Root and shoot susceptibility of peach/almond rootstock and pomegranate seedlings to pistachio bushy top syndrome isolates of *Rhodococcus* spp.

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Abstract

Approximately 30,000 acres of UCB-1 clonal pistachio rootstock recently planted in California were affected with pistachio bushy top syndrome (PBTS) (Figure 1) caused by *Rhodococcus fascians* (*Rf*) and a bacterium closely related to *Rhodococcus corynebacteriodes* (*Rc*) (Stamler et al, 2015). Orchards with high disease incidence were removed and replanted to pistachio or almond (Figure 2); consequently, the potential susceptibility of almond to PBTS isolates is of concern at replant sites. Additionally, due to the proximity of pomegranate research blocks to greenhouse facilities housing PBTS trials, the susceptibility of pomegranate to PBTS isolates is also a concern. The goal of this work was to determine the susceptibility of clonal peach/almond rootstock and pomegranate seedlings to PBTS isolates.

Figure 1: Pistachio rootstock symptomatic of PBTS.

Figure 2: Orchards with high PBTS disease incidence were replanted to pistachio or almond.

Objectives

1. Develop an improved selective medium for inhibition of foliar epiphytic fungi and enhanced detection of *Rhodococcus* spp.
2. Determine the susceptibility of peach/almond rootstock and pomegranate to PBTS isolates.
3. Assess the influence of foliar vs. root inoculation on symptom development of peach/almond rootstock.
4. Determine whether root inoculation of peach/almond rootstock results in aboveground plant colonization.
5. Determine the potential for inoculation of the phylloplane and rhizoplane to result in endophytic colonization of respective tissues.

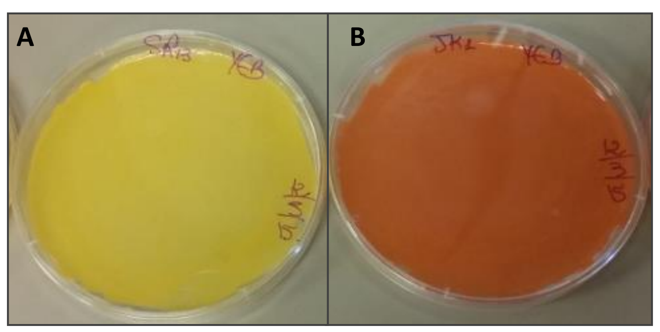
Materials and Methods

Objective 1: Amendment of D2 medium for enhanced selectivity of *Rhodococcus* spp. For suppression of epiphytic fungi, D2 medium (Kado and Heskett, 1970) was amended with either cycloheximide (2%) or pimaricin (0.4 mg/L), alone or in combination. Leaves from PBTS trees were depressed (abaxial and adaxial) on test media. Plates were incubated in the dark for 4 days and then colonies were enumerated. The experiment was run twice.

Objective 2: Susceptibility of peach/almond rootstock and pomegranate to PBTS isolates.

❖ **Plant material:** Clonal peach/almond rootstock ('Hansen 536') plantlets and open-pollinated pomegranate seedlings were used for pathogenicity trials.

❖ **Bacterial culturing:** PBTS isolates: *R. fascians* (PBTS2) (A) and *R. corynebacteriodes* (PBTS1) (B). Each isolate was grown on YEB plates (2 days, 28°C) and then suspended in 10 mM $MgCl_2$/10mM MES buffer (pH 7, OD (600nm) of 0.7). Plants were inoculated with a cocktail of both isolates. Two runs with 20 replicate plants were completed.

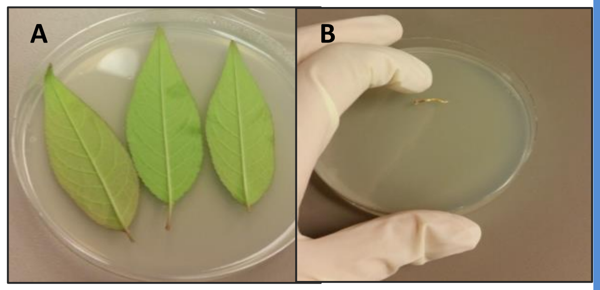


❖ **Inoculation treatments**:

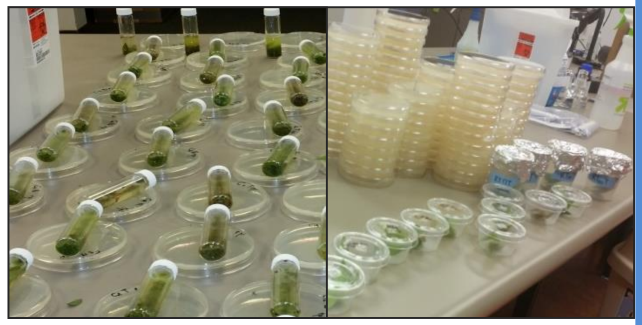
Peach/almond clonal plantlets	1) Uninoculated control (Buffer)	2) Root-inoculation	3) Foliar-inoculation
Pomegranate seedlings	1) Uninoculated control (buffer)	2) Inoculated seedlings (roots + shoots)	

❖ **Methods, objective 2 continued. Re-isolation of *Rhodococcus* spp. from peach/almond and pomegranate leaves and roots:**

1) Re-isolation of epiphytic *Rhodococcus* spp. from leaves **(A)** and roots **(B)** on amended D2 medium. Three leaves were sampled from two locations on the plant (apical and basal) for a total of 6 leaves/plant. Three 1cm root sections were excised from the root mass.



2) Detection of endophytic populations after surface-sterilization of leaves (0.5% NaOCl 20 sec, 1 min 70% EtOH, 3 times in sterile DIH_2O and roots (0.25% NaOCl 5 minutes, 3 times in sterile DIH_2O). Tissue was macerated in sterile water and the resulting suspension was spread on amended D2 medium.



Objective 3: Assess the influence of foliar vs. root inoculation on symptom development of peach/almond rootstock

Plant growth parameters measured in response to inoculation :

Peach/almond	**Plant height, #of nodes, # of leaves per node, # of nodes per cm, shoot and root biomass**
Pomegranate	Plant height, crown circumference, # of lateral shoots, total length of lateral shoots, # root curves, # of curves/g root biomass and root and shoot biomass

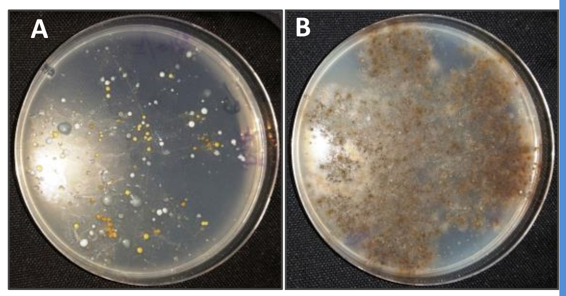
Objective 4: To determine the influence of root inoculation of peach/almond rootstock on aboveground plant colonization. Number of colony forming units (cfu) per leaf were recorded. Epiphytic and endophytic populations on lower leaves were compared with a two-tailed t-test.

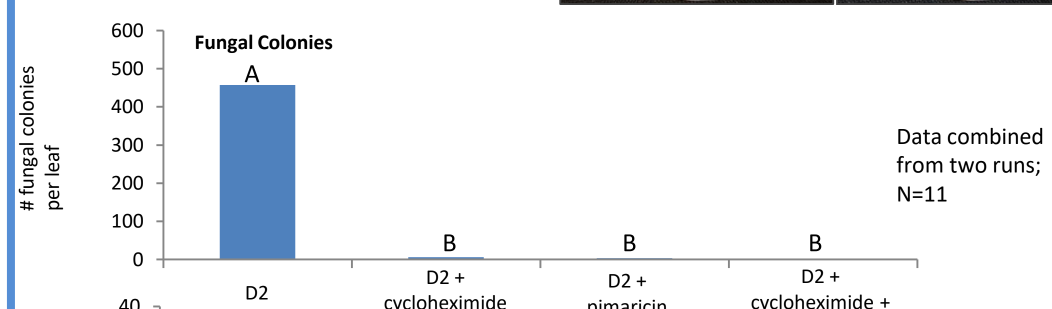
Objective 5: To determine the potential for inoculation of the phylloplane and rhizoplane to result in endophytic colonization of respective tissues, number of colony forming units (cfu) per leaf for the epiphytic and endophytic isolation were recorded.

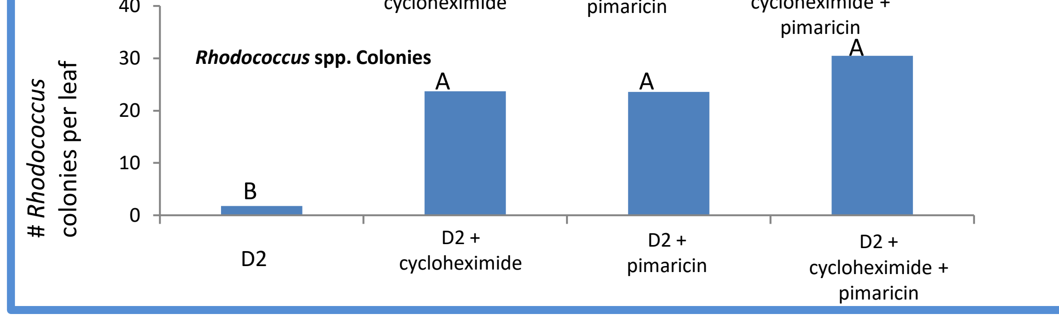
Asymptomatic almonds planted in a former PBTS orchard and adjacent to a row of remaining PBTS-symptomatic trees were sampled to determine the potential for pathogen transmission and infection of almond. Ten trees (non-adjacent to PBTS pistachio trees) were sampled for epi- and endophytic populations (2 leaves per tree).
Putative *Rhodococcus* isolates were subcultured. Bacterial genomic DNA was screened using chromosomal virulence -locus VicA primers (Nikoleva et al 2012).

Results

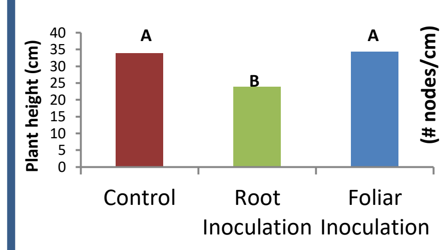
Objective1: Amendment of D2 medium for enhanced selectivity of *Rhodococcus* spp.
All test media containing cycloheximide and pimaricin, either alone or together, significantly suppressed growth of epiphytic fungi **(A)** (P≤0.001) and enhanced the detection of *Rhodococcus* spp. (P≤0.001) in comparison to unamended D2 medium **(B)**.



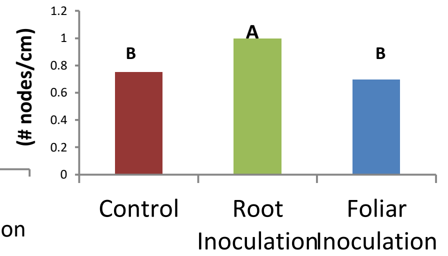




Objective 2: Susceptibility of peach/almond rootstock and pomegranate to PBTS isolates



Plants receiving root inoculations exhibited significant reduction in plant height P≤0.01)



Root-inoculated plants exhibited a significantly higher node density (P≤0.0001) then uninoculated or shoot-inoculated plants, resulting in a bushy morphology.

Objective2: Susceptibility of pomegranate seedlings to PBTS *Rhodococcus* spp.

Plant Metrics	Control	Inoculated
Plant height (cm)*	6.92	11.28
Girth (cm) *	172.58	174.04
∑ Lateral shoots/plant *	1.73	2. 94
∑ length lateral shoot/plant *	8.63	24.4
Shoot biomass (g)*	0.55	1.57
Root biomass (g)*	1.006	1.76
Epiphytic leaf population (cfu/leaf)*	35.07	66.08
Epiphytic root population (cfu/leaf)*	15.94	191.68
Endophytic leaf population (cfu/leaf)*	5.26	96.84
Endophytic root population (cfu/leaf)	17.36	71.05

* Denotes statistical difference t≤ 0.05

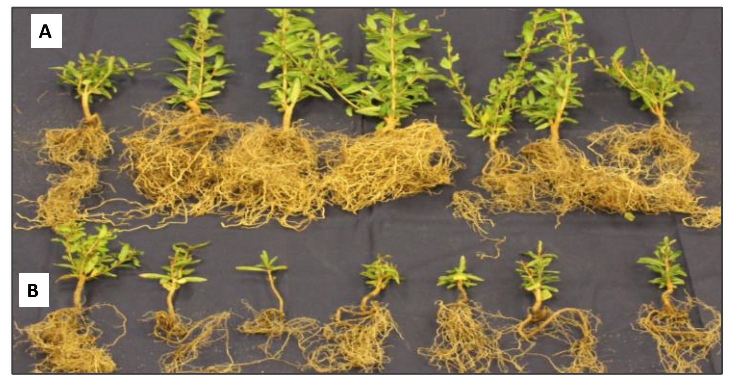


Figure 3: Inoculated (A) and uninoculated (B) pomegranate seedlings.

Objective3: Only Inoculation of peach/almond roots with PBTS isolates of *Rhodococcus* spp. had effect on plant growth and symptom development.

Objective 4: Root inoculation of peach/almond gave rise to endo- and epi-phytic colonization of lower and upper leaves. Eno- and epiphytic populations on foliage were statistically similar; however, epiphytic populations were higher on the lower leaves than on upper leaves.

Objective5: Phylloplane and rhizoplane inoculation resulted in endophytic colonization of respective tissues of pomegranate and peach/almond plants.

Discussion

The amended D2 medium effectively suppresses growth of epiphytic fungi, thus enhancing the detection of epiphytic *Rhodococcus* spp. colonization. The enhanced selectivity will allow for isolation of endemic *Rhodococcus* spp. in the landscape, providing opportunities for future ecological studies on these phytobacteria. The results of this study demonstrate that peach/almond rootstock and pomegranate are susceptible to infection by PBTS isolates of *Rhodococcus* spp.; however, the isolates have a plant growth promoting affect on pomegrante and a growth- suppressive affect on peach/almond rootstock.
Because pistachio and almond plants are often propagated in the same micropropopagation facilities, and young plants are particularly susceptible to *R. fascians* (Vereecke et al, 1999), sanitation is paramount to prevention of a PBTS-like outbreak on almond. Our results additionally suggest that almond blocks planted in former PBTS sites should be surveyed for presence of PBTS isolates of *Rhodococcus* spp.

Acknowledgements

Funding was provided by the Almond Board of California and the California Pistachio Research Board. We also thank grower cooperators for access to their orchards, Therese Kapaun (Lindcove Research and Extension Center), Craig Kallsen (UC Cooperative Extension in Kern County), and Mike Harvey (Mike Harvey Ag). Last we appreciate the collaborative assistance of Jennifer Randall (New Mexico State University), and Isolde Francis (CSU Bakersfield).

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