HHMI

Investigation of Biocontrol: Inhibition of *Agrobacterium rhizogenes* by Biofilming Organisms

H. Christy, J. Hall, A. Wiedemeier, Department of Biology, College of Arts and Sciences, The University of Louisiana at Monroe, Monroe, LA.

UNIVERSITY OF LOUISIANA MONROE

Abstract

This project focuses on the use of biofilms to inhibit infection of plant roots by *Agrobacterium rhizogenes* as a form of biocontrol. Biocontrol is defined as the control of destructive organisms through the disruption of the ecological status of that organism. This biocontrol is achieved through the use of other organisms that may be natural predators, poisons, or natural inhibitors. Biocontrol studies have occurred with other *Agrobacterium* species, specifically *tumefaciens* and *radiobacter*(1). This current study has focused on the inhibition of biofilm formation of *A. rhizogenes* because it provides an easy visual confirmation of infection upon examination. *A. rhizogenes* is a pathogenic bacterium that induces "hairy root" systems on host plants. This bacteria induces such affects by integrating its Ri plasmid into the host plant's DNA. This integration event increases the auxin sensitivity within the root, inducing root proliferation(2). In our study we used carrot discs as the model organism to examine the production of adventitious roots. *Bacillus subtilis* and *Bacillus cereus* were examined as the biocontrol organisms in this study. These bacteria were selected for this project because both are known to biofilm and were readily available lab strains. The data collected in this project shows that neither *Bacillus* strain inhibits the biofilm formation of *A. rhizogenes*. In the future, we will be analyzing the interaction of both *tumefaciens* and *rhizogenes* with other biofilming strains that can be isolated from Louisiana soil.

Background

Biocontrol is achieved through the use of organisms that may be natural predators, poisons, or natural inhibitors of microbes. An example of biocontrol is the introduction of ladybugs to croplands in order to protect from the aphid, an insect pest. The ladybug, a natural predator of the aphid, inhibits the aphid damage on the crops. In this case, biocontrol is an excellent natural alternative to the use of pesticides.

A biofilm is a dynamic colony of slime-encased microbial organisms. Biofilms form readily in various natural settings because the microorganisms produce extracellular polymeric substances including polysaccharides, glycolipids, proteins, glycoproteins, as well as DNA (3). Microbial organisms form biofilms to protect themselves from ultraviolet light as well as other harmful environmental agents. Biofilms often inhibit other organismal growth by introducing chemicals into the environment. In our project, we used the supernatants decanted from broth cultures of biofilming organisms to assess biocontrol activity. These supernatants were incorporated into the biofilm absorbance assays we used for analysis in this project.

If a suitable biocontrol agent was found, the next step in our project was to test it in vivo. Can we use a biofilming organism as an inhibitor to infection? We investigated numerous model systems of infection, such as infection with *Agrobacterium tumefaciens* on tomato, sunflower, carrot and *Arabidopsis thaliana*. Then, we also looked at the infection of *Agrobacterium rhizogenes* on tomato, carrot and *Arabidopsis thaliana*. Both *Agrobacterium* species are pathogenic microorganisms that use biofilming as an advantage. While both species of *Agrobacterium* produce noticeable illness within a plant, we focused on the use of *Agrobacterium rhizogenes* in carrot discs.

Data and Results

Absorbance Assays for Inhibition

Each assay included the following subsets:

1. **control supernatant**
2. ***A. rhizogenes* bacteria in its own supernatant**
3. ***A. rhizogenes* bacteria in *B. subtilis* supernatant**
4. ***A. rhizogenes* bacteria in *B. cereus* supernatant**

Biofilm formation can be determined by analyzing the absorbance spectrum from solubilized biofilms. Thicker biofilms will produce greater absorbance readings. The data below shows an average from three assays. Statistical values were computed using a One-way Analysis of Variance (ANOVA) in order to determine standard deviation and statistical significance . *B. cereus* exhibited negligible affects, yet *B. subtilis* had significant affect on the biofilm formation of *A. rhizogenes* in an additive, rather than an inhibitory manner.

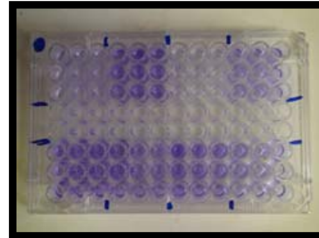

Figure 1
Pictured is a 96 well plate used in the biofilm absorbance assays. The biofilm material in this plate has been stained and solubilized. The darker wells represent a thicker biofilm formation and thus would correlate to greater absorbance readings.

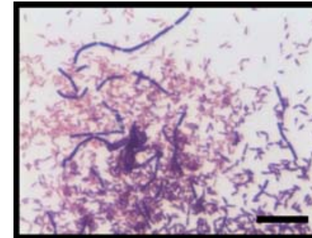

Figure 2
This picture shows the biofilm formation of *Agrobacterium* co-incubated with *B. subtilis*. Scale bar = 10um.

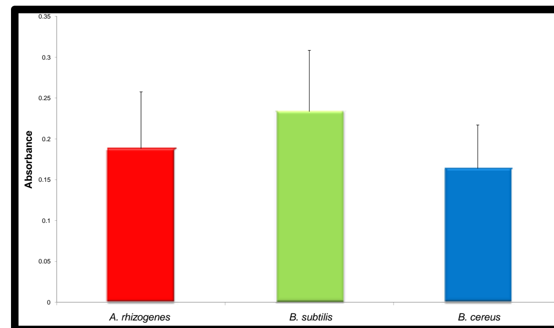

Figure 3
This graph shows data collected from our biofilm absorbance assays. Strains labeled on the horizontal axis represent the bacterial supernatant used in each experiment. All three experiments contained *A. rhizogenes* as the live biofilming organism. *B. cereus* does not show inhibition, nor does *B. subtilis*. However, *B. subtilis* did show a significant increase in the biofilm formation (p = .05).

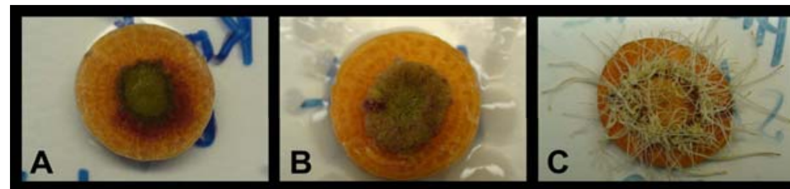

Figure 4
This panel illustrates the visual relationship between carrot discs used for control (A), infected with *A. tumefaciens* (B), and infected with *A. rhizogenes* (C). It is clearly much easier to visually confirm infection with *A. rhizogenes*, which was the primary reason for its use in this project.

Materials and Methods

Biofilm Absorbance Assay

Day 1:

Inoculate overnight cultures of *A. rhizogenes, B. subtilis , B. cereus grown in YMA broth.*

Day 2:

1. Using overnight culture, inoculate new YMA broth at 1:100 dilution. Allow 24 hour incubation. These cultures will be used to collect chemical supernatant.
2. Inoculate *A. rhizogenes* overnight culture into new YMA broth for use as organisms to be inhibited.

Day 3:

1. Centrifuge and filter supernatant broths to remove cellular material. Supernatant collected and used. If not used immediately supernatant frozen at 4˚C.
2. Set up biofilm assay in 96 well plate in sets that follow:
   a) 50/50 solution of *Agrobacterium* supernatant and fresh media to be used to set blank.
   b) *Agrobacterium* from overnight culture in its own supernatant.
   c) *Agrobacterium* from overnight culture in *B. subtilis* supernatant.
   d) *Agrobacterium* from overnight in *B. cereus* supernatant.
3. *Agrobacterium* cultures allowed to grow in the presence of supernatant either 24 or 48 hours.

Day 4/5:

1. Remove supernatant in each well, rinse with water and stain with gentian violet.
2. Solubilize biofilm material with 33.3% acetic acid.
3. Obtain absorbance readings of solution by spectrophotometry.

Carrot Disc Experiment:

1. Wash carrot with detergent, rinse with water, and allow to soak in 20% bleach solution.
2. Rinse x3 with water after removal from bleach.
3. While under laminar air flow hood, cut ¼ in. slices and place on plant agar (5) plate.
4. Smear pathogenic organism on upward facing surface of carrot disc.
5. Wrap plate with cloth tape and place under growth lamps for at least 2 weeks or until infection noticeable. (4)

Figure 5
Picture shows newly cut and uninfected carrot disc placed on plant agar plate.

Figure 6
***Agrobacterium rhizogenes* showing evidence of capsule production. Scale bar = 10 um.**

Conclusion

***Bacillus subtilis* and *Bacillus cereus* both failed to exhibit biocontrol properties on the biofilm formation of *A. rhizogenes*. These strains failed to inhibit biofilm formation at a 24 hour co-incubation period. However, incubation with *B. subtilis* supernatant exhibited absorbance values that were statistically significant. The affects of *B. subtilis* were additive, meaning that the chemicals within the supernatant did not inhibit, but rather helped the biofilm formation of *A. rhizogenes*.**

Future

Future projects will focus on biocontrol of *Agrobacterium* by organisms native to Louisiana soil. The bacterial strains will be isolated directly from the soil instead of using laboratory strains . Also, we will be taking a closer look at why *B. subtilis* supernatant exhibited additive affects on the biofilm formation of *A. rhizogenes*.

References

1. Moore, Larry W., and Guylyn Warren . "*Agrobacterium radiobacter* strain 84 and Biological Control of Crown Gall." *Annual Review of Phytopathology.* (1979): 163-179. Print.
2. Chabaud, Mireille, Aurelien Boisson-Dernier, Juan Zhang, Christopher G. Taylor, Oliver Yu, and David G. Barker. "*Agrobacterium rhizogenes*-mediated root transformation." *Medicago truncatula handbook* :(November 2006).
3. Willey, Joanne M. , Linda M. Sherwood, and Christopher J. Woolverton. Prescott's Microbiology. 8th ed. New York: Mcgraw-Hill, 2011. 183-185.
4. Carroll, Juliet E. *Learning Biology with Plant Pathology*. Reston, Virginia: National Association of Biology Teachers, 1994. 102-104.
5. Personal Communications with Tobias Baskin at the University of Massachusetts.

Acknowledgments

We would like to thank the following for their assistance with this project:
Dr. Debra Jackson for her expertise on biofilming organisms, Dr. Keith Jackson and Sayed Quadri for analysis of data, Dr. Thomas Sasek for donation of *Arabidopsis thaliana* seed and allowing use of his plant room, Dr. Anne Findley for donation of supplies. This project was supported in part by Howard Hughes Medical Institute and the ULM Biology department.