Studies on cellulolytic activity, ethanol tolerance and silver compound sensitivity of *Raffaelea lauricola*

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Abstract

Laurel wilt is caused by *Raffaelea lauricola*, a fungal pathogen transmitted by the invasive ambrosia beetle *Xyleborus glabratus*. The female ambrosia beetle bore into the trunk of a healthy or stressed tree and inoculate the galleries with ambrosia fungus (*R. lauricola*) on which the brood feeds. The fungus flourishes in the galleries of sapwood which clogs up the flow of water and nutrients in the tree. The fungus attacks the vascular system of trees in the Lauraceae family, and it has killed millions of trees since it was first found in the southeastern United States in 2004. Laurel wilt has been a devastating disease for avocados groves in Southern Florida and now has become a potential threat to the California avocado industry. The infected avocado trees can be saved by injecting them with a fungicide. But it is expensive and time-consuming and must be repeated every two or three year. Understanding of the biology of the pathogen may provide new insight into the control of the disease.

Although *Raffaelea lauricola* inside the galleries is known to consume and grow on wood, a clear demonstration of its cellulolytic ability has not been reported. In some ambrosia beetle-fungus relationship it has been reported that the beetle prefers stressed or dying trees, which have more ethanol in their tissue. This reliance on ethanol for host tree colonization promotes the growth of their ethanol tolerant fungal symbiont while inhibiting the growth of ethanol susceptible microbial competitors. Interestingly, *R. lauricola* is known to produce ethanol under laboratory conditions, but ethanol tolerance of *R. lauricola* has not been studied. Silver ions and silver based compounds have strong antimicrobial properties with multiple mode of action. Therefore, use of silver-based compounds may expand treatment options for the management of laurel wilt.

Methods

Cultures: *R. lauricola* strains #180256 and #180254 were obtained from Dr. Romania Gazis-Seregina, University of Florida Tropical & Research Education Center, Homestead, FL. The fungal cultures were grown on malt extract agar (MEA) amended with cycloheximide and maintained at 4 °C. *Trichoderma reesei* strain 180256, a cellulose degrading fungus was obtained from USDA culture collection and was grown on potato dextrose agar plates at room temperature. An unidentified cellulose degrading bacteria (RTCB) was grown on tryptic soy agar plates at 28 °C.

Ethanol tolerance:
The 2.5% and 5% Ethanol amended MEA media were prepared by adding appropriate volume of filter-sterilized ethanol to the autoclaved media after cooling to 50 °C. Sterile distilled water was used for the control. A mycelial plug of (3 mm in diameter) of *R. lauricola* was transferred to the center of each replicate plate. Inoculated plates were incubated at room temperature and were observed and photographed every 2 days.

Sensitivity to silver compound-x:
The silver compound-x was synthesized in Dr. Raptis's lab. Since the silver compound is light-sensitive, all laboratory work was performed under very low light conditions. A stock solution of silver compound-x was prepared using sterile deionized water just before amending it to sterile ME broth flasks and MEA agar plates. The concentrations treatments tested were 0 ppm, 15 ppm, 30 ppm and 45 ppm. Three-week-old MEA culture plates of strains 180256 and 180254 were used as inoculum source for the experiment. For *R. lauricola* inoculum preparation, 4 ml of sterile DI water was pipetted onto a culture plate and the fungal growth/spores were gently scraped using a sterile spreader. The liquid inoculum from the plates were collected into a sterile beaker. Each ME flask received 0.5 mL of inoculum and for MEA plates, the 0.5 mL inoculum was transferred on the agar surface and was evenly spread using a sterile spreader. The MEA plates were allowed to dry for 5 minutes inside the biological safety cabinet. Later a single cylindrical hole with a diameter of 6 mm was punched aseptically with a sterile cork borer, and 50 µL of silver compound stock solution was introduced into each well. All flasks and plates were wrapped in aluminum foil, the flasks were kept in a incubator shaker and plates were kept inside a clean cabinet at room temperature. After 7 days of incubation inhibition zone (if any on MEA plates) or level of turbidity in MEA liquid culture was recorded.

Cellulose Experiment:
Confirmation of cellulose-degrading ability of *R. lauricola* strains was performed by growing them on the cellulose Congo-Red agar media. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic activity. A mycelial plug of (3 mm in diameter) of *R. lauricola* strain was transferred to the center of each replicate plate. Inoculated plates were incubated at room temperature and were observed every 2 days. Similarly, as a positive control *T. reesei* and RTCB were also grown on cellulose congo-red agar plates. Colonies showing discoloration of Congo-Red around the colony were taken as positive cellulose-degrading.

Results

Ethanol Experiment:

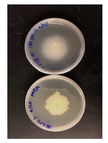

Figure 1: Shows strain 180256 in 2.5% ethanol compared to strain in MEA media after one week.

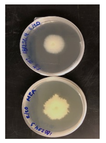

Figure 2: Shows strain 180256 in 5% ethanol compared to strain in MEA media after one week.

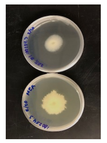

Figure 3: Shows strain 180254 in 2.5% ethanol compared to strain in MEA media after one week.

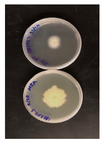

Figure 4: Shows strain 180254 in 5% ethanol compared to strain in MEA media after one week.

Silver Compound Plates Experiment:

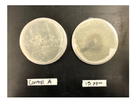

Figure 5: View of 15 ppm compared to Control A after one week.

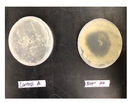

Figure 6: View of 30 ppm compared to control A after one week.

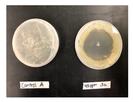

Figure 7: View of 45 ppm compared to Control A after one week.

Sample	Diameter (cm)
1a-15 ppm	0 cm
2a-30 ppm	6 cm
3a-45 ppm	7 cm

Silver Compound flasks Experiment:

Figure 8: View of 15 ppm compared to the control after one week.

Figure 9: View of 30 ppm compared to the control after one week.

Figure 10: View of 45 ppm compared to the control after one week.

Cellulose Experiment:

Sample	Cellulolytic Activity
RTCB	++++
T.ressi	++++
Strain 180254	++
Strain 180256	++

Conclusions

There was a distinct difference in *Raffaelea lauricola* ethanol tolerance between the two strains tested. At 5% ethanol both strains showed reduced growth compared to the control. The strain 180254 growth found to be less affected by ethanol than strain 180256 and its growth at 2.5% ethanol was comparable to that of control plates.

The silver compound-x was found to be more effective in inhibiting the growth of *R. lauricola* only at higher concentrations levels (30 and 45 ppm). At concentration 15 ppm, the silver compound-x caused minimal inhibition of *R. lauricola* growth on agar and no inhibition of growth in liquid culture. A very strong growth inhibition of *R. lauricola* on agar plates was observed at concentrations 30 ppm and 45 ppm. In liquid culture the *R. lauricola* growth was completely inhibited at concentrations 30 ppm and 45 ppm of silver compound-x. Demonstration of cellulolytic activity of *R. lauricola* was possible with the use of cellulose congo-red agar media. However, very slow growth rate of *R. lauricola* was a limiting factor while comparing its activity to that of fast-growing cellulolytic microorganisms. Future research will focus on repeating some the experiments and devising new experiments to help discover new research avenues that tackle the problem of laurel wilt.

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