Antibacterial Activity of Endophytic Fungi Isolated from Arkansas *Vitis aestivalis* (Norton / Cynthiana) Vegetative Tissue.

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Abstract

Grape production in the United States is a $162 billion business. *Vitis vinifera* is the major species in this industry, but less than desirable growing conditions for these grapes predominate in the US, making it critical that alternative species be identified for maximal production. One of these species, *Vitis aestivalis* (Cynthiana/Norton), is both drought and disease tolerant, making it an excellent candidate for widespread production. Previous research has revealed that *V. aestivalis* contains fungal endophytes. Other plant endophytes have been found to exhibit antibacterial properties; this project tested *V. aestivalis*' endophytes against bacteria known to be problems in food preservation and general sanitation. Two fungi (*Xylaria* and *Alternaria* or *Coniothyrium* species) exhibited inhibition consistently and other fungi showed erratic inhibition against the bacteria. More research is needed to test the remaining *V. aestivalis* endophytes and identify the antibacterial compounds the endophytes produce.

Table of Contents:

List of Tables

List of Figures

Introduction

Grapes in the United States are a $162 billion industry, including table, raisin, and wine. Internationally, *Vitis vinifera* grapes are most commonly used for wine-making, but the species is not well adapted to the environments that encompass the majority of the United States (MKF Research, 2007). This deficiency has driven significant efforts to hybridize varieties that can flourish in the range of environments that exist across the United States and produce grapes of acceptable quality. *Vitis aestivalis* (commonly known as Norton or Cynthiana) grapes are one of these hybrids. This grape is native to the United States and is much more hardy than its European (*V. vinifera*) counterparts (Stover *et al*., 2009). The relative hardiness and difficulty in propagating this variety has driven prior research on this grape and led to the current research.

Arnold *et al*. (2003) explains that all plants studied to date contain a fungal endophyte, and *V. aestivalis* is no exception. In Arnold *et al.* (2003), endophytes in tropical trees were studied and found that the endophyte helps decrease the damage caused by pathogens and prevent the tree from dying. While *V. aestivalis* is not a tropical tree, it does contain fungal endophytes, though the role of the grape's endophytes is undetermined. It is plausible though, that it provides protection to the plant similar to the tree endophyte, and is a factor in the grape's environmental tolerance. Furthermore, it is possible that this protection extends beyond the plant; fungal endophytes in other plants, such as *V. vinifera*, have been shown to have antibacterial activity (Núñez *et al*., 2012). It is, therefore, plausible that the endophytes found in *V. aestivalis* exhibit antibacterial activity.

Antibacterial compounds were discovered in nature and later synthesized by Man. Antibiotics made from natural products often are complex structures with substituents that target certain bacteria; synthetic drugs are made from chemical structures not found in nature and block specific bacterial mechanisms (Walsh 2003). Over the last few decades, antibiotic resistance has increased due to overuse by physicians and misuse by patients. As a result, multi-drug resistant (MDR) bacteria, which now threaten humans through both healthcare-related and foodborne illnesses, are rapidly increasing (Skariyachan *et al.*, 2016). Since microorganisms constantly adapt to their environment and we now expose microorganisms to manufactured antibiotics, new antibiotic discoveries are an important part of preventing complete antibiotic resistance in the entire population of microorganisms. This study seeks to determine whether the endophytes found in *V. aestivalis* grapevines exhibit antibacterial activity.

Four bacteria were chosen to test against for antibacterial activity. The first bacterium chosen was *Escherichia coli*. While *E. coli* is a normal member of human intestinal flora, when found outside the intestinal tract, the bacterium can cause a multitude of problems, including food poisoning. In the summer of 2018, an outbreak of *E. coli* O157:H7 infected Romaine lettuce occurred across thirty-six states, sickening 210 and killing five people (Multistate Outbreak, 2018). Additionally, *E. coli*, (specifically Shiga toxin-producing cells) can cause hemolytic uremic syndrome, which can result in loss of kidney function and may lead to permanent health damage and death (Hemolytic-uremic syndrome, 2018). Today, the *E. coli* population worldwide shows resistance to many popular antibiotics, including tetracycline, ampicillin, and gentamicin. Now, many countries are reporting *E. coli* strains resistant to fluoroquinolones. This resistance can

be attributed to both human use and agricultural overuse, with tests showing large populations of antibiotic-resistant *E. coli* in both chickens and pigs (Taiwo & South, 2016).

The second bacterium chosen was *Klebsiella pneumoniae*, a gram-negative bacterium commonly associated with healthcare-related infections like pneumonia, meningitis, and blood infections. This bacterium, though frequently found in the human intestine, causes significant health issues when found in or on other parts of the human body. Infections from the genus *Klebsiella* rarely spread to healthy individuals; the bacterium is more likely to infect people with compromised immune systems, on ventilators, or taking a long course of antibiotics. Like all bacterial infections, if a person is infected with a non-resistant bacterium, antibiotics can be prescribed. Administration instructions must be followed exactly, lest the bacteria gain resistance to the drugs leading to more virulent infections. If the person is infected with an antibiotic-resistant bacterium, treatment is much more difficult and lab testing must be accomplished to determine the most effective antibiotic to use for the infection (Klebsiella pneumoniae in Healthcare Settings, 2012). Unfortunately, as Kidd *et al.* (2017) reveal, some of the last lines of antibiotics against *K. pneumoniae* have lost effectiveness, leading to cases of community-onset infections.

Pseudomonas, an opportunistic pathogen, is the third genus of bacteria chosen for this study. First, many *Pseudomonas* species (spp.) can cause catastrophic damage to milk and dairy products, meat products, and water supplies. While milk and dairy products are pasteurized and many of the bacteria present are killed, *Pseudomonas* spp. produce enzymes capable of withstanding the pasteurization process. While

Pseudomonas spp. are common bacteria found in many animal intestinal tracts, mishandling of meats can allow the spread of this bacterium to the muscle of the animal and result in contamination of the meat. According to Raposo *et al.* (2016) *Pseudomonas* spp., specifically *P. aeruginosa,* is believed by many to be an excellent indicator of water supply contamination levels; the bacterium's origin in water is thought to be due to either sewage leaked into the water supply or agricultural runoff. The fact that *P. aeruginosa* is in the water supply is cause for concern, since ingestion is one of the main routes of transmission. Additionally, this genus has been linked to acute infections in the human population (Raposo *et al.*, 2016). What is more concerning, however, is the fact that *Pseudomonas aeruginosa* has become resistant to many different antibiotics in recent years and has become difficult to treat (Pseudomonas aeruginosa in Healthcare Settings, 2018). The Centers for Disease Control and Prevention (CDC) estimate that six thousand people are infected with a MDR strain of *P. aeruginosa* yearly and approximately four hundred people die due to the infection. The CDC has now deemed the threat level of MDR *P. aeruginosa* as serious (Pseudomonas aeruginosa in Healthcare Settings, 2018). Antibacterial activity against this organism by one of the *V. aestivalis* endophytes tested could possibly help fight the multi-drug resistance epidemic.

The last bacterium chosen for this study was *Staphylococcus aureus,* a gram-positive MDR bacterium that is found in about thirty percent of the human population's noses, with little harm to its hosts. However, *S. aureus* is responsible for both hospital and food borne infections. The bacterium can cause pneumonia, endocarditis, (infection of the heart valves), and osteomyelitis (a bone infection), all three of which can be fatal (Staphylococcus aureus in Healthcare Settings, 2011). Additionally, *S. aureus* can

produce toxins which lead to food poisoning. However, *S. aureus* food poisoning is rarely treated with antibiotics, since the bacterium itself does not cause the disease; a toxin produced by the bacterium causes the illness (Staphylococcal Food Poisoning, 2016). Nevertheless, *S. aureus* is a MDR bacterium and is now resistant to vancomycin, methicillin, and penicillin (along with many of its derivatives). As such, new antibiotics are needed to help prevent total resistance in the near future (Staphylococcus aureus in Healthcare Settings, 2011; MRSA in Healthcare Settings, 2016).

With antibiotic resistance on the rise, the question is where can the human population derive new antibiotics from and plant fungi may be the answer.

Hypotheses:

The hypothesis for this study is that some of the endophytic fungi found in Arkansas (AR)-grown *V. aestivalis* vegetative tissue will demonstrate antibacterial activity against at least one of four selected bacteria strains. The null hypothesis is that no antibacterial activity will be exhibited by any of the *V. aestivalis* fungal endophytes.

Methods:

Fungi were isolated from *Vitis aestivalis* (Norton / Cynthiana) grape vine cuttings from Altus, AR. Cuttings were taken from the vine and shipped to MTSU. The cuttings were surface sterilized by being soaked in 90% ethanol for two minutes, drained, then soaked in 2.5% bleach for two minutes, drained, and finally soaked in 70% ethanol for two minutes and drained. The sterilized pieces were then cut into sections before being plated on malt extract agar (MEA), laced with Neomycin (1 mL Neomycin per 1 L media) to inhibit bacterial growth. Cuttings were divided into three sections (A, B, and

C). The fungi were isolated and purified by plating fungal pieces in serial succession, until the fungal plates showed no visible sign of contamination on the MEA plates. Thirteen of these fungi were chosen based on various physical attributes (Table 1).

Table 1: AR vegetative tissue, fungal description, and name.

Plant Tissue	Section	Physical Attributes	Fungus Name
Leaf A	8C	Pink/White	Fungal Endophyte/ *Xylaria*
Leaf A	4D	Tan/Gray	*Fusarium profileratum*
Leaf B	4C	Blood colored	*Epicoccum nigrum*
Leaf B	7C	Green/Gray	*Alternaria tenuissima*
Leaf C	9C	White	*Fusarium esquiseti*
Leaf C	10A	White	*Pestalotiopis microspora*
Stem A	7C	Matte Peridot Green	*Coniothyrium/Alternaria*
Stem A	9C	White	*Fusarium esquiseti*
Stem B	6B	Rose	*Aureobasidium*
Stem B	9B	White	*Alternaria tenuissima*
Stem C	3D	Yellow Slime	*Aureobasidium*
Stem C	8C	Gray	Unknown, poss. *Alternaria*
Leaf C	10C	Dark Gray	*Curvularia geniculatus*

The procedure of dos Santos *et al.* (2015) was modified slightly as follows. New streaks of the thirteen AR fungi were plated on MEA and grown for six days at room temperature before being transferred to a 4° C refrigerator. While the fungi grew on the MEA plates, potato dextrose agar (PDA) was prepared, per the procedure of dos Santos *et al*. (2015). The fungi were then transferred onto the PDA plates and incubated for one day at 33° C followed by six days at approximately 30° C. Two plates of each fungus were prepared to ensure availability of a viable sample after seven days growth.

Four different bacteria (*E. coli, K. pneumoniae, P. aeruginosa*, and *S. aureus*) were acquired from Dr. Jeff Leblond (MTSU Biology Faculty) and grown on trypticase soy agar (TSA) for two days before being placed in the refrigerator. Mueller Hinton agar (MHA) plates were prepared for use during the initial testing of the fungi and bacteria.

Mueller Hinton agar plates were labeled based on the bacterium and the fungus placed within them. Bacteria were spread in a lawn on the corresponding plate and a fungal disc was then applied to the center of the bacterial lawn. The fungal discs were cut from the PDA plates, and contain both fungi and PDA. To ensure that the fungal discs placed on the plates were similar in size, plastic drinking straws (cut into approximately three-centimeter sections, with a diameter of 5 millimeters, and soaked in 70% isopropyl alcohol) were used to make discs of fungi; the straws were discarded after one use. For the first trial, fungal discs were placed fungus side down on the MHA plate. Once the fungi and bacteria were placed on MHA plates, the plates were placed in the incubator at 37° C for one day (for bacterial growth) and then 30° C for two more days (fungal growth).

After three days of growth, the plates were removed from the incubator and examined for zones of inhibition (ZOIs). All plates showing ZOIs, were set aside and the ZOI was measured from the edge of the fungal disc to the edge of the ZOI. The plates were placed in the refrigerator for storage for later review, if needed.

This process was repeated two more times, with slight variations. During the second trial, the fungal discs were placed agar side down on the MHA plates. In the third trial fungal discs were placed fungus side down. Additionally, in the third trial, plates that showed inhibition during one, but not both, of the previous trials were tested using

three plates and discs, as follows. One disc was placed fungal side down, one agar side down, and the third disc was placed either agar up or down depending on which previous trial showed inhibition.

For plates that showed inhibition on the third trial only or on only one of the three third trial plates, a fourth trial was performed. For the fourth trial, four plates per fungus were spread with bacteria; fungal discs were placed agar up on two and agar down on the other two.

Once the ZOIs were measured, statistical analysis was performed on the data to determine statistical significance of the results. Tests performed included a a Kruskal-Wallis One Way Analysis of Variance (KW) and a One Way Analysis of Variance (ANOVA) using Sigma Stat (v.3.1).

Results:

Replacement fungi:

After the first seven days of incubation, one fungus (AR Leaf B 4C, color of blood) had yet to grow on PDA media. As such, another fungus (AR Leaf C 10C Dark Gray) was chosen as a replacement sample.

Selection of Fungi:

Nine of the thirteen fungi for this experiment were chosen based on unique physical attributes such as unusual colors or textures. The *Xylaria* were pinkish white, the *Epicoccum nigrum* were blood colored, and the *Coniothyrium* or *Alternaria* were matte peridot green in hue. Both of the *Aureobasidium* genera were brightly colored and had unique textures. One was rose colored with a matte appearance and the other was yellow and slimy in appearance. The remaining four were of various shades of gray:

tanish gray (*Fusarium profileraum*), greenish gray (*Alternaria tenuissima*), dark gray (*Curvularia geniculatus*) and gray (unknown geneus).

Four additional fungi were chosen based on the abundance of white, fluffy fungi found as the AR endophyte (Table 1).

Bacterial Analysis:

Overall, most of the fungi tested in this research showed little to no inhibition of the chosen bacteria. Only two fungi showed consistent inhibition against *S. aureus* in every trial and only one fungus showed consistent inhibition against *E. coli*. Other fungi showed inhibition in some trials but not in others (Tables 2 - 5).

Table 2: Leaf fungal endophyte results of antibacterial activity.

Bacteria	Trial	Fungal Endophyte/ *Xylaria*	*F. profileratum*	*E. nigrum*	*A. tenuissima*	*F. esquiseti*	*P. microspora*	*C. geniculatus*
E. coli	Trial 1	~ 5mm	---	NG	---	---	---	---
	Trial 2	~ 4mm	---	NG	---	---	---	---
	Trial 3	T3	---	NG	---	---	---	---
K. pne	Trial 1	---	---	NG	---	---	---	---
	Trial 2	---	---	NG	---	---	---	---
	Trial 3	---	---	NG	---	---	---	---
P. aer	Trial 1	---	---	NG	---	---	---	---
	Trial 2	---	---	NG	---	---	---	---
	Trial 3	---	---	NG	---	---	---	---
S. aur	Trial 1	---	~ 2mm	NG	~ 1mm	---	---	~ 1mm
	Trial 2	~ 6mm	---	NG	~ 1mm	~ 1mm	~ 1mm	---
	Trial 3	T3	T3	NG	T3	T3	T3	T3

Inhibition Seen

T3 = See trial 3

NG = No growth on potato dextrose agar

--- = No growth on Muller Hinton agar

Table 3: Stem fungal endophyte results of antibacterial activity.

Bacteria	Trial	*Coniothyrium/Alternaria*	*F. esquiseti*	*Aureobasidium*	*A. tenuissima*	*Aureobasidium*	Unknown, poss. *Alternaria*
E. coli	Trial 1	---	---	---	---	---	---
	Trial 2	---	---	---	---	---	---
	Trial 3	---	---	---	---	---	---
K. pne	Trial 1	---	---	---	---	---	---
	Trial 2	---	---	---	---	---	---
	Trial 3	---	---	---	---	---	---
P. aer	Trial 1	---	---	---	---	---	---
	Trial 2	---	---	---	---	---	---
	Trial 3	---	---	---	---	---	---
S. aur	Trial 1	~ 1mm	---	---	---	---	---
	Trial 2	~ 1mm	---	---	---	---	---
	Trial 3	T3	---	---	---	---	~ 1mm

Inhibition Seen

T3 = See trial 3

NG = No growth on potato dextrose agar

--- = No growth on Muller Hinton agar

Table 4: Antibacterial activity of selected fungi.

Trial 3	Agar way	Fungal Endophyte/ *Xylaria*	*F. profileratum*	*A. tenuissima*	*F. esquiseti*	*P. microspora*	*C. geniculatus*	*Coniothyrium/Alternaria*
E. coli	Up	~ 6 mm	NPM	NPM	NPM	NPM	NPM	NPM
	Down	~ 5 mm	NPM	NPM	NPM	NPM	NPM	NPM
S. aur	Up	~ 4mm	---	---	---	---	~ 2 mm	~ 3 mm
	Up	NPM	---	---	NPM	NPM	---	NPM
	Down	~ 5 mm	---	---	~ 1mm	---	---	~ 2 mm
	Down	~ 5 mm	---	---	---	~ 1mm	NPM	NPM

Inhibition Seen

--- = No growth on Muller Hinton agar

NPM = No plate made

Table 5: Trial 4 of fungi with inconsistent inhibition.

Trial 4	Agar way	*F. esquiseti*	*P. microspora*	*C. geniculatus*	Unknown, poss. *Alternaria*
S. aur	Up	---	---	---	---
	Up	---	---	---	---
	Down	~ 1mm	---	---	---
	Down	---	---	---	---

Inhibition Seen --- = No growth on Muller Hinton agar

AR Leaf A 8C, pink/white, identified as a fungal endophyte likely to be of the genus *Xylaria,* showed constant inhibition against *E. coli*; the ZOI ranged from 4 mm to 6 mm. AR Leaf A 8C also showed inhibition against *S. aureus* on four of five plates and had a ZOI range of 4 mm to 6 mm (Tables 2, 4). AR Stem A 7C, matte peridot green, identified as either a *Coniothyrium* or *Alternaria* species, showed consistent inhibition against *S. aureus* and had a ZOI range of 1 mm to 3 mm (Tables 3, 4).

Two of the three *Fusarium* spp. showed slight inhibition against *S. aureus* (Tables 2 – 5). The *F. profileratum* showed inhibitory action on one of six plates. One *F. esquiseti* showed inhibition on three of nine plates, and the other showed no inhibition. On the fourth trial only one *F. esquiseti* plate showed a ZOI.

One of the *Alternaria tenuissima* samples exhibited inhibition against *S. aureus* on two of six plates, while the other showed no inhibitory properties against any of the bacteria (Tables 2 – 4).

Both the *Curvularia geniculatus* and the *Pestalotiopis microspora* showed inhibition against *S. aureus* on two of the nine plates (Tables 2, 4, 5).

The unknown fungal genera showed inhibition against *S. aureus* on one of seven plates. Neither of the *Aureobasidium* species showed any visible ZOIs during this experiment (Table 3).

Statistical Analysis

Both a KW and an ANOVA statistical analysis was performed on the collected data. The *Xylaria*, was shown to have a statistically significant inhibition against *E. coli* ($p < 0.001$) on both tests when compared to the other fungi on *E. coli*. The ANOVA test resulted in a power of 1.000.

The KW of *S. aureus* showed there was not a statistical difference between the inhibition effects of any of the fungi ($p = 0.057$). The ANOVA, however, indicated there was a statistical difference ($p = 0.004$; power of 0.883) between antimicrobial activity of the *Xylaria* and that of the other fungi.

Discussion:

No fungi exhibited inhibition against every bacterium tested and only a select few displayed consistent inhibition against one bacterium throughout every trial. The *Xylaria* spp. proved to exhibit antibacterial properties. Research conducted by Canli *et al.* (2016) showed that *Xylaria hypoxylon* inhibited both *E. coli* and *S. aureus*, but specified that only non–pathogenic *E. coli* was inhibited. More testing is needed to determine the exact *Xylaria* species extracted from the *V. aestivalis* tissue and the antibacterial compounds produced by said species.

The other fungus that showed consistent ZOIs was a fungus identified as either *Alternaria* (unknown species) or *Coniothyrium* (unknown species). Both genera have been shown to have antibacterial properties in previous research. Research performed by

Tomprefa *et al.* (2008) on *Coniothyrium minitans* found that the species produced an antibacterial compound known as macrosphelide A. This antibiotic was found to be highly effective against *S. aureus*. The other genus, *Alternaria*, includes species confirmed to have antibiotic capabilities. Hellwig *et al*. (2002), established that two species of *Alternaria* produced altersetin, which is chemically similar to equisetin, and was inhibitory toward several gram–positive bacteria (including *S. aureus*) and had little inhibition against gram–negative bacteria. This is similar to the present results. Because the exact genus and species of the fungi tested in the current research is unknown, more testing is needed to determine the identity and type(s) of antibiotic compounds produced.

Two different *Fusarium equiseti* samples were tested in this research, neither of which showed consistent antibacterial activity. Only three of twelve samples tested against *S. aureus* showed any inhibitory properties. While the current research demonstrated no definite inhibition, Burmeister *et al*. (1974) stated that *Fusarium equiseti* NRRL 5537 showed antibacterial activity against certain gram–positive bacteria. Vesonder *et al*. (1979) furthered the study on *F. equiseti* NRRL 5537 and found that it produced equisetin, which is active against several gram–positive bacteria and one gram–negative bacterium. It is possible that the fungi tested in this experiment produced conditional inhibition, which could have led to the inconsistent results found. It is also possible that the inconsistency observed was the result of human error. Only one of six plates of the other *Fusarium* species (*profileratum*) displayed ZOIs.

The *Alternaria* species tested did not display consistent ZOIs. However, it should be noted that Sandhu *et al*. (2014) established that *A. tenuissima* is inhibitory against a variety of bacteria using the well diffusion method. It is possible that the difference in

methods employed resulted in erratic inhibition results. Similarly, the *Pestalotiopis microspora* that was found in *V. aestivalis* grape tissue displayed little antibiotic activity despite the species (when found as an endophyte in mangrove trees) having been shown to inhibit *S. aureus* (Maria *et al.*, 2005).

Curvularia geniculatus and two *Aureobasidium* of unknown species were also tested and showed limited inhibitory action. However, unlike the other fungi, there is little published research on either genus and the antibacterial activity associated with them is, therefore, undetermined.

None of the fungi tested showed any inhibition against either *K. pneumoniae* or *P. aeruginosa*, which is not surprising given the recent development of MDR strains of both bacteria (Klebsiella pneumoniae in Healthcare Settings, 2012; Pseudomonas aeruginosa in Healthcare Settings, 2018). In 2009 it was noted that *K. pneumoniae* was becoming resistant to the antibiotic carbapenem and moving in the direction of resistance to all commonly used antibiotics (Hussein *et al.*, 2009). According to Kidd *et al*. (2017), recent studies have shown that the bacteria are now showing resistance to colistin, which was one of the last antibiotics effective against *K. pneumoniae*. Similarly, *P. aeruginosa* is the leading cause of hospital-borne infections and only a few antimicrobial agents are consistently effective against the bacterium. As a result, *P. aeruginosa* has become a public health threat with a disposition for developing resistance to antibiotics used to treat it (Carmeli *et al.*, 1999).

It would be beneficial for future research to determine whether and what antibacterial compounds these fungi secrete. It would also be beneficial to test the fungi

that displayed no ZOIs to see if any had antimicrobial activity that was not visually confirmed.

Conclusion:

Some of the fungi found in the *Vitis aestivalis* vegetative tissue showed antibacterial activity, similar to those found in many other plants' tissues. Since the ZOIs were shown to be statistically significant, future research should be directed toward identification and isolation of the antibacterial compounds produced.

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Appendices:

Pictures of selected ZOI for trials 2 and 3.

Pictures:

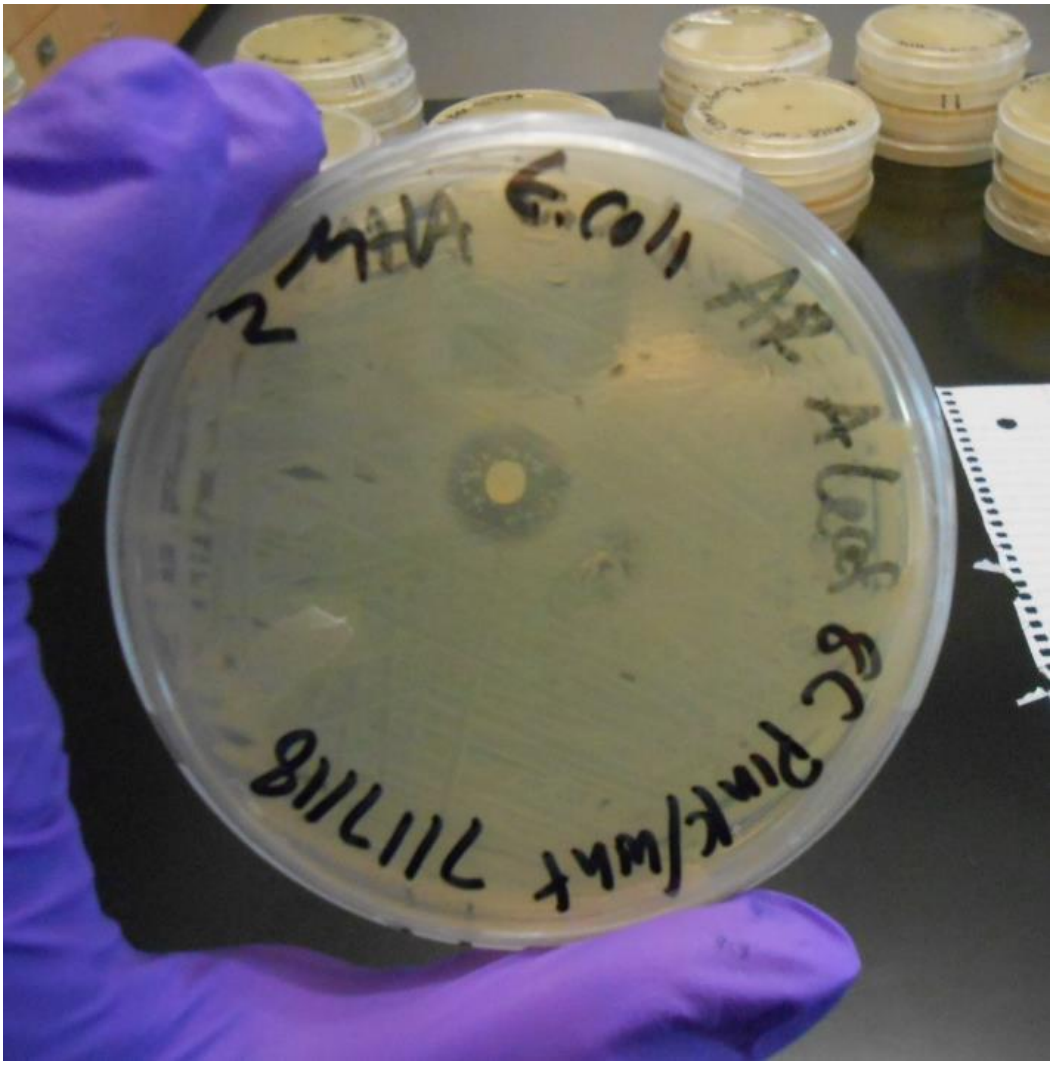

Figure 1: Xylaria ZOI, Trial 2, *E. coli*

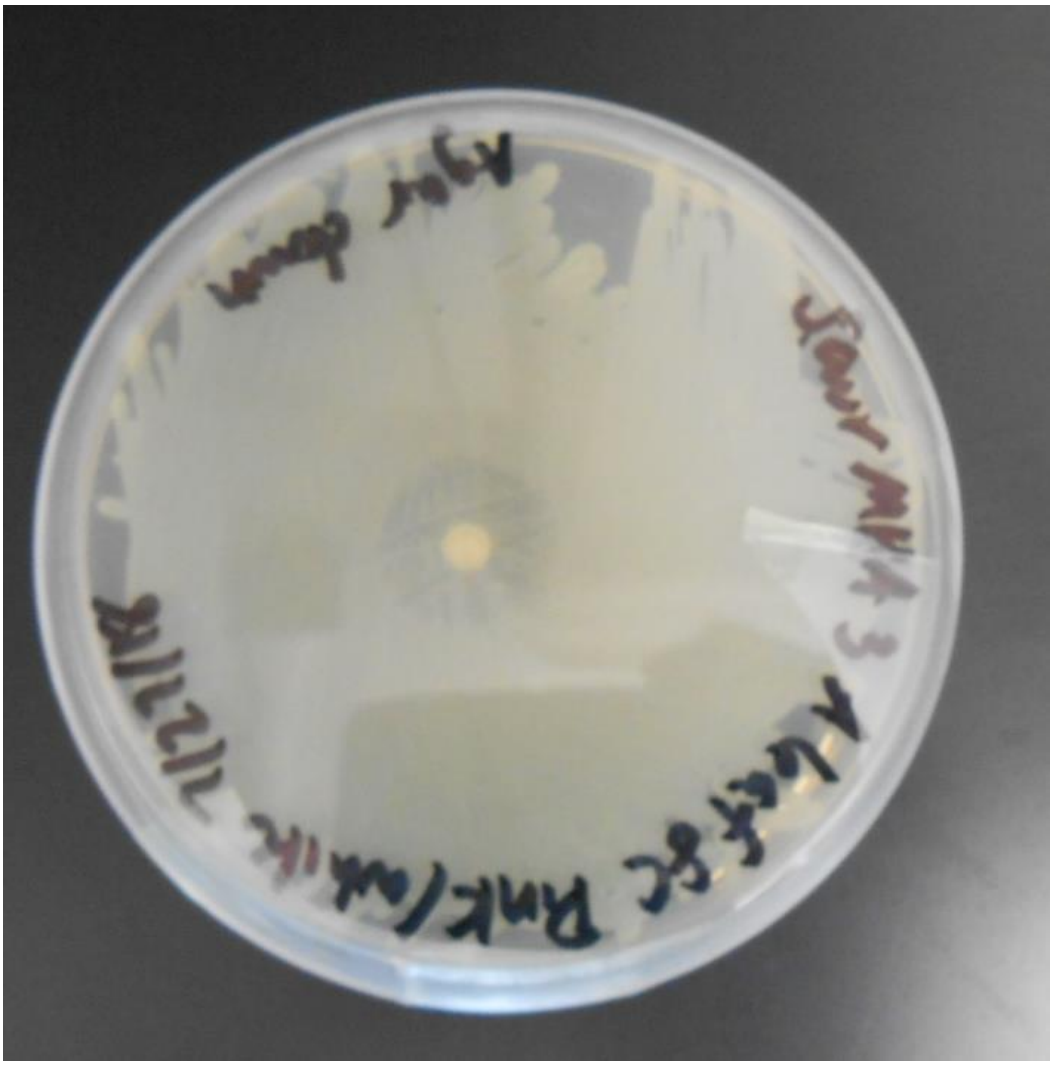

Figure 2: Xylaria ZOI, Trial 3, *S. aureus*

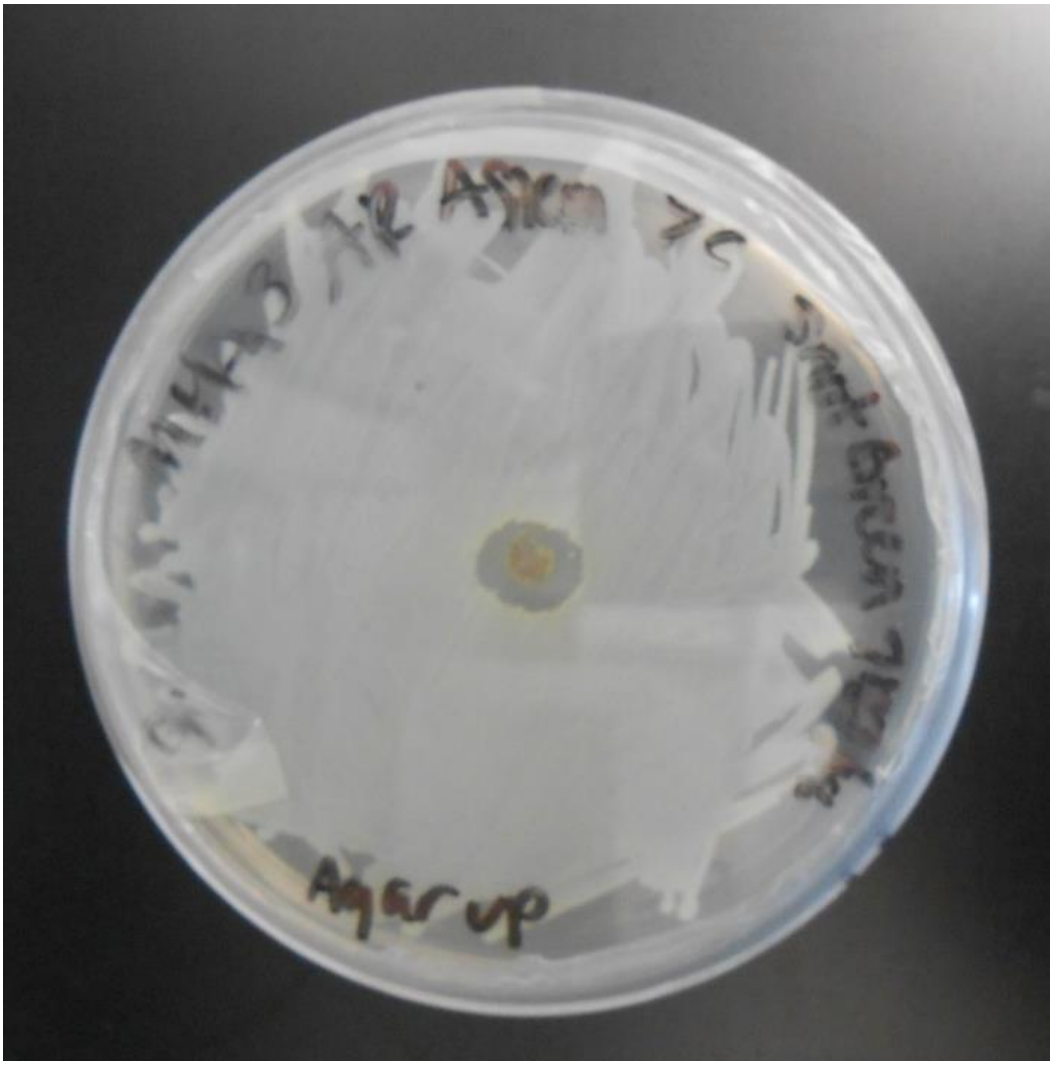

Figure 3: Alternaria or *Coniothyrium* ZOI, Trial 3, *S. aureus*