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A Study of an Uncharacterized Penicillium Species Isolated from a HYPERSALINE Pond in San Salvador, Bahamas

Virginia Elizabeth Hopkins
University of South Carolina - Columbia

A STUDY OF AN UNCHARACTERIZED *PENICILLIUM* SPECIES ISOLATED FROM A HYPERSALINE POND IN SAN SALVADOR, BAHAMAS

by

Virginia Elizabeth Hopkins

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Accepted by:

Anindya Chanda, Director of Thesis

Dwayne E. Porter, Reader

R. Sean Norman, Reader

Lacy Ford, Vice Provost and Dean of Graduate Studies

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ABSTRACT

A large number of facultative marine fungi are unexplored (Lai et al., 2007). They exist in the hypersaline environment with reduced growth and metabolism for an indeterminable period of time and are believed to have no ecological role. Here we report the discovery of one novel facultative *Penicillium* species that we named *Penicillium virginium*. We show that when transferred from its native ecosystem, a salt pond in San Salvador, Bahamas, to a laboratory fungal growth medium, *Penicillium virginium* demonstrates a higher growth rate in absence of salt than in presence of salt confirming that it is a facultative marine fungus. In an interaction experiment when *Penicillium virginium* was grown in a salt-free environment together with a known terrestrial saprophytic fungal pathogen model *Aspergillus parasiticus*, *Penicillium virginium* inhibited *Aspergillus*' growth and metabolism. We show that this inhibition was caused by bioactive compounds released into the growth medium that were not only inhibitory to *Aspergillus*' growth and metabolism, but also prevented growth of gram-positive bacteria including the methicillin resistant *Staphylococcus aureus* (MRSA). Our results therefore refute the current notion on the ecological role of the facultative marine fungi and implicate that in the current context of global climate change and severe weather events that can result in drastic drops in salinity of coastal waters and redistribution of marine microbes to habitats that support fungal growth, the bioactive potential of these fungi need to be thoroughly investigated.

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CHAPTER 1

LITERATURE REVIEW, RATIONALE, BACKGROUND, AND HYPOTHESIS

1.1 LITERATURE REVIEW

Marine fungi: A brief history

It is believed, based on fossil evidence, that the fungal kingdom came to existence about 1000 million years ago when they branched out from their eukaryotic ancestors and differentiated themselves from plants and animals (Zmitrovich, 2001). The existence of marine fungi was first reported during the middle of the 19th century. In 1849, Desmazieres reported the first facultative marine fungus, *Phaesosphaeria typharum* followed by the first report of an obligate marine fungus, *Pocedonia oceanica* in 1869. Since then about 1500 marine fungi have been reported that either inhabit the pelagic (open ocean waters) or the benthic (bottom) zones.

The high salt concentrations and the abrasion caused by waves and sand prohibit the development of fleshy fruiting bodies which are needed for needed to reproduce. Most marine fungi could not develop fruiting bodies beyond 3-4 mm, hence their role in the marine ecosystem remained mostly unappreciated (Rama 2013). The role of marine fungi in coastal ecosystems came to light in the 1970s when the symbiotic association of several fungi with algae and organic matter (plant or animal remains) was reported (Kohlmeyer and Kohlmeyer, 1979). Since then, the number of marine fungi reported has increased to about 1500 species (Blackwell, 2011). Since the tropical temperatures are conducive to fungal growth, most discoveries of novel marine fungi playing significant

roles in biodegradation have come from the tropical waters of the Atlantic and Pacific (Jones 1993; Kohlmeyer & Kohlmeyer 1979). Their most common habitats for marine fungi are submerged wood, inorganic matter, planktonic communities, marine plants (e.g. algae), invertebrates (e.g sponges and coral) and fishes. The majority of marine fungal species discovered belong to the phylum Ascomycota. Special appendages present on their spores help them to float, entrap humidity, and adhere to substrates, hence enabling them to survive the marine environment (Jones 1994). Currently, the widely accepted definition of a marine fungus is dependent on their ability to germinate and form a mycelium under normal marine conditions (Bugni and Ireland, 2004). The ones that grow exclusively under marine or estuarine conditions and have received most attention of marine mycologists are categorized as obligate marine fungi. The ones that originate from terrestrial or freshwater ecosystems, but have reached and are able to grow also under marine conditions, are categorized as facilitative marine fungi; these have received less attention from researchers because of the general notion that they have little or no contribution to their ecosystem (Lai et al., 2007). It is perceived that many facultative marine fungi do no interact with other microorganisms in their environment due their low rate of growth and lack of secondary metabolite production (Lai et al., 2007).

In this study, this general notion on facultative fungi has been questioned based on the observation made on the bioactive potential of a novel *Penicillium*, *Penicillium virginium*.

Ecological role of fungi:

Since fungi do not contain chlorophyll, they survive in their environment by acting as parasites, saprotrophs, or symbionts. They obtain their nutrition by secretion of an array of depolymerizing enzymes that digest the complex polymers in their environment to monomers that can then be absorbed into their cells osmotrophically. Also in order to protect themselves from the harsh environment that they often face, fungi develop chitin-rich cell walls and activate a large set of natural products (called secondary metabolites), virulent factors, and pigments that protect them from their predators and competitors in their ecosystem. These characteristics are the key to their ecological success. The synthesis of a variety of bioactive metabolite products (enzymes, small molecules, toxins and pigments) has greatly contributed to pharmaceutical and food biotechnological industries (Raghukumar, 2008). Yet fungal secondary metabolites have also caused significant agriculture loss and human illness worldwide (Keller et al., 2005). Most fungal impacts on environmental sustainability and health fall under one of the three categories:

Impacts as parasites in marine organisms: Harvell and coauthors (1999) have attributed 10% of all mass mortalities in marine environments to fungi. *Aspergilli, Cryptococci, Fusarium, Candida*, and a diverse set of Zygomycetes have been linked to infections in marine mammals as well as other organisms over the years (Higgins 2000). For example, sea fan corals have been infected by *Aspergillus sydowii* (Alker et al. 2001) and American lobsters have been diseased with the ascomycete fungus *Fusarium* (Cawthorn 2011). Several dozen ascomycete fungi are pathogenic to marine algae (Kohlmeyer & Kohlmeyer 1979) and many species are pathogenic to marine plants

including: *Cytospora rhizophorae*, *Phomopsis mangrovei*, and some phytophthoral species (Kohlmeyer and Kohlmeyer, 1979; Pegg et al., 1980; Weste et al., 1982; Maxwell, 1968; Garrettson-Cornell and Simpson, 1984; Hutchings and Saenger, 1987).

Impacts of fungi as saprophytes: Fungi have a critical role in detritus processing in marine ecosystems (Mann 1988, Raghukumar 2004) and hence provide essential nutrients (amino acids, polyunsaturated fatty acids, and sterols) to the marine food web (Phillips 1984). These help in the survival of detritivorous animals (Raghukumar 2004). Marine fungi can also degrade structures such as mollusk shells, burrow linings, and barnacle shells (Hyde et al. 1998). Endolithic fungi of the ascomycete phylum *Arenariomyces*, *Corollospora*, *Lindra*, *Remispora*, chytrids, *Dodgella priscus*, *Ostracoblabe* and *Lithopythium* play significant roles in degrading various calcareous structures in the marine environment (Spooner & Roberts 2005). Fungi also degrade lignocellulaose in marine mangrove ecosystems at reduced oxygen levels (Hyde et al. 1998).

Impacts as symbionts: Mutualistic symbiotic contributions of marine fungi in lichens, mycophycobioses, and mycorrhizas is already well established in the literature (Fletcher, 1975; Kohlmeyer and Kohlmeyer, 1979; McCarthy, 1991).

<u>The impact of climate change:</u>

Currently marine environments are subject to increasing anthropogenic impacts resulting from rapid urbanization and increased industrialization. Coastal and estuarine ecosystems have become vulnerable to sewage and industrial effluent discharge, oil spills, and pesticides containing leachates (Weinstein, 1996). For example, South

Carolina is currently experiencing a rapid urban growth and is projected to be one of the top retirement states in the U.S. (Deller, 1995). The sudden escalation in urban sprawl and the associated increase in unplanned development, especially in the coastal areas of South Carolina, is a cause of concern for the adjacent estuarine ecosystems. Such developmental pressures have consumed precious rural land resources, resulting in landscape alteration and pressure on the infrastructure. These changes have resulted in a sudden spike of a variety of domestic and industrial wastes, which challenge the neighboring estuarine ecosystems (Corbett et al., 1997, Kelsey et al., 2003). In addition, the increase of carbon dioxide as well as other greenhouse gases in the atmosphere have resulted in significant increase in the average global temperature over time; an increase in 0.6- 0.7°C has been reported in the last century (Bell et al., 2009, DiMichele et al., 2009, Kauserud et al., 2010, Kaas, 2009, Harsch et al., 2009). Due to this global climate change, we are currently experiencing severe weather events that are reorganizing the distribution and abundance of soil microbiomes, and their phenology, along with several species of the non-microbial kingdoms (Korner & Basler, 2010). How will such environmental perturbations impact the diversity of marine fungi and their ecological role in the environment?

1.2 Rationale, Background, and Hypothesis

Rationale:

Although a large dataset on the distribution of different marine fungi in various parts of the world is available, there are still many coastal ecosystems which have not been investigated; especially the areas in the tropical regions that contain dead and living coral,

mangroves, and sea grasses. There is little knowledge of the role of marine fungi in marine sediments. Also the facultative fungi in marine sediments, especially the typical fast-growing species (e.g *Aspergillus*, *Penicillium*) have long been neglected because of the general notion that they are carry-overs from terrestrial environments and remain dormant under marine conditions (Lai et al., 2007). Though these facultative marine species are considered to have no ecological role in their current environment, we theorize that their ecological influence could be stronger if redistributed by severe weather events to other non-marine aquatic or terrestrial ecosystems. Interruption of balance ecosystems by can have detrimental effects on the organisms of that ecosystem and eventually cause collapse of the ecosystem if the influence is potent enough. Therefore we want to determine what environmental health risks these facultative marine fungi pose and if these risks could lead to public health concerns.

Background:

In this project, one species of the genus *Penicillium* was isolated from a hypersaline pond (Salt Pond, 24°01′N, 74°27′W), in San Salvador, Bahamas (Petrisor et al., 2014). This marine ecosystem is well-characterized and is a home to many unique species of bacteria and fungi that are able to tolerate such a high salinity. For this study, the *Penicillium* species was randomly selected from a group facultative fungi that grew spontaneously upon transferring the sediment sample from an environment with a high salinity to fungal conducive growth medium.

Hypothesis:

The central hypothesis states that alteration of salinity levels in the environment alters growth rate and metabolite synthesis of facultative marine fungi which will activate its potential for synthesizing natural products. To address this central hypothesis, we used the novel *Penicillium* species as a model for our study and conducted a detailed study of its growth and metabolite synthesis in different levels of salinity. The overall work flow of the study is described in Figure 1.1:

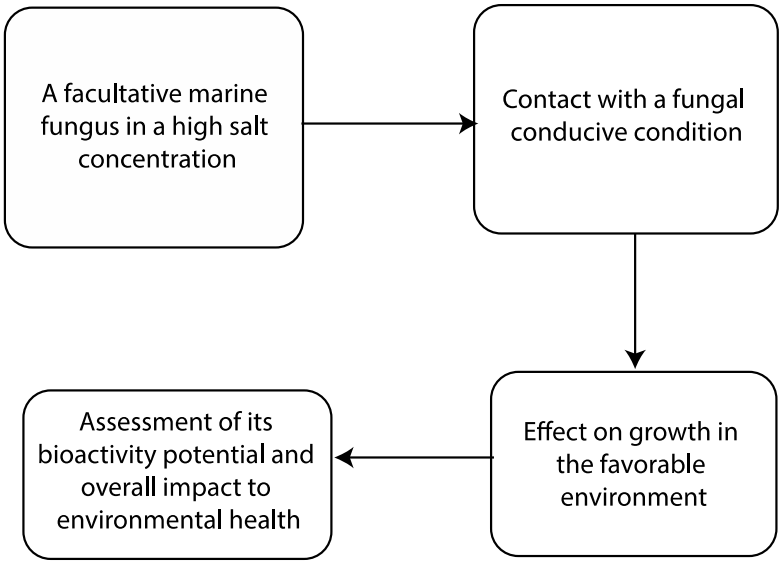


Figure 1.1: Overall work flow for this study

To establish the relevance of its metabolites on environmental health, we explored the impacts of the metabolites on the secondary metabolism of a known fungal pathogen, *Aspergillus parasiticus* B62 as well as gram negative and gram-positive bacterial strains. As will be described in details throughout this thesis, the following are highlights of the accomplishments in this study that support the central hypothesis:

(1) We establish that the hypersaline pond fungus chosen for this study is a novel *Penicillium* species; we name it *Penicillium virginium*

(2) We establish that removal of salt stress favors the growth rate of *Penicillium virginium*

(3) We show removal of salt stress activates natural product synthesis of *Penicillium virginium.*

(4) We discover that the metabolite pool of *Penicillium virginium* demonstrates bioactivity against the fungal pathogen *Aspergillus parasiticus* B62 and gram positive bacterium *Staphylococcus aureus* (including the methicillin resistance *Staphylococcus aureus*). This discovery refutes the general paradigm that facultative marine fungi are non-relevant to ecology and environmental health

(5) We have also optimized the conditions in the study for isolation of the fraction that demonstrates anti-*Aspergillus* activity.

In summary, we emphasize throughout this thesis that there is a need for initiating new efforts to investigate the diversity of facultative marine fungi and their potential role in ecology in the tropical coastal regions upon activation of their bioactive potential triggered by severe weather impacts resulting from ongoing climate change.

CHAPTER 2

ISOLATION AND CHARACTERIZATION OF *PENICILLIUM VIRGINIUM*

Introduction:

Historically fungi were classified based solely on identification of specific features, like spore-producing structures, using macroscopic and microscopic analysis. Relying solely on this process has many disadvantages while classifying marine fungi. One of the main disadvantages is posed by their general morphological characters that are often difficult to classify and sometimes misleading. On the contrary, molecular methods have proven more effective. Specifically, amplification of taxonomically specific gene markers from cDNA samples coupled with phylogenetic analyses has been used consistently and successfully and has accurately reflected the extensive complexity in fungal diversity (White et al., 1990). Many environmental mycologists have conducted ribosomal RNA (rRNA) gene array during environmental clone library analyses and focused their studies on the small subunit (SSU) rDNA sequence (Anderson et al. 2003, Bass et al. 2007, Jebaraj et al. 2009, Porter et al. 2008, Schadt et al. 2003, Vandenkoornhuyse et al. 2002). However this region of DNA is conserved between closely related fungal species and hence cannot be used to investigate fungal diversity beyond the higher taxonomic groups.

Fungal genomes have two sections of DNA called the Internal Transcribed Spacer (ITS) regions that are located between the small subunit (SSU) and Large Subunit (LSU) rRNA genes. The two ITS regions are separated by a 5.8S rRNA gene. In comparison with the SSU rRNA gene, this region is more variable and its sequences can be used to

classify a fungus to genus and species level (Bruns & Gardes 1993, Gardes & Bruns 1993, Horton & Bruns 2001) and accurately place an unknown fungus in the phylogenetic tree.

In this study, in order to confirm identity and novelty of the hypersaline fungal species *Penicillium virginium,* it was decided to incorporate phylogenetic analysis using ITS sequencing and verify the characteristics of the identified genus using macroscopic analysis coupled with light and electron microscopy. DNA was extracted and purified using a modified protocol from Janso et al. (2005), which included a combination of extraction buffer and phenol-chloroform-isoamyl alcohol solution. Amplification was done using a combination of ITS specific primers, from White et al. (1990) and Gardes & Bruns (1993), as well as custom designed primers. The ITS1-5.8S-ITS2 region of the rRNA gene was chosen because of its highly specific and highly conserved nature.

2.1 Molecular and phylogenetic analysis

Methods:

Penicillium virginium and *Aspergillus parasiticus* SU-1 were grown 100 mL of yeast extract sucrose media (Bacto™) in parallel. We used SU-1 as a positive control for the DNA extraction method, since our method has been consistently used previously to isolate SU-1 genomic DNA (Chanda et al., 2009). Also SU-1 DNA was used as a control to validate the accuracy of the phylogenetic analysis. Yeast extra sucrose media (YES) was prepared according to manufacturer's direction. Pellets from each culture were

harvested and then ground into a fine powder using liquid nitrogen. 800 µL of DNA extraction buffer, containing 1.2 M NaCl, 5 mM EDTA, and 0.2 M Tris HCl, and 20 µL of Proteinase K were added to each tube. The tubes were then vortexed and placed in a 50°C water bath for 18 hours. 1000 µL of 25:24:1 phenol, chloroform, and isoamyl alcohol solution was added to both tubes and they were then centrifuged at 10,000 rpm for 10 minutes at 4°C. The aqueous supernatant from each tube was removed and placed in fresh tube. 5 µL of 10 mg/mL RNAse solution was added and the tubes were placed into a 37°C water bath for 20 minutes. 500 µL of 200 proof alcohol was then added to each tube to precipitate out the DNA. The tubes were gently inverted and tilted by hand until the ribbon of DNA became visible. The tubes were then centrifuged at 15,000 rpm for 5 minutes at 4°C. The supernatant was discarded, leaving a DNA pellet on the bottom of the tube. The tubes were inverted for approximately 5 minutes to allow any remaining liquid to drip off and then turned upright for approximately an hour to allow all of the remaining moisture to evaporate. Finally, once the DNA pellets were dry, 100 µL of TE buffer was added to each tube. The samples were then stored at -20°C. A 0.8% agarose electrophoresis gel was run to confirm presence and determine the size of the DNA fragments that were extracted.

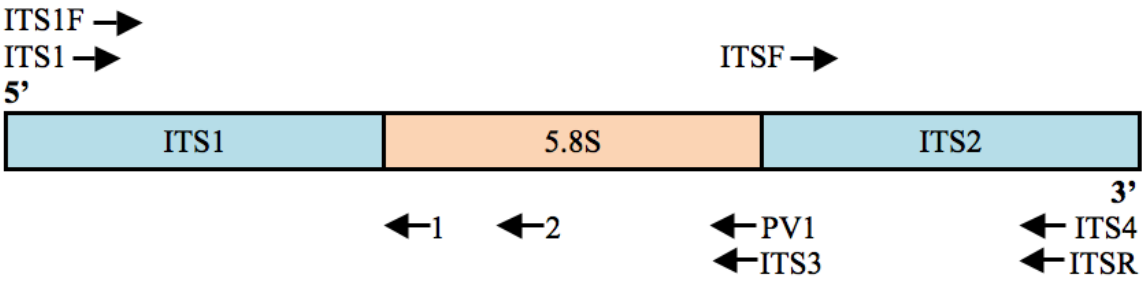


Figure 2.1 Primer annealing locations

Table 2.1 Primers used for DNA amplification

Primer name	Sequence (5' – 3')	Reference (if applicable)
ITS1	TCCGTAGGTGAACCTGCGG	White et al. (1990)
ITS1F	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns (1993)
ITSF	GTAAAACGACGGCCAGTGCATCGATGAAGAACGCAGC	Cheng et al. (2014)
ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
ITSR	CAGGAAACAGCTATGACTCCTCCGCTTATTGATATGC	Cheng et al. (2014)
ITS3	GCATCGATGAAGAACGCAGC	White et al. (1990)
PV1	CTTGAGGGCAGAAATGACG	-
1	CCCCGTCAATTCAAAAGTGT	-
2	TAGGAGCTTAGCTGCGGATT	-

To amplify the ITS1-5.8S-ITS2 region of rRNA genes, specific primers (Figure 2.1 and Table 2.1) and polymerase chain reaction (PCR) were used in combination. First, the primers were re-hydrated according to manufactures instructions with nucleus-free water to a concentration of 100µM. According to the New England BioLabs® Inc. protocol only 10µM primer solution was needed for the PCR, so 1:10 dilutions were made from the primer stocks. The New England BioLabs® Inc. protocol for 25 µL reaction should contain: 2.5 µL of 10x ThermoPol reaction buffer, 0.5 µL of 10mM dNTPs, 0.5 µL of 10µM forward primer, 0.5 µL of 10µM reverse primer, 100 ng of DNA, 0.125 µL of Taq DNA polymerase and then water up to the 25 µL line. A master mix for 4 samples containing: ThermoPol reaction buffer, dNTPs, Taq polymerase, primers and water was made and then aliquoted into 3 tubes. One microliter of DNA extract from SU-1 was added to the first tube, 1 µL DNA extract from *Penicillium*

virginium fungi was added to the second tube, and 1 µL of water was added to the last tube as a blank. The PCR conditions for Bio-Rad T100 thermal cycler were: initial denaturation at 94°C for 5 minutes followed by 33 cycles of melting temperature of 95°C for 30 seconds, annealing temperature of 57°C for 40 seconds, and extension temperature of 72°C for 1 minute, and ending with a final 5 minute extension time at 72°C (Luque et al., 2012). After the reaction was complete, samples were stored at -20°C. A 0.8% agarose electrophoresis gel was run to confirm the size of the DNA fragment that was amplified.

Penicillium virginium PCR amplicons were submitted to Selah Genomics Inc. for sequencing. Sequencing was completed using an Applied Biosystems® 3730 DNA Analyzer using manufacturer's protocols. Data were accessed using the chromatograph viewer FinchTV and then analyzed using Genbanks Basic Local Alignment Search Tool (BLAST). Using the BLAST aligner, the overlap between our sequence segments was determined and a complete sequence was assembled. Using BLAST, our sequences were analyzed for homology with other GenBank sequences to determine the most closely related species. The BLAST results from the complete sequence were also used to generate a neighbor joining tree, with a max sequence difference fraction of 0.75.

Results and Discussion:

DNA extraction

Extraction products were run on a 0.8% agarose gel to ensure that DNA was extracted from *Penicillium virginium* and *Aspergillus parasiticus* SU-1 (Figure 2.2). The gel image confirmed that sufficient DNA was extracted and that the product could be used for

amplification. Nucleic acid concentrations were measured using a NanoDrop 1000 spectrophotometer. *Penicillium virginium* extraction products contained 1463.3 ng/µL of nucleic acids and *Aspergillus parasiticus* SU-1 contained 1529.3 ng/µL of nucleic acids.

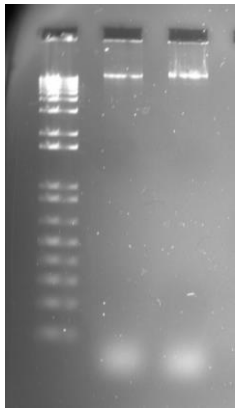
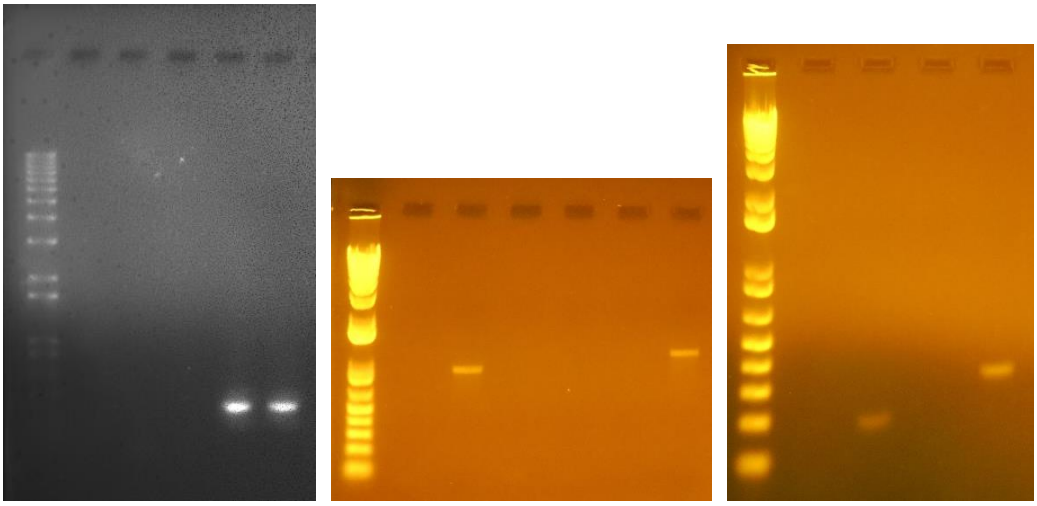


Figure 2.2 Gel image of DNA extraction products. Lane one contains a 1 kilobase (kb) ladder. Lane 2 contains 1 µL of DNA extraction product from *Penicillium virginium.* Lane 3 contains 1 µL of DNA extraction product from *Aspergillus parasiticus* SU-1.

PCR amplification

After each round of PCR the amplicons were run on a 0.8% agarose gel to ensure successful amplification and to determine the size of the products (Figure 2.3). The gel image for primer pair ITS1/ITS4 is not shown. The gel image for each PCR confirmed that our specified primers were indeed amplifying the *Penicillium virginium* extraction products. Nucleic acid concentrations were again measured using a NanoDrop 1000 spectrophotometer to ensure that the proper concentrations were being submitted for sequencing (concentrations not listed).



A B C

Figure 2.3 Gel images of *Penicillium virginium* PCR amplicons. Lane one in all images contains a 1 kb ladder. A) Lane 5 contains 1 µL of extraction product from *Aspergillus parasiticus* SU-1 and the primer pair ITSF/ITSR. Lane 6 contains 1 µL of extraction product from *Penicillium virginium* and the primer pair ITSF/ITSR. Both amplicons were approximately 200 base pairs (bps). B) Lane 3 contains 1 µL of extraction product from *Penicillium virginium* and the primer pair ITS1F/PV1. Lane 7 contains 1 µL of extraction product from *Penicillium virginium* and the primer pair ITS1F/ITS4. Both amplicons were between 800-1000 bps. C) Lane 3 contains 1 µL of extraction product from *Penicillium virginium* and the primer pair ITS1F/1. Lane 5 contains 1 µL of extraction product from *Penicillium virginium* and the primer pair ITS1F/2. Both amplicons were between 200-400 bps.

As the representative gel images show, the primers were efficient for PCR amplification. However, that was not the case for sequencing. Many of the sequencing results received only contained a sequence in one direction. It was suspected that this was due to mismatched primer sites. Primers that are not exact matches to their priming site can still function for PCR, but they will not function for sequencing. For this reason, many different conserved ITS specific primers were used as well as custom design primers to fill in the gaps. Once all the gaps were filled, the BLAST aligner was used to

align the sequence fragments and generate a complete sequence (Figure 2.4).

Penicillium virginium, 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence, Length 1228 bps

TCTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTATTAAG
TCTTTGCAGGCTGGAACATGATTCCTTCCGCAGTGTACACTTATCGAAGCC
TATGCAGCCACGCAAGTGGTAGGTCCAGACGACTCTAAACAAGTTGGTGC
CGCGATGCAAGTCCACCGGGGTGGGCGACACTTTTGAATTGACGGGGACA
CCCTAAAGCCGATCGCACCAACCTGTGTCGGGAAACCGCACGGGGGCCCA
TGGGAAATACATGGGGAAAGGTAACAGACGATCCGGATAGTTCTGCTTCA
GGCAGAGATCATGGGCAATCCGCAGCTAAGCTCCTACGGCCTCCGCGGCT
ACGGAGAAAGTCCACAGACTAAGTGGAAGTGGGTAGGATGACTAATCCT
GCTTAAGATATAGTCGGGCCGCCCGGGAAATCGGGTGGGCAAGTACACTG
TATGGGTTTCTACTGAGAGGTGCGACTTCCCATCTTGACAGTCGAATTGGC
AGGTACCTGTCCGAGTACCGTCCCGCCCCTGGCTCCCACCGGGTTAGGGC
TACTCGGACACGTGACTTCTTCTCTCAGAGATACTCAGTGAACCTCAAAA
AACCGTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGCGGACCC
CTCGCGGTCCAACCTCCCACCCGTGTCTACTTGAATACCCTGTTGCTTTGG
CGGGCCCACCGGGCCACCGGTCGCCGGGGGACGTCCCTGTCCCCGGGCCC
GTGCCCGCCGGAGCGCCCACAGAACCCTTTGTGAAGATGGACTGTCTGAG
CATGATTGATAATGAATCAAAACTTTCAACAATGGATCTCTTGGTTCCGGC
ATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATT
CCGTGAATCATCGAATCTTTGAACGCACATTGCGCTCCCTGGCATTCCGGG
GAGCATGCCTGTCCGAGCGTCATTTCTGCCCTCAAGCGCGGCTTGTGTGTT
GGGCGCTGGTCCCCCCTTCTCCCGGGGGGACCTGCCCGAAAGGCAGCGGC
GACGTTCCCGCCCAGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCGG
GACGGACCCGCGGGGCGTTGGTCATCCAACCCCATCTCTTTTTACGGTTGA
CCTCGGATCAGGTAGGAGTTACCCGCTGAACTTAAGCATATCAATAAGCG
GAGGAGTCATAGCTGTTTCCTA

Figure 2.4 Complete ITS1-5.8S-ITS2 sequence

Using BLAST to analyze for homology with other GenBank sequences, we were able to generate a table and neighbor joining tree to illustrate which species *Penicillium virginium* is most closely related to (Table 2.2 and Figure 2.5). Sequences with ≥98% homology can be assigned a species and sequences with between 95% - 98% homology can be assigned a genus (Cheng et al., 2014). The *Penicillium virginium* sequence only contained 92% homology with the closest related species, GenBank accession number:

HQ608123.1, but based on the ITS2 sequence the genus *Penicillium* could be assigned.

However, based on the GenBank database a specific species could not be assigned to this

Penicillium. Hence, we concluded that this was a novel *penicillium* species.

Table 2.2 10 Most closely related species to *Penicillium virginium* based on BLAST results of the ITS1-5.8S-ITS2 region of rRNA gene.

Taxonomic name	Percent Identity	Accession Number
Talaromyces purpurogenus isolate TR107	92%	HQ608123.1
Penicillium diversum strain KUC1284	92%	HM469392.1
Penicillium sp. 1 TMS-2011 voucher SC13d10p12-2	92%	HQ631054.1
Fungal endophyte voucher ARIZ:DM0110	91%	KF673654.1
Penicillium minioluteum strain E3	91%	GU566240.1
Penicillium minioluteum isolate IFV M04/029	91%	FR670332.1
Talaromyces purpurogenus strain: IAM15392	91%	AB872818.1
Penicillium samsonii strain CBS 137.84	91%	JN899369.1
Penicillium sp. NRRL 35186	92%	DQ123635.1
Talaromyces purpurogenus strain: IAM13755	91%	AB872819.1

A.

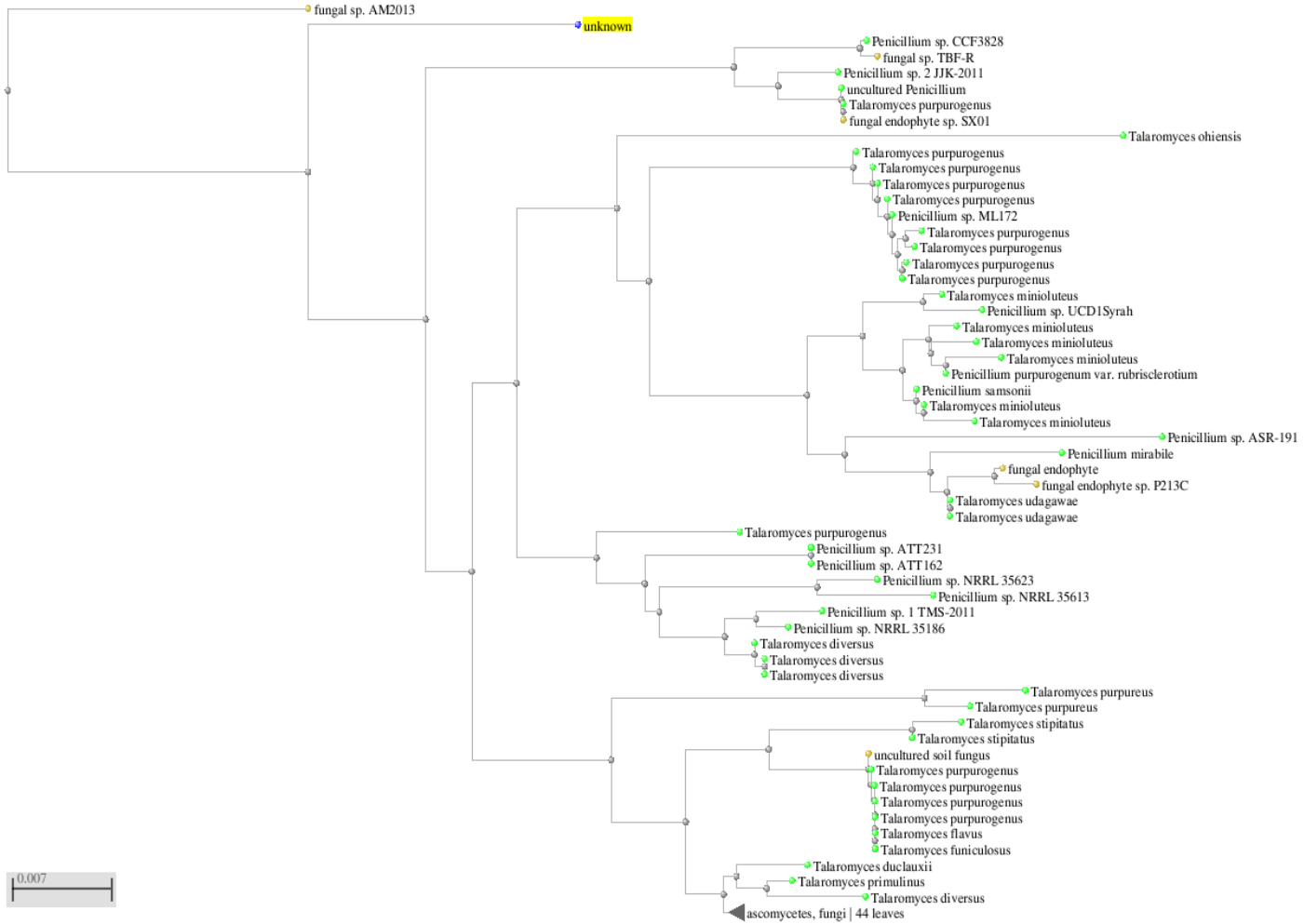


Figure 2.5 Neighbor Joining Tree generated from BLAST results of the ITS1-5.8S-ITS2 region of the rRNA gene of *Penicillium virginium*. *Penicillium virginium* is highlighted and label as unknown. B represents the 44 leaves not expanded at the bottom of A.

B.

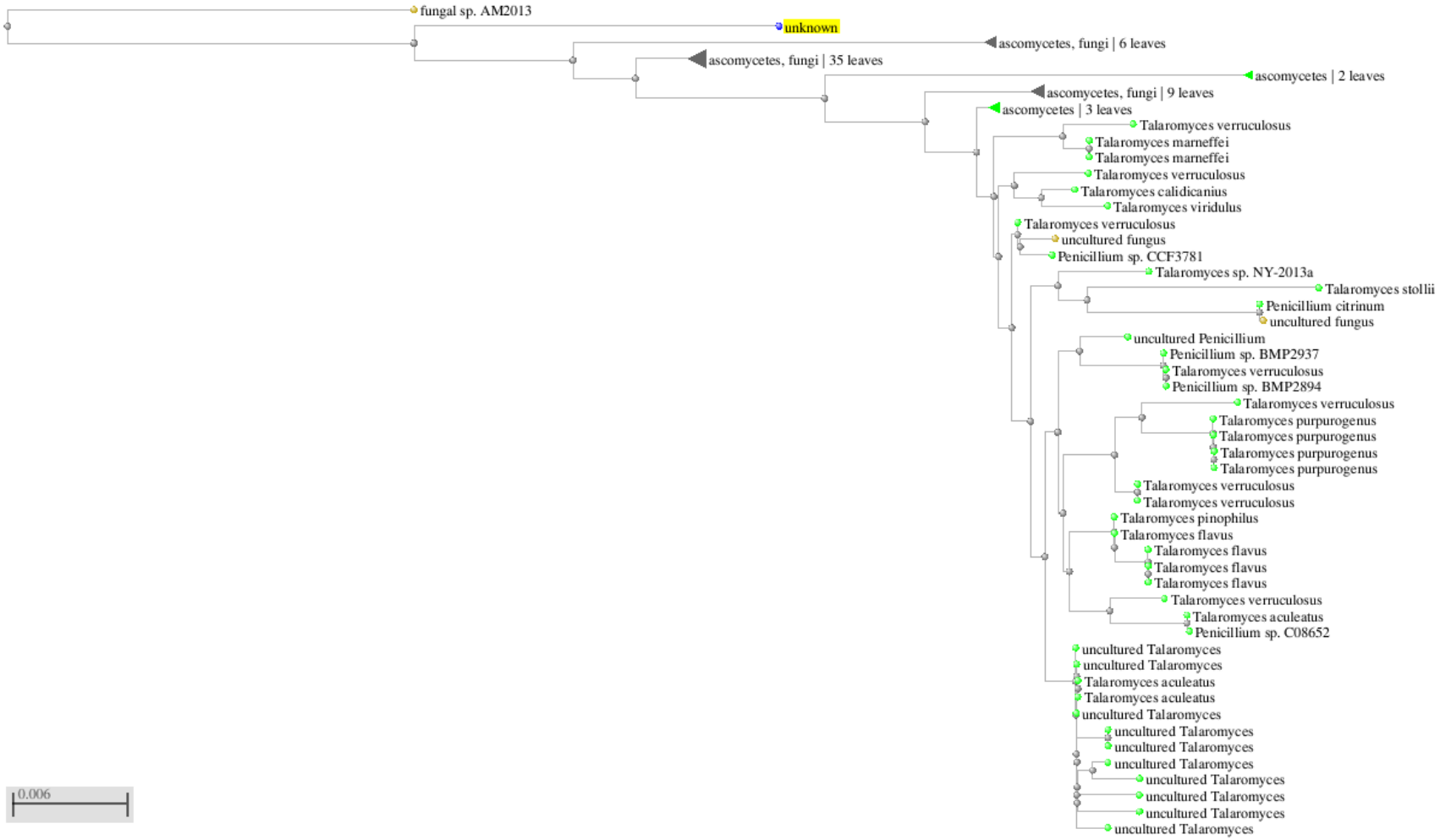


Figure 2.5 Neighbor Joining Tree generated from BLAST results of the ITS1-5.8S-ITS2 region of the rRNA gene of *Penicillium virginium*. *Penicillium virginium* is highlighted and label as unknown. B represents the 44 leaves not expanded at the bottom of A.

2.2 MORPHOLOGICAL ANALYSIS

Methods:

Generation of spore stock

The first step in macroscopical characterization of *Penicillium virginium* was to prepare a spore stock of a pure culture. This stock solution would be used to inoculate liquid and solid media throughout the remainder of the study. A month-old mature culture was used to make the stock spore solution. A magnetic stir bar and 10mL of 0.05% tween in phosphate buffer solution were placed into the flask with the culture. Using the stir plate and the stir bar inside the flask, the spores were removed from the culture and suspended in the PBS-tween solution. The spore containing solution was then filtered through a syringe containing glass wool and placed into a clean tube. The tube was centrifuged for 10 minutes at 3000 rotations per minute (rpm). The spores were then re-suspended in 90 mL of 50% glycerol solution. Using a hemocytometer we calculated the concentration of spores. Our final spore stock concentration was 10^7 spores per mm^3. The solution was then aliquoted, 1 mL per tube, into 1.5 mL Eppendorf tubes.

Growth conditions and sample preparation for light microscopy

To inoculate solid media, 100 µL of spore solution was pipetted onto the surface of a petri dish containing solid media. The closed dishes were then kept in a 29°C incubator. To inoculate liquid media 100 µL of spore solution was pipetted into a flask containing the liquid media. The flask was then swirled to mix the media and the spore solution and corked with a foam cork. The flasks were incubated in a 29°C shaking incubator (150 rpm).

Microscopy slides for optical microscopic analysis were prepared from mature fungal cultures, approximately 1 week old, grown in 10 cm petri dishes containing potato dextrose agar (Difco™). Potato dextrose agar (PDA) was prepared according to manufacturer's direction. An approximately 2 cm x 2 cm square, including both culture and media, was cut from the PDA plate using a sterile scalpel and placed into a sterile empty Petri dish. A microscopy slide cover slip was carefully laid on top of the culture. The Petri dish was then placed in an incubator for 2-3 days so that the culture could grow up onto the cover slip. 10 µL of phosphate buffered saline were placed onto a clean microscope slide and then the cover slip, which has been removed from the previously mentioned culture, was placed face down on top of PBS. 10-20 µL of PBS was added to the slide from the side of the cover slip. The slides were imaged using a Leica TIRF microscope at various magnifications and in differential interference contrast mode and fluoresce mode set to 561 nm.

Sample preparation for scanning electron microscopy

Samples for Scanning electron microscopy (SEM) were prepared from *Penicillium virginium* cultures that had grown for one week. The cultures used were grown on small circular pieces of PDA, diameter about 1 cm, cut from a 10 cm PDA plate. PDA was prepared according to manufacturer's direction. The PDA circles were inoculated with spores along one edge so that the culture would grow across the PDA. PDA and culture plugs were first fixed overnight in 0.1M cacodylate buffered (pH 7.2) glutaraldehyde at room temperature. Next they were washed several times with 0.1M cacodylate buffer (pH 7.2). The plugs were then placed into a solution of cacodylate buffered (pH 7.2) 1% osmium tetroxide for 1.5 hours at 4°C. They were then washed several times with 0.1M

cacodylate buffer (pH 7.2) and then dehydrated with a series of ethanol washes. Each plug was washed for 10 minutes each with 50%, 70%, 80%, and 95% ethanol. Finally the plugs were dried using a critical point dryer, mounted to SEM stubs, and gold coated. Samples were prepared and imaged in the University of South Carolina Electron Microscopy center using the Tescan Vega3 SBU Variable Pressure Scanning Electron Microscope.

Results and Discussion:

While counting spores on the hemocytometer, it was observed that the spores were ovular in shape and colorless. This observation differed greatly from the appearance that the spores had in solution during the spore prep process. When in solution the spores appeared dark green/grey colored. While preparing microscopy slides, the gross colony morphology of *P. virginium* was close examined. Primarily two colors were observed: yellow on the conidia, seen on the top, and red, which was underneath the culture in the agar (Figure 2.6). This prompted the hypothesis that the culture synthesizes at least 2 different metabolites: the yellow metabolite, which is localized to conidia, and the red metabolite, which is secreted to the medium..

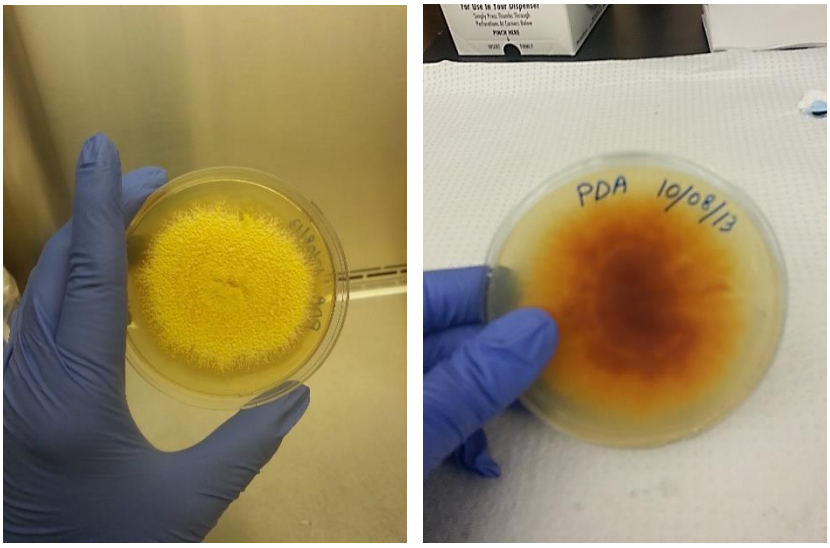


Figure 2.6 The yellow (left) and red (right) metabolites made by a 27 day old *Penicillium virginium* culture on PDA.

Microscopic analysis of the conidia under yellow light (561 nm) revealed that the surface of the conidia were indeed yellow in color (Figure 2.7). It was also observed that the conidia are produced in basipetal succession from phialides produced from branched metulae, which is very typical of a Penicillium species. This supports the conclusions made previously from the phylogenetic analysis results.

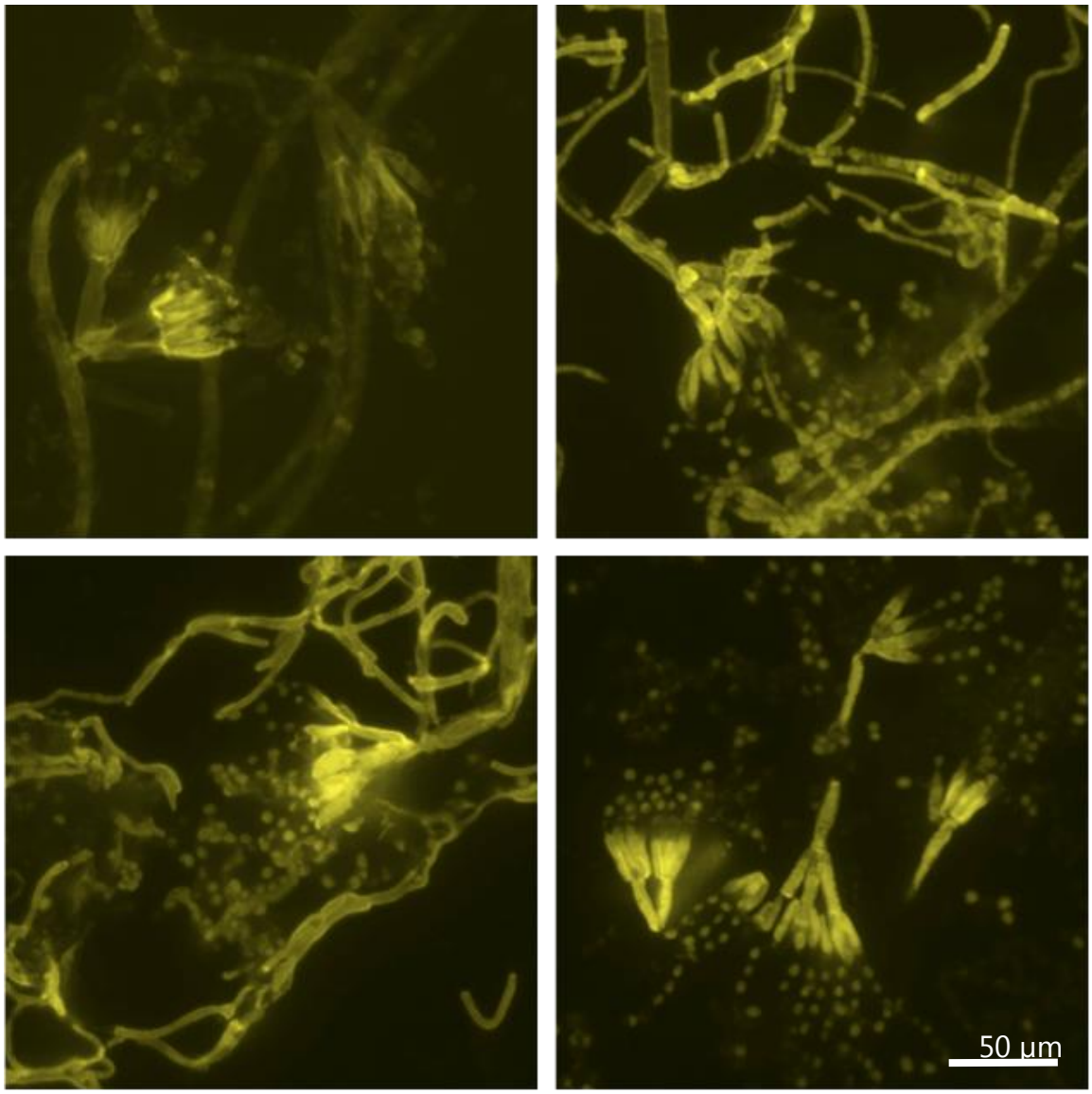


Figure 2.7 Microscopic images taken under light at wavelength 561 nm of the conidia and spores of *Penicillium virginium*. Images were taken at 100x magnification.

The SEM images collected confirmed, in great detail, many of the observations previously made. The images confirmed the ovular shape of the spores as well as the shape of branched conidia. The images also confirmed that the spores do indeed form in basipetal succession and that the hyphae are highly branched (Figure 2.8). All the gross morphological characteristics that were observed in SEM micrographs were very typical of the genus Penicillium and clearly supported our Phylogenetic analysis.

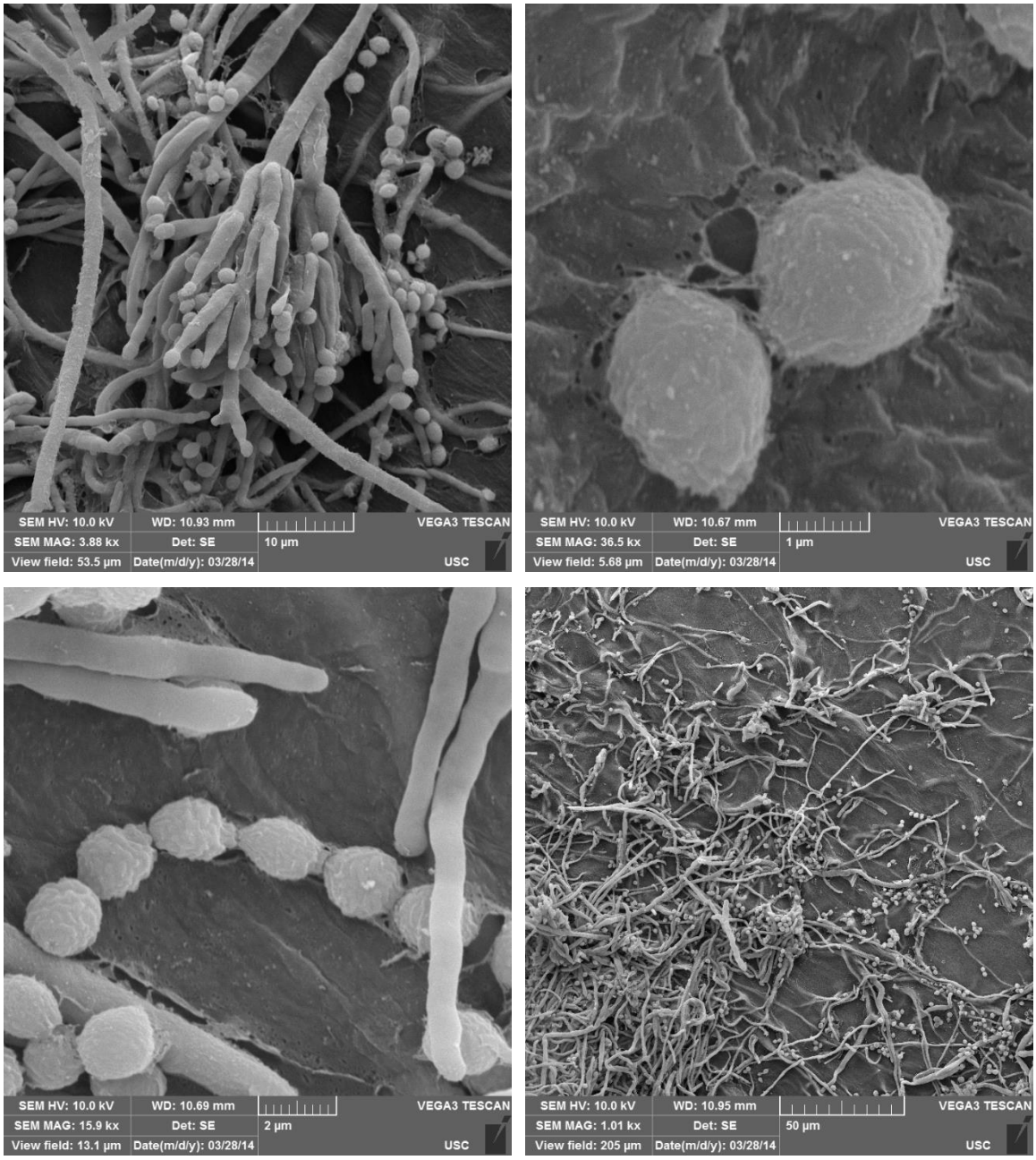


Figure 2.8 SEM images of the conidia (top left), the individual spores (top right), the spore chains (bottom left) and the highly branched hyphae (bottom right).

CHAPTER 3

ACTIVATIONS OF PRODUCTION OF BIOACTIVE METABOLITES BY *PENICILLIUM VIRGINIUM* UPON ENVIRONMENTAL MODULATION

Introduction:

Many studies have correlated global warming and climate change with increases in storm activity over the last few years (G. Holland & C.L. Bruyère, 2012). It is predicted that tidal influence and increased rainfall, resulting from such storm activities, will alter the composition of coastal ecosystem fungal communities as well as their metabolite production. In this study we tested the hypothesis that our model facultative fungus *Penicillium virginium* will demonstrate a significant alteration in growth and metabolism upon alteration of salinity in the growth medium. Since this fungus resides in a hypersaline ecosystem (Salt pond in San Salvador, Bahamas) , investigation of its phenotypic effects in response to change in salinity was a reasonable start-point to understand its response to the climatic perturbations

Since minimal salt levels are typical of terrestrial ecosystems, we challenged the fungus by growing it adjacent to one of the most popular and characterized pathogens, *Aspergillus parasiticus*, a filamentous fungus that synthesizes the naturally occurring hepatocarcinogen, aflatoxin B_1 (Cary et al., 2006). A construct of *A. parasiticus* that accumulates Norosolorinic acid (an orange metabolite) was an ideal choice for this interaction assay because its accumulation in the growth medium is easily visible. is easily visible.

3.1 Determination of the role of NaCl

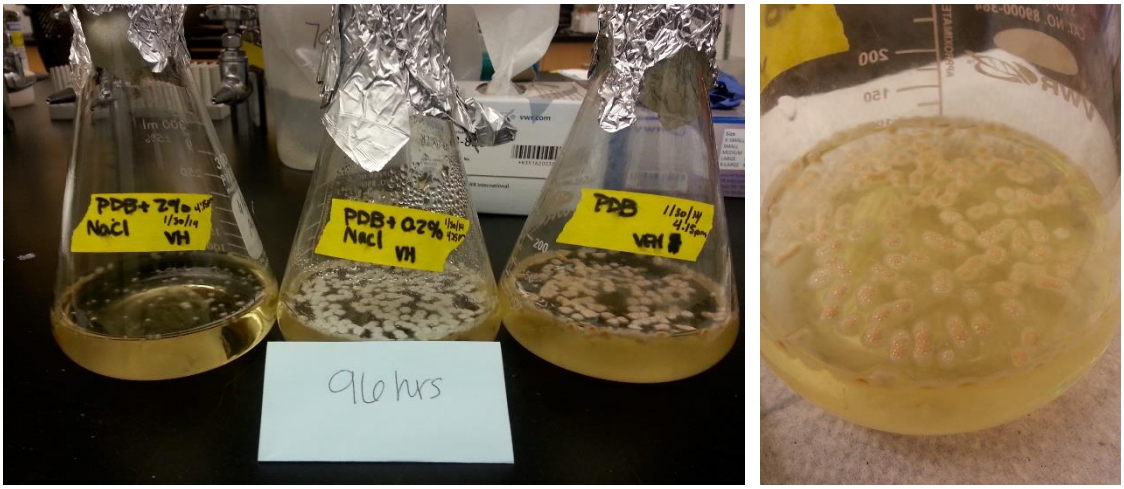
Methods:

Four flasks, containing 100 mL each, of potato dextrose broth (Difco™) were prepared according to manufacturer's instructions. Before the autoclave step, NaCl was added to 3 of the flasks. The 3 concentrations of NaCl added were: 2 PSU (0.2% NaCl), 20 PSU (2% NaCl), and 100 PSU (10% NaCl). 100 PSU was selected as the maximum level of salinity for this experiment because it mimics the average salinity of the hypersaline pond where *Penicillium virginium* was collected. 20 PSU and 2 PSU were chosen to mimic the possible salinity drops that could occur with increased tidal influence and storm activity. 0 PSU represents the salinity that *Penicillium virginium* would encounter if relocated to a terrestrial environment. Each flask was then inoculated with 100 µL of *Penicillium virginium* spore solution and placed into a 29°C incubator. A flask of marine broth (Difco™) was also prepared, inoculated, and placed in the 20°C incubator as an addition to the study. All of the flasks were monitored daily and, based on gross morphology and number of the mycelial pellets, a qualitative estimation of the level of growth was recorded. An image of each flask was captured at the conclusion of the study.

Results and Discussion:

At 96 hours from the point of inoculation in PDB media, the cultures exhibited different characteristics. As seen in Figure 3.1, the pellets grown only in PDB exhibited a red hue on their surface. Mycelial pellets in 2 PSU PDB although similar in size as in PDB only, demonstrated no color on their surface. Mycelial pellets in 20 PSU PDB demonstrated no

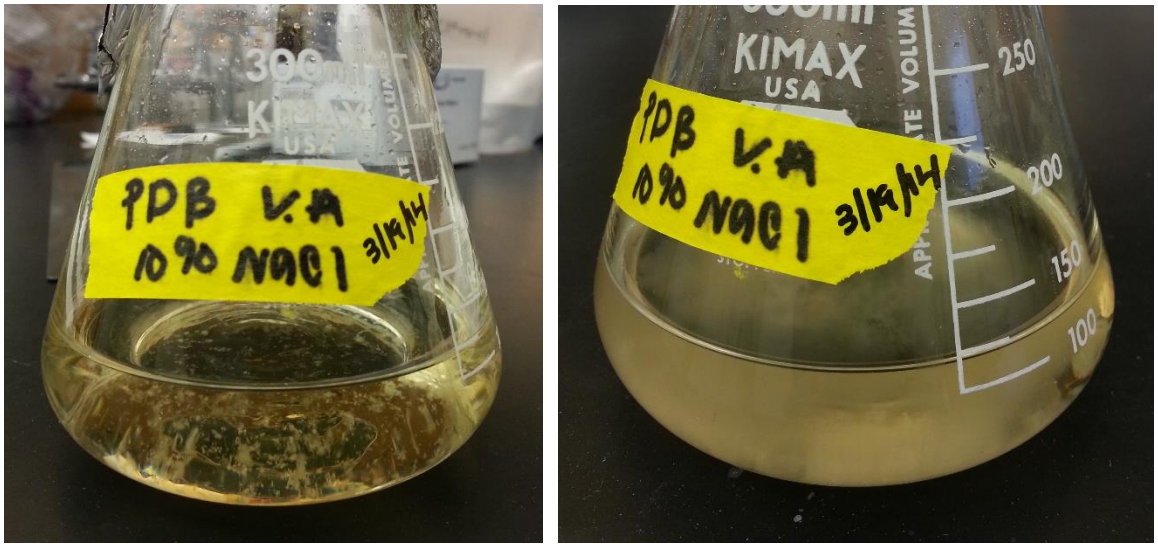
red color as well and were significantly smaller in size and numbers compared to the flasks with 2 PSU PDB and PDB only.



A B

Figure 3.1 *Penicillium virginium* cultures grown in various types of PDB for 96 hours. A) 20 PSU PDB (left), 2 PSU PDB (middle), and PDB with no NaCl added (right). B) Close up of red hued pellets in PDB with no NaCl added.

After 96 hours of incubation, the 100 PSU PDB flask had no visible growth. To observe whether the fungus demonstrates any visible growth in this salinity, the flask was incubated for an extended period of time. Figure 3.2 shows the growth level that was recorded after 18 days and then again after 75 days. In comparison to the 20 PSU PDB, the 2 PSU PDB, and the PDB only cultures, the rate of growth of pellets in 100 PSU PDB significantly less. After 18 days of incubation, mycleial pellets merged into a continuous biomass that was visible only against light. Based on this gross evaluation of morphology with time in presence of different NaCL concentrations it was concluded that increase in salinity reduces growth and alters the metabolism of the fungus (red color in PDB only *versus* no color in presence of salt).



A B

Figure 3.2 *Penicillium virginium* culture in 10 PSU PDB at 18 days of incubations (A) and 75 days of incubation. (B).

A comparison of mycleial morphologies was also conducted between the flasks that were incubated in a shaker incubator (at 150 rpm) *versus* the flasks incubated without shaking (similar to intertidal marine habitats with lesser waves). After 15 days of incubation the mycelia synthesized its characteristic red metabolite on the surface of the culture similar to the cultures grown in the shaking incubator.

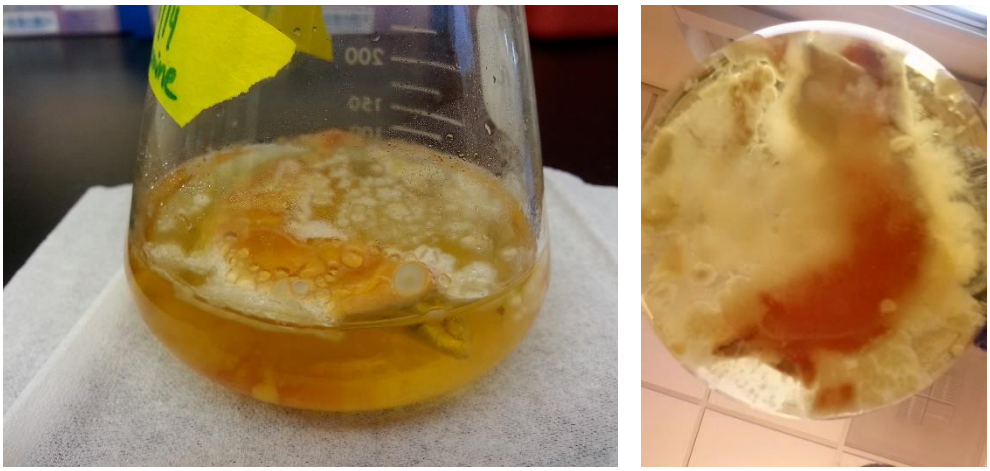


Figure 3.3 *Penicillium virginium* culture incubated in PDB for 15 days from the top (left) and bottom (right).

Similar to the 20 PSU PDB flask, the marine broth flask showed no growth after incubation for 96 hours. However, after 62 days the mycelia was visible (Figure 3.4), similar to those found in the 20 PSU PDB flask with no red color present. The marine broth has a concentration of 19.45 PSU of NaCl, which would explain the similarities to the 20 PSU PDB culture.

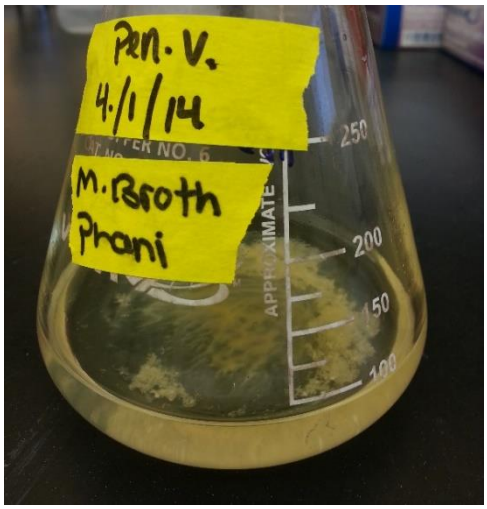


Figure 3.4 *Penicillium virginium* culture incubated in marine broth for 62 days.

Based on this comparative macroscopic study of the fungal growth and metabolism in presence of different levels of salinity, we concluded that growth and metabolite production of *Penicillium virginium* are dependent on the concentration of salt, in this case, NaCl. A decrease in the concentration of NaCl in the growth medium decreased fungal growth rate and altered its metabolism.

3.2 *PENICILLIUM VIRGINIUM* INTERACTION WITH *ASPERGILLUS PARASITICUS* B62 STUDY

Methods:

YES agar and PDA were prepared according to manufacturer's instructions and poured into 10 cm petri dishes. Petri dishes were taped off so that when the YES agar was

poured in, it only filled half of the plate. Once the YES agar had completely solidified, the tape was removed and the PDA was poured into the open side and allowed to set up. This produced a half YES agar and half PDA plate. The PDA side was inoculated with 50 µL of *Penicillium virginium* spore solution and placed in a 29°C incubator for 5 days. Then the YES agar side was inoculated with 1 µL of *Aspergillus parasiticus* B62 spore solution and the plate was placed back into the incubator for 48 hours. After 48 hours the interactions were observed and imaged. The *Penicillium virginium* cultures were allowed to incubate for a longer amount of time because they grow at slower rate compared to *Aspergillus parasiticus* B62 cultures.

Results and Discussion:

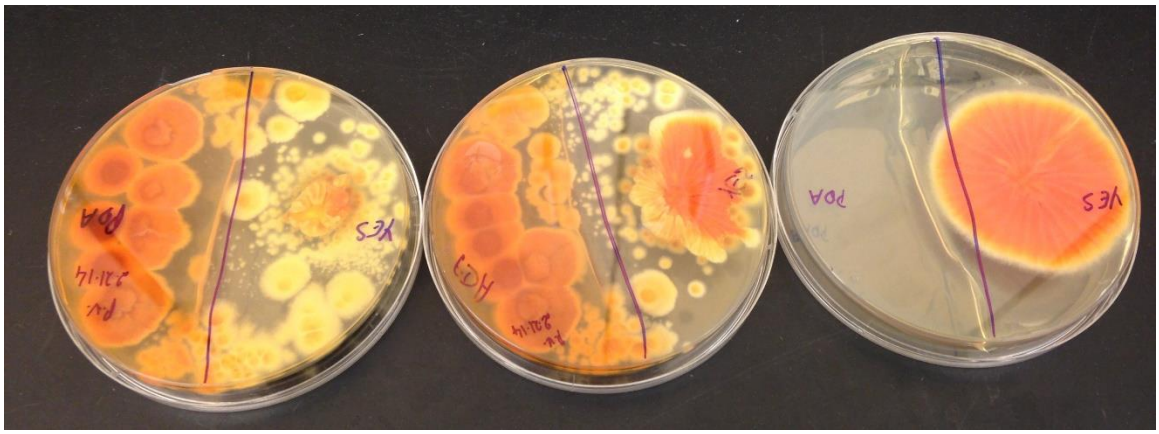


Figure 3.5 Petri dishes that each contain PDA and *Penicillium virginium* cultures in the left half and YES agar and *Aspergillus parasiticus* B62 cultures in the right each. The plate on the far right was used as a control and does not contain *Penicillium virginium* cultures.

After 7 days of incubation the colors and shapes of both the *Aspergillus parasiticus* B62 and *Penicillium virginium* cultures were closely examined. A significant difference in the growth morphology was observed between *Aspergillus parasiticus* B62 culture in a control plate (Figure 3.5 , far right) and B62 cultures growing adjacent to *Penicillium*

virginium cultures. The presence of *Penicillium virginium* and it metabolites hindered the growth of the *Aspergillus parasiticus* B62 as well as hindered the production of Norsolorinic acid (displayed as less orange color). It was also noted that *Aspergillus parasiticus* B62 demonstrated a tendency of growing away from the *Penicillium virginium* cultures. These results confirmed that *Penicillium virginium* have antifungal properties. In order to determine whether the *Penicillium* itself or its metabolites were antifungal, we designed our follow-up experiment (described below).

3.3 CHARACTERIZATION OF BIOACTIVITY OF EXTRACTED METABOLITES

3.3.1 Determination of the bioactivity of metabolites produced by *Penicillium virginium* on *Aspergillus parasiticus* B62 growth and norsolorinic acid production

Methods:

Metabolite extraction for *Penicillium virginium* in solid and liquid media

Penicillium virginium cultures were grown on PDA and PDB that was prepared according to manufacturer's instructions. The cultures were allowed to grow for at least 2 weeks before they were harvested to extract metabolites. To extract metabolites from the solid cultures, the complete contents of the petri dish was cut up and placed into a 50 mL plastic tube. Enough chloroform to cover the contents of the tube was then added; this averaged out to be about 15 mL of chloroform. The tube was then shaken vigorously for about 1 minute and then left to sit for 30-60 minutes. The chloroform was then poured or strained off into a labeled glass vial and left to evaporate overnight. Once all the chloroform evaporated, 1 mL of methanol was used to rehydrate the metabolites and the solution was transferred to a 1.5 mL Eppendorf tube. Tubes were stored at 4°C. To extract metabolites from the liquid culture, approximately 40 mL of media was

collected using a sterile syringe and placed in a 50 mL plastic tube. We were careful to not collect culture along with the media. The media was equally aliquoted into 4 tubes. 10 mL of chloroform, to match the 10 mL of media, was then added to each tube and tubes were shaken vigorously for 1 minute and then left to separate for 1 hour. Once the organic and aqueous layers had separated, the bottom organic layers were collected and placed in clean 20 mL glass vials. The vials were left out so that the chloroform could evaporate and once they were all dry, they were combined together using 1 mL of methanol. *Penicillium virginium* was grown both on solid and liquid media to mimic the environmental changes that could occur if spores were transferred from its current marine location to a terrestrial location.

Inoculation and treatment of *Aspergillus parasiticus* B62 cultures

YES agar was prepared according to manufacturer's instructions and poured into 10 small 3.5 cm petri dishes. The dishes were divided into three groups: blank, liquid and solid. Each group was assigned three 3.5 cm petri dishes. The blank dishes were inoculated with 100 µL of methanol by pipetting the methanol onto the surface of the dish then tilting and leaning the dish to ensure that the entire top surface of the media was coated with methanol. The liquid dishes were inoculated, in the same manner as the blank dishes, with 100 µL of extracted metabolites in methanol solution from the *Penicillium virginium* cultures grown on PDB. The solid dishes were inoculated, also in the same manner as the blank dishes, with 100 µL of extracted metabolites in methanol solution from the *Penicillium virginium* cultures grown on PDA. Once each plate was inoculated they were left open for a few minutes so that all the methanol would evaporate. Once the methanol was evaporate all nine dishes were centrally inoculated

with 1 µL of *Aspergillus parasiticus* B62 spore solution. The plates were then placed in a 29°C incubator for 48 hours. After 48 hours the culture sizes were measured and photographed.

Aspergillus parasiticus B62 metabolite extraction and TLC

In order to determine if the *Penicillium virginium* metabolites affect norsolorinic acid synthesis, the metabolites from the *Aspergillus parasiticus* cultures were extracted To do so, the complete contents of all the blank, liquid, and solid plates were cut up and put into individually labeled 50 mL plastic tubes. Enough chloroform to cover the contents was added to each tube then the tubes were each shaken vigorously for 30 seconds and then allowed to sit for 1 hour. The chloroform was then poured off into a 20 mL glass vials and left to evaporate overnight. Once all the chloroform had evaporated, 1 mL of methanol was added to each vial to collect all the material that was left behind. The methanol and metabolites were then transferred to a labeled 1.5 mL Eppendorf tube. The tubes were left open to dry for 1 hour and then 100 µL of 70% methanol was added to each tube.

To determine if norsolorinic acid or aflatoxin were present in the extraction, thin layer chromatography (TLC) was used. A 5 cm by 7.5 cm silica on PET polyester TLC plate (Sigma-Aldrich®) was loaded with 10 µL each of one of the blank extractions, one of the liquid extractions, and one of the solid extractions. The mobile phase used to run the plate was: 50% toluene, 30% ethyl acetate, 16% chloroform, and 4% acetic acid. The plate was imaged and analyzed using a UV light source.

Results and Discussion:

Figure 3.6 shows the nine *Aspergillus parasiticus* B62 cultures after they were incubated for 48 hours. Our comparative qualitative analysis (Figure 3.6) clearly indicated that metabolites collected from a solid culture of *Penicillium virginium* inhibited norsolorinic acid synthesis more effectively than the metabolites from a liquid culture (indicated by the color of the bottom of the cultures). This difference did coordinate with the norsolorinic acid TLC results shown later.

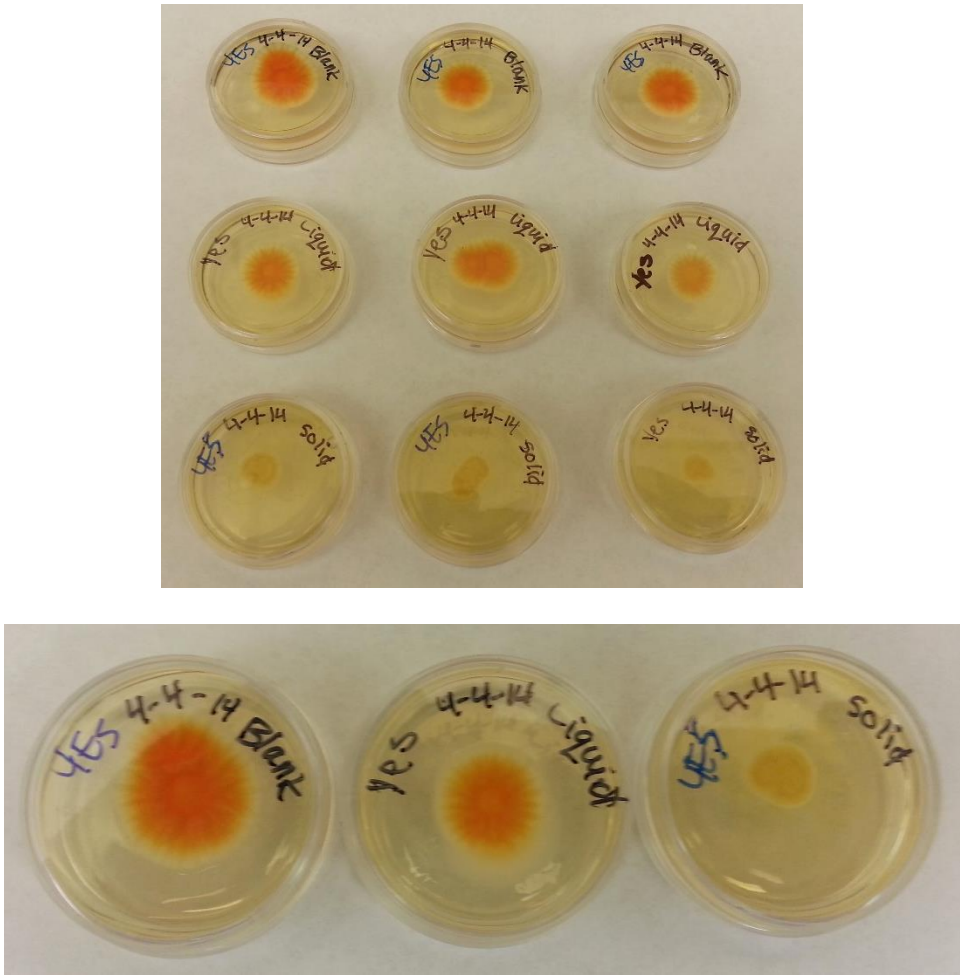


Figure 3.6 *Aspergillus parasiticus* B62 cultures incubated for 48 hours and treated with metabolites extracted from *Penicillium virginium* liquid and solid media cultures.

To decide whether the *Penicillium virginium* metabolites significantly changed the growth of the *Aspergillus parasiticus*, B62, we chose the criteria that a reduction of the area of the culture by at least 50% will be considered as a significant reduction in growth. When comparing the size of the cultures, the metabolites from liquid cultures reduced the area of B62 cultures by ~33% compared to the blank group (not significant), while those from the solid cultures resulted in 70% reduction in culture area (significant). Table 3.1 shows the diameter and areas of all of the cultures.

Table 3.1 Measurements collected and calculations for *Penicillium virginium* metabolite treated *Aspergillus parasiticus* B62 cultures.

	Diameter (cm)	Radius	Average diameter per group (cm)	Area (cm^3)	Average area per group (cm^3)
Blank 1	2	1	1.833333333	3.1415927	2.652027798
Blank 2	1.7	0.85		2.2698007	
Blank 3	1.8	0.9		2.54469	
Liquid 1	1.5	0.75	1.5	1.7671459	1.772381855
Liquid 2	1.6	0.8		2.0106193	
Liquid 3	1.4	0.7		1.5393804	
Solid 1	1	0.5	1	0.7853982	0.790634151
Solid 2	1.1	0.55		0.9503318	
Solid 3	0.9	0.45		0.6361725	

Figure 3.7 shows the TLC plate comparing the norsolorinic acid and aflatoxin levels between the groups. By comparing the TLC bands in each lane with each other, it can be seen that the liquid group (metabolites synthesized by B62 treated with *Penicillium virginium* metabolites collected from liquid culture) and blank group (metabolites synthesized by B62 treated with methanol) have similar bands showing presence of both norsolorinic acid and aflatoxin. However the bands from the solid group (metabolites synthesized by B62 treated with *Penicillium virginium* metabolites collected from solid culture) were significantly different. Norsolorinic acid and aflatoxin bands were not observed. These results suggest strongly that the *Penicillium virginium*

metabolites were capable of altering the metabolism or *Aspergillus parasiticus* B62. However more bioactive compounds or bioactive compounds with more efficacy were synthesized on a solid culture compared to a liquid culture.

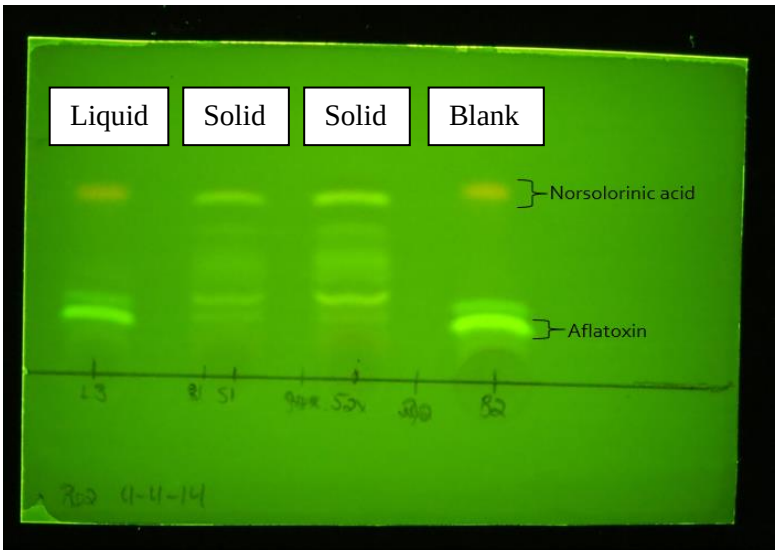


Figure 3.7 A TLC plate showing the metabolites extracted from *Penicillium virginium* metabolite treated *Aspergillus parasiticus* B62 cultures.

Our results also support that *Penicillium virginium* is a facultative marine fungus. The discovery of these antifungal properties implicates that *Penicillium virginium* is an ecologically relevant species and even demonstrates its bioactive potential in presence of environmental signals that are different from its native habitat (low salinity).

3.3.2 Determination of the bioactivity of metabolites produced by *Penicillium virginium* on growth of various MRSA strains

Methods:

HPLC separation of metabolite extracted from *Penicillium virginium*

Extracted metabolites from *Penicillium virginium* cultures grown on PDA were analyzed and separated into 2 fractions using and an Agilent 1100 high performance liquid chromatography system equipped with photo-diode array detection. Metabolites were

extracted using the process described in section 3.3.1. They were dried and rehydrated with acetone, then filtered through a syringe filter to remove any particulates. An injection volume of 100 µL, a flow rate of 0.5 mL/minute, and a mobile phase of 100% acetone were used to analyze the metabolites. Samples were analyzed using normal phase HPLC which separates compounds by polarity. In normal phase the more nonpolar compounds to come off the column first and the more polar compounds come off of the column last. The column used for this analysis was 800mm by 4.6mm and was packed with silica gel iatrobeads (Iatron labs Inc.) with a pore size of 10 µm. The column temperature was held at 35°C throughout analysis. Two peaks were identified on the chromatograph after analysis and the tubes containing the compounds associated with those 2 peaks were combined and labeled as peak 1 and peak 2. Peak 1 and peak 2 represent groups of compounds that all had similar polarities. They are not an isolated compound. Samples were dried and rehydrated in methanol.

Bacterial plate inoculation

Tryptic soy agar (TSA) was prepared according manufacturer's instructions and poured into 10 cm petri dishes. The plates were then streaked with the 3 strains of methicillin resistant Staphylococcus aureus: HA-MRSA (hospital acquired), CA-MRSA (community acquired), and 252-MRSA. Three small disks were placed on the surface of each plate using sterile forceps. One disk on each plate was inoculated with 30 µL of dimethyl sulfoxide. This disk would act as the control. The other 2 disks on each plate were inoculated with 20 µL (40 µg) or 30 µL (60 µg) of peak 1 from *Penicillium virginium* which had been dissolved in DMSO. The plates were placed upside down in a 32°C incubator overnight and then the zones of inhibition were measured and

photographed. Preliminary results showed that only peak 1 was reactive with gram-positive bacteria (data not shown), so plates were only streaked with strains of MRSA which are gram-positive bacteria. Peak 2 was nonreactive with both gram-negative and gram-positive bacteria so it was not used.

Results and Discussion:

The results, shown in Figure 3.8, show that the metabolites produced by *Penicillium virginium*, specifically the compounds that make up peak 1, have antibacterial properties to gram-positive bacteria like MRSA. As Table 3.2 shows, their effects are dose dependent- every increased dose resulted in an increase in the diameter of the inhibition zone. Also the inhibitory effect on MRSA also suggests strongly that the antibacterial component in peak 1 was not penicillin or a beta-lactam antibiotic. Discovering that these metabolites have antibacterial properties provides more evidence to our claim that *Penicillium virginium* is an ecologically relevant species.

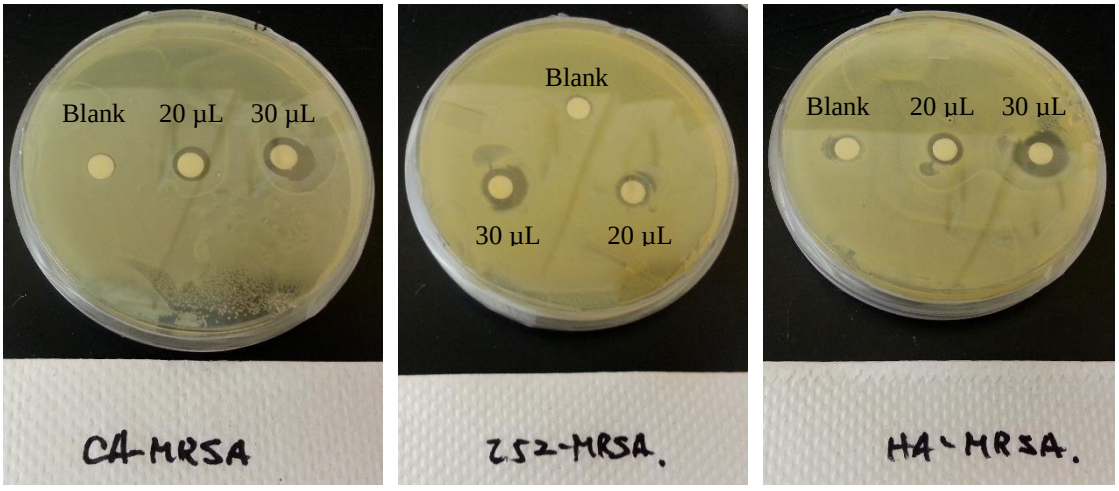


Figure 3.8 TSA plates streaked with various strains of MRSA and inoculated on the disks with metabolites extracted from *Penicillium virginium*.

Table 3.2 Zone of inhibition measurements for MRSA plates treated with and HPLC separated fraction of metabolites extracted *Penicillium virginium*.

	Dose (µL)	Diameter (mm)	percent diameter increase with 10 µL dose increase
CA-MRSA	20	8	75
CA-MRSA	30	14	
252-MRSA	20	11	27
252-MRSA	30	14	
HA-MRSA	20	10	40
HA-MRSA	30	14	

CHAPTER 4

SEPARATION AND ANALYSIS OF THE BIOACTIVE METABOLITE

Introduction:

"Fungal secondary metabolites are of intense interest to humankind due to their pharmaceutical (antibiotics) and/or toxic (mycotoxins) properties" (Yu & Keller, 2005). Fungi are the source for over 50% of metabolites used in pharmaceutical industry (Nielsen and Smedsgaard, 2002). They are also responsible for millions of wasted dollars each year due to contaminated food crops. Some of the most notable secondary metabolites are penicillin, aflatoxin, and statins. "The most obvious fungal natural products are the pigments—typically brown and black pigments referred to as melanins—giving color to spores, appressoria, sclerotia, sexual bodies, and other developmental structures" (Yu & Keller, 2005). Due to the fact that such a small section of the fungal kingdom has been explored and identified, many of the secondary metabolites have also not been identified. These unexplored secondary metabolites could have great importance from both a beneficial and harmful perspective. To determine what secondary metabolites are produced by *Penicillium virginium* and understand the function of these metabolites, experiments were designed to fractionate the metabolites so that the individual fractions could be analyzed. Fractions were generated first by solid phase extraction, or flash chromatography, and then bioactive fractions were separated using HPLC. As described later in this chapter, we developed an assay for gross qualitative characterization of the fungal growth inhibitory abilities of these initial

fractions obtained from flash chromatography. Later in this chapter we have optimized protocols that have identified more specific fractions that contain the antifungal properties. For characterizing those specific fractions we have developed a systematic protocol for quantatively comparing the bioactive potentials of these fractions with the less or non-bioactive controls.

4.1 SEPARATION AND CHARACTERIZATION OF BIOACTIVE METABOLITES USING SOLID PHASE EXTRACTION

Methods:

Solid phase extraction

Metabolites were extracted from fifteen 10 cm petri dishes containing *Penicillium virginium* on PDA using the solid media extraction process outlined in section 3.3.1. Samples were not rehydrated with methanol after the chloroform had evaporated. They were left dried so that they could be easily transported. Solid phase extraction was completed at the Hollings Marine Laboratory in Charleston, SC. Extraction products were rehydrated and combined together using a solution of 50% methanol and 50% dichloromethane. Rehydrated extraction products were bound to silica gel Iatrobeads using a rotary evaporator. A 1 inch wide by 17 inch tall, 500 mL reservoir column was packed with Iatrobeads and the extracted metabolite Iatrobead plug was loaded on top (see Figure 4.1). A sequence of 500 mL of each solvent was pushed through the Iatrobead plug, through the clean iatrobeads, and out of a spout at the bottom of the tube using nitrogen gas. The solvents were run in order from most non-polar to polar; this is called normal phase. The solvent order used was: hexanes, dichloromethane, ethyl acetate, and then methanol. The goal of this process was to use the polarity gradient to

divide up the metabolites into fractions that would span from nonpolar compounds to polar compounds. The fractions were dried using the rotary evaporator and transported back to our lab.

Figure 4.1 Images of the reservoir column packed with Iatrobeads in the body of the column and the plug of metabolites at the top. Dichloromethane is being pushed through in these photos.

Determining fraction concentrations

The fractions were all rehydrated with their respective solvent and the concentrations of each fraction was determined by weighing 100 µL of the fraction and comparing that to the weight of 100 µL of just the solvent (see Table 4.1). 1 µg/µL dilutions in DMSO were made from each of the fractions and used for the *Aspergillus parasiticus* B62 assays.

Table 4.1 Measurements collected and calculations made to determine the concentration of each solid phase extraction fraction

Fraction	Rehydration amount (mL)	Weight difference (g)	Concentration of fraction (µg/µl)
Methanol	10	0.0008	8
Ethyl acetate	9	0.00066	6.6
Hexane	7	0.00069	6.9
DCM	7	0.00155	15.5

Inoculating and treating plates *Aspergillus parasiticus* B62 plates

For the *Aspergillus parasiticus* B62 assays, YES agar was prepared according to manufacturer's instructions and poured into five 10 cm petri dishes. Dishes were labeled with blank, Meth, Et. Ac., Hex, and DCM to represent the 4 fractions and a blank. Using a marker to draw on the underside of the dish, the plate was divided into thirds. Within each third, a small circle was drawn to act as a marker for placement of a metabolite dose (see Figure 4.2). Each plate was centrally inoculated with 1 µL of *Aspergillus parasiticus* B62 spore solution and each of the three markers were inoculated with 10 µL of the 1 µg/µL DMSO fraction dilutions, which totaled up a 30 µg dose. The plates were then placed in a 29°C incubator for 72 hours. After 72 hours the culture sizes were measured and images.

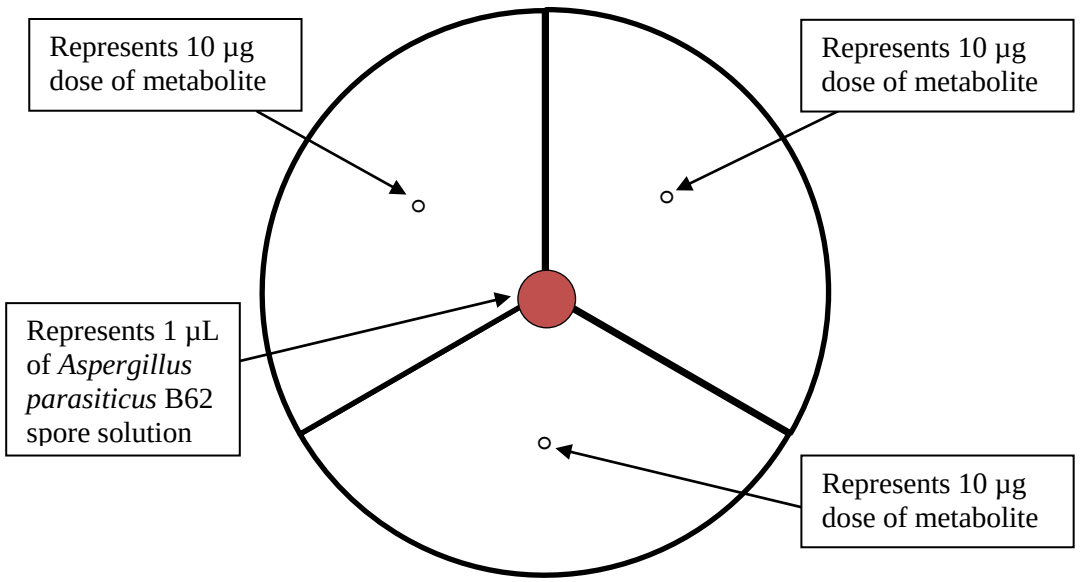


Figure 4.2 Representative diagram of *Penicillium virginium* metabolite fractions and *Aspergillus parasiticus* B62 interaction YES plates.

Norsolorinic acid levels were measured using the same extraction and TLC protocols outlined in section 3.3.1.

Results and Discussion:

After 72 hours, as Figure 4.3 shows, the colors of all the cultures were very similar. Visually the sizes were also very similar. The only plate that showed a visible effect as compared to the blank was the plate that was inoculated with metabolites from the methanol fraction. The shape of the culture on this plate was triangular compared to all the others which were relatively circular. This triangular shape of the culture resulting from the metabolites in the methanol fraction indicated that these metabolites inhibited the expansion of *Aspergillus parasiticus* B62 mycelia and hence decreased the growth rate of the mycelial fronts that were closest to the fractions. The diameters of the cultures were collected and the area of the cultures was calculated (Table 4.2). For the methanol plate 2 diameter measurements were collected and average to account for the shape

Figure 4.3 *Aspergillus parasiticus* B62 and *Penicillium virginium* metabolite fraction interaction plates (right). Comparison of the blank *Aspergillus parasiticus* B62 culture to the *Aspergillus parasiticus* B62 culture inoculated with metabolites from the methanol fraction.

Table 4.2 Measurements collected and calculations made for *Aspergillus parasiticus* B62 treated with *Penicillium virginium* metabolite fractions.

Fraction	Culture Diameter (cm)	Diameter % compared to control	Area (cm^2)	Area % compared to control
Blank	4.5	100	15.90431281	100
DCM	4.45	98.88888889	15.55284713	97.79012346
Hexane	4.65	103.3333333	16.98227179	109.1907587
Ethyl acetate	4.4	97.77777778	15.20530844	89.53636259
Methanol	3.75	83.33333333	11.04466167	72.63688017

Our results suggested that the fraction of metabolites that were extracted with methanol have antifungal properties. In our screening assay, these metabolites reduced the area of *Aspergillus parasiticus* B62 growth by about 30% but they did not stop its secondary metabolite production (see Figure 4.4).

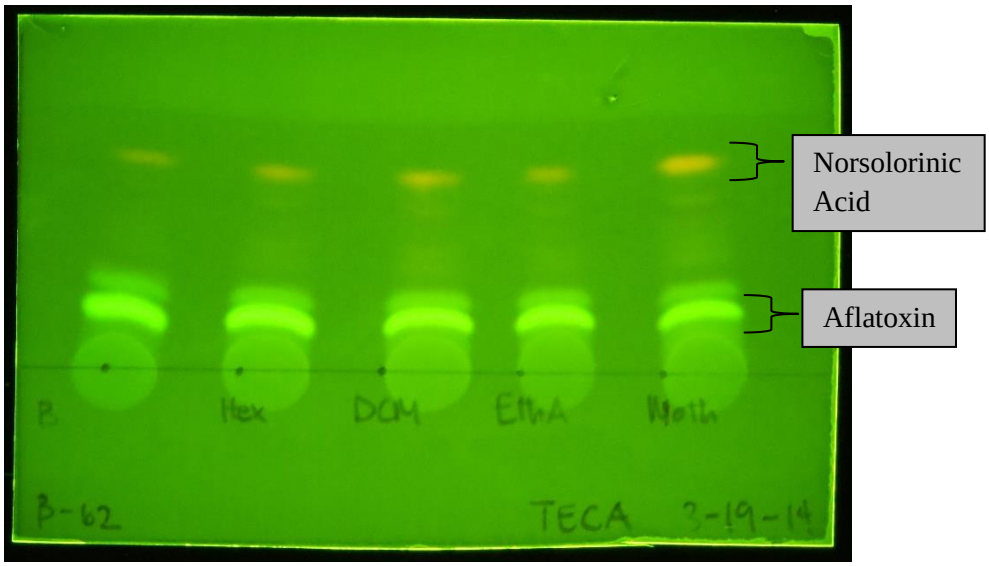


Figure 4.4 A TLC plate showing the metabolites extracted from *Penicillium virginium* metabolite fraction treated *Aspergillus parasiticus* B62 cultures.

To determine the composition of these antifungal metabolites more separation needed to be completed. During the first HPLC process discussed in 3.3.2 it was discovered that *Penicillium virginium* made too many compounds to analyze with just one round of separation. Hence, as described in section 4.2, we developed a systematic

methodology to generate sub-fractions from the methanol fraction to isolate a specific sub-fraction containing the major bioactive compound.

4.2 Separation and characterization of bioactive metabolites from the methanol fraction using HPLC

Methods:

HPLC Separation of the methanol fraction

Using a Waters® HPLC system consisting of: a Waters® 2767 Sample Manager, a Waters® 600 Pump, and Waters® 2996 Photodiode array detector set to from 210 to 400 nm, the remainder of methanol fraction was separated into 75 fractions. Looking at Figure 4.5, it can been seen that fractions 1-35 contained the bulk of the detectable compound levels. A mobile phase of 70% water and 1% formic acid and 30% acetonitrile and 1% formic acid was used in conjunction with a Waters® Xtera column that was 250 mm by 22 mm. All of the fractions were dried so that they could be transported back to Columbia.

A. Minute 1-75

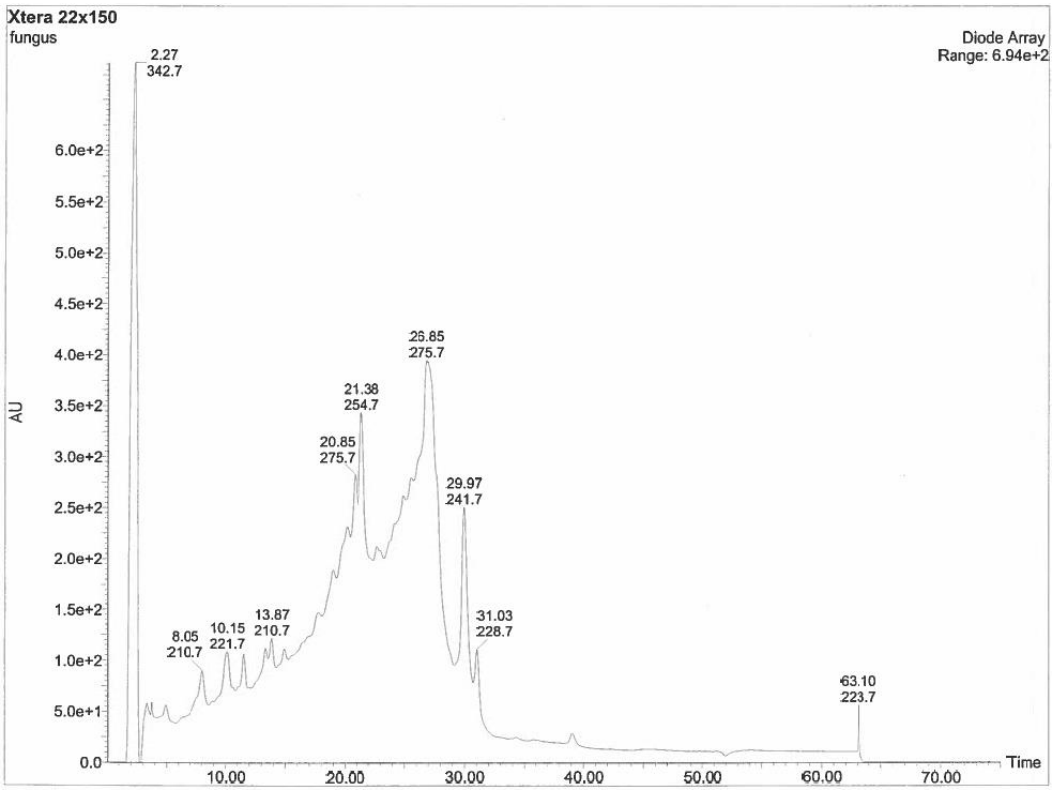


Figure 4.5 (A) Liquid chromatography chromatographs associated with the methanol fraction. The Y axis represents a given response from the instrument and the X axis represents the time of elution off of the LC column. The individual numbers above each represent the PDA signal (mas UV associated with the peak) and the nominal mass of the predominant ion under a given peak. Part A represents the reading collected during the entire 75 minute run and Part B represents the fractions collected during minutes 1-35.

B. Only minutes 1-35

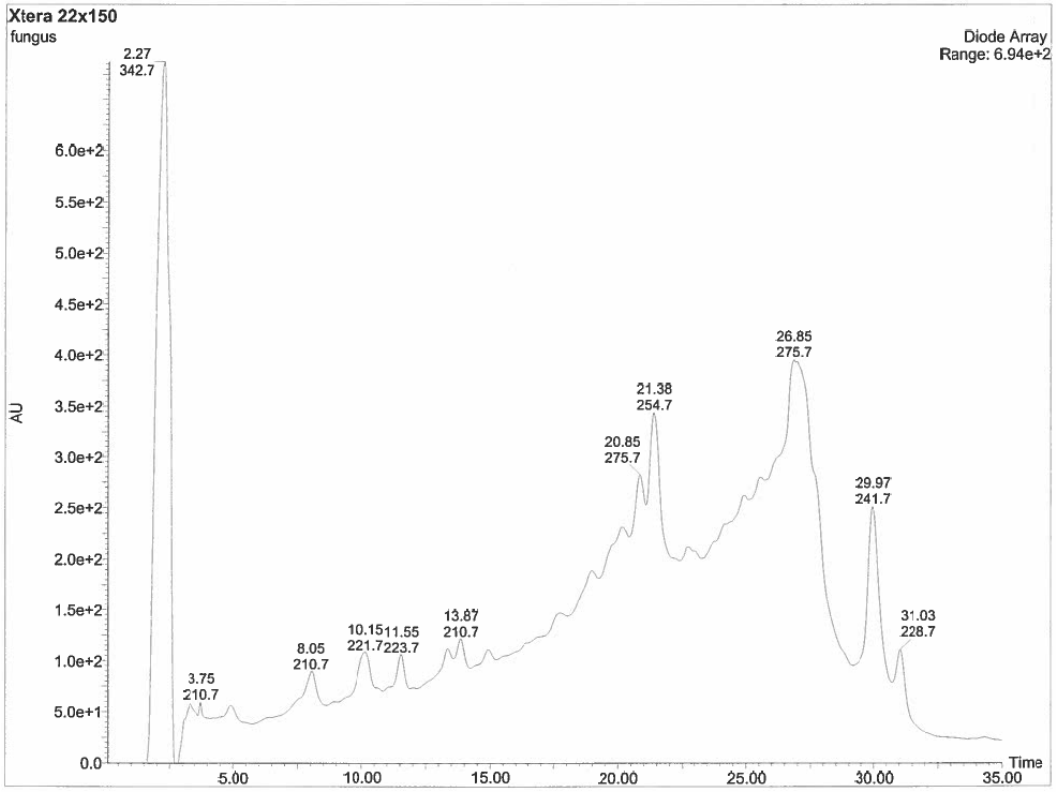


Figure 4.5 (B) Liquid chromatography chromatographs associated with the methanol fraction. The Y axis represents a given response from the instrument and the X axis represents the time of elution off of the LC column. The individual numbers above each represent the PDA signal (mas UV associated with the peak) and the nominal mass of the predominant ion under a given peak. Part A represents the reading collected during the entire 75 minute run and Part B represents the fractions collected during minutes 1-35.

Table 4.3 Concentrations of HPLC fractions. Concentration of fraction 4, 9, 11, 12, 16, and 27 not determined.

Sample Label	Rehydration amount (mL)	Weight difference(g)	Concentration of sample(µg/µl)	Concentration of diluted sample (µg/µl)	Dose per marker (µg)	Total dose per plate
1	3.5	0.00038	3.8	0.342	10.26	30.78
2	1	0.00044	4.4	0.352	10.56	31.68
3	1	0.00007	0.7	0.336	10.08	30.24
5	1	0.00009	0.9	0.3375	10.125	30.375
6	1	0.00004	0.4	0.336	10.08	30.24
7	1	0.00007	0.7	0.336	10.08	30.24
8	1	0.00023	2.3	0.345	10.35	31.05
10	1	0.00003	0.3	Not diluted	9	27
13	1	0.00008	0.8	0.336	10.08	30.24
14	1	0.00008	0.8	0.336	10.08	30.24
15	1	0.00007	0.7	0.336	10.08	30.24
17	1	0.00004	0.4	0.336	10.08	30.24
18	1	0.00011	1.1	0.3355	10.065	30.195
19	1	0.00015	1.5	0.3375	10.125	30.375
20	1	0.00011	1.1	0.3355	10.065	30.195
21	1.5	0.00006	0.6	0.336	10.08	30.24
22	1	0.00004	0.4	0.336	10.08	30.24
23	1	0.0001	1	0.335	10.05	30.15
24	1	0.00014	1.4	0.336	10.08	30.24
25	1	0.00027	2.7	0.3375	10.125	30.375
26	1	0.00024	2.4	0.336	10.08	30.24
28	1	0.00004	0.4	0.336	10.08	30.24
29	1	0.00011	1.1	0.3355	10.065	30.195
30	1	0.00005	0.5	0.335	10.05	30.15

Determination of fraction concentration and *Aspergillus* parasiticus B62 plate treatment and inoculation

Each fraction was individually rehydrated with methanol and, using the same method described in section 4.1, the concentrations were calculated (Table 4.3). 0.3 µg/µL dilutions were made with methanol from each of the fractions. Using the same protocol described in section 4.1, YES plates were treated with 30 µL (10 µg) of diluted fraction solution on each marker (totaling 30 µ, and inoculated with 1 µL of *Aspergillus* parasiticus B62 spore solution. Concentrations could not be calculated for some of the fractions because there was no detectable weight difference. For those fractions, 30 µL of the rehydrated solution was added to each marker. The plates were incubated for 72 hours at 29°C and then the cultures were imaged and measured. All of the plates were run in duplicates. The averages of the diameters from the duplicate cultures were used for assessing our results. As we decided previously, a reduction of the area of the culture by at least 50% was the criterion for considering the reduction of growth as significant.

Results and Discussion:

As shown in Table 4.4, many of the fractions hindered the growth of *Aspergillus parasiticus* B62. However, based on our criterion, significant reduction of growth was observed starting at fraction 16 and continued until fraction 27. The fractions that hindered growth the most were 25 and 26. Figure 4.6 shows images of these plates. This pattern correlated with the peaks seen on the HPLC chromatograph (Figure 4.5). These fractions represented the section of the chromatograph where the greatest peak heights were observed, signifying the presence of a compound in high quantity. It is possible that the fractions which hindered growth the most, fractions 16-27, contain many of the same

compounds. Since these fractions sequentially came off of the LC column, the compounds that they contain have similar polarities and could likely have similar compositions as well since the predominant molecular masses are very similar.

Table 4.4 Measurements collected and calculations made for HPLC fraction and *Aspergillus parasiticus* B62 interaction plates.

Sample	Plate 1 Diameter (cm)	Plate 2 Diameter (cm)	Plate diameter average (cm)	Diameter percentage compared to control	Average radius (cm)	Area of the culture (cm^2)	Area percentage compared to control
Blank	3	3.2	3.1	100	1.55	7.54767635	100
1	2.4	2.4	2.4	77.41935484	1.2	4.523893421	59.93756504
2	3.1	3	3.05	98.38709677	1.525	7.306166415	96.80020812
3	3.2	3.4	3.3	106.4516129	1.65	8.552985999	113.3194589
4	3.1	3.2	3.15	101.6129032	1.575	7.793113276	103.251821
5	3.1	3.2	3.15	101.6129032	1.575	7.793113276	103.251821
6	3.2	3.1	3.15	101.6129032	1.575	7.793113276	103.251821
7	3.1	3.2	3.15	101.6129032	1.575	7.793113276	103.251821
8	3.2	3.1	3.15	101.6129032	1.575	7.793113276	103.251821
9	2.9	2.7	2.8	90.32258065	1.4	6.157521601	81.58168575
10	2.9	2.8	2.85	91.93548387	1.425	6.379396582	84.52133195
11	2.7	2.7	2.7	87.09677419	1.35	5.725552611	75.85848075
12	2.4	2.4	2.4	77.41935484	1.2	4.523893421	59.93756504
13	2.7	2.7	2.7	87.09677419	1.35	5.725552611	75.85848075
14	2.7	2.7	2.7	87.09677419	1.35	5.725552611	75.85848075
15	2.6	2.5	2.55	82.25806452	1.275	5.107051557	67.66389178
16	1.8	1.8	1.8	58.06451613	0.9	2.544690049	33.71488033
17	1.9	2	1.95	62.90322581	0.975	2.986476516	39.56815817
18	2.3	2.4	2.35	75.80645161	1.175	4.337361357	57.46618106
19	2.3	2.6	2.45	79.03225806	1.225	4.714352476	62.46097815
20	2.1	2.2	2.15	69.35483871	1.075	3.63050301	48.10093653
21	1.7	1.7	1.7	54.83870968	0.85	2.269800692	30.07284079
22	1.4	1.6	1.5	48.38709677	0.75	1.767145868	23.41311134

Table 4.4 Continued Measurements collected and calculations made for HPLC fraction and *Aspergillus parasiticus* B62 interaction plates.

Sample	Plate 1 Diameter (cm)	Plate 2 Diameter (cm)	Plate diameter average (cm)	Diameter percentage compared to control	Average radius (cm)	Area of the culture (cm^2)	Area percentage compared to control
23	1.5	1.8	1.65	53.22580645	0.825	2.1382465	28.32986473
24	1.6	1.6	1.6	51.61290323	0.8	2.010619298	26.63891779
25	1.4	1.4	1.4	45.16129032	0.7	1.5393804	20.39542144
26	1.4	1.4	1.4	45.16129032	0.7	1.5393804	20.39542144
27	1.8	1.9	1.85	59.67741935	0.925	2.688025214	35.61394381
28	2.3	2.3	2.3	74.19354839	1.15	4.154756284	55.04682622
29	2.8	2.8	2.8	90.32258065	1.4	6.157521601	81.58168575
30	3	3	3	96.77419355	1.5	7.068583471	93.65244537

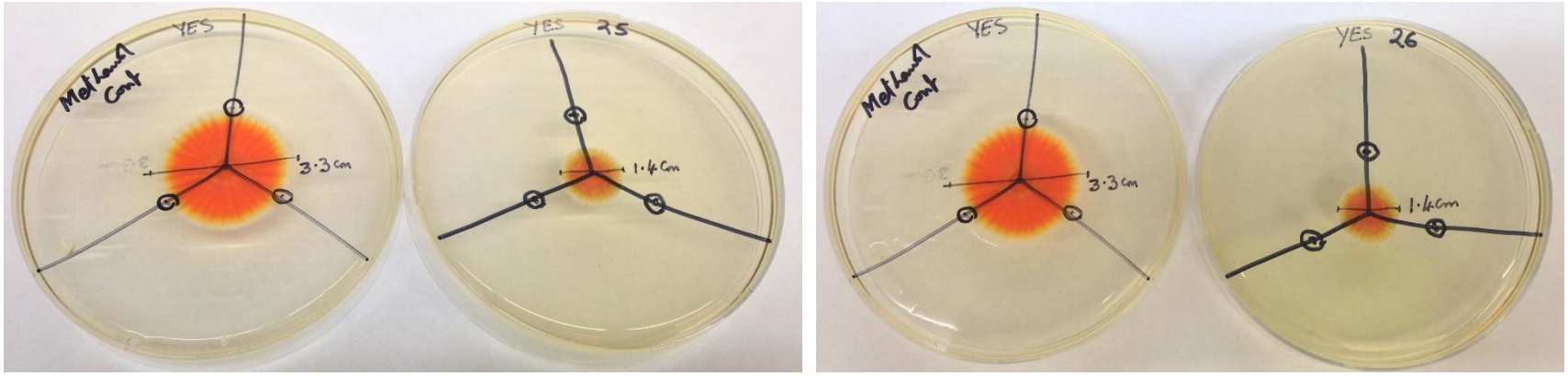


Figure 4.6 Images of HPLC fraction 25 and 26 and *Aspergillus parasiticus* B62 interaction plates. Plate on the left side of both images is the control which was treated with only methanol. Left image is fraction 25 and right image is fraction 26.

CHAPTER 5

FUTURE STUDIES, OVERALL SUMMARY, AND CONCLUSIONS

5.1 FUTURE STUDIES

Identification of bioactive secondary metabolites produced by *Penicillium virginium*

Interaction experiments completed as part of this thesis confirm that *Penicillium virginium* produces bioactive metabolites that have both antifungal and antibacterial properties. In order to identify the chemical structure and the intrinsic characteristics of the individual metabolites that responsible for the bioactive properties, further studies need to be completed. HPLC fractions 25 and 26, discussed in section 4.2, should be analyzed using liquid chromatography-mass spectrometry. If the fractions have been separated enough then predominant compounds can be identified and isolated. If the fractions still contain too many compounds to achieve good clean separation or compounds, then the fractions must be separated again using HPLC and then assayed using the *Aspergillus parasiticus* B62 assay described in section 4.1. Once bioactive fractions have been separated enough to identify and isolate the predominant compounds, mass and UV index information can be used to identify the compound. Isolates should also be submitted for nuclear magnetic resonance analysis to determine the structure. Identification of the bioactive metabolites is critical to understanding the ecological role of *Penicillium virginium* and the impact it could have if exposed to novel environmental signals that are more conducive for fungal growth.

5.2 Overall summary and Conclusions

In order to confirm identity and novelty of the *Penicillium virginium*, phylogenetic analysis, using ITS sequencing, and macroscopic analysis coupled with light and electron microscopy were combined. DNA was extracted and purified using a modified protocol from Janso et al. (2005), which included a combination of extraction buffer and phenol-chloroform-isoamyl alcohol solution. Amplification was done using a combination of universal ITS specific primers, from White et al. (1990) and Gardes & Bruns (1993), as well as custom designed primers. The ITS1-5.8S-ITS2 region of the rRNA gene was chosen because of its highly specific and highly conserved nature. DNA was sequenced and homology comparison analysis to sequences published in GenBank was completed using BLAST. Results confirmed novelty of the species and identified it as a part of the genus *Penicillium*. Optical and SEM microscopic analysis of the conidia and spores demonstrated the phylogenetic characteristics of a *Penicillium sp*.

To determine how drops in salinity, due to environmental changes would affect the growth and secondary metabolite production of *Penicillium virginium*, the strain was grown in PDB containing sodium chlorine corresponding to a range of PSU. Culture comparisons showed that growth and metabolite production of *Penicillium virginium* is dependent on the concentration of NaCl in the growth medium. When the concentration of NaCl decreased the growth as well as the metabolite production increased.

It is already well established that the ongoing changes in global climate will have profound impacts on the components of marine ecosystems (Beaugrand et al., 2014; Edwards and Richardson, 2004). Changes in temperature, wind and water circulation, precipitation, rise in sea-levels and fluctuations in salinity will influence their metabolism

as well, and alter their behaviors in unpredictable ways (Coelho et al., 2013). In addition, the past decade has witnessed severe weather events at frequent intervals. Comparison of microbial community structures in coastal ecosystems performed after hurricane Katrina (Amaral-Zettler et al., 2008; Sinigalliano et al., 2007) provide evidence in favor of the feasibility of reorganization of the microbial community structures in the flooded zones. It is possible that such severe weather disturbances will expose fungal communities to novel environmental signals (low salinity being the most prominent of these) and elevated interactions with pathogenic terrestrial and wastewater microbes. Hence understanding how *Penicillium virginium* interacts with other fungal and bacterial community members is essential to understanding how it could impact or imbalance other ecosystems. Interaction experiments with *Penicillium virginium* and *Aspergillus parasiticus* construct B62 showed that the presence of *Penicillium virginium* and it metabolites hindered its growth and the production of norsolorinic acid that it synthesizes as a part of secondary metabolism. Interaction studies with metabolites extracted from *Penicillium virginium* and *Aspergillus parasiticus* B62 showed that the extract collected from a solid culture (PDA) decreased B62 growth and norsolorinic acid more effectively than an extract collected from a liquid culture (PDB). A possible explanation of this difference could be that the fungus generates more of the antifungal secondary metabolites on a solid subtrate than in a liquid substrate. Interaction experiments with *Penicillium virginium* and methicillin resistant *Staphylococcus aureus* showed that metabolites produced by *Penicillium virginium* have antibacterial properties to gram-positive bacteria like MRSA. The discovery of these antifungal and antibacterial properties supports our notion that *Penicillium virginium* is an ecologically relevant and

has the ability to result significant alterations to the composition of a microbial community in an ecosystem. These changes in the community can affect their contribution to biogeochemical cycles. For example, without certain bacteria present in the soil, phosphorous cycle will not continue and the soil will become phosphorus poor, which would be detrimental to agricultural crops. In addition, the metabolites produced by *Penicillium virginium* could cause severe environmental health and public health concerns.

Identification of bioactive secondary metabolites produced by *Penicillium virginium* is necessary to understand what compounds would cause alterations to foreign ecosystems if relocated from its ecological niche. Extracted secondary metabolites from *Penicillium virginium* were separated into 4 polarity-based groups using solid phase extraction. Interaction studies between those groups and *Aspergillus parasiticus* B62 showed that the metabolites extracted with methanol were the most bioactive. This methanol fraction was then separated again using HPLC. The HPLC fractions were run through the *Aspergillus parasiticus* B62 interaction assay. The results showed that fractions 25 and 26 demonstrated the highest bioactivity. The findings from this project emphasizes the need for initiating new efforts to investigate the diversity of facultative marine fungi and their potential ecological role upon activation of their bioactive potential triggered by severe weather impacts resulting from ongoing climate change.

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