Spring 5-11-2019

See the forest for the leaves: foliar fungal community composition and volatile organic compound production

Joshua Copeland
jcopeland@dons.usfca.edu

See the forest for the leaves: foliar fungal community composition and volatile organic compound production

A thesis presented to the faculty
of the Department of Biology
at the University of San Francisco
in partial fulfillment of the requirements for the Degree of
Master of Science in Biology

Written by

Joshua Copeland
Bachelor of Science in Biology and Environmental Science
College of William and Mary
8/29/2019

See the forest for the leaves: foliar fungal community composition and volatile organic compound production

Thesis by Joshua Copeland

This thesis is written under the guidance of the Faculty Advisory Committee, and pending approval by all its members

Master of Science
in Biology
at
the University of San Francisco

Thesis Committee

Naupaka B. Zimmerman, PhD.
Research Advisor

Jennifer A. Dever, PhD.
Professor

John R. Paul, PhD.
Associate Professor

Marcelo Camperi, PhD.
Dean, College of Arts and Sciences

Acknowledgements

Although this thesis is ultimately the culmination of countless of hours of my labor, stress, and fear, I truly realize how impossible it would have been to get to this point without the support, knowledge and goodwill of the countless individuals that I have been blessed to cross paths with during my life and my time at USF. Due to my own paranoia and self-doubt, I often felt inadequate and frequently considered leaving all of the doors that this program could open for me, closed. Fortunately, those negative thoughts were overwhelmingly diluted with the knowledge that that my family, friends, peers, and USF faculty and staff wanted to see me succeed. For that, I am immensely grateful.

I am extremely appreciative of the two people who have instilled within me a love for research; Dr. Kurt Williamson and Dr. Naupaka Zimmerman. These men were excellent role models who convinced me that I could wholly succeed with my career goals. After Dr. Williamson convinced me to start down this metaphorical track, Dr. Zimmerman showed me how fun it was to run it. I truly hope that one day, I can be the same caliber of mentor to young minds as they were to me.

I am deeply thankful for my thesis committee members, Dr. Dever and Dr. Paul for their time, mentorship, insight, patience and understanding. My greatest regret about graduate school is not reaching out to you both more.

I am also very grateful for Jeff Oda and Matt Helm for their expertise on a wide variety of subjects and their willingness to help out whenever possible. They are truly an underrated resource of USF and deserve more accolades for all that they do.

I am so thankful to have worked with Amirtha Maria, Emre Övet, and Victoria Lamar as they began their research careers in the Zimmerman Lab. They were invaluable to helping me collect and process samples and although they might not have known it, they taught me some things as well. I am excited to watch them grow and develop into the world class researchers I know they are capable of becoming.

I am also thankful for the California Academy of Sciences for allowing me to collect herbarium samples that aided with data analysis. Through my interactions with Cal Academy staff, it was obvious that they have an immense love for increasing the accessibility and communication of science.

I consider myself lucky to have been selected as a recipient of the Whitehead Summer Research Scholarship in 2018. I am deeply grateful for Dr. and Mrs. Whitehead and would like to pay forward their gift by implementing a similar scholarship program later in my career.

I also want to thank my parents, Paul and Renee Copeland, as well as my whole family for the lifetime of love they have showed me. I will gladly spend the rest of my life trying to reciprocate all the affection that you poured into me to you and anyone I come in contact with. My mother would also be disappointed if I didn't, so I want to thank God for abundant mercy, grace and glory. I also appreciate anyone who took the time out of their day to read this work. Thank you!

Table of Contents

Abstract 7

Thesis Summary 8

Thesis Introduction 9

Chapter I: Poplar Foliar Community Structure and Phylogenetics 15

Introduction 15

Methods 17

Host Selection 17

Site Selection 18

Field Sampling 18

Surface Sterilization 19

Fungal Culturing 19

Fungal DNA Extraction 20

Fungal PCR Amplification and DNA Sequencing 20

Niles Canyon Tree DNA Extraction 21

Herbarium Sample DNA Extraction 21

Populus PCR Amplification and DNA Sequencing 23

Sequence Quality Control and Analysis 23

Bioinformatics Processing, Community and Statistical Analyses 24

Results 24

Culturing results 24

Tree Genotyping 25

Overall Fungal Community Analysis 25

Summer P. fremontii and P. trichocarpa Fungal Community Analysis 25

Seasonal P. fremontii Fungal Community Analysis 26

Discussion 26

Overall Fungal Community Analysis 26

Summer P. fremontii and P. trichocarpa Fungal Community Analysis 28

Seasonal P. fremontii Fungal Community Analysis 28

ITS Analysis and Phylogenetics 29

Culturing Issues 29

Chapter II: Volatile organic compound analysis of two commonly isolated putative pathogens and two commonly isolated putative endophytes 30

Introduction 30

Methods 33

Fungal Culturing 33

Solid Phase MicroExtraction 34

Gas Chromatography/Mass Spectroscopy Analysis 34

Chromatogram and Mass Spectra Examination 36

Results 36

Control VOC Profiles 36

Mycosphaerella sp. PB 2012 VOC Profiles 37

Penicillium glabrum VOC Profiles 37

Marssonina brunnea VOC Profiles 38

Cladosporium VOC Profiles 38

Discussion 39
Phenylethyl alcohol *39*
Styrene *39*
Aristolochene & Valencene *40*

Conclusions 41

Supplemental Tables and Figures 43
Table 1. Fungal endophytes isolated from first eight poplar trees surveyed in Niles Canyon on November 5, 2017. 43
Table 2. Fungal endophytes isolated from first eight poplar trees surveyed in Niles Canyon on July 12th, 2018. 43
Table 3. Fungal endophytes isolated from first remaining 13 poplar trees surveyed in Niles Canyon on July 5th, 2018. 44
Fig 1. PhyML Maximum Likelihood tree of extracted poplar ITS sequences 45
Fig 2. Mr. Bayes tree of extracted poplar ITS sequences. 46
Table 4. Total isolated and sequenced fungi isolated from November 2017 to July 2018. 47
Table 5. Total sequenced foliar fungi isolated from P. trichocarpa and P. fremontii in July 2018. 49
Table 6. Total sequenced foliar fungi isolated from P. fremontii in November 2017 and July 2018. 50
Fig 3. Box Plot of OTUs isolated from two poplar species in July 2018. 51
Fig 4. NMDS of foliar fungi microbiomes isolated from both poplar species in July 2018 52
Fig 5. Box Plot of OTUs isolated from P. fremontii in November 2017 and July 2018. 52
Fig 6. NMDS of foliar fungi microbiomes isolated from P. fremontii in November 2017 and July 2018 53
Fig 7. Organic volatile profile of SPME Fiber after being conditioned at 270°C for one hour. 54
Fig 8. Averaged VOC profile of SPME fibers. 55
Table 7. NIST 2017 database results of averaged peaks for SPME fiber. 55
Fig 9. Mass spectra for five most abundant SPME compounds 56
Fig 10. Chromatograms obtained from empty vial headspace analysis. 57
Fig 11. Mean chromatogram of empty vial headspace. 58
Table 8. NIST 2017 database results for Vial & SPME compounds 58
Fig 12. Mass spectra of top five library searches for vial and SPME compounds 59
Fig 13. Chromatograms of VOCs emitted from malt extract agar and sampling vials. 60
Fig 14. Average of four MEA, SPME and sampling vial chromatograms. 61
Table 9. NIST 2017 database results for MEA compounds 61
Fig 15. Mass Spectra for top five MEA VOCs 62
Fig 16. Chromatograms of VOCs emitted from Mycosphaerella sp. PB-2012b. 63
Fig 17. Mean chromatogram of Mycosphaerella VOCs 63
Fig 18. Averaged Mycosphaerella sp. PB-2012b chromatogram with abundance of control VOCs subtracted. 65
Table 10. NIST 2017 database results for Mycosphaerella compounds 65
Fig 19. Mass spectra for five most abundant Mycosphaerella sp. PB-2012b compounds 66
Fig 20. Chromatograms of VOCs emitted from four isolated Penicillium glabrum cultures 67
Fig 21. Average of four Penicillium glabrum sp. PB-2012b chromatograms. 68
Fig 22. Averaged Penicillium glabrum chromatogram with abundance of control VOCs subtracted. 69
Table 11. NIST 2017 database results for Penicillium compounds 69
Fig 23. Mass spectra for three most abundant Penicillium glabrum compounds and styrene monomer 70
Fig 24. Chromatograms of organic volatiles emitted by Marssonina brunnea. 71
Fig 25. Average of four Marssonina brunnea chromatograms. 72

Fig 26. Averaged Marssonina brunnea chromatograms with particle abundance of control VOCs subtracted. 73
Table 12. NIST 2017 database results for Marssonina compounds 73
Fig 27. Mass spectra for five most abundant Marssonina brunnea compounds 74
Fig 28. Chromatograms of VOCs emitted from Cladosporium sp. 75
Fig 29. Average of four Cladosporium sp. chromatograms. 76
Fig 30. Average of four Cladosporium sp. Chromatograms with control peaks removed. 77
Table 13. NIST 2017 database results for Cladosporium compounds 77
Fig 31. Mass spectra for five most abundant Cladosporium sp. compounds 78
Bibliography 79

Abstract

Endophytes are microbes that live within plants without causing detectable illness. These microorganisms can emit volatile organic compounds (VOCs), airborne signaling molecules that can significantly affect plant growth. Despite the prevalence of endophytes and their VOCs, little is known about these factors important to plant health. With this in mind I isolated fungal endophytes from leaves of *Populus trichocarpa* and *Populus fremontii,* tree species with economic and environmental uses, to determine if endophyte communities are reserved between the closely related tree species and over seasons. Surface sterilized leaf fragments were plated onto malt extract agar and the resulting fungi were DNA sequenced and vouchered once significant biomass grew. I sequenced the ITS region of rDNA to determine fungal and tree species. Ultimately, I found that the host species had a slight effect of endophyte community composition during the summer. I also found that there was no significant seasonal difference in foliar endophyte communities of *P. fremontii* during the summer and fall. Taxa of interest included *Penicillium, Mycosphaerella, Cladosporium,* and *Marssonina*. Following endophyte isolation, I assayed the VOC profiles of the aforementioned taxa. The microbial VOC profiles were assessed using solid phase microextraction (SPME) and GCMS techniques. SPME fibers were inserted airtight tubes containing malt extract agar and two weeks of fungal growth for an hour. Fibers were then subjected to a GCMS run where absorbed VOCs were desorbed into the GCMS to be identified via the NIST molecular library. *Penicillium* and *Cladosporium* released styrene, a compound known for its antimicrobial effects. *Marssonina* and *Mycosphaerella* emitted phenylethyl alcohol, a compound with multiple industrial uses. *Marssonina* also released aristolochene and valencene, known precursors to mycotoxins. Through this work, I have shown that poplar foliar microbiomes are influenced by hosts and that foliar microbes can produce volatile compounds with economic and botanical significance.

Thesis Summary

Symbiotic interactions are maintained by multifarious lifeforms worldwide. While these relationships are more apparent between multicellular organisms, multitudes more interspecific relations exist as endosymbiotic associations. In plants, fungi express genes and emit metabolites that can influence host phenotype. As research surrounding endosymbiotic relationships progresses, it is increasingly apparent that internally residing microorganisms affect host health and survival. **Through this thesis project, I examined and compared the foliar endophyte community composition of two closely related poplar species, *Populus trichocarpa* and *Populus fremontii*. I also surveyed the volatile organic profiles of poplar endophyte isolates.**

The first chapter of this thesis details the foliar fungal microbiome of surface sterilized poplar leaves. Out of 22 trees, seven autumn foliar microbiomes and 20 summer foliar microbiomes were sampled for foliar community analysis. Tree and fungal species were identified by sequencing the internal transcribed spacer (ITS) region of ribosomal DNA. Through the use of genetic analysis software and bioinformatic pipelines, I determined that while there was no significant difference in *P. fremontii* foliar endophyte communities between the summer and fall, there was a slight difference in community composition between *P. trichocarpa* and *P. fremontii* during the summer**.**

The second chapter of this work examines the volatile organic compound profile of fungi isolated from the first chapter. *Penicillium*, *Mycosphaerella, Cladosporium,* and *Marssonina* strains isolated from surface sterilized leaves were subjected to solid phase microextraction to isolate VOCs that were then analyzed on a GCMS system. Produced volatiles included styrene, phenylethyl alcohol, aristolochene and valencene; all compounds with documented uses in the economic sector.

During this project I found that while endophytes are unseen, they are diverse, chemically productive and widely dispersed according to a myriad of unknown factors. I hope that this work can inspire others to pursue studies involving microbial ecology, VOC production, and growth paradigms surrounding these enigmatic but important microorganisms.

Thesis Introduction

Symbiosis (from the Greek words *σύν* "together" and *βίωσις* "living") is crucial for the survival of countless species across all domains of life [1]. This term, defined as the close and sustained "living together of differently [classified] organisms", was coined by Heinrich Anton de Bary in 1879 to describe the parasitic (harmful), commensal (benign), or mutualistic (beneficial) interaction types maintained between dissimilar organisms [2]. One form of symbiosis, known as endosymbiosis, includes the "full spectrum of interaction types… [that apply] to organisms living anywhere within the host body" [3]. Generally, endosymbionts include microorganisms (fungi, bacteria or viruses) that can greatly influence the overall health and wellbeing of respective host organisms. While some inlying microbes like *Clostridium difficile* can induce fatal colon inflammation in humans, other species such as *Lactobacillus rhamnosus* can mitigate or even alleviate symptoms of *C. difficile* infections [4], [5]. As a result of the potentially significant effect of endosymbionts on host species, researchers strive to comprehend the complexities of endosymbiotic interactions across the planet. Through deciphering relationships between symbionts and their hosts, researchers can manipulate microorganisms for human benefit. **With this paradigm in mind, I undertook this thesis project to characterize fungal endosymbionts within the leaves of two tree species with social utility, *P. trichocarpa* and *P. fremontii*. I also quantified and analyzed the volatile organic compounds emitted from the isolated endosymbiotic fungi.** In order to influence endosymbiotic interactions for the greater good, one must first understand the micro-organic participants of endosymbiotic relationships, and their importance outside of these relationships.

Microbes are ubiquitous in the environment and constitute the majority of the biomass on the planet [6]. They can be unicellular or multicellular and exist in all three major domains of life: Archaea, Bacteria and Eukarya, where they feed as autotrophs, heterotrophs, or saprotrophs. The vast diversity of microbial life is estimated to be many times larger than that found for macroorganisms, and this variety of microbes has allowed these organisms produce a variety of metabolites and to inhabit mesophillic or extreme environments across the planet [7], [8], [9].

Despite the abundance and diversity of environmental microbes, early microbiologists mainly concerned themselves with pathology, the study of disease. Due to the high prevalence and mortality rates of individuals with "smallpox, cholera, diphtheria, pneumonia, typhoid fever, plaque, tuberculosis, typhus, [and] syphilis" [10], pathologists were highly interested in eradicating lethal infectious disease harbored within people for the benefit of all. However, as more information regarding the microbiome coalesced, it became evident that the planet also contains other microbes with valuable properties worth examining.

Microbial ecology is the study of microorganisms and their relation to each other, multicellular organisms, and the environments they inhabit. The shift from a wholly pathological perspective to an ecological perspective was spurred by findings that organisms were abundant in the environment. In the initial days of this discipline Martinus Willem Beijerinck (1851-1931) invented enrichment culture, a broth for supplementing microbial growth, in order to study aggregated cells gleaned from the environment [11]. This practice, still in use today, has significantly expanded and drastically furthered our ability to understand biological processes. Through the use of enrichment culture, researchers are able to grow ample amounts of microbes necessary for research purposes. With the advent of DNA sequencing technology, the ability to rapidly sequence nucleic acids with high accuracy and read lengths is important for the assessment of the planet's vastly diverse microbiome.

In additional to being found in almost all terrestrial and aquatic habitats of our planet, microbial organisms are abundant within multicellular organisms [12]. Within multicellular organisms, microbes can maintain parasitic, commensal or mutualistic relationships with their hosts. Differences in life strategies leads to high microbial diversity and abundance within multicellular organisms. Current estimates suggest there are as many bacterial cells in humans as there are host cells [13]. In some species, gut microbiomes have been shown to influence behavior [14]. For species such as the pea aphid and their associated *Buchnera* gut microbes, both parties have evolved to become dependent on one another [15]. In addition to pairwise host-microbe interactions, microbes can also interact with each other in mutualistic or antagonistic ways [16]. Symbiotic relationships are crucial for the health and fitness of many macroorganisms. Whilst

initial microbiome research initially focused on animal hosts, these relationships are also widespread in plant species as well.

Flora and fungi have interacted for hundreds of millions of years. In spite of the antiquity of these paradigms, these relationships are still not fully understood. In the early days of biology research, plants and fungi were considered to be more or less the same type of organism. Due to their apparent immobility, growth patterns, and propensity for moist environments, fungi were merely seen as simple, less advanced versions of plants. However, over the years, researchers learned this sentiment was false. The plant/fungi distinction was elucidated through various studies, which revealed that fungi have and cell walls mainly composed of chitin unlike the cellulose containing plant cell wall [17]. Fungi lack chloroplasts, necessary organelles found in plants used for performing photosynthesis. Fungal species can also exist as unicellular or multicellular organisms, while all plants species exist as multicellular entities. After the advent of sequencing technology, researchers found that plants and fungi diverged from each other some 1.1 billion years ago and that fungi are closer related to humans than to plants [18]. Despite the differences between plants and fungi, the relationships between these organisms are crucial to their survival and propagation, or expiration.

Generally, in the past, microbial taxa pathogenic to plants have been relatively well described due to their readily apparent negative effects on hosts. Fungal pathogens are fungous microorganisms that harm hosts in order to propagate themselves. If predators consume their prey in whole number units, then pathogens consume their hosts in decimal units. Pathogens can inhabit both the exterior and the interior tissue of their hosts. They are often the causes of plant diseases such as blight, leaf spotting, and mildew. Since they can remain latent and don’t usually outright kill their hosts, fungal pathogens are widespread and found wherever plant hosts reside. Fungal plant pathogens are likely to undergo mutation and gene flow, and as a result, they are both diverse in their genetics and life strategy [19]. They can inhabit virtually all types of plant tissue, where they secrete compounds like manganese peroxidases [20] or cellulases [21] that allow them to digest plant lignin and cellulose, respectively. They can also inhibit important plant metabolic pathways important for plant health, such as immune system response [22]. Although

fungal pathogens are the most visible to observers, unseen fungal mutualists are just as significant to host wellbeing.

Mycorrhizae are the symbiotic fungi best known by science. They inhabit the rhizosphere, or soil matrix surrounding the root system of plants. Soil-residing symbionts affect many factors that influence plant health. They can alter soil chemistry by secreting or consuming compounds that affect pH and dissolved organic carbon, in addition to other metrics, like soil nitrogen content, that can manipulate plant growth [23],[24]. Mycorrhizae can upregulate water and nutrient uptake into plants, conferring drought tolerance [25]. Rhizosphere fungi are also important to soil microbiome composition, as their primary and secondary metabolites can inhibit or enhance the growth of other microorganisms [26]. While root associated fungi are the most studied microbial plant symbionts, microbes of the phyllosphere, or above ground portion of plants, are less understood.

Fungal epiphytes are microbes that reside on the exterior surface of host plant tissue. Epiphytes can benefit hosts in various ways. Certain strains of epiphytes can attract or deter pollinators like bees to host [27]. Epiphytes can also inhibit the growth of pathogenic microbes on host tissue [28]. Despite these benefits, some species of epiphytes are pathogenic to hosts. Parasitic strains of epiphytes have been found to degrade tree wood for nutrients without providing any benefits to their hosts [29]. Others have been shown to cover leaf surfaces, thereby block incoming light and lower host ability to perform photosynthesis [30]. Ultimately, the benefit or harm conferred by an epiphyte is largely dependent on its strain.

Only recently have plant endosymbionts (those that live within healthy tissues, hereafter, endophytes) have gained substantial attention [31]. They are often largely asymptomatic and are consequently difficult to identify visually. As a result, endophytes are still largely uncharacterized. Endophytes can also affect multiple aspects of their hosts, including rates of nutrient uptake, the ability to persist in adverse soil pH conditions, and modulation of host immune responses to pathogens [32]. Foliar microbiomes have also been shown confer defensive [33] and nutritional benefits [34]. They can also prevent hosts from being grazed upon by herbivores [35]. Endophytic fungi interact in some systems to produce an important phytohormone known as indole-3-acetic acid [36]. Other studies have shown endophytes as the instigators for heightened

host drought tolerance, increased biomass production, and amplified pollinator recruitment [37],[38]. Despite their potentially beneficial effects, not much is known about the myriad mechanisms of interaction between fungal endophytes and the plant species they inhabit [39]. **Specifically, two concepts regarding plant endosymbionts that have received little attention are:**

(1) host effect on endophyte community composition

and

(2) endophyte-secreted volatile organic compounds.

Research shows that plant genotype can affect endophytic community composition. These effects have been shown to be result of genes that code for host immune response to internal microbes [40]. Poplar genes such as Mmd1 have been shown to affect resistance to rust pathogens [41]. Host secondary metabolite production can also create selection pressures that affect microbial community structure [42]. While many studies have compared the endophyte communities of cultivars within a species, fewer have assessed community composition between two closely related species residing within the same habitat.

Volatile organic compounds (VOCs) are organic molecules that have high vapor pressures in ambient temperatures that allows them to disperse readily into the environment. All smells and some flavors are the result of specific VOCs interacting with sensory organs [43]. Interestingly enough, endophytes are also known to secrete VOCs [37]. As a result of their potential for dispersal, they are a common currency used in plant/symbiont signaling [44]. They alert organisms that can sense them to the presence of the organisms that emitted them [45]. They can also deter growth by inhibiting biological pathways necessary for some organisms [46]. Fungal VOCs have been shown to induce positive growth responses in host plants [47] and to reduce pathogen presence [4849].

Through this thesis, I will investigate two related concepts; host effect on endophyte community composition and endophyte-secreted volatile organic compounds. In order to

accomplish this goal, I have two specific aims. My first aim is to identify and characterize focal fungi from naturally occurring plant hosts. Specifically, **in Chapter 1**, **I will characterize the foliar fungal microbiome of two co-occurring related plant taxa in the genus *Populus*. I will also construct a novel phylogenetic tree from field and herbarium obtained poplar leaf samples.** This genus contains multiple species of economic and ecological importance, and have been model systems for genetics, genomics, and ecology. I will specifically focus on two species, *Populus trichocarpa* and *P. fremontii*.

Once I have identified these focal endophytes, **in Chapter 2 I will assay the VOCs emitted by foliar fungi found within poplar**. These compounds have been shown to affect how plants grow and how organisms interact with each other [50]. To determine the VOCs secreted from the fungi isolated through work in the former chapter, I conducted targeted *in vitro* VOC gas chromatography assays coupled with mass spectrometry analysis.

Ultimately, I uncovered information concerning naturally occurring fungal endophytes that can potentially be used for societal and economic benefit. Since the beginning of the Green Revolution, society has implemented various artificial technologies and practices in order to increase crop yield. Although successful, practices like pesticide and herbicide use are often unsustainable and harmful to humans in the long term [51],[52]. **Microbes can be used to sustainably increase the yield and health of cultivated plants through their life strategies and production of metabolites. Plant derived microbes can also cheaply synthesize compounds with industrial, medical, and personal uses. Through the understanding, and eventual optimization, of factors affecting endophyte community composition and VOC emittance, people can harness them for the greater good.**

Chapter I: Poplar Foliar Community Structure and Phylogenetics

Introduction

Endosymbiotic interactions between plant species and microorganisms (viruses, bacteria, archaea, fungi and oomycetes) [53] of the endosphere (internal plant tissues) [54] have existed for hundreds of millions of years [55]. Vegetal tissue provides a "stable, nutrient-rich niche for associated microbes to thrive" and perpetuate symbiotic dynamics with plant hosts [56]. Per endosymbiotic theory, plants and other eukaryotic organisms descended from relationships between endosymbiotic bacteria and proto-eukaryotic hosts [57]. This is evident through the analysis of cpDNA unique to chloroplasts, vital plant organelles monophyletically derived from once free-living bacteria of the distant past [58],[59]. The evolution of once primordial endomutualisms into the eukaryotic organisms of the present clearly demonstrates the importance of endosymbiotic paradigms [60]. Endosymbiotic relationships are as crucial to the health and survival of *Plantae* as they were to their unicellular progenitors [61].

Endophytes are commensal or mutualistic microorganisms that inhabit most types of plant tissue [62]. Although fungal endophytes are often less abundant than their bacterial counterparts in most plant tissues, they are no less important [63]. Endophytic fungi can be found in roots [64], stems [65], leaves [66], flowers [37], and fruit [67] of host organisms. Within host, commensal endophytes can remain latent and grow once host tissue has senesced [68]. These types of fungi exist sapotrophically by feeding on already decaying leaf tissue and cycling its carbon back into the food web. In contrast, mutualistic endophytes can confer advantageous traits that aid in host survival. Endosymbiotic fungi have been shown to increase host growth [69], reduce host pathogen load, upregulate host nutrient uptake and even increase host heat tolerance [70]. Some species of plants, including the model organism *Arabidopsis thaliana*, do not grow as rapidly or as healthily in sterile conditions (without microbial presence) than their counterparts

grown in the presence of endophytic fungi [47]. While endophytic relationships have been known for some time, they are not well understood.

Some of the best-known plant/microbe endosymbiotic mutualisms occur within plant roots. Endomycorrhizal (from the ancient Greek ἔνδον (*éndon*, "inner; internal") + μύκης (*múkēs*, "fungus") + ῥίζα (*rhíza*, "root")) microbes are crucial to the growth of most species of plants [71]. Within this specific type of mycorrhizal mutualism, fungi help their hosts to derive important nutrients from their environment in exchange for resource compounds they may lack, such as carbon. They also can aid with beneficial soil microbiome community composition by spurring the secretion of phenolic acids such "coumarin, syringic acid and vanillic acid" that inhibit pathogen growth [72]. A lesser known type of endosymbiosis occurs with plant leaves. While foliar endophytes are much less understood than their endomycorrhizal counterparts, they are no less important.

Fungal foliar endophytes are non-clavicipitaceous, polyphyletic and tend to be ascomycetous [73]. Most (~75%) of foliar fungi belong to the classes *Dothideomycetes* and *Sordariomycetes* [74]. Within these classes, ~2500 genera of fungi exist. This diverse grouping of endophytes has been isolated in virtually all extant taxa of plant species. Inside hosts, endophyte foliar fungi are mostly advantageous. These microorganisms can alter patterns of nitrogen uptake and distribution in hosts [75]. They can also secrete compounds with antimicrobial and chitin degradation properties [76]. Foliar endosymbiotic dynamics can significantly influence the growth and development of their hosts [77].

Interestingly, research shows that host gene expression can affect plant microbiomes [78]. While plant immune responses to specific pathogenic microbes have been known for some time to have a genetic basis [79], endophyte community composition is more broadly modified by host gene expression and host tissue age [80]. A plant's genotype has been shown to affect the type of compounds it secretes into surrounding soil to recruit beneficial microbes [81]. Research also suggests that similar mechanisms occur in foliar microbiomes.

In order to further understand host genotype's effect on foliar microbiomes, I surveyed the fungal foliar communities of two closely related, but distinct poplar tree species, *Populus trichocarpa* & *Populus fremontii.* Through this work, I determined if the fungal endophyte communities harbored within healthy leaves were specific to the respective species of poplar. I also sought to determine if seasonality had an effect on these foliar fungal communities.

Methods

Host Selection

Members of the genus *Populus* diverged from the willow-containing genus *Salix* ~50 million years ago [82]. Poplar species tend to inhabit temperate climates and maintain deciduous characteristics, like leaf shed. Poplar are dioecious, with individuals possessing only one of two potential sexes. *Populus* species mostly spread through shoots originated from clonal roots known as suckers [83]. These characteristics make poplar unique among woody plant species.

In particular, Black cottonwood (*Populus trichocarpa*) and Fremont's cottonwood (*Populus fremontii*) are poplar species primarily distributed within northwestern and southwestern North America, respectively [84]. *Populus trichocarpa* usually grows in cooler, more damp environments while *P. fremontii* typically inhabits warmer, more arid climates. While these species tend to inhabit different climatological areas, they are known to hybridize in proximity of one another, producing hybrid offspring known as *Populus x parryi* [85]. These lineages are known for growth rates amongst the fastest of known tree species [83]. Consequentially, they have utility as quickly proliferating sources of softwood lumber [86]. Products made from poplar include "veneer and plywood, composite panels, structural composite lumber, pallets, furniture components, fruit baskets, containers, and chopsticks" [87]. Poplar is also sought for the creation of wood pulp. Products from poplar wood pulp include "specialty paper products, such as napkins, tissues, towels, fine paper, paper board for packaging…roofing felt… insulation board, ceiling tiles… groundwood, kraft, semi-chemical, and bleached sulfite" [87]. They can also be used in the synthesis of cellulosic biofuels, sustainable sources of energy that produce less waste and pollution than fossil fuel combustion [88]. Due to these factors, these species maintain a high degree of economic significance.

Populus trichocarpa and *P. fremontii* are also known to aid in environmental phytoremediation by incorporating potentially toxic compounds like toluene or benzene into their biomass and out of the environment. [89]. Due to their natural proclivity for river systems, their clonal roots are adept at holding soil substrate together. As a result of the aforementioned characteristics, these species are also commonly used for riparian restoration projects [90].

Unlike many other large woody plants, *P. trichocarpa* has the distinction of being the first tree with a fully sequenced genome [91]. The related but distinct genomes and hybridization potential of these two species (*P. fremontii* and *P. trichocarpa*) made them ideal for determining if host genotype has an effect on foliar microbiome composition.

Site Selection

Leaves from *P. trichocarpa* and *P. fremontii* were collected from trees within Niles Canyon. This 5-mile long ravine runs from Sunol, CA to Fremont, CA. This location was chosen for sampling because it exists as natural hybrid zone for the two targeted species of cottonwood [84]. The trees of the canyon are also subjected to similar climate, a metric known to affect the community composition of foliar microbiomes [92]. Additionally, Niles Canyon is located relatively close to the University of San Francisco, which made sample storage and transportation easier.

Field Sampling

Pilot study leaf collection occurred on November 5th, 2017. Eight *P. fremontii* trees were included within the initial survey. These initial eight trees were sampled again on July 12th, 2018. A final round of sampling occurred July 5th, 2018, when 14 previously unsurveyed trees were sampled. Overall, seven autumn foliar microbiomes and 20 summer foliar microbiomes were sampled for foliar community analysis. Three sets of leaves were collected from each tree; one group for fungal isolation, another for plant genotyping and species identification, and a third for visual species identification. In order to isolate beneficial foliar endophytes, trees with visible indicators of good health (green and abundant leaves) were selected over individuals with noticeable disease (i.e. wilted or decaying leaves with visible rust or necrotic spotting). I selected for outward facing sun leaves due to their higher levels of metabolic activity that may contribute

to endophytic growth [93]. Leaves located out of reach were obtained through the use of either a pruning pole (Fiskars Oyj) or an arborist slingshot (Notch Equipment). Gathered leaves were placed in plastic bags denoting when and what individual they were collected from. For each tree, I recorded metrics including tree height, diameter at breast height, leaf sample height, and GPS location. Tree height was measured using an EC II Digital Clinometer (Haglöf). Five extra trees putatively identified as *P. trichocarpa* with signs of noticeable pathogens rusts and powdery mildews were sampled for plant genotyping and leaf analysis. These extra leaves were not included within our analyses of endophytic communities. After labelling, leaf bags were then placed into a cooler with ice packs and transported for processing.

Surface Sterilization

Leaves were surface sterilized and plated the same day to minimize the significant alteration of their internal microbial communities. Surface sterilization allowed for the removal of epiphytes and other debris from the outer portions of leaves [94]. Leaves were first washed with diH_20 within a sterile petri dish. Following this initial wash, leaves were treated with a 95% ethanol wash for 10 seconds, a 0.5% NaOCl wash for two minutes, and then a 70% ethanol wash for two minutes to kill any surface microbes. Between each wash, the respective liquids were decanted from the petri dish to allow space for the newly added solutions. Following sterilization, dead or potentially diseased portions of leaves were trimmed and leaves were allowed to air dry in a sterile biosafety cabinet.

Fungal Culturing

Once dry, the remaining leaf portions were cut into 2 mm^2 fragments and placed into 1.5 mL slant tubes containing 2% Malt Extract Agar (MEA), a media type known to induce the growth of diverse fungi [95]. MEA consisted of 1 gram of agar (Fisher Scientific) and 1 gram of malt extract agar (VWR International, OXOID) for every 50 mL of diH_20 used. The resulting mixture was then autoclaved for 30 minutes and pipetted into slant tubes.

I cultured from 150 leaf tissue fragments for each of the trees sampled in November 2017. Slant tube cultures plated in November were allowed to grow for 16 days. After this initial growth period, isolation frequency (the number of tubes with fungal growth out of all tubes per tree) data

were collected. I recorded another round of isolation frequencies for these trees about five months after the original slant tube plating, in April 2018.

I cultured from 200 fragments for each tree sampled in July 2018. I increased the number of slant tubes for this round by 50 because I hypothesized that more endophytes could be isolated from healthier summer leaves than from older, senescing autumn leaves. Slant tube cultures plated in July 2018 were allowed to grow within container sealed plastics bags for about 35 days before the initial pass through and isolation frequency calculations. New isolation frequencies were recorded about seven months after initial slant tube plating, in February 2019.

After each pass though of each sampling, newly grown fungi were subcultured into larger 35 mm plates with 2% MEA. On these plates, fungi grew for six weeks before they were water vouchered in sterile culturing tubes. Vouchering facilitates survival of these strains for extended periods of time (more than a decade) [96]. Unvouchered fungal biomass was used for the following DNA extractions.

Fungal DNA Extraction

Extractions were performed with the REDExtract-N-Amp Plant kit (Millipore Sigma). For each extraction, 100 µL of extraction buffer was added to a tube containing a small amount of fungal biomass. Tubes containing fungal matter and extraction buffer were then subjected to one minute of bead beating using 1.0 mm diameter yttria-stabilized zirconium oxide beads (Next Advance) in a mini-Beadbeater 96 (Biospec Products). Sample tubes were then incubated at 95°C for 10 minutes. Following heating, 100 µL of dilution buffer was added to each sample to neutralize the extraction buffer. The extractions were then stored at 4°C until PCR.

Fungal PCR Amplification and DNA Sequencing

I sequenced the internal transcribed spacer ITS region of the nuclear ribosomal gene to identify targeted fungi. These non-coding barcode sequences have high rates of interspecific discrimination, which is helpful for denoting the species of fungal and plant species [97]. I used the REDExtract-N-Amp Plant Kit (Millipore Sigma) for PCR amplification of fungal ITS regions. Each PCR reaction contained 10 µL of amplification solution, 6.4 µL of PCR-grade H_20

(Millipore), 1 µL of BSA (Millipore Sigma), 0.8 µL of 20 mM of the fungal-specific ITS1F forward primer 5'– CTT GGT CAT TTA GAG GAA GTA A –3' , 0.8 µL of 20 mM ITS4 5'– TCC TCC GCT TAT TGA TAT GC–3', and 1 µL of template DNA. A negative control that included sterile water in place of the template was included for each batch of PCR. Reactions were run on either a C1000 Touch or T100 thermal cycler (BioRad). Cycle conditions were denaturation at 95°C for three minutes, followed by 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for one minute, and a final extension at 72°C for 10 minutes.

Three µL of PCR product from each sample were electrophoresed in 1% agarose gels with SYBR Safe DNA Stain (Invitrogen) at 100 volts for 30 minutes to verify amplification and the absence of bands in the negative controls. Successfully amplified samples were then treated with 1 µL of ExoSAP-IT High-Throughput PCR Product Cleanup reagent (Applied Biosystems) for every 17 µL of PCR Product. These mixtures were incubated at 37°C for 15 minutes to degrade excess nucleotides and primers and then at 80°C for 15 minutes to inactivate the ExoSAP-IT. Following PCR cleanup, samples were diluted with an additional 15 µL of PCR water per reaction. Samples with faint gel bands were not diluted. These cleaned PCR products were then sent for Sanger sequencing at MCLAB in South San Francisco, CA.

Niles Canyon Tree DNA Extraction

From the same trees, plant gDNA was also extracted for analysis. Fifty micrograms (approximately 0.050 g) of wet weight frozen leaf material from each tree was ground using autoclave sterilized pestles within sterile plastic tubes. Following pestle grinding, extractions were similarly performed with the REDExtract-N-Amp Plant kit as described above. Samples were stored at 4°C until PCR was performed.

Herbarium Sample DNA Extraction

Currently, no ITS sequence data of *P. fremontii* exists within NCBI's genetic database, Genbank. In order to ensure that samples isolated from Niles Canyon were appropriately genotyped, I extracted tree DNA from pressed samples of *P. trichocarpa* or *P. fremontii* procured from the California Academy of Science's herbarium. These pressed samples were chosen for this study

because they were visually identified by seasoned career botanists with extensive understanding of poplar foliar morphology.

Leaf tissue samples were taken from their respective exsiccatae sheets and placed in respectively labelled falcon tubes for transportation. Extractions were performed using a modified version of a DNeasy Plant Mini Kit protocol (Qiagen). Once transported to the lab for processing, I weighed .02 grams of each sample and added them to appropriately labelled tubes containing 1.0 mm diameter yttria-stabilized zirconium oxide beads (Next Advance). Tubes were then processed in a mini-Beadbeater 96 (Biospec Products) for three minutes. Following bead beating, 400μL of AP1 Lysis Buffer (Qiagen) was added to each tube along with 4 μL of a 1 molar solution of sodium metabisulfite, a compound used to help purify DNA extracted from leaves [98]. Tubes were then vortexed and incubated at 65°C for 45 minutes, with a physical inversion every 10 minutes. 130 μl of Buffer AP2 was then added to the lysate. The mixture was then vortexed and incubated for five minutes on a cold block. After incubation, tubes were centrifuged at 15,000 rpm for six minutes. The resulting lysates were then pipetted into Qiashredder mini spin columns (Qiagen) within 2 mL tubes and centrifuged for 2.5 minutes at 20,000 x g. Resulting flow through was then added into sterile tubes containing AP3/E Binding Buffer (Qiagen) and mixed by pipetting. Once mixed, 650 μLs of the resulting solutions were then transferred to respective DNeasy mini spin columns (Qiagen) within 2 mL tubes and centrifuged for one minute at 7000 rpm before decanting. This step was repeated until all of the previous mixture containing flow through and AP3/E Binding Buffer was spun through respective DNeasy mini spin columns. These columns were then placed in sterile tubes. 500 μL of AW Wash Buffer (Qiagen) was added to the columns before being centrifuged for one minute at 7000 rpm. Following this step, flow through was discarded and filter cleanliness was recorded. Columns were then subjected to another 500 μL of AW Wash Buffer and spun for 2.5 minutes at 20,000 rpms. 50 μL of Buffer AE was then directly added to the filters of columns placed in Lo Bind DNA tubes (Qiagen) and incubated at room temperature for 10 minutes. Following incubation, tubes were spun at 7000 rpm for one minute. Another 50 μL of Buffer AE was added to filters, incubated and spun under the same conditions for a final volume of 100 μL. Resulting extractions were then DNA quantified using Qubit HS fluorometric assays.

Populus PCR Amplification and DNA Sequencing

I sequenced the internal transcribed spacer ITS region of the nuclear ribosomal gene used to identify targeted tree species [99]. I used an REDExtract-N-Amp Plant Kit (Sigma-Aldrich) for PCR amplification for Niles Canyon plant ITS regions. Each PCR reaction contained 10 µL of amplification solution, 6.4 µL of PCR-grade H_20 Millipore, 1 µL of BSA, 0.8 µL of ITS5 forward primer 5'–GGA AGT AAA AGT CGT AAC AAG G–3', 8 µL of ITS4 reverse primer 5'– TCC TCC GCT TAT TGA TAT GC–3', and 1 µL of 1:10 dilution of template DNA in diH_20. This dilution helped to reduce PCR supression likely caused by plant inhibitors.

Reagents were run on either a C1000 Touch or a T100 thermal cycler (BioRad). Cycle conditions were 95°C for three minutes, followed by 35 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for one minute, and a final extension at 72°C for 10 minutes. Successful amplification was verified, and reactions were cleaned using 1 µL of ExoSAP-IT High-Throughput PCR Product Cleanup reagent (Applied Biosystems) for every 17 µL of PCR Product. Following PCR clean-up, samples were sent for Sanger sequencing as described above.

Sequence Quality Control and Analysis

Fungal and plant gDNA sequences were trimmed and checked for quality. Short (<300) base pairs sequences and poor-quality sequences removed. Remaining sequences were trimmed to a final length ~570 base pairs using Geneious software [100]. Bases with multiple Sanger specific fluorescence reads were reconciled through the use of degenerate bases that could account for either base represented. All sequences were then subjected to NCBI basic BLAST, an algorithm used for comparing DNA sequence information to a database of sequences submit by researchers across the globe [101].

Plant sequences were aligned using default Geneious Clustal W software [102]. Following alignment, sequences were used to infer a phylogeny with PhyML [103] with 5000 bootstraps and a GTR substitution model. Another phylogeny was constructed using Mr. Bayes software [104] with a GTR + I + gamma substitution model. Mr. Bayes chain length was set to 10,000,000, subsampling frequency to 5,000,000, its burn-in length to 1,000,003, and heated chains to 4 with a chain temp of .2. Through these phylogenies, I could determine the ITS genotype (species) of

each tree included within the survey by comparing them to Genbank poplar samples as well as herbarium extracted DNA reads.

Bioinformatics Processing, Community and Statistical Analyses

In order to visually fungal OTU abundance and distribution respective to tree species or seasonality, I generated box plots using RStudio [105]. I also generated NMDS ordinations to compare the microbiomes of different samples with similar treatments.

In order to determine if there was a species or seasonal effect on the fungal foliage communities of surveyed poplar species, I ran statistical test on each of datasets. Specifically, I used the Permanova and Wilcoxon signed-rank tests to determine if the variables had significant effects on foliar microbiomes. These tests were performed in RStudio using the vegan package [106].

Results

Culturing results

See supplemental table 1, pg 43

Out of all the sterilized leaf fragments plated in November 2017, 18.3%, or 220 out of 1200 tubes, exhibited growth after two weeks on MEA plates. Another 9.30%, or 112 tubes, out of 1200 were found to have grown roughly six months later. Overall 27.67%, or 332 out of 1200 MEA tubes exhibited growth after a five-month period. During the second pass through of the MEA tubes, it appeared that the bag inside of the plastic container for the fifth tree was contaminated with fungal growth. I discarded these tubes and excluded this sample from later analysis.

See supplemental table 2, pg 43

Out of leaf fragments sterilized and plated on July 12th, 2017, 2.25% (36 out of 1600 tubes), exhibited growth after two weeks on MEA plates. Another 8.81% (141 tubes out of 1600 tubes) were found to have grown roughly seven months later. Overall 11.1%, or 177 out of 1600 MEA tubes exhibited growth after a seven-month period. While inspecting the second pass through,

the plastic bags containing tubes from the fifth tree was also contaminated with slight fungal growth outside. These tubes were excluded from community analysis.

See supplemental table 3, pg 44

When surveyed on August 8th, 2018, 8.92% (232/2600) of the leaf fragments plated on July 5th, 2018 grew fungal mass. Once reevaluated seven months later, I found that 11.0% (287/2600) of tubes had fungal growth. Altogether, 20% of plated leaf fragments grew fungi after seven months. While inspecting the second pass through, the plastic bags containing tubes from the trees 11, 15, 19 and 20 were also contaminated. These tubes were also excluded from community analysis.

Tree Genotyping

See supplemental figures 1 & 2, pgs 45 and 46 respectively

Phylogenetically, sampled tree genotypes matched with expected leaf phenotype for each individual. Lighter, heart shaped leaves denoted *P. fremontii*, while smaller and darker leaves signified that a sample was from *P. trichcocarpa* [107].

Overall Fungal Community Analysis

See supplemental table 4, pg 47

Out of 466 successfully amplified and sequenced fungal ITS extractions, 77 different species were cataloged from nine *P. fremontii* and 11 *P. trichocarpa* trees. The fungal genera isolated in this survey have been shown to possess life histories ranging from endophytisim, to commensalism and obligate parasitism.

Summer *P. fremontii* and *P. trichocarpa* Fungal Community Analysis

See supplemental table 5, pg 49

This comparison was performed to determine difference between the fungal communities of both species while controlling for the season. 181 samples from targeted trees were successfully isolated, amplified and sequenced from 16 trees. Trees 2, 3, 6, 7, 8, 10 and 11 were determined to be *P. fremontii*. Trees 9 and 13 - 22 were *P. trichocarpa*. Out of 40 different foliar genera, 60 species were obtained. 14 successful extractions were obtained from *P. fremontii, and* seven of the 13 *P. fremontii* species in nine genera were unique to this specific host. *P. Trichocarpa* contained the other 160 fungi isolated.

Seasonal *P. fremontii* Fungal Community Analysis

See supplemental table 6, pg 50

Since cultures were successfully extracted from the same Fremont's cottonwood trees (trees 2,3,6 & 7) in November 2017 and July 2018. I compared the respective fungal communities isolated from these individuals. From these 196 samples, 32 separate species were isolated.

Discussion

Overall Fungal Community Analysis

The most abundant genera isolated in this study, *Mycosphaerella,* is known to contain over 10,000 different fungal species [108]. Many *Mycosphaerella* species were classified by morphological characteristics, before the propagation of gene sequencing technologies. When researchers began to sequence *Mycosphaerella* 28S nrDNA sequences responsible for coding ribosomal LSU, they found that the genus contained species that did not form a single, monophyletic group. Due to genetic analysis, this "genus" is understood to be polyphyletic, instead of monophyletic [109]. It contains about 30 true (distinctly monophyletic) genera that maintain diverse endosymbiotic relationship types with hosts. Some species have been shown to devastate important harvests, such as wheat crops [110]. Other species, such as one that inhabits *Psychotria horizontalis*, are endophytic and known to emit compounds that inhibit the growth of various human parasites such as malaria (*Plasmodium falciparum*), leishmaniasis (*Leishmania donovani*), and Chagas disease (*Trypanosoma cruzi*) [111]. In particular, the species isolated in this study *Mycosphaerella sp. PB-2012b* is not well known to science.

Penicillium species were the second most prominent taxa isolated from Niles Canyon poplar leaves. This monophyletic group contains over 300 unique species of fungi [112]. *Penicillium* have maintained ecological, economic and medical significance since being first described to science in 1809. These species are responsible for the decomposition of plant biomass, as well as carbon cycling within ecosystems. They are also used for the creation of products such as penicillin and cheese [113]. Like *Mycosphaerella*, *Penicillium* can exhibit endophytic, commensal or parasitic traits depending on host species and genotype. Some species have been found responsible for the decay of various fruits such as apples and strawberries. Other strains of *Penicillium* have been revealed to actively secrete phytohormones that upregulate plant growth genes during periods of increased environmental stress [114],[115]. The most bountiful *Penicillium* species isolated during this study, *Penicillium glabrum*, has been found as a pathogen in wide variety of plant fruits, including pomegranates, strawberries, and grapes. *Penicillium glabrum* also exists endophytically within *Bauhinia forficate,* a medicinal plant used for indigenous healing practices [116] . This species has been shown to either decompose hosts or produce compounds that help guard hosts against pathogen presence.

The third most abundant species isolated was *Marssonina brunnea*. This species is a well-known poplar pathogen that inflicts one of the most common foliar illnesses, leaf spot disease [117]. If unchecked, this pathogen can lead to premature defoliation of poplar trees before natural leaf shed. If combined with other stressful factors, this pathogen can contribute to the death of poplar trees [118].

Another fungal taxa isolated belonged to the genus *Cladosporium*. Due to advanced dispersal ability, *Cladosporium* are bountiful and can be found within what appears to be dust in indoor and outdoor environments alike [119],[120]. Species of this monophyletic genus can also exhibit traits ranging from endophytism to obligate parasitism. They have the ability exist parasitically on and within plant and animal species [121]. *Cladosporium* species can also exist endophytically by parasitizing other fungi that can harm host plant tissues [122]. Unlike the other prominent taxa isolated in this study, the BLAST results for this grouping was not specific to the species level. It

is mostly likely because the individuals isolated within this study has yet to be wholly described by science.

Other fungal species isolated from this study have many beneficial attributes. Foliar-isolated *Aureobasidium pullulans* is an endophyte that is used to stop the growth of pathogenic fungi on apples, grapefruit, tables grapes, cherry tomatoes and other popular produce products [123]. While *Penicillium expansum* is often pathogenic food contaminant, it produces the volatile geosmin that is a contributor to petrichor [124]. This earthy scent occurs after heavy rain and is enjoyed by people worldwide. *Penicillium spathulatum* produces the anticancer compound asperphenamate [125]. When treated with the common compound gold chloride, *Nigrospora oryzae* can create gold nanoparticles [126]. Strains of *Naganishia liquefaciens* secretes garbage lipase that can synthesize biodiesel from sludge [127]. Despite residing in seeming innocuous tree leaves, foliar microbes can be harnessed for diverse uses.

Summer *P. fremontii* and *P. trichocarpa* Fungal Community Analysis

See supplemental figures 3 & 4, pgs 51-52 respectively

OTU abundance was much greater in P. trichocarpa leaves than in its counterpart, P. fremontii. PERMANOVA and Wilcox tests for this grouping generated a p value of .049 and .057 respectively.

Seasonal *P. fremontii* Fungal Community Analysis

See supplemental figures 5 & 6, pgs 52-53 respectively

Despite the difference in amounts of fungal samples, summer and fall samples of P. fremontii contained roughly the same amount of OTU groups. The PERMANOVA and Wilcox tests had p-values of 0.963 and .9241, respectively, suggesting no significant between these sample groups. The NMDS ordination also failed to display any distinctive groupings in this dataset.

ITS Analysis and Phylogenetics

While both targeted species placed as expected within monophyletic clades in both phylogenies, the bootstrap supports for the Mr. Bayes tree were <90 and not sufficient enough for certainty. While the ITS region is known for its use to discriminate between species, it likely is not long or divergent enough for to be used as the sole gene used for phylogeny building in closely related and hybridizing species such as Fremont's and black cottonwood. In order to remedy poor bootstrap supports, I will couple ITS reads with non-coding chloroplast genes (e.g. trnL-F or trnH-psbA), known to help discriminate between poplar species [128].

The fourth tree had leaf characteristics of both species and was thought to be a member of the hybrid taxa *P. x parryii*. However, genetic testing of this individual and *x parryi* herbarium samples were inconclusive and unable to be supported consistently through phylogeny. Due to this occurrence, the fourth tree was left out of analyses.

Culturing Issues

Due to fungal contamination on the outside of slant tubes, I disposed of many cultures that potentially contained valuable data. The importance of culture vigilance and maintenance became increasingly apparent, especially during data analysis at the conclusion of this study. Fungal cultures should be assessed often for slightly uncapped tubes, mite presence, as well as media residues around lids. Through culture upkeep, a researcher can preserve data that can be extremely difficult or impossible to reobtain.

Chapter II: Volatile organic compound analysis of two commonly isolated putative pathogens and two commonly isolated putative endophytes

Introduction

Volatile organic compounds (VOCs) are carbon containing substances that maintain high vapor pressure (>0.01 kPa) and low molecular mass [129]. As a result of these defining traits, VOCS readily vaporize at ambient temperatures and atmospheric pressure [130] . Numerous types of compounds, such as “terpenes, alkanes, alkenes, alcohols, esters, carbonyls,… acids”, thiols, aromatics and more are able to naturally volatilize [131]. Once dispersed into the air column, VOCs can react with and significantly affect other compounds and entities that come in contact with them. Odors and some tastes are the direct result of VOCs interacting with olfactory and gustatory organs [43]. Since being discovered in the mid 20th century, VOCs have been shown to have important biological, environmental and economic impacts [132],[133]. Through the understanding and harnessing VOCs, people can utilize them for human gain and benefit.

Volatile organic compounds are produced by a wide array of diverse organisms. VOC emittance and perception are key biological features of bacteria, fungi, plants and animals. These easily vaporized compounds have many applications within the realm of biology. VOCs can be used for interspecific signaling [129]. Through emitting volatile organic compounds into the environment, organisms can be alerted to potential mutualisms and react accordingly. Without the production of appealing volatiles, many plant species reliant on pollinators would be unable to reproduce [44]. VOCs emitted from the bacteria *Bacillus subtilis* can alter the expression of genes related to antibiotic resistance and motility in the model organism *Escherichia coli* [134]. While these organisms are not sensing each other in a cognitive sense, these VOC-sensitive mechanisms have evolved because they aid in survival and propagation. Volatiles can also be used for intraspecific signaling. Depending on the situation, VOCs can relay messages quicker and more efficiently than sonic or visual forms of communication. Many social organisms like *Canis familiaris* analyze VOCs emitted from conspecific individuals to determine various

characteristics, such as age or sex of the emitter [135]. Mice have been shown to alter emitted VOC profiles according to social status [136]. Emitted volatiles have also been implicated in human food and mate preference [43]. VOCs are crucial signaling molecules for many different organisms.

Volatile organic compounds are utilized by an organism to defend itself or its partners in mutualisms. Many animals, such as skunks and rat snakes, produce a defensive musk that can distract, repel or overwhelm predators. Certain species of plants have the capacity to inhibit or kill contending vegetation through the use of VOCs. Despite their prominence in animals and plants, these types of interactions are not just limited to these organisms. Endophytic fungi have been shown to produce VOCs that inhibit the growth of competing microorganisms. Some endophytic species are known to emit volatiles that exterminate microbes that parasitize their hosts. Through the emission of volatiles, organisms can protect themselves and others.

VOCs have been shown to have substantial effects on human health. These compounds can incite human allergen responses and irritate mucosal membranes. They can also elicit headaches and sick building syndrome, a medical condition defined by the feeling of nauseous or unwellness in old or unclean buildings, is thought to be instigated by the inhalation of VOCs from various household molds. Some VOCs are carcinogenic to humans that have undergone extended exposure to these chemicals, such as benzene, formaldehyde and naphthalene. Others can inflict kidney, liver and central nervous system damage after prolonged exposure. While many VOCs can negatively impact human health, others can positively affect individuals. Volatiles emitted from foods can stimulate appetite and push people to seek out healthy foods like fruits, which aids in their seed dispersal. Although aromatherapy has not been proven to cure diseases, volatiles utilized during these treatments can improve the mood and mental health of individuals.

Volatile organic compounds are also abundant and important in environmental systems. Trees in forest ecosystems can produce hundreds of thousands of tons of terpene volatiles, compounds with medical and industrial use, a day [130]. Forest fires also discharge large amounts of VOCs into the atmosphere that can simultaneously harm human health while fertilizing soil. Oceans have also been shown to volatilize organic compounds into the atmosphere [130]. While there are substantial ecological sources of VOCs, the majority of volatile organic compounds emitted into

the atmosphere have anthropogenic sources. Household products, such as paints, aerosols cans and acetone can all release VOCs into the atmosphere. The combustion of fossil fuels emits millions of tons of greenhouse gases and VOCs into the atmosphere daily. Smog also contains VOC components that can negatively impact human health. As a result of these factors, many governments regulate VOC emissions and outright ban the use of specifically toxic VOCs. Volatile organic compounds have environment-level effects on the natural and anthropocentric worlds.

VOCs have multiple industrial uses. The antimicrobial effects of some VOCs are sought after for use in drug development research. The ability of VOCs to promote plant growth and stress tolerance has led researchers to consider them as natural fertilizers. VOCs can also act as naturally-occurring alternatives to insecticides, fungicides and herbicides. Organic volatiles can also be used in the production of biofuels. Researchers are also devising ways to grow microbes with specific VOC profiles that can be integrated into bioreactors to synthesize more complex compounds. This occurs in cycloprotenation bioreactors, where volatiles emitted from genetically modified *E. coli* are cheaply amassed to react with other compounds to form the more expensive compound cyclopropane [137]. Due to the potentially beneficial applications of VOCs, many organizations employ investigators as bioprospectors for novel organic volatiles.

The study of fungal-emitted VOCs is an area of rapidly growing interest. Fungi can produce multiple kinds of VOCs with beneficial characteristics. Some compounds, like 1,8-Cineole, isobutyric acid, and 1-Butanol-3-, methyl-, acetate have antifungal properties that can allow a fungus to inhibit the growth of fungal competition. Fungal produced VOCs like benzyl aldehyde, 6-Pentyl-a-pyrone and styrene are antibiotic and can more generally inhibit the growth of nonspecific microorganisms, either fungal or bacterial. Compounds like 1-octen-3-ol, allo-ocimene and C-6 aldehyde have been shown to upregulate genes that code for increased plant immune response against pathogens. Other fungal organic volatiles like b-Caryophyllene and 2-methyl-propanol have been revealed to elicit increased plant growth [138].

Plants and their associated fungal endophytes have evolved complex pathways of communication through the use of organic volatile compounds. Through VOC signaling, plants and fungal endophytes can relay chemical “messages” that spur actions beneficial to one or both

members of the mutualism. Endophytic VOCs can alert plant hosts to increase immune response during pathogen presence or metaphorically “reassure” plants that it is safe to grow in stressful or normally hostile environments. In turn, plants provide stable environments for the growth of these microbes. Plant immune systems also refrain from lysing endophytic fungi. Through these mutualisms, both organisms benefit.

Antimicrobial VOCs have various ways of inhibiting bacterial and fungal growth. These effects can be achieved through the significant modification or outright degradation of cell walls that lead to cellular lysis and deactivation. VOCs can also directly bypass microbial cell walls and disrupt vital organelles and enzymes needed to the sustain the viability of targeted cells. VOCs can also adversely affect the expression of vital enzymes, leading to cell lysis. Research also suggests that antimicrobial fungal VOCs can damage DNA of targeted organisms [139].

Despite the significance of endophyte-secreted VOCs, they are still vastly understudied. Through SPME (solid phase micro extraction) headspace analysis coupled with GCMS (gas chromatogram/mass spectroscopy) evaluation, I elucidated the volatile profiles of four endophytic fungi taxa isolated from the foliage of *P. trichocarpa* and *P. fremontii*, tree species known for their anthropocentric utility.

Methods

Fungal Culturing

Fungi included within this study were initially isolated from the summer leaves of trees residing within Niles Canyon, CA, covered in chapter 1. From these leaves, I isolated two commonly isolated potential pathogens, *Mycosphaerella sp. PB-2012b*, and *Marssonina brunnea.* I also isolated the taxa *Penicillium glabrum*, *Marssonina brunnea*, and an undetermined *Cladosporium* species. These species were chosen for this study because of their broad foliar distribution across hosts and season suggests they may secrete compounds important to plant host. These fungi were recultured from water vouchers onto 100 mm plates with autoclave-sterilized 2% malt extract agar and allowed to grow undisturbed for two weeks.

Once significant fungal growth had occurred, 100 mm fungal plates were placed within a biosafety cabinet. Sterile 40mL TraceClean clear borosilicate glass vials with PTFE-faced septa (VWR) were also added to the cabinet. These vials were uncapped under sterile biosafety cabinet conditions and 30 mLs of sterilized 2% MEA was pipetted into each vial, baring one empty control vial. Once media inside the vials cooled, agar plugs were removed from each fungal culture using sterile agar plungers and placed on respectively labeled vials. Once sealed, fungi were allowed to grow for two weeks before SPME & GCMS analysis. Overall, four replicates were created for each fungal strain, along with a MEA only control vial and empty vial control.

Solid Phase MicroExtraction

SPME techniques utilize industrial fibers coated in specific extracting phase to absorb analytes from desired samples [140]. Extracting phase consists of absorbent materials such as polydimethylsiloxane that acts as a sponge to "soak up" analytes, which can be "wrung out" through the high temperature and pressure maintained by a GC inlet. For this study, I utilized 1 cm 50/30 µL StableFlex DVB/CAR/PDMS fibers (Supelco), known for their affinity for absorbing a wide range of volatile organic compounds. Fibers were conditioned for one hour within a GC inlet at 270 °C before sampling to remove any compounds passively absorbed to the extraction phase. Once conditioned, fibers were inserted into septa and allowed to absorb headspace (airborne) volatiles for one hour. After an hour, fibers were desorbed into a GCMS for compound analysis. Once completed, fibers were conditioned again for one hour within a GC inlet at 270 °C before sampling new vials.

Gas Chromatography/Mass Spectroscopy Analysis

GCMS hardware consist of two separate machines, a gas chromatograph and a mass spectrometer, used in tandem to elucidate compounds. Gas chromatographs utilize an unreactive carrier gas (usually nitrogen or helium) to send compounds of interest through a heated and pressurized inlet that extracts compounds from extraction phase. Extracted compounds are then sent through an increasingly heated column containing solid stationary phase. Stationary phase, like extracting phase, can consist of various compounds that retain compounds of interest. As the column heats, the stationary phase releases molecules of interest at different times depending on their chemical composition and reactivity to stationary phase. The amount of time it takes for

molecules to eventually burn off of the stationary phase is known as the retention time. This metric is important because as long as the type of stationary phase and heat ramping protocol is kept constant, then identical molecules should also have a constant retention time. Due to this, researchers keep detailed libraries of the various retention times of specific compound on specific stationary phase. When unknown compounds are sent through a gas chromatograph, their retention times are monitored and compared to the retention times of already described substances. In order to further verify substances, GCs chromatograms containing all recorded retention times of a specific run are often paired with the mass spectra of Ms.

Once compounds have traveled through the GC column, they sent through the connected mass spectrometer. This device measures an ions mass-to-charge (m/z) ratio, a metric usually specific to a certain compound. In order to generate mass-to-charge data, compounds of interest are ionized by the removal of an electron from atoms. These newly positive ions are then accelerated to a constant speed and sent through a magnetic field. Charged ions are deflected from initial trajectory due to their mass, and that metric is recorded (mass/charge, or m/z). Deflected ion beams then have their charge recorded by an ion detector. With metrics for mass and ion charge recorded, a mass spectrum read is generated for each specific compound analyzed. When GS chromatograms are coupled with mass spectra, they can both be compared to the known GCMS results within compound libraries, and elucidated.

In order to elucidate the VOCs isolated from endophytic strains, I used an Agilent 6890 Series GC system (Hewlett Packard) coupled with a HP 5973 Mass Selective Detector (Hewlett Packard). The column used was a J&W HP-5 GC Column, 30 m, 0.32 mm, 0.25 μm, 7 inch cage (Agilent). GC inlet was maintained at 250 °C and set on splitless mode during analysis. Column temperature was initially 40°C and increased to 220°C at a rate of 4 °C /min for a total run time of 45 minutes. MS source temperature was 230°C, MS quad temperature was 150°C, and transfer temperature was 280°C. Scan range was from 10-700 amu.

Chromatogram and Mass Spectra Examination

Analytes were first generated using Chemstation software (Agilent). Chromatogram and mass spectra data were then exported and processed using Rstudio [105] and the packages eRAH [141] , enviGCMS [142], and ncdf4 [143]. Once processed, peaks were compared against the National Institute of Standards and Technology (NIST) 2017 mass spectra library using AMDIS 32 [144] to determine peak composition and metrics. All replicates from the various test groups were compiled and averaged. For averaged fungi and SPME/MEA/vial controls chromatograms, the five most abundant peaks from NIST 2017 were compiled with respective match factors, probability scores and retention times. Each averaged fungal chromatogram was the subjected to subtraction of averaged control chromatograms to determine pure fungal VOC emittance.

Generally, a match factor >900 is considered an excellent match, 800-900 a good match, 700-800 a fair match, and <600 a poor match to a library compound. Probability scores were also recorded for each of the most abundant VOCs. These scores first estimate if the unknown compound's spectrum is present in the library. It then is used to determine if the compound match is accurate by comparing it to adjacent hits. If two matches contain compounds that have highly similar structure, then it is harder to determine which is accurate through ion analysis and lowers the probability score of both compounds. While these metrics are useful, they must be combined with a robust understanding of chemical dynamics for accurate compound analysis.

Results

SPME analyses revealed the range of variation in VOC profiles between experimental groups. While specific organic volatiles were abundant in the cultures of some species, those same compounds were seemingly inexistent or untraceable in others.

Control VOC Profiles

See supplemental figure 14 & table 9, pg 61

SPME fibers and vials mostly emitted synthetic compounds such as Cyclohexasiloxane, dodecamethyl-, Silane, triethylfluoro, Ethylene glycol – Adiapte – Dietheylene glycol, and 2H-

Pyran-2,6(3H)-dione. While these synthetic compounds often had low matches and probabilities, they are likely honest, given the industrial origins of these sampling tools. Interestingly enough, the human cancer-causing compound tricholoromethane, or chloroform, was also emitted from MEA samples. The library hits of these control compounds are less important than their abundance and mass spectra data, which can be subtracted from fungal VOC profiles of interest.

Mycosphaerella sp. PB 2012 VOC Profiles

See supplemental figure 18 & table 10, pg 65

The five most abundant organic volatiles recovered from *Mycosphaerella sp. PB 2012* culture vials were trichloromethane, methane, bromodichloro, 1-butanol, 3-methyl-, benzeneacetic acid, methyl ester and phenylethyl alcohol. Baring 1-butanol, 3-methyl- and phenylethyl alcohol, all the rest of these compounds were also found in the controls. It is likely that these compounds are not fully subtracted out of mean chromatograms because of variability in control VOC abundance ranges between samples. Fibers can offgas more or less of their synthetic components between runs.

Phenylethyl alcohol maintained an excellent match score (955), a high probability score (81.80%) and a passable mass spectrum. It is likely that the signal for phenylethyl alcohol is accurate. 1-butanol, 3-methyl- had an excellent match score (925), but a lowered probability score (42.50%), and a poor mass spectrum match. While this library hit may be accurate, it is more likely that this compound was incorrectly identified or unknown to the NIST 2017 library.

Penicillium glabrum VOC Profiles

See supplemental figure 22 & table 11, pg 69

There were only three peaks with library hits for this *Penicillium* species. The three most abundant organic volatiles recovered from *Penicillium glabrum* headspace analysis were 1-pentene, cyclotrisiloxane hexamethyl-, and a third compound with multiple close hits (styrene, Bicyclo[4.2.0]octa-1,3,5-triene, or 1,3,5,7-Cyclooctatetetraene).

Cyclotrisiloxane hexamethyl- was isolated in control analyses, so it was disregarded as a fungal VOC. While had an excellent match score of 922, its probability score was low at 17.60%. It was also missing multiple mass spectrum peaks. Due to these factors, it is likely that this unknown compound was misidentified as 1-pentene.

The top hit for the third peak was styrene, followed by Bicyclo[4.2.0]octa-1,3,5-triene, and then 1,3,5,7-Cyclooctatetetraene. Despite having a match factor of 953 and a mass spectrum nearly identical to the library hit for styrene, its probability score was 37.00%. This uncharacteristically low probability score is due to the next two matches having a completely identical chemical formula (C8H8). In order to determine which of the three potential compounds were correct, I purchased styrene monomer (Tap Plastics). The compound was subjected to the same experimental procedure as the rest of the samples and analyzed using the same parameters. Ultimately, the styrene isolated from the fungi matched better to the library search than the styrene monomer itself. This is likely due to the lingering compounds used by industrial chemist to synthesize styrene.

Marssonina brunnea VOC Profiles

See supplemental figure 26 & table 12, pg 73

The five most abundant compounds isolated from *Marssonina* cultures were cyclopentane, cyclotrisiloxane hexamethyl-, phenylethyl alcohol, aristolochene, and valencen. Cyclotrisiloxane hexamethyl-, and cyclopentane were found in low amounts in controls. Through comparing match scores, mass spectra and probability scores, phenylethyl alcohol, aristolochene and valencene are likely accurate library matches.

Cladosporium VOC Profiles

See supplemental figure 30 & table 13, pg 77

Trichloromethane, cyclopentanone, cyclotrisiloxane hexamethyl-, and cyclohexasiloxane, 3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane dodecamethyl- were the most abundant compounds found in *Cladosporium* headspace. All these compounds were

found within control VOC profiles. The unidentified *Cladosporium* isolated within this study did not seem to secrete any detectable VOCs after two weeks of growth.

Discussion

The major volatile organic compounds isolated from fungal strains of interest were styrene, phenylethyl alcohol, valencene and aristolochene. *Cladosporium* and *Penicillium* emitted styrene, while *Mycosphaerella* and *Marssonina* released phenylethyl alcohol volatiles. *Marssonina* also volatilized valencene and aristolochene. All of the assayed compounds are well-known to science and have been shown to be emitted from fungal sources in previously published literature. Despite originating from fungi residing within leaves experiencing similar environmental factors, the isolated volatiles have varied properties and anthropocentric uses.

Phenylethyl alcohol

This compound, also known as 2-phenylethanol, is composed of a phenethyl group ($C_6H_5CH_2CH_2$) group attached to an OH. It is found in a variety of plant derived essential oils, and is known to have a light, floral aroma and taste that is similar to roses. Due to its pleasant odor, it is also found in a variety of skincare and soap products. Phenylethyl alcohol is also known for its antimicrobial properties [145]. This effect is achieved through the degradation of microbial cell walls, which allows for once impermeable compounds to enter into the cell [146]. This process leads to the degradation of DNA, and the eventual deactivation of the affect microorganism. Interestingly enough, this compound was isolated from both *Marssonina* and *Mycosphaerella,* two known fungal pathogens. It is likely it is used in interspecific competition for foliar biomass. This compound could also be used for the degradation of plant leaf tissue for nutrients in saprotrophic lineages.

Styrene

This chemical species, also known as phenylethene, maintains the formula $C_6H_5CH=CH_2$. This compound has a wide range of industrial uses. It is the flammable precursor to polystyrene, a relatively safe compound essential for the creation of plastics, rubber and insulation. It is a known carcinogen and has been shown irritate contacted mucosal membranes.

Penicillium glabrum was the only fungi in this study found to produce this compound. Researchers have isolated styrene from other cheese-making species of *Penicillium* in prior experiments, where the fungus is shown to convert phenylalanine into cinnamic acid, with styrene as a byproduct. [147]. Although it may mostly be a waste product, it maintains inadvertently antimicrobial qualities that can give *Penicillium* species advantageous traits.

Because these microbes make ample amounts of this compound, many researchers are seeking ways to utilize this byproduct. It can be used to make "green" plastics that are not derived from fossil fuels [148]. It can also be used in cyclopropanation bioreactors to cheaply craft more complex materials with industrial uses [137].

Aristolochene & Valencene

Aristolochene and valencene are compound produced by the pathogen *Marssonina* isolated within this study. These compounds are used as the precursor molecules for various mycotoxins from produced by fungi [149]. Mycotoxic compounds can cause serious illness or even death if consumed by humans [150]. Due to their deadliness, they have even been considered as agents of biological warfare [151]. One such mycotoxin formed from aristolochene is known PR toxin, a compound known to "damage to vital internal organs [and] alter the crucial processes like DNA replication, transcription, and translation" [152].

Volatiles organic compounds have been shown to have wide ranging effects and economic uses. While little is known about fungal endophytes, even less is understood about the VOC profiles they secrete into their environments. With SPME and GCMS techniques, researchers can sample VOCs and compare their desorption metrics to compound libraries to determine what chemical signals are released by fungi. Through the second chapter of this project, I found that 4 fungal taxa (*Cladosporium*, *Penicillium*, *Marssonina* & *Mycosphaerella*) isolated from poplar leaves can emit compounds such as styrene, phenylethyl alcohol, valencene and aristolochene. Through surveying the volatile profiles of the other 73 taxa isolated from this study, it is highly likely that more interesting and significant compounds can be isolated from the methods outlined within this body of work.

Conclusions

Microbes are found in nearly almost every environment surveyed. Within eukaryotic organisms, they can confer endosymbiotic benefits that aid both organisms with survival. Various factors can affect assemblages of microorganisms within eukaryotes, such as host genome and environmental factors. In light of this, I surveyed the foliar fungal microbiomes of two related species, *P. trichocarpa* and *P. fremontii* to determine if their distinctive genetics would lead to distinct fungal foliar community composition, despite close physical proximity to each other. I also compared foliar fungal microbiomes of the same *P. fremontii* individuals over the fall and summer to determine if season affected these fungal communities. Through the use of statistical analyses and ordination, I determined that host species had a slight effect on the composition of their foliar fungal microbiomes during the summer, while season did not.

With these findings, I hypothesize that there exists a set of genes in black and Fremont's cottonwood that can explain the slight difference in endophyte communities found within the leaves of the respective species. I also postulate that due to the mild climate of Niles Canyon, seasonal factors do not affect poplar foliar endophyte communities as much as they would in more variable environments.

In order to improve data for chapter 1, I will collect more fungal samples and plant genetic markers to further understand the dynamics concerning host-endophyte relationships. Whole genome sequencing and RNAseq analyses of hosts and endophytes may also lead to interesting discoveries that can help explain the findings detailed in the first chapter of this thesis.

Volatile organic compounds are ubiquitous and vital molecules with multiple purposes used by species across the domains of life. They can simultaneously aid hosts and endosymbionts engaged in mutualisms while deterring potential pathogens or predators. In plants, VOCs can be used as signaling molecules, as a form of biological currency for services rendered, and as potential weapons against pathogens and competitors. Likewise, microbes also utilize these compounds for identical reasons. Considering this, I surveyed the organic volatile profiles of endophytes residing within the leaves of previously surveyed *P. trichocarpa* and *P. fremontii*

samples. I found compounds that were characteristic of the genera isolated, but previously unknown to be organic volatiles of those species. The major volatile organic compounds sequestered during this project were styrene, phenylethyl alcohol, valencene and aristolochene. *Cladosporium* and *Penicillium* secreted styrene, a compound likely used to deter or deactivate host pathogens. *Mycosphaerella* and *Marssonina* released phenylethyl alcohol, hypothesized to aid with leaf decomposition in saprotrophic strains. *Marssonina* also emitted aristolochene and valencene, known precursors to mycotoxins.

Endophytic VOC secretion and the factors influencing these phenomena are still not well understood. In order to expand our knowledge of fungal VOC dynamics, I would perform further experiments on the foliar fungi isolated from this study. Genome and RNA sequencing of the isolated fungi held at different environmental conditions (temperature, photoperiod, etc.) could yield interesting results. Pairwise fungal VOC analysis and live monitoring of the four isolated strains could also further shed light on the nature and purpose of foliar endophyte VOC secretions. Another project could detail the effects of specific fungal VOCs on poplar seed germination. Better understanding of fungal-emitted VOCs and their effects on crop species can lead to innovative and sustainable advances in agricultural technology.

Endophytic fungi are abundant within plant species and the factors that influence their dispersal are still not well known to science. Research shows that microbial symbionts can be used to sustainably increase the yield and health of cultivated plants. Plant derived fungi can also cheaply synthesize compounds with industrial, medical, and personal uses. Through the understanding, and eventual optimization, of factors affecting fungal endophyte community composition and VOC emittance, people can harness them for the greater good.

Supplemental Tables and Figures

Tree #	1st pass Tubes with growth 11/21/17	2nd pass Tubes with growth 4/11/18	Total Tubes with growth	% of tubes from tree #
1	51	40	91	60.7%
2	56	3	59	39.3%
3	13	15	28	18.7%
4 (unused)	10	13	23	15.3%
5	6	0 – contaminated	6	4.00%
6	33	12	45	30.0%
7	8	16	24	16.0%
8	43	13	56	37.3%
total	220 (18.3%)	112 (9.33%)	332	27.7%

Table 1. Fungal endophytes isolated from first eight poplar trees surveyed in Niles Canyon on November 5, 2017.

Tree #	1st pass Tubes with growth 8/17/18	2nd pass Tubes with growth 2/6/19	Total Tubes with growth	% of tubes from tree #
1	1	0	1	0.50%
2	4	69	73	36.5%
3	10	32	42	21.0%
4	4	0	4	2.00%
5	2	0 – contaminated	2	1.00%
6	11	3	14	7.00%
7	2	34	36	18.0%
8	2	3	5	2.50%
total	36 (2.25%)	141 (8.81%)	177	11.1%

Table 2. Fungal endophytes isolated from first eight poplar trees surveyed in Niles Canyon on July 12th, 2018.

Tree #	1st pass Tubes with growth 8/8/18	Tubes with growth 2/6/19	Total Tubes with growth	% of tubes from tree #
9	3	10	13	6.50%
10	3	48	51	25.5%
11	13	0 – contaminated	13	6.50%
13	29	125	154	77.0%
14	31	6	37	18.5%
15	17	0 – contaminated	17	8.50%
16	9	90	99	49.5%
17	1	0	1	0.50%
18	37	1	38	19.0%
19	29	0 – contaminated	29	14.5%
20	3	2	5	2.50%
21	49	0 – contaminated	49	24.5%
22	8	5	13	6.50%
total	232 (8.92%)	287 (11.0%)	519	20.0%

Table 3. Fungal endophytes isolated from first remaining 13 poplar trees surveyed in Niles Canyon on July 5th, 2018.

.

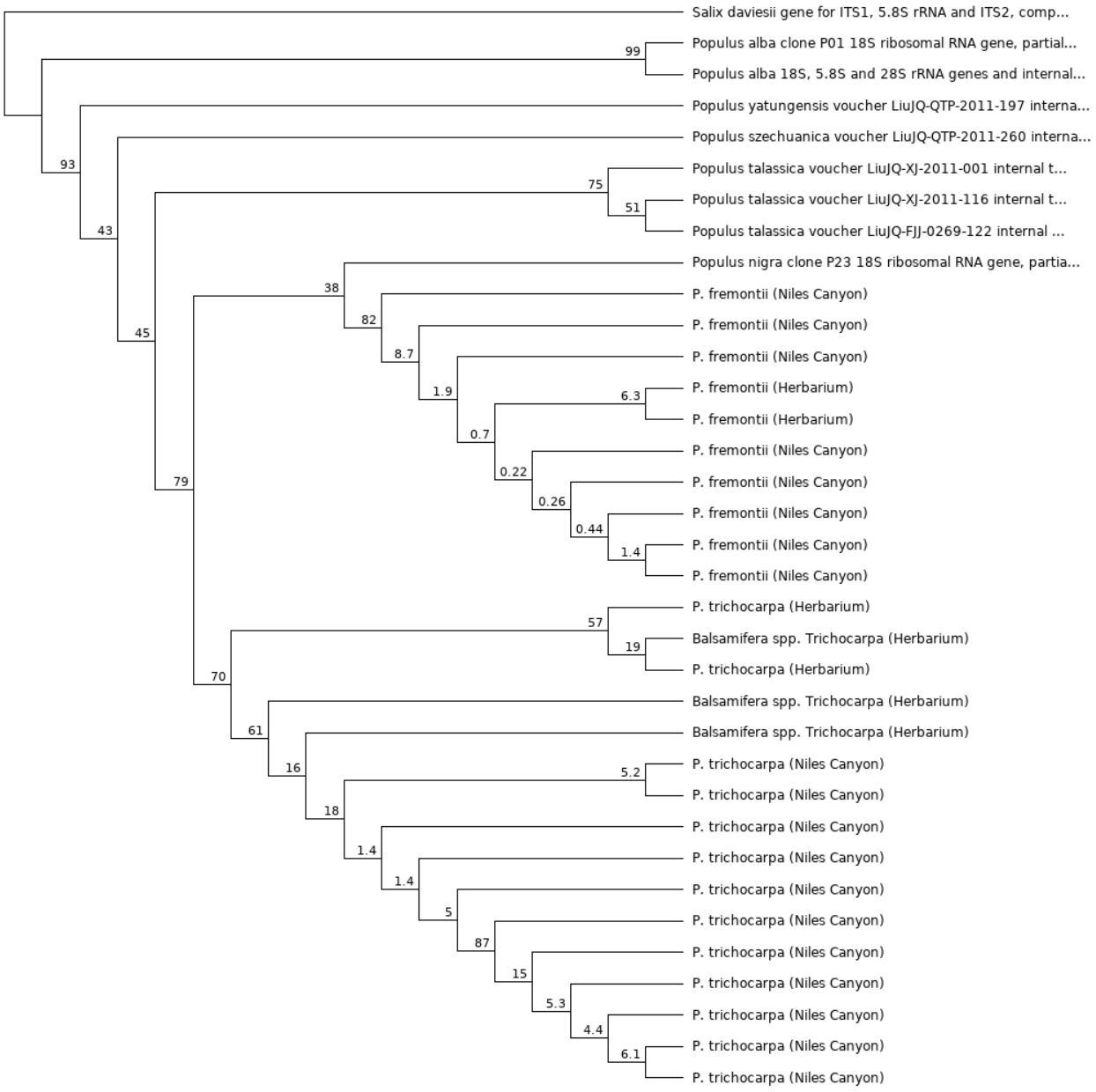

Fig 1. PhyML Maximum Likelihood tree of extracted poplar ITS sequences

Used PhyML software through Geneious with a GTR substitution model and 5000 replicates.

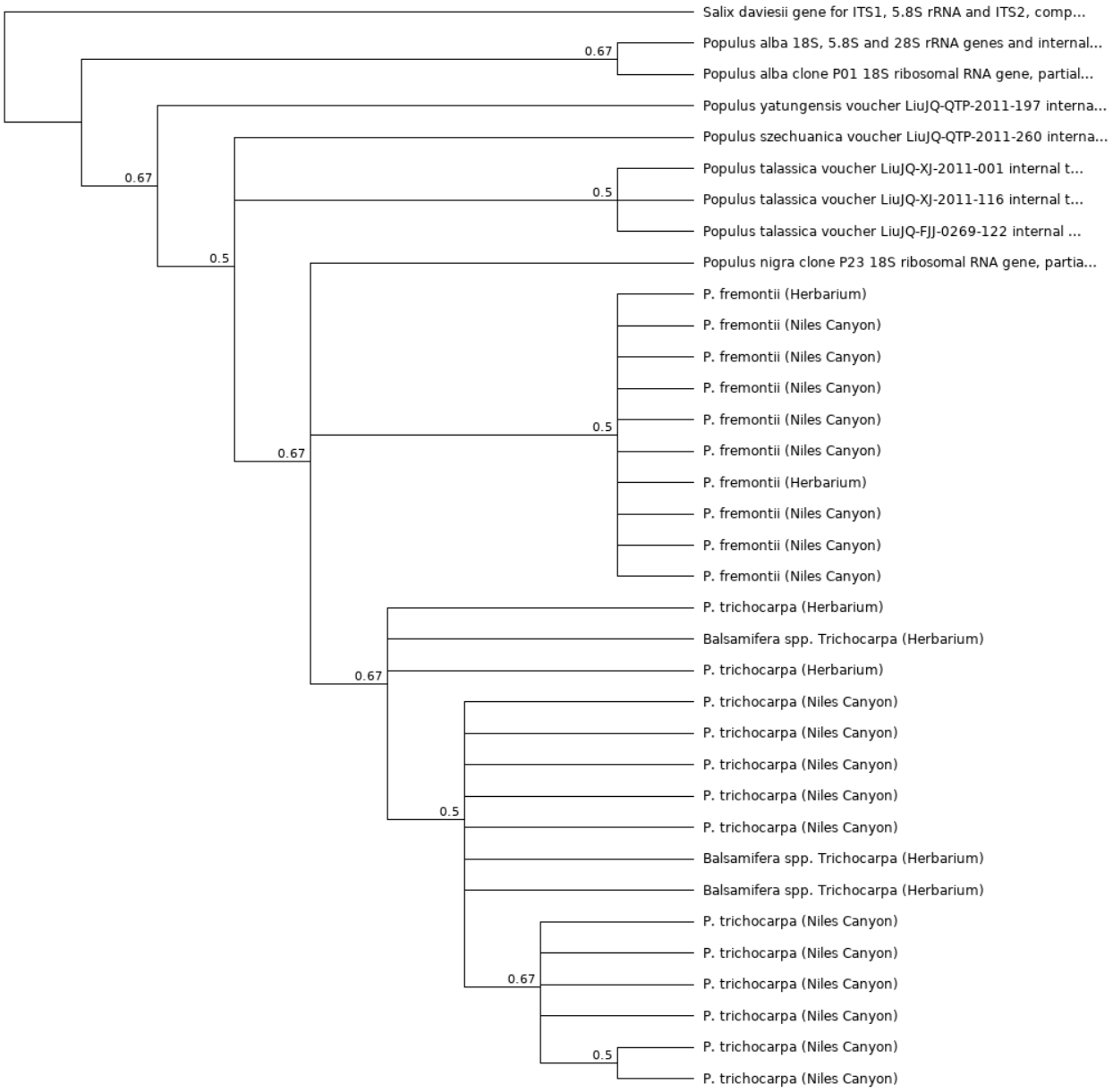

Fig 2. Mr. Bayes tree of extracted poplar ITS sequences.

Used Mr. Bayes software in Geneious with a GTR + I + gamma substitution model. Mr. Bayes chain length was set to 10,000,000, subsampling frequency to 5,000,000, its burn-in length to 1,000,003, and heated chains to 4 with a chain temp of .2.

	Count	Genus	Species		Count	Genus	Species
	140	*Mycosphaerella*	*sp. PB-2012b*		1	*Preussia*	*lignicola*
	49	*Marssonina*	*brunnea f. sp. "multigermtubi"*		1	*Preussia*	*australis*
	48	*Penicillium*	*glabrum*		1	*Periconia*	*sp. CY191*
	18	*Chaetomium*	*elatum*		1	*Penicillium*	*spathulatum*
	16	*Elsinoe*	*sp.*		1	*Penicillium*	*roseopurpureum*
	14	*Rosellinia*	*aquila*		1	*Penicillium*	*polonicum*
	12	*Alternaria*	*infectoria*		1	*Penicillium*	*echinulatum*
	11	*Alternaria*	*cf. infectoria*		1	*Penicillium*	*corylophilum*
	10	*Penicillium*	*expansum*		1	*Penicillium*	*chrysogenum*
	10	*Erysiphe*	*adunca*		1	*Penicillium*	*aurantiovirens*
	9	*Penicillium*	*brevicompactum*		1	*Nemania*	*sp. ARIZ AZ0890*
	9	*Aspergillus*	*sp.*		1	*Nemania*	*serpens*
	8	*Aspergillus*	*niger*		1	*Mycocalicium*	*victoriae*
	8	*Alternaria*	*atra*		1	*Lophiostoma*	*sp. 1 KB-2015*
	7	*Aspergillus*	*tubingensis*		1	*Lecanicillium*	*sp.*
	5	*Valsa*	*sordida*		1	*Geomyces*	*sp. 24MN18*
	5	*Penicillium*	*commune*		1	*Endosporium*	*aviarium*
	5	*Chaetomium*	*globosum*		1	*Dothideomycetes*	*sp. OUCMBI101101*
	4	*Sphaerulina*	*populicola*		1	*Cytospora*	*sp. 23 NA8*
	4	*Lewia*	*sp.*		1	*Coniochaeta*	*sp. Sib2-1-11*
	4	*Cladosporium*	*sp.*		1	*Clypeosphaeria*	*sp. D7a3b*
	3	*Pleosporales*	*sp. 19 KB-2015*		1	*Clypeosphaeria*	*sp. D4a2a2*
	3	*Neofusicoccum*	*parvum*		1	*Cladosporium*	*fusiforme*
	2	*Nigrospora*	*oryzae*		1	*Cladosporium*	*cf. cladosporiodes*
	2	*Naganishia*	*liquefaciens*		1	*Chaetomium*	*tectifimeti*
	2	*fungal*	*NLEndoHerit_021_2008N7-06-3C*		1	*Chaetomium*	*fimeti*
	2	*Elsinoe*	*salicina*		1	*Capnobotryella*	*sp. 130A*
	2	*Didymosphaeria*	*variabile*		1	*Candida*	*argentea*
	2	*Cladosporium*	*pulvericola*		1	*Botryosphaeria*	*sp. WF160*
	2	*Botrytis*	*sp.*		1	*Aureobasidium*	*sp.*
	2	*Aureobasidium*	*pullulans*		1	*Aspergillus*	*pseudoglaucus*
	2	*Alternaria*	*sp. BLE26*		1	*Aspergillus*	*fumigatus*
	2	*Alternaria*	*eureka*		1	*Apodus*	*oryzae*
	1	*Venturia*	*polygoni-vivipari*		1	*Apiognomonia*	*veneta*
	1	*Teichospora*	*rubriostiolata*		1	*Anthostomella*	*sp.*
	1	*Strattonia*	*insignis*		1	*Alternaria*	*sp. MFLUCC 13-0346*
	1	*Sordariomycetes*	*sp.11280; Sordariomycetes sp. 11328*		1	*Alternaria*	*alternata*
	1	*Roussoellaceae*	*sp. MUT 4886*		1	*Acanthophysellum*	*cf. lividocoerulem*
	1	*Rhodotorula*	*nothofagi*				
Total	428				38		
			Total cultures	466			
			total species	77			

Table 4. Total isolated and sequenced fungi isolated from November 2017 to July 2018.

Table 5	P. trichocarpa Counts	Genus	Species		P. fremontii Counts	Genus	Species
	17	*Acanthophysellum*	*elatum*		2	*Alternaria*	*alternata*
	16	*Alternaria*	*sp.*		1	*Penicillium*	*commune*
	14	*Alternaria*	*aquila*		1	*Penicillium*	*aurantiovirens*
	9	*Alternaria*	*glabrum*		1	*Penicillium*	*glabrum*
	10	*Alternaria*	*adunca*		1	*Penicillium*	*expansum*
	8	*Anthostomella*	*sp.*		1	*Penicillium*	*corylophilum*
	8	*Apiognomonia*	*niger*		1	*Coniochaeta*	*sp. Sib2-1-11*
	7	*Penicillium*	*glabrum*				
	8	*Apodus*	*atra*		1	*Chaetomium*	*globosum*
	7	*Aspergillus*	*tubingensis*		1	*Candida*	*argentea*
	2	*Aspergillus*	*commune*		1	*Aspergillus*	*sp.*
	4	*Aspergillus*	*globosum*		1	*Aspergillus*	*pseudoglaucus*
	4	*Aureobasidium*	*populicola*		1	*Aspergillus*	*fumigatus*
	2	*Aureobasidium*	*brevicompactum*		1	*Alternaria*	*alternata*
	4	*Botryosphaeria*	*sp. PB-2012b*				
	3	*Chaetomium*	*sordida*				
	2	*Chaetomium*	*parvum*				
	1	*Cladosporium*	*sp.*				
	2	*Clypeosphaeria*	*sp. 19 KB-2015*				
	1	*Clypeosphaeria*	*liquefaciens*				
	2	*Cytospora*	*salicina*				
	2	*Didymosphaeria*	*variabile*				
	2	*Dothideomycetes*	*sp. BLE26*				
	2	*Elsinoe*	*infectoria*				
	2	*Elsinoe*	*eureka*				
	1	*Endosporium*	*polygoni-vivipari*				
	1	*Erysiphe*	*rubriostiolata*				
	1	*Geomyces*	*insignis*				
	1	*Lecanicillium*	*sp. 11280*				
	1	*Lophiostoma*	*lignicola*				
	1	*Mycocalicium*	*australis*				
	1	*Mycosphaerella*	*sp. 19*				
	1	*Naganishia*	*sp. CY191*				
	1	*Nemania*	*polonicum*				
	1	*Nemania*	*sp. ARIZ*				
	1	*Neofusicoccum*	*serpens*				
	1	*Penicillium*	*victoriae*				
	1	*Penicillium*	*sp. 1 KB-2015*				
	1	*Penicillium*	*sp.*				
	1	*Penicillium*	*sp. 24MN 18*				
	1	*Periconia*	*aviarium*				
	1	*Pleosporales*	*sp. OUCMBI101101*				
	1	*Pleosporales*	*sp.*				
	1	*Preussia*	*sp. D7a3b*				
	1	*Preussia*	*sp. D4a2a2*				
	1	*Rosellinia*	*sp. WF160*				
	1	*Sordariomycetes*	*sp.*				
	1	*Sphaerulina*	*pullulans*				
	1	*Strattonia*	*oryzae*				

Table 5	P. trichocarpa Counts	Genus	Species		P. fremontii Counts	Genus	Species
	1	*Teichospora*	*veneta*				
	1	*Valsa*	*sp.*				
	1	*Venturia*	*cf. lividocoeruleum UC2023192*				
	1	*fungal*	*sp. NLEndoHerit_021_2008N7-06-3C*				
total	160		59		14		13
			Total cultures isolated	181			

Table 5. Total sequenced foliar fungi isolated from P. trichocarpa and P. fremontii in July 2018.

Fall Counts	Genus	Species		Summer Counts	Genus	Species
84	*Mycosphaerella*	*sp. PB-2012b*		2	*Cladosporium*	*Sp.*
37	*Penicillium*	*glabrum*		1	*Alternaria*	*alternata*
31	*Marssonina*	*brunnea f sp. Multigermtubi*		1	*Penicillium*	*commune*
9	*Penicillium*	*expansum*		1	*Penicillium*	*aurantiovirens*
4	*Penicillium*	*brevicompactum*		1	*Penicillium*	*glabrum*
1	*Cladosporium*	*sp.*		1	*Penicillium*	*expansum*
2	*Valsa*	*sordida*		1	*Penicillium*	*corylophilum*
2	*Nigrospora*	*oryzae*		1	*Coniochaeta*	*sp. Sib2-1-11*
2	*Cladosporium*	*pulvericola*		1	*Chaetomium*	*globosum*
1	*Roussoellaceae*	*sp. MUT 4886*		1	*Candida*	*argentea*
1	*Pleosporales*	*sp. 19 KB-2012*		1	*Aspergillus*	*sp.*
1	*Penicillium*	*spathulatum*		1	*Aspergillus*	*pseudoglaucus*
1	*Penicillium*	*roseopurpureum*		1	*Aspergillus*	*fumigatus*
1	*Naganishia*	*liquefaciens*				
1	*Cladosporium*	*cf. cladosporoides*				
1	*Chaetomium*	*tectifimeti*				
1	*Chaetomium*	*fimeti*				
1	*Capnobotryella*	*sp. 130 A*				
1	*Aureobasidium*	*pullulans*				
182		19		14		13
		Overall count	196			
		Overall Species	32			

Table 6. Total sequenced foliar fungi isolated from P. fremontii in November 2017 and July 2018.

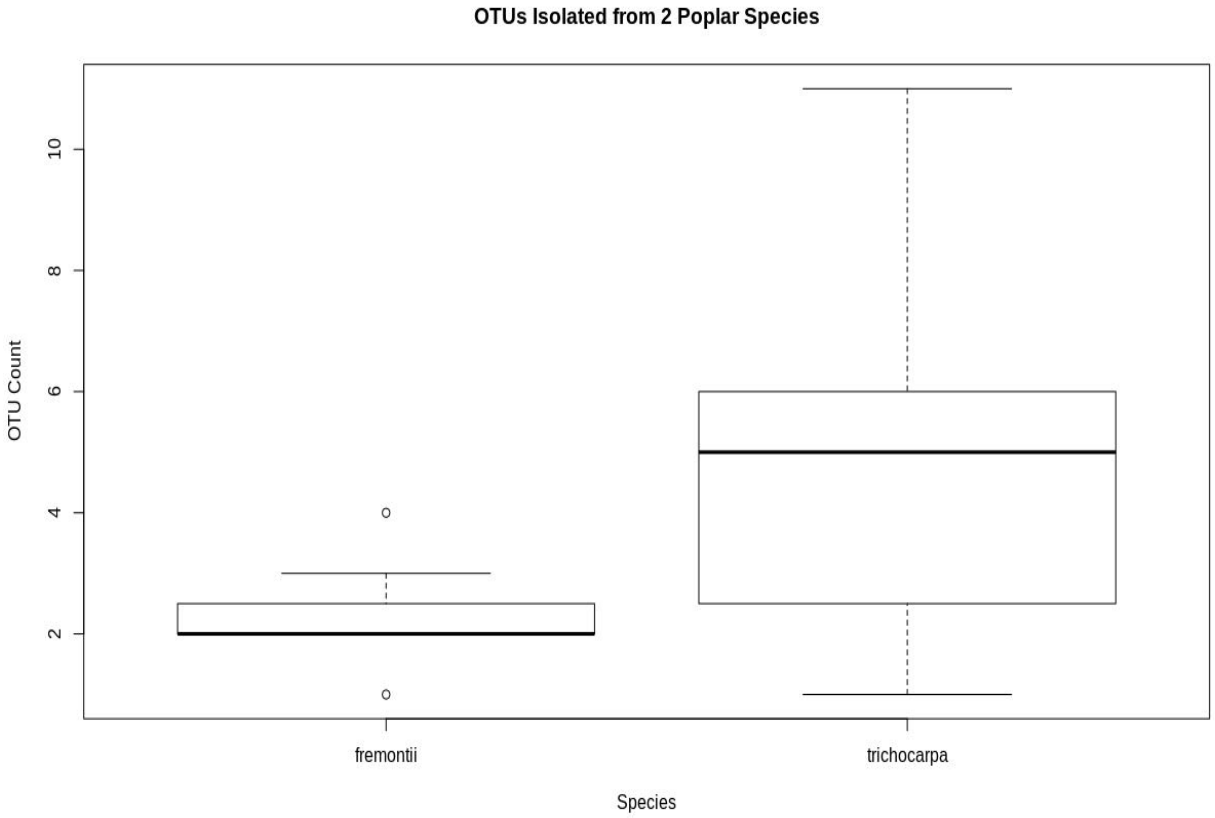

Fig 3. Box Plot of OTUs isolated from two poplar species in July 2018.

OTU abundance was much greater in P. trichocarpa leaves than in its counterpart, P. fremontii.

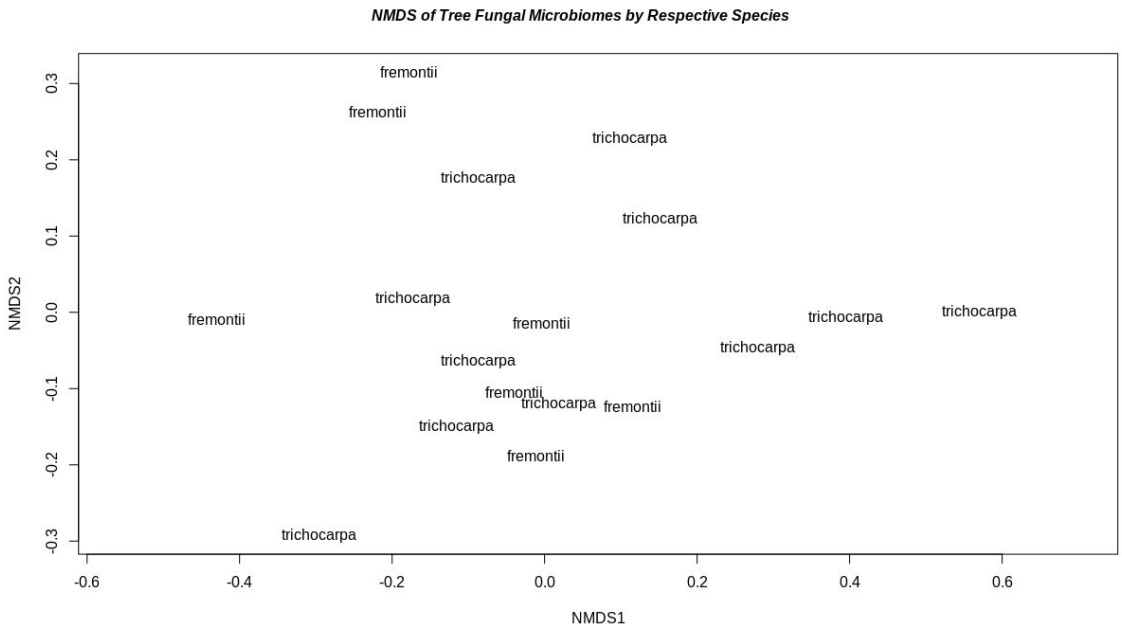

Fig 4. NMDS of foliar fungi microbiomes isolated from both poplar species in July 2018

PERMANOVA and Wilcox tests for this grouping generated a p value of .049 and .057 respectively.

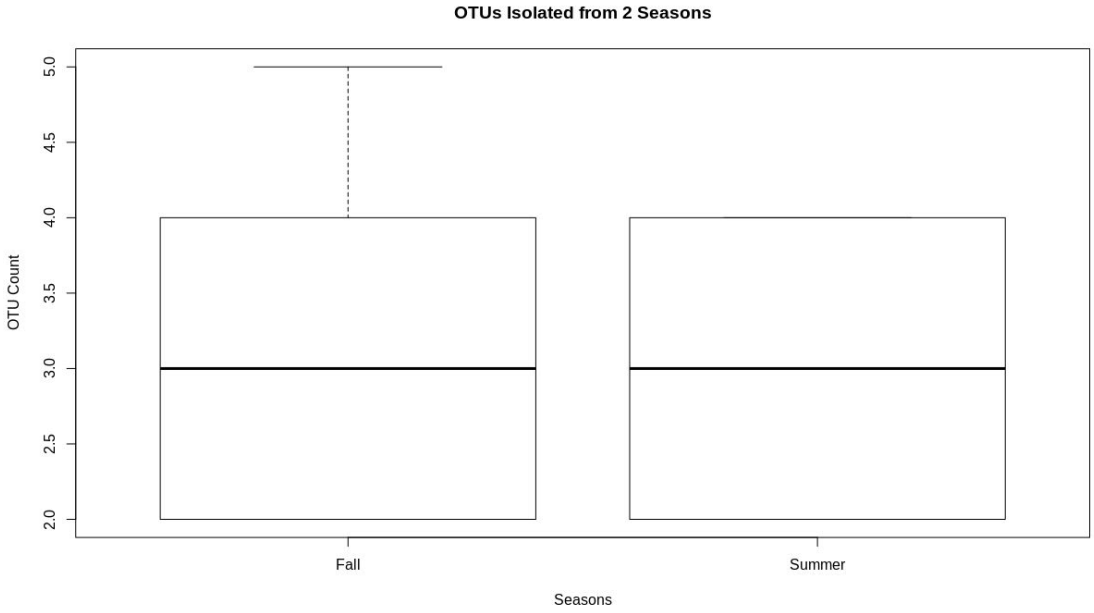

Fig 5. Box Plot of OTUs isolated from P. fremontii in November 2017 and July 2018.

Despite the difference in amounts of fungal samples isolated, summer and fall samples of P. fremontii contained roughly the same amount of OTU groups.

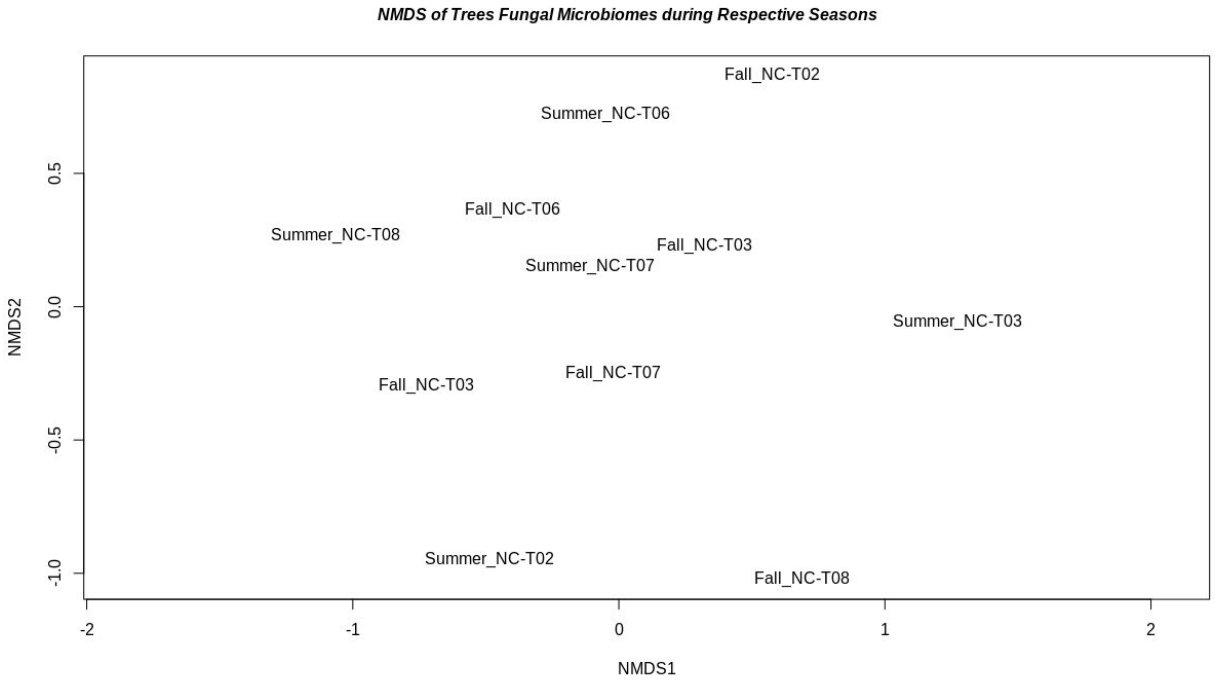

Fig 6. NMDS of foliar fungi microbiomes isolated from P. fremontii in November 2017 and July 2018

The PERMANOVA and Wilcox tests had p-values of 0.963 and .9241, respectively, suggesting no significant between these sample groups. The NMDS ordination also failed to display any distinctive groupings in this dataset.

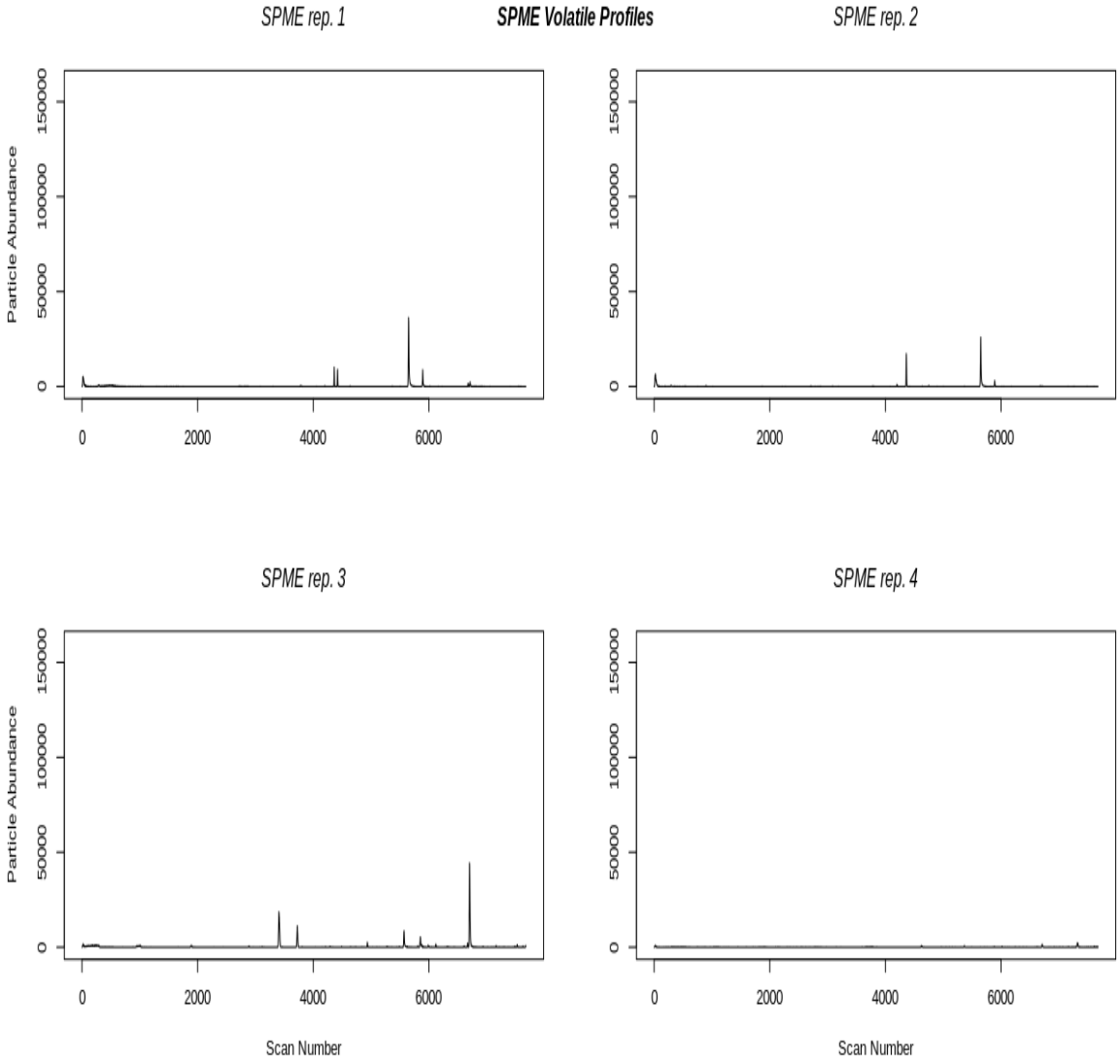

Fig 7. Organic volatile profile of SPME Fiber after being conditioned at 270°C for one hour.

Fibers emit low amounts of synthetic polymers that are produced when the fiber is heated. These compounds were measured so that the resulting chromatograms may be subtracted from fungal VOC profiles.

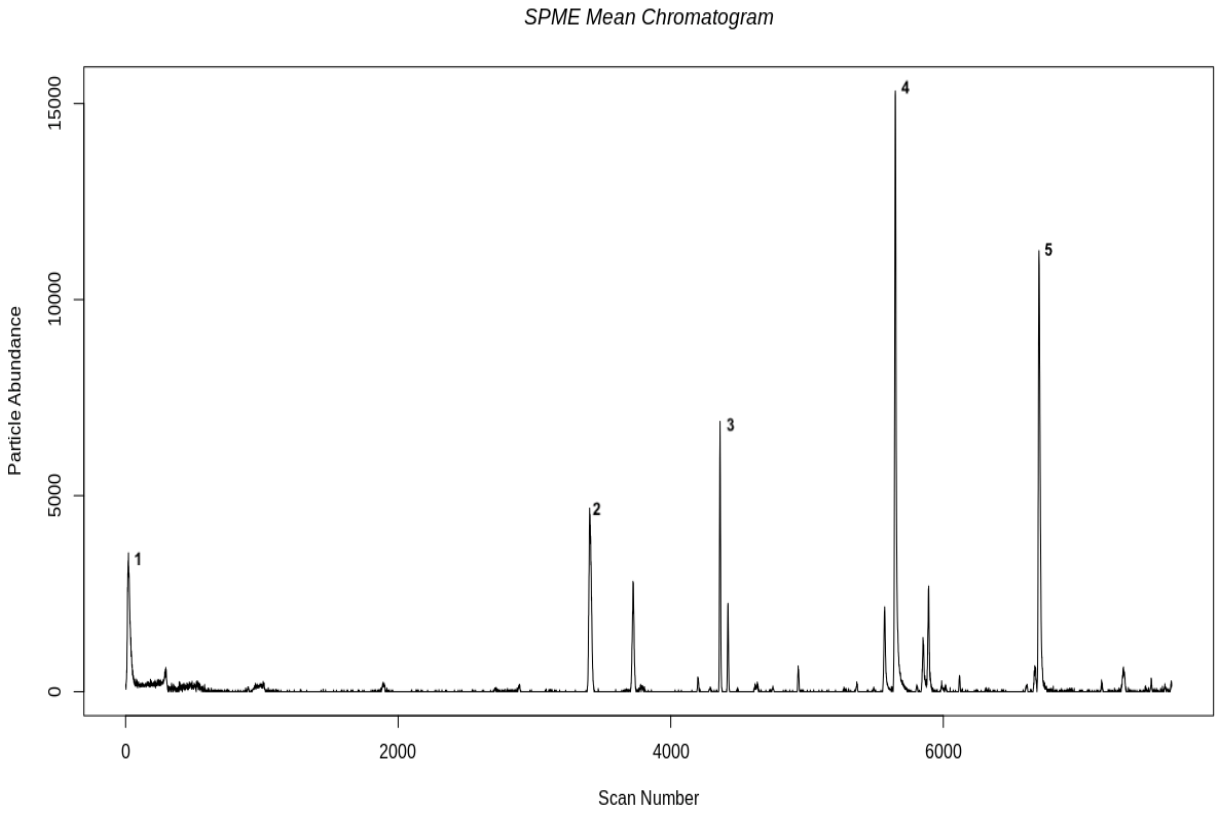

Fig 8. Averaged VOC profile of SPME fibers.

Overall, there were low amounts of VOCs produced from the fiber alone. This chromatogram was used to subtract control chromatogram and spectra data from averaged fungal VOC chromatograms.

Compound Hit	Match Factor	Probability	Retention time in minutes (Scan number)
#1 3-Methylpyridazine	813	26.60%	1.200 (21.00)
#2 2H-Pyran-2,6(3H)-dione	887	88.00%	20.56 (3404)
#3 Ethylene glycol – Adiapte – Dietheylene glycol	689	40.00%	26.04 (4362)
#4 Dimethyl palmitamine	653	5.16%	33.39 (5648)
#5 Phenol, 4,4'-(1-methylethylidene)bis-	899	72.50%	39.47 (6710)

Table 7. NIST 2017 database results of averaged peaks for SPME fiber.

SPME VOC Mass Spectra

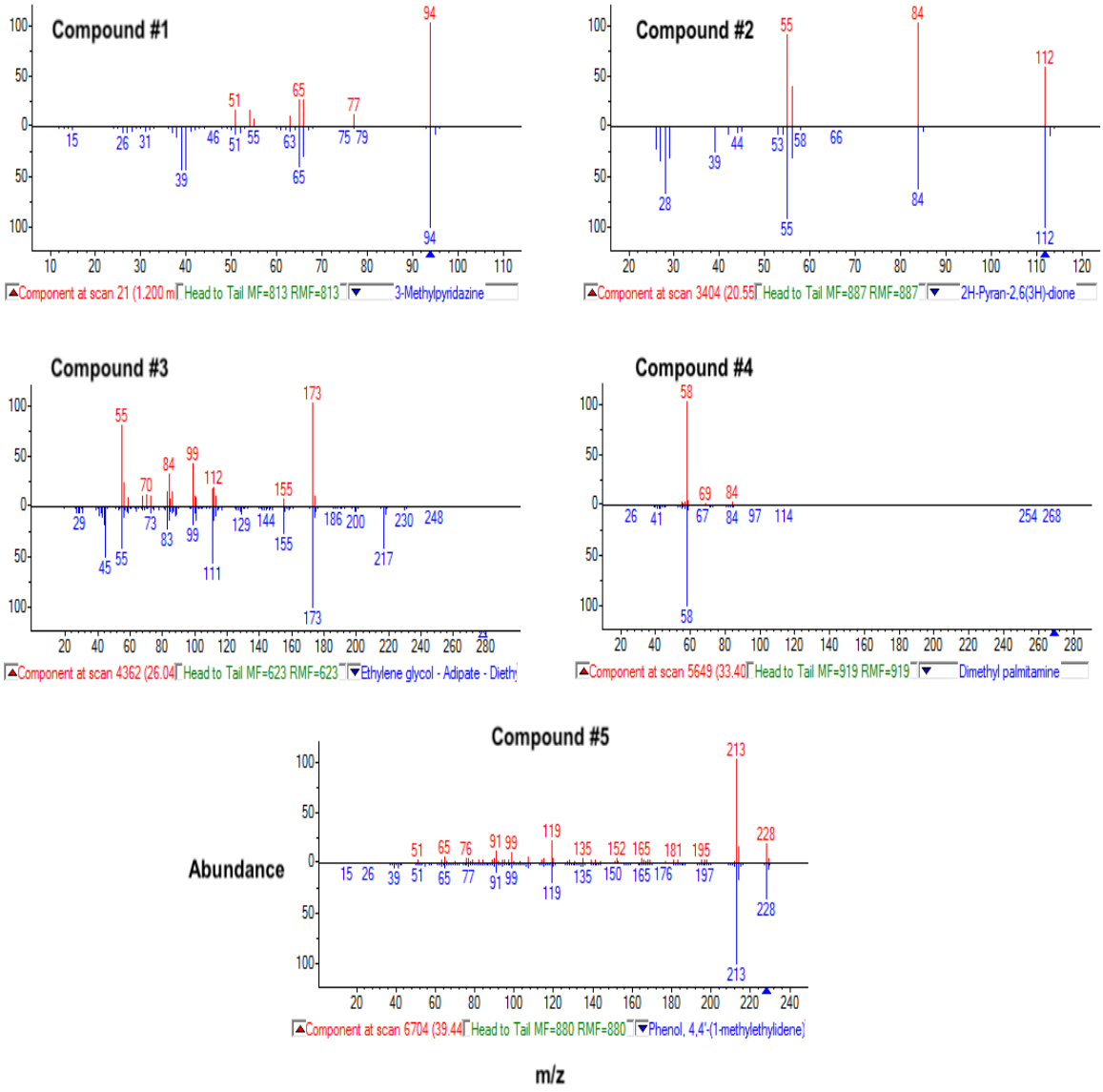

Fig 9. Mass spectra for five most abundant SPME compounds.

Red spectra denote unknown compounds isolated in this analysis, while blue spectra are derived from library matches. Generally, optimal matches will contain each ion represented on the m/z axis. However, because library compounds are potentially surveyed using different protocols,, they are not wholly necessary.

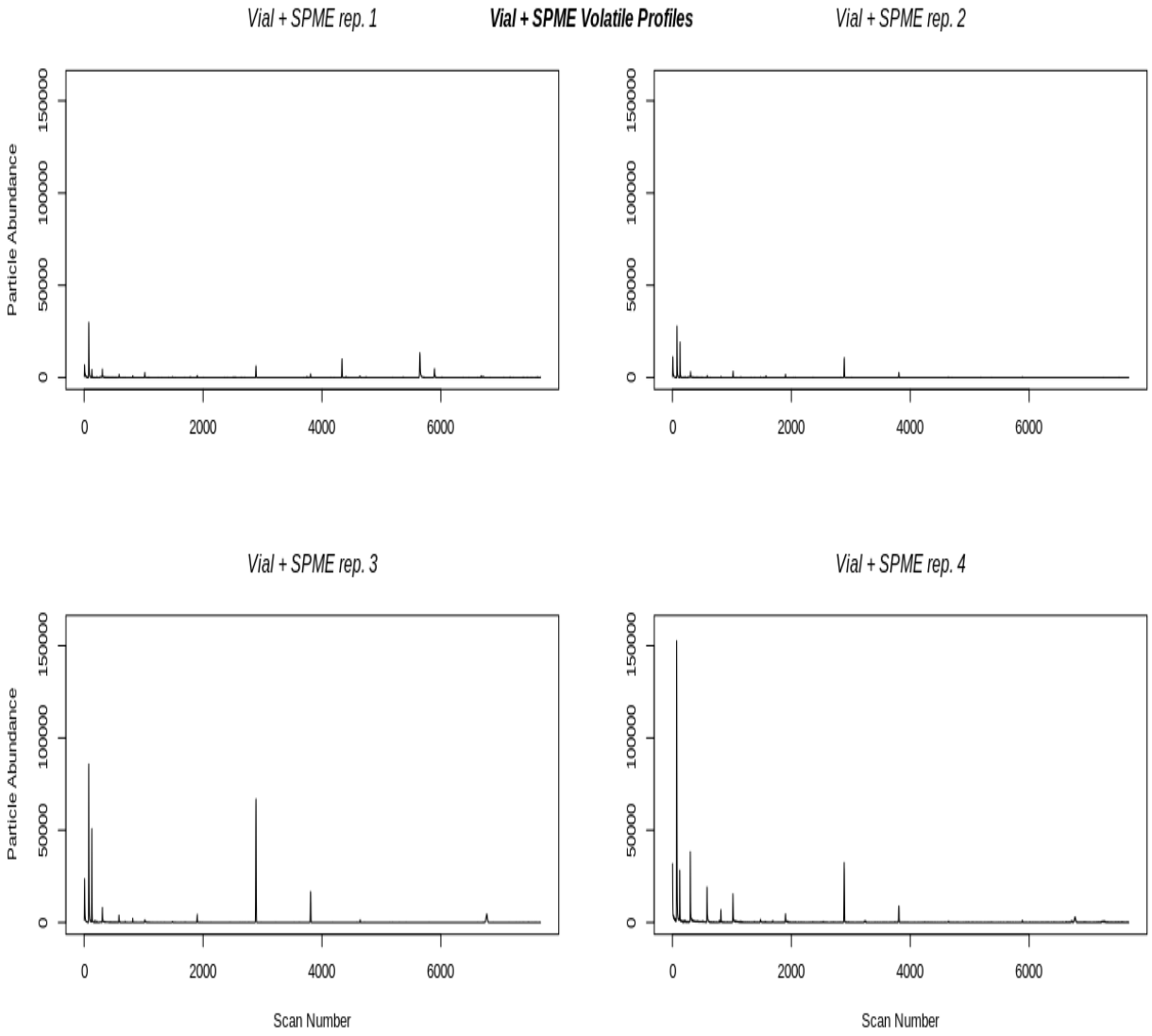

Fig 10. Chromatograms obtained from empty vial headspace analysis.
Empty vials were opened under a sterile biosafety cabinet to maintain consistency with the vials that MEA was pipetted into.

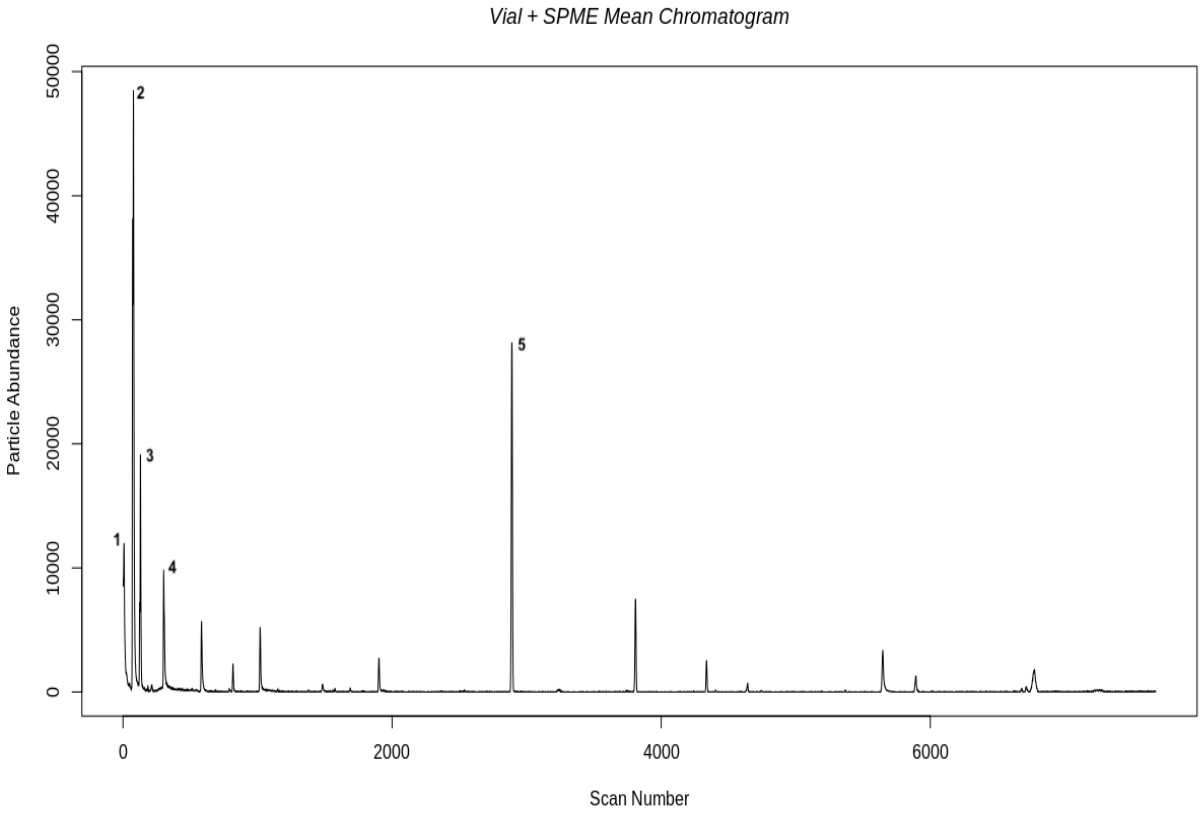

Fig 11. Mean chromatogram of empty vial headspace.

This chromatogram was used to subtract abundance and spectra data from averaged fungal VOC chromatograms.

Compound Hit	Match Factor	Probability	Retention time in minutes (Scan number)
#1 Hydrazine, 1, 1-dimethyl-	990	20.3%	1.098 (3.000)
#2 Silanediol, dimethyl-	946	91.1%	1.492 (91.10)
#3 Silane, triethylfluoro	813	20.60%	1.824 (130.0)
#4 Cyclotrisiloxane	950	90%	2.809 (302.0)
#5 Cyclohexasiloxane, dodecamethyl-	920	95.4%	17.62 (2891)

Table 8. NIST 2017 database results for Vial & SPME compounds

Vial + SPME VOC Mass Spectra

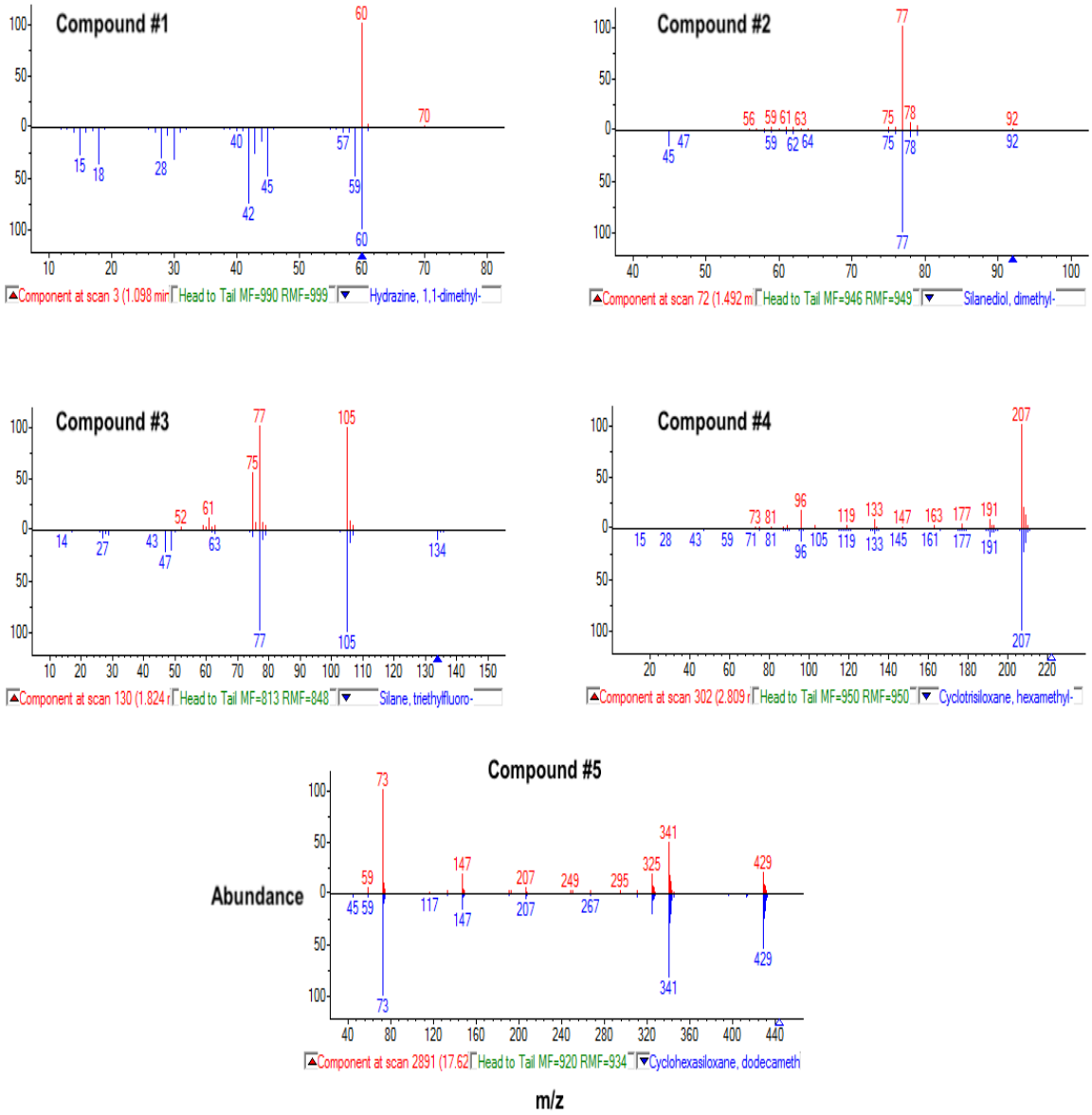

Fig 12. Mass spectra of top five library searches for vial and SPME compounds

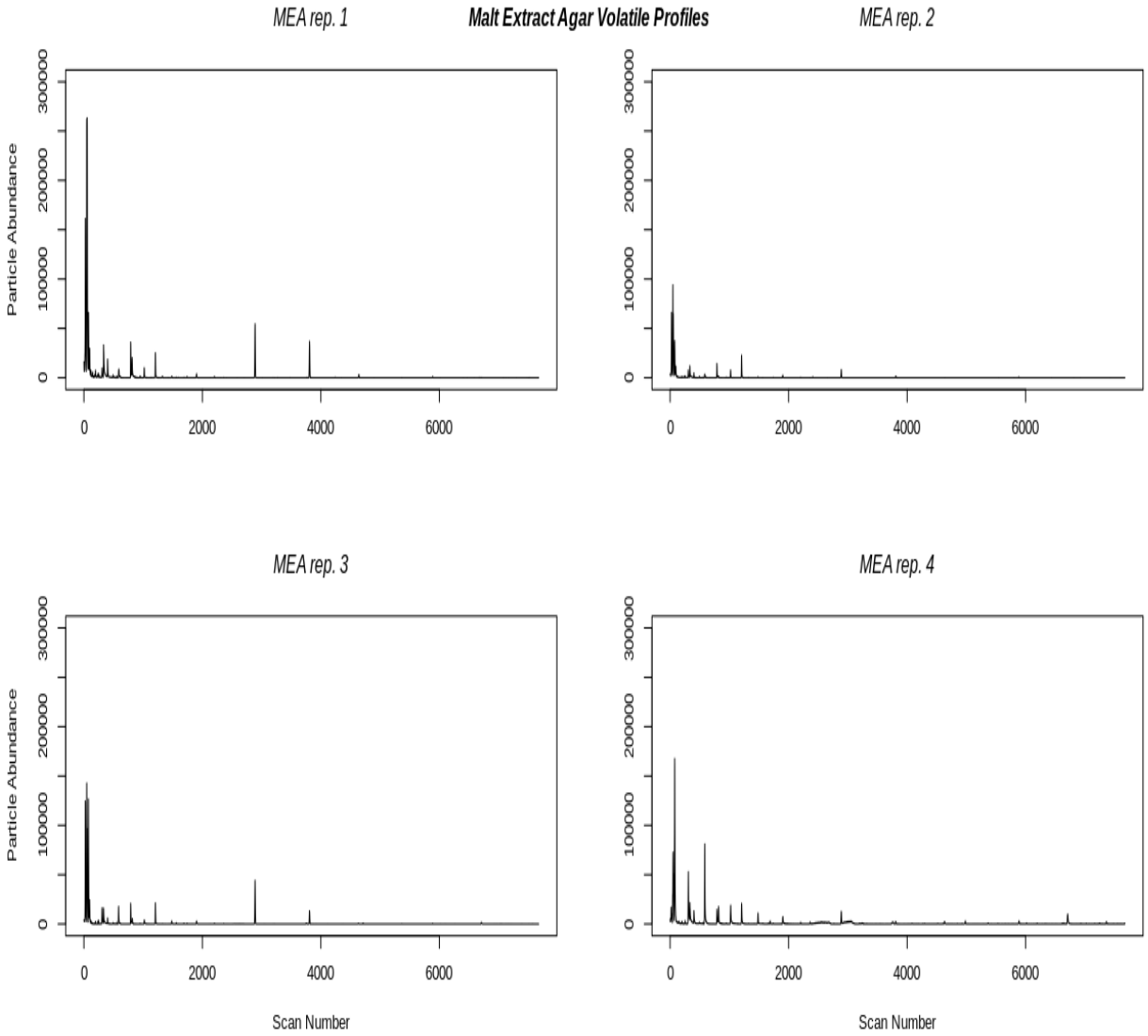

Fig 13. Chromatograms of VOCs emitted from malt extract agar and sampling vials.
Each rep corresponds to the MEA control used for each full run of sampling, which included the aforementioned MEA vial, four fungal samples and control vial without any media.

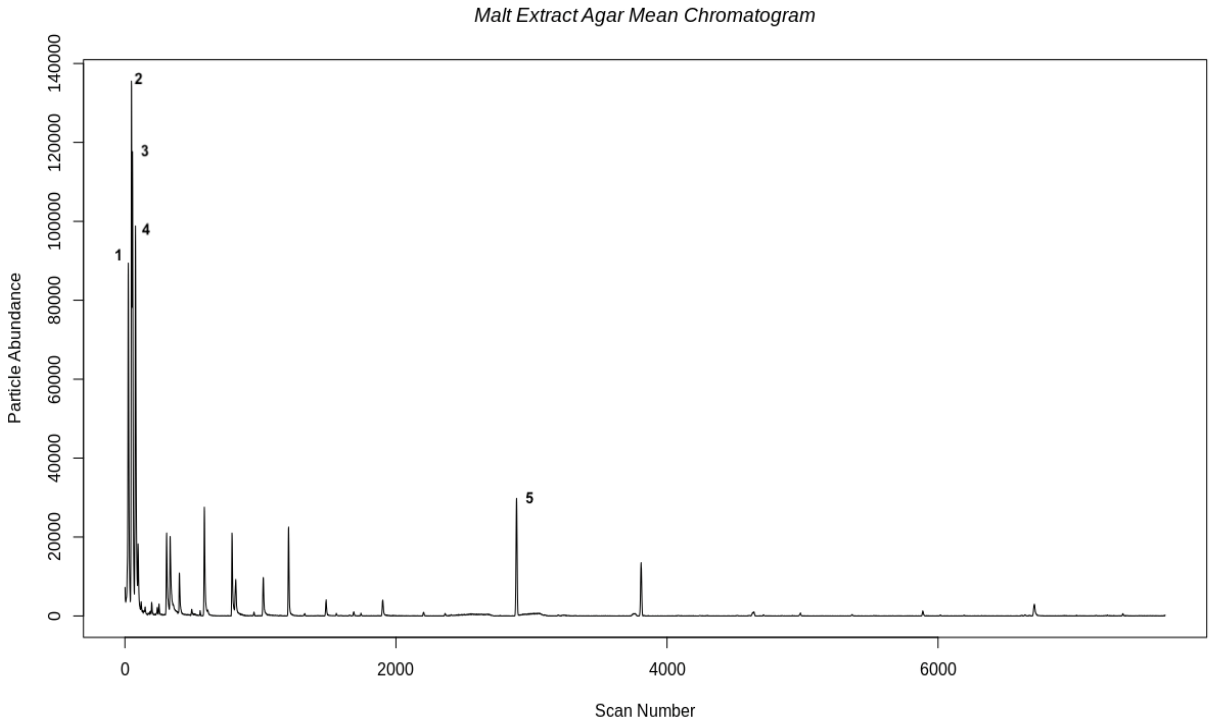

Fig 14. Average of four MEA, SPME and sampling vial chromatograms.

This chromatogram is used to subtract chromatogram and spectra data from averaged fungal VOC chromatograms for determining fungal-emitted VOCs.

Compound Hit	Match Factor	Probability	Retention time in minutes (Scan number)
#1 Trichloromethane	945	78.00%	1.225 (25.00)
#2 Butanal, 3-methyl-	923	76.50%	1.361 (49.00)
#3 Butanal, 2-methyl-	915	61.60%	1.406 (57.00)
#4 Silanediol, dimethyl-	962	93.60%	1.530 (78.00)
#5 Cyclohexasiloxane, dodecamethyl-	905	94.19%	17.62 (2890)

Table 9. NIST 2017 database results for MEA compounds

MEA VOC Mass Spectra

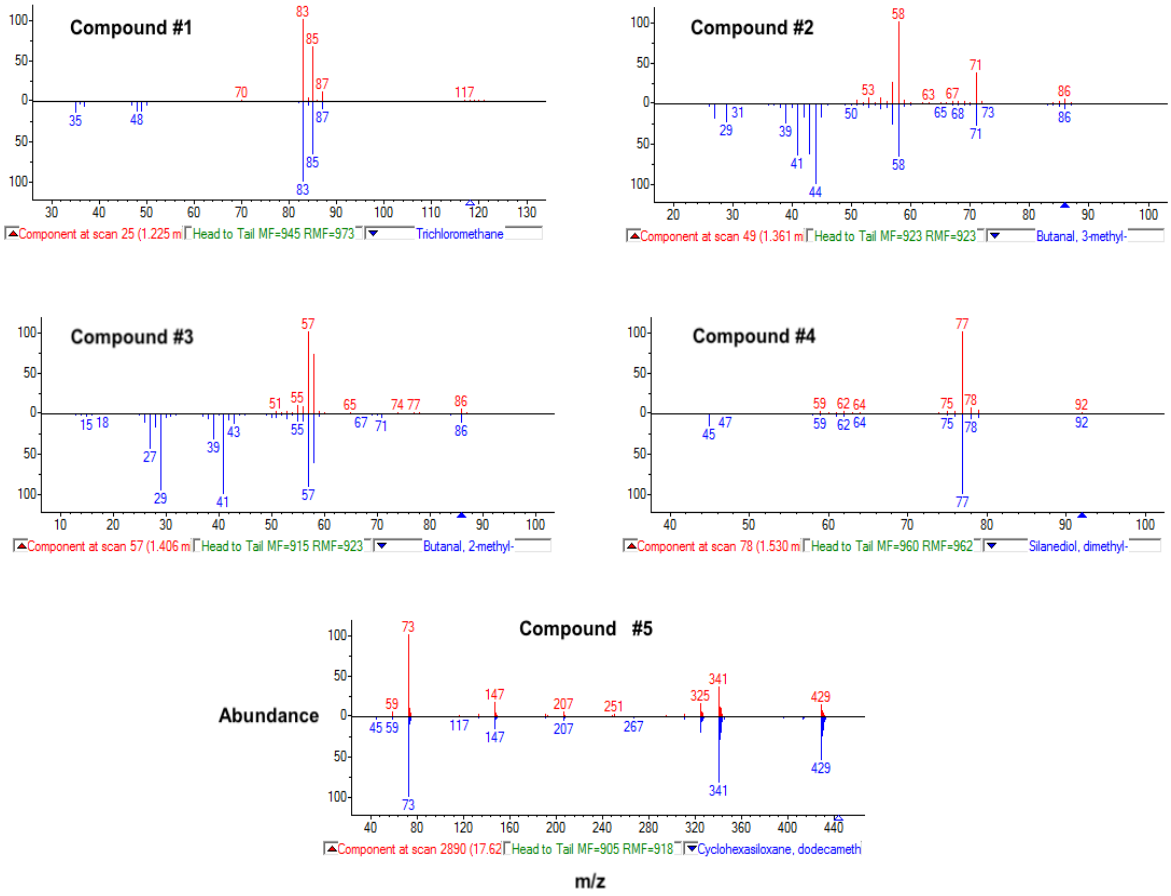

Fig 15. Mass Spectra for top five MEA VOCs

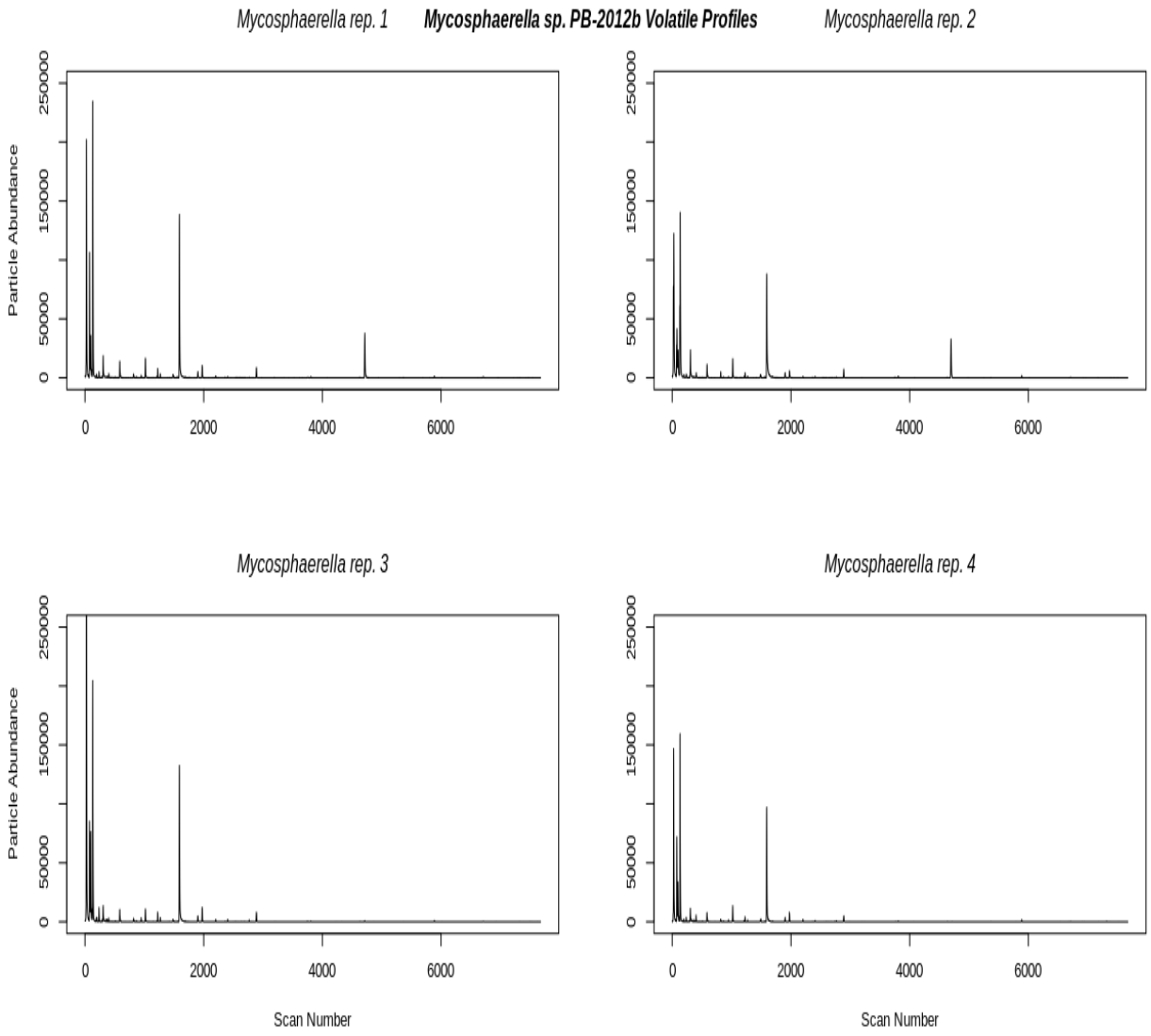

Fig 16. Chromatograms of VOCs emitted from Mycosphaerella sp. PB-2012b.

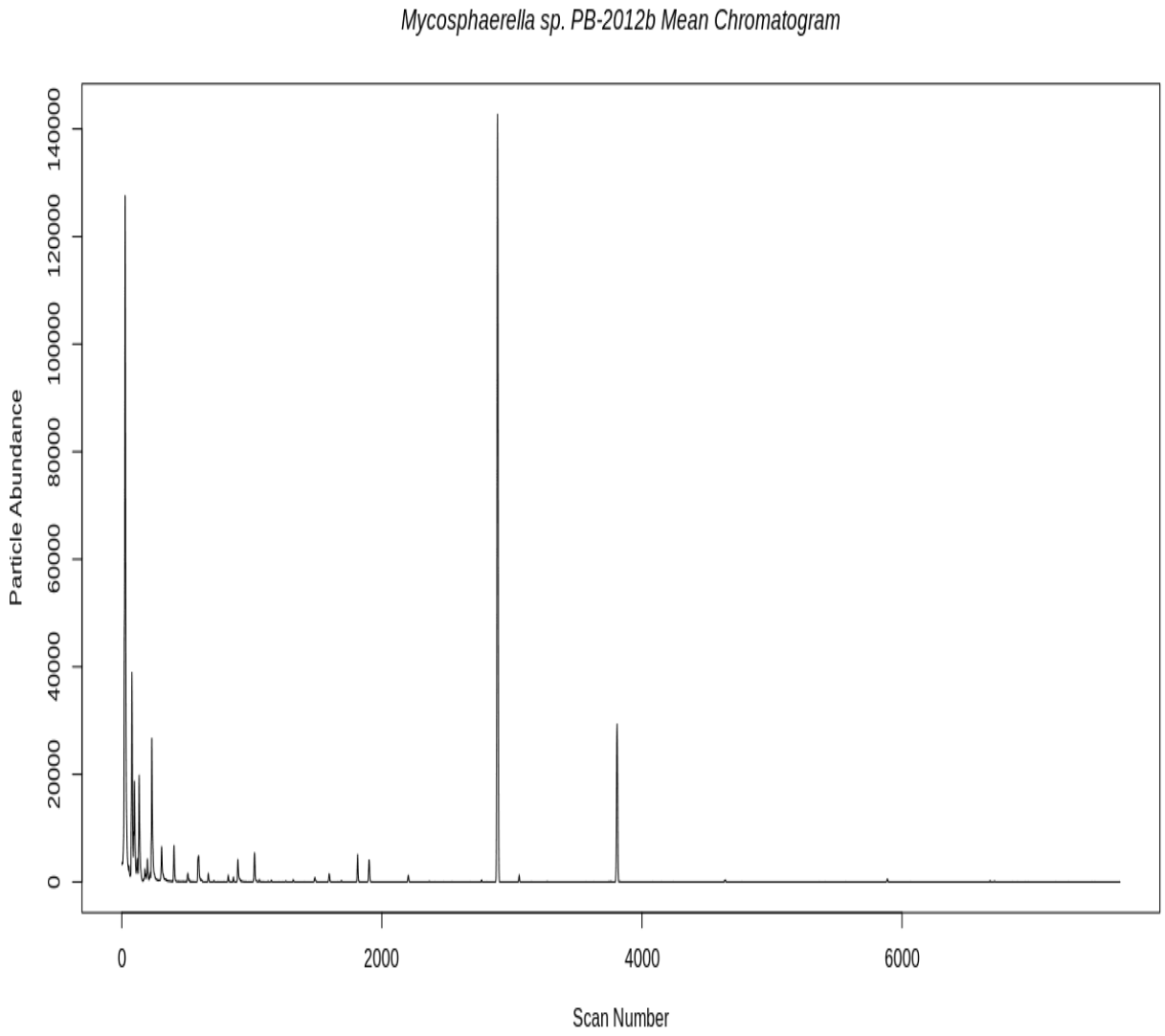

Fig 17. Mean chromatogram of Mycosphaerella VOCs

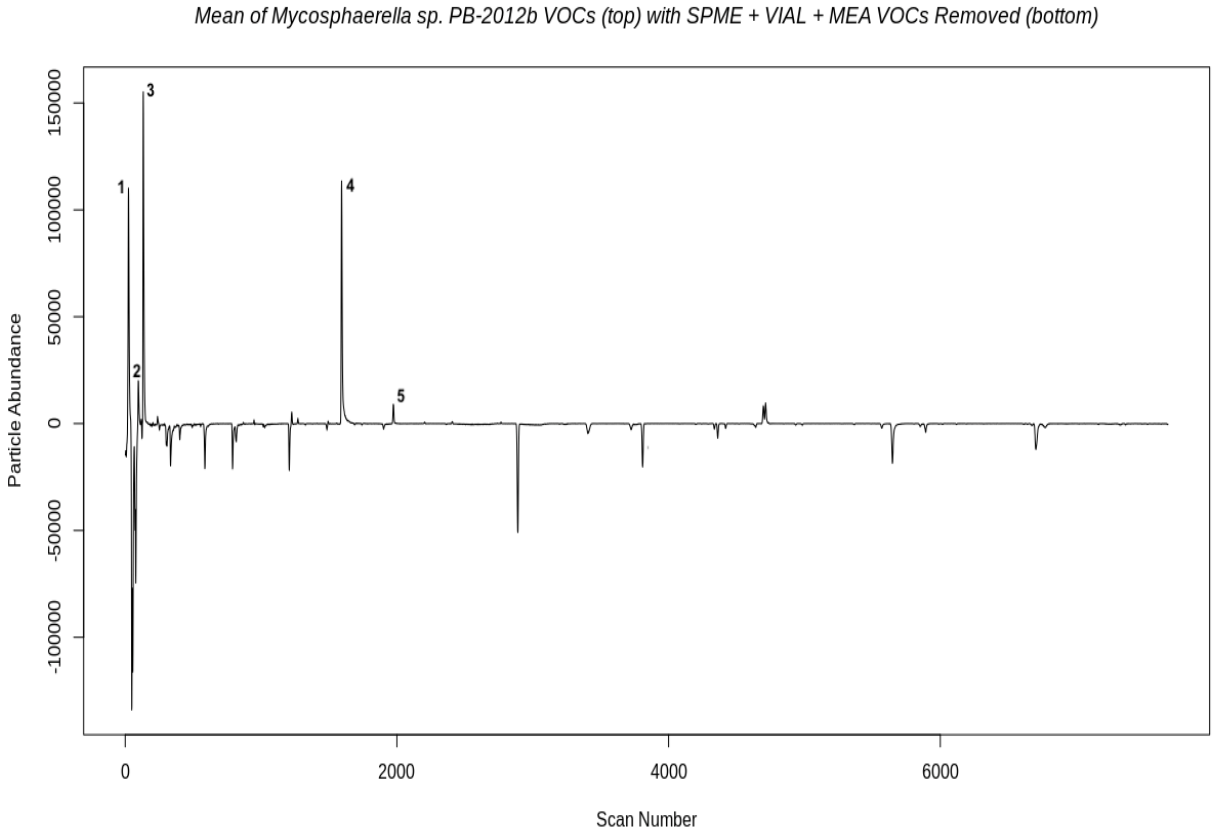

Fig 18. Averaged Mycosphaerella sp. PB-2012b chromatogram with abundance of control VOCs subtracted.

Compound Hit	Match Factor	Probability	Retention time in minutes (Scan number)
#1 Trichloromethane	950	79.70%	1.220 (25.00)
#2 Methane, bromodichloro	960	95.80%	1.636 (91.10)
#3 1-Butanol, 3-methyl-	925	42.50%	1.824 (133.0)
#4 Phenylethyl alcohol	955	81.80%	10.19 (1593)
#5 Benzeneacetic acid, methyl ester	890	72.30%	12.382 (1975)

Table 10. NIST 2017 database results for Mycosphaerella compounds

Mycosphaerella sp. PB-2012b VOC Mass Spectra

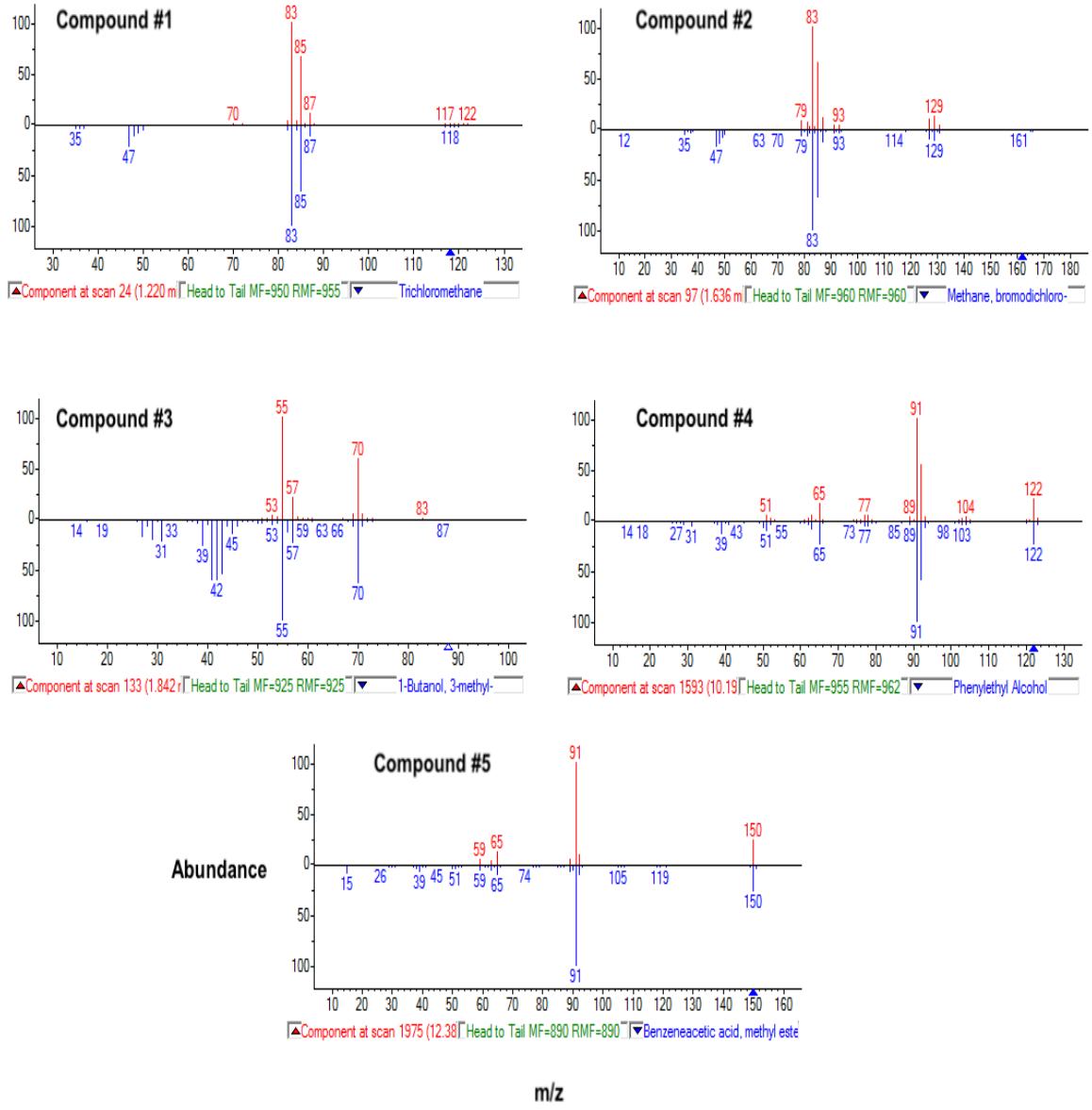

Fig 19. Mass spectra for five most abundant Mycosphaerella sp. PB-2012b compounds

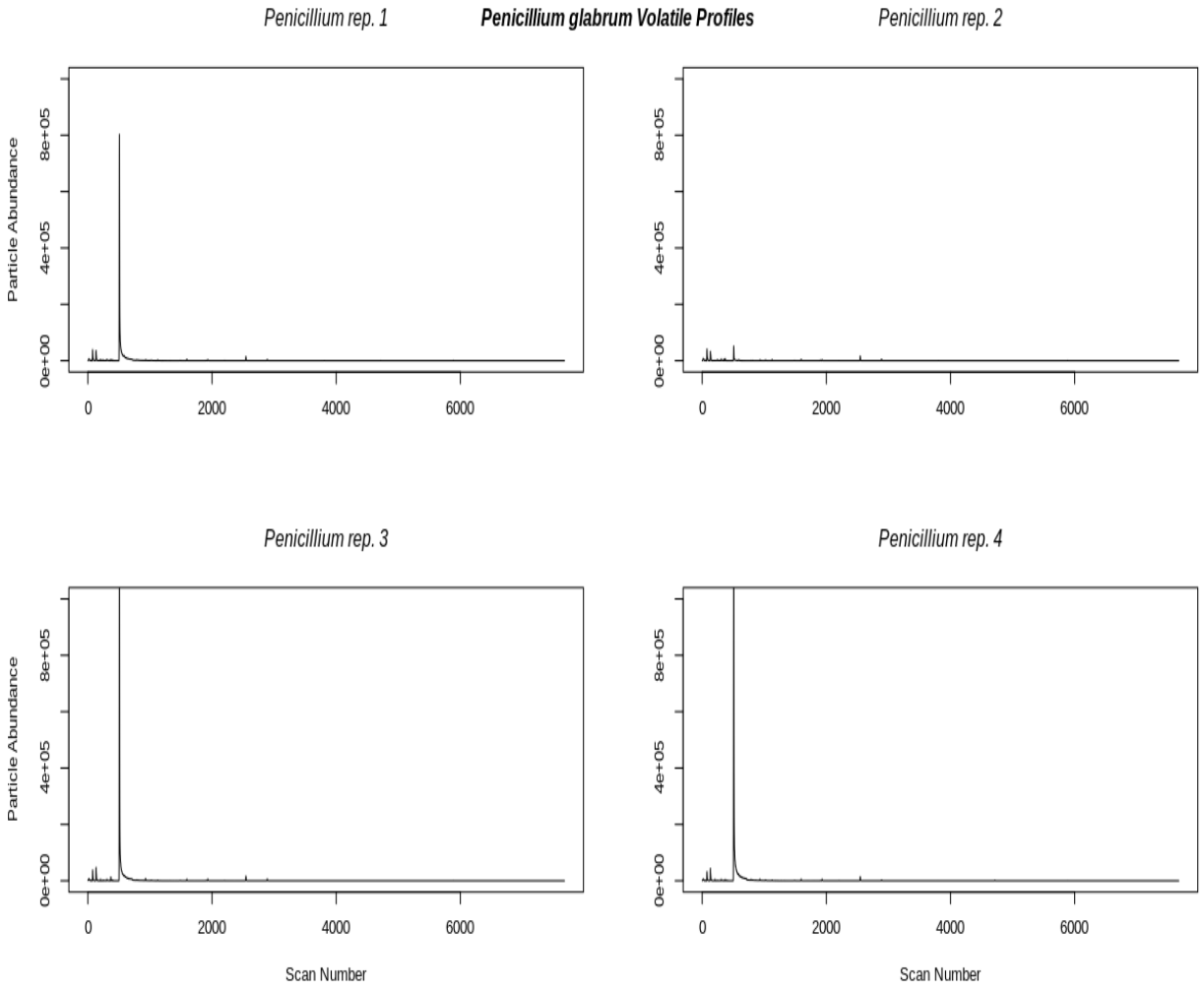

Fig 20. Chromatograms of VOCs emitted from four isolated Penicillium glabrum cultures

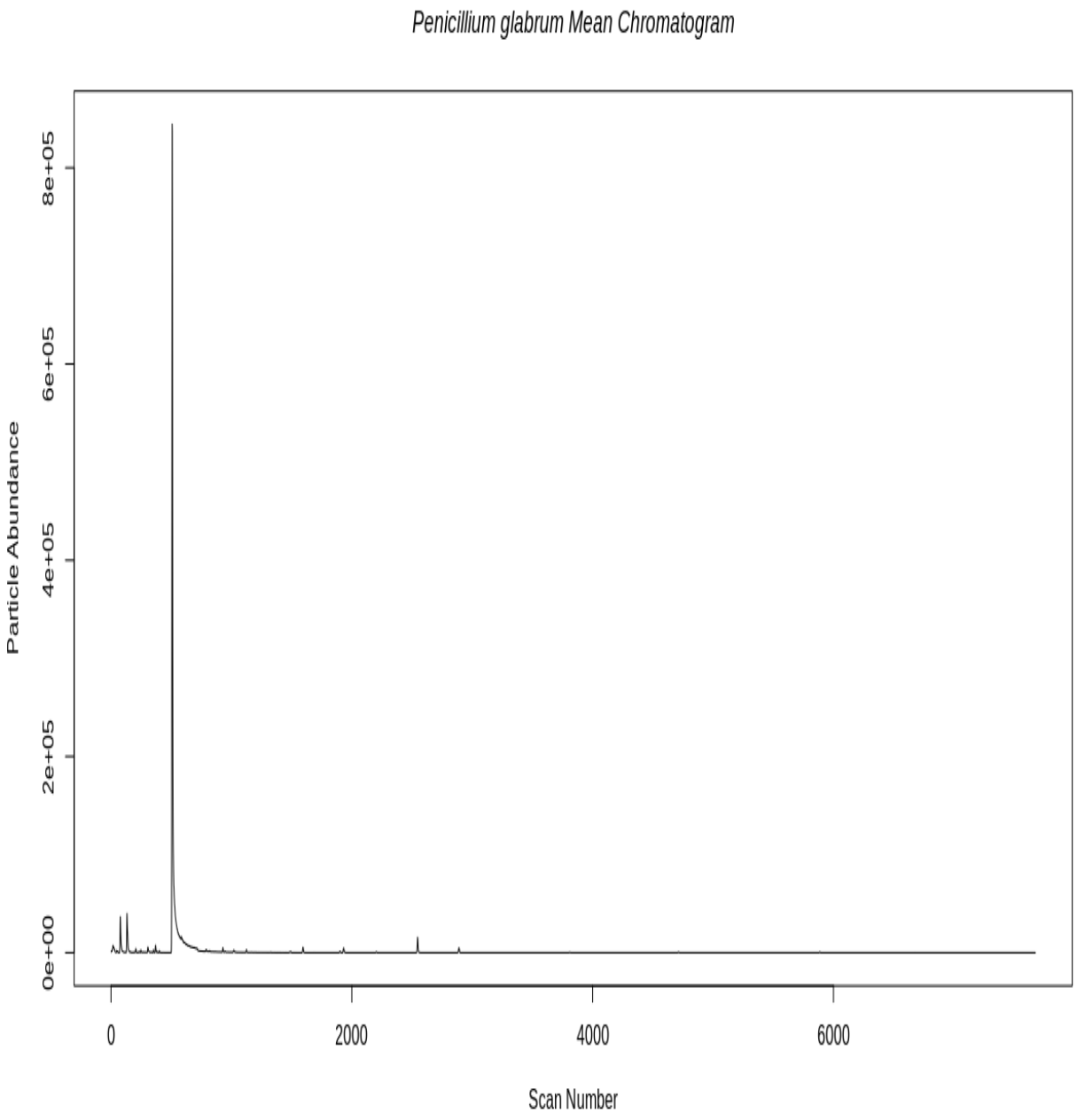

Fig 21. Average of four Penicillium glabrum sp. PB-2012b chromatograms.

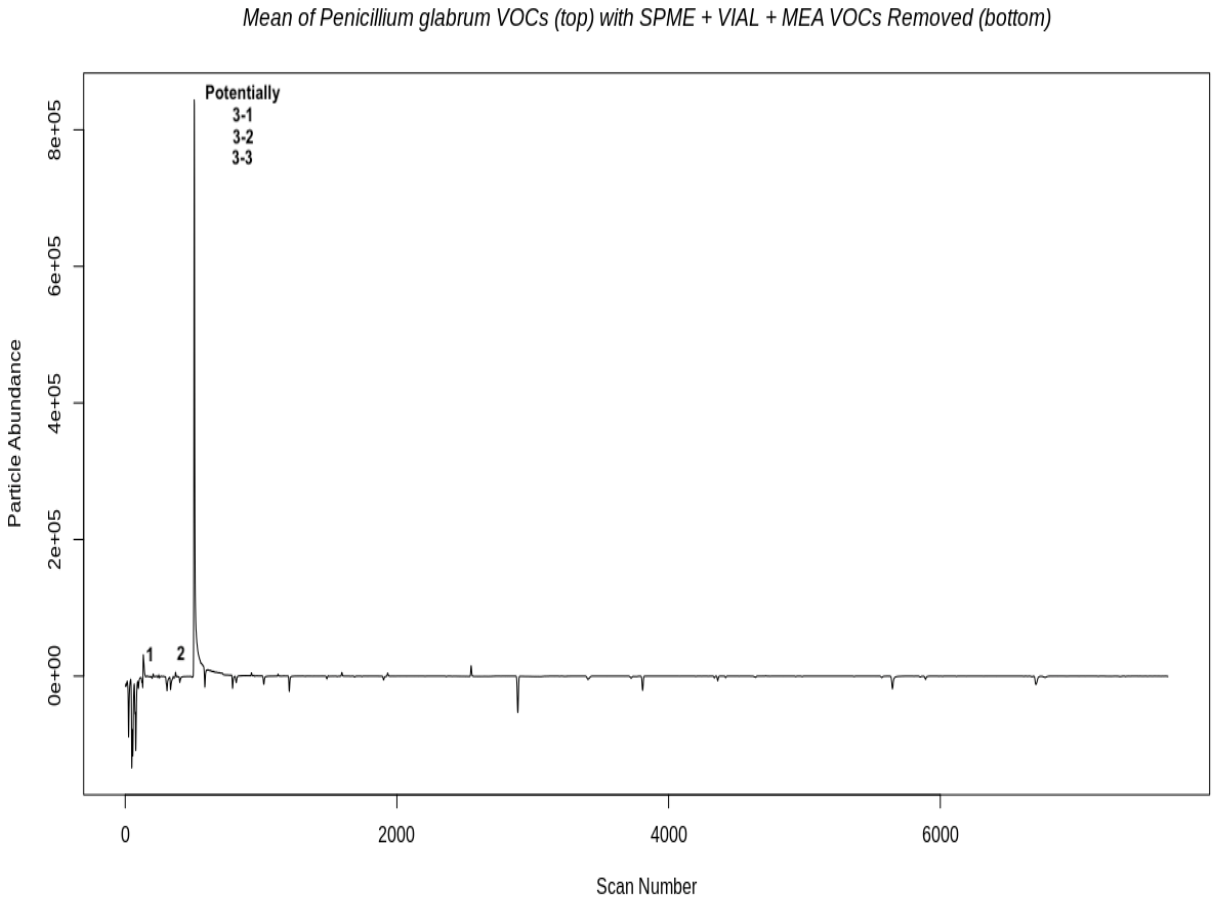

Fig 22. Averaged Penicillium glabrum chromatogram with abundance of control VOCs subtracted.

Compound Hit	Match Factor	Probability	Retention time in minutes (Scan number)
#1 1-Pentene	922	17.60%	1.845 (133.0)
#2 Cyclotrisiloxane hexamethyl-	896	69.10%	2.834 (307.0)
#3-1 Styrene	953	37.00%	3.993 (509)
#3-2 Bicyclo[4.2.0]octa-1,3,5-triene	961	33.90%	3.993 (509)
#3-3 1,3,5,7-Cyclooctatetetraene	938	12.40.30%	3.993 (509)

Table 11. NIST 2017 database results for Penicillium compounds

Penicillium glabrum VOC Mass Spectra

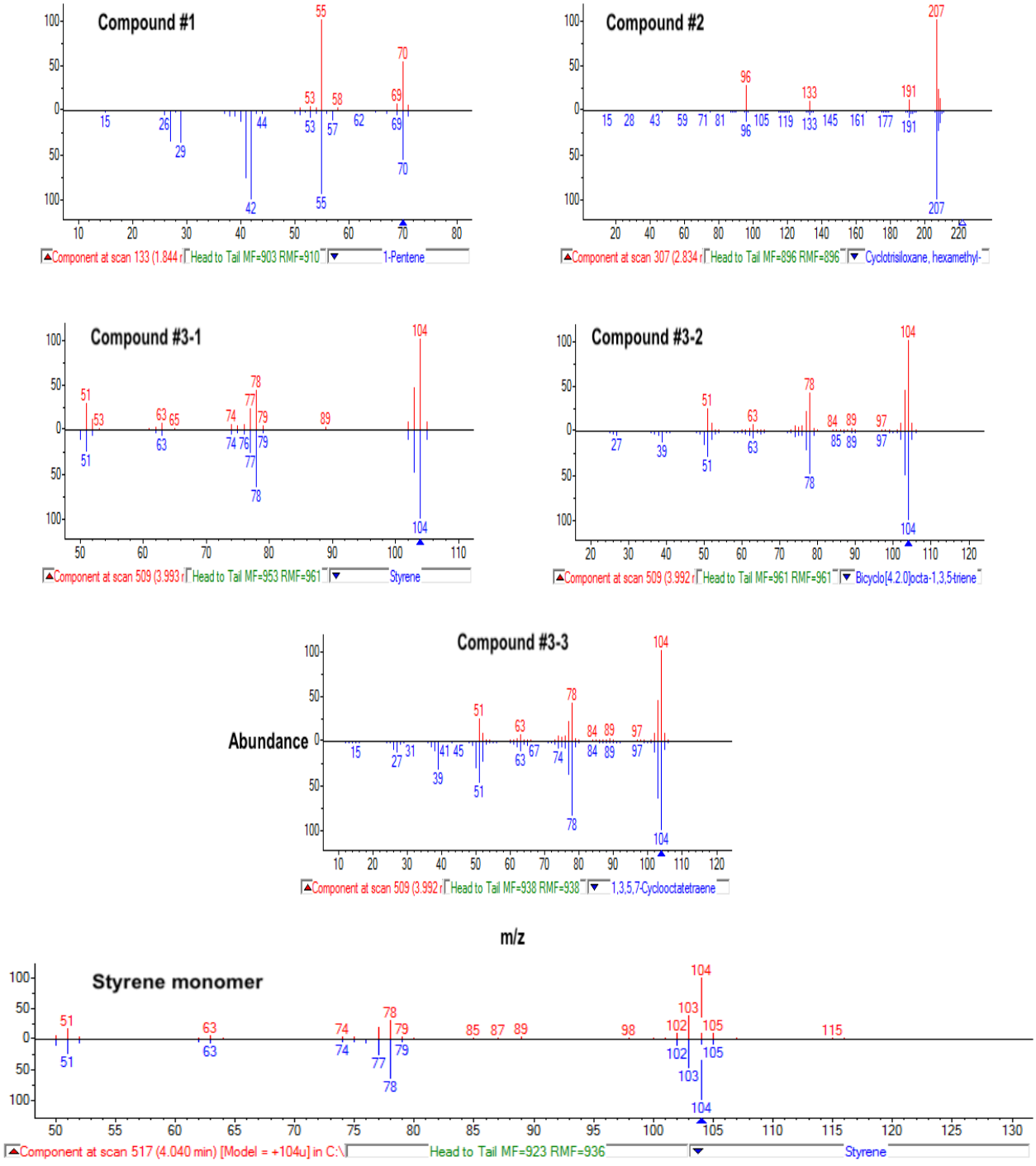

Fig 23. Mass spectra for three most abundant Penicillium glabrum compounds and styrene monomer

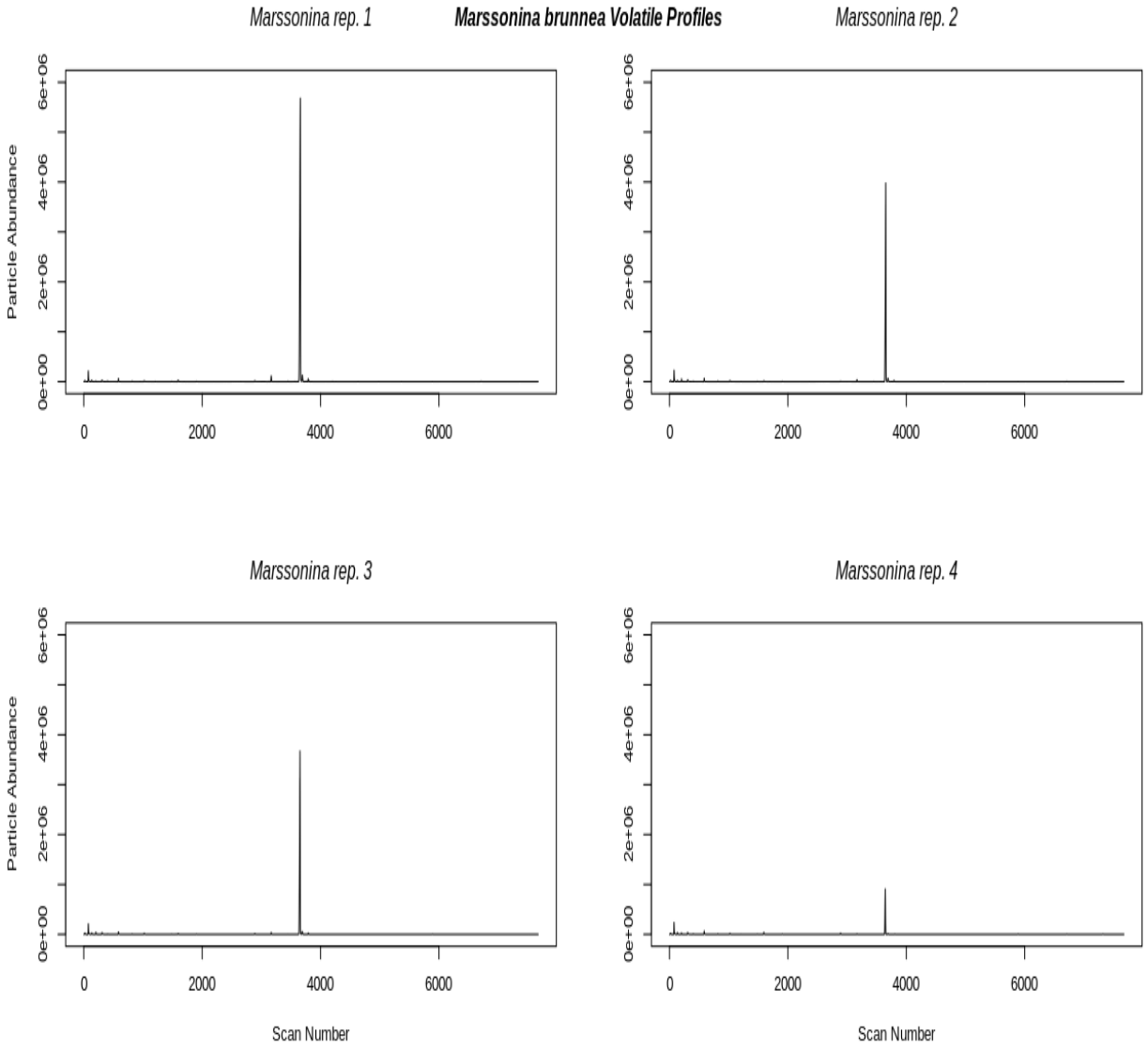

Fig 24. Chromatograms of organic volatiles emitted by Marssonina brunnea.

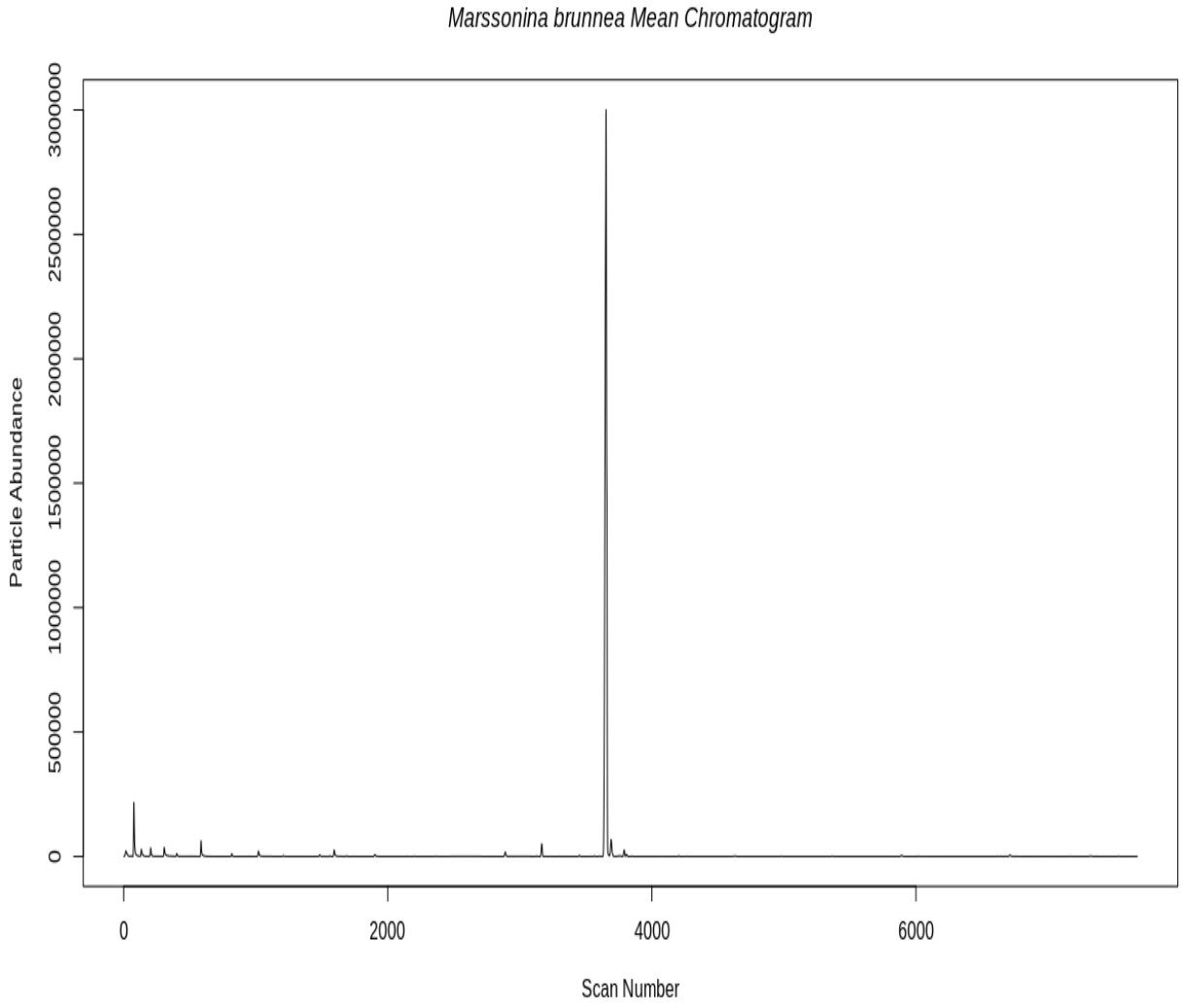

Fig 25. Average of four Marssonina brunnea chromatograms.

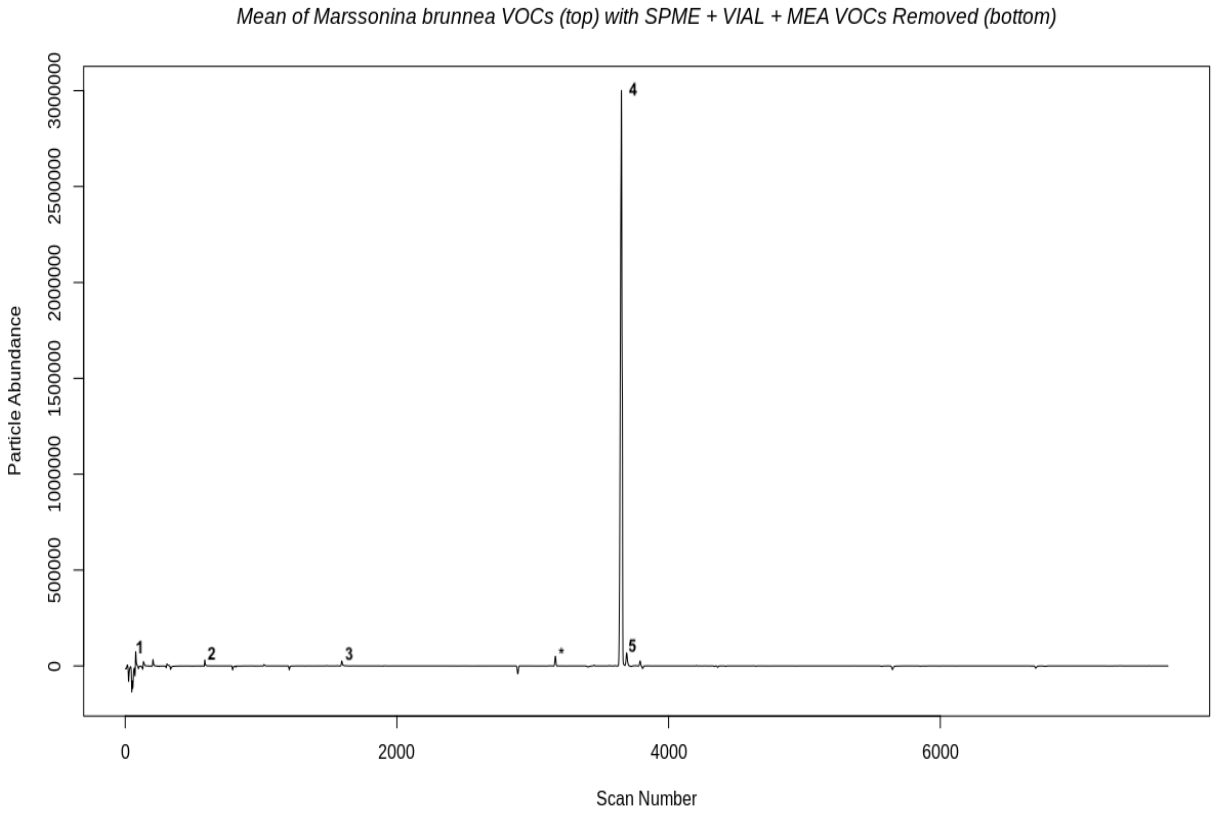

Fig 26. Averaged Marssonina brunnea chromatograms with particle abundance of control VOCs subtracted.

Compound Hit	Match Factor	Probability	Retention time in minutes (Scan number)
#1 Cyclopentane	930	16.20%	1.846 (134.0)
#2 Cyclotrisiloxane hexamethyl-	953	90.40%	2.836 (307.0)
#3 Phenylethyl alcohol	954	85.40%	10.21 (1594)
#4 Aristolochene	963	63.10%	21.94 (3645)
#5 Valencen	869	49.00%	22.20 (3690)

Table 12. NIST 2017 database results for Marssonina compounds

Marssonina brunnea VOC Mass Spectra

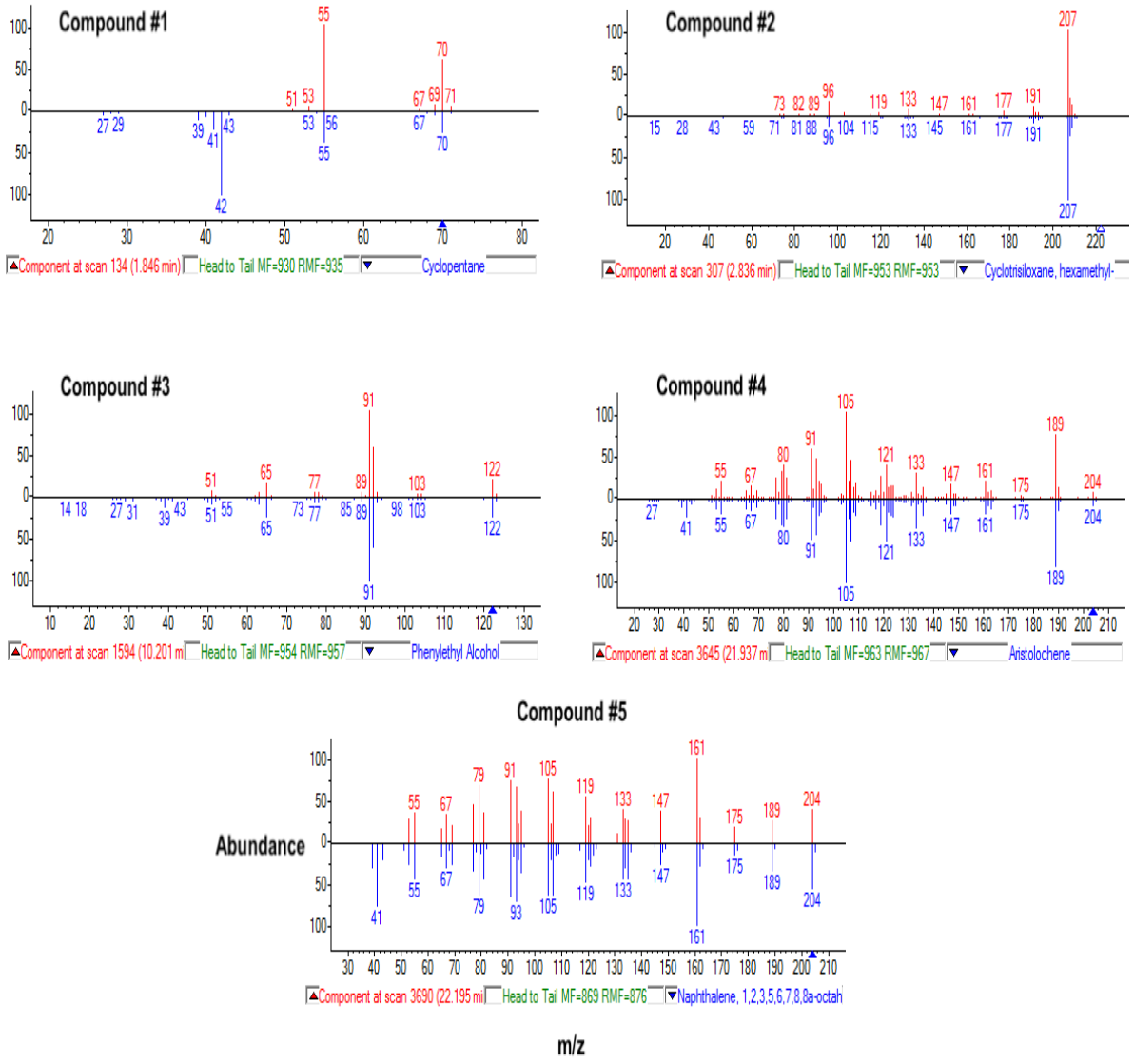

Fig 27. Mass spectra for five most abundant Marssonina brunnea compounds

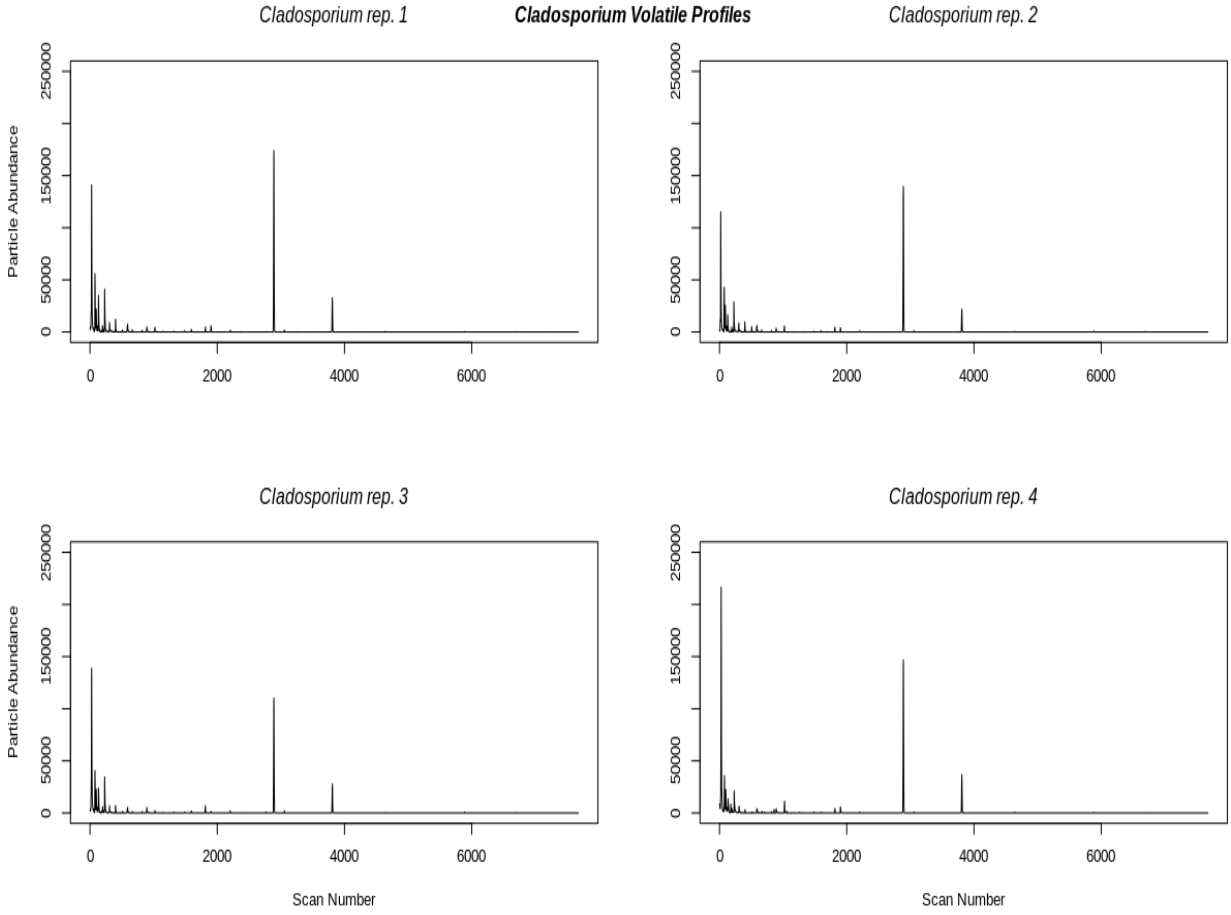

Fig 28. Chromatograms of VOCs emitted from Cladosporium sp.

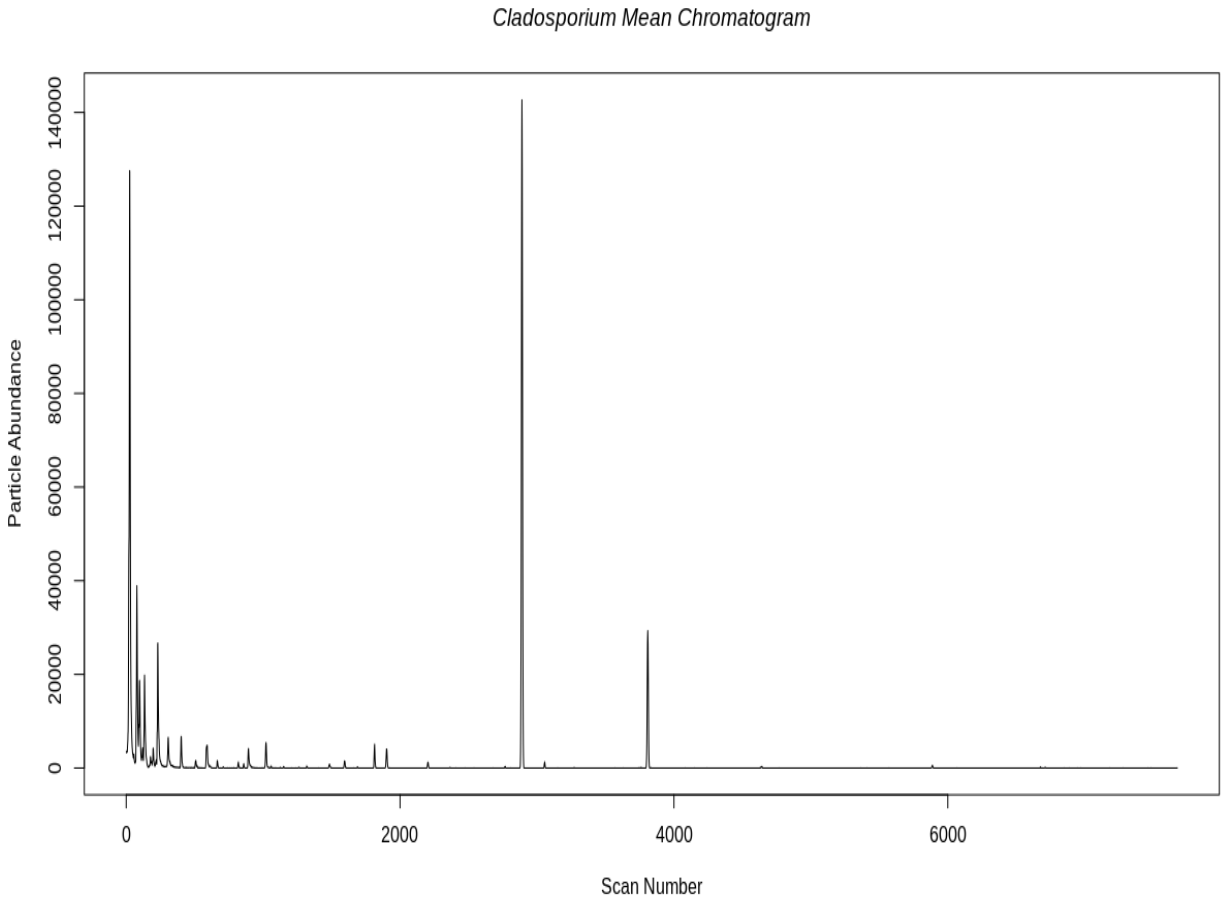

Fig 29. Average of four Cladosporium sp. chromatograms.

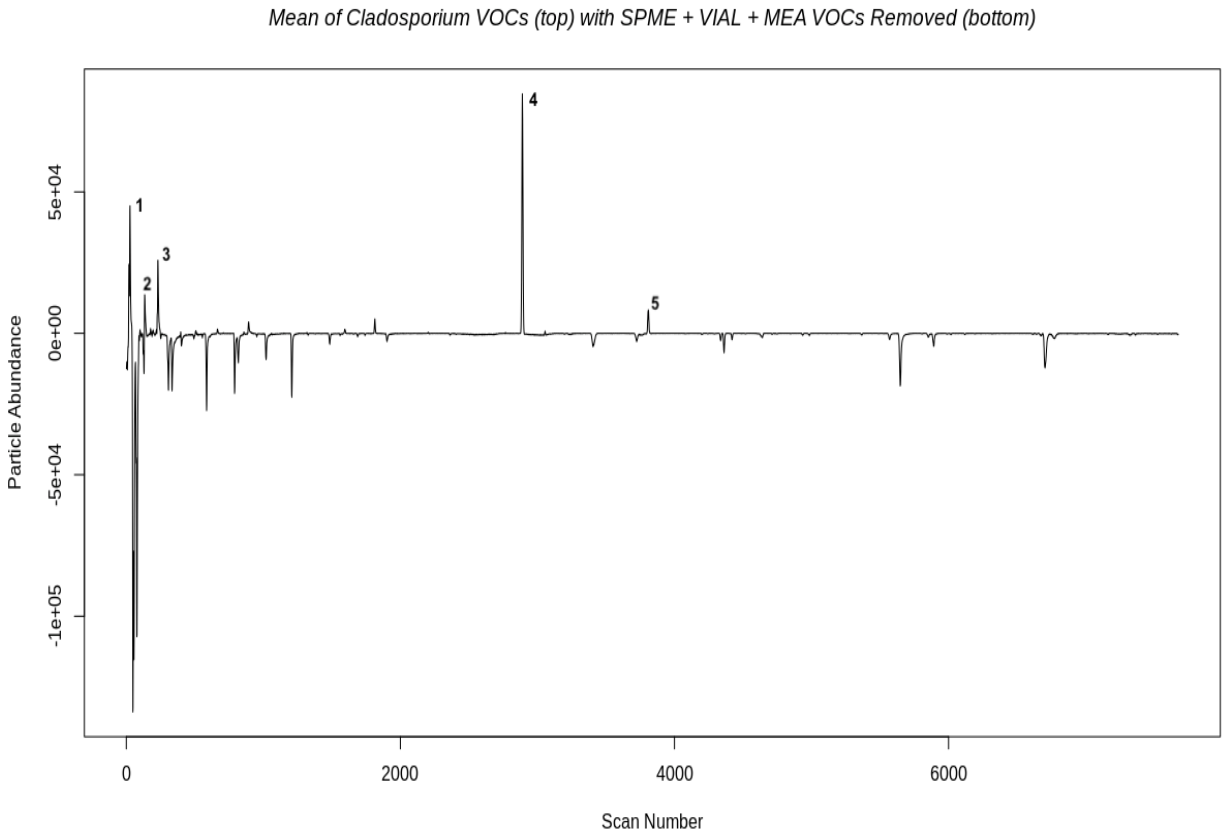

Fig 30. Average of four Cladosporium sp. Chromatograms with control peaks removed.

Compound Hit	Match Factor	Probability	Retention time in minutes (Scan number)
#1 Trichloromethane	951	79.50%	1.226 (25.00)
#2 Cyclopentanone	962	79.50%	2.403 (231.0)
#3 Cyclotrisiloxane hexamethyl-	953	90.40%	2.836 (307.0)
#4 Cyclohexasiloxane, dodecamethyl-	901	95.6%	17.618 (2890)
# 3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	713	44.00%	22.88 (3810)

Table 13. NIST 2017 database results for Cladosporium compounds

Cladosporium sp. VOC Mass Spectra

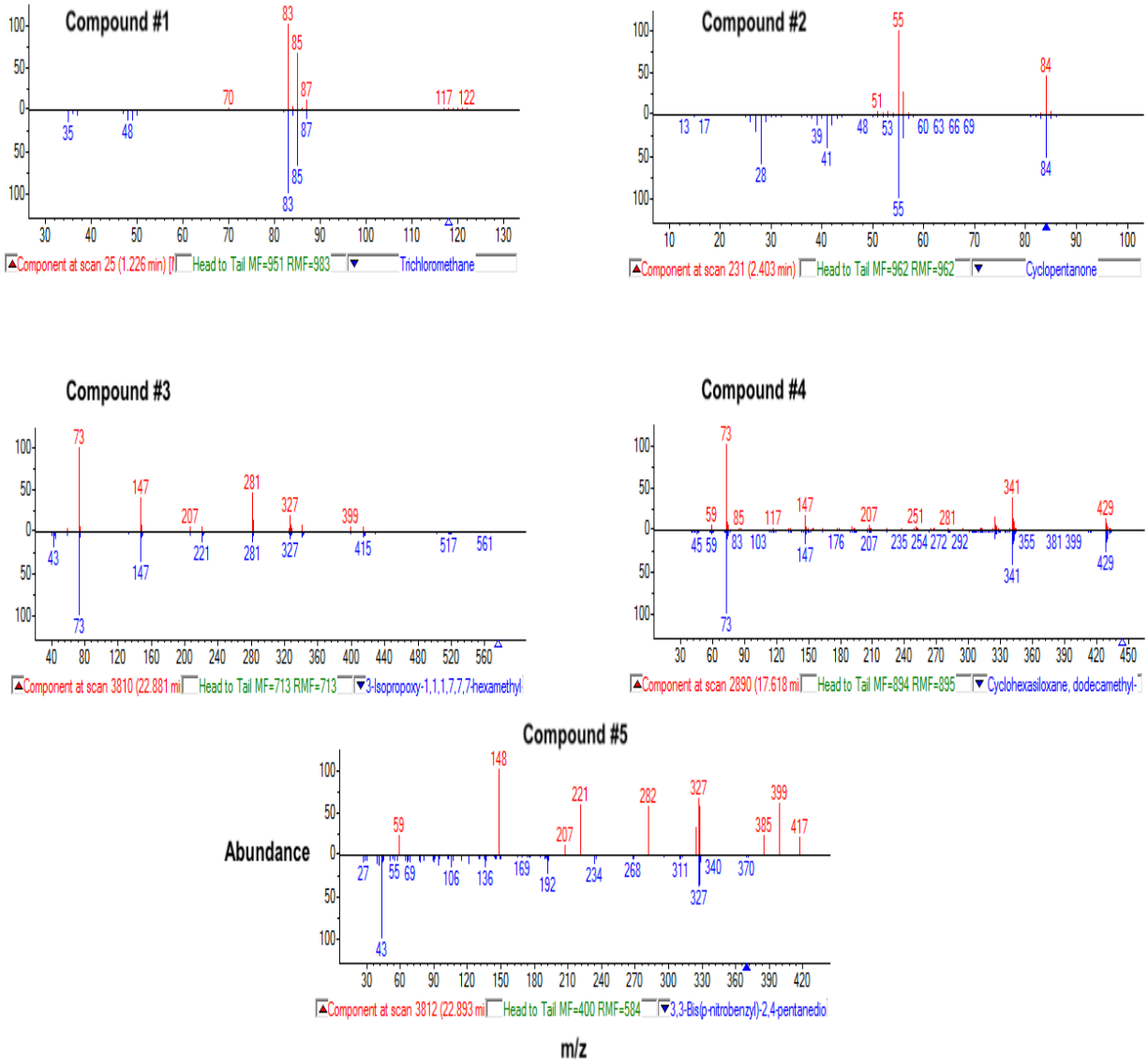

Fig 31. Mass spectra for five most abundant Cladosporium sp. compounds

Bibliography

1. McFall-Ngai, M. Are biologists in ‘future shock’? Symbiosis integrates biology across domains. *nrmicro* **6,** 789–792 (2008).
2. Oulhen, N., Schulz, B. J. & Carrier, T. J. English translation of Heinrich Anton de Bary’s 1878 speech, ‘Die Erscheinung der Symbiose’ (“De la symbiose”). *Symbiosis* 1–10 (2016). doi:10.1007/s13199-016-0409-8
3. Wernegreen, J. J. Endosymbiosis. *CURBIO* **22,** R555–R561 (2012).
4. Bakken, J. S. *et al.* Treating Clostridium difficile Infection With Fecal Microbiota Transplantation. *Clinical Gastroenterology and Hepatology* **9,** 1044–1049 (2011).
5. Na, X. & Kelly, C. Probiotics in Clostridium difficile Infection. *Journal of Clinical Gastroenterology* **45,** S154–S158 (2011).
6. Whitman, W. B., Coleman, D. C. & Wiebe, W. J. Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences* **95,** 6578–6583 (1998).
7. Pace, N. R. A Molecular View of Microbial Diversity and the Biosphere. *Science* **276,** 734–740 (1997).
8. Finlay, B. J. & Clarke, K. J. Ubiquitous dispersal of microbial species. *Nature* **400,** 828–828 (1999).
9. Rižner, T. L., Adamski, J. & Žakelj Mavrič, M. Expression of 17β-hydroxysteroid dehydrogenases in mesophilic and extremophilic yeast. *Steroids* **66,** 49–54 (2001).
10. Adedeji, W.2016. The treasure called antibiotics. *ncbi.nlm.nih.gov*
11. Kamminga, H. Martinus Willem Beijerinck - His life and his work: by G. van Iterson Jr., L. E. den Dooren de Jong and A. J. Kluyver, Science Tech, Inc. 1983. (xxix + 181 pages) ISBN 0 910 23902 9. *Trends in Biochemical Sciences* **9,** 407 (1984).
12. Bordenstein, S. R. & Theis, K. R. Host Biology in Light of the Microbiome: Ten Principles of Holobionts and Hologenomes. *PLoS Biol* **13,** e1002226 (2015).
13. Sender, R., Fuchs, S. & Milo, R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol* **14,** e1002533–14 (2016).
14. Ezenwa, V. O., Gerardo, N. M., Inouye, D. W., Medina, M. & Xavier, J. B. Animal Behavior and the Microbiome. *Science* **338,** 198–199 (2012).
15. Smith, A. H. *et al.* Patterns, causes and consequences of defensive microbiome dynamics across multiple scales. *Mol Ecol* **24,** 1135–1149 (2015).
16. Rosenblueth, M. & Martínez-Romero, E. Bacterial Endophytes and Their Interactions with Hosts. *MPMI* **19,** 827–837 (2006).
17. Crini, G. Historical review on chitin and chitosan biopolymers. *Environmental Chemistry Letters* **116,** 1–21 (2019).
18. Crespo, A., of, P. D. T. T.2014. *Fungi: hyperdiversity closer to animals than to plants*. (Sinauer Associates).
19. Burdon, J. J., Phytopathology, J. S.1997. Sources and patterns of diversity in plant-pathogenic fungi. *Am Phytopath Society* **87,** 664–669 (1997).
20. Ding, C., Wang, X. & Li, M. Evaluation of six white-rot fungal pretreatments on corn stover for the production of cellulolytic and ligninolytic enzymes, reducing sugars, and ethanol. 1–12 (2019). doi:10.1007/s00253-019-09884-y

21. Kothari, N. *et al.* Cellulose hydrolysis by Clostridium thermocellumis agnostic to substrate structural properties in contrast to fungal cellulases. *Green Chem.* **21,** 2810–2822 (2019).
22. Jones, J. D. G. & Dangl, J. L. The plant immune system. *Nature* **444,** 323–329 (2006).
23. Bago, B., Vierheilig, H., Phytologist, Y. P. N.1996. Nitrate depletion and pH changes induced by the extraradical mycelium of the arbuscular mycorrhizal fungus Glomus intraradices grown in monoxenic culture. *Wiley Online Library*
24. Högberg, M. N. & Högberg, P. Extramatrical ectomycorrhizal mycelium contributes one-third of microbial biomass and produces, together with associated roots, half the dissolved organic carbon in a forest soil. *New Phytologist* **154,** 791–795 (2002).
25. Ruiz-Lozano, J. M., Azcón, R., Microbiol, M. G. A. E.1995. Effects of arbuscular-mycorrhizal glomus species on drought tolerance: physiological and nutritional plant responses. *Am Soc Microbiol* doi:10.1007/s00572-002-0215-4
26. Azcón-Aguilar, C., Jaizme-Vega, M. C., in, C. C. M. T.2002. The contribution of arbuscular mycorrhizal fungi to the control of soil-borne plant pathogens. *Springer* doi:10.1007/s10311-019-00901-0
27. Russell, A. L. & Ashman, T.-L. Associative learning of flowers by generalist bumble bees can be mediated by microbes on the petals. *Behavioral Ecology* **30,** 746–755 (2019).
28. Liu, Y., Yao, S., Deng, L., Ming, J. & Zeng, K. Different mechanisms of action of isolated epiphytic yeasts against Penicillium digitatum and Penicillium italicum on citrus fruit. *Postharvest Biology and Technology* **152,** 100–110 (2019).
29. Büttner, E., Gebauer, A. M., Resour, M. H. M.2019. Draft Genome Sequence of Xylaria longipes DSM 107183, a Saprotrophic Ascomycete Colonizing Hardwood. *Am Soc Microbiol* **8,** 457 (2019).
30. Sand-Jensen, K. Effect of epiphytes on eelgrass photosynthesis. *Aquatic Botany* **3,** 55–63 (1977).
31. Brader, G. *et al.* Ecology and Genomic Insights into Plant-Pathogenic and Plant-Nonpathogenic Endophytes. *Annu. Rev. Phytopathol.* **55,** 61–83 (2017).
32. Hacquard, S. *et al.* Microbiota and Host Nutrition across Plant and Animal Kingdoms. *Cell Host and Microbe* **17,** 603–616 (2015).
33. Madej, C. W. & Clay, K. Avian seed preference and weight loss experiments: the effect of fungal endophyte-infected tall fescue seeds. *Oecologia* **88,** 296–302 (1991).
34. Oono, R., Lefèvre, E., Simha, A. & Lutzoni, F. A comparison of the community diversity of foliar fungal endophytes between seedling and adult loblolly pines (Pinus taeda). *Fungal Biology* **119,** 917–928 (2015).
35. Peñuelas, J. & Terradas, J. The foliar microbiome. *Trends in Plant Science* **19,** 278–280 (2014).
36. Hoffman, M. T., Gunatilaka, M. K., Wijeratne, K., Gunatilaka, L. & Arnold, A. E. Endohyphal Bacterium Enhances Production of Indole-3-Acetic Acid by a Foliar Fungal Endophyte. *PLOS ONE* **8,** e73132 (2013).

37. Rering, C. C., Beck, J. J., Hall, G. W., McCartney, M. M. & Vannette, R. L. Nectar-inhabiting microorganisms influence nectar volatile composition and attractiveness to a generalist pollinator. *New Phytologist* **40,** 6 (2017).
38. Bulgarelli, D. *et al.* Structure and Function of the Bacterial Root Microbiota in Wild and Domesticated Barley. *Cell Host and Microbe* **17,** 392–403 (2015).
39. Saunders, M., Glenn, A. E. & Kohn, L. M. Exploring the evolutionary ecology of fungal endophytes in agricultural systems: using functional traits to reveal mechanisms in community processes. *Evolutionary Applications* **3,** 525–537 (2010).
40. Cregger, M. A. *et al.* The Populus holobiont: dissecting the effects of plant niches and genotype on the microbiome. 1–14 (2018). doi:10.1186/s40168-018-0413-8
41. Phytopathology, G. N.1998. Association of Mmd1, a Major Gene for Resistance to Melampsora medusae f. sp. deltoidae, with Quantitative Traits in Poplar Rust. *Am Phytopath Society* **88,** 114–121 (1998).
42. Levy, M., Blacher, E. & Elinav, E. ScienceDirect Microbiome, metabolites and host immunity. *Current Opinion in Microbiology* **35,** 8–15 (2017).
43. Young, B. D. Smelling phenomenal. 1–9 (2014). doi:10.3389/fpsyg.2014.00713/abstract
44. Gervasi, D. D. L. & Schiestl, F. P. Real-time divergent evolution in plants driven by pollinators. *Nat Comms* **8,** 14691 (2017).
45. Zhu, J., Bean, H. D., Kuo, Y.-M. & Hill, J. E. Fast detection of volatile organic compounds from bacterial cultures by secondary electrospray ionization-mass spectrometry. *J. Clin. Microbiol.* **48,** 4426–4431 (2010).
46. Syed-Ab-Rahman, S. F. *et al.* Soil bacterial diffusible and volatile organic compounds inhibit Phytophthora capsici and promote plant growth. *Science of the Total Environment* **692,** 267–280 (2019).
47. Jalali, F., Zafari, D. & Salari, H. Volatile organic compounds of some Trichoderma spp. increase growth and induce salt tolerance in Arabidopsis thaliana. *Fungal Ecology* **29,** 67–75 (2017).
48. Humphris, S. N., Wheatley, R. E. & Bruce, A. The Effects of Specific Volatile Organic Compounds Produced by Trichoderma Spp. on the Growth of Wood Decay Basidiomycetes. *Holzforschung* **55,** 307
49. Kai, M., Effmert, U., Berg, G. & Piechulla, B. Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen Rhizoctonia solani. *Arch Microbiol* **187,** 351–360 (2006).
50. Hung, R., Lee, S. & Bennett, J. W. Fungal volatile organic compounds and their role in ecosystems. *Appl Microbiol Biotechnol* **99,** 3395–3405 (2015).
51. Dixit, S., Srivastava, M. P. & Sharma, Y. K. in *Handbook of Research on the Adverse Effects of Pesticide Pollution in Aquatic Ecosystems* (eds. Wani, K. A. & Mamta) **1,** 85–104 (IGI Global, 2018).
52. Gasnier, C. *et al.* Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology* **262,** 184–191 (2009).
53. Leach, J. E., Triplett, L. R., Argueso, C. T. & Trivedi, P. Communication in the Phytobiome. *Cell* **169,** 587–596 (2017).
54. Turner, T. R., James, E. K. & Poole, P. S. The plant microbiome. *Genome Biol* **14,** 249–10 (2013).

55. McFadden, G. I. Endosymbiosis and evolution of the plant cell. 1–7 (1999).
56. Martin, F. M., Uroz, S. & Barker, D. G. Ancestral alliances: Plant mutualistic symbioses with fungi and bacteria. *Science* **356,** eaad4501–11 (2017).
57. Provorov, N. A. Symbiogenesis as Evolution of Open Genetic Systems. *Russ J Genet* **54,** 888–896 (2018).
58. Sakamoto, W. & Takami, T. Chloroplast DNA Dynamics: Copy Number, Quality Control and Degradation. *Plant and Cell Physiology* **59,** 1120–1127 (2018).
59. Margulis, L. Symbiosis in cell evolution: Life and its environment on the early earth. (1981).
60. Embley, T. M. & Martin, W. Eukaryotic evolution, changes and challenges. *Nature* **440,** 623–630 (2006).
61. Gilbert, S. F., Sapp, J., of, A. T. T. Q. R.2012. A SYMBIOTIC VIEW OF LIFE: WE HAVE NEVER BEEN INDIVIDUALS. *journals.uchicago.edu* **87,** 325–341 (2012).
62. Kusari, P., Kusari, S., Spiteller, M. & Kayser, O. Endophytic fungi harbored in Cannabis sativa L.: diversity and potential as biocontrol agents against host plant-specific phytopathogens. *Fungal Diversity* **60,** 137–151 (2012).
63. Wang, W., Zhai, Y., Cao, L., Tan, H. & Zhang, R. Endophytic bacterial and fungal microbiota in sprouts, roots and stems of rice (Oryza sativa L.). *Microbiological Research* **188-189,** 1–8 (2016).
64. Schulz, B. J. E., Boyle, C. J. C. & Sieber, T. N. *Microbial Root Endophytes*. (Springer Science & Business Media, 2007).
65. Tian, X. *et al.* Infection of Plasmodiophora brassicae changes the fungal endophyte community of tumourous stem mustard roots as revealed by high-throughput sequencing and culture-dependent methods. *PLOS ONE* **14,** e0214975–15 (2019).
66. Busby, P. E., Aime, M. C. & Newcombe, G. Foliar pathogens of Populus angustifolia are consistent with a hypothesis of Beringian migration into North America. *Fungal Biology* **116,** 792–801 (2012).
67. Marinho, A. M. D. R., Rodrigues-Filho, E., Moitinho, M. D. L. R. & Santos, L. S. Biologically active polyketides produced by Penicillium janthinellum isolated as an endophytic fungus from fruits of Melia azedarach. *Journal of the Brazilian Chemical Society* **16,** 280–283 (2005).
68. Promputtha, I. *et al.* A Phylogenetic Evaluation of Whether Endophytes Become Saprotrophs at Host Senescence. *Microb Ecol* **53,** 579–590 (2007).
69. Khan, A. L. *et al.* Endophytic fungal association via gibberellins and indole acetic acid can improve plant growth under abiotic stress: an example of Paecilomyces formosus LHL10. *BMC Microbiol* **12,** 1–14 (2012).
70. Márquez, L. M., Redman, R. S., Rodriguez, R. J.2007. A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. *science.sciencemag.org* doi:10.1126/science.1137195
71. Chaparro, J. M., Sheflin, A. M., Manter, D. K. & Vivanco, J. M. Manipulating the soil microbiome to increase soil health and plant fertility. *Biol Fertil Soils* **48,** 489–499 (2012).
72. Sugiyama, A. & Yazaki, K. Flavonoids in plant rhizospheres: secretion, fate and their effects on biological communication. *Plant Biotechnology* **31,** 431–443 (2014).

73. Rodriguez, R. J., White, J. F., Jr, Arnold, A. E. & Redman, R. S. Fungal endophytes: diversity and functional roles. *New Phytologist* **182,** 314–330 (2009).
74. Arnold, A. E. Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal Biology Reviews* **21,** 51–66 (2007).
75. Moyes, A. B. *et al.* Evidence for foliar endophytic nitrogen fixation in a widely distributed subalpine conifer. *New Phytologist* **210,** 657–668 (2016).
76. Deanoff. Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India. 1–14 (2005).
77. Hmielowski, T. The fascinating tale of tall fescue. *Crops, Soils, Agronomy News* **61,** 4–9 (2016).
78. Wille, L., Messmer, M. M., Studer, B. & Hohmann, P. Insights to plant-microbe interactions provide opportunities to improve resistance breeding against root diseases in grain legumes. *Plant Cell Environ* (2018). doi:10.1111/pce.13214
79. Chisholm, S. T., Coaker, G., Day, B. & Staskawicz, B. J. Host-Microbe Interactions: Shaping the Evolution of the Plant Immune Response. *Cell* **124,** 803–814 (2006).
80. Wagner, M. R. *et al.* Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. *Nat Comms* **7,** 12151 (2016).
81. Reeve, J. R. *et al.* Effects of soil type and farm management on soil ecological functional genes and microbial activities. *ISME J* **4,** 1099–1107 (2010).
82. Dai, X. *et al.* The willow genome and divergent evolution from poplar after the common genome duplication. *Nature Publishing Group* **24,** 1274–1277 (2014).
83. National Research Council Canada. *Biology of Populus and Its Implications for Management and Conservation*. (NRC Research Press, 1996).
84. Eckenwalder, J. E. Natural intersectional hybridization between North American species of Populus(Salicaceae) in sections Aigeirosand Tacamahaca. II. Taxonomy. *Can. J. Bot.* **62,** 325–335 (1984).
85. Eckenwalder, J. E. Natural intersectional hybridization between North American species of Populus (Salicaceae) in sections Aigeiros and Tacamahaca. I. Population studies of P. × parryi. *Can. J. Bot.* **62,** 317–324 (1984).
86. Porth, I. *et al.* Populus trichocarpa cell wall chemistry and ultrastructure trait variation, genetic control and genetic correlations. *New Phytologist* **197,** 777–790 (2013).
87. Balatinecz, K. Properties and utilization of poplar wood: CHAPTER 9. 1–16 (2002).
88. Beckers, B. *et al.* Lignin engineering in field-grown poplar trees affects the endosphere bacterial microbiome. *Proceedings of the National Academy of Sciences* **113,** 2312–2317 (2016).
89. Moore, F. P. *et al.* Endophytic bacterial diversity in poplar trees growing on a BTEX-contaminated site: The characterisation of isolates with potential to enhance phytoremediation. *Systematic and Applied Microbiology* **29,** 539–556 (2006).
90. Grabau, M. R. *et al.* Direct seeding for riparian tree re-vegetation: Small-scale field study of seeding methods and irrigation techniques. *Ecological Engineering* **37,** 864–872 (2011).
91. Cronk, Q. C. B. Plant eco-devo: the potential of poplar as a model organism. *New Phytologist* **166,** 39–48 (2005).

92. Zimmerman, N. B. & Vitousek, P. M. Fungal endophyte communities reflect environmental structuring across a Hawaiian landscape. *Proceedings of the National Academy of Sciences* **109,** 13022–13027 (2012).
93. Lichtenthaler, H. K. *et al.* Photosynthetic activity, chloroplast ultrastructure, and leaf characteristics of high-light and low-light plants and of sun and shade leaves. *Photosynthesis Research* **2,** 115–141 (1981).
94. Raja, S., Subhashini, P. & Thangaradjou, T. Differential methods of localisation of fungal endophytes in the seagrasses. *Mycology* **7,** 112–123 (2016).
95. Bills, G. F., Sydowia, J. P.1992. *Recovery of endophytic fungi from Chamaecyparis thyoides*. *kbd.kew.org*
96. Arpan. Field Collection Guidelines. 1–56 (2014).
97. Gutzmer, R. Rapid identification and differentiation of fungal DNA in dermatological specimens by LightCycler PCR. *Journal of Medical Microbiology* **53,** 1207–1214 (2004).
98. Peterson, D. G., Boehm, K. S. & Stack, S. M. Isolation of milligram quantities of nuclear DNA from tomato (<Emphasis Type='Italic'>Lycopersicon esculentum</Emphasis>), A plant containing high levels of polyphenolic compounds. *Plant Mol Biol Rep* **15,** 148–153 (1997).
99. Feng, J. *et al.* Barcoding Poplars (Populus L.) from Western China. *PLOS ONE* **8,** e71710–8 (2013).
100. Kearse, M., Moir, R., Wilson, A., Stones-Havas, S.2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *academic.oup.com*
101. Pevsner, J. in *Bioinformatics and Functional Genomics* 87–125 (John Wiley & Sons, Inc., 2005). doi:10.1002/047145916X.ch4
102. Thompson, J. D., Higgins, D. G., research, T. G. N. A.1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix …. *academic.oup.com*
103. Guindon, S. & Gascuel, O. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Systematic Biology* **52,** 696–704 (2003).
104. Ronquist, F. & Huelsenbeck, J. P. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19,** 1572–1574 (2003).
105. Inc, R. S. R., Boston, Massachusetts2012. *RStudio: integrated development environment for R*.
106. Dixon, P. VEGAN, a package of R functions for community ecology. *Journal of Vegetation Science* **14,** 927–930 (2003).
107. Critchfield, W. B. LEAF DIMORPHISM IN POPULUS TRICHOCARPA. *American Journal of Botany* **47,** 699–711 (1960).
108. Quaedvlieg, W. *Re-evaluating Mycosphaerella and allied genera*. (Utrecht University, 2014).
109. Crous, P. W., Braun, U. & Groenewald, J. Z. Mycosphaerella is polyphyletic. *Studies in Mycology* **58,** 1–32 (2007).

110. Goodwin, S. B. *et al.* Finished Genome of the Fungal Wheat Pathogen Mycosphaerella graminicola Reveals Dispensome Structure, Chromosome Plasticity, and Stealth Pathogenesis. *PLoS Genet* **7,** e1002070–17 (2011).
111. Moreno, E. *et al.* Chemical Constituents of the New Endophytic Fungus Mycosphaerellasp. nov. and Their Anti-parasitic Activity. *Natural Product Communications* **6,** 1934578X1100600–6 (2011).
112. Kirk, P. M., Cannon, P. F. & David, J. C. *Dictionary of the Fungi*. (2001).
113. Papagianni, M., Ambrosiadis, I. & Filiousis, G. Mould growth on traditional greek sausages and penicillin production by Penicillium isolates. *Meat Science* **76,** 653–657 (2007).
114. Waqas, M., Hussain, J., Al-Harrasi, A., Hamayun, M. & Lee, I.-J. Phytohormones enabled endophytic fungal symbiosis improve aluminum phytoextraction in tolerant Solanum lycopersicum: An examples of Penicillium janthinellum LK5 and comparison with exogenous GA3. *Journal of Hazardous Materials* **295,** 70–78 (2015).
115. Khan, S. *et al.* Plant growth promotion and Penicillium citrinum. *BMC Microbiol* **8,** 231–10 (2008).
116. Bezerra, J. D. P. *et al.* Endophytic fungi from medicinal plant Bauhinia forficata: Diversity and biotechnological potential. *Braz. J. Microbiol.* **46,** 49–57 (2015).
117. Zhu, S., Cao, Y. Z., Jiang, C., BMC, B. T.2012. Sequencing the genome of Marssonina brunnea reveals fungus-poplar co-evolution. *bmcgenomics.biomedcentral.com* doi:10.1186/1471-2164-13-382
118. Busby, P. E. *et al.* Leaf endophytes and Populus genotype affect severity of damage from the necrotrophic leaf pathogen, Drepanopeziza populi. *Ecosphere* **4,** 1–12 (2013).
119. Xing, M. *et al.* Antifungal Activity of Natural Volatile Organic Compounds against Litchi Downy Blight Pathogen Peronophythora litchii. *Molecules* **23,** 358–15 (2018).
120. Bensch, K. *et al.* Cladosporium species in indoor environments. *Studies in Mycology* **89,** 177–301 (2018).
121. Bensch, K., Braun, U., Groenewald, J. Z. & Crous, P. W. The genus Cladosporium. *Studies in Mycology* **72,** 1–401 (2012).
122. Tsuneda, A., Pathology, Y. H. C. J. O. P.1979. *Mode of parasitism of a mycoparasite, Cladosporium gallicola, on western gall rust, Endocronartium harknessii [pines]*. *agris.fao.org*
123. Mounir, R. *et al.* Production, formulation and antagonistic activity of the biocontrol like-yeast Aureobasidium pullulans against Penicillium expansum. *Biotechnol Lett* **29,** 553–559 (2007).
124. Mattheis, J. P. & Roberts, R. G. Identification of Geosmin as a Volatile Metabolite of Penicillium expansum
. *Applied and Environmental Microbiology* **58,** 3170–3172 (1992).
125. Frisvad, J. C., Houbraken, J., Popma, S. & Samson, R. A. Two new Penicillium species Penicillium buchwaldiiand Penicillium spathulatum, producing the anticancer compound asperphenamate. *FEMS Microbiol Lett* **339,** 77–92 (2012).

126. Kar, P. K., Murmu, S., Saha, S., Tandon, V. & Acharya, K. Anthelmintic Efficacy of Gold Nanoparticles Derived from a Phytopathogenic Fungus, Nigrospora oryzae. *PLOS ONE* **9,** e84693–9 (2014).
127. Selvakumar, P. & Sivashanmugam, P. Ultrasound assisted oleaginous yeast lipid extraction and garbage lipase catalyzed transesterification for enhanced biodiesel production. *Energy Conversion and Management* **179,** 141–151 (2019).
128. Yousefzadeh, H., Colagar, A. H., Yousefi, E., Badbar, M. & Kozlowski, G. Phylogenetic relationship and genetic differentiation of Populus caspica and Populus alba using cpDNA and ITS noncoding sequences. *Journal of Forestry Research* **30,** 451–461 (2018).
129. Bennett, J. W., Hung, R., Lee, S. & Padhi, S. in *Fungal Associations* 373–393 (Springer Berlin Heidelberg, 2012). doi:10.1007/978-3-642-30826-0_18
130. Goldstein, A. H. & Galbally, I. E. Known and Unexplored Organic Constituents in the Earth's Atmosphere. *Environ. Sci. Technol.* **41,** 1514–1521 (2007).
131. Kesselmeier, J. & Staudt, M. Biogenic Volatile Organic Compounds (VOC): An Overview on Emission, Physiology and Ecology. *Journal of Atmospheric Chemistry* **33,** 23–88 (1999).
132. Strobel, G. Harnessing endophytes for industrial microbiology. *Current Opinion in Microbiology* **9,** 240–244 (2006).
133. Fiedler, K., Schütz, E. & Geh, S. Detection of microbial volatile organic compounds (MVOCs) produced by moulds on various materials. *International Journal of Hygiene and Environmental Health* **204,** 111–121 (2001).
134. Kim, K.-S., Lee, S. & Ryu, C.-M. Interspecific bacterial sensing through airborne signals modulates locomotion and drug resistance. *Nat Comms* **4,** 1809–12 (1AD).
135. Preti, G., Muetterties, E. L., Furman, J. M., Kennelly, J. J. & Johns, B. E. Volatile constituents of dog (Canis familiaris) and coyote (Canis latrans) anal sacs. *J Chem Ecol* **2,** 177–186 (1976).
136. Thoß, M. *et al.* Author Correction: Regulation of volatile and non-volatile pheromone attractants depends upon male social status. *Sci Rep* **9,** 6185–14 (2019).
137. Wallace, S. & Balskus, E. P. Interfacing Microbial Styrene Production with a Biocompatible Cyclopropanation Reaction. *Angew. Chem.* **127,** 7212–7215 (2015).
138. Morath, S. U., Hung, R. & Bennett, J. W. Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. *Fungal Biology Reviews* **26,** 73–83 (2012).
139. Alpha, C. J., Campos, M., Jacobs-Wagner, C. & Strobel, S. A. Mycofumigation by the Volatile Organic Compound-Producing Fungus Muscodor albus Induces Bacterial Cell Death through DNA Damage. *Applied and Environmental Microbiology* **81,** 1147–1156 (2015).
140. Sawoszczuk, T., Syguła-Cholewińska, J. & del Hoyo-Meléndez, J. M. Optimization of headspace solid phase microextraction for the analysis of microbial volatile organic compounds emitted by fungi: Application to historical objects. *Journal of Chromatography A* **1409,** 30–45 (2015).
141. Domingo-Almenara, X., Brezmes, J., Analytical, M. V.2016. eRah: a computational tool integrating spectral deconvolution and alignment with quantification and identification of metabolites in GC/MS-based metabolomics. *ACS Publications* **88,** 9821–9829 (2016).

142. Yu, M. *et al.* Evaluation and reduction of the analytical uncertainties in GC-MS analysis using a boundary regression model. *Elsevier* **164,** 141–147 (2017).
143. Pierce, D., ncdf4, U. H. C. R.-P. O. P.2012. *ncdf4: Interface to Unidata netCDF (version 4 or earlier) format data files*.
144. Johnson, S. G. *NIST Standard Reference Database 1A v17*. (2014).
145. Zhu, Y.-J. *et al.* Antityrosinase and antimicrobial activities of 2-phenylethanol, 2-phenylacetaldehyde and 2-phenylacetic acid. *Food Chemistry* **124,** 298–302 (2011).
146. Silver, S. & Wendt, L. Mechanism of action of phenethyl alcohol: breakdown of the cellular permeability barrier. *Journal of Bacteriology* **93,** 560–566 (1967).
147. Pagot, Y., Belin, J.-M., Husson, F. & Spinnler, H.-E. Metabolism of phenylalanine and biosynthesis of styrene in Penicillium camemberti. *Journal of Dairy Research* **74,** 180–185 (2007).
148. Azeem, M., Borg-Karlson, A. K. & Rajarao, G. K. Sustainable bio-production of styrene from forest waste. *Bioresource Technology* **144,** 684–688 (2013).
149. Demyttenaere, J., Moriña, R. M., A, P. S. J. O. C.2003. Monitoring and fast detection of mycotoxin-producing fungi based on headspace solid-phase microextraction and headspace sorptive extraction of the volatile …. *Elsevier* **985,** 127–135 (2003).
150. Bennett, J. W. & Klich, M. Mycotoxins. *Clinical Microbiology Reviews* **16,** 497–516 (2003).
151. Casadevall, A. & Pirofski, L.-A. The weapon potential of human pathogenic fungi. *Med Mycol* **44,** 689–696 (2006).
152. Dubey, M. K. *et al.* PR Toxin – Biosynthesis, Genetic Regulation, Toxicological Potential, Prevention and Control Measures: Overview and Challenges. *Front. Pharmacol.* **9,** 263–19 (2018).