°C for 4-10 days. Bacterial growth was then re-streaked for single colonies and then further analyzed.

Bacterial Genomic DNA Isolation. Bacterial genomic DNA was isolated from orange and yellow-orange colonies that were gram positive and pleomorphic in structure using UltraClean Microbial DNA Isolation Kit (MO Bio Laboratories, Carlsbad, California). The isolated DNA was stored at -20°C and DNA was visualized on a 1% agarose gel stained with ethidium bromide and visualized on a GelDoc-It imager (UVP Upland, CA).

Molecular Tests performed with Bacterial Genomic DNA. Bacterial genomic DNA was screened for the chromosomal virulence locus *vic A* (Vereecke et al., 2002) using published primers and cycle times (Nikolaeva et al., 2012). Further analysis was performed with PCR primers previously developed for *R. fascians* virulence genes including *fas-1*: RF 229/RF 408 (Nikolaeva et al., 2012); *FasF/FasR* and *p450F/R* (Serdani et al. 2013). Sequencing reactions were performed using Big Dye Terminator v3.1 and run on the ABI 3100 Genetic Analyzer (Life Technologies Carlsbad, CA).

Nearest-neighbor phylogenetic trees were generated with the HKY algorithm (Geneious v6.0.6 Auckland, New Zealand).

Replant Studies. Two studies are currently underway to determine risk of disease transmission from rhizosphere soil of replant holes to healthy plants. Replant soils were collected from a Tulare County and a Kern County orchard within two weeks of removal of bushy-top symptomatic plants. At each site, soils were sampled from 20 individual holes to the depth of a shovel, thus targeting collection of soil within the rhizosphere of PBTS plants. Soils from each site were bulked and homogenized in a cement mixture. A portion of each batch of homogenized soil was steamed for one hour on two consecutive days to kill potential infestation with *Rhodococcus* spp.

UCB-1 clonally propagated plantlets were transplanted into replant soils or steamed replant soils. Additionally, a positive control treatment was established by dipping roots of healthy plants into a bacterial suspension containing each of the two isolates utilized for completion of Koch's postulates by the Randall laboratory. Plantlets with inoculated roots were then transplanted into naturally infested field soil. Twenty replicate plants are included in each of the three treatments. Replant trial 1 (Tulare County soil) was initiated on November 27, 2014; replant trial 2 (Kern County soil) was initiated on December 15, 2014. Plants will be monitored for symptom development over time.

Pruning-transmission Study. An ongoing study is designed to address the potential for transmission of *Rhodococcus* spp. to healthy plants via infested pruning shears.

Symptomatic PBTS tissue was collected from a Tulare County orchard to serve as a

natural source of inoculum. Pruners were purposely sliced into symptomatic tissue prior to cutting two nodes down from the apex of healthy UCB-1 plantlets. Similarly, healthy plantlets were also clipped with surface disinfested pruners (dipped in 95% ethanol and flamed) to establish a negative control treatment. Last, to establish a positive control treatment, pruners were dipped in a bacterial suspension containing the two isolates utilized in the Randall laboratory for completion of Koch's postulates. Twenty replicate plants were established of each treatment, and the experiment was initiated on November 27, 2014. Plants will be monitored for symptom development.

RESULTS

Pathogenicity Testing. A number of pathogenicity assays on pea and tobacco seedlings were performed at New Mexico State University and Oregon State University using the *Rhodococcus* isolates from symptomatic PBTS trees. The pea assay results from NMSU were not statistically significant in regards to an affect of the *Rhodococcus* isolates on the pea seedlings. The pea results from OSU are preliminary and incomplete. The first pea assay performed with *Rhodococcus* isolates from UCB-1 showed some effects on peas, including stunting and increased number of stalks (data not shown). The results from the second assay did not show a strong effect by the bacteria. The *N. benthamiana* assay showed reduced root growth on some plants inoculated with *Rhodococcus* sp. 1 (data not shown). This assay was performed once, and additional assays with both pea and *N. benthamiana* are ongoing at OSU.

Koch's Postulates. *Rhodococcus* isolates from PBTS trees were tested on small UCB-1 clonal rootstock trees. Three trials of were performed. Four treatments were applied to

these trees. 1. Control trees (mock-inoculated with buffer); 2. Trees inoculated with *Rhodococcus* isolate 1; 3. Trees inoculated with *Rhodococcus* isolate 2; and 4. Trees inoculated with an equal volume of *Rhodococcus* isolate 1 and *Rhodococcus* isolate 2.

The control trees grew at a consistent rate throughout the experiment and maintained an approximately constant internode length. The control trees were apically dominant and had normal root morphology at 100 days post inoculation. Trees that were inoculated with *Rhodococcus* isolate 1 had similar growth rates for the first 40 days post-inoculation (data not shown). After 80 days post-inoculation the trees inoculated with *Rhodococcus* isolate 1 were on average 10 cm (3.9 inches) shorter than the control trees and this size difference was significant at 100 days post-inoculation when the trees were on average 15 cm (6 inches) shorter (Figure 1). Similar results were also observed with trees inoculated with *Rhodococcus* isolate 2 in regards to growth rates. However, trees inoculated with *Rhodococcus* isolate 2 exhibited sylleptic branching from lateral buds, small leaves that are a light green color, and had swollen lateral buds compared to control trees at 100 days post-inoculation (Figure 2). Trees that were inoculated with equal titers of both *Rhodococcus* isolate 1 and *Rhodococcus* isolate 2 exhibited severe stunting by day 40 post-inoculation (data not shown). At 100 days post-inoculation with the two *Rhodococcus* isolates the trees had an average height difference of 37 cm (14.5 inches) compared to the mock-inoculated trees (Figure 1 and Figure 2). Root systems from the *Rhodococcus* inoculated plants were less developed and displayed abnormal twisting of structural roots compared to control trees. To complete Koch's postulates, both *Rhodococcus* isolates were re-isolated from inoculated plants and their identity confirmed

through sequencing analysis.

Disease Transmission And Replant Studies. Initiated studies are in progress.

Characterization Of *Rhodococcus* Isolates. Complete genome sequencing accomplished. The complete genomes for both isolates are currently being analyzed and compared to known *R. fascians* isolates (Creason et al., 2014).

CONCLUSIONS AND PRACTICAL APPLICATIONS.

Inoculation of UCB-1 trees with *Rhodococcus* isolates resulted in symptoms consistent with PBTS. These symptoms include severe stunting, shortened internodes, loss of apical dominance, small light green colored leaves, swollen lateral buds, and root abnormalities. Time and additional experiments are required to determine if the formation of stem galls and cracked bud-unions will develop on these inoculated trees.

Growers with PBTS trees need to sanitize their tools between trees when pruning or grafting to avoid spreading the bacteria to nearby unaffected trees. Although it is unclear at the moment if *Rhodococcus* can be mechanically transmitted in a field setting, common sense dictates extra caution while the details of this new disease are elucidated. Current publications recommend the use of disinfectants with quaternary ammonium compounds for sanitizing pruning tools or hard surfaces in greenhouse settings (Putnam and Miller, 2007).

For growers who have removed PBTS trees, it is currently unknown if additional soil treatment or fumigation is required to prevent infection of replants. Currently experiments are in progress to monitor *Rhodococcus* persistence in soil and trees re-planted in PBTS orchards are being screened for the presence of *Rhodococcus*.

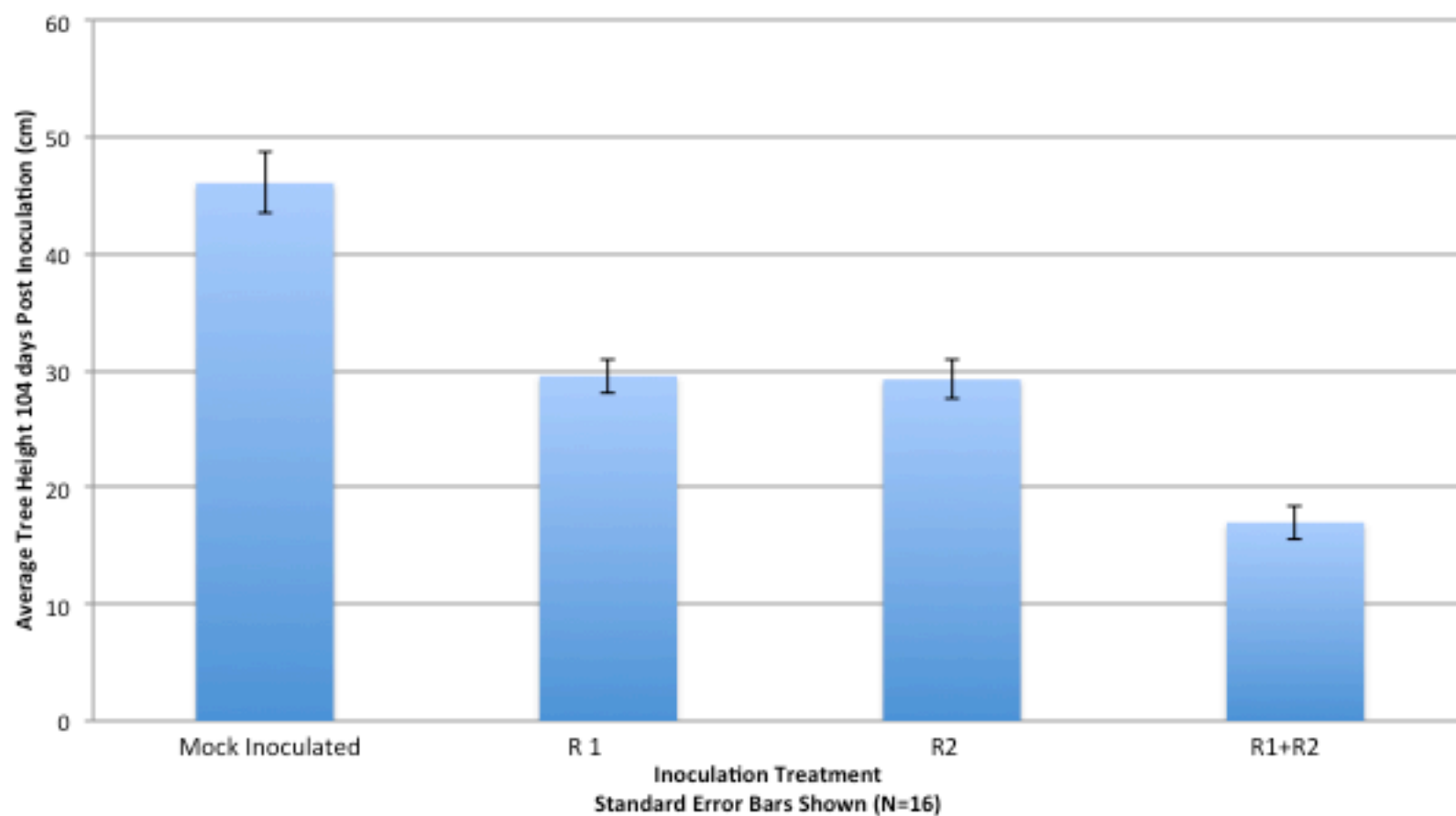
Acknowledgements. The authors would like to thank several Pistachio growers for allowing us to tour their affected orchards and providing samples of their trees. The authors would also like to acknowledge the technical help of Jordan Martin. The authors would like to acknowledge the New Mexico State University Experimental Station and the California Pistachio Research Board for funding this study.

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***Rhodococcus* isolates inhibit 'UCB-1' (*Pistacia* sp.) Root Stock Growth**



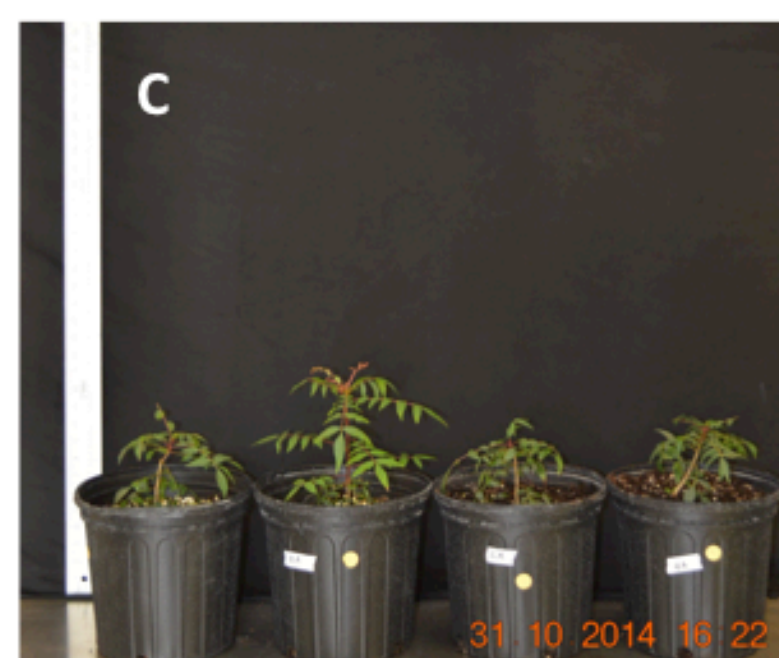
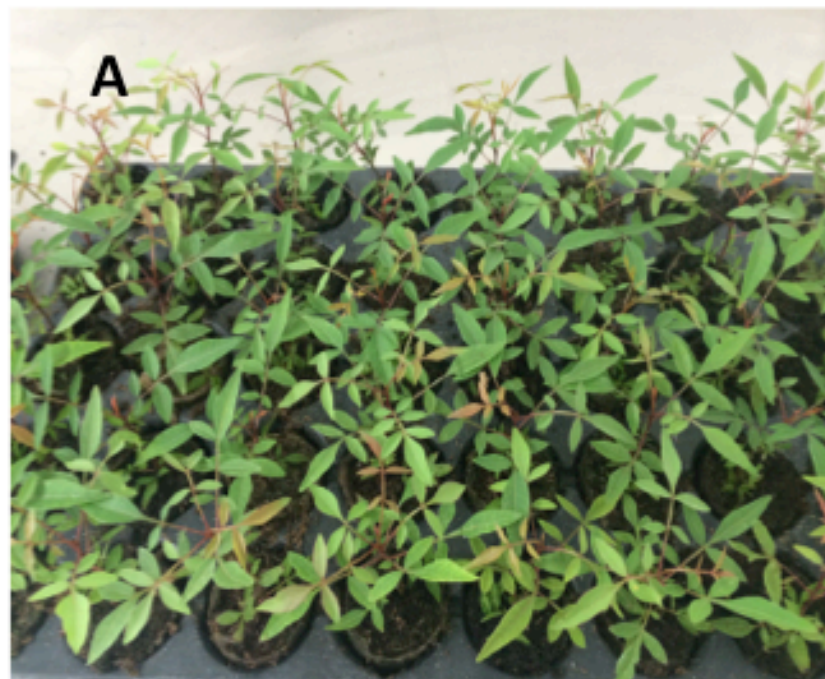


Figure Legends for California Pistachio Research Board Report

Figure 1. *Rhodococcus* isolates cause stunting on clonally propagated UCB-1 trees. Three trials of Koch's postulates were performed with clonal UCB-1 trees with the *Rhodococcus* isolate 1 (R1) and *Rhodococcus* isolate 2 (R2) isolates. Four treatments from a single trial are shown in this data. The control plants are UCB-1 trees that were mock-inoculated with buffer. Trees were inoculated with R1, R2, and with both R1+R2. The average height of each treatment is shown with standard error bars at 104 days post inoculation. Statistical significance between control and inoculated treatments at $p \leq 0.05$ was determined using the Tukey-Kramer test with Satterthwaite adjustment (SAS 9.2; SAS Institute Inc, Cary, NC) mixed linear model procedure.

Figure 2. Symptoms observed during Koch's Postulates with *Rhodococcus* spp. **A.** Healthy, uniform trees used for experiments. **B.** UCB-1 trees mock-inoculated with buffer at 104 days post inoculation. **C.** UCB-1 trees inoculated with both *Rhodococcus* isolate 1 (R1) and *Rhodococcus* isolate 2 (R2) at 104 days post inoculation. **D.** Control tree on the left with R1 + R2 inoculated tree on the right. **E.** Light green color and reduced leaf size in R2 inoculated UCB-1 tree. **F.** Swollen lateral nodes in R2 inoculated UCB-1 tree. **G.** Sylleptic branching and loss of apical dominance in R2 inoculated UCB-1 tree. **H.** Root system of R2 inoculated tree.