Endophytic Fungi of *Juniperous ashei*

November 1, 2019

Moriah Sandy, Michelle Bao, Alenna Jamal, Joel Raj

Abstract

The aim of this project is to examine the benefits of *Juniperous ashei*, specifically looking at the role of endophytic fungi of *Juniperous ashei* play in helping it and other Texas plants thrive, providing a home of choice for the Golden Cheeked Warbler, preventing rot in the wood of *Juniperous ashei* and whether or not the medicinal properties of the tree are also derived from the fungal inhabitants. This project is a collaboration between the City of Austin Wildlands Biologists, Lisa and Jim O'Donnell, and the UT Austin College of Natural Sciences Freshman Research Initiative (FRI) Bioprospecting Stream.

We sampled above ground plant tissue (bark and leaves) of Juniper trees in the Vireo preserve including old growth – Golden-cheeked Warbler nesting sites – and burned and rehabilitation sites – non-Golden-cheeked Warbler nesting sites. Plant tissue was used for culturing endophytes from leaves, also some plant tissue will be frozen for metabolomic and future proteomic and environmental DNA (culture independent DNA analysis) studies. Endophytes were grouped by morphology, assigned temporary IDs and be analyzed to determine whether endophyte population and/or plant tissue significantly influences populations of endophytic fungi in *J. ashei*. Additionally, endophytes were screened for antifungal and antibacterial activity. Current work is underway to genetically characterize endophytes that were found to have biological activities, including antifungal activity against the Oak Wilt fungus, *Ceratocystis fagacearum*.

Introduction

Juniperous ashei is a drought-resistant, evergreen tree native to the south central United States and North-eastern Mexico. *Juniperous ashei* has a rich history in central Texas. Its wood is rot resistant and was used by early settlers for building homes, fences and fuel. Today, the wood is used to extract essential oils which are used in aromatherapy and medicinally to treat skin rashes, arthritis, and rheumatism.

Juniperous ashei is an integral part of the native Central Texas flora, and it is responsible for generating a large majority of the rich topsoil in Central Texas, essential for many other plant species to thrive. In fact, several species of plants including, Cedar Sage (*Salvia roemeriana*), Cedar Rosette Grass (*Dichanthelium pedicillatum*) and Texas Madrone (*Arbutus xalapensis*), will only grow in close proximity to *Juniperous ashei* trees. Root systems of *Juniperous ashei* help stabilize the surrounding earth, preventing landslides and directing water into underground springs. Additionally, *Juniperous ashei* is a special home to the endangered Golden Cheeked Warbler, which is only found in Central Texas and only resides in mixed Juniper and Oak woodlands in canyons and ravines.

Juniperous ashei also gets a 'bad rep.' Its pollen is the cause of 'cedar fever' and due to poor land management practices, in some ranch lands *Juniperous ashei* has out competed native grasses decreasing livestock productivity. While the positive features of *Juniperous ashei* far outnumber

the negative features, *Juniperous ashei* is constantly coming up against the chopping block, and commercial development and expansion of cities pose threats to clear large, currently protected areas of land where *Juniperous ashei* thrives. If allowed to happen, the outcome could be disastrous – no underground springs, floods, and a loss of other native flora and endangered birds are possible outcomes of Juniper removal.

Endophytic fungi reside in plant tissue without causing visible signs of harm. Endophytes are transmitted to above ground plant tissue either vertically from seed, or horizontally, through the surrounding environment. Endophytic fungi are emerging as key to plant survival, providing chemical defense against microbial pathogens and herbivores, and increasing plant tolerance to drought and salt stress. The goal of our project is to isolate and study endophytes from bark and leaves of *Juniperous ashei* and probe the role endophytes play in mediating known ecological and medicinal values of their plant host. For example, we are interested in whether endophytic fungi of *Juniperous ashei* influence Golden Warbler nesting site selection and whether endophytic fungi exhibit antimicrobial properties which may confer to medicinal and rot-resistant properties of *Juniperous ashei*. Herein, we report our work examining populations of endophytic fungi in Golden-cheeked warbler nesting and non-nesting sites, and screening endophytic fungi for antimicrobial activities.

Materials and Methods

Sample collection and fungal isolation

The fungal endophytes were isolated from the bark and leaves of *Juniperus Ashei* located at two sites (A and B) on the Vireo preserve in Austin, TX, USA (**Appendix Figure 1**). Nine trees were sampled in site A, three trees from three different locations on a hillside (MS2A N 30.31145 W 97.81517, MS5A N 30.31158, W97.81510, MS8A N 30.31168, W 97.81503). Site A, a previous burn site on a hillside, contains younger trees and is less densely shaded. Ten trees were sampled in site B, at four different locations (MS1B N 30.31246, W 97.81517, MS5B N30.31245, W 97.81592, MS6B N 30.31242, W 97.81602, and MS9B N 30.31218, W 97.81680). Site B, contains trees that are much older and taller in an area with a dense canopy cover providing much shade. Site B is also a documented nesting site for the Endangered Golden-cheeked Warbler. Leaf (3 small bunches of needles) and bark tissue (3 strips of bark) from nine trees in each site was collected in September 2018.

Endophyte culturing from plant tissue

Twelve potato dextrose agar (PDA) with 100 uM ampicillin and 34 uM chloramphenicol plates were prepared per tree. For each tree, nine plates were set aside for leaf tissue, with each plate divided into eight sections - six leaf fragments and two surface swipe controls and three plates for bark tissue.

Small pieces of bark and leaves were washed with water for 30 s prior to surface sterilization. Plant tissue was surface sterilized by immersion in 70% ethanol for 1 minute, 10% bleach for 3 minutes for leaves (or 2 minutes for bark), and 90% ethanol for 30 seconds. The tissue was quickly washed with sterile nanopure water before being cut into small 3 mm x 10 mm fragments and

plated onto a PDA (+AMP, +CM) agar plate. 4 3 mm x 10 mm fragments were plated onto individual sections of one plate. Controls were performed to ensure that leaf fragment surfaces were sterile by swiping a fragment on a control section of an agar plate, prior to plating the leaf fragment on a different section.

If any growth was observed in the control sections, any corresponding growth from the plating sections was discarded. Growth observed from plating sections (and where corresponding control sections had no growth) were subcultured onto new PDA (+AMP, +CM) plates to obtain pure, single isolates. The pure, single isolates were grown at room temperature and once fungal growth had covered most of the plate, or reached a stationary phase of growth, pure cultures were grouped by morphology. Three plates from each morphology group were selected for DNA isolation and analysis. Singleton isolates that could not be characterized with morphology groups were grouped alone.

Preservation of isolated fungi

Water and cellophane stocks were made before DNA isolation for preservation purposes. For water stocks, 3 fungal plugs were cut out of the growth edges of each culture using the top of a 1 mL pipette tip. Fungal plugs were placed in 1 mL of sterile nanopore water in 1.5 mL tubes and stored at room temperature. For cellophane stocks, one fungal plug was cut out of each plate and subcultured onto a PDA + AMP plate which had been covered with a piece of autoclaved, sterile cellophane. Once the fungus reached stationary phase, squares of cellophane with fungal growth were cut out and placed in 1.5 mL cryogenic tubes and stored at -80°C.

Statistical Analysis

Univariate ANOVAs were used to examine endophyte populations as a function of plant site and plant tissue type. Plant site and tissue type were treated as fixed factors. Multivariate Partial least squares-discriminant analysis (PLS-DA) was performed to visualize patterns between populations of fungi isolated from leaves, bark, and plant site.

Extraction Methods

Fungal plate cultures were extracted by using a scalpel to slice the agar fungal culture into small pieces, which were then extracted two times in 25 mL. Briefly, culture pieces were submerged and subjected to continuous smashing in ethyl acetate for 15 minutes, after which the solution was filtered using filter paper and a glass funnel. The filtrate was concentrated to dryness under vacuum using a Rotovap. The dried extract was then re-dissolved in 500 µL MeOH and stored at -20°C for further bioactivity and LCMS analysis.

Antimicrobial assays

<u>Antibacterial assays</u>

Fungi were screened for antibiotic activity against *Escherchia coli* and *Staphylcoccus aureus*. Bacteria were grown in 5 mL liquid cultures overnight at 30°C with shaking at 180 RPM. Next, a full lawn of bacteria was prepared on LB agar plates, following which a fungal plug was immediately placed on the center of the plate. The assay plates were incubated overnight at 30°C. The assay plates were then checked for a ring of inhibition around the fungal plug (i.e. no bacterial growth).

Antifungal assays

Antifungal assays were performed from fungi which had been isolated and characterized from UT Austin Oak Trees which had previously been identified as having the Oak wilt disease. We were unable to isolate oak wilt from these trees, however in Summer 2019, we were kindly provided strains of Oak Wilt, *Ceratocystis fagacearum*, from the Texas A&M Plant Disease Diagnostic Lab. Approximately 100 Juniper fungal isolates (selected from each morphology group) were tested for antifungal activity against the different Oak fungal isolates and Oak wilt.

Competition assays: Antifungal assays were performed by creating a competition plate of two fungi, where an agar plate was divided into two sections, and a Juniper fungal isolate was placed equal distance from an Oak fungal isolate, control plates were created for both the Juniper fungal isolate and competing Oak fungal isolates. Measurements of growth area of both fungi on each plate (assay and controls) were measured three times a week for two weeks. Differences in morphology, growth area, and growth rate were analyzed. Antifungal activity is defined as 1) preventing growth of competing fungus, 2) slowing down growth rate 3) reducing area of plate covered by competing fungus as compared to single fungus controls.

Cellophane assays: Antifungal assays were performed by first covering a PDA (+AMP, +CM) plate with a piece of sterile cellophane. Juniper fungal isolates were then subcultured onto the cellophane -PDA plate and allowed to grow until the plate was covered or the fungus had reached stationary phase of growth. The cellophane was then removed, leaving a cell-free PDA plate with the excreted metabolome of the Juniper fungal isolate. The oak fungus was then plated onto the cellophane-less plate and growth was observed compared to an oak fungus plated on a non-cellophnae PDA plate. Measurements of growth area of both fungi on each plate (assay and controls) were measured three times a week for two weeks. Differences in morphology, growth area, and growth rate were analyzed. Antifungal activity is defined as 1) preventing growth of competing fungus, 2) slowing down growth rate 3) reducing area of plate covered by competing fungus as compared to single fungus controls.

Results and Discussion

Leaves and bark of Juniper trees in the Vireo preserve were sampled and cultured for endophytic fungi from old growth trees (Golden-cheeked Warbler nesting sites) and burned and rehabilitation sites (non-Golden-cheeked Warbler nesting sites). Endophytes were grouped by morphology, assigned temporary IDs. 305 fungal isolates were grouped into 98 different morphology groups (**Figure 1**).

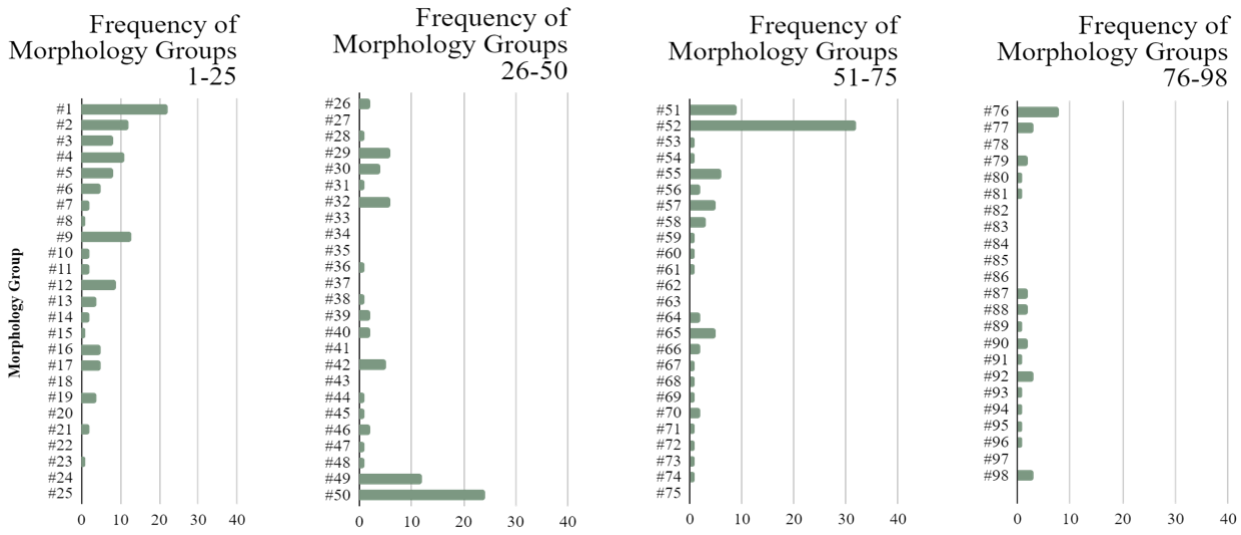


Figure 1. Frequency Distribution of Morphology Groups

Endophytes were further analyzed to determine whether endophyte population differs by plant tissue type, plant sample site and/or an interaction of the tissue type and sample site. Supervised PLS-DA analysis, selecting for site location and tissue type explained 30% and 32% of variation respectively (**Figure 2**). PERMANOVA results suggest that Juniper endophyte populations are influenced by plant site (*MS* 8.977, *pseudo F* 3.0043, $P = 0.056$), plant tissue type (*MS* 8.977, *pseudo F* 10.6831, $P = 0.002$), and their interaction (*MS* 13.719, *pseudo F* 4.5917, $P = 0.015$). **NOTE**: A limitation of these preliminary results is that analysis is based on fungal morphology types, rather than genetic characterization. Most likely genetic identification using rDNA finger printing will reduce the diversity pool to ~15-30 genotypes.

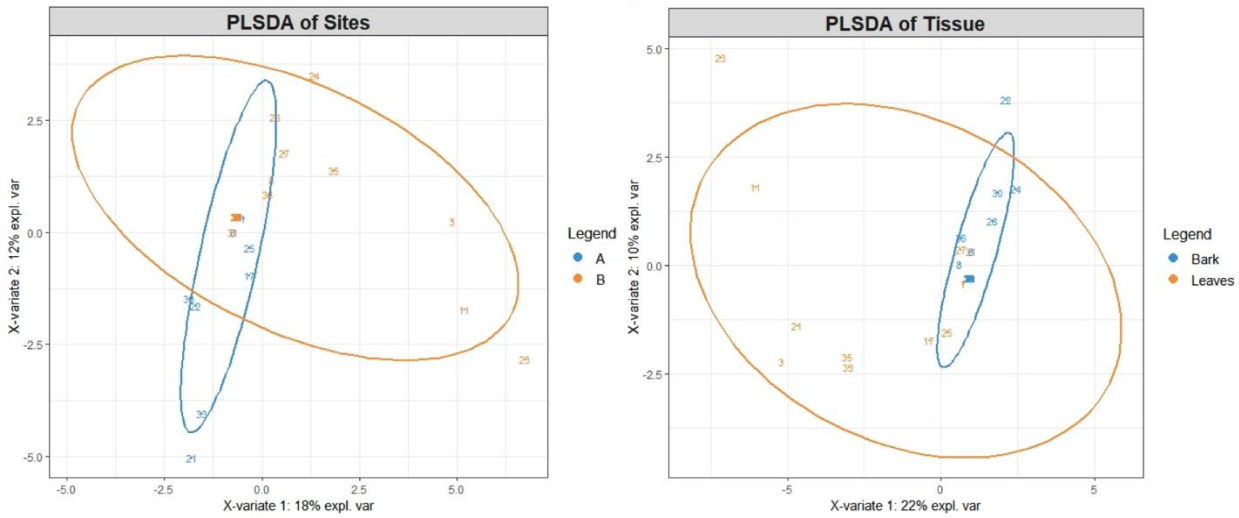


Figure 2. Partial Least Squares - Discriminant Analysis (PLS-DA) score scatter plot showing cluster separation of endophyte populations by site and by tissue type.

In Spring 2019, fungi from each morphology group were screened for antibacterial and antifungal activity. Six antibacterial activity against *E. coli* and *S. aureus*, and fourteen isolates were found to have antifungal activity against the Oak wilt fungus *Ceratocystis fagacearum* in both whole fungi and fungal extract antimicrobial assays (**Table 1**). An example antifungal assay (isolate 112 vs oak wilt) is shown in **Figure 3**. So far, we have identified four of the active isolates (43, 101, 112, 133) as *Tricoderma* species. While *Tricoderma* species are There is precedent in the literature for using *Tricoderma* species as antifungal agents in agricultural food crops (Zhang *et al*, *Pesticide Biochemistry and Physiology*, **2018**, *147*, 59). Future studies will be focused on genetically characterizing the other bioactive Juniper fungi. In parallel, we are working to isolate, purify and structurally characterize the active molecules in all biologically active Juniper isolates.

Table 1. Juniper fungal isolates with antibacterial and antifungal activities.

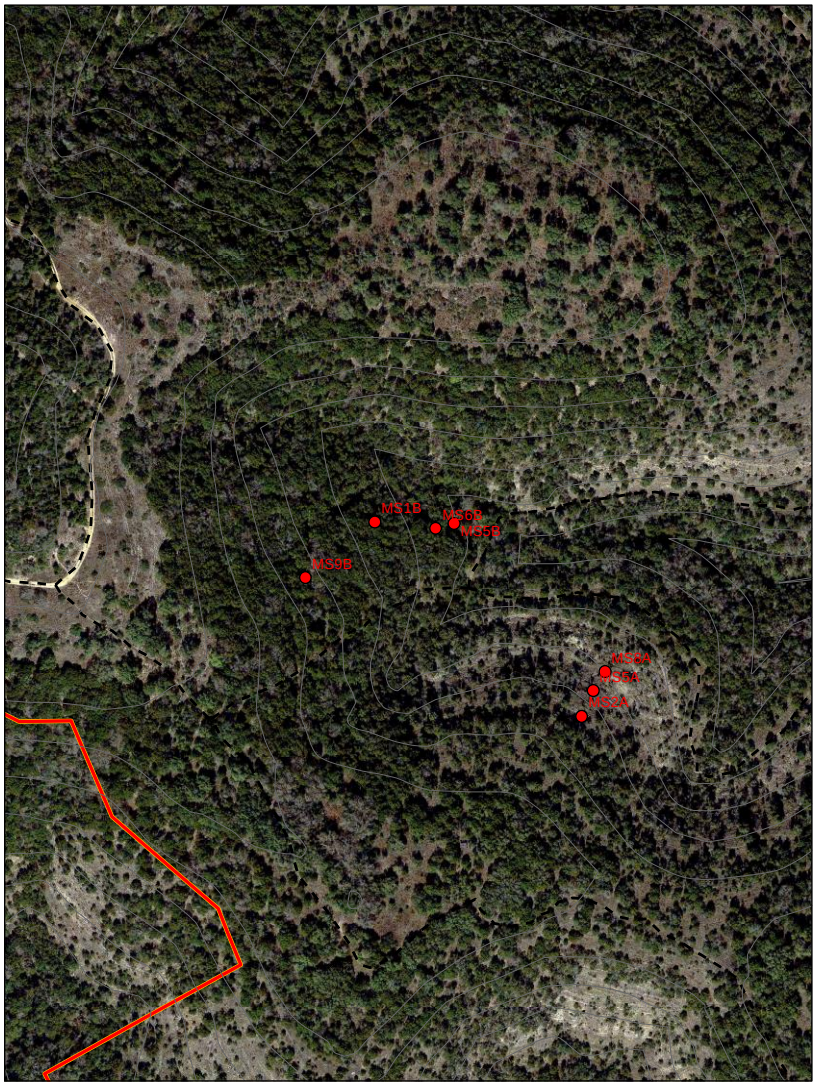
Juniper Fungus ID	Antibacterial Activity (Y/N)	Antifungal Activity (Y/N)
2	Y	N
43 (*Tricoderma)*	N	Y
50	N	Y
60	N	Y
85	N	Y
92	N	Y
101 (*Tricoderma)*	N	Y
103	N	Y
109	Y	N
110	N	Y
111	Y	N
112 (*Tricoderma)*	N	Y
128	N	Y
131	N	Y
133 (*Tricoderma*)	N	Y
134	Y	N
135	Y	Y
143	Y	N
148	N	Y



Figure 3. Antifungal assays of Juniper fungal isolate 112 *Tricoderma* sp. (right) against oak wilt (left) over 6 days (culture plates left to right, day 1, day 3, day 6).

Conclusions – In conclusion, we have found that endophyte populations differ by site location and plant tissue type. 305 fungal isolates were grouped into 98 different morphology groups, and fungi from each morphology group were screened for antibacterial and antifungal activity. Ongoing work is focused on 1) identifying the bioactive fungal isolates using 18S rDNA sequencing, 2) identifying the bioactive molecules responsible for antifungal activity. Future work will focus on developing an assay to test the effectiveness of anti-oak wilt fungi and anti-oak wilt molecules against diseased trees.

Appendix



Appendix Figure 1. Map of *Juniper Ashei* sampling sites (A and B) at Vireo Preserve, Austin TX.

Appendix Table 1. Detailed morphology descriptions of each morphology group.* *Note: this table will be updated with genus species information once DNA sequence analysis is complete.

Morphology Group	Description Top	Description Bottom
1	White, foamy-looking body. Growth covers the plate. Move heavily concentrated growth at the center, with dissipation towards the outer edges. Looks like a thin layer of white lint.	Salmon-colored. Uniform growth. No filaments. Is a bit lighter in the center and gets marginally darker after about an inch
1i	black agar, visible white clumps	light peachy agar
2	Body is white and looks a bit like short animal fur. There is more growth separation toward the origin, but it then begins to condense towards the outer edges.	salmon-colored, similar to group 1.
3	White/peach colored growth that appears grainy. The center of some of the samples are dark green/brown, but disperse as a gradient into the sandy white color. This is not present in all of the samples.	Salmon-colored as well. The undergrowth is not as thick, or as uniform as groups 1 and 2. There is a bit more patchiness in the growth.
\	Fuzzy white body with dark, muddy green color with white sporadic edges. Small growth diameter, covers about 1/4 or less of plate area.	White body color but two have orange tint in some areas. Dark green fungal color on fungal plug area.
6	raised, white, snow-like growth that covers the entire plate. Some plates have darker portions, but the majority of the plate is white and soft/fuzzy	opaque white or dark orange/brown with one plate in between these two colors
8	Very raised, small stem-like structures that protrude upward on at least half the plate; grayish	1-2 medium-sized opaque black regions; the rest of the plate is kind of a solid light white/gray
10	fuzzy white border, dark gray/brown center, dense circular growth	flat (no protruding) but with translucent border and deep inner white circle
11	small deep circle on top of agar. frosted/ snow like	flat (no protruding) but same with small frost inner white circle
12	white fuzzy. Not very dense	light grey
13	Covers the entirety of plate with cloud-like white fungi. One plate has growth that resembles hair while another one resembles more like carpet. Dark green center	One plate has a whiter bottom with an edge of brown tint while the other plate has almost an entirely light brown colored bottom plate.

14	Fungi is growing radially. New parts of the fungi are very translucent. Old fungi developed white fluffy stuff.	Fungi gets less and less translucent near the middle (where the oldest part of the fungi is)
15	Slightly darkened white color. Solid color. Growth is somewhat radial but not quite. In between a radial growth and a 3-leaf clover	Looks exactly like top
17	Fungi grew radially but has no pigment. Hard to even see the fungi grown. Very smooth except near the middle some hyphae seem to be growing up. It's a very small amount though.	Same as top
19	flat; gray with some white growth throughout	opaque white with some dark yellow/brown regions
21	opaque dark forest green raised growth that covers the plate; some translucent pale yellow scattered throughout. While doing stocks and maintenance subculture, noticed the agar was a lot softer, falling apart, more liquid (no other plate seems to have that issue)	light yellow growth with some opaque and some translucent regions
22	For the most part, radial growth. Wave like concentric circles. Working from edge to center colors were white, dull yellow, buttercup yellow, reddish-orange.	All the colors shown on the top are on the bottom but are more darker and richer in color
23	white frosty entire border. tan tint near center of circle, but still very white	light peach/tan white circular growth
24	Fungi grew radially and is opaque. For some reason after growing flat for most of the time near the end a ring kind of formed. Mostly white throughout but near the middle it gets slightly darker	Middle is light tan and the edges are white. Everything else the same as top
25	Light gray in color. Growth is radial. Newer fungal growth is lighter in color and older fungi turns a light gray. The fungi grow flat but after some time it starts to grow upwards	Solid off-white color. Slight closer to a tan color
26	opaque gray growth that covers the plate with fuzzy white protrusions	about half the plate is opaque and very dark (either jet black or black surrounded with brown); the rest of the

		plate is either a light gray/brown or opaque white
28	dark light brown, light tan border on top	black surrounding
29	white frosty entire border. tan tint near center of circle, but still very white	light yellow growth with some opaque and some translucent regions
30	very thick layer of white frost (resembles like skiing resort). entire plate is covered, rises from on top agar	translucent brown color, same color as the original agar (plate #2 has a very small visible black dot)
31	Growth was radial. Most of the fungi is white but near the center the white is more yellowish	There are black streaks spread throughout the fungi on the underside
32	Faint green body color with white outer edges. The growth covers ~1/2 of plate. There is also a slight red outer glow coming from each fungus	Reddish/Orange tint to all plates. Dark red/brown fungal plug center. Resembles shape and color of an apricot
34	dark green brown circular rings	dark green/black middle, light yellow outer ring
35	growth all over plate with white spots on top	bright yellow
36	Radial growth with translucent edges. Growth is in concentric circles with each circle having a distinct darker color. Overall, fungi have a coffee color.	Translucent orange with certain darkened spots
37	white spots with dark blue centers throughout plate	spots are yellow from bottom, still can see the center
38	Fungi is growing radially and is very dense making it opaque. It appears to be almost a true white near the middle and a dull white near the edge.	The bottom just seems to be slightly darker...it may even be the same shade as above.
39	Dark gray, almost black. Fungi started of growing radially but at the edges there are more random growth. Fungi near the edge have white tops while fungi near the middle are solid dark gray.	A definite darkened line is seen where the fungi switched from growing radially to random growth. Translucent dark gray with edges that are white
40	white, filamentous. White spots randomly throughout.	light yellow/gray underside.
41	Circular, orange body. White border that gradually fades. Dark orange plug. Fungal grew radially.	Same radial growth. Darker orange body color with light orange/yellow outer edge. Resembles sliced grapefruit

42	Nebulous growth with large "petals" of cotton-like fungi coming out at the edges. White in color with slight yellow tint at petal edges.	Nebulous growth. All white color
43	Radial growth. Orange body color but has hair-like white fungi growing outwards above the orange color. Small yellow edge.	Deep orange color that radiates radially into a yellow color. Almost like a sun!
44	very dark brown/black agar color, light orange ring on top, with orange fungal plug center	very dark large black inner circle, thin outer orange ring
45	cranberry/wine agar color with large grey/white alternating rings center	whole plate is cranberry/wine color
46	white, filmy top. blurry.	red tint.
47	Light distribution of a white top. Fungi grew radially	Deep red-orange color. Barely translucent.
48	If looks exactly like how a water freezes on top... Translucent gray. Hyphae are climbing upwards. Growth is radial.	Translucent gray
49	Light and pale. stretches of hyphae	Translucent white
50	Rose petal, fluffy and white	Translucent light pink
51	Web like white hyphae	Translucent yellow tint
52	White powder	Translucent yellow tint
53	White and green mountain; slow growth; gray/green center with fuzzy white border	black (or incredibly dark green) with the same distinct white border
54	White and green mountain; faster growth	black
56	Irregular, flat growth with curled margins; very faint pink color and very fuzzy green undertone (moss like)	Extremely dark brown irregular growth with pink areas.
57	Irregular, mostly flat growth with filamentous margins; very faint green hue with white cotton like growth above it	Faint yellowish color; dark green plug center
58	fuzzy brown growth with white protrusions; white center, brown middle ring, white border	opaque white with brown or black regions
59	Irregular growth, translucent white with a small pink hue area. Little white fuzz growing on top	Very faint yellow with an orange-pink circular area

60	Faint green hue that covers entire plate; Small circular white mossy "island" in the middle with dark green faint middle	Faint yellow that covers entire plate; gets progressively darker brown when getting closer to middle
61	All over the place white patches, sort of like a marshmallow colony. Light brown spots on each small clump	Colony like, sporadic. Faint yellow color
62	Light brown inner ring with textured outer ring	Translucent white/yellow tint
63	Hyphae grow like white snow/ice crystals	Translucent white with black spot in middle
64	Charcoal color with white webs on top	Black
65	Layers of white fuzz	Yellow tint
66	Slow growing pink and white fuzz	Salmon pink
67	White hyphae grow upward. Middle is green	White outer ring. Green then dark green inner ring
68	White upward growing hyphae with gray layer underneath	Gray
69	Many 2mm diameter lime green/white spots	Light green
70	Layer of white fuzz with small white cotton ball structures	White
71	Slow growing light gray fuzz	Pink-brown
72	Sporadic stretches of lime green fuzz	Yellow Green
73	Slow growing gray fuzz	Gray rings
74	Textured manila paper; snowflake edges	Yellow with orange tint
75	distinct inner white ring, then light frost on outside	white ring, peachy border
76	yellow inner circle, light white border, visible white hyphae	dark mustard yellow inner circle, light yellow border
77	light grey ring with frosted white tops	entire surface manila/peachy
78	small whit frosted ring, protruding upward	small area is white/manila color
79	large white protruding hyphae, small outer black ring	entire surface white/manila color
80	small region of white growth, flat hyphae	small surface area of manila/peachy color
81	very slow growing radial fungi. red colored PDA. Fully white on top can see like small rings	Dark in the middle and surrounded by white
82	Textured gray and white with red secondary pigment	Opaque gray

83	Gray-Green fuzz with white powder on top	Black inner ring with gray outer ring
84	Slow growing flat white flower-like pattern	White
85	Gray, lots of long hyphae grow to lid	Yellow tint
86	Slow growing white radial lines with concentric circles	White
92	clear tint of agar, light brown inner ring	same thing
93	dandelion orange, white tint border,	same thing
94	white frosted fluff, bright pink center	white frosty hyphae, dark reddish black center
95	large white frosted ring, red center	light peachy color, light pink/reddish center
96	frosted grey tint on top of lightly red agar	peach white blots, with red center
97	multiple blotches of light green white frosted on top	same thing
98	light grey on bottom, then white spider-web like frost	light grey background, peachy
99	very translucent agar, black fungal plug	light peach background