Evaluation of Induced Cells of Rhodococcus Rhodochrous to Inhibit Fungi

Muzna Saqib

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Evaluation of Induced Cells of *Rhodococcus Rhodochrous* to Inhibit Fungi

Muzna Saqib

Under the Direction of Dr. George Pierce
Acknowledgements

I would like to thank the Georgia State University Honors College for giving me the opportunity to complete an Honors Research Thesis. I would also like to acknowledge Dr. George Pierce for providing me the opportunity to conduct research in his lab, and for his guidance in advancing me as a researcher. Furthermore, I would also like to thank Dr. Courtney Barlament, Kelly Cannon, Joshua Renfroe, and all members of the Pierce Lab for their guidance and willingness to help me in completing this thesis.
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Introduction:

Even though fruits and vegetables harvested in the US are stored and transported in refrigeration with improved packaging, absorbents and ethylene receptor blockers, 40% of them are still never consumed and go to waste (Cantu 2008). As plants, especially fruits, continue to ripen after harvest and gain a higher risk of ripening during transportation and temperature changes, there is a high need to develop methods to delay this ripening and reduce post-harvest loss. This will lead to an availability of more fruits and vegetables without the need to increase production (Wilson 1985).

*Rhodococcus rhodochrous* is an aerobic, non-pathogenic, gram-positive bacterium that is often used in industries as a biocatalyst. *R. rhodochrous* DAP 96253 is capable of exhibiting contact-independent inhibition of selected fungal pathogens. The use of *R. rhodochrous* as a potential biocontrol agent against plant and animal fungi will be examined and the focus of this study will be to employ representative species of fungi, and each species will be studied to establish the effect of dose (g/cells) and time of exposure to *R. rhodochrous*.

To develop a standard for the trials, a cell paste was prepared from induced *R. rhodochrous* fermentation cells. The trials were done with different types of co-cultures at variant temperatures in petri plates, and controls were used. The amount of cell paste used varied from 1-3 grams and the dosing time varied from 1-15 days. The time and g/cells it takes to inhibit each fungi was used to establish the standards, as some of the species to be tested are quite resistant. Therefore, the grams of cell paste it takes and the dosage time were crucial to the development of a biocontrol agent. This data was then applied to develop a non-contact biocontrol agent that was tested on fruits such as, bananas and tomatoes.

The fungi tested were *Botrytis cinerea, Pseudogymnoascus destructans, Aspergillus flavus, Fusarium oxysporum 'Cigar Tip', Rhizopus stolonifer 'D1',* and other species isolated from berries. *B. cinerea* is associated with fruit ripening, especially berries, and more ripening makes fruit more susceptible to it and other pathogens (Cantu 2008). The strain of *B. cinerea* used was NRRL 1650. *P. destructans* causes white nose syndrome in bats, and is leading to bat extinction (Cornelison 2014). The strain of *P. destructans* used was PD71, Southeastern Cooperative Wildlife Disease Study, UGA, clinical isolate. *A. flavus* makes aflatoxins and effects nuts, grains, and cotton (Geiser, 1998). Berry isolates (D1 and D2) were isolated from different berries and cigar tip (banana isolate) was isolated from bananas. These fungal species were evaluated for inhibition by using different types of co-culture methods.
Methods:

Spore Germination Inhibition Dosing Test

The dose being tested was determined by using previous data and moving from higher values to lower values based on whether they work or not.

Specific amounts of fermentation paste were weighted out in empty 35 mm petri plates and set up in 100 mm plates with inoculated agar plates with specific amounts of spores used for inoculation. The amounts were increased or decreased based on various trials.

A first trial was done with 1g, .7g, .5g, .3g and .1g of fermentation paste (From March 25th 2016) set up against $10^3 P. destructans$ spores (Figure 2.1). A second trial was done with .3g, .4g and .5g of fermentation paste (from February 5th 2016) were set up against $10^3 P. destructans$ spores to determine the minimum amount of fermentation paste needed to inhibit the fungi(Figure 2.2). A third trial was done with .4g, .5g and .6g of fermentation paste (from April 8th 2016) were set up against $10^3 P. destructans$ spores(Figure 2.3).

.1g, .25g and .5g of fermentation paste (from February 5th 2016) were set up against $10^3 B. cinerea$ spores to determine the minimum amount of fermentation paste needed to inhibit the fungi(Figure 2.4).

The first dose tested was 1.5g, .2g, .5g and 3g of fermentation paste (from March 25 2016) were set up against $10^3 A. flavus$ aflatoxin positive spores to determine the minimum amount of fermentation paste needed to inhibit the fungi(Figure 2.5). A second trial was done with 3g of cell paste (from February 5th 2016) confirm the level of inhibition 3g of fermentation paste inhibit has against $A. flavus$ aflatoxin positive(Figure 2.6). Once it was determined that 3g of fermentation paste successfully inhibit fungi and 2g has been shown not to inhibit fungi, intervals between the two value were set up. 2.25g, 2.50g and 2.75g of fermentation paste (from April 8th 2016) were set up in contact independent antifungal tests against $A. flavus$- aflatoxin positive spores(Figure 2.7). After determining that 2.25g of fermentation paste (from April 8th 2016) successfully inhibited fungi, 2g of fermentation paste was tested again to evaluate the level of inhibition(Figure 2.8). Antifungal inhibition tests were repeated (with fermentation paste from

---

**Figure 1.1. Contact Independent Antifungal Inhibition of Spore Germination.**

Spore Solution on SDA

Rr 96253
10/1/2016) using 1g and 3g of fermentation paste against *A. flavus*- aflatoxin positive (Figure 2.9).

The first dose tested was 1.5g, 2g, 2.5g and 3g of fermentation paste (from March 25 2016) were set up against $10^3$ *A. flavus* tox negative and tox positive spores to determine the minimum amount of fermentation paste needed to inhibit the fungi (Figure 2.10). A second trial was done with 3g of cell paste (from February 5th 2016) confirm the level of inhibition 3g of fermentation paste inhibit has against *A. flavus*- aflatoxin negative spores (Figure 2.11). Once it was determined that 3g of fermentation paste successfully inhibit fungi and 2g has been shown not to inhibit fungi, intervals between the two value were set up. 2.25g, 2.50g and 2.75g of fermentation paste (from April 8th 2016) were set up in contact independent antifungal tests against *A. flavus*- aflatoxin negative spores (Figure 2.12). After determining that 2.25g of fermentation paste (from April 8th 2016) successfully inhibited fungi, 2g of fermentation paste was tested again to evaluate the level of inhibition (Figure 2.13). Antifungal inhibition tests were repeated (with fermentation paste from 10/1/2016) using 1g and 3g of fermentation paste against *A. flavus*- aflatoxin negative (Figure 2.14).

1g and .5g of fermentation paste (of 5/16/2016) were tested to determine the lowest dose required to inhibit the growth of D1 (Figure 2.15). Two different fermentation pastes were used to confirm results. .5 g and 1g of (from 6/6/2016) fermentation paste was set up against 10 ul of 1x10^5 D1 (Figure 2.16).

**Mycelial Growth Inhibition Test with Plugs (Agar Diffusion)**

Antifungal plugs were done by replacing a core of agar in an identical core with the desired fungi growing on it. Plug was pushed into the inoculated petri dish. The amount of fermentation paste being tested was weighed out and placed in a shared airspace with the fungal plate.

1g and .5g of fermentation paste (from February 5th 2016) was tested against *B. cinerea* plugs (Figure 3.1).

3g of fermentation paste (from February 5th 2016) was tested against *A. flavus* aflatoxin positive plugs (Figure 3.2).

3g of fermentation paste (from February 5th 2016) was tested against *A. flavus* tox negative and tox positive plugs (Figure 3.3).

3g and 1g of cell paste was tested against cigar tip plugs. Tests with fermentation paste (from May 6, 2016) with both SDA and PDA were done to monitor any variability (Figure 3.4 and 3.5).

3g and 1g of cell paste was tested against *P. destructans* Plugs. Tests with fermentation paste from July 23, 2016 and from July 29, 2016 with both SDA were done to monitor any variability (Figure 3.6 and 3.7).

**Antifungal Inhibition Test with Frozen Fermentation Paste**

Fermentation paste was flash frozen in liquid nitrogen for 30 minutes and tested against different fungi. Cell paste was weighed out into conical tubes, dropped in liquid nitrogen (-270°C) and left for 30 minutes. After flash freezing the tubes were either stored in -80°C, -20°C or
thawed immediately. Before testing all frozen fermentation pastes were thawed on ice for 30 minutes to reduce cell lysis. The stored paste was tested at different timed intervals of 1 week, 1 month and by months.

Fermentation paste from 1/29/2016 was thawed on ice for 30 minutes. 1g of fermentation paste stored at -80°C and -20°C was used against 1 x 10³ P. destructans spores and tested for inhibition(Figure 4.1). Frozen Fermentation paste from 2/5/2016 was thawed on ice for 30 minutes. 1g of fermentation paste stored at -80°C and -20°C was used against 1 x 10³ P. destructans spores and tested for inhibition(Figure 4.2). Frozen Fermentation paste from 3/25/2016 was thawed on ice for 30 minutes. 1g of fermentation paste stored at -80°C and -20°C was used against 1 x 10³ P. destructans spores and tested for inhibition(Figure 4.3).

Fermentation paste (from February 5th 2016) was flash frozen in liquid nitrogen for 30 minutes and then thawed on ice for 30 minutes(Figure 4.6). 1g of cell paste was used against 1 x 10³ B. cinerea spores and tested for inhibition. Fermentation paste and ‘thawed’ fermentation paste was also used for testing.

**Image Study of Exposed fungal Spores**

Slides for image study were prepared by pouring Sabaroud Dextrose Agar onto slides and letting it solidify. Once they solidified, glycerol solution of spores was used to inoculate the slides. Co-cultures with fermentation paste were combined and incubated. Microscopy images were captured post-inoculation.

![Image of exposed fungal spores](image_url)

**Figure 1.2.** Set ups for antifungal inhibition for exposed fungal spores.(Left : Exposed fungal spore control, Right: exposed fungal spores and 1g Rr 96253)

10³ Aspergillus flavus spores of tox negative and tox positive were spread onto SDA plates and co-cultures with fermentation paste and incubated at room temperature and 30°C(Figure 5.1 and 5.2). All were run in triplicate. Images were captured 5 days post-inoculation. 10⁵ D1(Berry Isolate) and D2 (Berry Isolate) spores were spread onto SDA plates co-
cultured with fermentation paste and incubated at room temperature (Figure 5.3 and 5.4). All were run in triplicate. Images were captured 2 days’ post-inoculation.

$10^3$ Aspergillus flavus spores of tox negative and tox positive were spread onto SDA plates and co-cultures with fermentation paste and incubated at room temperature and 30˚C. All were run in triplicate. Images were captured 5 days post-inoculation.

$10^5$ D1 (Berry Isolate) and D2 (Berry Isolate) spores were spread onto SDA plates co-cultured with fermentation paste and incubated at room temperature. All were run in triplicate. Images were captured 2 days’ post-inoculation.

**Spore Harvesting for Glycerol Stocks**

Spores were harvested from plate a fungal plate by adding 10ml of candida spore harvesting solution (Appendix A) and 10ml of Phosphate Buffered Saline (PBS) (Appendix A) or 0.9% NaCl, and pipetted using a sterile pipette into a sterile conical tube. The spore suspension was then centrifuged at 3000 rpm for 10 minutes up to 3 times, and supernanent was dispensed each time and pellet resuspended in fresh PBS. The suspension was then poured through a sterile funnel with glass wool and rinsed with 5ml of PBS. Spores were counted via microscopy by using a hemocytometer. Spores were counted in grids as shown in picture below on both sides of the hemocytometer and the values averaged.

\[
A + B + C + D + E = \text{Slide total. (5 squares / 25 Squares) Dilution x Desired Concentration.}
\]

\[
\frac{\text{Slide 1} + \text{Slide 2}}{2} = \text{Total} = \text{Average spores /10 uL (50000) = Final spores per ml.}
\]

Both were added and average was found. Multiplied by 50,000 to get the final spore count/ml. It was then diluted to obtain desired concentration if making glycerol stocks. The amount to dilute was found by the dilution equation. The final amount was diluted with PBS to make a total of 15ml of the desired concentration. 15ml of glycerol were added to made stocks for storage.
Results:

Spore Germination Inhibition Dosing Test with *P. destructans*:

Antifungal dosing tests were done with contact independent use of fermentation paste and inoculated agar plates. The aim of the dosing test was to find the minimum amount of fermentation paste needed to inhibit each fungi. Different doses were tested to determine the lowest dose needed.

10⁴ *P. destructans* Spores  1g Fermentation Paste  .7g Fermentation Paste  .5g Fermentation Paste  .3g Fermentation Paste  1g Fermentation Paste

![Figure 2.1 Spore Germination inhibition test with 1g,.7g, .5g,.3g and .1g of fermentation paste from March 25th 2016 and 10³ *P. destructans* spores.](image)

10⁵ *P. destructans*  .3g Fermentation Paste  .4g Fermentation Paste  .5g Fermentation Paste

![Figure 2.2. Spore Germination inhibition test with .3g,.4g and .5g of fermentation paste from February 5th and 10³ *P. destructans* spores.](image)
Figure 2.3. Spore Germination inhibition test with .4g,.5g and .6g of fermentation paste from April 8th 2016 and $10^3$ P. destructans spores.
Spore Germination Antifungal Dosing Test with *B. Cinerea*:

$10^5 \text{ } B.\text{cinerea} \text{ Spores} \quad .1\text{g Fermentation Paste} \quad .25\text{g Fermentation Paste} \quad .5\text{g Fermentation Paste}$

**Figure 2.4.** Spore Germination inhibition test with .1g, .25g and .5g of fermentation paste and $10^3 \text{ } B.\text{cinerea}$ spores.
Spore Germination Inhibition Dosing Test with *A. flavus*- aflatoxin positive:

$10^3$ *A. flavus* aflatoxin positive 1.5g Fermentation Paste  2g Fermentation Paste  2.5g Fermentation Paste  3g Fermentation Paste

**Figure 2.5.** Spore Germination Inhibition test with 1.5g,2g,2.5g and 3g of fermentation paste and *A. flavus* - aflatoxin positive spores.

$10^3$ *A. flavus* aflatoxin positive  3g Fermentation Paste

**Figure 2.6.** Spore Germination Inhibition test with 3g of fermentation paste and *A. flavus*- aflatoxin positive spores.
Figure 2.7. Spore Germination Inhibition test with 2.25g, 2.5 and 2.75g of fermentation paste and A. flavus- aflatoxin positive spores.

Figure 2.8. Spore Germination Inhibition test with 2g of fermentation paste and A. flavus-aflatoxin positive spores.
Figure 2.9. Spore Germination Inhibition test with 1g and 3g of fermentation paste from October 1st 2016 and *A. flavus*- aflatoxin positive spores.
Spore Germination Inhibition Antifungal Dosing Test with *A. flavus*- aflatoxin negative:

<table>
<thead>
<tr>
<th></th>
<th>1.5g Fermentation Paste</th>
<th>2g Fermentation Paste</th>
<th>2.5g Fermentation Paste</th>
<th>3g Fermentation Paste</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^3 <em>A. flavus</em> aflatoxin negative</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.10.** Spore Germination Inhibition test with 1.5g, 2g, 2.5g and 3g of fermentation paste March 25th 2016 and *A. flavus* - aflatoxin negative spores.

<table>
<thead>
<tr>
<th></th>
<th>3g Fermentation Paste</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^3 <em>A. flavus</em> aflatoxin negative</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.11.** Spore Germination Inhibition test with 3g of fermentation paste February 2nd 2016 and *A. flavus* - aflatoxin negative spores.
Figure 2.12. Spore Germination Inhibition test with 2.25 g, 2.50 g and 2.75 g of fermentation paste from March 25th 2016 and *A. flavus*- aflatoxin negative spores.

Figure 2.13. Spore Germination Inhibition test with 2g of fermentation paste from April 8th 2016 and *A. flavus*- aflatoxin negative spores.
Figure 2.14. Spore Germination Inhibition test with 1g and 3g of fermentation paste from October 1st 2016 and A. flavus- aflatoxin negative spores.
Spore Germination Inhibition Dosing Test with D1:

| 10^5 D1 (Berry Isolate) spores | 1g Fermentation Paste | 3g Fermentation Paste |

Figure 2.15. Spore Germination Inhibition test with 1g and 3g of fermentation paste from May 6\textsuperscript{th} 2016 and D1.

| 10^5 D1 (Berry Isolate) spores | 1g Fermentation Paste | .5g Fermentation Paste |

Figure 2.16. Spore Germination Inhibition test with 1g and .5g of fermentation paste from June 6\textsuperscript{th} 2016 and D1.
Figure 2.17. Spore Germination Inhibition test with .5g and 1g of fermentation paste and D1.
Mycelial Growth Inhibition Test with *B. cinerea* Plugs (Agar Diffusion):

| 10³ *B. cinerea* plate | 1g Fermentation Paste | .5g Fermentation Paste |

**Figure 3.1.** Mycelial Growth Inhibition test with .5g and 1g of fermentation paste from February 5th 2016 and *B. cinerea* Plugs.
Mycelial Growth Inhibition Test with *A. flavus* aflatoxin positive Plugs (Agar Diffusion):

\[10^3 A. \text{flavus} \text{ aflatoxin positive} \quad 3g \text{ Fermentation Paste}\]

**Figure 3.2.** Mycelial Growth Inhibition test with 3g of fermentation paste from February 5th 2016 and *A. flavus* aflatoxin positive Plugs.
Mycelial Growth Inhibition Test with *A. flavus* aflatoxin negative Plugs (Agar Diffusion):

3g of fermentation paste (from February 5th 2016) was tested against *A. flavus* tox negative and tox positive plugs.

\[10^3 \text{A. flavus aflatoxin negative} \quad 3\text{g Cell Paste}\]

**Figure 3.3.** Mycelial Growth Inhibition test with 3g of fermentation paste from February 5th 2016 and *A. flavus* aflatoxin negative Plugs.
**Mycelial Cell Inhibition Test with Banana Isolate ‘Cigar tip’ Plugs (Agar Diffusion):**

<table>
<thead>
<tr>
<th>Cigar tip plug</th>
<th>1g Fermentation Paste</th>
<th>3g Fermentation Paste</th>
</tr>
</thead>
</table>

![Image of agar plates with different treatments]

**Figure 3.4.** Mycelial Growth Inhibition test with 1g and 3g of fermentation paste from May 6\textsuperscript{th} 2016 and cigar tip (banana isolate)

<table>
<thead>
<tr>
<th>Cigar tip plug</th>
<th>3g Cell Paste</th>
</tr>
</thead>
</table>

![Image of agar plates with different treatments]

**Figure 3.5.** Mycelial Growth Inhibition test with 3g of fermentation paste from May 6\textsuperscript{th} 2016 and cigar tip (banana isolate).
Mycelial Growth Inhibition Test with *P. destructans* Plugs (Agar Diffusion):

![Figure 3.6](image1.png)

**Figure 3.6.** Mycelial Growth Inhibition test with 1g and 3g of fermentation paste from July 23rd 2016 and *P. destructans* Plugs.

![Figure 3.7](image2.png)

**Figure 3.7.** Mycelial Growth Inhibition test with 1g and 3g of fermentation paste from and *P. destructans* Plugs.
Antifungal Inhibition Test with *P. destructans* and Frozen Fermentation Paste

<table>
<thead>
<tr>
<th>$10^3 \text{P. destructans}$</th>
<th>Paste Only</th>
<th>Freeze Only</th>
<th>Thawed only</th>
<th>Frozen and Thawed</th>
</tr>
</thead>
</table>

**Figure 4.1.** Antifungal Inhibition Test with fermentation paste only, frozen paste, thawed only paste and frozen and thawed paste.

<table>
<thead>
<tr>
<th>$10^3 \text{P. destructans}$</th>
<th>1g Cell Paste</th>
<th>1g cell paste stored at -20°C</th>
<th>1g cell paste stored at -80°C</th>
</tr>
</thead>
</table>

**Figure 4.2.** Antifungal Inhibition Test with 1g Frozen Fermentation paste, frozen fermentation paste stored at -20°C and -80°C and of *P. destructans* spores.
Figure 4.3. Antifungal Inhibition Test with frozen fermentation paste stored at -20°C and -80°C, and of *P. destructans* spores.

Figure 4.4. Antifungal Inhibition Test with 1g Frozen Fermentation paste from February 5th 2016 frozen fermentation paste stored at -20°C and -80°C, and of *P. destructans* spores.
Figure 4.5. Antifungal Inhibition Test with 1g Frozen Fermentation paste, frozen fermentation paste stored at -20°C and -80°C, and of *P. destructans* spores.
Antifungal Inhibition Test with \textit{B. cinerea} and Frozen Fermentation Paste

![Image of Petri dishes showing antifungal inhibition test]

**Figure 4.6.** Antifungal Inhibition Test with 1g Fermentation Paste, 1g thawed only, 1g frozen and thawed cellpaste with \textit{B. cinerea} spores.
Image Study of *A. flavus* aflatoxin positive exposed fungal spores:

10^3 *A. flavus* tox Positive control slide 10^3 *A. flavus* tox Positive slide with 3g Ferm Paste

![Image study of *A. flavus* aflatoxin positive exposed fungal spores](image.png)

**Figure 5.1.** Image study of *A. flavus* aflatoxin positive exposed fungal spores with 3g fermentation paste.

Image study of *A. flavus* aflatoxin negative slides:

10^3 *A. flavus* tox negative control slide 10^3 *A. flavus* negative slide with 3g Ferm Paste

![Image study of *A. flavus* aflatoxin negative exposed fungal spores](image.png)

**Figure 5.2.** Image study of *A. flavus* aflatoxin negative exposed fungal spores with 3g fermentation paste.
Figure 5.3. Image study of D1 exposed fungal spores with 3g fermentation paste.

Figure 5.4. Image study of D2 exposed fungal spores with 3g fermentation paste.
Results for Spore Harvesting for Storage in Glycerol Stocks

*B.cinerea* Spore Harvest Results:

10 ml of harvesting solution and 10 ml of 0.9% NaCl were added to a *B.cinerea* plate. A sterilized hockey stick was used to gently scrape spores, and the solution was pipetted into a sterile conical tube. The solution was then spun at 3000 rpm for 10 minutes twice, Supernatant was poured off and the pellet was re-suspended in sterile PBS. The suspension was poured through a funnel with glass wool and rinsed with approximately 5 ml of PBS. Spores were counted and diluted by the following method:

\[
\text{Slide 1 + Slide 2} = \frac{\text{Total}}{2} = \text{Average spores /ml}
\]

\[
1811 + 1747 = 1779 \\
(\text{Average}) (50,000) = (1779)(50,000) = 88,950,000 \text{ Spores / ml}
\]

To make glycerol stocks were rounded up to 8 x 10^7 CFU/ml.

1st dilution \(\frac{1}{4}\):

\[
1 \text{ ml spore solution or } \frac{1}{1000} \text{ of spore solution.}
\]

\[
3 \text{ ml of 0.9% NaCl (3+1)}
\]

2nd dilution 1/100:

\[
0.1 \text{ ml of } 2 \times 10^7 \text{ spore solution or } \frac{1}{100} \text{ of } 2 \times 10^7 \text{ spore solution.}
\]

\[
9.9 \text{ ml 0.9% NaCl (3+1)}
\]

resulted in 2 x 10^5 cfu/ml of spore solution.

3rd dilution \(\frac{1}{2}\):

\[
10 \text{ ml of } 2 \times 10^5 \text{ cfu/ml solution or } \frac{1}{2} \text{ of } 2 \times 10^5 \text{ cfu/ml solution.}
\]

\[
10 \text{ ml 60% glycerol (3+1)}
\]

resulted in a final count of 10 x 10^5 CFU/ml spore solution.

*A.Flavus* aflatoxin-negative Spore Harvest Results:

10 ml of harvesting solution and 10 ml of 0.9% NaCl was added to a *A.Flavus* aflatoxin-negative. Sterilized hockey stick was used to gently scrape spores and the solution was pipetted into a sterile conical tube. The solution was then spun at 3000 rpm for 10 minutes twice, Supernatant was poured off and the pellet was suspended in sterile PBS. The suspension was poured through a funnel with glass wool and rinsed with approximately 5 ml of PBS. Spores were counted and diluted by the following method:

\[
\text{Slide 1 + Slide 2} = \frac{\text{Total}}{2} = \text{Average spores /ml}
\]
(161+184)/2 = (172)(50,000) = 8,600,000 Spores / ml
(8.6*10^6)(x)=(1*10^5/ml)(15ml)
x=.175 ml

.175 ml of the spore solution was diluted with 14.825 ml of PBS. 15 ml of glycerol solution was added to make stocks.

**Flavus aflatoxin-positive Spore Harvest Results:**

10 ml of harvesting solution and 10 ml of 0.9% NaCl was added to a A.Flavus aflatoxin-positive. Sterilized hockey stick was used to gently scrape spores and the solution was pipetted into a sterile conical tube. The solution was then spun at 3000 rpm for 10 minutes twice, Supernatant was poured off and the pellet was suspended in sterile PBS. The suspension was poured through a funnel with glass wool and rinsed with approximately 5 ml of PBS. Spores were counted and diluted by the following method:

\[
\frac{\text{Slide 1} + \text{Slide 2}}{2} = \frac{\text{Total}}{2} = \text{Average spores/ml}
\]

\[
\frac{(209+277)}{2} = (243)(50,000) = 12,150,000 \text{ Spores / ml}
\]

\[
(1.2*10^7)(x)=(1*10^5/ml)(15ml)
\]

\[x=8.1 ml\]

8.1 ml of the spore solution was diluted with 6.9 ml of PBS. 15 ml of glycerol solution was added to make stocks.

**D1 Spore Harvest Results:**

10 ml of harvesting solution and 10 ml of 0.9% NaCl was added to a D1 plate. Sterilized hockey stick was used to gently scrape spores and the solution was pipetted into a sterile conical tube. The solution was then spun at 3000 rpm for 10 minutes twice, Supernatant was poured off and the pellet was suspended in sterile PBS. The suspension was poured through a funnel with glass wool and rinsed with approximately 5 ml of PBS. Spores were counted and diluted by the following method:

\[
\text{Slide Section 1:}
\]

\[
\frac{\text{E} : 21}{\text{Slide 1} + \text{Slide 2}} = \frac{\text{Total}}{2} = \text{Average spores/ml}
\]

\[
\frac{(142 + 127)}{2} = 134
\]

\[
(Average) (50,000) = (134)(50,000) = 6725 \text{ Spores / ml}
\]

To make glycerol stocks round up the spores/ml count to 7 \( \times \) \( 10^6 \) cfu/ml
2.14 ml of the solution was diluted with 12.86 ml of PBS and then with 15 ml of 60% glycerol solution.

**R.stolonifer Spore Harvest Results:**
10 ml of harvesting solution and 10 ml of 0.9% NaCl was added to a DJ plate. Sterilized hockey stick was used to gently scrape spores and the solution was pipetted into a sterile conical tube. The solution was then spun at 3000 rpm for 10 minutes twice. Supernatant was poured off and the pellet was suspended in sterile PBS. The suspension was poured through a funnel with glass wool and rinsed with approximately 5 ml of PBS. Spores were counted and diluted by the following method:

\[
\text{Slide 1 + Slide 2 = \frac{\text{Total}}{2} = \text{Average spores /ml}}
\]

\[
(30+24)/2 \times 50,000 = 1,350,000 \text{ Spores / ml}
\]

\[
(1.4 \times 10^6)(x) = (1 \times 10^6/x)(15ml)
\]

\[
x = 1.07 \text{ ml}
\]

1.07 ml of the spore solution was diluted with 13.9 ml of PBS. 15 ml of glycerol solution was added to make stocks.
Discussion:

Spore Germination Dosing test were done with *P. destructans*, *B. cinerea*, *A. flavus*-aflatoxin positive, *A. flavus*- aflatoxin negative and D1(berry isolate). Spore Germination dosage for *P. destructans* was found to be between .4g -.5g depending on the strength of the paste(figures 2.1-2.3). The minimum dosage for *B. cinerea* was found to be .5g(Figure 2.3). *A. flavus*- aflatoxin positive and *A. flavus*- aflatoxin negative were inhibited with 2g of fermentation paste(Figure 2.5-2.14).D1(berry isolate) was inhibited with usage of 1g fermentation paste(Figure 2.15-2.17). The trials will be repeated with different future fermentation pastes to insure the minimum values previously determined and find the unknown minima for D1(berry isolate).

It was found that with the March 25th paste,.5g of fermentation paste was the minimum dose needed to inhibit the growth of *P. destructans* spores(Figure 2.1). Due to contradicting results with .5g the trial was repeated with different intervals and different fermentation pastes. It was found that .3g,.4g and .5g all failed to inhibit the fungi.1g of cell paste is already proven to work against *P. destructans* Figure 2.2). Therefore, an effective dose between .5g and 1g is being evaluated. There was a minimal amount of sporulation observed with .4g of fermentation paste. Therefore,.5g was confirmed as the minimum dose of fermentation paste needed to inhibit *P. destructans* spore growth (Figure 2.3).

The doses of fermentation paste (from March 25 2016) failed to completely inhibit *A. flavus* aflatoxin positive spores. Although, decrease in sporulation was observed starting at 3g with *A. flavus* aflatoxin positive(Figure 2.5). The second trial showed that 3g of fermentation paste from February 2nd 2016 completely inhibited the growth of *A. flavus* aflatoxin positive spores (Figure 2.6). It was concluded from other experiments that the fermentation paste from March 25 2016 was ‘weaker’. Therefore, it failed to inhibit the growth of fungi at doses which have otherwise shown to successfully inhibit fungi. All doses of fermentation paste including 2.25g successfully inhibited *A. flavus*- aflatoxin positive growth. 2g fermentation paste significantly decreased the sporulation but did not completely inhibit the growth of fungi as little sporulation could be seen on the agar(Figure 2.8). Therefore, it was determined that 2.25g of fermentation paste will completely inhibited the *A. flavus*- aflatoxin positive growth(Figure 2.9).

All doses of fermentation paste including 2.25g successfully inhibited *A. flavus*- aflatoxin negative spores. 2g fermentation paste significantly decreased the sporulation but did not completely inhibit the growth of fungi as little sporulation could be seen on the agar((Figure 2.10). Therefore, it was determined that 2.25g of fermentation paste will completely inhibited the *A. flavus*- aflatoxin negative spores. 1g and 3g of fermentation paste (from 10/1/2016) failed to inhibit the growth of *A. flavus*- aflatoxin negative spores(Figure 2.11). 1g and 3g of fermentation paste (from 10/1/2016) failed to inhibit the growth of *A. flavus*- aflatoxin negative spores(Figure 2.12).

After determination of minimum dosage for the target fungi, the tests were repeated with the use of antifungal agar diffusion tests or plugs. The values that were inhibitory to spores were not the same for the mycelial plugs. *B. cinerea* plugs were not inhibited with the use of 1g fermentation paste( figure 3.1) and *A. flavus*- aflatoxin positive and *A. flavus*- aflatoxin negative were not inhibited with 3g of fermentation paste(Figure 3.2-3.3). Additional mycelial plugs (agar diffusion) was done with cigar tip (banana isolate). No inhibition was observed but there was a decrease in sporulation with 3g of fermentation paste(Figure 3.4-3.5). Antifungal plugs (agar diffusion) test with *P. destructans* showed complete inhibition of mycelial expansion with 3g of fermentation paste(Figure 3.6 -3.7).
The antifungal inhibition test with *P. destructans* and *B. cinerea* with frozen fermentation paste showed inhibition with fermentation paste stored at -80°C is active for up to 5 months (Figure 4.4) and fermentation paste stored at -20°C is active for up to 4 months (Figure 4.5). Pastes stored at -20°C and -80°C are being tested after storage of 1 week, 1 month, and other time intervals against *B. cinerea*. The flash frozen paste was tested against $10^3$ *P. destructans* spores. As controls paste that had nothing done to it, paste that was flash frozen in liquid nitrogen only and paste that was only thawed on ice for 30 minutes was tested. All of the pastes successfully inhibited the growth of fungi. The flash frozen paste successfully worked against *P. destructans* spores. Pastes stored at -20°C and -80°C are being tested after storage of 1 week, 1 month, and other time intervals against *P. destructans*. Fermentation paste from 1/29/2016 failed to inhibit the growth of *P. destructans*. Fermentation paste from 2/5/2016 stored at -20°C failed to inhibit the growth of $10^3$ *P. destructans*. However, the fermentation paste stored at -80°C successfully inhibited the growth of $10^3$ *P. destructans*. Fermentation paste from 3/25/2016 successfully inhibited the growth of *P. destructans*.

Image study of *A. flavus* aflatoxin positive and negative, D1 (berry isolate) and D2 (berry isolate). *A. flavus* aflatoxin positive and negative showed inhibition and decrease in spore formation (Figure 5.1-5.2). D1 and D2 were not inhibited by the fermentation paste (Figure 5.3-5.4). This could be due to error of contamination or in inoculation. Repetition will be done to show more conclusive results.

Spore harvests were done to normalize amount of spores used in all trials done with different types of fungi.

These results can then be applied to the production of a biocontrol agent for industrial applications for reduction of plant wastage and treatment of white nose syndrome. To reduce plant wastage applications for strawberries, blueberries, tomatoes, corn, and other plants are being tested. The results will also be applied towards treatment of white nose syndrome by application of potential biocontrol agent in bat caves during migration season (Cornelison 2014).
Bibliography:


Appendix A:

PBS solution:

1L H₂O
8g NaCl
2g KCl
1.44 Na₂HPO₄
.24 KH₂PO₄

All were dissolved and PH was adjusted to 7.4. Autoclave to Sterilize.

Candida Harvesting Solution:

1L H₂O
500 ul tween80
9g NaCl

All were dissolved and autoclaved to sterilize.