Diversity and Effects of the Fungal Endophytes of the Liverwort *Marchantia polymorpha*

by

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Paul S. Manos

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
Biology in the Graduate School
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ABSTRACT

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Abstract

Fungal endophytes are ubiquitous inhabitants of plants and can have a wide range of effects on their hosts, from pathogenic to mutualistic. These fungal associates are important drivers of plant success and therefore contribute to plant community structure. The majority of endophyte studies have focused on seed plants, but in order to understand the dynamics of endophytes at the ecosystem scale, as well as the evolution of these fungal associations, investigations are also necessary in earlier-diverging clades of plants, such as the non-vascular bryophytes (mosses, liverworts, and hornworts). This dissertation presents a survey of the diversity of fungal endophytes found in the liverwort *Marchantia polymorpha* L. and develops a gnotobiotic experimental system for testing the effects of these fungi on their liverwort host. The survey reveals a diverse community of fungi in *M. polymorpha,* with some fungi that are associated with this host across geographically distant sites. The laboratory experiments demonstrate that culturable endophytes of *M. polymorpha* can, in isolation, cause positive, negative, or neutral effects on host success and that these effects change in response to nutrient levels and the presence of multiple endophytes. The experimental system developed in this dissertation has great potential in the growing field of plant microbiota research to answer questions that range in scale from molecular mechanisms to ecosystem function.

Dedication

I dedicate this dissertation to my fiancé, Duncan Hauser, who has been my amazingly patient and supportive partner for the past six and a half years. Duncan encouraged my developing interest in bryophytes when we were in college together and has been encouraging me in my pursuit of science ever since. When we graduated college, he was crazy enough to pick up and move across the country with me. Over the five years of my PhD work, he has assisted me in so many ways: in the lab, in the field, as editor, scientific sounding board, and moral support. He has kept me from giving up and I could not have accomplished this dissertation without him.

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Northwest looking for the ever-elusive “common” liverwort. Also thanks to Tom Streeter for hosting us during our field work in Vermont and giving us a precious two days of vacation in the midst of frenetic graduate school work.

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since she helped in the lab the longest and her senior thesis project is the topic of the last chapter of this dissertation.

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1 Introduction

The concept of symbiosis has been tied to intimate microbial relationships since the first uses of the term in the 1870s to describe the association of algae and fungi in a lichen (Sapp, 1994). Although the term has been used to refer to mutualisms in particular, it more literally refers to organisms living in close association and does not specify the impacts of the partners on each other (Margulis, 1990; Saffo, 1992; Sapp, 1994). In the time since the introduction of the term, it has become clear that symbiotic associations are not an intriguing but relatively rare curiosity, but rather underpin the ecology of most life and indeed the very origin of major eukaryote organelles. The endosymbioses that led to mitochondria and chloroplasts were suspected at the beginning of the 20th century, but the idea fell out of favor until its resurgence in the 1970s, bolstered by the work of Lynn Margulis (Mereschkowsky, 1905; Margulis, 1990; Martin & Kowallik, 1999; McFall-Ngai, 2008). Now, with advances in molecular technologies, the field of microbial symbiosis is burgeoning and we continue to discover that the reductionist ideas of macro-organisms as individuals occasionally interacting with a single pathogen or mutualist that have historically prevailed are a poor reflection of the true complexity present (McFall-Ngai, 2008; Relman, 2008; Vandenkoornhuyse *et al.*, 2015). None of the macroscopic eukaryotes (and few, if any, microscopic ones, for that matter) exist in isolation; instead they carry complex microbial communities that

augment their physiology, metabolism, and immunity (McFall-Ngai, 2008; Vandenkoornhuyse *et al.*, 2015; Shi & Mu, 2017). To fit this new understanding, the idea of the 'holobiont,' a host with all its associated microbes, has been developed (Gordon *et al.*, 2013). Our understanding of organismal function cannot be complete without considering holobionts rather than individual host-symbiont interactions. So far, the most progress on this front has been made in research on the human microbiome.

Progress in describing the dynamics of the human gut microbiome has revolutionized our understanding of our own health and physiology (Thomas *et al.*, 2017). Following behind the human research is a similar development of our understanding of plant microbiomes. The success of crops and other plants has been revealed to be much more complex than interactions with abiotic soil conditions and individual pests. Just as for mammalian gut microbiota, plant microbiota appear to be key to host physiology, nutrition, and immunity (Porras-Alfaro & Bayman, 2011; Vandenkoornhuyse *et al.*, 2015; Mitter *et al.*, 2016). In the "omics" age, we now have the tools to investigate plants as holobionts and ecosystems in themselves so the new field of plant microbiota is developing quickly, but challenges in detecting the signal through the noise in these complex systems remain (Hacquard & Schadt, 2015; Vandenkoornhuyse *et al.*, 2015).

For obvious reasons, much of the research on plant microbial communities and symbioses to date has focused on crop plants, forest trees, and model angiosperms. But there is increasing evidence that microbiomes are important in determining plant community structure and ecosystem properties, and are therefore key to understanding natural ecosystem ecology as well (Rudgers *et al.*, 2004; Afkhami & Strauss, 2016; Aguilar-Trigueros & Rillig, 2016). In order to understand the roles that plant microbiomes play in organizing ecosystems, it is necessary to expand research to include a wider diversity of host plants.

The non-vascular bryophytes (mosses, liverworts, and hornworts) are arguably one of the groups most distinct in physiology and ecology from the commonly studied angiosperms and gymnosperms and their microbial communities have not been widely investigated. The relationships between these three bryophyte groups are still debated (Cox *et al.*, 2014; Wickett *et al.*, 2014), but they are united by lacking true xylem and phloem and having gametophyte-dominant life cycles with maternally dependent, unbranched sporophytes (Ligrone *et al.*, 2000; Shaw *et al.*, 2011). They all produce spores by meiosis and are more tied to water than seed plants because they retain swimming sperm and are poikilohydric (Paolillo, 1981; Raven, 2002). The need for water films for fertilization and the lack of supportive conducting tissues have provided pressure for bryophytes to stay small in stature and, unfortunately, their size often leads to them

being overlooked (Paolillo, 1981). They have been called "lower plants" and considered primitive relicts of early land plant forms, but they are in fact significant members of most modern terrestrial and aquatic biomes and are even the dominant vegetation in certain boreal ecosystems (Lindo & Gonzalez, 2010). Bryophytes contribute to regulating the movements of water, heat, and nutrients through ecosystems, and provide habitat for a diverse community of small arthropods as well as eukaryotic and prokaryotic microbes (Lindo & Gonzalez, 2010). The differences in structure and physiology of bryophytes from vascular plants, such as their lack of roots and minimal cuticles (Budke *et al.*, 2011; Jones & Dolan, 2012), likely present different microbial niches and their interactions with microbiota may be quite different from those in vascular plants. Any commonalities identified would also be key information, demonstrating conservation of microbial interaction across the plant tree of life.

There are currently few bryophyte model systems available, the most prominent being the moss *Physcomitrella patens.* However, mosses appear to be unusual in their microbial interactions. Whereas genes that control mycorrhizal symbioses in the roots of vascular plants are functionally conserved in liverworts and hornworts, they have diverged in mosses (Wang *et al.*, 2010) and the mosses appear to lack obvious mycorrhiza-like associations (Pressel *et al.*, 2010). Research on microbes associated with mosses has instead focused mostly on parasites and pathogens (Döbbeler, 1997; Davey

& Currah, 2006; Akita *et al.*, 2011). Most of the existing work on bryophyte microbial interactions focuses instead on liverworts since they host a variety of fungal symbioses similar to mycorrhizae.

Use of the liverwort *Marchantia polymorpha* as a molecular model system is currently developing and its genome is now sequenced (Bowman *et al.*, 2016). This liverwort has a broad distribution spanning both hemispheres (Bischler-Causse, 1993). This species appears to have three genetic groups within it and, based on morphology and isozyme genetic assessment, these have been designated as *M. polymorpha subspp. polymorpha, ruderalis,* and *montivagans* (Bischler-Causse & Boisselier-Dubayle, 1991; Boisselier-Dubayle *et al.*, 1995). These three subspecies are reported to have different ecologies: subspecies *ruderalis* tends to grow in disturbed habitats, especially ones impacted by humans, including greenhouses where it can become a horticultural pest; subspecies *montivagans* also inhabits disturbed areas, but more often ones influenced by natural phenomena like fires (Graff, 1936; Bradbury, 2006); and subspecies *polymorpha* tends to be found in streams and other wet natural areas (Bischler-Causse, 1993). The subspecies used for the recent genome sequence and molecular work is *ruderalis,* but resequencing to investigate the diversity of the broader species is underway. The broad range and variable ecology of *M. polymorpha* make it an ideal candidate for studies on how microbiome assembly depends on geography, disturbance, and other ecological

factors. The development of molecular tools for genetic manipulation and the ease of growing *M. polymorpha* axenically in the lab also make it a promising model for experimental investigation of microbial interactions (Bowman *et al.*, 2016).

The microbiome of *M. polymorpha* is only just beginning to be investigated. Alcaraz et al. (2017) have recently presented an assessment of the bacterial community in *M. polymorpha* and its sister species *Marchantia paleacea.* Previous work on the fungi inhabiting the relatives of *M. polymorpha*, the members of the complex thalloid liverwort clade (Marchantiopsida), has mostly been limited to individual mycorrhiza-like symbioses though one study has investigated the metagenome of *Conocephalum conicum* (Knack *et al.*, 2015). Nutrient sharing symbioses have been demonstrated in *M. paleacea* and *Neohodgsonia mirabilis* with Mucoromyota fungi, including the arbuscular mycorrhizal Glomeromycotina (Humphreys *et al.*, 2010; Field *et al.*, 2012; Field *et al.*, 2016). Much of the mycorrhizal symbiosis work on liverworts has not focused on *M. polymorpha* itself since it is not often found to be colonized by arbuscular mycorrhizal fungi (Ligrone *et al.*, 2007; Pressel *et al.*, 2010). Differences in fungal colonization between the three subspecies of *M. polymorpha* have been reported, with Glomeromycotina associations only detected in *subsp. montivagans* (Ligrone *et al.*, 2007). As is clear from developments in the study of plant microbiomes, the communities in liverworts contain many more fungi than the morphologically obvious mutualists. The taxonomic and

functional diversity of the fungal microbiome inhabiting *M. polymorpha* has not been previously assessed.

In this dissertation, I present a series of studies that provide a foundation for using *M. polymorpha* as a model for investigations of fungal endophyte diversity and function. In Chapter 1, I survey the diversity of fungal endophytes found in *M. polymorpha* in sites in the United States and Canada by culturing and culture-independent methods. In Chapter 2, I present methodology for assaying the effects of individual cultured endophytes on *M. polymorpha* growth and assess the growth effects for 100 isolated fungi. In Chapter 3, I use the experimental system presented in Chapter 2 to test whether the growth promoting effects of two fungi are dependent on the amount of nutrients present. In Chapter 4, I use this experimental system to test how inoculation of *M. polymorpha* with multiple endophytes changes the outcome of the interactions. I conclude with a discussion of future directions for the experimental system I have developed, including comments on its potential in undergraduate research participation.

2 Chapter 1: Fungal endophyte diversity in the liverwort *Marchantia polymorpha* L. based on culturing and culture-independent amplicon sequencing

2.1 Introduction

Plant symbioses with fungi are ancient and likely played an important role in the evolution of plants (Heckman *et al.*, 2001; Taylor & Krings, 2005; Delaux *et al.*, 2012). Fungi found inside healthy plant tissues are termed fungal endophytes and form diverse communities in virtually every plant examined across the land plant phylogeny (Stone *et al.*, 2000; Rodriguez *et al.*, 2009) and additionally in some chlorophyte and charophyte algae (Cui *et al.*, 2010; Knack *et al.*, 2015). Many definitions of the term endophyte have been used in the literature, but we choose to use the broad sense that includes any microbe that can be found in healthy plant tissue (Stone *et al.*, 2000). This definition for fungal endophytes includes mycorrhizal fungi, latent pathogens and saprobes, and a diverse array of additional fungi with functions across the spectrum from mutualist to parasite (Schulz & Boyle, 2005; Porras-Alfaro & Bayman, 2011). Endophytes have a wide range of effects on host success including changes in growth, interactions with other species, and resistance to pathogens or stressors (Porras-Alfaro & Bayman, 2011). The many effects of fungal symbionts make them important, inconspicuous drivers of plant

community structure and therefore also broader ecosystem structure (Rudgers *et al.*, 2004; Afkhami & Strauss, 2016; Aguilar-Trigueros & Rillig, 2016).

Endophyte communities have been surveyed across a wide diversity of host plants and habitats, but investigations of endophytes in the non-vascular bryophytes have been limited. The bryophytes consist of three clades of debated relationship to each other: mosses (Bryophyta), liverworts (Marchantiophyta), and hornworts (Anthocerotophyta) (Cox *et al.*, 2014; Wickett *et al.*, 2014). The approximately 20,000 species in these three groups are united by having gametophyte dominant life cycles with maternally dependent unbranched sporophytes (Shaw *et al.*, 2011). Bryophytes are ubiquitous members of terrestrial and aquatic biomes and comprise a significant percentage of the total biomass in some ecosystems, especially in the boreal zone (Lindo & Gonzalez, 2010). Bryophytes are important in regulating temperature, moisture, and nutrient movement in ecosystems and provide habitat for microbes and small arthropods, some endemic to this habitat (Lindo & Gonzalez, 2010).

Because of their early-diverging position in the embryophyte clade, bryophytes are also important in the study of land plant evolution, including the role of fungal symbioses. Associations with fungi are thought to have been an important factor in allowing plants to colonize land (Heckman *et al.*, 2001; Taylor & Krings, 2005; Delaux *et al.*, 2012). Parts of the cellular machinery for these interactions appear to have originated

before the origin of embryophytes (Delaux *et al.*, 2015) and the functions of some of these components have been highly conserved from bryophytes to angiosperms (Wang *et al.*, 2010). Functionality of some common symbiosis genes and the ability to form mycorrhiza-like associations are conserved in liverworts and hornworts, but not in mosses (Pressel *et al.*, 2010; Wang *et al.*, 2010).

Liverworts include both leafy and thalloid morphologies (Shaw *et al.*, 2011), and these correspond to divergent clades that also appear to have different types of mycorrhiza-like symbioses (Kottke & Nebel, 2005). The Haplomitriopsida, which include leafy and thalloid forms, are sister to all other liverworts and, after their divergence, the complex thalloids (Marchantiopsida) are sister to the remaining clades which include the simple thalloids (Pelliidae and Metzgeriidae) and the speciose crown group of leafy liverworts (Jungermanniidae) (Shaw *et al.*, 2011). The Haplomitriopsida and complex thalloids engage in mutualistic nutrient-transfer interactions with Glomeromycotina and Mucoromycotina fungi (Kottke & Nebel, 2005; Humphreys *et al.*, 2010; Field *et al.*, 2012; Field *et al.*, 2015), some simple thalloids can associate with Glomeromycotina and Basidiomycete symbionts (Kottke & Nebel, 2005), and a range of Ascomycete and Basidiomycete endophytes are detectable by microscopy in leafy liverworts (Duckett *et al.*, 1991; Duckett & Read, 1995; Duckett *et al.*, 2006). In contrast to work on specific mycorrhiza-like associations, only a few studies have attempted to

characterize the diversity of the whole fungal communities found in liverworts (Davis *et al.*, 2003; Davis & Shaw, 2008; Knack *et al.*, 2015).

In this study, we sampled fungal endophytes from the liverwort *Marchantia polymorpha* L. This liverwort belongs to the complex thalloid clade and is widely distributed in naturally and anthropogenically disturbed habitats. It has been used as a model for well over a century, but is now also emerging as a molecular model system with established protocols for transformation and a recently published genome sequence (Bowman *et al.*, 2016). We survey the endophytes of *M. polymorpha* with both culture collections and culture-independent methods, because previous work has indicated that microbial communities found with these two types of methods differ markedly, with many taxa recovered from only one of the two (Arnold, 2007; Siddique *et al.*, 2017). The goals of this study were (1) to characterize the fungal endophyte communities of wild populations of *M. polymorpha*, (2) to compare the fungi in *M. polymorpha* to those in previously studied bryophyte and vascular plant hosts, (3) to directly compare the results of culture dependent and independent methods, and (4) to determine how fungal endophyte communities vary with geographic location for this liverwort host.

2.2 Materials and Methods

2.2.1 Collection locations

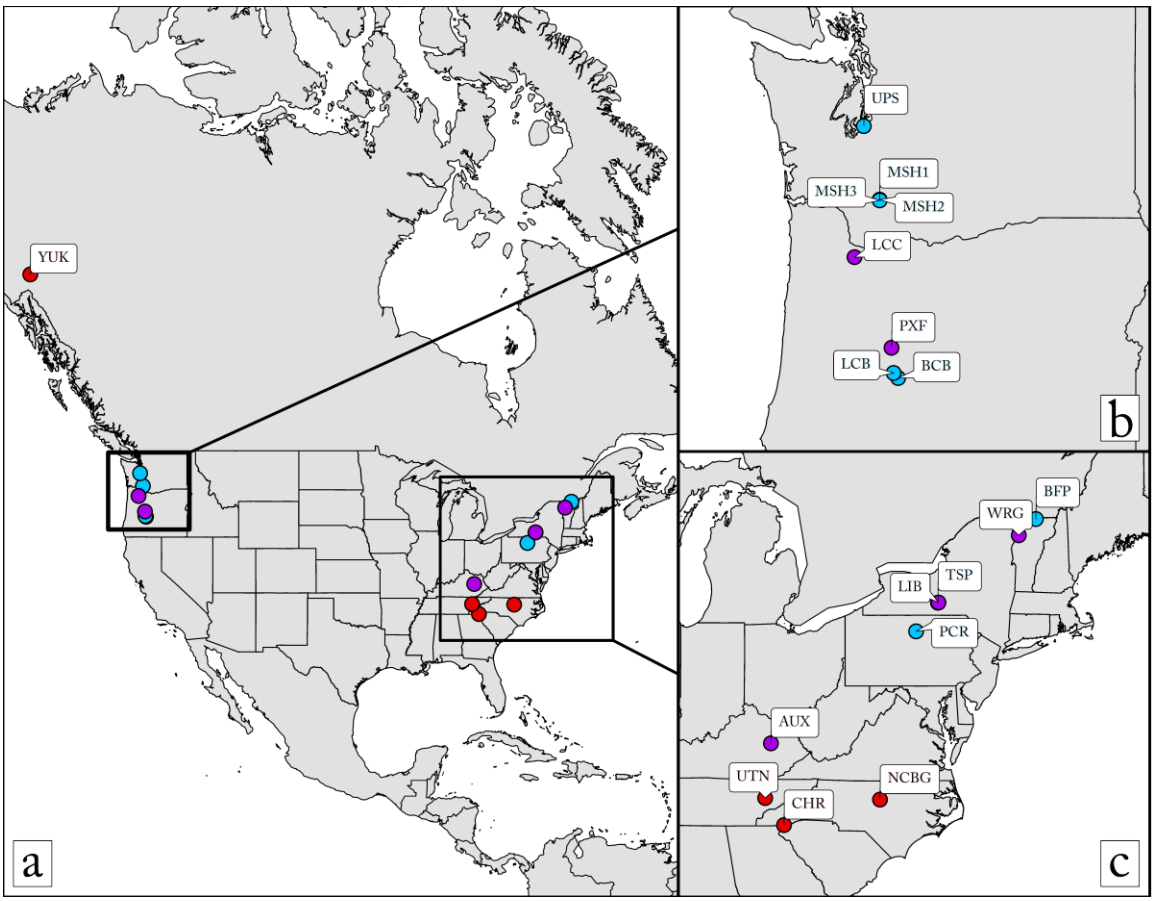

Figure 1: Map of sampling locations.
Points in red only had culture sampling, blue points only had culture-independent amplicon sampling, and purple points had both kinds of sampling. Detailed locality information for each of the sites is provided in Appendix B, Table 5.

Twenty-four patches of *M. polymorpha* were collected from 16 sites in eight US states and one Canadian territory (Fig. 1). The plants were collected from October 2013 to August 2015. Each collection locality was given a unique three letter code and

multiple patches collected at the same locality are distinguished by numbers added to the codes (Fig. 1). Collection details are shown in Appendix B, Table 5. All plant voucher specimens were deposited into the L.E. Anderson Bryophyte Herbarium at Duke University (DUKE).

2.2.2 Host sampling and endophyte isolation

Patches of *M. polymorpha* plants were collected together with adhering substrate and stored in plastic bags. Samples were refrigerated until being cleaned and processed, no longer than two days. Plants were washed in sterile deionized water then surface sterilized. Plants used for culture-independent sampling were sterilized whole, while plants used for culturing were first dissected into pieces approximately 2 mm^2 in area. Surface sterilization was based on the methods of Arnold (2002). Liverwort pieces were surface sterilized by being submerged in 95% ethanol for 30 seconds, 10% bleach (Sodium hypochlorite) for two minutes, and 70% ethanol for two minutes, then air dried on a Kimwipe treated with 70% ethanol in a sterile air environment. This and similar protocols have previously been demonstrated to effectively remove epiphytic microbes (Schulz *et al.*, 1993; Arnold, 2002).

For culture-independent sampling, surface sterilized plants were transferred to sterile 2 mL tubes and stored at -80° C. Substrate samples were taken from patches collected in the summer of 2015: soil or decomposing plant matter adhering to each

liverwort patch was removed and stored in a sterile 15 mL tube at -80° C. During storage, the freezer failed without an alarm, leaving samples to thaw for an unknown number of days, but no more than seven. Samples remained in sealed tubes and were transferred back to -80º as soon as the failure was discovered.

For culture-dependent sampling, pieces were cut from liverwort thalli, rhizoids (unicellular holdfasts), and gametangiophores (gametophytic structures that elevate the antheridia in male plants and the archegonia and later sporophytes in female plants). Surface sterilized 2 mm^2 liverwort tissue pieces were placed on slants of 1.5% malt extract agar (MEA) and left at room temperature for up to one year. Once hyphal growth was visible on a slant, the culture was transferred to a 1.5% MEA plate and allowed to grow. Plate cultures were sub-cultured to separate distinct morphologies. In addition, a few cultures were initiated from fungal fruiting bodies found on *M. polymorpha* plants. Fruiting bodies were cleaned in 70% ethanol and cultured on 1.5% MEA. All cultures were vouchered live in sterile double distilled water stored at room temperature.

2.2.3 Culture identification

A small amount of hyphal tissue was sampled from each pure culture plate into a 1.2 mL tube for DNA extraction. The tissue was disrupted with two 3.5 mm stainless steel balls by submerging tubes in liquid nitrogen, then shaking them at 500 strokes per

minute for one to two minutes on a Geno/Grinder 2000 (SPEX SamplePrep). The resulting pulverized tissue was suspended in 300 μL of 2% CTAB solution containing either 0.2% or 1% β-mercaptoethanol. The tubes were then incubated at 65 °C for at least 30 minutes and an equal volume of chloroform:IAA (24:1) was added and mixed to form an emulsion. The phases were separated by centrifugation for five minutes at 5750 rcf and the aqueous phase removed to a fresh tube. An equal volume of cold isopropanol was added and DNA was precipitated at -20°C. DNA was pelleted by centrifugation for 20 minutes at 5750 rcf. Pellets were washed once with 70% ethanol, allowed to dry overnight, and resuspended in 50 μL sterile molecular biology grade water.

DNA extracts were diluted to 10% with sterile molecular biology grade water. PCR reactions were then run on the diluted extracts using the ITS1f (Gardes & Bruns, 1993) and LR3 (Vilgalys & Hester, 1990) primers. Each 25 uL PCR reaction contained 1 uL template; 2.5 uL each of 10 mg/mL bovine serum albumin (BSA), 10x PCR buffer with 15 mM $MgCl_2$ (Denville), and dNTPs (2 mM of each); 1.25 uL of each primer; and 0.15 uL *Taq* DNA polymerase (Denville, Choice Blue). PCR conditions were as follows: three minutes at 94°C; 35 cycles of 30 seconds at 94°, 30 seconds at 55°, and two minutes at 72°; and a final 10 minutes at 72°.

PCR products were checked for amplification by running on a 1% agarose gel containing SYBR Safe gel stain (Invitrogen). The products that showed clear bands were

cleaned with an ExoSAP protocol by adding 0.2 uL each of Exonuclease I and Antarctic Phosphatase (New England BioLabs) with 2.6 uL PCR water and incubating at 37° for 30 minutes, then 80° for 20 minutes.

Cleaned PCR products were then prepared for Sanger sequencing in 10 uL reactions containing 1 uL cleaned PCR product, 1 uL sequencing buffer, 1 uL 2 μM primer, and 0.5 μL BigDye version 1.1. The BigDye reactions used the following protocol: 2 minutes at 96°, then 25 cycles of 30 seconds at 96°, 15 seconds at 50°, and 4 minutes at 60°. Samples were sequenced using a capillary Sanger sequencing machine at the Duke University Department of Immunology DNA Analysis Facility. Sanger sequences were cleaned and forward and reverse sequences joined in MEGA (Kumar *et al.*, 2008).

Fungal isolates were identified by comparing to the NCBI GenBank database with BLAST searches. A species name was assigned if the sequence matched one and only one species with at least 97% identity and full coverage. When an isolate could not be identified to species, it was labeled with a unique species number. When multiple isolates without species identifications were at least 97% similar, they were considered the same species. In one case (*Coniochaeta* sp. 2), multiple isolates formed a grade such that all samples were 97% similar to some but not all isolates in the group, with no

independent groupings that could be split out. These samples were therefore assigned to one species number.

2.2.4 DNA extraction from *M. polymorpha*

Frozen whole liverwort thalli were ground in 1.2 mL tubes using two 3.5 mm stainless steel balls and a mix of 2mm and 0.7mm zirconia beads on a Geno/Grinder. Then 500 μL of 2% CTAB buffer with 2% β-mercaptoethanol was added to each sample. Thereafter, the extraction followed the same protocol as described above for fungal culture extractions.

2.2.5 DNA extraction from substrates

DNA was extracted from substrate samples for 15 of the *M. polymorpha* patches using a protocol modified from the "Method 2" extraction of Carrigg et al. (2007). We made slight changes in the grinding step and added a final cleaning step at the end of the protocol. For grinding our samples, we used 300 mg of a mixture of 2 mm and 0.7 mm zirconia beads and 0.1 mm silica beads for each tube and processed them with a Geno/Grinder at 500 strokes per minute for two minutes. Tubes were centrifuged to reduce the foam that formed during shaking. DNA pellets after the extraction were not

completely clean and some were brown in color. Therefore, resuspended extracts were centrifuged to pellet debris and the cleaner supernatant retained.

2.2.6 Amplicon sequencing

Extracts from whole *M. polymorpha* plants and substrates were used in PCR targeting the fungal ITS region (ITS1, 5.8S, ITS2) using the primers ITS1f (Gardes & Bruns, 1993) and ITS4 (Innis *et al.*, 2012) and part of the large ribosomal large subunit using primers LROR (Bunyard *et al.*, 1994) and LR3 (Vilgalys & Hester, 1990). DNA extract concentrations were determined using a Qubit 2.0 fluorimeter (Thermo Fisher) with the High Sensitivity DNA assay. All extracts with a concentration higher than 8 ng/μL were diluted to 5 ng/μL. Extracts were tested for amplification success with PCRs using the ITS1f-ITS4 and LROR-LR3 primer pairs. These test PCRs were used to determine which samples would have libraries prepared for Illumina sequencing. All samples passing the test PCR had libraries prepared in duplicate.

Two mock community positive controls were created to run with the samples for assessing quality and biases in the library preparation and sequencing process (Nguyen *et al.*, 2015). Both mock communities included DNA from a set of 19 taxonomically diverse endophytes isolated from *M. polymorpha* (Appendix B, Table 6). For the first

mock community, equal ng amounts of raw fungal DNA extract were used and for the second community, equal ng amounts of an amplicon from ITS1f to LR3 were used.

Libraries of amplicons were prepared from liverwort samples, negative controls (PCR water instead of DNA sample as starting material), and mock community controls in three separate PCR phases using frame-shifted primers, based on the methods of Lundberg et al. (2013). In the first phase, all samples were amplified using standard primer pairs for 15 cycles to enrich for the target region. In the second stage, Illumina sequencing adaptors were added in 10 cycles of PCR using 2 μL of enriched sample. Adaptor primers in this step were mixes of six frameshifted primers to increase sequence diversity for Illumina sequencing. In the third stage, unique barcodes and Illumina adaptors were added to each sample in a final 10 cycle PCR. This final barcode step was set up in a sterile flow hood to prevent cross-contamination. PCR recipes and programs are provided in Appendix A.

After library preparation, concentrations of all samples were determined with a Qubit fluorimeter. Samples were used if they had at least 5 ng/μL concentration. This resulted in 99 usable amplicon samples from the original 140 plants and 15 substrate samples, of which all samples had at least one replicate for LSU and 77 samples had at least one for ITS. Four to 10 plants were represented from each collected site. Two replicates of each of the two mock communities and two replicates of a negative control

were also included in the library. Amounts of DNA from all samples were normalized and pooled to 50 ng per sample based on Qubit readings and the pooled amplicons were cleaned using Agencourt AmpPure XP beads at a 1.2:1 ratio of bead solution to sample to remove sequences shorter than the target amplicons. The prepared library was sequenced on an Illumina MiSeq at the Duke Center for Genomic and Computational Biology Sequencing and Genomic Technologies Shared Resource.

2.2.7 Amplicon data analysis

Reads obtained from the MiSeq library were demultiplexed using QIIME (Caporaso *et al.*, 2010). Reads were split by primer and cleaned using cutadapt (Martin, 2011), cleaned of adaptors and quality filtered using Trimmomatic (Bolger *et al.*, 2014), and filtered and dereplicated with VSEARCH (Rognes *et al.*, 2016). Singletons were removed using VSEARCH. Operational taxonomic units (OTUs) were clustered at 97% similarity with VSEARCH and those with fewer than 10 reads were removed. Chimeras were detected and filtered with VSEARCH using *de novo* chimera detection. Reads were assigned to OTUs with VSEARCH using a 97% threshold. For ITS reads, taxonomy was assigned with mothur implemented in QIIME (Schloss *et al.*, 2009) using the UNITE database (Abarenkov *et al.*, 2010). For LSU reads, taxonomy was assigned with the RDP classifier web portal (Wang *et al.*, 2007). Identifications for taxa detected in mock

communities and the most abundant taxa in sample communities were refined using individual BLAST searches of the NCBI database. Code for data processing is provided in Appendix D1.

In order to filter out unreliable data, individual replicates (i.e. PCR products with unique barcodes) with fewer than 500 reads and OTUs with fewer than 10 reads were removed from the OTU tables. When both technical replicates of a sample passed this initial filter, their OTU abundances were added to combine the data for a sample. Any sample with fewer than 1000 reads (i.e. those with only one remaining replicate with fewer than 1000 reads) was removed from the table at this point. Numbers of reads were compared between mock and sample community data to estimate the levels of leakage of reads between samples and determine a noise filtering threshold. Based on this, all values (i.e. the abundance for one OTU in one sample) under 10 were removed from the OTU tables and all values that represented less than 1% of all reads in the same OTU were also removed. Any samples that dropped below 1000 reads and any OTUs that dropped to 0 abundance from this noise filtering were removed.

The LROR-LR3 primer pair is not strictly fungal specific, so the LSU dataset was split by assigned taxonomy into fungal and non-fungal subsets for further analysis. Since there were fewer non-fungal reads, samples with at least 500 reads were used. OTU tables were normalized in R 3.3.1 (R Core Team, 2014) to account for differences in

read counts between samples. Any OTUs with mean abundances under 0.0001% after normalization were excluded. Bray-Curtis and Jaccard indices were calculated for samples in R using the vegan package (Oksanen *et al.*, 2017). Principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) using the MASS package (Venables & Ripley, 2002) were conducted in R and visualized with ggplot2 (Wickham, 2009) and plyr (Wickham, 2011) packages. Significance of separation between communities from different sites was determined with a PERMANOVA using the vegan package in R.

To calculate alpha diversity for plant samples, datasets were rarefied to 1000 reads and Shannon diversity indices were computed in R using the vegan package. The significance of differences between Shannon diversities of fungal communities from plants in different sites and regions was tested with ANOVA in R. For non-fungal reads from the LSU dataset, samples were rarefied to 500 reads since there were fewer non-fungal reads. R code used for data analysis and visualization is provided in Appendix D2.

2.2.8 Fungal phylogeny

A phylogenetic analysis of the sequences from the culture collection and amplicon datasets was conducted using the online T-BAS portal applying maximum

likelihood to place query sequences into a reference fungal phylogeny (Carbone *et al.*, 2017). All LSU OTUs retained after filtering and all cultures were included in an analysis based on the T-BAS nucLSU dataset.

2.3 Results

2.3.1 Culture collection

A total of 95 fungal isolates were obtained from *M. polymorpha* plants, six from surficial fruiting bodies and the remainder from surface-sterilized tissues. Fungi were obtained from 67% of all plants sampled (29 of 43 plants). Pieces from a single plant yielded up to six fungal species. Fungi were isolated from 15% of all the 2 mm^2 surface-sterilized plant pieces, with 89 of 592 plant fragments yielding fungi (Appendix B, Table 7). This plant fragment fungal isolation rate varied between sites, from 2.4% recovery from two Oregon sites (PXF and LCC with only one isolate each), to 68.4% isolation rate from one Kentucky collection (Appendix B, Table 7). Plant tissues also had differing yields with gametangiophores yielding the highest fragment isolation percentage (29.1%), thalli intermediate (14.4%), and rhizoids the lowest (8.3%) (Appendix B, Table 7).

The vast majority of the fungal cultures were Ascomycetes; only two Basidiomycetes were obtained. Five Ascomycete classes were represented:

Saccharomycetes, Pezizomycetes, Dothideomycetes, Leotiomycetes, and, most abundantly, Sordariomycetes (Fig. 2). The isolated fungi represented at least 52 distinct species and only 12 species were isolated more than once (Appendix B, Table 8). Of these 12, seven were found at more than one site: *Candida* sp. 1 (2 sites), *Colletotrichum truncatum* (2), *Hypoxylon* sp. 1 (2), *Hypoxylon submonticulosum* (2), *Nemania* sp. 1 (2), *Phoma herbarum* (3), and *Xylaria cubensis* (3). Of these, only *Candida* sp. 1 and *P. herbarum* were found in plants from both eastern and western sites.

As indicated by the small number of species found at more than a single site, there was little species overlap between sampling locations, but there were some patterns at higher taxonomic ranks. Five genera had more than one species represented in our collections: *Colletotrichum* (3 species), *Coniochaeta* (2), *Hypoxylon* (2), *Nemania* (3), and *Xylaria* (2). The family isolated most frequently was Xylariaceae; these fungi were found in six sites in the southeast and northeast. One fungal family, Coniochaetaceae, was unique to one site: Auxier Ridge (AUX) in Kentucky. Coniochaetaceae fungi were found in different AUX patches and in both years the site was sampled. No other family that was isolated multiple times was restricted to a single location.

Some repeatedly isolated fungi were only found in a particular tissue. *Phoma herbarum* and *Colletotrichum* spp. only came from gametangiophores. Species isolated

from different tissues were mostly non-overlapping with only one identified isolate shared between rhizoids, gametangiophores, and thalli: *Xylaria cubensis.*

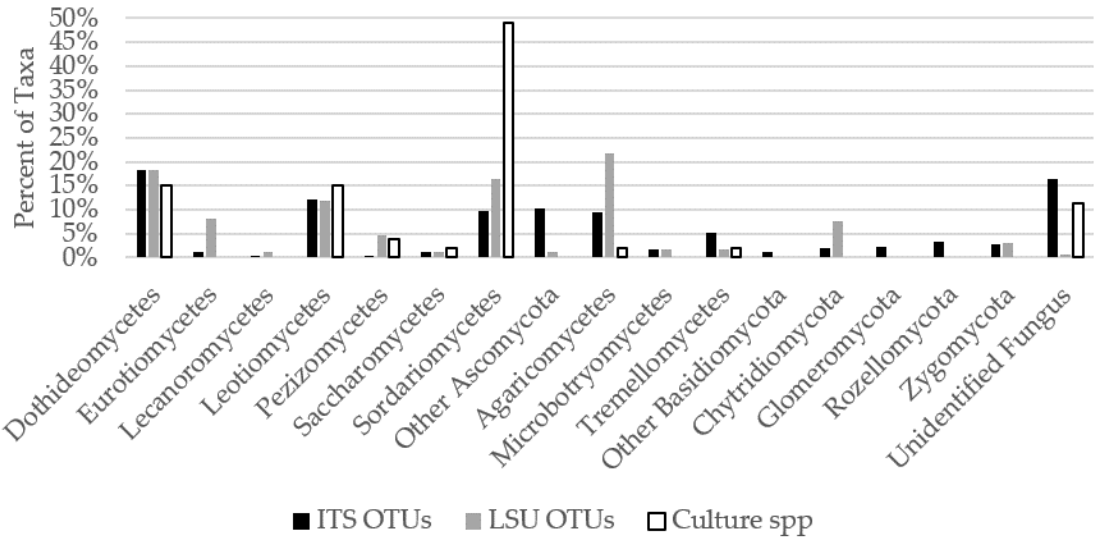

Figure 2: Taxonomic diversity of *M. polymorpha* fungal endophytes as assessed by culturing and Illumina amplicon sequencing.
All fungal classes that account for at least 1% of OTUs of one of the datasets are displayed. Bar height shows percent of OTUs or culture isolates belonging to the class listed. Black shows ITS amplicons, grey shows LSU fungal amplicons, and white shows culture collection species.

2.3.2 Mock community recovery

Taking into account the less accurate identifications from the LSU amplicons, all 19 mock community members were recovered in the MiSeq dataset (Appendix B, Table 6). Some taxa were only detected with one of the amplicon regions: *Cercospora, Coniochata,* and *Pholiota* were detected with LSU but not ITS and *Colletotrichum* was detected with ITS but not LSU. Read numbers varied widely between taxa. The ITS reads for both mock communities were dominated by *Candida* while this pattern was only seen in the raw extract mock community (mock 1) for LSU reads. *Microsphaeropsis* and *Colletotrichum* were poorly detected overall. The PCR product mock community (mock 2) LSU reads were instead dominated by *Pholiota.* For ITS and LSU, taxa in the Xylariaceae tended to have many more reads in the PCR product mock community than in the raw extract one. When amplification of the original DNA samples was checked, only one sample did not amplify (*Xylaria cubensis* raw extract with ITS primers), so the bias observed did not result from degraded DNA samples.

2.3.3 Culture-independent sampling

The Illumina MiSeq run yielded 22,837,264 raw paired-end reads. After filtering, 59 plants yielded at least 1000 reads for the LSU amplicon and 57 plants yielded at least 1000 for ITS. Two substrate samples, both from the Pacific Northwest, yielded reads for

both LSU and ITS. After filtering, the LSU and ITS datasets contained 469,491 and 1,747,873 reads, respectively. The LSU data had 249 OTUs with at least 100 reads and the ITS data had 417. The OTU accumulation curves for both amplicon data sets approached but did not reach a plateau (Appendix B, Fig. 19). The LSU dataset leveled off more at a lower OTU number, likely due to lower sequence divergence in the LSU region relative to ITS (Appendix B, Fig. 19).

Shannon diversity indices show more diverse fungal communities inhabiting individual plants in eastern US sites as compared to western sites in both ITS and LSU datasets ($p < 0.01$). One of the New York sites (LIB) has the highest Shannon diversity, closely followed by the Pennsylvania (PCR) and Vermont (WRG) sites (Fig. 3).

Both ITS and LSU datasets were dominated by Ascomycete fungi, Basidiomycete fungi were next in abundance, and a small number of earlier-diverging fungi also were detected. Some Chytridiomycota and Mucoromycotina were detected in both ITS and LSU datasets at low abundances (<2% of fungal reads) (Fig. 2). Some Glomeromycotina and Rozellomycota were detected in the ITS dataset but both at less than 1% read abundance. Of the Ascomycete fungi, Dothideomycetes, Leotiomycetes, and Sordariomycetes were most abundant (Fig. 2). The ITS and LSU amplicons yielded different read abundances of these most common classes, with the Dothideomycetes

being much more dominantly abundant in the ITS data and Sordariomycetes being more significant relative to Dothideomycetes in the LSU data (Fig. 2).

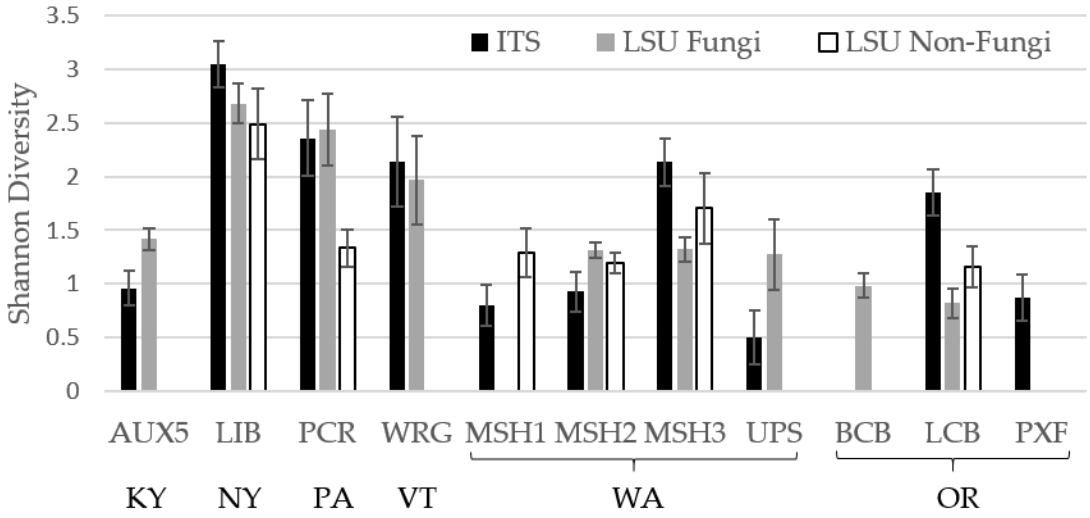

Figure 3: Shannon diversity of microbial communities in individual *M. polymorpha* plants across sites.
Bar height shows the average Shannon diversity value for an individual plant at the site in question. Error bars show one standard error in each direction. Black bars are ITS data, grey bars are LSU fungal data, and white bars are LSU non-fungal eukaryote data. Site codes and states are listed on the x-axis. Sites are included for a data subset (ITS, LSU fungi, LSU non-fungi) if at least three plants had usable data.

The identities (determined by BLAST searches) and read frequencies for the 20 most abundant OTUs from each amplicon are shown in Tables 1 and 2. No OTU was found with significant read numbers at all sites and many taxa were unique to a single site (Tables 1, 2). The taxa that were detected most abundantly and in multiple sites with both gene regions were from Didymellaceae and in the ITS dataset the didymellaceaous fungus was identified as a species of *Phoma.* Both amplicon datasets detected *Pezoloma marchantiae* but the LSU data showed it as more abundant and present at more sites. The *Marchantia*-associated agaric *Loreleia marchantiae* was detected by ITS amplicons as abundant at two Washington sites, and one Oregon site. Some abundant taxa could not be identified even to fungal phylum based on BLAST searches (Table 2). The most abundant of these OTUs came from the Little Cultus Bog (LCB) site in Oregon and the Mt. St. Helens (MSH) sites in Washington (Tables 1, 2).

Table 1: Twenty most abundant LSU fungal OTUs.
Most abundant OTUs were determined for plant samples i.e. not taking substrate communities into account. Ascomycete taxon names are green and Basidiomycete names are blue. Each abundance column is labeled either P for plant sample or S for substrate sample. Sites are coded by first letter of site code and a number if there was one in the original code. Fill of cells is coded by number of reads after filtering. Darker greens show higher read numbers. Any values below 100 are filled in orange.

Region	SE	NE				NW							
State	KY	PA	NY		VT	OR				WA			
Site	A	P	L	T	W	B	L	L	P	M1	M2	M3	U
BLAST Taxon ID	P	P	P	P	P	P	P	S	P	P	P	P	P
Pezoloma marchantiae	10294	0	0	0	0	4105	23647	0	0	1695	830	17431	2566
Didymellaceae sp.	5990	1466	0	0	0	0	0	0	0	4370	2318	0	19165
Fusarium sp.	0	11447	0	0	0	0	0	0	0	0	0	0	0
Didymellaceae sp.	1750	324	129	0	0	0	0	0	0	1474	743	0	6148
Botryosphaeriaceae sp.	0	0	0	0	9891	0	0	0	0	0	0	0	0
Helotiales sp.	0	2671	1382	223	4832	0	106	150	144	0	0	0	0
Pleosporales sp.	0	243	254	0	0	0	0	0	0	96	6276	0	0
Helotiales sp.	0	88	3455	0	2837	0	0	0	0	0	0	0	0
Eurotiomycete sp.	0	0	0	0	0	0	3057	0	57	0	600	0	1801
Sebacina sp.	0	0	0	0	640	0	0	0	4163	0	0	0	0
Dothideomycete sp.	597	667	743	0	1120	0	0	0	0	0	0	987	665
Fusarium sp.	0	4442	0	0	0	0	0	0	0	0	0	0	0
Nectriaceae sp.	0	0	0	4380	0	0	0	0	0	0	0	0	0
Cladosporiaceae sp.	0	2446	405	0	140	176	206	376	0	0	0	795	0
Penicillium sp.	3623	0	0	0	0	0	0	0	0	0	0	0	0
Helotiales sp.	577	0	0	0	0	198	1482	0	0	96	0	1030	148
Lachancea kluyveri	0	0	0	0	3017	0	0	0	0	0	0	0	0
Russula sp.	0	0	0	0	0	0	0	0	0	0	0	2867	0
Dipodascaceae sp.	362	0	0	2408	66	0	0	0	0	0	0	0	0
Thelephoraceae sp.	0	0	326	0	2205	0	0	0	0	139	0	0	0

Table 2: Twenty most abundant ITS fungal OTUs.
Most abundant OTUs were determined for plant samples i.e. not taking substrate communities into account. Ascomycete taxon names are green and Basidiomycete names are blue. Each abundance column is labeled either P for plant sample or S for substrate sample. Sites are coded by first letter of site code and a number if there was one in the original code. Fill of cells is coded by number of reads after filtering. Darker greens show higher read numbers. Any values below 100 are filled in orange.

Region	SE	NE				NW								
State	KY	PA	NY		VT	OR				WA				
Site	A	P	L	T	W	B	L	L	P	M1	M1	M2	M3	U
BLAST Taxon ID	P	P	P	P	P	P	P	S	P	P	S	P	P	P
Phoma sp.	121502	0	0	0	0	0	0	0	0	91069	6899	60120	0	130615
Pyrenochaeta sp.	0	0	0	0	0	0	0	0	0	0	0	109882	0	0
Hypocreales sp.	0	69028	0	0	0	0	0	0	0	0	0	0	0	0
Fungus sp.	0	0	0	0	0	0	0	0	0	0	0	60944	0	0
Loreleia marchantiae	0	0	0	0	0	13711	0	0	0	21104	0	20170	0	0
Fungus sp.	0	0	0	0	0	0	44124	0	0	0	0	6934	0	0
Phialophora sp.	0	37318	0	0	4275	0	0	0	0	0	0	0	0	0
Pezoloma marchantiae	0	0	0	0	0	0	0	0	0	19798	0	7684	0	0
Dothideomycete sp.	0	0	0	0	0	0	25071	0	0	0	0	0	0	0
Sebacina sp.	0	0	0	0	0	0	0	0	25061	0	0	0	0	0
Nectriaceae sp.	0	0	0	24077	0	0	0	332	0	0	0	0	0	0
Tetracladium sp.	0	642	9290	0	7568	0	0	0	0	0	0	0	0	0
Botryosphaeriaceae sp.	0	0	0	0	16807	0	0	0	0	0	0	0	0	0
Sebacinales sp.	16714	0	0	0	0	0	0	0	0	0	0	0	0	0
Loreleia marchantiae	0	0	0	0	0	0	0	0	0	1972	0	12662	0	0
Tremellomycetes sp.	0	0	438	0	0	0	7435	8171	0	1692	3906	824	1644	0
Dactylaria sp.	0	2336	0	292	9311	0	0	0	0	0	474	0	0	0
Helotiales sp.	0	220	0	10826	0	0	0	0	0	0	0	0	0	0
Cladosporium sp.	0	6065	1527	0	0	0	193	660	0	145	207	0	2816	0
Didymellaceae sp.	0	8264	0	0	197	0	0	0	0	0	0	0	0	0

Since PCoAs with Bray-Curtis and Jaccard indices gave similar patterns and our mock community data showed biases in dataset read abundances, we present results from the presence-absence Jaccard index. PCoA analyses of amplicon data indicate that very little variation is explained by the first two axes (<11%) (Fig. 4), but scree plots of the eigenvalues show that these first axes do explain significantly more variation than the remaining axes. Despite the low signal, PCoAs of both ITS and LSU show a clear pattern with a few sites distinct from the others and there is significant separation among sites ($p < 0.01$) (Fig. 4). The Kentucky and Pennsylvania sites are the most distinct, each separated along one of the first two axes. (Fig. 4 a, b). The samples show some separation between Northeast, Southeast, and Northwest regions and the difference is significant based on PERMANOVA ($p<0.01$) (Fig. 4). This distinction is more pronounced for the LSU dataset (Fig. 4 d,f).

2.3.4 Phylogeny of cultures and amplicons

The fungi detected in *M. polymorpha* come from across the fungal tree of life with few major groups lacking representatives. Fungal orders frequently recovered by both culturing and amplicon methods include Xylariales, Helotiales, and Pleosporales (Fig. 5). Very few clades were exclusively found in eastern or western populations of plants and individual OTUs detected in both regions are scattered across most of the phylogeny

(Fig. 5). Most OTUs detected in the Eurotiomycetes, Lecanoromycetes, and Lichinomycetes were only found either in eastern or western plants.

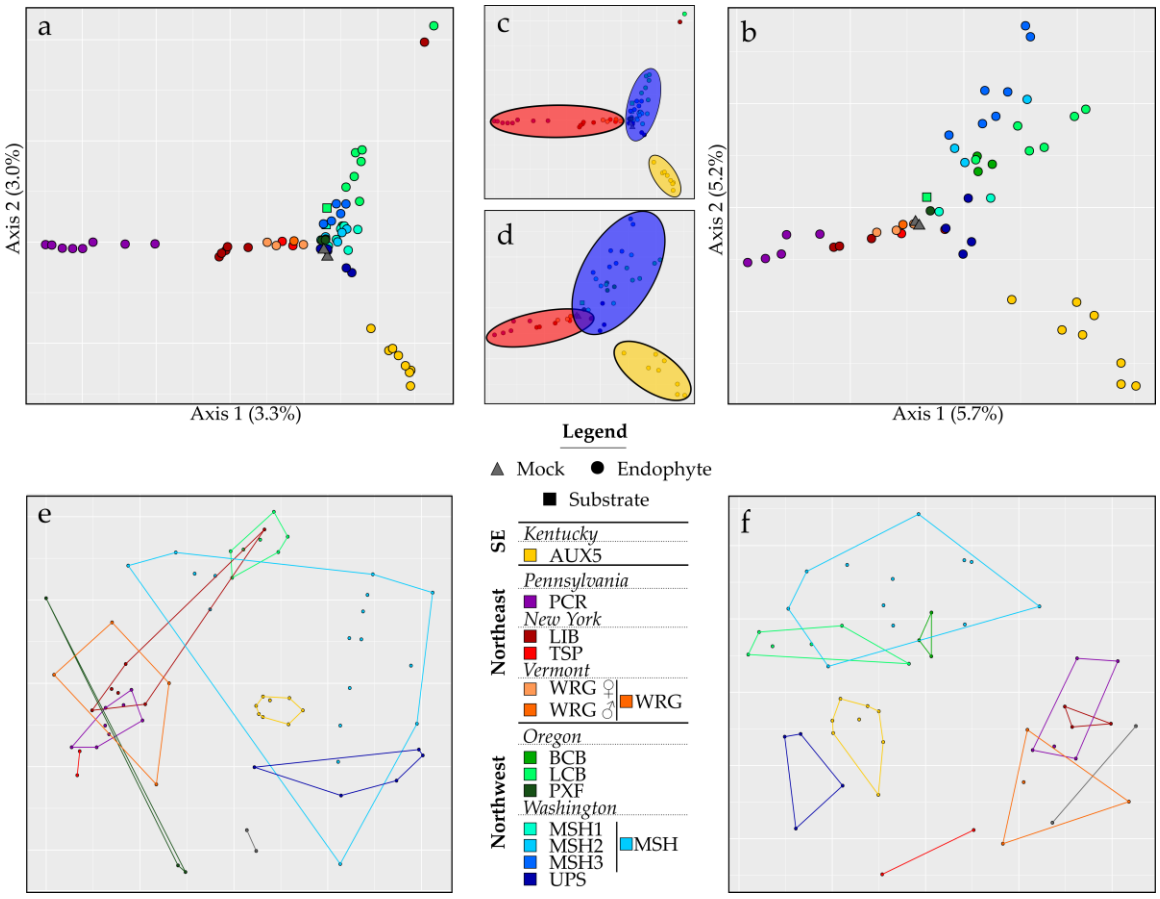

Figure 4: Ordinations of fungal community data.
All ordinations use Jaccard index to calculate distance. (a) PCoA using ITS data. (b) PCoA with LSU fungal data. (c) Three sampling regions mapped onto ITS PCoA. Red is Northeast, Yellow is Southeast, and Blue is Northwest. (d) Three sampling regions mapped onto LSU PCoA. (e) NMDS using ITS data. (f) NMDS using LSU fungal data.

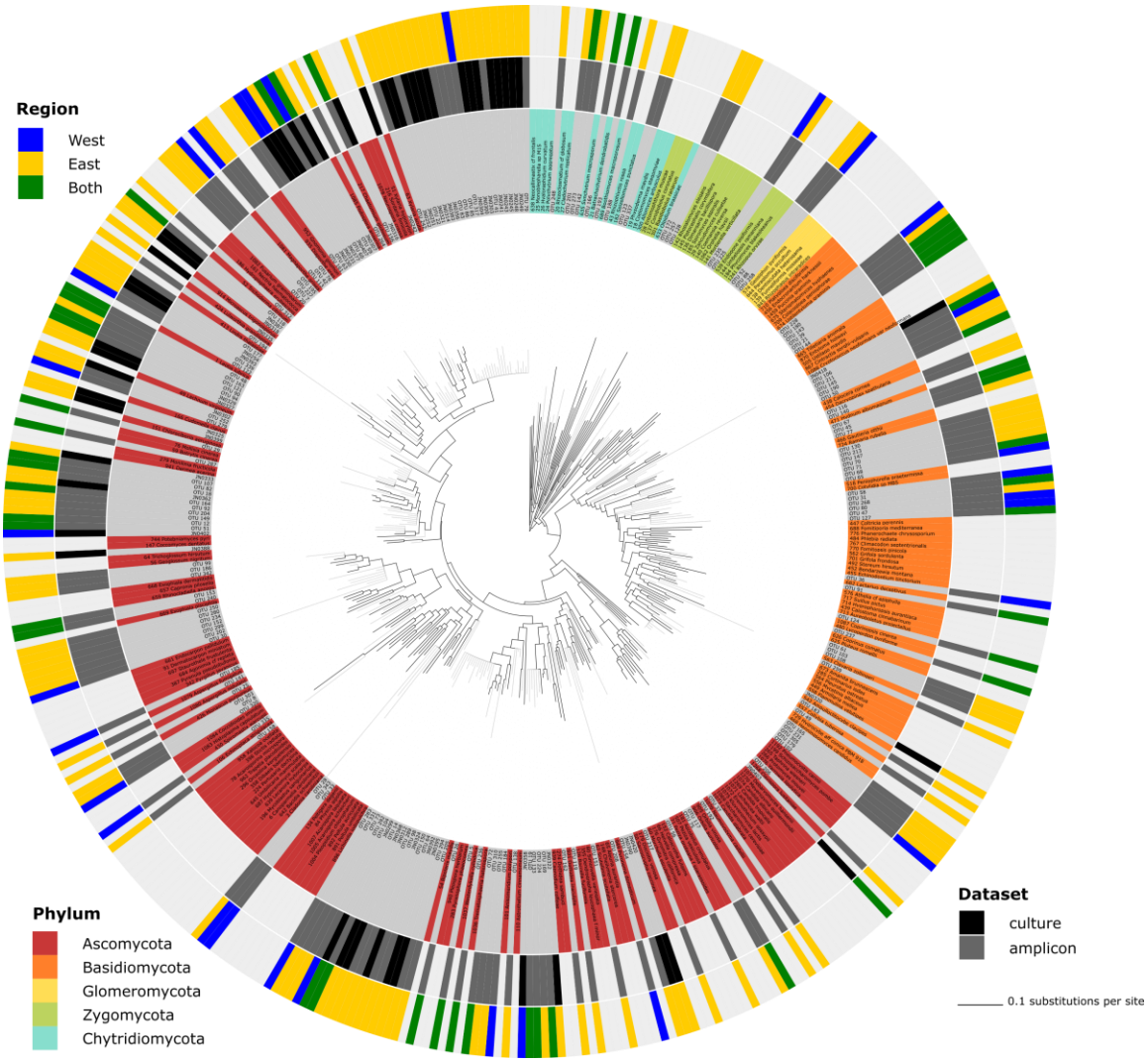

Figure 5 : Phylogeny showing the relationships between fungi detected by both sampling methods.

The inner ring shows phyla of reference sequences used. The middle ring shows the dataset each sequence from this study comes from: black for culture, dark grey for LSU amplicon. The outer ring shows which fungi were found in sites in the eastern United States (Kentucky, North Carolina, Tennessee, Pennsylvania, New York, or Vermont) or in northwestern North America (Oregon, Washington, or the Yukon). Fungi detected only in the eastern region are colored yellow, ones only found in the west are blue, and ones found in both regions are green.

2.3.5 Non-fungal associates

Non-fungal taxa detected by LSU sequencing included oomycetes, chlorophyte algae, mites, nematodes, and other microbial eukaryote groups. Reads from Chlorophyte algae and Metazoa were detected across all sites and Ciliates were also found in many collections (Table 3). Chlorophyta accounted for 4% of reads and 9% of OTUs and Metazoa for 19% of reads and 24% of OTUs. As with the fungal data, the non-fungal communities show separation between Northeastern and Northwestern regions that is significant by PERMANOVA ($p < 0.01$) (Fig. 6). No difference in alpha diversity of non-fungal communities between east and west was detected ($p = 0.136$). Non-fungal communities generally had similar diversity to fungal communities from the same site if they were detected in high enough abundance to be assessed (Fig. 3).

Table 3: Abundances of non-fungal taxa detected by LSU sequencing.
Read abundance of non-fungal phyla are displayed. Color gradient shows abundances with dark green being high and red being absent.

Region	**SE**	**NE**				**NW**						
State	**KY**	**PA**	**NY**		**VT**	**OR**			**WA**			
Site	**AUX5**	**PCR**	**LIB**	**TSP**	**WRG**	**BCB**	**LCB**	**PXF**	**MSH1**	**MSH2**	**MSH3**	**UPS**
Chlorophyta	648	539	1122	36	11	40	233	304	86	5829	4189	197
Ciliophora	110	879	145	0	48	0	4108	546	79	136	0	0
Metazoa	696	29165	1511	134	1236	1411	7315	1830	15073	2813	2255	1745
Mycetozoa	0	0	0	0	0	0	59	0	0	0	0	0
Stramenopiles	1560	1243	500	283	282	83	4263	0	0	0	0	0
Tubulinea	0	0	0	0	0	0	0	0	12	14	31	0

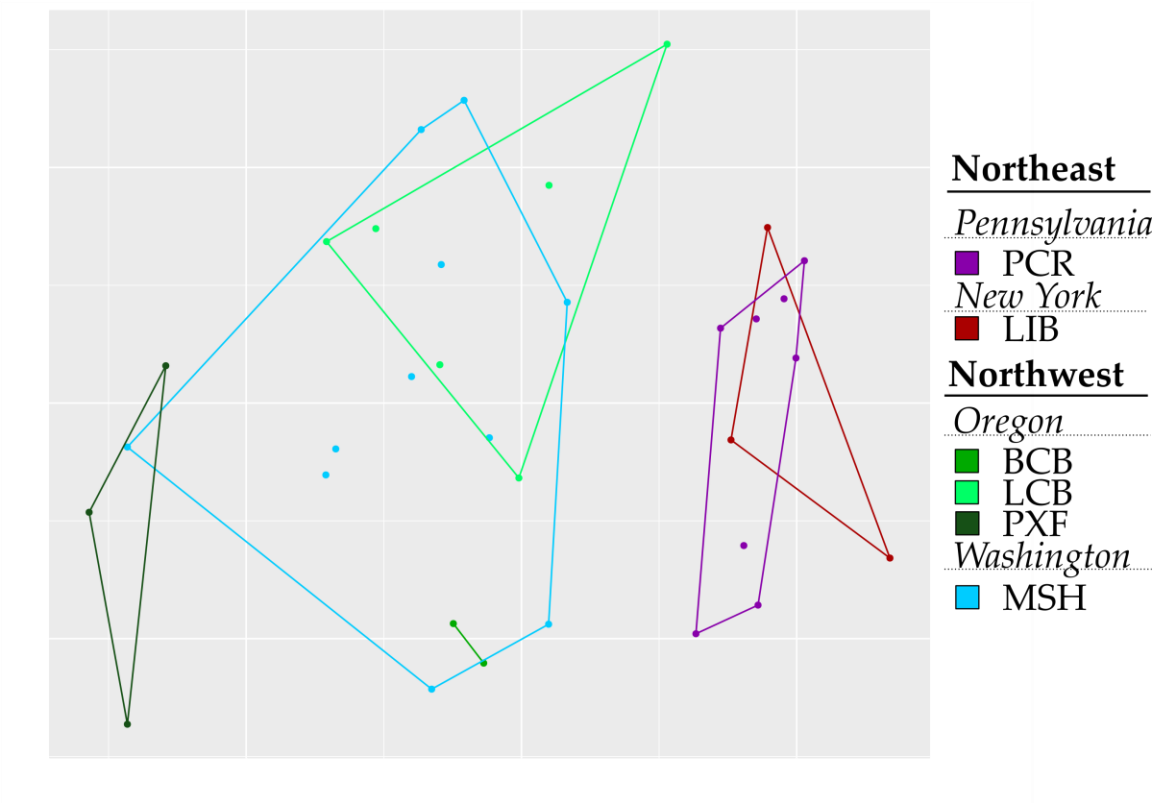

Figure 6: NMDS of non-fungal taxa detected by LSU sequencing.
NMDS used Jaccard's index to calculate distances between non-fungal eukaryote communities in plants from the six sites that had significant amounts of non-fungal reads.

2.4 Discussion

Our sampling of *M. polymorpha* endophytes indicates a diverse community of fungi with high variation across space and even between habitats in close proximity to each other. Some of the abundantly detected fungi from the LSU dataset have no close relatives in sequence databases, making *M. polymorpha* a potential source for discovering new fungi at high taxonomic ranks. From our sampling, some of the sites in the Pacific

Northwest were particularly promising as sources of poorly known taxa: a bog in Oregon (LCB) and the Mount St. Helens pumice plain (MSH).

2.4.1 Comparison of culture and culture-independent sampling of *M. polymorpha* endophytes

Comparison of high taxonomic ranks between sampling methods shows a bias toward Ascomycete fungi and Sordariomycetes in particular in the culture sampling relative to amplicon data. As shown for other liverworts, we isolated an abundance of xylariaceous fungi in culture (Davis *et al.*, 2003), but these taxa were rare in the amplicon data. It is possible that the library preparation is biased against these taxa. This is supported by the observation that in our mock community made with raw DNA extracts, Xylariaceae were poorly recovered. In addition to almost exclusively recovering Ascomycetes, the culture-based sampling missed some classes within this phylum that were recovered by amplicon sequencing: Eurotiomycetes, Lecanoromycetes, and Lichinomycetes.

At sites where both culturing and culture-independent sequencing were used, the taxa recovered by the two methods did not frequently overlap. Three sites yielded enough amplicon and culture data to make comparisons: AUX in Kentucky, LIB in New York, and WRG in Vermont. In Kentucky, orders that were detected by both methods were Xylariales, Pleosporales, Saccharomycetales, and Helotiales. In New York,

Xylariales and Helotiales were also detected in both datasets and in Vermont Pleosporales and Pezizales were the overlapping clades between methods.

Because mock community members were all from the cultures collected from *M. polymorpha*, comparison of OTUs between mock community and environmental samples allowed more specific direct comparison of parts of the culture and culture-independent data sets. The ITS dataset showed clear recovery in environmental plant samples of the mock community taxa *Cadophora luteo-olivacea, Epicoccum nigrum, Phoma herbarum,* and *Plectosphaerella* sp. while the other recovered mock community taxa were represented by very few reads, if any, in the environmental samples. The LSU dataset had less taxonomic resolution, but also showed some overlap in OTUs between mock and environmental samples: *Coniochaeta, Plectosphaerella, Hypoxylon submonticulosum,* a Leotiomycete (likely *Cadophora*), Didymellaceae sp., and a Xylariaceae sp. were all overlapping between the two. Detection of these fungi by both methods increases the confidence with which we can say that these fungi are consistent members of the *M. polymorpha* fungal endophyte community.

2.4.2 Factors organizing the fungal community in *M. polymorpha*

Although the signal of geographic organization was weak in ordination analyses of the fungal communities, there was separation between the three sampled regions

(Southeast, Northeast, and Northwest). Factors determining the composition of endophyte communities are complex and differ between host groups and geographic regions, along elevation gradients and successional sequences, and through time (Davis & Shaw, 2008; Davey *et al.*, 2012; Davey *et al.*, 2013; Davey *et al.*, 2014; Peršoh, 2015). Since we were attempting to cover a broad area with our sampling, we could not control for all the possible factors that might cause differences in fungal community composition. More intensive sampling would be needed to determine the spatial scales and abiotic environmental conditions structuring the diverse fungal communities in *M. polymorpha.* Since *M. polymorpha* is often found in disturbed sites and is an early colonizer after fire (Graff, 1936; Bradbury, 2006), investigating its fungal communities along disturbance and successional gradients may be particularly interesting.

Despite the weak patterns at the regional scale, there were clear differences in presence of specific taxa between sites. Some particularly strong differences in taxon presence are apparent for the rarer non-Dikarya groups. Mortierellales, known plant associates, were only detected in significant abundance at the Little Cultus Bog (LCB) site in Oregon and one of the Mount St. Helens sites (MSH3). More than 1000 ITS reads for Rozellomycota occurred in each of three sites: AUX in Kentucky, LCB in Oregon, and Pine Creek Rail Trail (PCR) in Pennsylvania. Microsporidia were abundant only at LCB. Glomeromycotina fungi were detected with more than 1000 ITS reads at only 2 sites

(AUX and MSH1) and Chytridiomycota were only found in this abundance at one Oregon site, Proxy Falls (PXF). Among the Dikarya in both datasets, many OTUs were unique to a single site and abundant there. This high number of unique taxa likely explains the difficulty in aligning a high percentage of the variation in the amplicon community data set along any one ordination axis.

Another factor that can structure endophyte communities is tissue specificity. Most of the current knowledge about fungal endophyte communities comes from conifer and angiosperm hosts, focusing on sporophyte leaves and roots—structures not present in bryophytes. In our culturing, we separated *M. polymorpha* thalli, rhizoids, and gametangiophores and found that there was some indication of tissue-specificity of certain fungi. Other studies have previously observed tissue specificity in culturable fungi from vascular hosts. Junker et al. (2012) found dramatic distinctions between genera obtained from stems, seed pods, and flowers for endophytes of the angiosperm model *Arabidopsis thaliana* in one of two sample years. Yuan et al. (2009) observed specificity of certain endophytes to leaf or root tissues in an orchid and Wu et al. (Wu *et al.*, 2013) found distinct fungal assemblages in yew leaves, branches, and bark. Even without the complex, highly differentiated tissues found in vascular plants, bryophytes may present multiple niches to their fungal inhabitants. Our observations suggest that differences in even the simpler tissues of a liverwort may still be significant for

structuring fungal communities at the small scale of bryophytes, but more tissue-specific sampling would be necessary to confirm this.

2.4.3 Dominant members of the *M. polymorpha* mycobiome

Our amplicon data showed high abundances of fungi known to fruit specifically on *M. polymorpha*, namely *Pezoloma marchantiae* (Garcia & Van Vooren, 2005; Egertová *et al.*, 2015) and *Loreleia marchantiae* (Bresinsky & Schotz, 2006). This observation confirms these specialists to be important components of the *M. polymorpha* endophyte community. The two substrate samples show negligible numbers of reads for these two fungi, further suggesting their close association with live *M. polymorpha* hosts in the environment. The sequencing results were corroborated by observations of these two fungi associated with collected plants. Fruiting bodies with the macroscopic morphology of *P. marchantiae* were observed on plants collected from the Kentucky site in 2015 (Appendix B, Fig. 20 a), one of the sites with high recovery of *P. marchantiae* OTUs in the LSU dataset. Mushrooms matching the macroscopic characteristics of *L. marchantiae* were found in the MSH2 site in Washington at the time of collection (Appendix B, Fig. 20 b), and Mt. St. Helens was the location where this taxon was abundant in the ITS amplicon dataset.

Pezoloma marchantiae is a close relative of the ericoid mycorrhizal fungus *P. ericae* and other *Pezoloma* species are known to associate with bryophytes (Stenroos *et al.*, 2010). These bryophyte-associated *Pezolomas* have been hypothesized to be saprobes specializing on bryophytes (Stenroos *et al.*, 2010), but the recent demonstration of growth enhancement and nutrient transfer by *Pezoloma ericae* in two leafy liverwort hosts indicates that some fungi in the group could also function as mutualists (Kowal, 2016). Formation of fruiting bodies on plant hosts is often interpreted as evidence of a parasitic function (Döbbeler, 1997), but it is also possible that *P. marchantiae* is a mutualist of *M. polymorpha* as seen with *P. ericae* in other liverworts.

Loreleia marchantiae can be found fruiting on a number of complex thalloid liverworts and infects the rhizoids of *M. polymorpha* (Bresinsky & Schotz, 2006). This fungus has been observed to infect both smooth (live at maturity) and pegged (dead at maturity) rhizoids (Guminska & Mierzenska, 1992). Infection with *L. marhcantiae* also appears to change growth patterns and other microbial associations in *M. polymorpha*. Guminska and Mierzenska (1992) observed that infected plants formed more oil cells and more smooth rhizoids and attracted colonies of cyanobacteria to gather among their rhizoids.

Species of *Phoma* and other taxa in the Didymellaceae also seem to be dominant members of the *M. polymorpha* mycobiome. *Phoma herbarum* was isolated in culture from

three sites, all distant from each other: YUK in the Yukon, Winooski River Gorge (WRG) in Vermont, and AUX in Kentucky. This species was isolated from AUX in both 2014 and 2015. From YUK, *P. herbarum* was isolated from surface-sterilized tissue and from a fruiting body that developed on plants maintained in the laboratory after collection. In addition, an OTU of *Phoma* was abundant in the ITS dataset and was detected clearly at all but three sites. This *Phoma* OTU included the *P. herbarum* used in the mock community, indicating that the same or a similar taxon to the one cultured can be widely detected with culture-independent methods as well.

Phoma herbarum is a widespread fungus in habitats ranging from terrestrial plants to marine fish (Ross *et al.*, 1975). A strain of this species isolated as a soybean endophyte was demonstrated to promote growth of rice and soybean (Hamayun *et al.*, 2009). A close relative of *P. herbarum*, *Phoma muscivora,* is a pathogen identified in boreal mosses (Davey & Currah, 2009; Davey *et al.*, 2009). *Phoma muscivora* produces enzymes capable of degrading bryophyte cell walls and may function as an opportunistic saprobe as well as a pathogen of live mosses (Davey & Currah, 2009). It is possible that *P. herbarum* is an opportunistic pathogen or saprobe in *M. polymorpha*, but both culturing and sequencing methods indicate it can be abundant in healthy plants as well. It promotes growth in other hosts (Hamayun *et al.*, 2009), so it may have a beneficial relationship with *M. polymorpha* too.

2.4.4 Comparison of *M. polymorpha* fungal communities to those of other bryophytes

Culture-based approaches investigating leafy and simple thalloid liverwort hosts showed a particular enrichment of fungi in the order Xylariales (Davis *et al.*, 2003; Davis & Shaw, 2008). We also found enrichment in Xylariales, but to a lesser degree; 67% of isolates obtained by Davis and Shaw (2008) belonged to this order, but only 33% of our isolates were Xylariales. In contrast, we found higher percentages of Pleosporales (12% vs. 2.5%) and Sordariales (18% vs. 1%) than Davis and Shaw (2008). A metagenomic study which included the complex thalloid liverwort *Conocephalum conicum* revealed colonization of this host with a variety of fungal taxa, most of which were also detected in our sampling of *M. polymorpha* (Knack *et al.*, 2015). Of the fungal taxa from *C. conicum* identified to genus in the study of Knack et al. (2015), we detected *Cladosporium, Davidiella, Mortierella, Ochrocladosporium,* and *Phaeosphaeria,* but not *Dicellomyces*, *Glomus, Myrothecium,* or *Saccharomyces* (Knack *et al.*, 2015). The lack of *Glomus* and the low recovery of Glomeromycotina in general from our study is unsurprising given previous reports that at least some subspecies of *M. polymorpha* do not form arbuscular mycorrhizal type associations (Ligrone *et al.*, 2007). Where we did detect ITS reads of Glomeromycotina fungi, they were not abundant.

More studies have examined the fungal communities of mosses than of liverworts or hornworts. Fungi in mosses have been catalogued with culturing (U'Ren *et*

al., 2010; Osono & Trofymow, 2012; U'Ren *et al.*, 2012), clone libraries (Kauserud *et al.*, 2008), and 454 pyrosequencing (Davey *et al.*, 2012; Davey *et al.*, 2013; Davey *et al.*, 2017). Of 37 genera of fungi cultured in our study of *M. polymorpha* and Osono and Trofymow's (2012) study of the moss *Kindbergia oregana*, only three were recovered in both: *Cladosporium, Epicoccum,* and *Phoma.* Since we obtained very few cultures from plants sampled in the Pacific Northwest, part of this disparity could be due to geographic differences in available fungi, but could also signal host specificity of fungal communities in bryophytes, or simply very diverse communities insufficiently sampled to show the true taxon overlap. We also compared our ITS amplicon results to two previous culture independent studies of moss-associated fungi: Kauserud et al. (2008) used ITS clone libraries to assess fungi in *Hylocomium splendens, Pleurozium schreberi,* and *Polytrichum commune* and Davey et al. (2017) used 454 pyrosequencing to identify fungi in *Dicranum scoparium, H. splendens,* and *P. schreberi.* All three datasets showed slightly over half the OTUs to be Ascomycota. Both moss studies had approximately 30% of OTUs in Basidiomycota and less than 6% in basal fungal phyla, but *M. polymorpha* had only 19% Basidiomycota OTUs and 10.2% of OTUs in basal phyla, including Chytridiomycota, Mucoromycota, and Rozellomycota. The *M. polymorpha* fungal community included fewer Chaetothryales, Helotiales, and Agaricales, but more Pleosporales relative to the two moss studies (Appendix B, Fig. 21).

Individual parasites and pathogens of bryophytes have been identified and studied for a wider range of hosts (Döbbeler, 1997; Döbbeler, 2002; Davey & Currah, 2006; Akita *et al.*, 2011). There are many ascomycetes that fruit on mosses and liverworts and are considered parasites of these bryophytes (Döbbeler, 1997; Döbbeler, 2002). Of the Ascomycete genera containing specialized bryophyte parasites, we detected *Hymenoscyphus*/*Pezoloma, Bryochiton* (Döbbeler, 1997), and *Cladophialophora* (Davey & Currah, 2007). Fewer of these bryophilous Ascomycetes have been found in thalloid liverworts than in mosses and leafy liverworts, so not detecting a wide diversity of these genera does not come as a surprise (Döbbeler, 1997), and it is also possible that some of these parasites were present, but only on the liverwort surface and were not detected after surface sterilization. We also detected fungi belonging to genera containing known pathogens of mosses: *Alternaria*, *Arrhenia, Cladosporium, Coniochaeta, Phoma,* and *Epicoccum* (Davey & Currah, 2006; Davey & Currah, 2009; Davey *et al.*, 2010; Akita *et al.*, 2011).

2.4.5 Comparison of *M. polymorpha* fungal communities to those of vascular plants

Some of the genera recovered in our culture collection are also found in vascular plant endophyte culture studies. We compared the genera in our culture collections to a set of 12 studies that provided easily comparable lists of cultured endophyte identities

for 13 angiosperm hosts, two conifers, and a horsetail (Appendix B, Table 9) (Arnold, 2002; Qi *et al.*, 2009; Yuan *et al.*, 2009; Li *et al.*, 2010; Quilliam & Jones, 2010; Yuan, Z-l *et al.*, 2011; Yuan, ZL *et al.*, 2011; Junker *et al.*, 2012; Pancher *et al.*, 2012; Quilliam & Jones, 2012; Oono *et al.*, 2015; Yao *et al.*, 2017). The genus found both in our study of *M. polymorpha* and in the majority of the vascular hosts from the studies listed above was *Cladosporium*. Additional genera found in both *M. polymorpha* and other vascular hosts were *Biscogniauxia* (1 of the vascular hosts), *Colletotrichum* (7), *Coniochaeta* (2), *Daldinia* (2), *Epicoccum* (5), *Hypoxylon* (3), *Microsphaeropsis* (1), *Nemania* (2), *Phoma* (4), *Plectosphaerella* (1), *Toxicocladosporium* (1), and *Xylaria* (4). The information summarized from these previous studies is provided in Appendix B, Table 10. These genera found in *M. polymorpha* as well as vascular hosts likely represent ubiquitous plant associates with very little host specificity, at least at the fungal genus level. *Cladiosporium, Epicoccum,* and *Phoma* in particular are likely widespread since they were common in this comparison as well as shared in the previously presented comparison to culturable fungi of mosses.

A number of common endophyte genera cultured frequently from diverse vascular hosts were conspicuously absent from our collections. These included *Alternaria, Fusarium, Penicillium* and *Tricoderma*. *Alternaria* and *Penicillium* were detected in relatively low abundance by the amplicon sequencing and OTUs of *Fusarium* were

abundantly detected in the LSU data set at the Pennsylvania site which had no culture sampling. In contrast, no *Trichoderma* were identified in the amplicon data. *Trichoderma* cultures might have been discarded as contamination due to their fast-growing "green mold" morphology, but this would not have changed the amplicon sampling and the absence of this genus that is common in soils and plants is notable (Harman *et al.*, 2004).

In addition to comparing genera from previous culture collections, we also assessed the taxonomic similarity at higher ranks between amplicon data from our study and other next generation sequencing studies of plant mycobiomes. A meta-analysis of deep-sequenced fungal communities using the ITS1 region showed that the most OTU-rich classes of fungi in tree phyllosphere samples were Dothideomycetes and Sordariomycetes (Meiser *et al.*, 2014). In our ITS dataset, Dothideomycetes were also the dominant class by OTU count (18%), but Leotiomycetes were next in OTU number (12%) and Sordariomycetes were third (10%). We also compared our results to three endophyte studies of vascular plants published subsequent to Meiser et al. (2014) that provided supplemental OTU tables with assigned taxonomy. These studies sampled fungal endophytes with ITS1 amplicon libraries for needles of *Picea glauca* in Alaska (Eusemann *et al.*, 2016), leaves of *Fagus sylvatica* in Germany (Siddique *et al.*, 2017), and roots of *Onosma echioides* in Italy (Muller & Hilger, 2015). The community of fungi we detected with ITS data in *M. polymorpha* was overall similar to the other three

mycobiomes at the fungal phylum and class ranks (Appendix B, Fig. 22). All communities were dominated by Ascomycota, except the *O. echioides* root study that had a high abundance of Glomeromycotina reads (53% of reads). In all but *O. echioides* roots, Dothideomycetes were the dominant class by read abundance. The fungi of *M. polymorpha* were similar to those in *O. echioides* roots in that they both had higher abundances of Agaricomycetes present (16% and 23% respectively). *Marchantia polymorpha* also had a higher abundance of Sordariomycetes than the vascular plants.

2.4.6 Non-fungal associates of *M. polymorpha*

A number of non-fungal eukaryotes were detected in high abundance in the LSU dataset. The most abundant of these include chlorophyte algae and mites (Acari). Two OTUs likely representing mites even appeared in the more fungal-specific ITS data set. It is possible that some of these organisms were not inside the liverwort tissues, but rather adhering to the surface strongly enough to resist the washing and sterilization treatments. Microarthropods could also shelter in the air pores on the liverwort's surface, for example. But it is possible that some of the detected organisms, particularly the green algae, could be inside the plant tissues as well. Intracellular *Coccomyxa*-like algae have been reported to be widespread in *Ginkgo biloba* (Trémouillaux-Guiller & Huss, 2007) and green algal endophytes have also been reported from a number of

mosses (Reese, 1981; Reese, 1992). It is likely that there is a rich community of non-fungal eukaryotes inhabiting *M. polymorpha*, but the true composition of this community and its effects on the plant are as yet unknown.

2.4.7 Concluding remarks

Overall, *M. polymorpha* fungal communities seem to follow similar trends to those in other plants at high taxonomic ranks and share some ubiquitous endophyte genera, but also are distinguished by a few specialist fungi and the absence of certain common endophytes. The diverse set of fungi we detected in *M. polymorpha* could potentially be acting as mutualists, commensals, saprobes, or pathogens, based on previous knowledge of the taxa. The communities were weakly structured by geographic region. More sampling will be needed to circumscribe the microbiome of this model liverwort, especially since it has such a wide distribution, and to determine the ecological roles of the members of its diverse microbial community. Out study provides context for further investigation of liverwort microbiomes and use of the model *M. polymorpha* to answer questions about of the ecology and molecular-level interactions of fungi in bryophytes and other plants.

3 Chapter 2: A novel experimental system using the model liverwort *Marchantia polymorpha* L. reveals diverse effects of fungal endophytes in a bryophyte host

3.1 Introduction

Fungal symbioses are ubiquitous in plants, and these relationships range from pathogenic to mutualistic. These symbioses are also ancient, as fossil and molecular evidence suggest that fungal symbionts may have helped plant ancestors colonize dry land (Heckman *et al.*, 2001; Taylor & Krings, 2005; Delaux *et al.*, 2012). This ancient origin and its strong impact on selection is also indicated by the observation that core genes involved in symbiotic interactions with mycorrhizal fungi (and nitrogen-fixing rhizobia) are functionally conserved from basal to crown groups of the land plant evolutionary tree (Wang *et al.*, 2010; Delaux *et al.*, 2014). Some of these symbiotic genes are found in chlorophyte and charophyte algae as well, indicating pre-adaptation for terrestrial symbioses in the green lineage (Delaux *et al.*, 2015).

Fungal endophytes, which live inside the healthy tissues of plants, have been found in virtually every plant tested, from bryophytes to angiosperms (Stone *et al.*, 2000; Rodriguez *et al.*, 2009) as well as in chlorophytes and charophytes (Cui *et al.*, 2010; Knack *et al.*, 2015). Fungal endophytes can affect host growth and success in many ways,

thereby playing a critical role in structuring terrestrial ecosystems (Rudgers *et al.*, 2004; Afkhami & Strauss, 2016; Aguilar-Trigueros & Rillig, 2016). Many definitions of the term endophyte have been used; here we use the broad sense that includes any microbe that can be found living in healthy plant tissue (Stone *et al.*, 2000). Under this broad definition, endophytes can be anywhere on the spectrum from mutualist to pathogen, as long as they have at least a brief asymptomatic phase of colonization. While this definition technically includes mycorrhizal relationships, we do not focus on mycorrhizae in this study as the fungi we isolated do not belong to mycorrhizal taxa and we did not observe mycorrhiza-like infection structures.

The most intensively studied endophyte symbioses are the clavicipitaceous endophytes in grasses (Poaceae), which garnered early attention because of their agricultural significance. These fungi (Clavicipitaceae, Hypocreales) densely infect above-ground grass tissues and are vertically transmitted (Rodriguez *et al.*, 2009). In contrast, the non-clavicipitaceous endophytes (hereafter simply 'endophytes') are found in hosts from all major plant groups, can infect any tissue, are usually horizontally transmitted, and represent a wide taxonomic range of fungi (Rodriguez *et al.*, 2009). These fungi can be highly diverse within a single plant, with dozens of culturable fungi living in a single host species in the same geographic site (Higgins *et al.*, 2007; U'Ren *et*

al., 2010). Endophytes have so far been revealed to have diverse ecological roles, but many remain unstudied (Rodriguez *et al.*, 2009).

Existing studies of fungal endophyte functions have revealed diverse effects on the success of plant hosts. Some fungi enhance plant growth (Banhara *et al.*, 2015), competition through allelopathy (Aschehoug *et al.*, 2014), secondary metabolite production (Prasad *et al.*, 2013), or nutrient uptake and use efficiency (Yang *et al.*, 2014). Endophytes can protect plants from biotic stressors such as attack by pathogens (Arnold *et al.*, 2003; Tellenbach & Sieber, 2012) or herbivores (Jaber & Vidal, 2010; Gange *et al.*, 2012). Certain endophytes also have been found to increase plant resistance to abiotic stressors such as drought (Murphy *et al.*, 2015; Azad & Kaminskyj, 2016; Molina-Montenegro *et al.*, 2016), high salt concentrations (Azad & Kaminskyj, 2016; Leitão & Enguita, 2016), or the presence of heavy metals (Yamaji *et al.*, 2016; Khan *et al.*, 2017). In contrast, some endophytes can be latent pathogens or saprobes (Schulz & Boyle, 2005; Porras-Alfaro & Bayman, 2011). Additionally, studies are beginning to reveal how the beneficial effects of certain endophytes are dependent on environmental conditions such as nutrient supplies (Hiruma *et al.*, 2016; Qin *et al.*, 2017) or soil moisture (McCormick *et al.*, 2001), as well as levels of exposure to the fungus (Swett & Gordon, 2017). Observations of context-dependency support the notion that plant-endophyte

interactions are dynamic and they hint at the complexity of plant microbiome functioning yet to be revealed.

The study of plants as holobionts composed of host and complex microbiome is still in the early stages (Vandenkoornhuyse *et al.*, 2015), with investigations directly testing the effects of various endophytes so far mostly limited to angiosperm and conifer hosts. The earlier-diverging non-seed plants make up a considerable proportion of species in most ecosystems, but there is much less known about their endophyte interactions. Understanding the evolution of fungal symbioses and their current ecosystem roles requires studying endophytes in these earlier clades of plants as well.

So far, studies have surveyed endophyte communities of some liverworts (Davis *et al.*, 2003; Davis & Shaw, 2008), mosses (U'Ren *et al.*, 2010; Davey *et al.*, 2012; Osono & Trofymow, 2012; U'Ren *et al.*, 2012; Davey *et al.*, 2013; Davey *et al.*, 2017), lycophytes (Higgins *et al.*, 2007), and ferns (Muthukumar & Prabha, 2012; Del Olmo-Ruiz & Arnold, 2017). Relatively few experiments have tested the effects of endophytes on these earlier-diverging plant hosts. Freeberg (1962) demonstrated that an unidentified endophytic fungus from *Lycopodium obscurum* could infect and increase growth of other *Lycopodium* species in the lab. Investigations of fungal effects on mosses has focused on pathogenic interactions (Akita *et al.*, 2011; Lehtonen *et al.*, 2012; Reboledo *et al.*, 2015). Some endophyte infections of liverworts have been resynthesized in the lab (Duckett & Read,

1995; Pressel *et al.*, 2008) and display host specificity (Duckett *et al.*, 2006) and effects on host morphology (Duckett *et al.*, 1991; Pressel *et al.*, 2008). More functional work has focused on arbuscular mycorrhizal fungal infection (Glomeromycotina) in liverworts and, to some extent, hornworts, demonstrating that these symbioses can be resynthesized in bryophyte hosts (Schüßler, 2000; Fonseca & Berbara, 2008; Silvani *et al.*, 2012) and that nutrient transfer occurs between partners (Humphreys *et al.*, 2010; Field *et al.*, 2012). Similar symbioses have also been shown between liverworts and fungi belonging to the Mucoromycotina (Field *et al.*, 2015; Field *et al.*, 2016). In addition, mutualism involving plant growth enhancement and nutrient transfer between the ericoid mycorrhizal fungus *Pezoloma ericae* and two species of leafy liverworts was recently demonstrated (Kowal, 2016).

For this study, we have developed a new experimental system for testing the effects of culturable endophytic fungi on a bryophyte host using the model liverwort, *Marchantia polymorpha* L. This liverwort has a global distribution where it grows in habitats with moderate natural or anthropogenic disturbance. It belongs to the complex thalloid group of liverworts, a basal branch of the liverwort phylogeny. As in other bryophytes, the perennial stage of the life cycle is the haploid gametophyte, and like most complex thalloid liverworts, it has gametophytes with separate sexes. *Marchantia polymorpha* has several features that make it particularly useful for experimental

investigations. Many replicate clones of the same plant genotype can be grown axenically from a single wild plant using surface sterilization of the liverwort's naturally produced asexual propagules (gemmae). It can be propagated in small Petri dishes on defined agar medium indefinitely by fragmentation of thalli. The liverwort can be grown to full size in a Petri dish and induced to form sexual structures. These features allow high-throughput experiments with fully grown hosts in which dozens of fungi can be screened for their effects using relatively little space and resources. *Marchantia polymorpha* is also a developing molecular model with a recently published genome sequence and established transformation procedures (Bowman *et al.*, 2016). At 230 Mb, the *M. polymorpha* genome is relatively small and has low gene redundancy (i.e. fewer genes filling each biochemical function) (Kato *et al.*, 2015; Berger *et al.*, 2016). These features of *M. polymorpha* and the resources now becoming available give it great potential for genetic investigations, including ones on symbioses.

In addition, studying a liverwort model presents the opportunity to study symbioses with potentially ancient origins. Liverworts are estimated to have diverged from mosses (prior to the origin of vascular plants) approximately 493 million years ago (Mya) (Cooper *et al.*, 2012). The major crown groups of fungi were diverging at a similar time, with Ascomycota originating some 531 Mya (Prieto & Wedin, 2013). The genus *Marchantia* is estimated to be approximately 126 Ma old and *M. polymorpha* to be 5 Ma

old (Villarreal *et al.*, 2016). The main classes of fungi tested in our experiments have estimated crown ages in a similar range to genus *Marchantia*; Dothideomycetes at 174 Ma, Leotiomycetes at 133 Ma, and Sordariomycetes at 130 Ma (Prieto & Wedin, 2013).

Here we present the results of two experiments testing the effects of fungi isolated from wild *M. polymorpha* plants on axenically grown wild-collected liverwort clones. The first experiment screened for growth effects of 100 fungal endophyte isolates and the second experiment examined the growth effects of 30 of those isolates with greater replication. The isolates represent a range of Ascomycete fungi and display experimental effects from completely killing *M. polymorpha* to significantly enhancing growth.

3.2 Materials and Methods

3.2.1 Endophyte collections

Fungal endophytes were cultured from surface sterilized fragments of *Marchantia polymorpha* collected from 17 sites in six USA states and one Canadian territory (Appendix C, Table 11). Endophytes were isolated separately from different plant parts: thalli, rhizoids, and, when present, reproductive gametangiophores. Tissue of origin was recorded for all isolates (Appendix C, Table 11). Endophyte cultures were initiated,

processed, and vouchered as described in Chapter 1. Origin and identity information for all isolates used in this study are provided in Appendix C, Table 11.

The fungi used in the growth experiments were all Ascomycetes belonging to the orders Dothideomycetes, Leotiomycetes, Pezizomycetes, Saccharomycetes, and, most frequently, Sordariomycetes. These isolates represented at least 45 fungal species (Appendix C, Table 11). Five isolates are of unknown identity because we were not able to obtain DNA sequences from them with the standard protocol we used.

A phylogeny of relationships between the fungal isolates was estimated based on amplicons of the full ITS ribosomal region and the beginning of the LSU region with Bali-Phy (Suchard & Redelings, 2006) using default settings with a GTR substitution model. We ran eight parallel MCMC chains and combined their results.

3.2.2 Liverwort axenic cultures

The *M. polymorpha* plants used in these experiments were collected from a weedy population at the North Carolina Botanical Garden in Chapel Hill, NC (NCB) in October 2013. Gemmae (asexual propagules) were collected from four plants, two female and two male gametophytes, to start axenic cultures. The gemmae were surface sterilized for two minutes in a 1:30 bleach solution, then rinsed with sterile deionized water (Miller, 1964). Gemmae were then transferred to modified Hatcher's agar (Hatcher, 1965) (see

Appendix C, Table 12 for recipe) and grown under lights with 12 hour day length at approximately 20° C.

Plants were checked for fungal and bacterial contamination visually and by extracting DNA with a CTAB protocol (Chapter 1) and using PCR to amplify fungal ITS and LSU and bacterial 16S DNA. The fungal primer pair was ITS1f (Gardes & Bruns, 1993) and LR3 (Vilgalys & Hester, 1990) and the bacterial primer pair was 27F and 1492R (Suzuki & Giovannoni, 1996). No amplification of fungi or bacteria was detected in any of the four plant clones and no contaminants were observed growing on the plants.

3.2.3 Experiment one: Fungal effect screen

Replicates of the four *M. polymorpha* individuals from the NCB site were initiated by punching circles five mm in diameter from axenically grown thalli. Plant replicates were grown on modified Hatcher's agar supplemented with 0.15% malt extract to allow initial fungal growth. Replicate plants were randomly assigned to experimental or control treatments.

One hundred fungal isolates were tested for their effects on *M. polymorpha* growth. Each fungus was inoculated onto one of each of the four plants from the NCB collection and an empty Hatchers+0.15% MEA plate (fungal control). The fungal controls were not measured or analyzed, but served as comparison in case unusual fungal

morphologies occurred in plant treatments. Fungi were inoculated onto plates in agar plugs 3 mm in diameter, approximately 1 cm away from the liverwort. Plates were kept at approximately 20° C with a 12 hour day length. During experiments, plate locations on growth shelves were shuffled to control for effects from any slight differences between or within shelves.

Eleven experimental plates were eliminated because of contamination and two were not used because the fungus grew so slowly it never met with the plant. This left eight fungal treatments with fewer than four tested plants. Since not all fungal DNA sequences were obtained before the beginning of the experiment, some of the 100 fungi ended up being identical by ITS1F-LR3 sequence despite morphological differences in MEA cultures. Data from fungi with identical ITS1f-LR3 sequences that had been isolated from the same starting plant fragment were pooled for calculations. Therefore, the final number of fungal treatments was 81, with 13 having more than four replicates. Table 11 (Appendix C) provides sample sizes for all isolates used.

3.2.4 Experiment one data processing

Data were collected in the form of digital photographs of plates every other day. Photos were captured with a Canon Powershot S100 camera mounted on a copy stand. The date when fungal growth first touched the liverwort thallus ("meeting day") was

recorded for each plate. Data were taken from each plate for 28 days following the recorded meeting date.

The area of green liverwort tissue in each photo was automatically measured with a custom macro using color thresholding in Fiji running ImageJ 1.51g (Schindelin *et al.*, 2012) (Fig. 7, Appendix D3). Dates and times of image acquisition were extracted from the metadata of each image using BR's EXIFextractor (www.br-software.com/extracter.html). Measured areas were plotted against time of photos to graph growth curves for each plant (Fig. 7). Points that appeared aberrant (e.g., were much higher or lower than points flanking them) in the growth curves were checked and plant areas manually re-measured if necessary. The most common reason for this was condensation on dish lids that obscured plants in photos.

On or soon after the ending day for each experimental plate, the plant and fungal growth was examined under 100x magnification while still in the plate using an Olympus BX41 microscope with mounted DP25 camera. Any features of plant morphology that appeared different from the control plants were noted and representative structures were photographed.

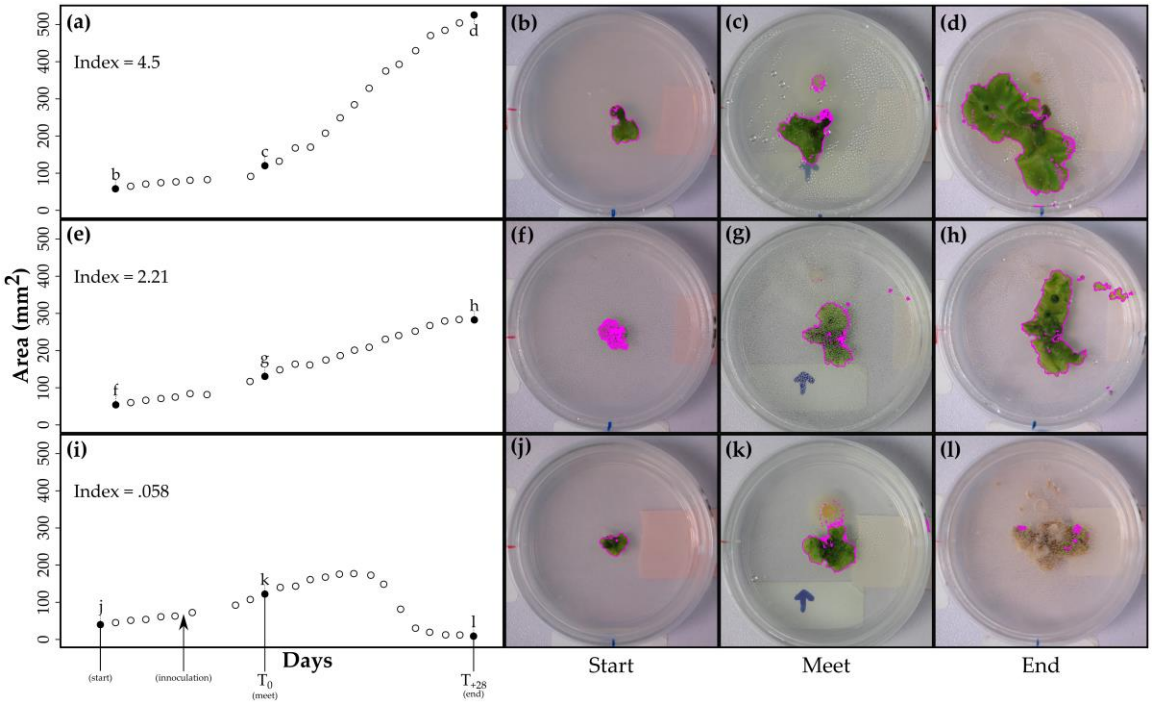

Figure 7: Measurement and evaluation of *M. polymorpha* growth curves, showing fungal treatments with positive, neutral, and lethal effects.
Graphs (a,e,i) show plant area measurements over the course of Experiment two for three plates. Photographs of each plate are shown, in order from left to right, from the first day of the experiment, the day the fungus first met the plant, and the final day of the experiment, 28 days after the meeting day. Outlines from plant area measurements with a custom ImageJ macro in Fiji are displayed in pink. (a-d) show interaction with a fungus that increased the plant's growth rate, (e-h) show a treatment that did not affect the plant growth rate, and (i-l) show a fungus that killed the plant. In order to describe the differences in these growth curve shapes, an index was calculated: $\frac{A_{max}}{A_{meet}} \times \frac{A_{min}}{A_{meet}}$

where A_{meet} is the plant size on the meeting day, A_{max} is the largest size from the meeting day onward, and A_{min} is the smallest size from the meeting day onward. Calculated growth indices for each plate are shown on the growth curve graphs. Points are filled in on the graph corresponding to the locations of data from the displayed images.

3.2.5 Experiment one data analysis

The shapes of plant growth curves were assessed by calculating an index that summarizes the amount and pattern of growth after the time the plant and fungus met. This was calculated as $\frac{A_{max}}{A_{meet}} \times \frac{A_{min}}{A_{meet}}$ where A_{meet} is the plant area on the meeting day, A_{max} is the largest area from the meeting day onward, and A_{min} is the smallest area from the meeting day onward (see Appendix D4 for R code). The time after meeting was the 28 day period for all plants, except those that were killed by their fungus before 28 days. An index value of 0 indicates that the plant died, a value between 0 and 1 indicates that the plant lost tissue area, a value of 1 indicates no net growth, and values above 1 indicate growth. Examples of values of this growth index for different growth curve shapes are presented in Fig. 7.

Growth indices were calculated for control plants by using the median meeting day of all fungal treatment plates as their meeting day. The means of all fungal treatments were compared to the distribution of growth index values for the controls to bin them into growth effect types. If the treatment mean was greater than the control mean plus two standard deviations, it was labeled “positive”; if the mean was between the control mean plus one and two standard deviations, it was labeled “weak positive”; if the mean was within one standard deviation of the control mean in either direction, it was considered “neutral”; and any mean less than 1 indicated death of plant tissue and

the treatment was considered “negative”. This classified all treatments since the control mean minus one standard deviation and the control minimum were both approximately one.

For treatments having at least 11 replicates based on pooling of ITS1f-LR3 identical fungi, an Analysis of Variance (ANOVA) was conducted in R 3.3.1 (R Core Team, 2014) to test whether the growth index of the fungal treatments differed significantly from those of a randomly selected set of 12 control plates. Before the ANOVA, normality of the data set was confirmed with a Shapiro-Wilk normality test and a qq-plot in R. A Bartlett test was used to check for homogeneity of variances and showed difference between groups ($p=0.006$).

3.2.6 Experiment two: Testing selected fungi

To screen a large number of fungal isolates, low replication was used in Experiment one. For Experiment two, 30 fungal isolates were selected to test with greater replication. Isolates with negative effects in Experiment one were not used because their detrimental impacts were unambiguous even with low replication. Instead, isolates for Experiment two were selected from those that showed growth promotion, no detectable effect, or particularly variable effects in Experiment one. Closely related fungi with differing effects were of particular interest. For one fungal

isolate (JN0315), two morphologies were separated in the culture after Experiment one, so each was tested separately in Experiment two.

Plant replicates were prepared and inoculated with fungi as in Experiment one. Each fungus was inoculated onto twelve plant plates (experimental plates) and two empty plates (fungal controls). The twelve plant replicates in a treatment included three each of the four plant clones used for Experiment one. Plates were kept in a temperature-controlled growth chamber at 15° C. Four plates from one fungal treatment were excluded because the fungus grew so slowly that it never met with the plant during the time of the experiment (see Table 11 in Appendix C for final sample sizes).

Photographs were collected and meeting days recorded as for Experiment one. To improve the efficiency of photo analysis, plates were labeled with unique QR codes. Photos were automatically renamed based on QR code labels and photo metadata using a custom shell script (Appendix D5) which automatically identified the plate based on its QR code via Zbarimg (zbar.sourceforge.net), as well as extracting date information using exiftool (http://www.sno.phy.queensu.ca/~phil/exiftool/). Measurement of plant areas, extraction of metadata, and growth curve plotting were conducted as in Experiment one. Post-experiment morphologies were not examined for Experiment two.

3.2.7 Experiment two data analysis

Growth index values were calculated as in Experiment one. Differences between index values for treatments and controls were assessed with an ANOVA implemented in R. The glht command in the R package multcomp (Hothorn *et al.*, 2008) was used to compute p-values for pairwise comparisons between each of the treatments and the control group. Since the data were not completely normal and variances were not homogeneous between groups, a Kruskal-Wallis test and Dunn's test of multiple comparisons were run as well, but gave similar results to ANOVA. The ANOVA results were more conservative in assigning significance to treatments, so we present them rather than the non-parametric results. For the purpose of comparison to Experiment one, fungal treatments with growth index values significantly greater than those of the controls ($p<0.01$) were considered to have a positive effect, any treatments with a mean growth index below one were labeled negative, and the rest were labeled as neutral.

In addition, ANOVAs and t-tests were implemented in R to check data from both experiments for any overall differences between plant genotypes or sexes and to test whether variability in responses to individual fungal isolates corresponded to plant genotypes or pooled fungal isolate identity. When ANOVAs produced significant F values, Tukey's honestly significant difference post-hoc test was used to obtain p-values.

3.3 Results

3.3.1 Overall liverwort growth

In Experiment one, the two male clones of *M. polymorpha* had growth significantly different from each other when grown as controls (p=0.034), but there were no other significant differences between controls. When all the fungal-treated plants were examined together, this genotype difference in growth was not observed (p=0.183). There was no difference in growth between any of the plant clones in Experiment two (p=0.241).

3.3.2 Experiment one growth effects

The majority of fungi did not change plant growth in comparison to the controls, but 19 isolates promoted growth (Fig. 8). The strongest growth promoting effects were observed for *Biscogniauxia mediterranea, Microsphaeropsis arundinis, Nemania* spp., and *Lasiosphaeriaceae* sp. 1 (Fig. 8). Weaker growth promotion was also observed from *Colletotrichum truncatum,* two species from *Conichaetaceae, Daldinia loculata, Helotiales* sp. 2, *Sordariales* sp. 1, *Hypoxylon* sp. 1, and *Sordariomycete* sp. 2 (Fig. 8).

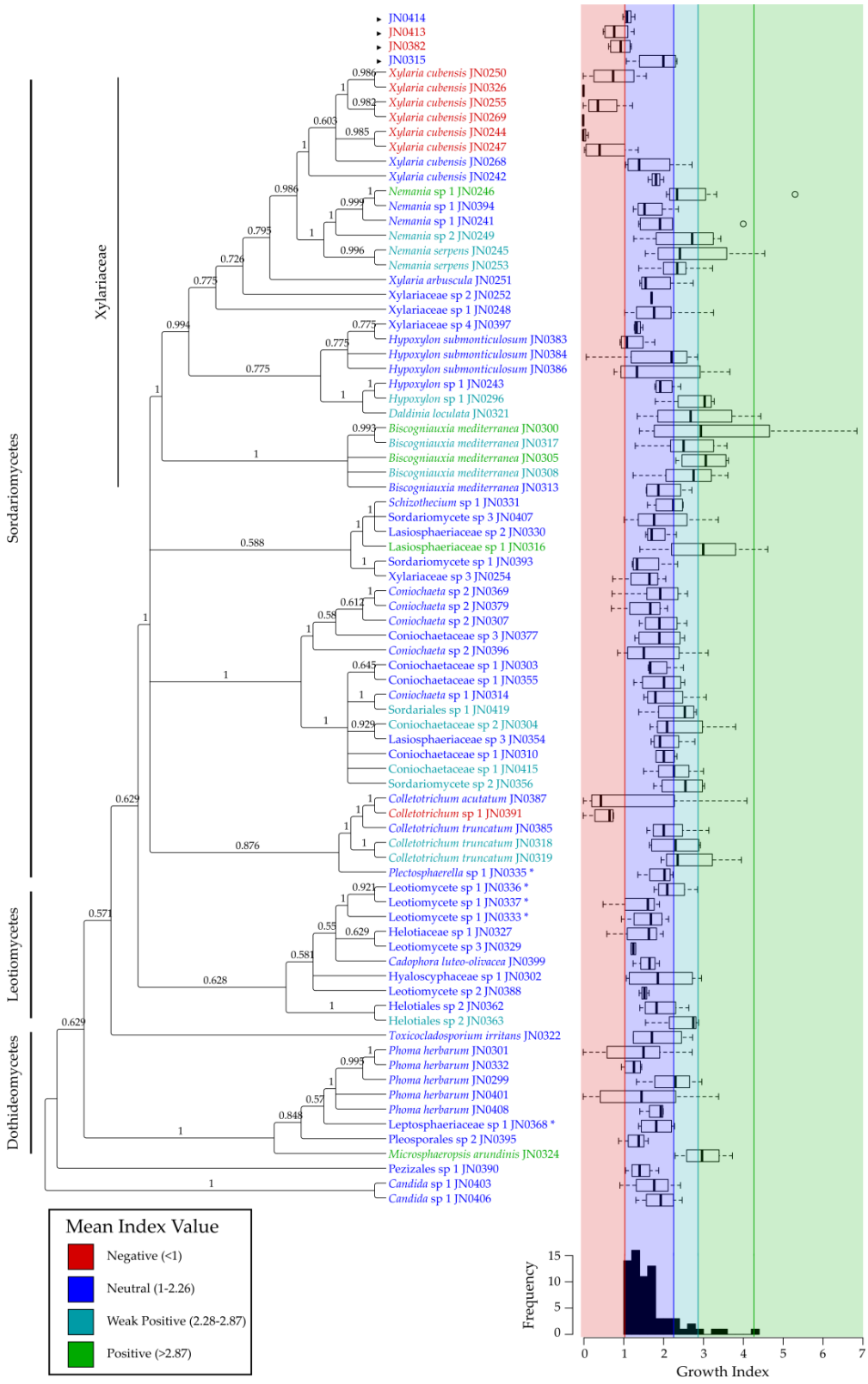
JN0414
JN0413
JN0382
JN0315
Xylaria cubensis JN0250
Xylaria cubensis JN0326
Xylaria cubensis JN0255
Xylaria cubensis JN0269
Xylaria cubensis JN0244
Xylaria cubensis JN0247
Xylaria cubensis JN0268
Xylaria cubensis JN0242
Nemania sp 1 JN0246
Nemania sp 1 JN0394
Nemania sp 1 JN0241
Nemania sp 2 JN0249
Nemania serpens JN0245
Nemania serpens JN0253
Xylaria arbuscula JN0251
Xylariaceae sp 2 JN0252
Xylariaceae sp 1 JN0248
Xylariaceae sp 4 JN0397
Hypoxylon submonticulosum JN0383
Hypoxylon submonticulosum JN0384
Hypoxylon submonticulosum JN0386
Hypoxylon sp 1 JN0243
Hypoxylon sp 1 JN0296
Daldinia loculata JN0321
Biscogniauxia mediterranea JN0300
Biscogniauxia mediterranea JN0317
Biscogniauxia mediterranea JN0305
Biscogniauxia mediterranea JN0308
Biscogniauxia mediterranea JN0313
Schizothecium sp 1 JN0331
Sordariomycete sp 3 JN0407
Lasiosphaeriaceae sp 2 JN0330
Lasiosphaeriaceae sp 1 JN0316
Sordariomycete sp 1 JN0393
Xylariaceae sp 3 JN0254
Coniochaeta sp 2 JN0369
Coniochaeta sp 2 JN0379
Coniochaeta sp 2 JN0307
Coniochaetaceae sp 3 JN0377
Coniochaeta sp 2 JN0396
Coniochaetaceae sp 1 JN0303
Coniochaetaceae sp 1 JN0355
Coniochaeta sp 1 JN0314
Sordariales sp 1 JN0419
Coniochaetaceae sp 2 JN0304
Lasiosphaeriaceae sp 3 JN0354
Coniochaetaceae sp 1 JN0310
Coniochaetaceae sp 1 JN0415
Sordariomycete sp 2 JN0356
Colletotrichum acutatum JN0387
Colletotrichum sp 1 JN0391
Colletotrichum truncatum JN0385
Colletotrichum truncatum JN0318
Colletotrichum truncatum JN0319
Plectosphaerella sp 1 JN0335 *
Leotiomycete sp 1 JN0336 *
Leotiomycete sp 1 JN0337 *
Leotiomycete sp 1 JN0333 *
Helotiaceae sp 1 JN0327
Leotiomycete sp 3 JN0329
Cadophora luteo-olivacea JN0399
Hyaloscyphaceae sp 1 JN0302
Leotiomycete sp 2 JN0388
Helotiales sp 2 JN0362
Helotiales sp 2 JN0363
Toxicocladosporium irritans JN0322
Phoma herbarum JN0301
Phoma herbarum JN0332
Phoma herbarum JN0299
Phoma herbarum JN0401
Phoma herbarum JN0408
Leptosphaeriaceae sp 1 JN0368 *
Pleosporales sp 2 JN0395
Microsphaeropsis arundinis JN0324
Pezizales sp 1 JN0390
Candida sp 1 JN0403
Candida sp 1 JN0406
Xylariaceae
Sordariomycetes
Leotiomycetes
Dothideomycetes
Mean Index Value
Negative (<1)
Neutral (1-2.26)
Weak Positive (2.28-2.87)
Positive (>2.87)
Frequency
Growth Index

Figure 8: Endophyte effects relative to fungal relationships, from experiment one. Phylogeny was estimated with BAli-Phy and posterior probabilities are shown for nodes. Fungal classes to which the majority of the fungi belong are labeled at the left of the cladogram. The most common family among the isolates, Xylariaceae, is also indicated. Taxa marked with * were isolated from fruiting bodies found on *M. polymorpha* thallus surfaces. Taxon name and boxplot shading color codes correspond to effect: negative (red) indicates a mean growth index value below 1, neutral (blue) has a mean value within one standard deviation of the control mean, weakly positive (teal) has a mean between one and two standard deviations above the control mean, and positive (green) have a mean above two standard deviations. The histogram shows the distribution of growth index values for all control plants. The boxplot shows the index value distributions for each fungal treatment. The green line on the boxplot shows the maximum growth index value in the controls, the teal line shows two standard deviations above the control mean, the blue line shows one standard deviation above the control mean, and the red line shows the minimum control value as well as one standard deviation below the control mean.

Some fungi killed host liverwort tissues. While some tissue death was observed in most treatments, including control plants, this was at least in part due to natural senescence of thalli. This did not impact effect calls as natural senescence never resulted in the plant shrinking to a smaller size than its measured meeting day area. Twenty-one isolates belonging to seven fungal species killed tissue of at least two of their four host plants. Of these 21, the fungi with strongest pathogenic effects were strains of *Xylaria cubensis* and two species of *Colletotrichum* (Fig. 8). The remaining taxa displayed less consistent negative effects and included *Hypoxylon submonticulosum*, *Phoma herbarum*, and two unidentified isolates.

Notably, one of the strains of *H. submonticulosum* appeared to kill plant tissue before visibly contacting it in both experiments, after which the plants continued growing from undamaged tissue regions. This pre-contact effect is not well reflected by the growth index values as they are calculated starting on the meeting day, but this effect only occurred in one isolate and was easily identified from the images and plotted growth curves.

Four fungal treatments had twelve or more replicates in Experiment one so they could be statistically evaluated using the same methods applied for Experiment two: *Phoma herbarum* (JN0301), *Coniochaeta* sp. 2 (JN0369), *Xylariaceae* sp. 1 (JN0248), and *Nemania serpens* (JN0253). When tested with ANOVA, only *N. serpens* differed from the controls ($p = 0.029$), showing a growth promoting effect. This is consistent with the results obtained from the binning by mean method used for the rest of the data set.

3.3.3 Experiment two growth effects

Most of the fungi tested in Experiment two showed growth promotion with growth indices significantly higher than the controls ($p<0.01$) (Fig. 9). Among the many growth promoting effects in Experiment two, an isolate of *Nemania serpens* (Xylariaceae) showed the strongest promotion (Fig. 9). Two strains of *X. cubensis* and one of *H. submonticulosum* showed negative effects, but their growth indices were not significantly

different from the controls. Natural thallus senescence was rare in this experiment, so we considered any obvious tissue death to indicate detrimental effects.

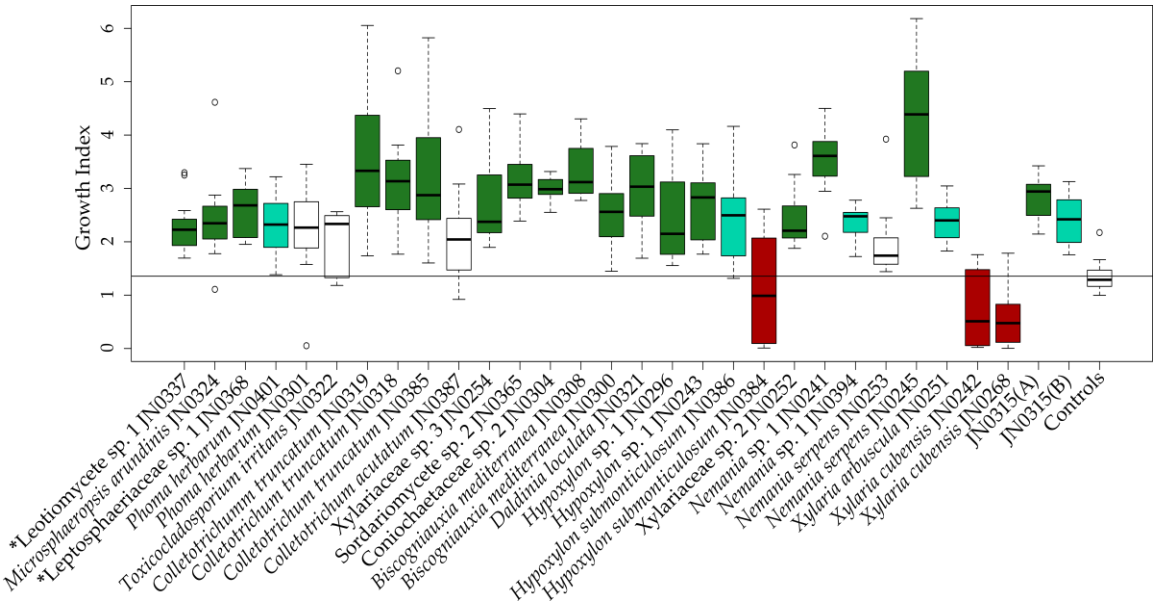

Figure 9: Endophyte growth effects on *M. polymorpha*, from experiment two. Boxplot represents growth index value distributions for treatments with each of 30 fungal isolates as well as 12 randomly selected control samples. ANOVA was used with the glht() command in R to compare each treatment to the control group. Green boxes indicate fungi that significantly increased plant growth at $p < 0.01$, teal indicates additional treatments with growth increases only significant at $p < 0.05$, and the red boxes are treatments with means less than one which indicates tissue killing. None of the treatments with means less than one were statistically significant, but any tissue death indicates negative effects. Taxa marked with * were originally isolated as fruiting bodies from *M. polymorpha* surfaces while all others were endophytes isolated from surface sterilized liverwort tissues.

3.3.4 Variability in isolate effects

Some isolates showed variable effects within a single experiment, with different plant replicates responding to the same fungus with either enhanced growth or tissue death. Taxa that had isolates with this level of effect variability were *Hypoxylon submonticulosum*, *Coniochaeta* sp., *Colletotrichum acutatum*, and *Phoma herbarum* (Fig. 8).

The variable effects of *P. herbarum* were especially visible within the JN0301 pooled isolate group in Experiment one (Fig. 8). In this group, the most negative effects are all from only one of the pooled sub-isolates (JN0416). Sub-isolate JN0416 produced growth indices lower than all other sub-isolates in its pooled group and this difference was significant when comparing to two of the other four sub-isolates ($p=0.446$, $p=0.020$). JN0416 was not the only sub-isolate that displayed tissue killing behavior, so it does not completely explain the variability in effect. Another isolate group of *P. herbarum* (JN0401 and JN0412) showed variation between sub-isolates and the two sub-isolates caused significantly different growth ($p=0.007$) with JN0401 showing weak positive effects and JN0412 showing negative ones.

Pre-contact killing behavior was only seen from one sub-isolate of *H. submonticulosum* (JN0409) in Experiment one, and not the other sub-isolate from the same plant piece (JN0384). Similarly, *H. submonticulosum* sub-isolate JN0386 caused tissue death in all treated plants while its paired sub-isolate JN0411 caused no tissue

death. In Experiment two, the JN0409 sub-isolate of *H. submonticulosum* showed striking effect variability: six of the twelve plant replicates stayed healthy with little or no tissue death, while the other six were killed or nearly so. The healthy and diseased *M. polymorpha* plants did not differ consistently in sex ($p=0.404$) though some non-significant differences were seen between plant clones ($p=0.119$). The plant clones that were most negatively affected by this fungal sub-isolate differed between Experiments one and two.

3.3.5 Effects of fungi on host morphology

Examination of plants at the conclusion of Experiment one revealed that infection with some fungal isolates produced morphological changes aside from tissue death. Dark spots were observed in plant thalli or individual thallus cells were browned (Fig. 10a-c). These thallus effects were most evident for *Xylaria cubensis* and other xylariaceous taxa, often accompanied by some tissue death (Appendix C, Table 11). The most striking interaction effects were observed in host rhizoids. *M. polymorpha* rhizoids are normally hyaline and belong to two types: smooth rhizoids are alive at maturity and pegged rhizoids are dead at maturity (Fig. 10d). Some fungi caused rhizoids to have melanized walls or dark internal inclusions (Fig. 10e-g). The most consistent melanization was in response to isolates of *Hypoxylon* species: seven of eight plants

inoculated with *Hypoxylon* sp. 1 had some brown rhizoids and six of 20 inoculated with *Hypoxylon submonticulosum* also showed rhizoid browning. In a few cases, fungal hyphae could be seen penetrating individual rhizoids (Xylariaceae sp. 1, *Phoma herbarum*) or apparently growing inside them (Pleosporales sp. 2) (Fig. 10h,i). Two fungi (*Biscogniauxia mediterranea, Colletotrichum* sp. 1) resulted in irregular granular inclusions in the lumen or on the walls of rhizoids (Fig. 10j,k). In addition, brown peg structures were observed in otherwise smooth rhizoids in plants treated with Pleosporales sp. 2 (Fig. 10l). Because of the large number of plates to be checked in the experiment, these morphological features were not rigorously quantified, but notes on observations are provided in Table 11 (Appendix C) and examples of morphologies are shown in Fig. 10.

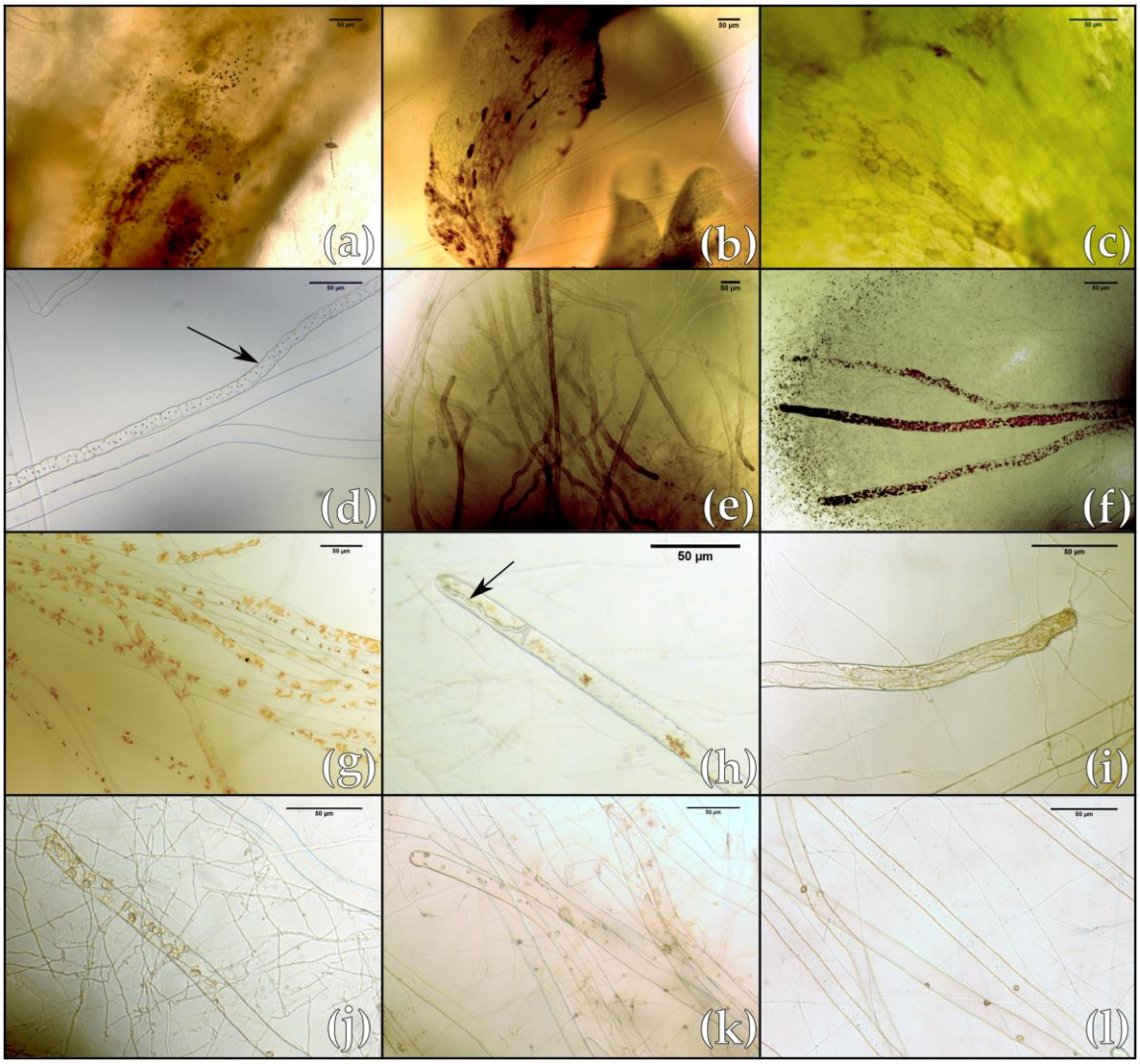
(a)
(b)
(c)
(d)
(e)
(f)
(g)
50 µm
(h)
(i)
(j)
(k)
(l)

Figure 10: Effects of fungal inoculation on *M. polymorpha* morphology, from experiment one.
Scale bars in upper right corners of images are all 50 μm (a) Dark spots in thallus of plant treated with *Xylaria cubensis* (JN0247). (b) Thallus cells filled with dark material after treatment with Xylariaceae sp. 4. (c) Scattered browning of thallus cells after treatment with Xylariaceae sp. 1 (JN0352). (d) Normal *M. polymorpha* rhizoids including both pegged rhizoids (arrow) that are dead at maturity and smooth rhizoids that are alive at maturity (all other rhizoids in image). (e) Melanized rhizoids after treatment with *Hypoxylon* sp. 1 (JN0243). (f) Dark spots in rhizoids after treatment with Xylariaceae sp. 1 (JN0352). (g) Brown inclusions in rhizoids after treatment with *Phoma herbarum* (JN0366). (h) Hypha (arrow) of *P. herbarum* (JN0301) penetrating tip of smooth rhizoid. (i) Hyphae of Pleosporales sp. 2 growing inside a rhizoid. (j) Inclusions in a rhizoid of a plant treated with *Colletotrichum* sp. 1. (k) Inclusions in a rhizoid of a plant treated with *P. herbarum* (JN0366). (l) Brown pegs on the walls of a rhizoid from a plant treated with Pleosporales sp. 2.

3.4 Discussion

The *M. polymorpha* endophyte experimental system presented here allowed screening of a large number of fungal isolates as well as the detection and quantification of their growth effects. These experiments demonstrate that fungal endophytes isolated from wild *M. polymorpha* plants can produce a wide range of effects on their liverwort host, from lethal to strongly growth promoting. Across the two experiments, 20 fungal species showed some growth promotion, five fungi showed some pathogenic impacts, including the ability to completely kill *M. polymorpha*, and 23 fungi demonstrated no growth effects.

3.4.1 Comparison of growth effects between experiments one and two

Between the two experiments, 60% of fungi were categorized into the same effect class (positive, neutral, or negative) and the remainder shifted in strength one way or the other. Eight of the isolates tested changed from being assessed as neutral in Experiment one, to positive in Experiment two; 19 isolates stayed the same; and three isolates showed more detrimental effects—two neutrals showed negative effects, and one positive showed neutral effects.

In Experiment two, significant positive effects outnumbered neutral ones and fungi that had shown no detectable effect in Experiment one displayed significant effects. It is possible that the greater replication in Experiment two allowed more sensitive detection of weak growth promotion or that slightly different growth conditions between experiments shifted the observed effects. Experiment two was run in a temperature-controlled growth chamber while Experiment one was run in a less controlled laboratory room. In both rooms, humidity varied with season and the two experiments were run at different times of year, Experiment one from November to February at a time of low humidity and Experiment two from June to August when ambient humidity was highest. The shift of effects between experiments could also have to do with maintaining fungal inocula over time in the lab, since there was a gap of six months between the inoculations of the two experiments. Even so, across both

experiments a few taxa stand out as having consistently stronger growth promotion, in particular, *Colletotrichum truncatum* and *Nemania* species.

3.4.2 Morphological effects of fungi

The morphological impacts of fungal colonization observed in Experiment one show rhizoids as an important interface for liverwort interactions with fungi. This is consistent with previous studies of the infection patterns of fungal associates of leafy liverworts (Duckett *et al.*, 1991; Duckett & Read, 1995; Duckett *et al.*, 2006; Upson *et al.*, 2007; Pressel *et al.*, 2008; Newsham & Bridge, 2010). Notably, not all fungal isolates originally derived from *M. polymorpha* rhizoid samples showed obvious infection morphologies of rhizoids in these experiments. Conversely, the morphological effects on rhizoids were not limited to rhizoid-derived isolates, but this could be due to some rhizoids ending up in fragments of thallus and gametangiophore tissues during initial endophyte isolation. *Marchantia polymorpha* gametangiophores have grooves containing bundles of rhizoids running through them and the mats of rhizoids attached to the underside of the thalli are difficult to completely separate from the thallus tissue.

The morphological effects of fungi in Experiment one also clearly indicate that not all the fungi tested colonize strictly asymptomatically. This might be an artifact of inoculating plants with only a single fungus under laboratory conditions, but it may also

hint at the continuum of effects that endophytic fungi can have on their hosts. Some of the morphologies could be signs of stress responses in the host plants. The browning of scattered thallus cells could indicate a defense response to localized fungal attack. Similar browning was observed in the cells of the model moss *Physcomitrella patens* when infected with the pathogen *Colletotrichum gloeosporioides* and the authors proposed that the brown coloration could indicate accumulation of phenolic compounds (Reboledo *et al.*, 2015).

The brown peg-like structures observed in *M. polymorpha* rhizoids in response to Pleosporales sp. 2 look similar to structures observed in protonema and rhizoids of the moss *Funaria hygrometrica* when associated with fungi (Martínez-Abaigar et al., 2005). Martínez-Abaigar *et al.* (2005) concluded that these dark pegs were entry points of infecting fungal hyphae, and they may also indicate fungal penetration in our study though we cannot confirm that. Previous studies of basidiomycete and ascomycete associations with leafy liverworts have also reported peg-like wall ingrowth structures at hypha penetration locations and the authors suggest that these could represent nutrient-exchange interfaces and indicate the potential for mutualism (Duckett *et al.*, 1991; Kottke *et al.*, 2003).

3.4.3 Pathogenic effects

Some of the isolated fungi acted as aggressive pathogens in these experiments even though they were originally isolated from healthy plants. Similar observations have previously been reported for fungal endophytes of *Arabidopsis thaliana* and *Centaurea stoebe* (Newcombe *et al.*, 2009; Junker *et al.*, 2012). This could be due to the artificial growth conditions in a greenhouse and axenic culture in a petri dish or to the amount of fungus to which the plants were exposed. Swett and Gordon (2017) found that a known pine pathogen could also cause growth-enhancement and disease resistance in its host depending on amount and duration of exposure to fungal inoculum. They also suggest that part of the growth enhancement could result from increased mycorrhization of roots observed in the plants treated with the fungal pathogen (Swett & Gordon, 2017), pointing out the importance of interactions between plant-associated microbes in determining plant success. A possible explanation for our observation of strong negative symptoms from fungi isolated from asymptomatic tissue could be that the interactions between members of plant microbiomes in the wild mediate the effects of fungi that would otherwise act as pathogens. This is supported by studies that show certain bacterial or fungal endophytes can inhibit colonization and growth of plant pathogens or increase mycorrhization (Arnold *et al.*, 2003; Tellenbach & Sieber, 2012; Adame-Álvarez *et al.*, 2014; Hugentobler *et al.*, 2014; Hacquard & Schadt,

2015; Terhonen *et al.*, 2016; van Overbeek & Saikkonen, 2016). Future work in experimental systems for endophyte biology should investigate the complex interplay of interacting microbiome members and environmental factors that shapes plant success. The *M. polymorpha* system presented here is amenable to inoculation with multiple fungi and one such experiment is presented in Chapter 4 of this dissertation.

3.4.4 Variability of growth effects

We observed high variability in plant responses to some of the fungal species tested. Variation between strains of the same species of fungi in their effects on hosts has been previously documented for some dark septate endophytes (Mandyam *et al.*, 2012; Mandyam *et al.*, 2013), but has not often been investigated. In some cases, we also found variability between morphotypes of the same species isolated from the same plant fragment. It is possible that separately maintained cultures changed over time in the lab, leading to these different effects. Fungal cultures that were split into multiple sub-isolates had variable morphologies that prompted the sub-culturing initially, so it is possible that these particular species are inherently more variable in interactions with plants as well as growth morphologies.

3.4.5 Fungi fruiting on *M. polymorpha*

Fungi that fruit on bryophyte surfaces have been generally classified as parasites even if they do not produce visible harm to their hosts (Döbbeler, 1997). The five fungi that we obtained from fruiting bodies all produced no detectable effects in Experiment one and the two that we tested further in Experiment two showed growth promotion. We did not observe any formation of fruiting bodies in our experiments. This suggests that the parasitic interactions of these bryophilous fungi may be context-dependent. It is possible that fruiting on bryophyte surfaces is one part of a more complex plant-fungal interaction.

3.4.6 Comparison to similar fungi in other hosts

Some of the fungal isolates used in these experiments are known associates of other plant species. For example, various *Colletotrichum* species are known as aggressive plant pathogens that cause anthracnose and leaf spot in many agriculturally important plants (Freeman *et al.*, 1998; Latunde-Dada, 2001), but *Colletotrichum tofeldiae* has been found to be a beneficial endophyte for *Arabidopsis thaliana* (Hiruma *et al.*, 2016). *Colletotrichum tofeldiae* and its pathogenic relative *Colletotrichum incanum* provide a useful system for studying the mechanisms of fungal endophytes with contrasting effects in *A. thaliana* (Hacquard *et al.*, 2016). Similarly, studying the opposing effects on *M. polymorpha*

of *C. acutatum* and *C. truncatum* observed in our experiments could help reveal how liverworts respond to fungi on both ends of the endophytic effect continuum.

Phoma herbarum seems to be a widespread associate of *M. polymorpha* (Chapter 1), and our experiments do not clearly categorize it as either a mutualist or pathogen in this liverwort.*Phoma herbarum* can be found in vascular hosts such as soybean and has been shown to promote rice and soybean growth (Hamayun *et al.*, 2009). A close relative of *P. herbarum*, *Phoma muscivora,* is a moss pathogen that can degrade bryophyte cell walls (Davey & Currah, 2009; Davey *et al.*, 2009).

Forty of the 100 endophyte isolates used in these experiments belong to the family Xylariaceae (Ascomycota, Sordariomycetes, Xylariales). Xylariaceous fungi are common culturable members of endophytic communities (Yuan, ZL *et al.*, 2011; Osono *et al.*, 2013; Thomas *et al.*, 2016) and are especially prominent in culture collections from liverwort hosts (Davis *et al.*, 2003; Davis & Shaw, 2008). Many of these fungi are known as wood-rotters (Johannesson *et al.*, 2001; Balasuriya & Adikaram, 2009) and some may have lifestyles that involve cycling between endophytic and wood-decomposing stages (Thomas *et al.*, 2016). In addition, some of the xylariaceous fungi included in this study are known pathogens in woody angiosperms. *Biscogniaxia mediterranea* causes charcoal disease of oaks (*Quercus*) and other hardwoods (Rostamian *et al.*, 2016) and *Xylaria arbuscula* has been linked to disease in *Macadamia* trees (Ko & Kunimoto, 1991). The

results of our experiments indicate that liverworts may also have varied relationships with xylariaceous fungi, including taxa that act as mutualists and pathogens.

It is important to note that knowing the effects of particular fungi in vascular plant hosts did not allow prediction of their impact on a liverwort host. The lack of correspondence between fungal effects in phylogenetically divergent hosts highlights the need to study endophyte functions across a wide range of host plants. Fungi that are pathogens in certain vascular plants may be important mutualists in non-vascular hosts. Recent work is beginning to show that the same fungal isolates can have very different effects on different vascular hosts, including fungi traditionally considered pathogens acting as mutualists on other hosts (Redman *et al.*, 2001; Kia *et al.*, 2017). In addition, Lehtonen *et al.* (2012) showed that fungal isolates pathogenic to two mosses have variable effects on vascular crop plant hosts, demonstrating that there are indeed differences between vascular and non-vascular hosts in responses to fungal colonizers. Continuing to study fungal interactions in an evolutionarily broad sampling of plants will expand understanding of the ecologies of these plant-associated fungi, likely revealing that labeling individual fungi as only mutualist or pathogen is insufficient to capture their true roles.

It remains to be seen if there are consistent differences in endophyte effects between major plant lineages, especially as the mechanisms behind most of these effects

have yet to be elucidated. Parts of the common symbiosis pathway are conserved across most major plant groups from liverworts to angiosperms (Wang *et al.*, 2010). However, recent research on molecular mechanisms of endophyte effects indicates that they do not all rely on this core pathway for mycorrhizal and rhizobial symbioses (Banhara *et al.*, 2015). Therefore, expanding genetic analyses of plant-microbial symbioses is likely to reveal a wealth of previously undescribed interaction mechanisms.

3.4.7 Concluding remarks

We propose that *M. polymorpha* can provide an important tool in future investigations of the functions, molecular mechanisms, and evolution of plant-fungal symbioses. *Marchantia polymorpha* is an attractive genetic model since it has many of the same pathways as vascular plants, but with less genetic redundancy (Kato *et al.*, 2015). The genomic resources becoming available for *M. polymorpha* combined with the evidence for a wide range of fungal interactions demonstrated in this study, make this liverwort system promising for studying symbiosis mechanisms. Since some of the symbiosis mechanisms in liverworts are conserved all the way to angiosperms (Wang *et al.*, 2010), results from experiments with *M. polymorpha* have the potential to be applicable beyond bryology. As more work on angiosperm models explores plant microbiomes, it will also be important to have non-vascular comparisons like *M.*

polymorpha to build knowledge of the evolution of these symbioses that are so central to the existence and ecology of terrestrial plants.

4 Chapter 3: Impacts of fungal endophytes on the liverwort *Marchantia polymorpha* L. depend on nutrient levels

4. 1 Introduction

Plants harbor diverse communities of fungi inside their healthy tissues, including different kinds of mutualistic mycorrhizal fungi, latent pathogens, and many other endophytic fungi with diverse functions. These fungal symbionts are important drivers of plant success since they can affect growth (Banhara, Ding, Kühner, Zuccaro, & Parniske, 2015), competition (Aschehoug, Callaway, Newcombe, Tharayil, & Chen, 2014), nutrient use (Yang et al., 2014) and responses to biotic and abiotic stressors in their hosts (Partida-Martinez & Heil, 2011; Porras-Alfaro & Bayman, 2011). Through these impacts, endophytes contribute to determining plant community structure and thereby broader ecosystem properties as well (Afkhami & Strauss, 2016; Aguilar-Trigueros & Rillig, 2016; Rudgers, Koslow, & Clay, 2004).

Plant-fungal interactions are also dynamic, with the outcomes depending on timing, dosage, environmental conditions, and complex signaling between partners (Johnson, Graham, & Smith, 1997; Partida-Martinez & Heil, 2011). The beneficial effects of certain non-mycorrhizal endophytes have been shown to depend on nutrient availability (Hiruma et al., 2016; Qin et al., 2017), soil moisture (McCormick, Gross, &

Smith, 2001), and level of plant exposure to the fungus (Swett & Gordon, 2017). Individual fungi may have interactions with the same plant host that span a wide range on the continuum from pathogen to mutualist; some fungi may be asymptomatic endophytes at one point in their life cycle, but pathogens or saprobes at another time (Porras-Alfaro & Bayman, 2011; Schulz & Boyle, 2005). Fungi traditionally considered pathogens can act as mutualists in other hosts (Chapter 2) (Redman, Dunigan, & Rodriguez, 2001). In addition, closely related fungi can show opposite effects on the same host and it appears that such evolutionary transitions have occurred often (Fesel & Zuccaro, 2016) and can happen with very few genetic changes (Freeman & Rodriguez, 1993).

Since nutrient sharing is a key component of mycorrhizal symbiosis, dependence of interactions on nutrient conditions has been of particular interest in investigating the dynamic nature of plant-fungal symbioses. In mycorrhizae and other nutritional mutualisms, it is commonly accepted that plants benefit only under nutrient limitation and lower mycorrhization is often observed in higher nutrient environments (Breuillin et al., 2010; Partida-Martinez & Heil, 2011; Treseder, 2004). Meta-analyses of context dependency in mycorrhizal symbiosis indicate that nitrogen and phosphorus availability and the interplay between them is important in determining the outcome of the interaction (Hoeksema et al., 2010; Treseder, 2004). Nutrient dependencies have also

been demonstrated for non-mycorrhizal endophytes. Fitness benefits for *Arabidopsis thaliana* from the root endophyte *Colletotrichum tofieldiae* depend on the availability of phosphorus, with the fungus acting as a phosphorus-transferring mutualist only under low phosphorus conditions (Hacquard et al., 2016; Hiruma et al., 2016). Growth enhancement of grasses by certain endophytes can disappear at low soil nutrient levels or even switch to growth impairment, potentially because the plant and fungus begin to compete for resources (Cheplick, Clay, & Marks, 1989; Ren et al., 2011). Qin et al. (2017) demonstrated that the type of nitrogen source, organic or inorganic, can change the effects of certain endophytes belonging to the Pleosporales. Fungal inoculation tended to show neutral or negative effects on plant hosts when only inorganic nitrogen was provided, but when in the presence of organic nitrogen, most of the fungi promoted plant growth (Qin et al., 2017). Such changes in interaction outcomes based on abiotic conditions have been hypothesized to indicate that harboring the endophytes in question has some cost for the host plant (McCormick et al., 2001; Rasmussen, Parsons, Fraser, Xue, & Newman, 2008).

Most investigation of context dependency in the outcomes of fungal endophyte symbioses has focused on seed plants, but earlier diverging plants, including ferns, lycophytes, mosses, liverworts, and hornworts, also host rich fungal endophyte communities (Davey et al., 2017; Davis & Shaw, 2008; Pressel, Bidartondo, Field,

Rimington, & Duckett, 2016; Pressel, Bidartondo, Ligrone, & Duckett, 2010). Nutrient exchange mutualisms with dependence on CO_2 levels have been demonstrated for four liverworts and a fern (Field et al., 2012; Field et al., 2016; Humphreys et al., 2010), but to the authors' knowledge, no other studies have investigated the effects of environmental factors on fungal interactions with non-spermatophyte hosts.

Here we present the results of an experiment testing the nutrient dependency of fungal endophyte growth promotion of a liverwort. We have established an experimental system for investigating culturable fungal endophyte effects on the model liverwort *Marchantia polymorpha* and have previously screened for growth effects of 100 fungal strains originally isolated from wild *M. polymorpha* plants (Chapter 2). In the present study, we use two growth-promoting isolates identified as *Nemania* sp. and *Colletotrichum truncatum* to test whether the growth effects change with overall nutrient availability. If these endophytes promote growth in a nutrient-dependent fashion, we would predict to see loss of growth benefits at either high or low nutrient levels. Loss at high nutrients would be in line with a nutrient exchange mechanism like mycorrhizae while loss at low nutrients would indicate a different promotion mechanism and possible competition between partners. In addition to the growth experiment, we present microscopy of the infection morphologies of these two endophytes.

4.2 Materials and Methods

4.2.1 Plant and fungal materials

The *M. polymorpha* plants used in these experiments were originally collected in Chapel Hill, NC in October 2013. Axenic cultures were initiated from surface-sterilized gemmae of a male gametophyte and propagated by sterile fragment transfers.

Fungi were isolated from surface-sterilized *M. polymorpha* plants collected in North Carolina. (See Chapter 1 for detailed methods). For this experiment, we used two Sordariomycete fungi that strongly promoted *M. polymorpha* growth in previous screens (Chapter 2): a strain of *Nemania* sp. (Xylariaceae, JN0241) and one of *Colletotrichum truncatum* (Glomerellaceae, JN0319).

4.2.2 Growth experiment

Replicate plants were grown from gemmae derived from lab-grown axenic *M. polymorpha* clones of a single collected gametophyte. Gemmalings were grown in petri plates on media with four different levels of modified Hatcher's (Hatcher, 1965) mineral nutrients: normal nutrient levels used for previous experiments (1x) as well as 2x, 1.5x, and 0.5x. All nutrient level media were supplemented with 0.15% malt extract as a limited carbon source for the fungi (Chapter 2). Once established, gemmalings were inoculated with a 3 mm agar plug from a culture of *Nemania* or *Colletotrichum*, or left

uninoculated as controls. Based on the numbers of gemmalings that successfully established, all combinations of nutrient level and treatment had five plant replicates except the 1x nutrient level, for which all treatments had four replicates.

Growth of *M. polymorpha* plants was tracked by taking photographs of all plates three times per week. For each experimental plate, the date when the fungus first touched the plant was recorded ("meeting day"). Photographs started 14 days before inoculation and ended for each plate 28 days after its meeting day.

4.2.3 Data processing and analysis

The area of green liverwort tissue was measured in each photograph with a custom macro in Fiji running ImageJ 1.51g (Schindelin et al., 2012) (Chapter 2, Appendix D3). Date and time metadata were extracted from each photograph using BR's EXIFextractor (www.br-software.com/extracter.html). Growth curves of time versus size were plotted for each plant and checked for aberrant points i.e. ones that had very different measurements from their two flanking points. Any erroneous measurements were corrected by manually re-measuring the photograph in Fiji.

Growth responses to fungi were assessed using a model that estimated how much plant growth after meeting with the fungus deviated from linear growth predicted based on pre-meeting growth: $Y_t = \alpha + \beta_0\, t+1_{(t>0)}\, (\beta_1 t + \gamma_1 t^2) + \epsilon_t$. Time (t) was calculated as

days since meeting day, so the meeting day was t = 0. The model fit a linear equation for the pre-meeting growth ($\alpha + \beta 0t$) and added additional terms including a quadratic one to describe post-meeting growth ($\beta 1t + \gamma 1t2$). The model result was summarized by the linear contrast $c(t) = \beta 1 + \gamma 1t$. This contrast calculated at the final time represents the amount of deviation of the actual growth from projected linear growth and the value of this contrast is represented hereafter as $\hat{c}$. Both $\beta 1$ and $\gamma 1$ should be 0 for a growth curve with an unchanging linear trajectory, so $\hat{c} = 0$ would indicate no change in growth. Negative $\hat{c}$ values indicate less growth than expected from the linear projection and positive values indicate more than expected growth. For control plants, t = 0 was set to the median meeting day for inoculated plants. The final day for all fungus-treated plants was t =28 while the controls, which were run for the full length of the experiment and all set to the same t = 0, had final time of t = 36. R code for calculating $\hat{c}$ is provided in Appendix D6.

Values of $\hat{c}$ were compared between nutrient levels, fungal treatments, and the interaction of these two factors using Analysis of Variance (ANOVA) implemented in R 3.3.1 (R Core Team, 2014). Before ANOVA, the data were checked for normality with a Shapiro-Wilk test and for homogeneity of variances with a Bartlett test. In order to fit these assumptions of ANOVA, the $\hat{c}$ values were adjusted to all be positive by adding the same integer value to all and adjusted to a normal distribution by a square root

transformation. A Tukey's Honestly Significant Difference test was run to determine significance of comparisons between factor combination groupings.

4.2.4 Visualizing fungal colonization of *M. polymorpha* host plants

After the end of the growth experiment, plants were removed from their experimental plates to examine the patterns of fungal colonization in their tissues. Whole liverworts were fixed in 3:1 95% ethanol to glacial acetic acid (Clark, 1981) and stained with wheat germ agglutinin (WGA) conjugated to fluorescent CF 488A dye (Biotium). This stain binds N-acetylglucosamine, a monomeric unit in chitin, and sialic acid residues, thereby selectively staining fungal tissues. Fixed specimens were vacuum infiltrated with phosphate buffered saline (PBS) then incubated for 20 minutes. Then specimens were vacuum infiltrated with WGA CF 488A in PBS three times and incubated overnight at room temperature in the dark. Liverwort thalli were mounted in PBS and examined with a FitC filter on a Leica DMRBE fluorescence microscope. Specimens were also examined under brightfield to correlate fluorescence with plant cell structures.

4.3 Results

4.3.1 Growth Experiment

The fungal treatment main effect was significant ($p < 0.001$), the nutrient main effect was not ($p = 0.0531$), and the interaction between fungal treatment and nutrient level was significant ($p = 0.0167$). The post-hoc Tukey test showed that the growth impacts of *Nemania* and *Colletotrichum* were not different from each other at any of the individual nutrient levels ($p > 0.9$), but both fungal treatments differed from the controls at all nutrient levels except the lowest, 0.5x (Fig. 11). The growth of control plants did not differ across nutrient levels ($p > 0.4$) nor did the absolute size of control plants differ between nutrient treatments ($p = 0.497$).

4.3.2 Microscopy

Fungal hyphae were observed colonizing treated plants. Both *Nemania* and *Colletotrichum* produced hyphae that grew tightly appressed to the liverwort surface, sometimes following the margins between plant cells (Fig. 12). Some hyphae appeared to run between cells within the liverwort tissue, but none were observed penetrating plant cells. Hyphae also wrapped around some rhizoids and a few seemed to be running inside the rhizoids. Colonization by both fungi was patchy so it was unclear whether

there were any morphological distinctions between infection on the different nutrient media. No fungal structures were observed on control plants.

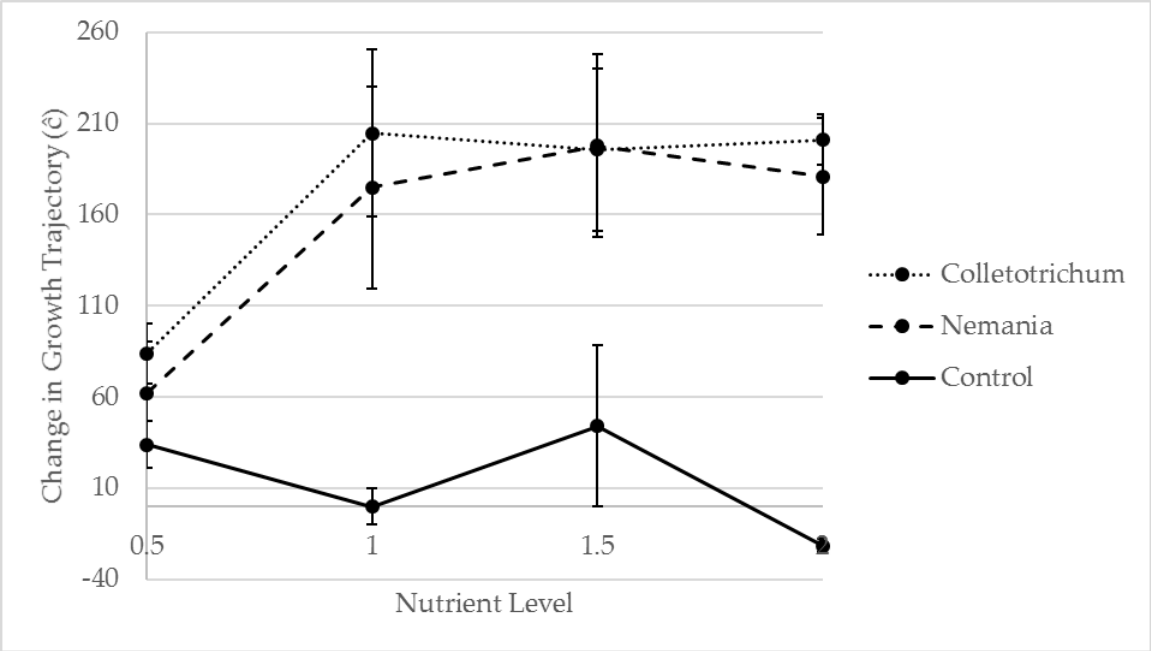

Figure 11: *M. polymorpha* growth changes in response to fungi across nutrient levels. Results for inoculation with *Nemania* sp. are shown with a dashed line, for inoculation with *Colletotrichum truncatum* with a dotted line, and for uninoculated control plants with a solid line. Dots show the means of model outputs representing changes in liverwort growth after meeting with the fungus (ĉ) and error bars are one standard error in each direction.

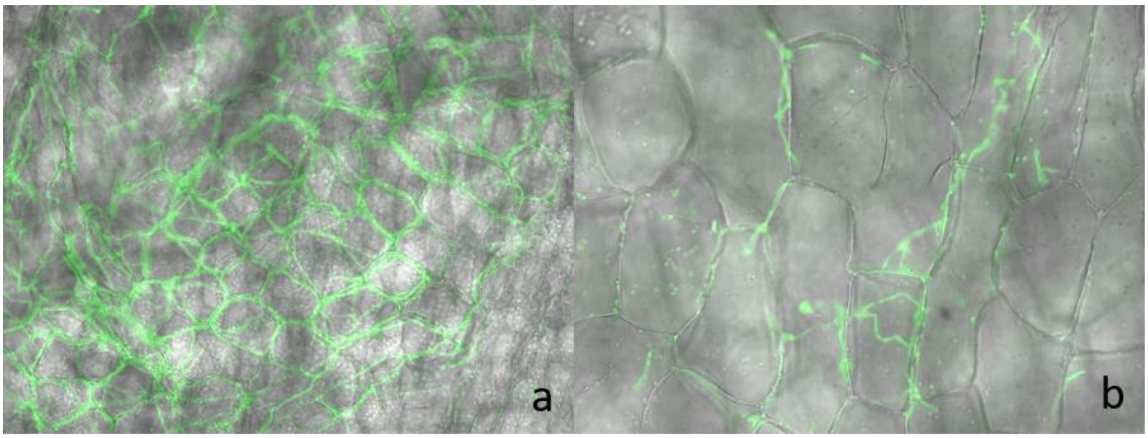

Figure 12: Colonization structures of fungi in *M. polymorpha*
Fungal hyphae are shown in green over light micrographs of liverwort thallus cells. A. *Colletotrichum truncatum* with plant growing on 1.5x nutrient medium. B. *Nemania* sp. with plant growing on 2x nutrient medium.

4.4 Discussion

Despite their different taxonomic affinities, *Nemania* and *Colletotrichum* both showed the same pattern in growth promotion across nutrient levels, with similar strong growth promotion across the normal and high nutrient levels (1x, 1.5x, 2x) that disappeared at the low (0.5x) nutrient level. Based on investigations of nutrient-dependence in mycorrhizal and other nutrient-sharing symbioses (Hiruma et al., 2016; Partida-Martinez & Heil, 2011; Treseder, 2004), this result suggests that *Nemania* and *Colletotrichum* are not promoting *M. polymorpha* growth by enhancing nutrient scavenging ability. In line with this hypothesis, both fungi displayed infection patterns consistent with endophytes rather than nutrient-transfer symbioses like mycorrhizae

(Clay, 1990; Peterson & Massicotte, 2004); no arbuscules or other nutrient-transfer interface structures were observed. Also in contrast to the expected dynamics of nutrient-sharing symbioses, uninoculated *M. polymorpha* showed no growth change across the range of nutrient levels. Bryophytes tend to have low nutrient requirements and some even prefer laboratory media with no added nutrients (Duckett et al., 2004), so the lack of nutrient limitation observed in our experiment is unsurprising.

Other possible mechanisms for growth promotion by fungal endophytes include plant hormone production by fungi and induced changes in plant gene expression or metabolism (Wani, Ashraf, Mohiuddin, & Riyaz-Ul-Hassan, 2015). Lower nutrient levels could impair fungal ability to produce compounds that promote plant growth or put the plant and fungus in direct competition for resources, switching the balance of their relationship. This latter possibility has been suggested in the case of grass endophytes that are observed to reduce growth under low nutrient conditions but enhance it when nutrients are plentiful (Cheplick et al., 1989).

Future investigations sampling transcriptomes and metabolomes of *M. polymorpha* with *Nemania* or *Colletotrichum* could elucidate the basis of the growth promotion induced by these fungi and the factors that change the balance at low nutrient levels. Similar investigations into the mechanisms of endophyte growth promotion in other host systems will be necessary to build an understanding of the

diversity of functioning in endophyte relationships. Comparisons of findings from the *M. polymorpha* system with those from angiosperm models will help in constructing a framework for understanding the evolution of endophyte symbioses across the plant tree of life and the dynamics of these fungi in modern ecosystems.

5 Chapter 4: The liverwort *Marchantia polymorpha* L. displays different growth responses to inoculation with individual versus combined fungal endophytes

5.1 Introduction

Plants host diverse fungal endophyte communities inside them (Stone *et al.*, 2000). This term "fungal endophyte" covers fungi that range from parasitic to mutualistic with their plant hosts, including nutrient-gathering mycorrhizal fungi and latent pathogens and saprobes (Schulz & Boyle, 2005; Porras-Alfaro & Bayman, 2011). Non-mycorrhizal endophytes, which are both taxonomically and functionally diverse, can impact the success of their plant hosts in many ways including providing resistance to drought (Molina-Montenegro *et al.*, 2016), salt stress (Azad & Kaminskyj, 2016), heavy metals (Yamaji *et al.*, 2016), herbivores (Jaber & Vidal, 2010), or pathogens (Busby *et al.*, 2016). Some fungal endophytes promote host growth (Banhara *et al.*, 2015), change plant metabolism (Prasad *et al.*, 2013), increase nutrient use efficiency (Yang *et al.*, 2014), or strengthen allelopathic competition with neighboring plants (Aschehoug *et al.*, 2014). These many effects on plant hosts make endophytes important in structuring plant communities and contributing to ecosystem functioning (Rudgers *et al.*, 2004; Afkhami & Strauss, 2016; Aguilar-Trigueros & Rillig, 2016).

A variety of methods have been used to document the impacts of endophytic fungi on their host plants. One strategy is to conduct experiments inoculating axenic plants with individual endophytes. Such experiments reveal that fungi isolated from healthy plants can have a range of effects on their host in isolation, from growth promotion and protective functions to pathogenesis (Chapter 2) (Redman *et al.*, 2001; Newcombe *et al.*, 2009; Junker *et al.*, 2012; Rinu *et al.*, 2014). The reductionist approach of individual inoculation is useful for ensuring that the effects observed can be traced to a particular fungus, but endophytes in nature are part of diverse microbiome communities. Therefore, inoculation with individual isolates is unlikely to yield realistic assessments of endophyte effects.

Studies have also tested pairwise interactions between endophytes, especially inhibition of pathogens. One commonly used approach is *in vitro* inhibition tests on pairs of microbes in laboratory culture (Combès *et al.*, 2012; Díaz *et al.*, 2013; Rinu *et al.*, 2014; Orlandelli *et al.*, 2015), but it has been demonstrated that these results do not correlate strongly to the protective effects of endophytes against pathogens *in planta* (Shittu *et al.*, 2009; Hugentobler *et al.*, 2014; Terhonen *et al.*, 2016). Experiments with dual inoculations of plant hosts show that certain endophytes can protect against bacterial, fungal, or oomycete pathogens (Campanile *et al.*, 2007; Tellenbach & Sieber, 2012; Adame-Álvarez *et al.*, 2014; Terhonen *et al.*, 2016), but these effects can be variable based

on timing and inoculation method (Campanile *et al.*, 2007; Adame-Álvarez *et al.*, 2014). Few studies have investigated pairwise interactions beyond pathogen inhibition, but these few show that interactions between multiple fungi can shift the balance of the overall effects on the host plant. For example, the effects of the root endophyte *Chaetomium elatum* on host avocado trees depends on the presence of an arbuscular mycorrhizal fungus and light levels (Violi *et al.*, 2007).

Pairwise comparison studies still only focus on a small fraction of the microbes that would naturally be present in a host. Some studies address this problem of extremely reductionist approaches by instead testing the effects of natural microbial communities from soil inocula or large synthetic communities assembled from culture collections (Bonito *et al.*, 2014; Rinu *et al.*, 2014; Müller *et al.*, 2016). Different complex inocula produce different growth effects in host plants and enrichment of certain endophytes can shift the composition of the overall plant microbiome. These large community methods work with more realistically diverse microbial assemblages, but also yield data that are more difficult to interpret and can require subsequent combinatorial methods to determine whether the observed effects are based on certain individual taxa or interactions between many.

Few studies take an intermediate approach to studying fungal endophyte interactions that uses small synthetic communities. This can be done by inoculating with

mixtures of multiple endophytes for pathogen challenge experiments (Arnold *et al.*, 2003) or assembling all possible combinations of a small number of endophytes to test how their interactions impact host response (Reininger *et al.*, 2012; Aguilar-Trigueros & Rillig, 2016). Expanding work with controlled small communities has great potential for informing about the dynamics of endophytic microbiota.

Whatever their approach, most studies on the effects of fungal endophytes have focused on angiosperm and conifer hosts, but diverse endophyte communities also inhabit the non-vascular bryophytes (mosses, liverworts, and hornworts). Bryophytes are present in most terrestrial and aquatic ecosystems and are even the dominant vegetation in parts of the boreal zone (Lindo & Gonzalez, 2010). They are important in the cycling of water, heat, and nutrients through ecosystems; provide important habitat for many small arthropods and microbes; and are important early colonists after ecological disturbance (Bradbury, 2006; Cornelissen *et al.*, 2007; Lindo & Gonzalez, 2010). The fungal endophyte communities in mosses and liverworts have similar diversity to the those of vascular plants (Davis & Shaw, 2008; U'Ren *et al.*, 2012; Davey *et al.*, 2017), but the taxonomic compositions of these communities in bryophytes differ somewhat from those of vascular plants in the same area and instead bear more resemblance to those of nearby lichens (U'Ren *et al.*, 2010). Since bryophytes are significant components of many ecosystems, investigating their microbiome interactions is important to

understanding ecosystem functions. Yet the impacts of non-mycorrhizal fungal endophytes in bryophytes have rarely been investigated and the interactions between members of these endophyte communities have not been tested.

In this study, we build on a previously developed experimental system using the model liverwort *Marchantia polymorpha* L. and a collection of its culturable endophytes (Chapter 2) to test interactions between different fungal endophytes. This study aimed to address the lack of microbial community interaction studies in bryophytes and to demonstrate the utility of the *M. polymorpha* experimental system for synthetic community experiments. We use replicate clones of a single axenically grown *M. polymorpha* host and inoculate with all possible combinations of three fungi originally isolated from that same plant in the field. We selected fungi which had differing effects in a previous screen of effects using individual inoculations. We find that co-inoculation changes the growth response of *M. polymorpha*. In particular, we observe loss of growth promotion when an individually beneficial fungus is co-inoculated with others.

5.2 Materials and Methods

5.2.1 Plant and fungal materials

Marchantia polymorpha plants were collected in October 2013 growing as weeds at the nursery of the North Carolina Botanical Garden in Chapel Hill, NC. Axenic plant

cultures were initiated by surface sterilizing gemmae (asexual propagules) in a bleach solution and rinsing in sterile double distilled water (Miller, 1964) after which the gemmae were placed on modified Hatcher's agar (Chapter 2) (Hatcher, 1965). Clones of these plants were maintained in the lab by sterile transfer of thallus fragments to fresh media. Fungi were isolated, as described in Chapter 1, by surface sterilization of 2 mm^2 plant fragments and grown on 1.5% malt extract agar (MEA).

Fungi were selected for the experiments presented here based on a previous screen of effects of 100 isolates from wild *M. polymorpha* on the growth of axenically grown *M. polymorpha* from the Chapel Hill collection (Chapter 2). Three fungi that were all isolated from one female gametophyte in that collection and displayed different effects in the screen were chosen: *Hypoxylon* sp. 1 which increased growth, *Xylariaceae* sp. 1 which had no effect on growth, and *Xylaria cubensis* which killed host tissues (Chapter 2).

5.2.2 Co-inoculation growth experiment

Replicate clones of the plant from which the three selected fungi were originally isolated were propagated by punching 5 mm discs from axenically grown thalli. Thallus punches were placed on plates containing modified Hatcher's medium augmented with

0.15% MEA. Plants were allowed to grow for 49 days before being inoculated with fungi.

In order to investigate the effects of co-inoculation with the three selected fungi, we split plants into eight inoculation treatments: no fungus, each fungus individually, all possible pairs of fungi, and all three fungi together. Each treatment had seven replicate plants. In order to standardize inoculations, fungi were pipetted onto plants in a blended slurry. Each fungus was grown in malt extract broth with constant swirling for one week. The resulting mycelia were rinsed in sterile double distilled water, then ground in a sterile Waring blender for 30 to 40 seconds to lightly homogenize them. Samples of the inoculum were checked under a light microscope to ensure that hyphae were fragmented but still partially intact. 90 μL of fungus slurry was added to each plant. If two fungi were being added to a plant, 45 μL of each was used, and when all three fungi were added, 30 μL of each was used. Inocula were also put on agar plates without plants to confirm the viability of the fungi.

5.2.3 Follow-up experiment on *Xylaria cubensis*

One of the three fungi used in the growth experiment, *Xylaria cubensis,* did not produce the same growth effect as it had in previous screens (Chapter 2). We hypothesized that this could be due to the different inoculation method used in the

initial screens—placing a solid plug from a fungal culture at a distance from the plant—or from the culture changing over the year it was maintained in the laboratory between the initial screen and the co-inoculation experiment. To determine the source of the difference, we conducted a follow-up experiment using liquid and solid inoculations of the fungus directly on and at a distance from the plant, as well as solid inoculation with a culture grown from the original culture of the fungus preserved as a live voucher in sterile water at room temperature. Eight plant replicates were used for each of seven treatments: uninoculated controls, solid inoculum of the vouchered fungus directly adjacent and distant from the plant, and liquid or solid inoculum of the lab strain directly on or distant from the plant.

5.2.4 Growth data acquisition and processing

We tracked the growth of plants by taking digital photographs and measuring the area of green plant tissue, as previously described (Chapter 2). For the initial co-inoculation experiment, photos began 25 days before inoculation. For the follow-up experiment photos were taken for 11 days before inoculation. Each plant was photographed for 33 (co-inoculation) or 28 (follow-up) days after its meeting with the fungus. The meeting day was the inoculation day if the fungus was directly applied or the day physical contact of the fungus with the plant was first observed if inoculation

was at a distance. When plants died before their designated ending day, i.e. had no remaining green tissue, no more photographs were collected.

5.2.5 Data analysis

Changes in growth trajectories of plants were assessed with a model fitting a linear equation to the pre-meeting growth and a quadratic one to the post-meeting growth, as previously described (Chapter 3, Appendix D6). Differences in growth as summarized by this model were compared between treatments using Analysis of Variance (ANOVA) in R 3.3.1 (Team, 2014). For the co-inoculation experiment, the various combinations of fungi were analyzed as distinct treatments and a second time with three factors representing presence/absence for each of the three fungi (Table 4, Appendix D6). For the follow-up experiment, factors included whether a fungus was inoculated directly or at a distance ("distance"), whether the fungus was inoculated as a liquid slurry or a solid plug ("inoculum"), and which fungal strain was used ("fungus"). Tukey Honestly Significant Difference tests were used to determine significance of pairwise comparisons between the various factors.

Table 4: Analysis of co-inoculation of *M. polymorpha* with three fungi.
One ANOVA used the combined factor and compared all combinations of fungi to the control treatment and each other and a separate ANOVA analyzed the fungi separated into three factors to look for interactions between them.

Fungi present	Combined Factor	*Hypoxylon* sp. Factor	*X. cubensis* Factor	Xylariaceae sp. Factor
None (control)	A	0	0	0
All	B	1	1	1
X. cubensis and Xylariaceae sp.	C	0	1	1
Hypoxylon sp. and Xylariaceae sp.	D	1	0	1
Hypoxylon sp. and *X. cubensis*	E	1	1	0
Xylariaceae sp.	F	0	0	1
X. cubensis	G	0	1	0
Hypoxylon sp.	H	1	0	0

5.2.6 Confirmation of fungal colonization

Colonization of plants was confirmed by transferring two new tips of each plant to sterile Hatcher's media lacking any carbon source after the co-inoculation experiment and by PCR to detect fungal DNA in the plants. DNA was extracted from plants using a previously described CTAB protocol (Chapter 1). A part of fungal ribosomal RNA gene including the ITS and part of the LSU region was amplified using the ITS1f (Gardes & Bruns, 1993) and LR3 (Vilgalys & Hester, 1990) primers as previously described (Chapter 1). Samples were checked for amplification on a 1% agarose gel with SYBR Safe

gel stain (Invitrogen). Three controls, two samples of each single inoculation treatment, and one sample of each mixed inoculation treatment were checked for amplification. To confirm that the fungi in the plants were the same as those initially inoculated, we re-sequenced two samples of plants inoculated with each fungus individually. Samples were prepared for Sanger sequencing with an ExoSAP clean-up and BigDye version 1.1 reaction, as previously described (Chapter 1). Samples were sequenced at the Duke University Department of Immunology DNA Analysis Facility.

5.2.7 Microscopy

To examine fungal colonization morphology, some plants were harvested at the end of the co-inoculation experiment and stained with Rose Bengal. Individual thalli were soaked in 0.5% Rose Bengal in 95% ethanol for 15 seconds, then rinsed and mounted with water. Seven plants were examined, one from each of the fungal inoculation treatments. To compare microscopic characteristics of the two strains tested in the follow-up experiment, thallus pieces were mounted in water without staining. Three plants treated with each of the two strains were examined. Slides were viewed using an Olympus BX41 microscope with mounted DP25 camera.

5.3 Results

5.3.1 Co-inoculation experiment

Growth of *M. polymorpha* varied significantly between fungal inoculation treatments ($p < 0.01$). Plants inoculated with only *Hypoxylon* sp. or only *Xylaria cubensis* showed growth enhancement relative to control plants ($p < 0.01$ and $p = 0.02$, respectively) (Fig. 13). *Hypoxylon* sp, also enhanced growth relative to all other combinations of fungi, except *X. cubensis* alone and all three fungi together. Individual inoculation with Xylariaceae sp. and all co-inoculations did not significantly change plant growth relative to controls (Fig. 13). When three factors representing the presence of each fungus were used in ANOVA, *Hypoxylon* sp. ($p = 0.04$), the interaction between *Hypoxylon* sp. and *Xylaria cubensis* ($p < 0.01$) and the three-way interaction between all fungi ($p < 0.01$) were significant.

All but two plant fragments showed outgrowth of fungi when thallus tips were transferred to sterile mineral nutrient media, and these two were from different plants. This showed colonization of fungi beyond their initial inoculation point in all plants. When checked with PCR, the selected control plants showed no amplification of fungal DNA and all selected inoculated plants showed strong amplification.

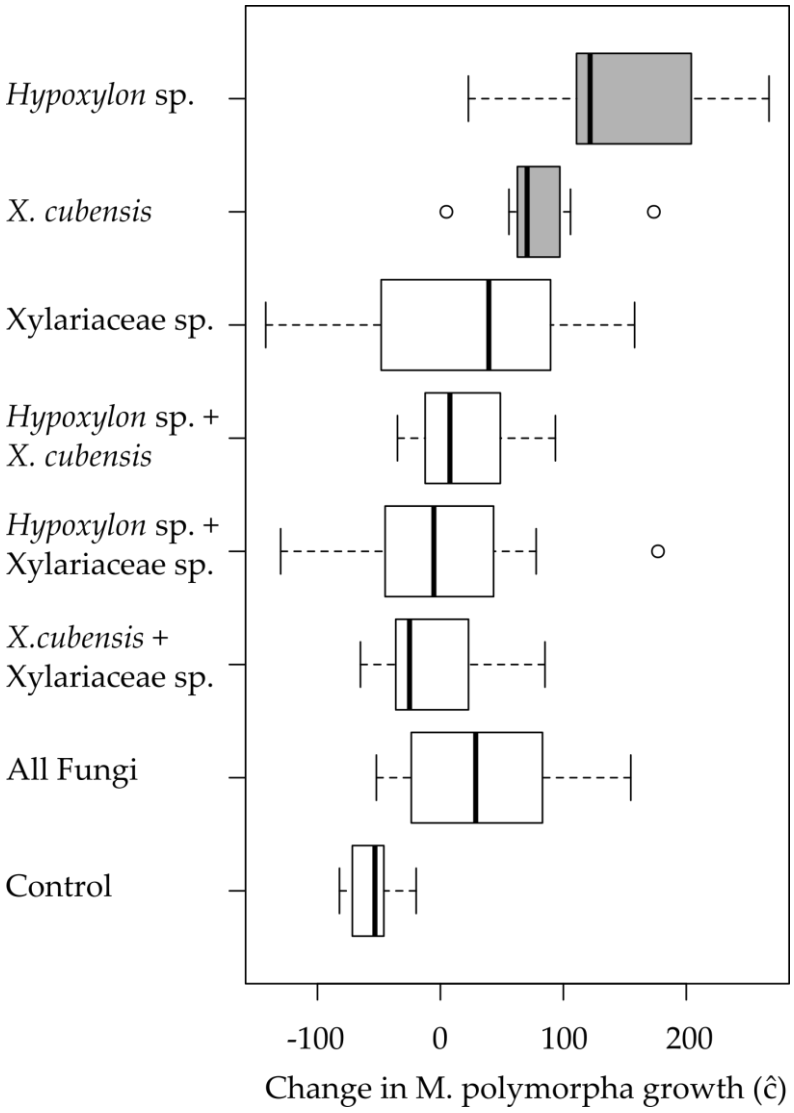

Figure 13: Effect on *M. polymorpha* growth from inoculation with three fungi individually and in combination.
Shaded boxes show fungal treatments that caused growth significantly different from control plants. Positive values of ĉ indicate increase in growth rate after contact with the fungus while negative values show decrease of growth after meeting day.

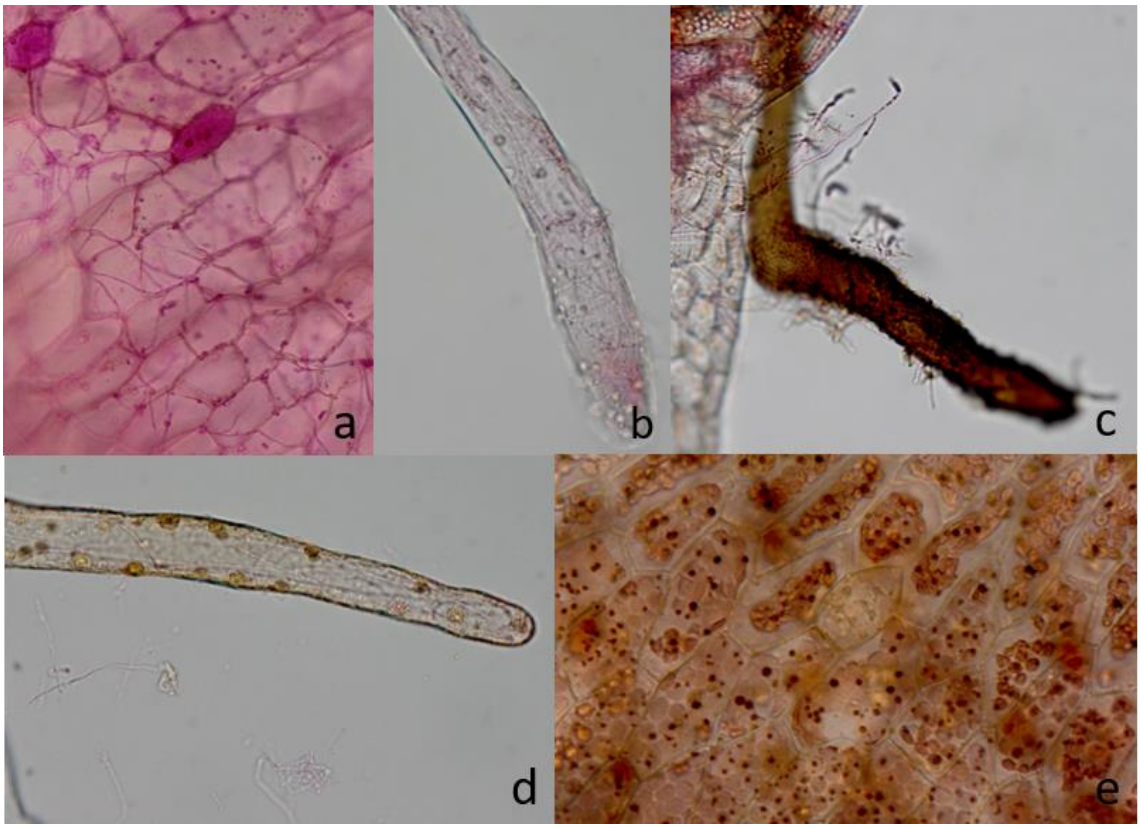

Figure 14: Colonization morphologies of *M. polymorpha* by three endophytes. Specimens were stained with Rose Bengal. (a) Hyphae of Xylariaceae sp. on thallus. Darkly staining cells are *M. polymorpha* oil cells. (b) Rhizoid colonized by Xylariaceae sp. (c) Melanized rhizoid on a plant colonized by *Hypoxylon* sp. and Xylariaceae sp. (d) Rhizoid with amber inclusions and hyphae of *Xylaria cubensis.* (e) Melanized spots in *M. polymorpha* thallus cells in a plant inoculated with *Hypoxylon* sp. and Xylariaceae sp.

Fungi colonized both thalli and rhizoids of *M. polymorpha* (Fig. 14 a-d). It was unclear if colonization of the thalli was internal, but hyphae were observed inside rhizoids (Fig. 14 b,d). Most affected rhizoids were the smooth type which are alive at maturity rather than the pegged type that are dead at maturity. Melanization either of whole rhizoids or consisting of dark spots within rhizoids were observed in treatments

including *Hypoxylon* sp. (Fig. 14c). Treatments that included *X. cubensis* showed round amber-colored inclusions within rhizoids (Fig. 14d). Small melanized spots in thallus cells were observed for fungal treatments including Xylariaceae sp. (Fig. 14e).

5.3.2 Follow-up experiment

The growth of *M. polymorpha* was significantly different between fungal treatments ($p < 0.01$), mostly due to differences between the two strains of *Xylaria cubensis* used. The *X. cubensis* voucher strain killed plant tissue, while the strain maintained in the lab promoted plant growth slightly relative to controls ($p = 0.03$). Significant growth promotion by the lab strain only occurred in the treatment in which the fungus was inoculated in liquid distant from the plant (Fig. 15). There was no significant difference overall in growth between plants inoculated with solid or liquid inoculum ($p = 0.16$) or adjacent or distant from the plant ($p = 0.14$).

Some of the treatments also displayed different levels of variation among replicates, reflected in the variances calculated for each. The growth responses were more variable for liquid than solid inoculation with the lab strain of the fungus (and not comparable with the voucher strain since it was only inoculated with solid plugs). Growth was also more variable when either fungus was inoculated far from the plant versus directly on or beside it.

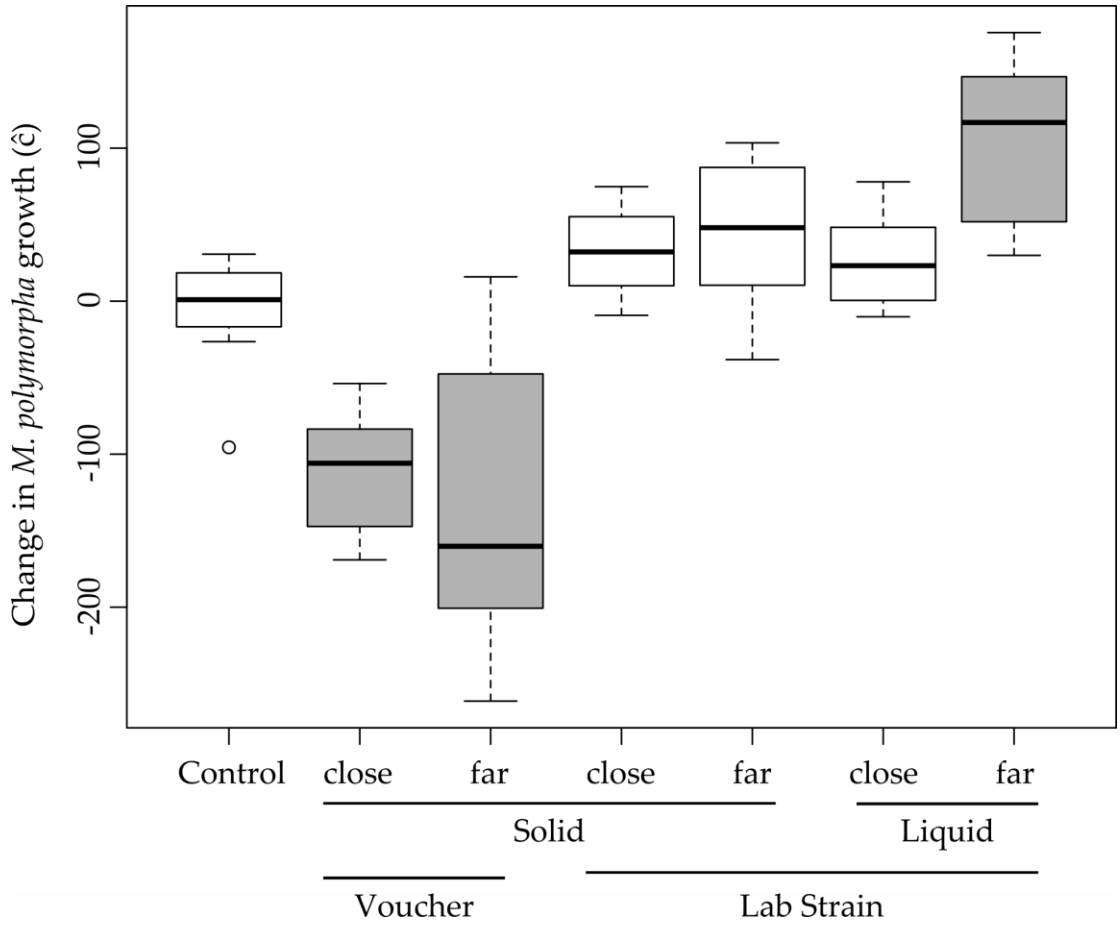

Figure 15: Growth of *M. polymorpha* in response to different methods of inoculation with *Xylaria cubensis*.
Two fungal cultures derived from the same original isolate were used: one from a voucher at the time of isolation and one from a culture maintained in the laboratory. The lab strain fungus was inoculated by either pipetting a liquid suspension of mycelium or with a solid agar plug. The voucher culture was only inoculated on solid plugs. All inoculation types were placed either directly on/adjacent to the plant or at a distance from the plant (close vs. far). Shaded boxes show inoculation treatments for which *M. polymorpha* growth was significantly different from controls.

In addition, the macroscopic morphologies of the voucher and lab strain fungi differed. The vouchered strain had more aerial hyphae and produced horn-like fruiting bodies while the lab strain appeared thinner and made no fruiting bodies (Fig. 16). Both strains resulted in similar microscopic morphologies, with round darkly pigmented inclusions found in thallus cells and rhizoids (Fig. 17). Dense collections of the dark material were found in both dead and live thallus cells. Both strains colonized inside *M. polymorpha* rhizoids (Fig. 17).

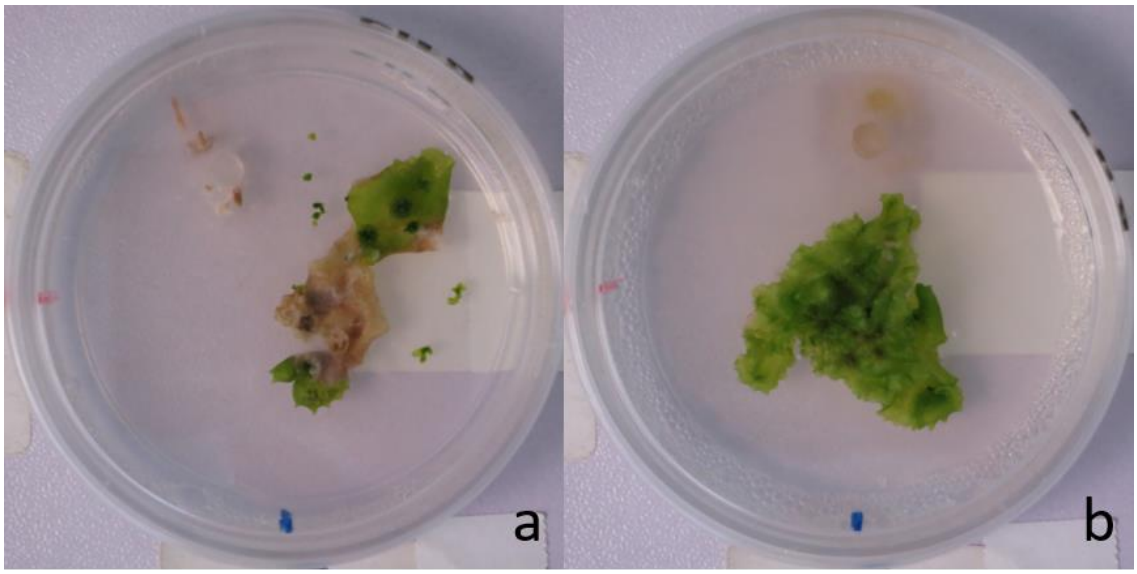

Figure 16: Changes in *X. cubensis* characteristics after a year of growing in the lab. (a) The vouchered fungus (not maintained in laboratory culture) was pathogenic to *M. polymorpha*, produced thicker mycelium above the agar surface, and produced horn-like fruiting bodies. (b) The same fungal isolate maintained in the lab was avirulent, appeared thinner, and did not produce fruiting bodies. Images are from the same day of the follow-up experiment.

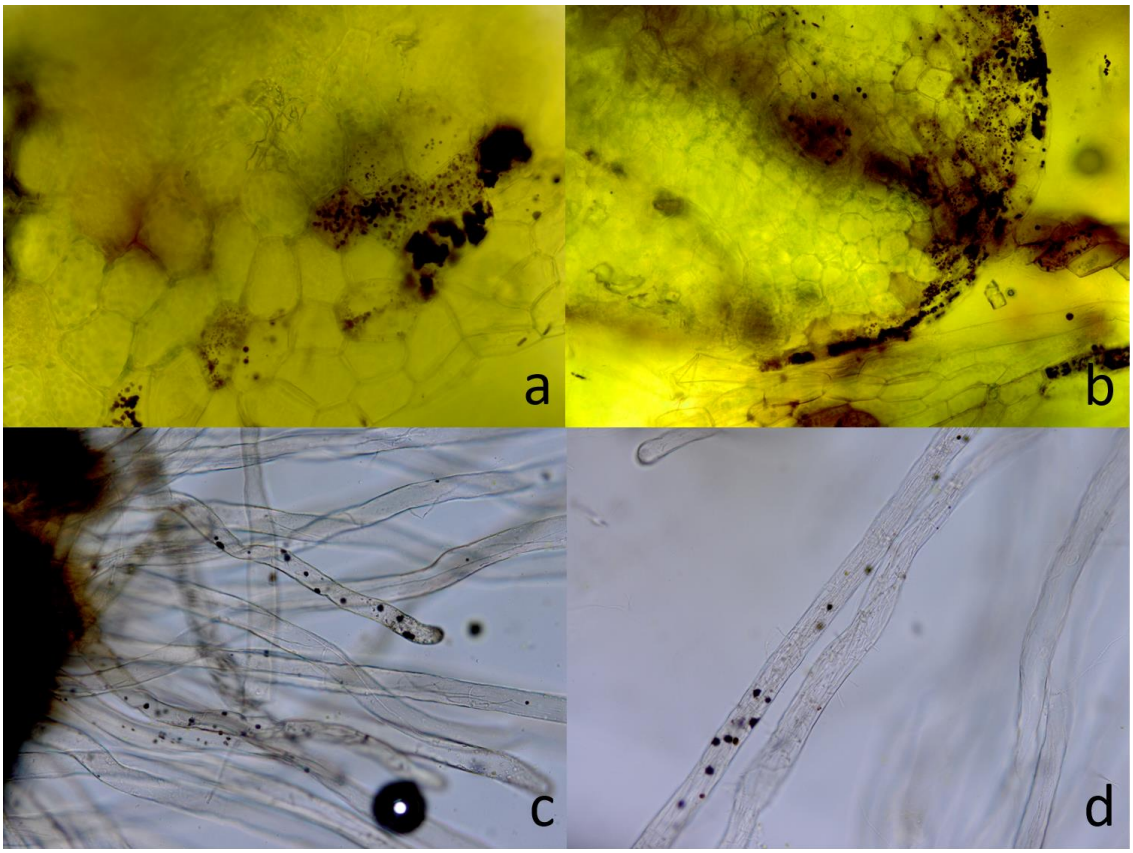

Figure 17: Microscopic effects of *X. cubensis* colonization of *M. polymorpha*.
All plants are unstained. Dark inclusions were observed in cells of green thalli for both the voucher strain (a) and the lab-maintained strain (b). Hyphae and dark inclusions were also observed in rhizoids of plants inoculated with the voucher (c) and lab (d) strains.

5.4 Discussion

The co-inoculation experiment presented here demonstrates that interactions between multiple fungi change the impacts on *M. polymorpha* host growth. In particular, we observed that two fungi promoted host growth only when alone. When the two

growth-promoting fungi were combined, we did not observe an additive effect. Instead, the growth promotion was lost, perhaps indicating that the two fungi interfere with each other in some way. Previous *in vitro* studies have indicated that such interference between fungi could be from competition for resources, changes in metabolism, or direct mycoparasitism (Bailey *et al.*, 2008; Chagas *et al.*, 2013; Orlandelli *et al.*, 2015). This result also clearly shows that classifying the effects of endophytes based only on individual inoculation experiments provides incomplete information on the interaction between that fungus and its plant host; the effects of individual microbiome members also depend on other members of the community. Future studies inoculating *M. polymorpha* with synthetic communities of microbes and measuring changes in the holobiont metabolome and transcriptome could elucidate how various endophytes enhance or interfere with the effects of other microbes in this host.

Xylaria cubensis was pathogenic in previous screens (Chapter 2), but in the multiple inoculation experiment, it showed no negative impacts and even slightly enhanced host growth. Because of this change, we could not determine with this co-inoculation experiment whether other endophytes would mediate the negative effects of this pathogenic fungus, but the inconsistent effect of *X. cubensis* was an opportunity to study the loss of virulence in our laboratory strain. Resequencing of extracts from plants inoculated with this culture confirmed that it was the same fungus, not a contaminant

and the follow-up experiment demonstrated that the fungus grown from a voucher retained virulence. Over the course of a year being maintained in the lab, the morphology of *X. cubensis* changed and its ability to kill *M. polymorpha* disappeared. This could be due to a genetic change in the culture or perhaps the loss of an endohyphal bacterium. A mutation at a single locus can be enough to switch a fungus from pathogen to protective endophyte (Freeman & Rodriguez, 1993). Endohyphal bacteria occur in many endophytic fungi and can drastically change the metabolic profile of the fungus, including the production of plant hormones (Hoffman, 2010; Li *et al.*, 2017). These bacterial associations tend to be lost in laboratory culture over time, which would be consistent with the changes we observed (Hoffman, 2010). Further study of the virulent and avirulent strains of this fungus to reveal what changed in the culture could elucidate one potential pathway for rapid switching of fungi between pathogenicity and mutualism.

The follow-up experiment also demonstrates that the experimental system used is generally robust to changes in inoculation method. This system can easily be used for a variety of microbiome interaction studies. In addition to a diverse set of fungal endophytes (Chapter 1), *M. polymorpha* also hosts endophytic bacteria (Nelson, unpublished data), surficial cyanobacteria (Guminska & Mierzenska, 1992; Adams & Duggan, 2008), arthopods, green algae, and other "protists" (Chapter 1). Few studies

have investigated the interactions between the various types of microbes that comprise plant microbiota and the compact *M. polymorpha* experimental system has great potential for addressing these kinds of holistic microbiome ecology questions.

6 Conclusion

6.1 Potential of the M. polymorpha experimental system for microbiome research

The system established in this dissertation allows for a broad range of experiments on plant-microbe interaction in a compact and inexpensive format. Experiments on adult plants can be completed in approximately three to four months from starting growth of plant replicates to finishing data analysis. The system is robust to different methods of inoculation and can be used to study interactions between multiple microbes.

The work begun on *M. polymorpha* can easily be expanded to include other species in *Marchantia* and in the complex thalloid clade that are also amenable to axenic culture. Experiments with more species could be used to test host specificity of fungal interactions. I already am growing *Marchantia paleacea* and *Marchantia inflexa* axenically and have tested the effects of a subset of the *M. polymorpha* endophytes I collected for their effects on *M. paleacea.* This preliminary investigation indicates that fungi may have host specific effects, with these two sister *Marchantia* species having different resistances to potentially pathogenic endophytes (Radhakrishnan and Nelson, unpublished data). I also have a set of different genotypes of *M. polymorpha* from various sites in the United States in culture that could be used to investigate intraspecific variation in responses to endophytes.

One important extension of the *M. polymorpha* system will be establishing a method to cultivate axenic plants on more natural substrate. *Marchantia polymorpha* can be grown on organic or clay substrates in a greenhouse setting. Creating soil microcosm systems with *M. polymorpha* would open up opportunities for experiments with more direct relevance to natural conditions. Microcosm experiments have been used for vascular plant ecosystems, such as grasslands, to demonstrate how endophytes can shift plant community compositions (Aguilar-Trigueros & Rillig, 2016). A similar approach could be used with *M. polymorpha* and other early successional bryophyte species to look at smaller scale plant community dynamics and how they are influenced by microbiota. Such soil systems for *M. polymorpha* would also allow trap plant experiments and bioassays with natural soils to study the assembly of the liverwort's microbiome.

6.2 Utility of the M. polymorpha system in undergraduate research training

In addition to its potential to further plant microbiota research, the *M. polymorpha* experimental system presented here can also be a useful tool in research training for university undergraduate students. The system is very modular and experiments can comfortably be designed and implemented with an undergraduate student in one or two semesters. This dissertation project has supported two undergraduate senior thesis projects, an independent study project, a work-study student, and three volunteer

students. The products of one of the senior thesis projects, with some added follow-up, are presented as Chapter 4 of this dissertation. The co-inoculation experiment was designed and implemented by myself and the undergraduate student over two semesters. Another student volunteer formulated a question about how plant rhizoids were responding to fungal inoculation and we carried out an experiment to test it within the one semester she was volunteering. The resulting answer was that rhizoids were not responding, but the student was able to go through the full scientific process in a few months using the *M. polymorpha* system. Multiple students were trained in acquiring and processing data for the *M. polymorpha* growth experiments and helping with the fungus and culture maintenance to support the project. These skills were simple enough to train students in quickly and all students were able to work independently on such tasks after initial training.

The experimental system could also be scaled up for the laboratory section of an upper-level undergraduate course. With a single growth room, a class could participate in running a large interaction experiment from design to completed data analysis in one semester. Plants could be started before the semester for students to choose from. Depending on the class level, students could do the inoculations themselves or the instructor and a technician could do the sterile work. Students could easily learn to take data, check microscopic morphology changes, and analyze growth. This experimental

system lends itself to discrete, manageable questions that could be investigated from beginning to end in the time available in a single course.

6.3 Future investigations

The findings of this dissertation research about the *M. polymorpha* microbiome can provide a foundation for more investigations of bryophyte microbiomes. Culture-independent methods for assessing microbiome diversity have only been applied to a few bryophyte species from a few geographic locations so far and not at all to hornworts. In addition to their diverse fungal endophyte communities that are only beginning to be surveyed, bryophytes also host a variety of other microbes including bacteria and non-fungal eukaryotic microbes. In contrast to the situation for vascular plants and mammalian research, bryophyte bacterial communities are even more poorly known than the fungal ones. Very little is known about green algal endophytes, though they do occur in some vascular plants and bryophytes (Reese, 1981; Trémouillaux-Guiller & Huss, 2007). I detected abundant sequences of a few green algae and also obtained some cultures of unicellular green algae from some of the *M. polymorpha* cultures I started from surface-sterilized gemmae. Further investigation of culturable microbes beyond fungi in *M. polymorpha* could lead to some interesting discoveries and help develop *M. polymorpha* as a system for testing interactions between disparate

groups of endophytic organisms and how these relationships play into larger-scale plant ecology.

There is already some scattered evidence that interactions between various microbial groups can be significant in bryophytes. For example, there seem to be associations between cyanobacteria and fungi in *M. polymorpha* and *Sphagnum* mosses (Guminska & Mierzenska, 1992; Kostka *et al.*, 2016). To understand plant functioning, research needs to continue its expansion past the focus on exchanges between pairs of symbionts to the holobiont community scale. *M. polymorpha* and other bryophytes could provide ideal systems for this holobiont-focused method of study since they are compact and can be grown to maturity in shorter time periods using fewer resources than needed for many common vascular plant models. Assembling reasonably complete synthetic communities for individual bryophytes will likely be more manageable than doing the same for a microbial community of a tree, for instance.

In addition, much more work is needed to determine how bryophyte microbiomes differ in composition and function from those of vascular plants. It is possible that the different ecologies, scales, and physiologies of bryophytes lead to fundamentally different microbial interactions than those found in vascular plants. Bryophytes may be interacting much more directly with their microbes since they have

much less barrier between them and the outside. The difference between outside and inside may be much less for a plant with many tissues that are only one cell layer thick.

6.4 Concluding remarks

The research presented in this dissertation demonstrates that *M. polymorpha* hosts a diverse community of endophytic fungi that have a range of effects on host success. These effects can be dynamic based on abiotic conditions like nutrient supplies and the biotic environment of other microbes in the same host. The experimental system established and refined in this dissertation has great potential for future investigations in the emerging field of plant microbiota research. This work on the *M. polymorpha* microbiome, combined with this liverwort's developing molecular model resources and its wide geographic and ecological distribution, will allow investigation of questions at many scales of endophyte interaction, from molecular mechanisms to broad ecosystem functioning.

Appendix A: Chapter 1 Supplemental Methods

Protocol: Illumina Amplicon Sequencing Library Preparation

This methodology is based on protocols from the Vilgalys and Bonito labs which were modified from Lundberg et al. (2013). DNA was first extracted from whole *M. polymorpha* plants using a CTAB protocol. ITS and LSU amplicons were used (primer pairs ITS1f-ITS4 and LROR-LR3).

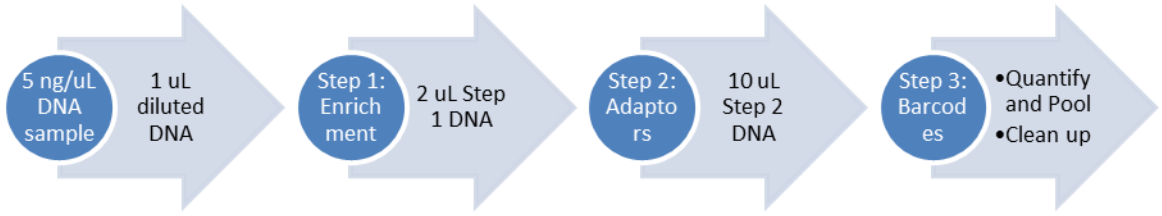

Figure 18: Summary of library preparation protocol for one sample

Step 1: Enrich Target DNA

The target region was amplified with ordinary primers in fifteen PCR cycles using the specifications shown below. Each reaction uses 1 uL of DNA sample. Based on preliminary PCR tests, I used samples diluted to approximately 5 ng/uL. If a sample had a concentration less than 8 ng/uL, it was not diluted.

LSU master mix	
Ingredient	**uL per sample**
Water	13.35
10x Buffer*	2.5
$MgCl_2$ (50 mM)*	0.5
dNTPs (2 mM each)	2.5
BSA (10 mg/mL)	2.5
LROR (10 uM)	1.25
LR3 (10 uM)	1.25
Taq	0.15

ITS master mix	
Ingredient	**uL per sample**
Water	13.6
10x Buffer*	2.5
$MgCl_2$ (50 mM)*	0.25
dNTPs (2 mM each)	2.5
BSA (10 mg/mL)	2.5
LROR (10 uM)	1.25
LR3 (10 uM)	1.25
Taq	0.15

* Note: The buffer contains 15 mM $MgCl_2$ and 500 mM KCl. The amount of buffer added to these reactions results in a Mg^{2+} concentration of 1.5 mM. Based on preliminary PCR tests, the LSU mix has been adjusted to an overall Mg^{2+} concentration of 2.5 mM and the ITS to a concentration of 2 mM.

LSU Step 1 PCR Program

95° C 5 minutes
15 cycles of:
    95° C 1 minute
    62° C 45 seconds
    72° C 1 minute
72° C 7 minutes
4° C hold

ITS Step 1 PCR Program

95° C 5 minutes
15 cycles of:
    95° C 1 minute
    58° C 45 seconds
    72° C 1 minute
72° C 7 minutes
4° C hold

Step 2: Ligate Frameshift Primers and Sequencing Adaptors

The region was amplified while adding adaptors and Illumina primers in ten PCR cycles using the specifications below. The primers are each a mix of six different frame-shifted versions. Each reaction had 2 uL of the product from step 1 added to 23 uL master mix.

.**LSU master mix**	
Ingredient	**uL per sample**
Water	12.35
10x Buffer	2.5
$MgCl_2$ (50 mM)	0.5
dNTPs (2 mM each)	2.5
BSA (10 mg/mL)	2.5
LROR (f1-6) (10 uM)	1.25
LR3 (f1-6) (10 uM)	1.25
Taq	0.15

ITS master mix	
Ingredient	**uL per sample**
Water	12.6
10x Buffer	2.5
$MgCl_2$ (50 mM)	0.25
dNTPs (2 mM each)	2.5
BSA (10 mg/mL)	2.5
ITS1f (f1-6) (10 uM)	1.25
ITS4 (f1-6) (10 uM)	1.25
Taq	0.15

LSU Step 1 PCR Program

95° C 5 minutes
10 cycles of:
    95° C 1 minute
    62° C 45 seconds
    72° C 1 minute
72° C 7 minutes
4° C hold

ITS Step 1 PCR Program

95° C 5 minutes
10 cycles of:
    95° C 1 minute
    58° C 45 seconds
    72° C 1 minute
72° C 7 minutes
4° C hold

Step 3: Ligate Barcodes

Unique barcodes were on the reverse primers. The forward primer was the same for all reactions and therefore was added to the master mix. The DNA samples and barcode primers were added in a sterile environment. Ten PCR cycles were run with the following specifications plus 10 uL product from step 2 and 0.5 uL barcoded reverse primer (5 μM).

Barcoding master mix	
Ingredient	**uL per sample**
Water	8.35
10x Buffer	2.5
dNTPs (2 mM each)	2.5
BSA (10 mg/mL)	0.5
Forward primer (5 uM)	0.5
Taq	0.15

Barcode PCR Program

95° C 5 minutes
10 cycles of:
    95° C 1 minute
    63° C 1 minute
    72° C 1 m, 30 s
72° C 10 minutes
4° C hold

Appendix B: Chapter 1 Supplemental Figures and Tables

Table 5: *M. polymorpha* collection information.
Column abbreviations are as follows. C: whether a patch was used for culture sampling, A: whether a patch was used for culture-independent amplicon sequencing (Y indicates yes and N indicates no), Nat: country, R: region of collection (SE = southeastern US, NE = northeastern US, NW = northwestern US or western Canada), St: state or province abbreviation.

Patch	C	A	Nat	R	St	Location	Date	Locality	Habitat
AUX2	Y	N	USA	SE	KY	37.8189833, -83.6794333	5/20/16	Near Auxier Ridge trailhead, Daniel Boone National Forest	on soil among leaf litter
AUX3	Y	N	USA	SE	KY	37.8190333, -83.6788833	5/20/16	Near Auxier Ridge trailhead, Daniel Boone National Forest	on soil in shade of log, among *Atrichum*
AUX4	Y	N	USA	SE	KY	37.8188833, -83.6791167	5/20/16	Near Auxier Ridge trailhead, Daniel Boone National Forest	on soil between two logs, among litter
AUX5	Y	Y	USA	SE	KY	37.81899, -83.67944	7/11/15	Near Auxier Ridge trailhead, Daniel Boone National Forest	on soil sheltered on downslope side of log
BCB	N	Y	USA	NW	OR	43.72352, -121.79533	8/5/15	Deschutes National Forest, off of Wickup Reservoir Rd	along side channel of stream by burned area
BFP	N	Y	USA	NE	VT	44.97348, -72.38688	7/1/15	Big Falls State Park, north of Troy, VT	on river bank, near current waterline
CHR1	Y	N	USA	SE	NC	35.01564, -83.12671	5/21/14	W side Bull Pen Road Bridge over Chatooga River Gorge	on edge of roadside ditch
LCB	N	Y	USA	NW	OR	43.79757, -121.8847	8/5/15	bog near Little Cultus Lake	edge of tiny water channel
LCC	Y	Y	USA	NW	OR	45.44962, -122.67003	8/15/15	Lewis & Clark College, Portland, OR	among edging stones of gravel path
LIB	Y	Y	USA	NE	NY	42.39997, -76.53836	6/27/15	Lick Brook lower bowl, Ithaca, NY	on rock ledge in stream

Patch	C	A	Nat	R	St	Location	Date	Locality	Habitat
MSH1	N	Y	USA	NW	WA	46.25019, -122.1667	7/31/15	Mount St Helens pumice plain	edge of small side stream
MSH2	N	Y	USA	NW	WA	46.25177, -122.16563	7/31/15	Mount St Helens pumice plain	tiny bog, on ~24cm thick peat of mosses & *Marchantia*
MSH3	N	Y	USA	NW	WA	46.24313, -122.16515	7/31/15	Mount St Helens pumice plain	Shaded edge of path
NCB	Y	N	USA	SE	NC	35.898996, -79.034389	10/24/13	Chapel Hill, North Carolina Botanical Garden	on weed mat below nursery benches
PCR	N	Y	USA	NE	PA	41.48655, -77.49085	6/25/15	North of Slate Run, PA	along bike path at edge of ditch
PXF	Y	Y	USA	NW	OR	44.1616, -121.92743	8/4/15	Proxy Falls	on, in mist from falls
TSP	N	Y	USA	NE	NY	42.396838, -76.560536	6/27/15	Robert H. Treman state park	below waterfall
UPS	N	Y	USA	NW	WA	47.26383, -122.48361	8/10/15	University of Puget Sound, Thompson Hall	bare soil
UTN	Y	N	USA	SE	TN	35.95713, -83.92493	3/2/14	University of Tennessee in Knoxville, TN	Ken McFarland's bryophyte garden
WRG1	Y	Y	USA	NE	VT	44.48071, -73.11571	6/30/15	Winooski River Gorge, just below dam in park, Williston	on sandy soil
WRG2	Y	Y	USA	NE	VT	44.48067, -73.11578	6/30/15	Winooski River Gorge, just below dam in power company park, Williston, VT	on sandy soil on ledge above wet rocks
YUK1	Y	N	CA	NW	YT	60.6841667, -135.2375	6/13/14	alongside Fish Lake Road, ~10 km W-SW of Whitehorse, Yukon Territory	flooded area alongside seepage stream
YUK2	Y	N	CA	NW	YT	60.6502778, -135.24	6/13/14	Fish Lake, ~12 km NW of Whitehorse, Yukon Territory	Seepy ground near lake shore, north end of lake

Table 6: Mock community members and sequencing recovery.
Taxonomic rank names are abbreviated. Phyla are shown in column P: A=Ascomycota and B=Basidiomycota. Class, Order, and Family names are shown with the common suffixes removed. For full class names, add "mycetes"; for orders, add "ales"; and for families, add "aceae". Read and OTU counts are for taxa in the amplicon datasets identified to the same genus.

Original Taxon	P	Class	Order	Family	ITS Reads	ITS OTU	LSU Reads	LSU OTU
Cercospora sp.	A	Dothideo	Capnodi	Mycosphaerell	0	0	431	1
Phoma herbarum	A	Dothideo	Pleospor	Didymell	1155	1	2099	3
Microsphaeropsis arundinis	A	Dothideo	Pleospor	Montagnul	130	1	216	2
Epicoccum sp.	A	Dothideo	Pleospor	Pleospor	13066	1	216	2
Cadophora luteo-olivacea	A	Leotio	Heloti	Unknown	67	1	686	2
Candida sp. 1	A	Saccharo	Saccharomycet	Saccharomycet	164612	3	9477	3
Colletotrichum truncatum	A	Sordario	Glomerell	Glomerell	61	1	0	0
Plectosphaerella sp.	A	Sordario	Phyllachor	Plectosphaerell	3662	1	1167	2
Coniochaeta sp.	A	Sordario	Sordari	Coniochaet	0	0	442	1
Biscogniauxia mediterranea	A	Sordario	Xylari	Xylari	853	1	526	1
Daldinia loculata	A	Sordario	Xylari	Xylari	346	1	158	1
Hypoxylon sp.	A	Sordario	Xylari	Xylari	731	1	408	1
Hypoxylon submonticulosum	A	Sordario	Xylari	Xylari	642	1	447	1
Nemania serpens	A	Sordario	Xylari	Xylari	7487	3	1767	1
Nemania sp.	A	Sordario	Xylari	Xylari	7487	3	1767	1
Xylaria arbuscula	A	Sordario	Xylari	Xylari	6763	1	423	1
Xylaria cubensis	A	Sordario	Xylari	Xylari	6763	1	423	1
Pholiota highlandensis	B	Agarico	Agaric	Strophari	0	0	73003	4
Bulleromyces albus	B	Tremello	Tremell	Tremell	2884	1	746	2

Table 7: Isolation frequencies of fungal endophytes from *M. polymorpha*.
Column labels are abbreviated as follows. I: number of isolates, P: number of pieces, %: percent of samples yielding isolates, S: number of unique species, A: number of plants for which isolation was attempted, Y: number of plants that yielded cultures, T: number of species found in a patch, and M: maximum number of species in one plant.

	Gametangiophore				Rhizoid				Thallus				Total			All Plants			Spp	
Patch	**I**	**P**	**%**	**S**	**I**	**P**	**%**	**S**	**I**	**P**	**%**	**S**	**I**	**P**	**%**	**A**	**Y**	**%**	**T**	**M**
AUX1	2	4	50.0	1	0	4	0.0	0	3	12	25.0	2	5	20	25.0	2	2	100.0	3	3
AUX2	1	4	25.0	1	0	4	0.0	0	3	12	25.0	3	4	20	20.0	2	2	100.0	4	3
AUX3	1	4	25.0	1	2	4	50.0	2	9	12	75.0	5	12	20	60.0	2	2	100.0	7	6
AUX4	1	3	33.3	1	3	4	75.0	2	9	12	75.0	9	13	19	68.4	2	2	100.0	10	5
AUX5	3	12	25.0	3	0	12	0.0	0	4	30	13.3	4	7	54	13.0	3	3	100.0	7	4
CHR	5	10	50.0	4	1	12	8.3	1	1	36	2.8	1	7	58	12.1	3	3	100.0	6	4
LCC	0	0		0	1	12	8.3	1	0	30	0.0	0	1	42	2.4	3	1	33.3	1	1
LIB	0	12	0.0	0	1	12	8.3	1	3	30	10.0	3	4	54	7.4	3	2	66.7	4	2
NCB	5	11	45.5	5	3	16	18.8	3	8	28	28.6	5	16	55	29.1	4	3	75.0	10	6
PXF	0	0		0	0	12	0.0	0	1	30	3.3	1	1	42	2.4	3	1	33.3	1	1
UTN	0	0		0	1	18	5.6	1	2	42	4.8	1	3	60	5.0	6	2	33.3	2	1
WRG	6	24	25.0	6	0	24	0.0	0	4	60	6.7	4	10	108	9.3	6	4	66.7	9	6
YUK1	0	0		0	0	6	0.0	0	4	14	28.6	4	4	20	20.0	2	1	50.0	4	4
YUK2	1	2	50.0	1	0	4	0.0	0	0	14	0.0	0	1	20	5.0	2	1	50.0	1	1
Totals	25	86	29.1		12	144	8.3		52	362	14.4		88	592	14.9	43	29	67.4		

Table 8: Fungal isolates from *M. polymorpha*

Any isolates with identical patch, plant, tissue, and rep (replicate) values are derived from the same plant fragment. Any isolates with the same patch and plant values came from pieces of the same *M. polymorpha* plant.

Isolate	Fungal Taxon	Patch	Plant	Tissue	Rep
JN0300	*Biscogniauxia mediterranea*	AUX1	2	thallus	B
JN0305	*Biscogniauxia mediterranea*	AUX3	1	thallus	D
JN0308	*Biscogniauxia mediterranea*	AUX2	2	thallus	A
JN0313	*Biscogniauxia mediterranea*	AUX4	1	thallus	B
JN0317	*Biscogniauxia mediterranea*	AUX4	1	rhizoid	B
JN0418	*Bulleromyces albus*	LIB	12	rhizoid	B
JN0399	*Cadophora luteo-olivacea*	LIB	13	thallus	C
JN0403	*Candida* sp. 1	LCC	11	rhizoid	A
JN0406	*Candida* sp. 1	AUX4	1	thallus	E
JN0417	*Candida* sp. 1	AUX5	11	thallus	G
JN0358	*Cladosporium sphaerospermum*	YUK1	2	thallus	F
JN0387	*Colletotrichum acutatum*	WRG	13	receptacle	C
JN0391	*Colletotrichum* sp. 1	WRG	13	receptacle	A
JN0318	*Colletotrichum truncatum*	CHR	2	receptacle	A
JN0319	*Colletotrichum truncatum*	CHR	3	receptacle	A
JN0385	*Colletotrichum truncatum*	WRG	12	receptacle	C
JN0314	*Coniochaeta* sp. 1	AUX4	1	thallus	E
JN0311	*Coniochaeta* sp. 2	AUX3	2	thallus	E
JN0323	*Coniochaeta* sp. 2	AUX4	2	thallus	F
JN0369	*Coniochaeta* sp. 2	AUX3	1	thallus	F
JN0376	*Coniochaeta* sp. 2	AUX3	1	rhizoid	B
JN0379	*Coniochaeta* sp. 2	AUX3	2	thallus	C
JN0396	*Coniochaeta* sp. 2	AUX5	12	thallus	C
JN0303	Coniochaetaceae sp. 1	AUX3	1	thallus	A
JN0310	Coniochaetaceae sp. 1	AUX3	2	thallus	D
JN0355	Coniochaetaceae sp. 1	AUX4	2	thallus	C
JN0415	Coniochaetaceae sp. 1	AUX3	1	thallus	C
JN0304	Coniochaetaceae sp. 2	AUX3	1	thallus	C

Isolate	Fungal Taxon	Patch	Plant	Tissue	Rep
JN0377	Coniochaetaceae sp. 3	AUX3	1	rhizoid	B
JN0321	*Daldinia loculata*	AUX2	1	thallus	C
JN0392	*Epicoccum* sp. 1	WRG	16	receptacle	A
JN0327	Helotiaceae sp. 1	AUX2	2	thallus	E
JN0325	Helotiales sp. 1	CHR	2	receptacle	B
JN0362	Helotiales sp. 2	CHR	2	receptacle	B
JN0402	Helotiales sp. 3	PXF	3	thallus	I
JN0302	Hyaloscyphaceae sp. 1	AUX2	2	receptacle	A
JN0243	*Hypoxylon* sp. 1	NCB	female 2	receptacle	A
JN0296	*Hypoxylon* sp. 1	UTN	5	rhizoid	B
JN0383	*Hypoxylon submonticulosum*	LIB	13	thallus	E
JN0384	*Hypoxylon submonticulosum*	WRG	11	receptacle	D
JN0386	*Hypoxylon submonticulosum*	WRG	13	thallus	H
JN0316	Lasiosphaeriaceae sp. 1	AUX4	1	rhizoid	A
JN0330	Lasiosphaeriaceae sp. 2	YUK1	2	thallus	C
JN0354	Lasiosphaeriaceae sp. 3	AUX3	1	thallus	C
JN0333	Leotiomycete sp. 1	UTN	1	surface	A
JN0334	Leotiomycete sp. 1	UTN	1	surface	F
JN0336	Leotiomycete sp. 1	UTN	6	surface	B
JN0337	Leotiomycete sp. 1	UTN	6	surface	E
JN0388	Leotiomycete sp. 2	LIB	12	thallus	K
JN0329	Leotiomycetes sp. 3	CHR	3	thallus	E
JN0368	Leptosphaeriaceae sp. 1	AUX4	lab grown	surface	
JN0324	*Microsphaeropsis arundinis*	CHR	1	receptacle	B
JN0245	*Nemania serpens*	NCB	female 2	thallus	A
JN0253	*Nemania serpens*	NCB	male 2	thallus	G
JN0241	*Nemania* sp. 1	NCB	female 1	receptacle	D
JN0246	*Nemania* sp. 1	NCB	female 2	thallus	B
JN0394	*Nemania* sp. 1	AUX5	11	thallus	J
JN0249	*Nemania* sp. 2	NCB	male 2	receptacle	C
JN0390	Pezizales sp. 1	WRG	13	thallus	J
JN0320	*Pholiota highlandensis*	AUX1	2	thallus	E

Isolate	Fungal Taxon	Patch	Plant	Tissue	Rep
JN0299	*Phoma herbarum*	AUX1	1	receptacle	B
JN0301	*Phoma herbarum*	AUX1	2	receptacle	B
JN0332	*Phoma herbarum*	YUK2	1	receptacle	A
JN0367	*Phoma herbarum*	YUK2	lab grown	surface	
JN0398	*Phoma herbarum*	WRG	11	receptacle	D
JN0401	*Phoma herbarum*	AUX5	12	receptacle	C
JN0335	*Plectosphaerella* sp. 1	UTN	6	surface	A
JN0312	Pleosporales sp. 1	AUX3	2	receptacle	A
JN0395	Pleosporales sp. 2	AUX5	11	receptacle	A
JN0420	Pyronemataceae sp. 1	WRG	13	thallus	B
JN0331	*Schizothecium* sp. 1	YUK1	2	thallus	F
JN0419	Sordariales sp. 1	AUX4	2	thallus	C
JN0393	Sordariomycete sp. 1	AUX5	11	thallus	C
JN0356	Sordariomycetes sp. 2	AUX4	3	thallus	C
JN0407	Sordariomycetes sp. 3	YUK1	2	thallus	C
JN0322	*Toxicocladoporium irritans*	AUX4	2	thallus	B
JN0315	Unseqeunced	AUX4	1	receptacle	B
JN0357	Unseqeunced	AUX4	1	rhizoid	B
JN0382	Unseqeunced	AUX4	1	thallus	C
JN0389	Unseqeunced	WRG	13	thallus	G
JN0413	Unseqeunced	AUX1	1	Thallus	C
JN0414	Unseqeunced	NCB	2	Receptacle	B
JN0251	*Xylaria arbuscula*	NCB	male 2	thallus	C
JN0242	*Xylaria cubensis*	NCB	female 1	thallus	F
JN0244	*Xylaria cubensis*	NCB	female 2	receptacle	D
JN0247	*Xylaria cubensis*	NCB	female 2	thallus	C
JN0250	*Xylaria cubensis*	NCB	male 2	thallus	B
JN0255	*Xylaria cubensis*	NCB	male 2	rhizoid	C
JN0268	*Xylaria cubensis*	UTN	3	thallus	B
JN0269	*Xylaria cubensis*	UTN	3	thallus	G
JN0326	*Xylaria cubensis*	CHR	2	rhizoid	A
JN0248	Xylariaceae sp. 1	NCB	female 2	rhizoid	B

Isolate	Fungal Taxon	Patch	Plant	Tissue	Rep
JN0252	Xylariaceae sp. 2	NCB	male 2	thallus	F
JN0254	Xylariaceae sp. 3	NCB	male 2	rhizoid	B
JN0397	Xylariaceae sp. 4	AUX5	13	receptacle	D

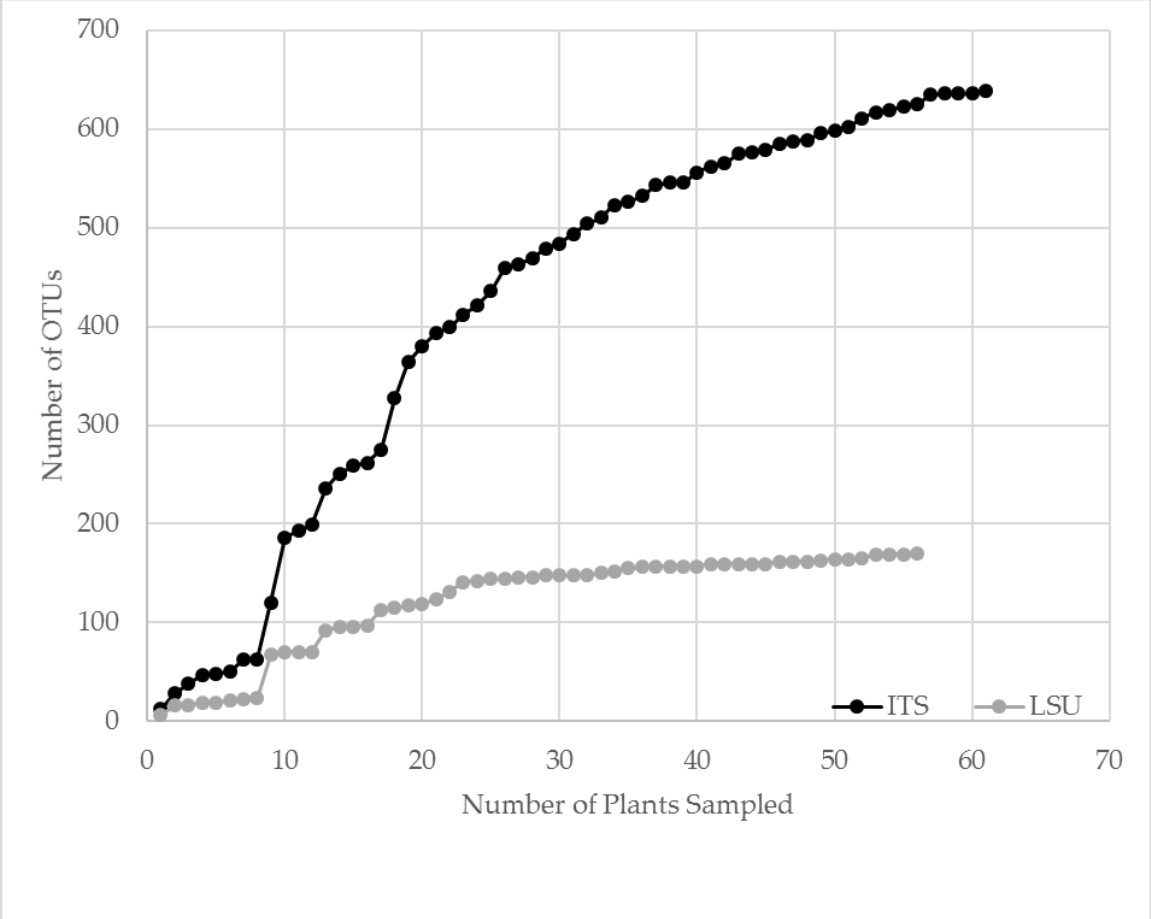

Figure 19: Operational taxonomic unit accumulation curves for ITS and LSU amplicon datasets.
Substrate samples and mock communities are excluded and post-filtering data are used. Samples are ordered by region moving right from Southeast, to Northeast, to Northwest. Black shows the ITS dataset and grey shows the fungal OTUs from the LSU dataset.

Figure 20: Fungal fruiting bodies on *M. polymorpha*.
(a) Putative *Pezoloma marchantiae* from AUX5 collection in Kentucky. (b) Putative *Loreleia marchantiae,* from near MSH2 collection in Washington.

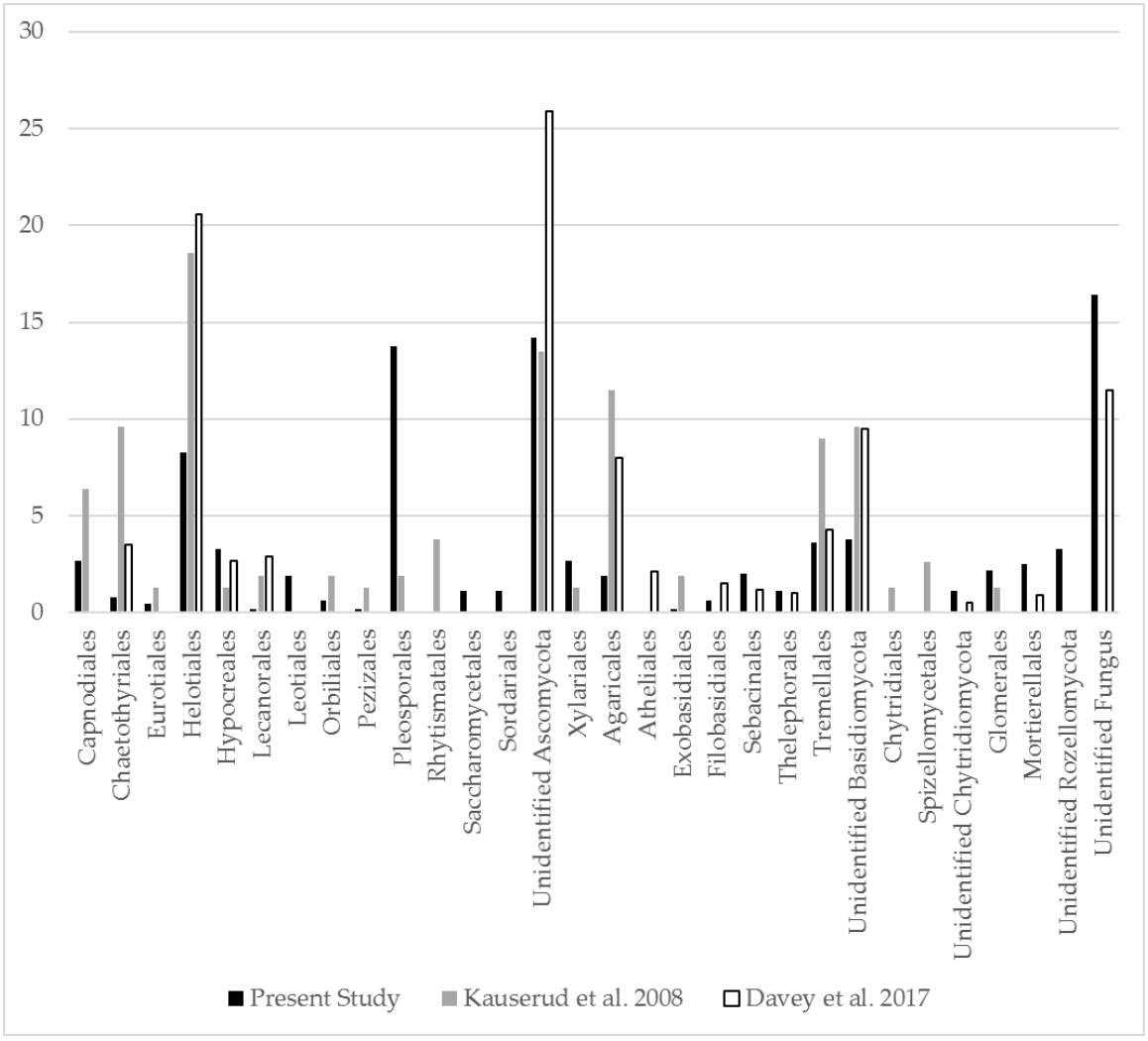

Figure 21: Comparison of *M. polymorpha* fungal community to those recovered from boreal mosses.

Bars show percentage of total study OTUs belonging to each listed order. *M. polymorpha* is shown in black, mosses assessed by Kauserud et al. 2008 with an ITS clone library are shown in grey, and mosses assessed by Davey et al. 2017 with 454 pyrosequencing of ITS are shown in white. Orders are shown if at least one of the three studies had at least 1% of OTUs assigned them.

Table 9: Studies of vascular hosts compared to *M. polymorpha* endophyte culture collection.

Source	Location	Host
Arnold 2002	Panama	*Laetia thamnia*
Junker et al. 2012	Braunschweig, Germany	*Arabidopsis thaliana*
Li et al. 2010	Yunnan Province, China	*Impatiens chinensis*
Li et al. 2010	Yunnan Province, China	*Ottelia acuminata*
Li et al. 2010	Yunnan Province, China	*Myriophyllum verticillatum*
Li et al. 2010	Yunnan Province, China	*Equisetum arvense*
Li et al. 2010	Yunnan Province, China	*Cardamine multijuga*
Oono et al. 2015	Duke Forest, NC, USA	*Pinus taeda*
Pancher et al. 2012	Trentino, Italy	*Vitis vinifera*
Qi et al. 2009	Harbin, China	*Acer ginnala*
Quilliam and Jones 2010	Gwynedd,UK	*Drosera rotundifolia*
Quilliam and Jones 2012	Gwynedd,UK	*Pinguicula vulgaris*
Yao et al. 2017	Guangxi Province, China	*Sophora tonkinensis*
Yuan et al. 2009	Yunnan Province, China	*Dendrobium nobile*
Yuan et al. 2011 a	Yunnan Province, China	*Oryza granulata*
Yuan et al. 2011 b	Zhejiang Province, China	*Abies beshanzuensis*

Table 10: Comparison of *M. polymorpha* endophyte culture collection to cultures from vascular hosts.
Host names are abbreviated by the first letter of genus and species names. Refer to Table 9 for full names and sources. Mp is *Marchantia polymorpha* from the present study. Presence of a fungal genus in a culture collection for a particular host is indicated by a 1 and a shaded box. Column S shows the number of vascular plant studies in which the fungal genus in question was isolated. Sums for each genus across all vascular hosts are coded with a color gradient with green showing genera frequently isolated from multiple hosts and red showing genera not isolated from any of the investigated vascular hosts but present in the present study's culture collection from *M. polymorpha.*

Host	*Pt*	*St*	*Ic*	*Oa*	*Mv*	*Ea*	*Cm*	*Lt*	*Og*	*Dn*	*Pv*	*Dr*	*Ag*	*Ab*	*At*	*Vv*	S	*Mp*
Absidia																1	1	
Acaromyces														1			1	
Acremonium									1								1	
Alatospora												1					1	
Alternaria	1	1		1	1				1				1	1	1	1	9	
Annulohypoxylon	1																1	
Anthostomella	1																1	
Aposphaeria				1		1											2	
Arthrinium									1						1		2	
Articulospora												1					1	
Ascochyta			1												1		2	
Aspergillus		1	1	1					1					1		1	6	
Astrosphaeriella	1																1	
Aureobasidium		1													1	1	3	
Beaveria									1					1			2	
Biscogniauxia	1																1	1

Host	*Pt*	*St*	*Ic*	*Oa*	*Mv*	*Ea*	*Cm*	*Lt*	*Og*	*Dn*	*Pv*	*Dr*	*Ag*	*Ab*	*At*	*Vv*	S	*Mp*
Bjerkandera														1			1	
Botryosphaeria								1	1	1							3	
Botrytis															1	1	2	
Bulleromyces																	0	1
Cadophora																	0	1
Candida																	0	1
Capnobotryella																	0	
Catinula			1			1											2	
Cephalosporium						1		1									2	
Cercospora	1							1	1								3	
Ceriporia														1			1	
Chaetomella				1													1	
Chaetomium		1															1	
Chaetophoma						1											1	
Chaetosphaeria		1															1	
Chalara															1		1	
Chaunopycnis														1			1	
Cladosporium	1	1	1	1	1	1	1		1		1		1	1	1	1	13	1
Clonostachys										1							1	
Cochliobolus	1								1								2	
Coleophoma															1		1	
Colletotrichum	1	1						1	1	1				1	1		7	1
Coniochaeta	1													1			2	1

Host	*Pt*	*St*	*Ic*	*Oa*	*Mv*	*Ea*	*Cm*	*Lt*	*Og*	*Dn*	*Pv*	*Dr*	*Ag*	*Ab*	*At*	*Vv*	S	*Mp*
Coniothyrium															1		1	
Coprinellus														1			1	
Corydyceps								1									1	
Cryptosporiopsis		1												1			2	
Cylindrocladium								1									1	
Cytospora	1														1		2	
Daldinia	1							1									2	1
Debaryomyces																1	1	
Diaporthe	1							1									2	
Dicarpella	1																1	
Doratomyces															1		1	
Dothistroma	1																1	
Epicoccum	1	1											1		1	1	5	1
Exophiala								1									1	
Fomitopsis		1															1	
Fusarium	1	1	1		1	1	1		1	1			1		1	1	11	
Fusicoccum							1	1									2	
Gaeumannomyces															1		1	
Geniculsporium															1		1	
Geotrichum			1	1	1	1	1										5	
Gliocladium															1		1	
Gloeosporium						1											1	
Glomerella								1									1	

Host	*Pt*	*St*	*Ic*	*Oa*	*Mv*	*Ea*	*Cm*	*Lt*	*Og*	*Dn*	*Pv*	*Dr*	*Ag*	*Ab*	*At*	*Vv*	S	*Mp*
Guignardia	1							1		1							3	
Hainesia					1												1	
Hansenula																1	1	
Harpophora									1								1	
Hypocrea		1															1	
Hypoxylon	1							1		1							3	1
Lachnellula														1			1	
Lasiodiplodia		1															1	
Leohumicola											1						1	
Leptosphaeria	1								1						1		3	
Lophodermium	1																1	
Macrophomina	1																1	
Melasmaia							1										1	
Meira														1			1	
Metarhizium		1															1	
Microdochium									1						1		2	
Microsphaeropsis															1		1	1
Monochaetia														1			1	
Mortierella		1														1	2	
Mucor		1		1	1		1									1	5	
Muscodor	1								1					1			3	
Mycosphaerella	1													1			2	
Myrothecium		1															1	

Host	*Pt*	*St*	*Ic*	*Oa*	*Mv*	*Ea*	*Cm*	*Lt*	*Og*	*Dn*	*Pv*	*Dr*	*Ag*	*Ab*	*At*	*Vv*	S	*Mp*
Nemania	1									1							2	1
Neonectria											1						1	
Neurospora													1			1	2	
Nigrospora	1																1	
Nodulisporium	1							1						1	1		4	
Ochroconis	1																1	
Oedocephalum			1				1										2	
Ophiocordyceps	1																1	
Paecilomyces								1	1					1			3	
Paraconiothyrium	1													1			2	
Penicillium		1	1	1					1	1		1	1	1		1	9	
Peniophora	1													1			2	
Pestalotia						1											1	
Pestalotipsis								1		1				1			3	
Periconia															1		1	
Peyronellaea	1																1	
Pezicula														1			1	
Phacidiopycnis														1			1	
Phaeomoniella	1																1	
Phaeosphaeria									1							1	2	
Phaerochaete														1			1	
Phialocephala		1												1			2	
Phialophora		1															1	

Host	*Pt*	*St*	*Ic*	*Oa*	*Mv*	*Ea*	*Cm*	*Lt*	*Og*	*Dn*	*Pv*	*Dr*	*Ag*	*Ab*	*At*	*Vv*	S	*Mp*
Pholiota																	0	1
Phoma	1	1											1		1		4	1
Phomopsis								1		1			1	1	1		5	
Phyllosticta				1		1								1			3	
Pichia																1	1	
Pithomyces																1	1	
Plectosphaerella															1		1	1
Pleurocytospora															1		1	
Podospora	1															1	2	
Pseudomassariella	1																1	
Pycnoporus														1			1	
Pyrenochaeta														1			1	
Purpureocillium		1															1	
Ramichloridium									1								1	
Rhexocercosporidium		1															1	
Rhinocladiella								1									1	
Rhizoctonia										1					1		2	
Rhizopus															1	1	2	
Rhizosphaera	1													1			2	
Rhytidhysteron		1															1	
Saccharomyces																1	1	
Sagenomella		1															1	
Schizophyllum		1												1			2	

Host	*Pt*	*St*	*Ic*	*Oa*	*Mv*	*Ea*	*Cm*	*Lt*	*Og*	*Dn*	*Pv*	*Dr*	*Ag*	*Ab*	*At*	*Vv*	S	*Mp*
Schizothecium																	0	1
Schizosaccaromyces																1	1	
Sclerotinia																1	1	
Scytalidium								1									1	
Seiridium								1									1	
Septoria	1														1		2	
Septorioides	1																1	
Sirodesmium			1			1	1										3	
Sordaria															1		1	
Sphaceloma							1										1	
Stachybotrys									1								1	
Stagnospora															1		1	
Talaromyces		1															1	
Teratosphaeria	1																1	
Thysanophora														1			1	
Tilletiopsis														1			1	
Torula						1									1		2	
Toxicocladosporium	1																1	1
Trametes														1			1	
Trichoderma		1					1			1	1	1	1		1	1	8	
Trichosporon		1															1	
Ulocladium															1		1	
Umbelopsis							1									1	2	

Host	*Pt*	*St*	*Ic*	*Oa*	*Mv*	*Ea*	*Cm*	*Lt*	*Og*	*Dn*	*Pv*	*Dr*	*Ag*	*Ab*	*At*	*Vv*	S	*Mp*
Verticillium								1	1								2	
Virgaria	1																1	
Volutella											1				1		2	
Xylaria	1								1	1				1			4	1
Yarrowia																1	1	
Zygorhyncus																1	1	
Zygosaccaromyces																1	1	

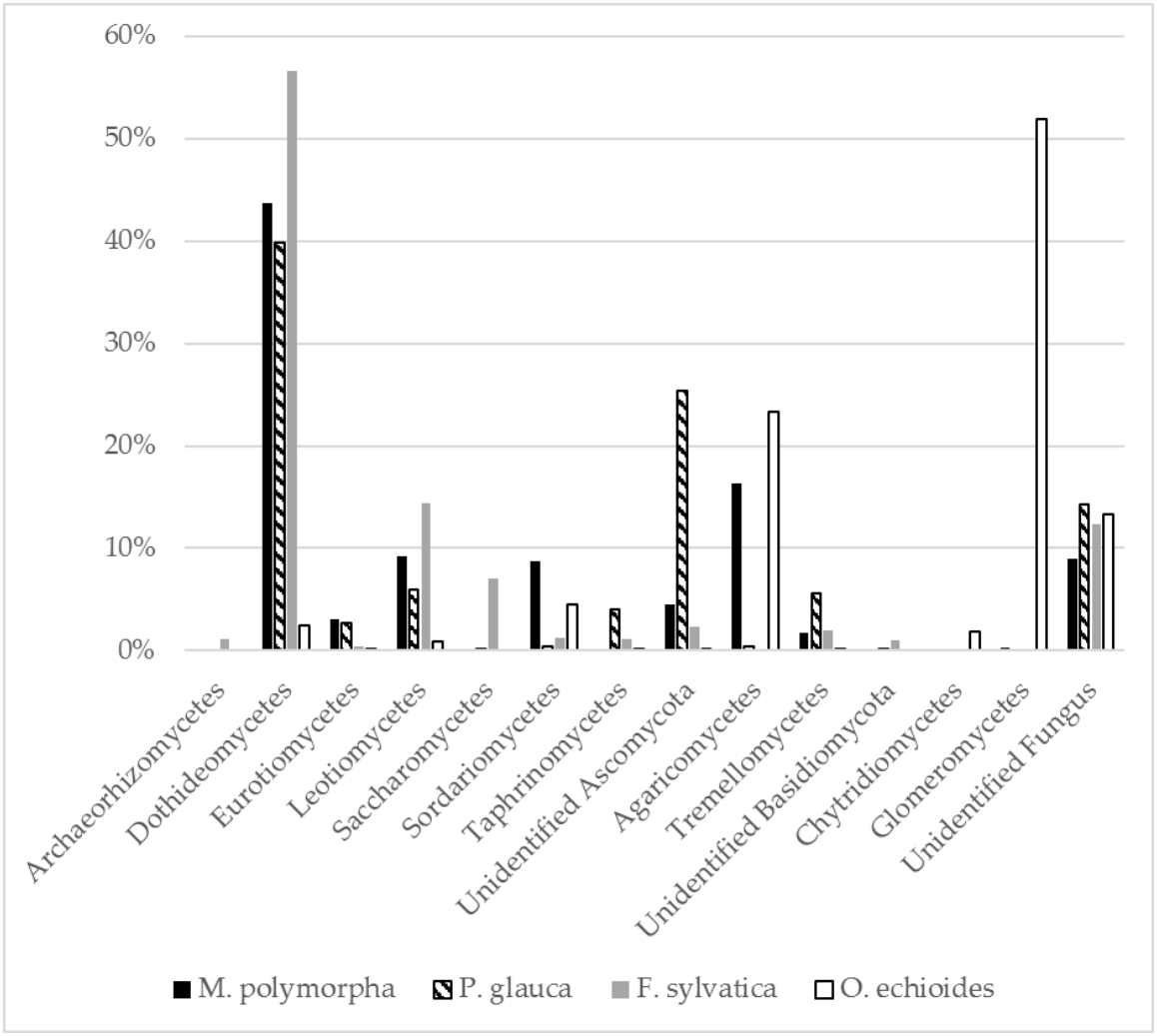

Figure 22: Comparison of *M. polymorpha* ITS amplicon data to previous studies of vascular plant endophytes.
Height of bars shows the percent of ITS OTUs belonging to a fungal class. A class is shown if at least one of the studies recovered at least 1% of their OTUs from that class.

Appendix C: Chapter 2 Supplemental Figures and Tables

Table 11: List of all fungal isolates used in this study, summarizing original collection information, taxon identities, experiment sample sizes, and experimental effects on growth and host morphology.
Taxa labeled as unsequenced did not yield usable DNA sequences from our standard methods. Site codes correspond to those in Table 5 in Appendix B. Column Sub shows numbered sub-isolates of the same original plant fragment. *Collection*: Abbreviations used in the tissue column (T) indicate the liverwort tissue source: G = gametangiophore, T = thallus, R = rhizoid, and S = surface. Surface isolations used fungal fruiting bodies found on plants. *Results and Parameters:* Effects calls, p-values, and sample sizes (SS) are given for Experiment one (E1) and Experiment two (E2). Effects of fungal inoculation are abbreviated as follows: + = positive i.e. growth promotion, W = weak positive, N = neutral i.e. no difference in growth from controls, and - = negative i.e. plant tissue was killed, making the plant shrink. N/- indicates fungi that were quantitatively categorized as neutral but still killed tissues. P-values are given for ANOVA comparing growth of treated plants with control plants. Sample sizes are numbers of *M. polymorpha* plants treated with each fungus. *Morphological:* Morphologies of plants observed at the conclusion of Experiment one are recorded for rhizoids and thalli. Rhizoid morphology categories are as follows: B = brown/melanized rhizoids, S = dark spots inside rhizoids, H = hyphae of fungus observed penetrating rhizoids, and I = irregular inclusions observed inside rhizoids. Thallus morphology categories are: D = some tissue death, S = dark spots in cells, and B = browning of individual cells. Numbers in these columns indicate how many plants the morphology was noted in.

					Results and Parameters						**Morphological**						
			Collection		**Effect**		**P-val**		**SS**		**Rhizoids**				**Thalli**		
Isolate	**Sub**	**Fungus Name**	**Site**	**T**	**E1**	**E2**	**E1**	**E2**	**E1**	**E2**	**B**	**S**	**H**	**I**	**D**	**S**	**B**
JN0241		*Nemania* sp. 1	NCB	G	N	+		<0.001	4	12					1		
JN0242		*Xylaria cubensis*	NCB	T	N	-		0.400	4	12	1				4	1	
JN0243		*Hypoxylon* sp. 1	NCB	G	N	+		<0.001	4	12	4				1		
JN0244		*Xylaria cubensis*	NCB	G	-				4						4		

Isolate	Sub	Fungus Name	Site	T	E1	E2	E1	E2	E1	E2	B	S	H	I	D	S	B
JN0245		*Nemania serpens*	NCB	T	W	+		<0.001	4	12					1		
JN0246	263	*Nemania* sp. 1	NCB	T	+				8								
JN0247		*Xylaria cubensis*	NCB	T	-				4						4	3	
JN0248	351, 352	Xylariaceae sp. 1	NCB	R	N		0.559		12		2	6	1		3	1	1
JN0249		*Nemania* sp. 2	NCB	G	W				4						1	1	
JN0250		*Xylaria cubensis*	NCB	T	-				4			1			4		
JN0251		*Xylaria arbuscula*	NCB	T	N	N		0.014	4	12	2				2		
JN0252		Xylariaceae sp. 2	NCB	T	N	+		0.009	2	12					2		
JN0253	264, 267	*Nemania serpens*	NCB	T	W	N	0.029	0.447	11	12					1	1	
JN0254		Xylariaceae sp. 3	NCB	R	N	+		<0.001	4	12					2		
JN0255		*Xylaria cubensis*	NCB	R	-				4			1			3	2	
JN0268		*Xylaria cubensis*	UTN	T	N	-		0.172	4	12					3	2	
JN0269		*Xylaria cubensis*	UTN	T	-				4						4		
JN0296		*Hypoxylon* sp. 1	UTN	R	W	+		0.009	4	12	3						
JN0299	359	*Phoma herbarum*	AUX1	G	N				8					1	3		
JN0300	353	*Biscogniauxia mediterranea*	AUX1	T	+	+		0.003	8	24	2				3		
JN0301	364, 365, 366, 416	*Phoma herbarum*	AUX1	G	N	N	0.999	0.091	20	12	3	6	1	1	11		
JN0302		Hyaloscyphaceae sp. 1	AUX2	G	N				4						1		
JN0303		Coniochaetaceae sp. 1	AUX3	T	N				4						1		
JN0304		Coniochaetaceae sp. 2	AUX3	T	W	+		<0.001	4	12							
JN0305		*Biscogniauxia mediterranea*	AUX3	T	+				4								
JN0307		*Coniochaeta* sp. 2	AUX3	R	N				4								
JN0308	350	*Biscogniauxia mediterranea*	AUX2	T	W	+		<0.001	8	12					1		
JN0310		Coniochaetaceae sp. 1	AUX3	T	N				4								

Isolate	Sub	Fungus Name	Site	T	E1	E2	E1	E2	E1	E2	B	S	H	I	D	S	B
JN0313		*Biscogniauxia mediterranea*	AUX4	T	N				4						2		
JN0314		*Coniochaeta* sp. 1	AUX4	T	N				4			1			2	1	
JN0315	A in E2	(Unsequenced)	AUX4	G	N	+		<0.001	4	24					2	1	
JN0315	B in E2	(Unsequenced)	AUX4	G	N	N		0.015	4	24					2		
JN0316		Lasiosphaeriaceae sp. 1	AUX4	R	+				4						2		
JN0317	357	*Biscogniauxia mediterranea*	AUX4	R	W				8			1			1		
JN0318		*Colletotrichum truncatum*	CHR	G	W	+		<0.001	4	12					3		
JN0319		*Colletotrichum truncatum*	CHR	G	W	+		<0.001	4	12		1			3		1
JN0321		*Daldinia loculata*	AUX2	T	W	+		<0.001	4	12					2		
JN0322		*Toxicocladosporium irritans*	AUX4	T	N	N		0.541	4	8					2	1	
JN0324		*Microsphaeropsis arundinis*	CHR	G	+	+		0.009	4	12					2		
JN0326		*Xylaria cubensis*	CHR	R	-				4						3		
JN0327		Helotiaceae sp. 1	AUX2	T	N				4								1
JN0329		Leotiomycete sp. 3	CHR	T	N				2						2	1	
JN0330		Lasiosphaeriaceae sp. 2	YUK1	T	N				4						2		1
JN0331		*Schizothecium* sp. 1	YUK1	T	N				4						1	2	
JN0332		*Phoma herbarum*	YUK2	G	N				4						4	4	
JN0333		Leotiomycete sp. 1	UTN	S	N				4						2		1
JN0335		*Plectosphaerella* sp. 1	UTN	S	N				4						2		
JN0336		Leotiomycete sp. 1	UTN	S	N				4						1		
JN0337		Leotiomycete sp. 1	UTN	S	N	+		<0.001	4	12	1				3		
JN0354		Lasiosphaeriaceae sp. 3	AUX3	T	N				4						1		
JN0355		Coniochaetaceae sp. 1	AUX4	T	N				4						1		
JN0356		Sordariomycete sp. 2	AUX4	T	W	+		<0.001	8	12					1		

Isolate	Sub	Fungus Name	Site	T	E1	E2	E1	E2	E1	E2	B	S	H	I	D	S	B
JN0362		Helotiales sp. 2	CHR	G	N				4						1		
JN0363		Helotiales sp. 2	CHR	G	W				3								
JN0368		Leptosphaeriaceae sp. 1	AUX4	S	N	+		<0.001	4	12	1				1		
JN0369	373, 375	*Coniochaeta* sp. 2	AUX3	T	N		0.493		11						2		
JN0377		Coniochaetaceae sp. 3	AUX3	R	N				4						1		
JN0379		*Coniochaeta* sp. 2	AUX3	T	N				4								
JN0382		(Unsequenced)	AUX4	T	-				4						3		
JN0383		*Hypoxylon submonticulosum*	LIB	T	N				4		2				1		
JN0384	409	*Hypoxylon submonticulosum*	WRG	G	N/-	-		1.000	8	12	1				4		
JN0385	410	*Colletotrichum truncatum*	WRG	G	N	+		<0.001	8	12					1		
JN0386	411	*Hypoxylon submonticulosum*	WRG	T	N	N		0.011	8	12	3				4		
JN0387		*Colletotrichum acutatum*	WRG	G	N/-	N		0.239	4	12					2		
JN0388		Leotiomycete sp. 2	LIB	T	N				3						2		
JN0390		Pezizales sp. 1	WRG	T	N				4						2	1	
JN0391		*Colletotrichum* sp. 1	WRG	G	-				4					1	3		1
JN0393		Sordariomycete sp. 1	AUX5	T	N				4						2		
JN0394		*Nemania* sp. 1	AUX5	T	N	N		0.019	4	12							
JN0395		Pleosporales sp. 2	AUX5	G	N				4				2	1	1		
JN0396		*Coniochaeta* sp. 2	AUX5	T	N				4						1		
JN0397		Xylariaceae sp. 4	AUX5	G	N				4						1	3	
JN0399		*Cadophora luteo-olivacea*	LIB	T	N				4						1		
JN0401	412	*Phoma herbarum*	AUX5	G	N	N		0.033	8	12	1	1			6	2	
JN0403		*Candida* sp. 1	LCC	R	N				4						1		
JN0406		*Candida* sp. 1	AUX4	T	N				4						2		

Isolate	Sub	Fungus Name	Site	T	E1	E2	E1	E2	E1	E2	B	S	H	I	D	S	B
JN0407		Sordariomycete sp. 3	YUK1	T	N				2			1			1		
JN0408		*Phoma herbarum*	WRG	G	N				4								
JN0413		(Unsequenced)	AUX1	T	-				4						3		
JN0414		(Unsequenced)	NCB	G	N				3						1		
JN0415		Coniochaetaceae sp. 1	AUX3	T	W				4						1		1
JN0419		Sordariales sp. 1	AUX4	T	W				4						1		

Table 12: Recipe for modified Hatcher's agar.
Rightmost column shows the concentration of each nutrient salt in the final medium mix used for growing plants.

Stock	Stock Vol	Ingredient	in Stock	Final Concentration
A	2 L	$MgSO_4 * 7H_2O$	14.7 g	2.98 mM
A	2 L	$Ca(NO_3)_2 * 4H_2O$	5.2 g	1.10 mM
A	2 L	Na_2SO_4	4 g	1.41 mM
A	2 L	$NaNO_3$	1.6 g	941 μM
A	2 L	KCl	1.3 g	872 μM
A	2 L	KH_2PO_4	3.3 g	1.21 mM
B	1 L	H_2SO_4	0.5 mL	0.0015%
B	1 L	$MnSO_4 * 4H_2O$	3 g	40.3 μM
B	1 L	$ZnSO_4 * 7H_2O$	500 mg	5.22 μM
B	1 L	H_2BO_3	500 mg	24.7 μM
B	1 L	$CuSO_4 * 5H_2O$	25 mg	300 nM
B	1 L	$Na_2MoO_4 * 2H_2O$	25 mg	309 nM
B	1 L	$CoNO_3$	25 mg	409 nM
Iron	0.5 L	$FeC_6H_5O_7 * 7H_2O$	1.25 g	6.74 μM

Appendix D: Code

1. Amplicon Sequence Processing

These commands use the programs QIIME, cutadapt, Trimmomatic, and VSEARCH. They were run in a Linux environment.

```
##Amplicon Data Processing##

#Demultiplexing with QIIME
split_libraries_fastq.py -i JN_3952-L1_S1_L001_R1_001.fastq.gz -m
map_corrected.txt -b JN_3952-L1_S1_L001_I1_001.fastq.gz --
store_qual_scores --store_demultiplexed_fastq --barcode_type 10 -
o ForwardQIIME/ --rev_comp_mapping_barcodes -q 19 -p .5

#Split ITS1 and LSU datasets and remove primers with cutadapt
cutadapt -g ITS1=CTTGGTCATTTAGAGGAAGTAA -g LROR=ACCCGCTGAACTTAAGC
-e .1 -o {name}-F_cutadapt.fastq.gz seqs.fastq --discard-
untrimmed --match-read-wildcards

#Trimmomatic filtering to remove adapters
java -jar ./Trimmomatic-0.36/trimmomatic-0.36.jar SE -phred33
ITS1-F_cutadapt.fastq.gz ITS1-F_trimm.fq.gz ILLUMINACLIP:TruSeq3-
PE_ed.fa:2:30:7 MINLEN:100 SLIDINGWINDOW:4:20 MINLEN:36
java -jar ./Trimmomatic-0.36/trimmomatic-0.36.jar SE -phred33
LROR-F_cutadapt.fastq.gz LROR_trimm_superextreme.fastq.gz
ILLUMINACLIP:TruSeq3-PE_ed.fa:2:30:7 MINLEN:100
SLIDINGWINDOW:4:28 MINLEN:36 TRAILING:28

#vsearch filtering
vsearch --fastq_filter ITS1-F_trimm.fq.gz --fastaout
ITS1_filt.fasta --fastq_truncqual 10 --fastq_minlen 180
vsearch --fastq_filter LROR_trimm_superextreme.fastq.gz --
fastaout LROR_filt_suext.fasta --fastq_truncqual 10 --
fastq_minlen 180

#Vsearch dereplication
vsearch --derep_fulllength ITS1_filt.fasta --output
derep_ITS1_filt.fasta --sizeout
vsearch --derep_fulllength LROR_filt_suext.fasta --output
derep_LROR_filt_suext.fasta --sizeout
```

```
#Vsearch sort dereplication by size, remove singleton reads.
vsearch --sortbysize derep_ITS1_filt.fasta --output
derepm2_ITS1_filt.fasta -minsize 2
vsearch --sortbysize derep_LROR_filt_suext.fasta --output
derepm2_LROR_filt_suext.fasta -minsize 2

#removeChimeras
vsearch --uchime_denovo derepm2_ITS1_filt.fasta --nonchimeras
ITS_nonchim_97.fasta
vsearch --uchime_denovo derepm2_LROR_filt_suext.fasta --
nonchimeras LSU_suext_97_nonchim.fasta

#vsearch OTU picking
vsearch --cluster_fast ITS_nonchim_97.fasta --id 0.97 -iddef 2 --
relabel OTU --centroids ITS1F_centroid_cd.fasta --otutabout
ITS1F_clusters_cd.txt --sizein --sizeout
vsearch --cluster_fast LSU_suext_97_nonchim.fasta --id 0.97 -
iddef 2 --relabel OTU_ --centroids
LROR_centroid_97_suext_cd.fasta --otutabout
LROR_clusters_97_suext_cd.txt --sizein --sizeout

#sort by size and remove OTU tail
vsearch --sortbysize ITS1F_centroid_cd.fasta --output
m10_ITS1F_centroid_cd.fasta -minsize 10 --relabel OTU_ --sizeout
vsearch --sortbysize LROR_centroid_97_suext_cd.fasta --output
m10_LROR_centroid_97_suext_cd.fasta -minsize 10 --relabel OTU_ --
sizeout

#regular expression to edit the input file so that the region
filtering works properly. Delimiters weren't too happy otherwise.
REGEX for making modified region_filt file in jedit (probably can
use sed to script it) (this is done on the ITS1_filt.fasta file)
Find .
Replace _

Find _\d* M00
Replace M00

#get read counts from original database
vsearch --usearch_global ITS1_filt_modified.fasta -db
m10_ITS1F_centroid_cd.fasta -id 0.97 -biom
./output/ITS/ITS1F_global_cd.biom --sizeout --otutabout
./output/ITS/ITS1F_global_m10_cd.txt
vsearch --usearch_global LROR_filt_modified.fasta -db
m10_LROR_centroid_97_suext_cd.fasta -id 0.97 -biom
LROR_global_m10_97_suext_cd.biom --sizeout --otutabout
LROR_global_m10_97_suext_cd.txt
```

```
#assign taxonomy for ITS
assign_taxonomy.py -i m10_ITS1F_centroid_cd.fasta -m mothur -r
./sh_qiime_release_s_20.11.2016/sh_refs_qiime_ver7_97_s_20.11.201
6.fasta -t
./sh_qiime_release_s_20.11.2016/sh_taxonomy_qiime_ver7_97_s_20.11
.2016.txt -o ./mothur_ITS_taxonomy
#LSU taxonomy assignments was done by uploading the centroid file
to that LSU database.
```

2. Amplicon Data Analysis and Visualization

These commands were written in R version 3.3.1. They are not fully independent scripts and commands were run individually for the analyses presented in this dissertation. Commands specific to file names and paths have been filled in with pseudo-coded names.

```
####Analysis of amplicon data####

setwd("file/path")#insert appropriate path

#load packages
library("vegan", lib.loc="~/R/win-library/3.3")
library("ggplot2", lib.loc="~/R/win-library/3.3")
library("plyr", lib.loc="~/R/win-library/3.3")
library("MASS", lib.loc="C:/Program Files/R/R-3.3.1/library")


###Data Preparation and Filtering###

#load OTU table
otu.tab<-read.csv("file.csv",header=TRUE)#insert file name

#filter out samples with too few reads
otu.fil<-otu.tab[rowSums(otu.tab[,2:ncol(otu.tab)])>999,]
#used 499 instead of 999 for non-fungal dataset

#normalize OTU table
otu.test<-otu.fil
otu.test[]<-lapply(otu.test, function(x) if(is.numeric(x))
scale(x,center=FALSE,scale=sum(x)) else x)
```

```
#remove nas
otu.ready<-otu.test
cut<-sapply(otu.ready, function(x) all(is.nan(x)))
otu.clean<-otu.ready[,!cut]

#remove taxa with mean abundance below 1 per million
otu.final<-
otu.clean[,colMeans(otu.clean[,2:nrow(otu.clean)])>(1/1000000)]

#extract sample names from filtered table
Sample<-otu.final$Sample


###Ordinations###

#calculate Bray-Curtis index and create distance matrix
BC.index<-vegdist(otu.final[,2:ncol(otu.final)],method="bray")

#calculate Jaccard index and create distance matrix
J.index<-vegdist(otu.final[,2:ncol(otu.final)],method="jaccard")

##PCoA##
#PCoA for both indices
pcoa.bc<-cmdscale(BC.index,k=4,eig=TRUE,add=TRUE)
pcoa.j<-cmdscale(J.index,k=4,eig=TRUE,add=TRUE)

#scree plots
plot(pcoa.bc$eig/sum(pcoa.bc$eig))
plot(pcoa.j$eig/sum(pcoa.j$eig))

#get percentage variance explained on each axis
pcoa.bc$eig[1]/sum(pcoa.bc$eig)
pcoa.bc$eig[2]/sum(pcoa.bc$eig)

pcoa.j$eig[1]/sum(pcoa.j$eig)
pcoa.j$eig[2]/sum(pcoa.j$eig)

#add labels back in
pcoa.bc.df<-data.frame(pcoa.bc$points)
pcoa.bc.lab<-cbind(Sample,pcoa.bc.df)

pcoa.j.df<-data.frame(pcoa.j$points)
pcoa.j.lab<-cbind(Sample,pcoa.j.df)
```

```
#graph PCoA results#
#shows one example for plotting one PCoA result set

#load design file
meta<-read.csv("metadata.csv",header=TRUE)#fill in file name
#metadata should include sample names in one column named
“Sample” and additional information in subsequent columns.

#trim metadate file to include just the sites in the data
index<-meta$Sample %in% pcoa.j.lab$labels
meta.sub<-meta[index,]

#make plot with shapes to indicate soil vs. endophytic and colors
to indicate collected plant patch
plot<-ggplot(pcoa.j.lab,aes(X2,X1))
geom_point(aes(colour=factor(meta.sub$Patch),shape=factor(meta.su
b$Compartment),size=1))

##NMDS##
nmds.j<-isoMDS(J.index,k=2)

#add sample names to results
nmds.j.df<-data.frame(nmds.j$points)
nmds.j.lab<-cbind(Sample,nmds.j.df)

#plot with color code by site
plot + geom_point(aes(colour=factor(meta.sub$Site),size=1))

#plotting polygons
df<-merge(x=nmds.j.lab,y=meta,by="Sample",all.x=TRUE)

find_hull <- function(df) df[chull(df$X1, df$X2), ]
hulls <- ddply(df, "Site", find_hull)

plot <- ggplot(data = df, aes(x = X1, y = X2, colour=Site,
fill=Site )) +
  geom_point() +
  geom_polygon(data = hulls, alpha = 0.5)
plot

##PERMANOVA##
##Check for significance of groups based on distance matrix

#subset metadata
index<-meta$Sample %in% otu.final$Sample
meta.sub<-meta[index,]
```

```
#run permanova
perm<- adonis(J.index ~ Site, data=meta.sub, permutations=999,
method ="jaccard")
summary(perm)

perm$aov.tab

#look for difference between regions
perm.reg<- adonis(J.index ~ Region, data=meta.sub,
permutations=999, method ="jaccard")
perm.reg$aov.tab


###Alpha diversity###

#extract sample labels
Meta<-otu.tab$Sample

#convert to matrix
otu.mat<-data.matrix(otu.tab)

#rarify OTU table
otu.rar<-rrarefy(otu.mat, sample=1000)

#calculate Shannon diversity
otu.div<-diversity(otu.rar, index="shannon")
otu.div.lab<-cbind(Meta,otu.div)

#save, exclude sites with 2 or fewer plants, and read back in
write.csv(otu.div.lab,"Shannon_file.csv") #give file name
otu.trim<-read.csv("Shannon_Trim.csv",header=TRUE)#fill in file

#check data characteristics
hist(otu.trim$Shannon)
bartlett.test(otu.trim$Shannon~otu.trim$Site)
shapiro.test(otu.trim$Shannon)

#ANOVA by collection site
otu.aov<-aov(otu.trim$Shannon~otu.trim$Site)
summary(otu.aov)
TukeyHSD(otu.aov)

#ANOVA by region
otu.aov2<-aov(otu.trim$Shannon~otu.trim$Region)
summary(otu.aov2)
```

3. Automated measurement of liverwort area

This is a macro for the Fiji distribution of Image J.

```
macro "Liverwort auto"{

// Make sure each image is labeled with a date and plate code
before running this!
//make sure to label the calibration image "Ruler.jpg"

//The color thresholding min and max values can be adjusted to
fit the color temperature and white balance of different image
conditions.

//set measurements to show only area and image name
run("Set Measurements...", "area display redirect=None
decimal=3");

//set output folder
output = getDirectory ("Pick output folder");

//set input folder
input = getDirectory ("Pick input folder");

//calibrate measurements
ruler = input + "Ruler.jpg";
open(ruler);
file = getTitle();
setTool("line");
waitForUser("draw a 10mm line on the ruler");
run("Set Scale...", "known=10 unit=unit global");
close(file);


// Color Thresholder 2.0.0-rc-41/1.50d
function colorThreshold(){
      min=newArray(3);
      max=newArray(3);
      filter=newArray(3);
      a=getTitle();
      run("HSB Stack");
      run("Convert Stack to Images");
      selectWindow("Hue");
      rename("0");
      selectWindow("Saturation");
      rename("1");
      selectWindow("Brightness");
```

```
      rename("2");
      min[0]=40;
      max[0]=126;
      filter[0]="pass";
      min[1]=64;
      max[1]=255;
      filter[1]="pass";
      min[2]=0;
      max[2]=185;
      filter[2]="pass";
      for (i=0;i<3;i++){
            selectWindow(""+i);
            setThreshold(min[i], max[i]);
            run("Convert to Mask");
      if (filter[i]=="stop")  run("Invert");
      }
      imageCalculator("AND create", "0","1");
      imageCalculator("AND create", "Result of 0","2");
      for (i=0;i<3;i++){
       selectWindow(""+i);
       close();
      }
      selectWindow("Result of 0");
      close();
      selectWindow("Result of Result of 0");
      rename(a);
      }
// End Colour Thresholding-------------

//go through files and measure
list = getFileList(input);

for (i = 0; i < list.length; i++){
      open (input + list[i]);
      name=getTitle();
      if (name != "ruler.jpg"){
            colorThreshold();
            run("Threshold...");
            run("Create Selection");
            run("Measure");
            check = getTitle();
            save(output + name);
            close(check);
            close(name);
      }else {
                  close();
            }
}
```

```
//save results table
saveAs("measurements", output + "autoareas.csv");

//clean up
selectWindow("Results");
run("Close");

selectWindow("Threshold");
run("Close");

print("All done!");

}
```

4. Growth index calculation

These analyses were conducted in R 3.3.1. Prior to this, formulas for time since meeting day for each experimental plate were input manually in excel. Subsequent standard ANOVA analyses of calculated indices are not shown.

```
###Comparing Growth Curves###
##Jessica Nelson, 2017

##your input file should have these columns
  #plate (the plate number)
  #area (area measurement)
  #time (the calculated time relative to meeting day)

#set working directory and load library
setwd("file/path") #insert file path to directory

#load packages
library(multcomp)

#load data file
data<-read.csv("file.csv", header=T, sep=",") #insert file name
attach(data,warn.conflicts=FALSE)
```

```
#subset data from 28 days after meeting
afterdata<-subset(data,time>=0 & time<29,select=plate:area)

#split data by plate number
splitdata<-split(afterdata, afterdata$plate)

#create empty dataframe in which to store outputs
resulttable<-data.frame()

#for loop to calculate growth index each plate's data
for(i in splitdata){

  attach(i, warn.conflicts=FALSE)

  endtime<-time[length(time)]

  high<-max(i$area)
  low<-min(i$area)

  meet<-i$area[1]

  index<-(high/meet)*(low/meet)

  #Grab plate info
  plateid<-plate[1]

  #create vector to put results in and add results
  resultvector<-vector()
  resultvector<-c(plateid, high, low, index,endtime)

  #add vector to data table
  resulttable<-rbind(resulttable,resultvector,deparse.level=0)
}


#replace default column names with sensible labels
N<-c("plate","max","min", "index","endtime")
colnames(resulttable)<-N

#save data table to csv file
write.csv(resulttable, file="fileOut.csv") #fill in file name
```

5. Automated renaming of data photographs

This shell script requires the installation and pathing of ZBar bar code reader, ExifTool, and ImageMagick. It will rename images using a QR code in each image to determine the name and the image metadata to determine the date. If there are multiple photos with the same QR code, these will be given numbers to distinguish them. The script also rotates the photos 180° since our image acquisition apparatus resulted in upside-down images.

```
#!/bin/bash
for file in IMG_*.JPG;
do
     echo "rotating $file"
     magick $file -rotate 180  $file;
done

for file in *.JPG
do
    name="`zbarimg --raw -q $file`_"
     mod_date="`exiftool -d "%m_%d_%Y" -CreateDate "$file"
| awk '{print $4}'`"
     picname="$name$mod_date"
     count="`find . -name "$name*.JPG" | wc -l`"
     append="_$count"
     picnamedup="$picname$append"
if [ "$name" == "Ruler_" ]
then
     echo "Ruler image found, renaming to Ruler.JPG"
     mv -n $file Ruler.JPG
elif [ $count -ge 1 ]
then
    echo "Duplicate found, renaming to $picnamedup"
    mv -n $file $picnamedup.JPG
else
    echo "Renaming $file to $picname"
    mv -n $file $picname.JPG
fi
done
```

6. Model calculations and analysis

```
###Comparing Growth Curves, Model###
##Jessica Nelson, 2017

#set working directory and load library
setwd("path") #fill in path
library(multcomp)

#load data file
data<-read.csv("file.csv", header=T, sep=",") #fil in file name
attach(data,warn.conflicts=FALSE)

#split data by plate number
splitdata<-split(data, data$plate)

#create empty dataframe in which to store outputs
resulttable<-data.frame()

#for loop to analyze each plate's data
for(i in splitdata){

  attach(i, warn.conflicts=FALSE)

  #Create dummy variable for model
  afterContact = ifelse(time>=0,1,0)

  #define the model
  model<-lm(area ~ 1 + time + afterContact * time + afterContact
* time^2)

  #grab the last day time value
  endtime<-time[length(time)]

  #calculate stats for the model parameters
  m<-matrix(c(0,0,1,endtime),1)
  E<-glht(model,linfct=m)

  #call the estimate and error values from the glht
  result<-summary(E)$test$coefficients
  attr(result, "names")<-NULL

  error<-summary(E)$test$sigma
  attr(error, "names")<-NULL

  #Grab plate info
  plateid<-plate[1]
```

```
  #create vector to put results in and add results
  resultvector<-vector()
  resultvector<-c(plateid, result, error)

  #add vector to data table
  resulttable<-rbind(resulttable,resultvector,deparse.level=0)
}

#replace default column names with sensible labels
N<-c("plate","estimate","stder")
colnames(resulttable)<-N

#save data table to csv file
write.csv(resulttable, file="Out.csv") #fill in file name


##Analysis of calculated model outputs##

#read in output with treatment levels added in
out<-read.csv("Labeled.csv", header=T) #fill in file name
attach(out)


##ANOVA##
#example from multiple inoculation experiment

#plot c-hat estimates to look at normality
hist(estimate)
qqnorm(estimate)
qqline(estimate)
boxplot(estimate~group)

#test normality and homogeneity of variances
shapiro.test(estimate)
bartlett.test(estimate~group)

aov.reg<-aov(estimate~group)
summary(aov.reg)

TukeyHSD(aov.reg)
##3-factor ANOVA##
#used in multiple inoculation experiment

aov3<-aov(estimate~f1*f2*f3)
Summary(aov3)
```

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Biography

Jessica Nelson was born in Santa Barbara, CA on June 19, 1991. She received her Bachelor of Arts in Biology from Lewis & Clark College in May 2012. She is co-author on one article published in the Journal Molecular Ecology: "Plant host and soil origin influence fungal and bacterial assemblages in the roots of woody plants." Jessica has been awarded research grants from Highlands Biological Station, Duke University Biology Department, the American Bryological and Lichenological Society, and Sigma Xi. She also received a National Science Foundation Doctoral Dissertation Improvement Grant, a summer research fellowship from Duke University, and the Duke Biology Department Teaching Assistant Award. Jessica is an associate member of Sigma Xi. In addition to her biology PhD, Jessica is in the process of completing certifications in college teaching and botanical art and illustration.