TRUFFLES IN MICHIGAN: IMPACTS OF HERBICIDES ON THEIR GROWTH, EFFECTIVENESS OF IN-FIELD INOCULATIONS, AND THE DISCOVERY OF A LOCAL TRUFFLE (TUBER RUGOSUM)

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ABSTRACT

With the advent of promising truffle cultivation techniques, there are many new truffle plantations (truffière) being established and managed throughout the world; one of the biggest challenges these plantations face is weed management. There is little known about the impact popular herbicides have on truffle mycelial growth. Here, I discuss how the use of herbicides in management may impact mycelial growth. Pure culture growth assays were performed to assess the impacts glyphosate and glufosinate chemistries on three species of truffle, two species of morels, and two members of the Mortierellaceae. While there were variable responses by each species tested, most fungi experienced growth inhibition near or above the manufacturer's recommended application rates for general weed use.

I also assess how introducing exotic truffle species, local ectomycorrhizal species, and commercial mycorrhizal inoculants in post-methyl bromide fumigated bare-root conifer tree nursery impacts seedling growth. To test whether inoculum benefits seedling health and growth, exotic, and commercial ectomycorrhizal inoculua were added at the time of planting for three species of conifer. The results showed a high mycorrhizal diversity on bare-root seedling roots, which generally differed from those of the added inoculum. Additionally, there were no growth or health improvements over uninoculated control blocks compared to treatment blocks.

Finally, I will showcase the taxonomic discovery of a new species of truffle native to Michigan, *Tuber rugosum*. Also, observing slug fungivory of this new truffle led to an improved ascus technique for performing scanning electron microscopy of truffle spores. Collectively, this thesis aims to inform herbicide use in myco-agricultural lands, to inform the use of added mycorrhizal inoculants in bare-root conifer tree nurseries, and to improve our knowledge on Michigan’s native truffle species.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BI	Bayesian inference
BLAST	Basic Local Alignment Search Tool
CIPRES	Cyberinfrastructure for Phylogenetic Research
DNA	Deoxyribonucleic Acid
EF1α	elongation factor 1α
EPSPS	5-enolpyruvyl shikimic acid-3-phosphate synthase
ES	Extraction Solution
FAME	Fatty acid methyl esters
Fig	Figure
H3PO4	Phosphoric acid
HCl	Hydrogen chloride
INVGAMMA	Inverse gamma distribution
ITS	Internal Transcribed Spacer
KCl	Potassium chloride
KOH	Potassium hydroxide
MCMC	Markov chain Monte Carlo
MCMCMC	Metropolis-coupled Markov Chain Monte Carlo
MEB	Malt Extract Broth
ML	Maximum likelihood
MSU	Michigan State University
NCBI	National Center for Biotechnology Information

OTU	Operational Taxonomic Unit
PBS	Phosphate-buffered saline
PDA	Potato dextrose agar
pH	Potential of Hydrogen
p-value	Probability value
RAxML	Randomized Axelerated Maximum Likelihood
rDNA	Ribosomal Deoxyribonucleic Acid
RNA	Ribonucleic acid
RPB2	RNA polymerase II
RPS	Rotations per second
RTSF	Research Technology Support Facility
SEM	Scanning electron microscopy
sp.	Species
Sp. Nov.	Species nova (new species)
TM	Tuber Media
VA	Veterans Administration
YE	Yeast Extract

Chapter 1

Introduction

Problem statement

As with any cropping system, conifer trees and nut trees face many challenges. These tree producers share many risks, from failed crops to increasing disease pressure with few pesticide options available (Frampton et al., 2018; Pettersson et al., 2017). Conifer trees, for example, take seven to 15 years to grow before harvest and represent a substantial time investment before any returns are possible (Zinati et al., 2016). When a conifer fails to sell, or a nut tree is inadequately pollinated for example, there are few if any avenues by which the grower may earn income from that tree. Michigan produces over four million conifers each year and annually, over 25 million conifers are purchased in the United States grown on roughly 350,000 acres (*National Christmas Tree Association*, 2019). Hazelnut production has also been increasing, and as of 2021 there are over 60,000 nut bearing acres in the United States (USDA, 2022). These trees represent important jobs and revenue; maturing into a diverse agroforestry system could strengthen this delicate sector.

One potential revenue source for plantations such as conifer tree farms, may be found growing on the roots as edible mushroom or truffle forming ectomycorrhizal fungi. By pairing ectomycorrhizal fungi that form edible mushrooms or truffles with compatible tree species, some of the risk associated with growing conifers or nut trees in Michigan may be mitigated through increased revenue and decreased pathogen presence within their stands. However, there are several challenges such as weed plants and non-target fungi which need to be addressed for growers to have a chance at success. Researching alternative revenue sources and their challenges is critical for developing a more robust agroforestry plantation.

Background

Co-cropping with Ectomycorrhizal Fungi

Co-cropping, a practice where two or more crops are grown together, has garnered much interest as this technique offers to increase yields by adding an additional source of revenue, trapping unwanted pests, or even by improvements to the soil. Unlike plant-based co-cropping systems, fungi do not compete for sunlight. One group of fungi in particular form beneficial symbiotic relationships with particular host trees. These fungi, collectively called ectomycorrhizal fungi, interact with the roots of their host tree. This root-fungal interface serves as the point of nutrient exchange between the two organisms. Economically important trees within the Betulaceae, Fagaceae, Juglandaceae, and Pinaceae families form mutually beneficial relationships with many genera of ectomycorrhizal fungi (Tedersoo et al., 2009). Evidence of these symbiotic relationships have been long established between some plant and fungal species dating back millions of years.

Fossil records from 50 million years ago (Eocene Princeton chert) provide evidence of well-developed ectomycorrhiza on the roots of *Pinus* which show the Hartig net, a pseudoparenchymous mantle, and extramatrical hyphae (Lepage et al., 1997). Ectomycorrhizal fungi are characterized by the formation of these unique morphological structures and the interactions this group of fungi has with compatible plants. A Hartig net is the site of nutrient exchange between fungi and host; it consists of fungal hyphae growing outside of, and between root cortical cells of the host plant. The Hartig net can be seen by placing thin cross sections of colonized root tips on a microscope slide for close inspection. An ectomycorrhizal mantle is a sheath composed of pseudoparenchyma hyphae surrounding host plant root tips. The densely packed puzzle-like pseudoparenchymal hyphae which form the mantle may act as a physical

barrier and be one of the means by which ectomycorrhizal fungi impart protection against invading pathogens. Variations in the formation and structure of the mantle such as the shape and density of the hyphae can be a tool by which the absence or presence of target ectomycorrhizal fungi can be ascertained. Extending beyond the mantle out into the surrounding soil environment are determinant hyphae called cystidia. The morphology of cystidia can also aid in the identification of the fungi present, for example, *Tuber borchii* Vittad. short, awl-shaped hyaline cystidia, but *Tuber aestivum* Vittad. produces curled, interwoven hyaline cystidia (Giomaro et al., 2000; Molinier et al., 2016). These host interactions cannot occur with any host however, compatibility between ectomycorrhizal fungi and host plant can be highly specific.

Ectomycorrhizal Host Specificity

Though many ectomycorrhizal fungi are generalists in that they can form these symbiotic relationships with multiple different host species; it is wise for any targeted ectomycorrhizal tree producer to ensure the targeted fungi are compatible (G. M. Bonito et al., 2010). *Suillus* for example, are well known for showing high levels of host specificity amongst *Pinus*, *Pseudotuga*, and *Larix* (Pérez-Pazos et al., 2021). The edible North American truffle *T. lyonii* Butters and recently described *T. brennemanii* A. Grupe, Healy & M.E. Sm. and *T. floridanum* A. Grupe, Sulzbacher & M.E. Sm. dominate the roots of pecan (*Carya illinoinensis* (Wangenh.) K.Koch) trees (G. Bonito et al., 2011; Grupe et al., 2018) but can be found under oak trees as well. Many tree species depend on these symbionts to survive. Some of these symbioses have been shown to promote growth and health benefits.

Picea A. Dietr. trees, which form symbiotic relationships with the ectomycorrhizal fungus *Laccaria bicolor* (Maire) P.D. Orton, are known to obtain significant increases in biomass after ectomycorrhizal colonization compared to those grown in the same soils without

fungal colonization (Quoreshi & Timmer, 2000). The most commonly grown tree in Northern nurseries, *Picea abies* (L.) H.Karst., has been shown to be resistant to one of the most damaging root rot causing pathogens, *Heterobasidion annosum* P.K. Buchanan, when its roots are colonized by various ectomycorrhizal forming fungi (Velmala et al., 2017). It is also clear that the presence of ectomycorrhizal fungi on the roots of trees has a positive influence on the growth and health of trees even when soil conditions are less than favorable.

Pinus L. trees inoculated with the ectomycorrhizal fungi *Pisolithus* Alb. & Schwein., *Cenococcum* Moug. & Fr., and *Laccaria* Berk. & Broome have been shown to grow significantly better in poor soils which contain high concentrations of heavy metals (Zong et al., 2015). Additionally, chestnut trees have been shown to greatly benefit from ectomycorrhizal colonization when growing on retired and contaminated coal mined landscapes (Bauman et al., 2018). Where there are contaminated soils, the pairing of edible ectomycorrhizal fungi with host trees should be avoided as it has been noted that the fruiting body of certain ectomycorrhizal fungi may act as a toxin sink for the fungi. (Cocchi et al., 2006) found that in many mushroom species such as the ectomycorrhizal fungi *Boletus edulis* Bull., there were arsenic and cadmium levels equal to or exceeding the maximum weekly dosage recommended by the World Health Organization (Cocchi et al., 2006).

Economically and Culinarily Valuable Ectomycorrhizal Fungi

Beyond some of the direct benefits to the tree crop through growth promotion, pathogen resistance, or resilience against heavy metal toxicity, there are many ectomycorrhizal fungi which form edible mushrooms or truffles which often fetch very high market prices. Matsutake (*Tricholoma matsutake* (S. Ito & S. Imai) Singer), porcini (*Boletus edulis* Bull.), chanterelle (*Cantharellus* sp. Adans. ex Fr.), saffron milk cap (*Lactarius deliciosu*s (L.) Gray), and truffles

(*Tuber* sp.) are among those ectomycorrhizal fungi possessing high economic and culinary value. Though there have been recent advances, many of the highly prized edible mushroom producing ectomycorrhizal fungi such as *Tricholoma matsutake* (Yamanaka et al., 2020), *Lactarius deliciosus* (Guerin-Laguette et al., 2014; Wang et al., 2021), and *Cantharellus anzutake* W. Ogawa, N. Endo, M. Fukuda & A. Yamada (Ogawa et al., 2019) have little commercial success in cultivation and their entire presence in the market is based on wild foraging efforts (Yun & Hall, 2004). Other edible ectomycorrhizal fungi of interest such as *Lactarius deliciosus* (L.) Gray, *Suillus luteus* (L.) Roussel and *Suillus variegatus* (Sw.) Richon & Roze have been successfully cultivated (González-Ochoa et al., 2003). The apparent inability to cultivate economically important fungi highlights how little is understood about these organisms and how much there is remaining to be discovered in regard to their cryptic lifecycles. The most economically valuable ectomycorrhizal fungi are truffles (Ascomycota, Pezizales, *Tuber*aceae) in the genus *Tuber*.

What are Truffles?

Tuber species, commonly called truffles, are ectomycorrhizal forming fungi which are best known for their aroma with many species fetching high prices in international markets. Though some European truffles command high prices and garner much media attention, there is a rich diversity of truffle species in North America with many species yet to be described (Bonito et al., 2010; Healy et al., 2016). There are a few famous North American truffles, *Tuber canaliculatum* Gilkey, *Tuber gibbosum* Harkn., *Tuber lyonii*, and *Tuber oregonense* Trappe, Bonito & P. Rawl. contributing greatly toward building the culinary reputation of North American truffles. Many of these truffle species are known as a prized delicacy due to the wide

range of complex aromatic compounds they release and can be differentiated based on these compounds alone (Culleré et al., 2013).

Unlike many sought after fungal fruiting bodies such as morels (*Morchella* sp. Dill. ex Pers.), truffles are a sequestrate hypogeous fungi which would ideally be located by a trained animal with a good sense of smell to ensure that the maturity of the mushroom is such that the aromatic qualities the market demands are present (Grupe et al., 2018; Pieroni, 2016). Immature truffles will not produce the desired aroma and are not considered to have much culinary value. Using a meta-analysis of global ITS rDNA, it has been predicted that there are over 180 *Tuber* species (Bonito et al., 2010). Despite the abundance of truffle species, most are not economically important and only a dozen or so species are harvested in economic quantities.

Some of the most sought-after truffle species including *Tuber aestivum*, *T. melanosporum* Vittad., and *T. borchii,* have long culinary histories in Europe where there have been documented efforts to cultivate truffles dating back to the 18th century (Reyna & Garcia-Barreda, 2014; Yun & Hall, 2004). *Tuber melanosporum* alone accounts for tens of millions of dollars of direct revenue for producers within Europe every year (Reyna & Garcia-Barreda, 2014). The cultivation of these truffles is no simple task as the life-cycle of these fungi are complex and partially unknown. As an obligate symbiont, germination of truffles spores occurs after the presence of root exudates are intercepted (Ali & Jackson, 1988; Yun & Hall, 2004). The germinated spores will then grow and a mycorrhizal connection with the host tree will take place. Truffles are heterothallic organisms and compatible mating types must interact before completing their reproductive cycle (Rubini et al., 2011), and as such, most truffle tree production is spore-based.

Mycorrhizal Tree Production

One of the most widely used methods for inoculating trees in a nursery with ectomycorrhizal species is through the use of spores (Reyna & Garcia-Barreda, 2014), though other ectomycorrhizal species seem to perform better with mycelial inoculation. *Suillus* mycorrhizas can be synthesized by spore-based inoculation, but some ectomycorrhizal fungi such as *Lactarius* Pers. show greater success colonizing nursery trees from vegetative, mycelium-based inoculation techniques (González-Ochoa et al., 2003). *Lactarius deliciosus*, for example, has been successfully cultivated by layering mycelium into the pots of nursery trees in *Pinus* species (Guerin-Laguette et al., 2014). Still, spore-based inoculation is the current standard with *Tuber* species. The precise methodology of spore inoculation will vary based on the target fungal species.

Truffle trees start their life as seedlings potted with *Tuber* spores in sterile or pasteurized soil in a greenhouse until they are mature enough to plant in the field (Iotti et al., 2012). Growing truffle trees by the pot can be expensive due to environmental, labor, and material costs. Roughly 500,000 truffle trees are produced every year in nurseries (Murat, 2014). Ensuring the spores being applied come from the correct target fungus is of critical importance. Best practices require nurseries to purchase fresh mushrooms or truffles for positive identification and quality checks to ensure that no time is wasted inoculating with a non-target species. Buying pieces and scraps of poor quality truffles may increase the chances of less desirable truffle species becoming part of the inoculum.

After positive identification of the truffle species, the ascoma containing spores are often placed in a blender with an abrasive such as ice or sand to help break the asci and release the spores from the asci (Iotti et al., 2012). These spores are then homogenized into the substrate the

trees are grown in. One key to successfully cultivating the correct target fungi is cleanliness in both the soil, the roots of the tree, the growing environment, and even the water source. The time needed between inoculation and good colonization may extend through an entire growing season before mycorrhization checks can be made and identification of the ectomycorrhizal fungus on the roots can be verified (Benucci et al., 2012). Non-target ectomycorrhizal fungi such as *Sphaerosporella* (Svrček) Svrček & Kubička may dominate the pot and make inoculation efforts ineffective (Iotti et al., 2012). In truffle tree production, it is standard to wait six months after inoculation before attempts are made to verify the presence of truffle on the roots (Benucci et al., 2012; Iotti et al., 2012). Verifying that the desired truffle species is on the roots of the host tree is important before out-planting since it may be five years or longer before truffle production begins and this would constitute a considerable loss of time for the grower anticipating truffle production. Pairing the right truffle with the right tree is also of considerable importance. Even though truffles are often thought of as generalists, there is some host specificity which needs to be considered.

What Tree Plantations May Benefit Most?

A pecan grower would be interesting in knowing that the European truffles *T. aestivum* and *T. borchii* form good ectomycorrhizal relationships with pecan roots in a nursery setting and should therefore consider these as viable options (Benucci et al., 2012). Additionally, the chestnut (*Castanea* sp. Mill.) industry is young in North America and in a prime position to begin looking at the possibility of adding value through the use of valuable, edible ectomycorrhizal fungi. Chestnut trees are in the Fagaceae family along with an important truffle producing trees in the *Quercus* genus. *Quercus* trees are known to be natural hosts to many ectomycorrhizal truffles such as *T. borchii, T. aestivum*, and *T. melanosporum* (García-Montero

et al., 2014; Reyna & Garcia-Barreda, 2014). It therefore appears that since *Castanea* spp. have been shown to host ectomycorrhizal relationships with many fungi, and that they are closely related to genera known to strongly associate with truffle species, that these trees may be a good candidate determining the potential of co-cropping with these high-value fungi.

Additionally *Corylus* L. nut producers may also consider ways to increase the value of their farm since these trees are also ectomycorrhizal forming. Both *T. magnatum* Picco, the Piedmont white truffle, *T. aestivum*, and *T. borchii* are known to grow on the roots of trees in the *Corylus* genus (Bonito et al., 2010; Wang & Marcone, 2011). These trees have been widely used in establishing truffle orchards in Australia where the goal is to produce *T. melanosporum*, the European black truffle (Bradshaw, 2005). As this tree is susceptible to a myriad of disease pressures such as the devastating Eastern Filbert Blight which as caused by *Anisogramma anomala* (Peck) E. Müll., efforts to increase the value of these trees should be of great interest to growers looking to mitigate the risks associated with dependence on the full production of this nut (Pscheidt et al., 2018). For *Corylus* and all of the other trees mentioned here, the time to consider inoculations should begin while at the nursery stage since the best time to sway the community of the roots on a plant is when they are uncolonized and before they enter the diverse environment of the tree orchard.

In the tree cropping systems utilizing compatible trees, there is an opportunity to greatly improve the value of these trees by forming a system whereby targeted, high-value fungi are cultivated on their roots. Understanding the need to verify the target ectomycorrhizal fungi prior to planting by looking at the colonization percentage and morphology based on some of the characteristics described in this thesis may prove to be important as North America matures its ectomycorrhizal cultivation. In conifer and nut tree nurseries, the benefits ectomycorrhizal fungi

impart on their host in terms of pathogen defense and nutrient and water uptake has generated much interest. The potential for increasing the value of these tree crops through the use of edible ectomycorrhizal fungi is too great to ignore and throughout Europe a great deal of work has been done to produce edible ectomycorrhizal producing trees. By adapting European methods for producing truffle trees in Michigan and for its tree crops, farms may benefit through the added revenue these high-value fungi will provide. In addition to providing a second crop, particularly culinarily valuable truffle species may also impart other benefits to the plantation in which they are grown.

Weed Management

The summer truffle, *T. aestivum*, can be found growing on the roots of *Pinus* and *Quercus* L. species. Growth of this truffle on both tree species results in the formation of a brûlé. A brûlé is used to describe the burned look seen in the vegetation growing in competition to the host tree and is associated with certain truffles species such as *T. aestivum* (García-Montero et al., 2014). It has been noted that the brûlé associated with *T. aestivum* is different in both size and intensity based on the associated tree species, for example, on *Pinus*, the brûlé is known to be over double the diameter that what has been seen under *Quercus* trees (García-Montero et al., 2014). This effect on the vegetation surrounding the host plant is the result of phytotoxic compounds produced by the truffle mycelium and the reduction in plant competition serves as one of the mutualistic returns this ectomycorrhizal fungus provides to the host tree (García-Montero et al., 2014). Beyond the cost of specialized equipment and herbicide chemicals, there is the ever-climbing cost of fuel and labor producers must consider, thus, a high-value edible mushroom producing ectomycorrhizal fungus that has an affinity for killing weeds probably sounds too good to be true. In practice, though reduced, weed management practices are still

needed since these brûlé formations are not impervious to all weed growth, nor are these formations uniform in dispersal beneath the trees since the colonization by *T. aestivum* may not be uniform.

Weed plants reduce colonization and truffle production (Mamoun & Oliver, 1997; Olivera et al., 2011) and therefore many orchard operators manage weeds either mechanically (Olivera et al., 2014) or chemically (Gómez-Molina et al., 2020). Offering significant savings in time and labor over manually removing weeds, many cropping systems utilize herbicides to increase crop productivity. Herbicides play important roles in agriculture by reducing competition for nutrients, water, and sunlight. This type of pesticide can also prove critical for use in controlling invasive plant species which in turn aids in conservation efforts to preserve native ecosystems. Though there has been work to develop fungal based bioherbicides (Julia et al., 2022), most herbicides are produced with synthetic chemicals. Despite these benefits, there are also many potential side effects related to the use of herbicides and many unknown outcomes especially in relation to fungal cultivation.

Herbicide Sensitivity in Fungi

There have been numerous studies showing that fungi can be sensitive to chemical exposure. For example, glyphosate inhibits 5-enolpyruvyl shikimic acid-3-phosphate synthase (EPSPS) which is one reason it is such an effective herbicide (Steinrücken & Amrhein, 1980). However, in addition to plants, both bacteria and fungi have the shikimic acid pathway (Gupta & Crissman, 2013). As such, it stands to reason that herbicide overspray containing glyphosate may impact organisms other than the targeted weed plant. Currently there have been no studies directly exploring fungal EPSPS interactions with glyphosate (Hammerschmidt, 2018). Paraquat (1,10-dimethyl-4,4-bipyridinium dichloride), has been shown to interfere with biofilm and

melanin production in *Cryptococcus* Vuill. (Castelo-Branco et al., 2022). Flurochloridone and prosulfocarb inhibit conidial germination of *Beauveria bassiana* (Bals.-Criv.) Vuill. (Celar & Kos, 2016). *Pleurotus ostreatus* (Jacq.) P. Kumm. experiences mycelial growth reduction with glyphosate (Connelly et al., 2019), and glufosinate ammonium has been shown to reduce mycelial growth of *Guignardia bidwellii* (Ellis) Petr. by nearly 80% (Albrecht & Kortekamp, 2009) to name a few examples. There has been sparse work done investigating conventional herbicide-fungal interactions, and even less investigating organic herbicide-fungal interactions. These sorts of interactions should be further investigated as many agroforestry mushroom growers may consider organic herbicides as a safer alternative, but it is unclear how these herbicides impact growth or consumer safety.

With any pesticide, there is also the possibility residuals may be found on or in mushrooms which were previously exposed. This could have great health implications as many pesticides are not intended for human consumption. Recent publications outline reliable methods to identify over 100 pesticides in mushroom tissue (Cao et al., 2016; Le et al., 2021). One alarming finding Le et al. (2021) found was that various pesticides were detected in every *Lentinula edodes* (Berk.) Pegler, *Agaricus bisporus* (J.E. Lange) Imbach, and *Pleurotus ostreatus* mushrooms purchased for this study (Le et al., 2021). This includes the herbicides Atrazine, MCPA, Dicamba, Diuron, 2,4-D, and Cyhalofop Butyl (Le et al., 2021). Le et al. (2021) postulate that these pesticides enter the mushroom via residuals on the substrate from which these fungi are cultivated. Pesticide chemistries, their interactions with edible fungi and human health implications will vary product to product suggesting an area potentially rich for further study. Despite its use, there are still many questions about how the use of herbicides may impact fungi including those beneficial to plant growth, ectomycorrhizal fungi, and even saprotrophic fungi.

Herbicide-fungal interactions are discussed more in Chapter 2, "Impacts of Glufonisate-Ammonium and Glyphosate Herbicides on Mycelial Growth of Truffles, Morels, and Plant Growth Promoting Fungi."

Fungal Priority Effects

Additionally, growers must also contend with non-target weed fungi if they are trying to cultivate a specific edible mycorrhizal species in their agroforestry system. As with weed plants, weed fungi compete for space, nutrients, and water. Priority effects also play an important role in the establishment of target species and subsequent development. Fumigated bare-root outdoor tree nurseries offer an opportunity to investigate priority effects. However, as this is an outdoor cropping system, there are many opportunities for non-target fungal species to take advantage of priority effects. There is a need to characterize and investigate priority effects in bare-root tree nurseries as well as a need to learn which native ectomycorrhizal species may be present and able to disrupt target-species colonization. These are further discussed in Chapter 3, "Does Ectomycorrhizal Inoculum After Methyl Bromide Field Applications in Bare-Root Conifer Nursery Seedlings Establish and Persist?" The need to better understand non-target ectomycorrhizal fungi does not stop at the plantation though, there are a wealth of new species to be discovered in native tree stands as well.

New Ectomycorrhizal Species

Characterizing fungal species include examining spore size, spore shape, spore ornamentation, and determining that purported new species yields a unique genetic sequence. Many truffle species are yet to be described and a global meta-analysis was conducted by Bonito et al. (2010) of over 2000 *Tuber* ITS rDNA sequences to offer a framework by which future studies may be built. This 2010 study predicted between 180 and 230 *Tuber* species globally and

used numerical designations for species not yet described (Bonito et al., 2010). Truffles, as with many fungi, often use the internal transcribed spacer (ITS) as the primary region used to differentiate species. There are limitations to building *Tuber* phylogenies with the ITS region and therefore primer sets have been developed for assist with multigene phylogenies including elongation factor 1a (EF1α_*Tuber*_f, EF1α_*Tuber*_r), and 2nd subunit of RNA polymerase (RPB2_*Tuber*_f, RPB2_*Tuber*_r)(Bonito et al., 2013; Bonito et al., 2010). Together with morphological characteristics, phylogenetics offer a powerful tool for researchers to better understand how taxa relate to each other.

There are numerous reasons why it is important to describe novel taxa. Firstly, we know the importance fungi play in nearly every ecosystem. By expanding our knowledge of fungal diversity we better understand their ecological roles and functions in sustaining the health of ecosystems. Secondly, fungi provide countless industrial and medical applications which improve our daily lives. Through understanding and exploring fungal species diversity, we have the opportunity to discover solutions to problems we face. Third, by describing new species, we better develop fungal taxonomy. This is important because it helps us develop better understanding of evolutionary relationships which in turn helps with the identification of species. Finally, as our climate is changing and ecosystems shrink or disappear, describing novel taxa gives us the chance to conserve threatened or endangered species through outreach and conservation efforts.

Research focus

The three primary goals pursued in this thesis are:

1. *Determine mycelial growth in media containing herbicides*

Hypothesis: (H1) Herbicides containing either glyphosate or glufosinate will impact the mycelial growth of truffles, morels, and plant growth promoting fungi. (H2) There will be variability of response between saptrotrophic and ectomycorrhizal ecologies. (H3) Both herbicides act fungistatically at concentrations near or above the manufacturers recommended application rate for field use.

2. *Investigate ectomycorrhizal priority effects in methyl bromide fumigated bare-root Christmas tree nurseries.*

Hypothesis: (H4) *Pseudotsuga menziesii* (Mirb.) Franco, *Picea abies* (L.) H.Karst., and *Pinus sylvestris* L. can be colonized by exotic ectomycorrhizal fungal species. (H5) There will be little to no significant increases in above ground biomass, tree height, or root collar diameter based on inoculant. (H6) Either by persisting through fumigation or through blowing in, local ectomycorrhizal species will dominate the root systems of *Pseudotsuga menziesii, Picea abies,* and *Pinus sylvestris*.

3. *Confirm and describe a new species of truffle in the Tuber genus*

Hypothesis: (H7) Morphology and molecular analysis will support *Tuber rugosum* Rennick B, Benucci GMN, Du Z, Healy, & Bonito as a novel species in the Rufum clade. (H8) This new species will have a unique fatty acid profile. (H9) Utilizing observed fungivory in slugs may provide an alternative ascus removal for high quality scanning electron microscopy. (H10) Based on numerous new species within the Rufum clade, an updated taxonomic key can be produced.

Value of this research

The research outlined within this thesis may serve to inform management of truffières and other fungal cultivation systems that may consider the use of herbicides to combat weed plants. Many truffle plantations are already using herbicides and will be interested to know how

over applying may lead to fungistatic situations. Furthermore, there is a growing interest in morel cultivation in fields between agronomic crops, this work begins to inform sensitivities of edible fungi to herbicides of which there may still be residuals.

Other research covered within this thesis outlines what outcomes may occur if a post methyl bromide fumigated bare-root Christmas tree nursery is interested in adding mycorrhizal inoculum to their fields. As a potentially cost prohibitive activity, growers will be interested in knowing that there are a strong group of ectomycorrhizal beneficial symbionts either persisting through fumigation or blowing in from nearby tree stands. This research also provides evidence that while in-field inoculation of exotic *Tuber* species is possible, the rates are far below standards set by the truffle tree industry. Decades of efforts to cultivate truffles in North America have proved highly challenging.

Understanding competing ectomycorrhizal fungi requires that little or unknown species are properly described and subsequently identified to further make sense of how their ecologies and ranges may impact a truffières ability to establish and persist. Beyond the value describing new ectomycorrhizal species has to truffières, there are many potential benefits gained by understanding their ecological roles and functions. Developing better fungal taxonomy helps improve our understanding of evolutionary relationships and may inform our ability to identify rare taxa for conservation purposes. Cohesively, research presented in this thesis will impact our understanding on the ecology, taxonomy, scanning electron microscopy preparation, herbicide sensitivity, and priority effects of truffles.

Chapter 2

Impacts of Glufonisate-Ammonium and Glyphosate Herbicides on Mycelial Growth of Truffles, Morels, and Plant Growth Promoting Fungi

Abstract

Herbicides are frequently used to control weeds in agricultural and residential settings, however, herbicides can also impact fungi. Understanding how weed management impacts soil fungi may help reduce unintended consequences of these chemicals in agricultural settings. In this study we assessed the impacts of glyphosate [N-(phosphonomethyl)-glycine] (Roundup) on vegetative growth of soilborne edible and plant growth promoting fungi including *Benniella erionia, Linnemannia elongata,* two species of morels (*Morchella*), six species of truffles (*Tuber*). We also assess the impact of glufosinate-ammonium (Liberty), on vegetative growth of two species of morels (*Morchella*), three species of truffles (*Tuber*), and two soil fungal species within Mortierellaceae (*Benniella* and *Linnemannia)*. Each fungal isolate was grown in a common medium containing an herbicide ranging in concentrations spanning above and below the application rate recommended by the manufacturer. To determine the impact of these chemistries on fungal growth, hyphal growth rates on agar media and fungal biomass in liquid media were collected. The fungi grew unimpeded at low herbicide concentrations but were completely inhibited at higher herbicide concentrations. The recommended working glyphosate concentration was found to be near the point of fungal growth inhibition *in-vitro*. However, the fungi tested here were far less sensitive to glufosinate. Low amounts of this foliar spray would be expected to drench the soils these fungi inhabit when applied during typical application. As such, the use of these herbicides as a foliar spray at recommended rates during typical application is unlikely to interfere with the mycelial growth of the fungal species assayed in this experiment. Nonetheless, use of these herbicides at concentrations higher than recommended could inhibit mycelial growth and fungal biomass in soil environments.

Introduction

Glyphosate, a systemic pesticide, is the active compound in popular commercial herbicides, and is intended for application to plant photosynthetic surfaces where it is highly phototoxic (Coupland & Caseley, 1979). Within fungi, bacteria, and plants, the shikimic acid pathway feeds into the production of the amino acids tryptophan, tyrosine, and phenylalanine (Gupta & Crissman, 2013). Glyphosate is an inhibitor of 5-enolpyruvyl shikimic acid-3-phosphate synthase (EPSPS) and as such, has great value as an effective herbicide (Steinrücken & Amrhein, 1980). Glufosinate, a contact pesticide, is used in many popular herbicides. Glufosinate on the other hand, is a contact herbicide, that inhibits the production of glutamine synthetase, which leads to the buildup of ammonia in cells leading to cell death (Gupta & Crissman, 2013; Herrmann & Weaver, 1999). Glufosinate does not appear to inhibit the growth of the oomycete *Pythium aphanidermatum* (Edson) Fitzp., but has shown fungicidal activity against *Rhizoctonia solani* J.G. Kühn and *Sclerotinia homoeocarpa* F.T. Benn. indicating variability in response should be expected (Liu et al., 1998).

Many agricultural production systems rely upon beneficial soil fungi to sustain crop yields, while others may be specifically focused on the production of fungal fruiting bodies. For example, there have been many successes in the cultivation of truffles (*Tuber* spp.) and other ectomycorrhizal fungi (*Lactarius* spp.) over the past several decades (Chevalier & Pargney, 2014; Wang et al., 2012). Notable successes in truffle cultivation include productive *T. melanosporum* Vittad. plantations (truffières), particularly in Italy, France, Spain, Australia and New Zealand (Hall et al., 2017). In 2019 and 2020, *T. magnatum* Picco was detected and harvested in truffières outside the natural range in France (Bach et al., 2021). Similarly, new outdoor cultivation of edible and medicinal morels (*Morchella* spp.) have had recent successes

(Dissanayake et al., 2021; Xu et al., 2022). As with many plant cropping systems, fungi cultivated in soil must contend with weed plants for water and nutrients (Olivera et al., 2011, 2014a). For instance, truffle cultivation involves planting seedlings inoculated with *Tuber* into prepared calcareous field soils, which must be specifically managed to improve and maintain the production of truffles (Chevalier & Pargney, 2014). Standard management of truffle orchards requires that weeds be removed around host trees to aid in moisture retention and to reduce other forms of biotic competition within the soil (Chevalier & Pargney, 2014; Olivera et al., 2011). While mechanical weed removal is the standard method in truffle orchards, chemical management of weeds in truffle orchards, such as with glyphosate and other herbicides, is becoming more common due to its low cost and high efficacy (Gómez-Molina et al., 2020).

There is still little known about the effects of this herbicide on fungal growth. For example herbicides may enhance or suppress fungal disease development, in part due to the influence that the application of the herbicide may have over all facets of the disease triangle (Hammerschmidt, 2018). In particular, rust fungi are inhibited in crops where glyphosate is used, however, suppression is possibly linked to the obligate biotrophic relationship these fungi have with their host plants as these fungi require living plant cells to live (Hammerschmidt, 2018).

However, during herbicide application, herbicides also come into contact with the soil and though it quickly adheres to soil minerals, some herbicide can make its way deeper into the soil (Jonge et al., 2000). Both glyphosate and glufosinate have also been found to be translocated through the plant from the foliage down into the roots and where it is exuded into the surrounding rhizosphere (Feng et al., 1999; Laitinen et al., 2007; Steckel et al., 1997). Soil organisms, especially mycorrhizal fungi, may then be exposed to these xenobiotic compounds. For example, (de Novais et al., 2019) found herbicide exposure reduced extramatrical hyphal

density, explored area, length, instances of anastomosis, and hyphal branching among of mycorrhizal networks of *Cichorium intybus* L. by *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler. In addition, glyphosate has been shown to decrease arbuscular mycorrhizal fungi colonization among *Abelmoschus* (Brindhavani et al., 2018), *Trifolium* (Zaller et al., 2014), *Festuca* (Helander et al., 2018) and to decrease *T. melanosporum* spore viability amongst truffle inoculated trees (Gómez-Molina et al., 2020). There is far less information available regarding glufosinate interactions with soil inhabiting fungi. To address this gap of knowledge, this study aimed to further investigate direct impacts of the commercially available products glyphosate (Roundup) and glufosinate (Liberty) herbicides on the vegetative growth of diverse fungi including morels (*Morchella* spp.), truffles (*Tuber* spp.) and select soil-inhabiting fungi. We hypothesize that fungi would be sensitive to both chemistries, but that mycorrhizal fungi would be less sensitive than saprotrophic fungi as other studies have indicated established *Tuber* colonies persist after recommended rates of glyphosate applications (Bonet et al., 2006; Gómez-Molina et al., 2020; Olivera et al., 2011).

Methods

Direct impacts of commercially available glufosinate and glyphosate herbicides on fungal vegetative growth were tested on pure culture isolates through two experiments. In the first experiment, herbicides were incorporated into an agar-based plate assay to assess fungal growth rates in this semi solid matrix. In the second experiment, a broth-based assay was used to assess fungal biomass at different concentrations of herbicide. *Benniella erionia* Liber & Bonito, *L. elongata* (Linnem.) Vandepol & Bonito, *M. americana* Clowez & Matherly, *M. importuna* M. Kuo, O'Donnell & T.J. Volk, *T. borchii* Vittad., *T. canaliculatum* Gilkey, *T. floridanum* A. Grupe, Sulzbacher & M.E. Sm., *T. gibbosum* Harkn., *T. lyonii* Butters, and *T. rugosum* Rennick

B, Benucci GMN, Du Z, Healy R, & Bonito G. were chosen for this study to represent several trophic ecologies as described in detail below and summarized in Table 1. Additionally, *Rhizopus oryzae* Went & Prins. Geerl., *Serendipita indica* (Sav. Verma, Aj. Varma, Rexer, G. Kost & P. Franken) M. Weiss, F. Waller, Zuccaro & Selosse, *Stropharia rugosoannulata* Farl. ex Murrill, and *Umbelopsis* sp. were used in a pilot experiment at lower replication, further detailed in FIGURE 2.6.

Table 2.1 - Fungal isolates and herbicides used in this study.

Experiment 1: Solid Media

Species	Isolate Voucher	Trophic Ecology	Repititions with Roundup	Repititions with Liberty
Benniella erionia	GB_AUS_27b	Saprotrophic	3	3
Linnemannia elongota	GB_AUS_24	Endophytic	3	3
Morchella americana	BR3	Saprotrophic	3	3
Morchella importuna	GB772G	Saprotrophic	3	3
Tuber borchii	BR11	Ectomycorrhizal	5	5
Tuber borchii	GMNB230	Ectomycorrhizal	5	5
Tuber gibbosum	GMNB46	Ectomycorrhizal	5	5

Experiment 2: Liquid Media

Species	Isolate Voucher	Trophic Ecology	Repititions with Roundup
Benniella erionia	GB_AUS_27b	Saprotrophic	10
Linnemannia elongota	GB_AUS_24	Endophytic	10
Morchella americana	BR3	Saprotrophic	10
Morchella importuna	GB772G	Saprotrophic	10
Tuber borchii	BR25	Ectomycorrhizal	5
Tuber canaliculatum	BR7	Ectomycorrhizal	5
Tuber floridanum	BR61c	Ectomycorrhizal	5
Tuber lyonii	GB17	Ectomycorrhizal	5
Tuber rugosum	BR64a	Ectomycorrhizal	5

Pilot Trial: Solid Media

Species	Isolate Voucher	Trophic Ecology	Repititions with Roundup	Repititions with Liberty
Rhizopus oryzae	NVP158	Saprotrophic	1	1
Serendipita indica	NVP157	Endophytic	1	1
Stropharia rugosoannulata	BR81	Saprotrophic	1	1
Umbelopsis sp.	TLT204	Saprotrophic	1	1

Experiment 1 Solid Media

Solid Media Preparation

Assays were carried out on half strength potato dextrose agar (PDA½) and quarter strength potato dextrose agar (PDA¼). The PDA½ recipe per liter is as follows: 12.0g potato dextrose broth (PDB), 1.0g yeast extract (YE), 10.0g agar, double distilled water filled to 1000.0mL, then autoclaved. The PDA¼ recipe per liter is as follows: 6.0g PDB, 1.0g YE, 10.0g agar, double distilled water filled to 1000.0mL, then autoclaved.

Glyphosate herbicide (Roundup Ready-to-Use Weed and Grass Killer) was filter sterilized at 0.2 microns, and once media were cooled to 45°C, was added at the following concentrations 0.001 mL/L, 0.01 mL/L, 0.1 mL/L, 0.0 mL/L, 1.0 mL/L, 10.0 mL/L, and 40.0 mL/L. The herbicide bottle provided an application rate of 30fl oz/70ft^2 (887mL/6.5m^2). The surface area of a Petri dish is 78.54cm^2 thus an application rate of 1.07mL/78.54cm^2 was chosen as a central concentration. Glufosinate (Liberty 280 SL) herbicide was too viscous to filter sterilize and was therefore added to the agar mediums at 55°C in the following concentrations: 0.001 mL/L, 0.01 mL/L, 0.1 mL/L, 0.0 mL/L, 1.0 mL/L, 10.0 mL/L, and 40.0 mL/L. The herbicide bottle provided an application rate of 20fl oz/ac (650.6mL/4046.9m^2). Disposable 100 mm Petri dishes were filled to 25.0 mL using a serological pipette. No contamination was observed.

A 4.0 mm cork borer was used to transfer fungal isolates including *M. americana* (BR3), *M. importuna* (GB772G), *B. erionia* (GB_AUS_27b), and *L. elongata* (GB_AUS_24) onto the center of PDA½ media containing glufosinate or glyphosate herbicides at the concentrations described above. The agar removed from the mother culture was such that the front of the colony's growth was centered across the diameter of the plug cut by the cork borer. Similarly,

with a 4.0 mm cork borer, fungal isolates including *Tuber borchii* (BR11), *Tuber borchii* (GMNB230), *Tuber gibbosum* (GMNB46) were transferred onto the center of PDA¼ media containing glufosinate or glyphosate also at concentrations of 0.001 mL/L, 0.01 mL/L, 0.1 mL/L, 0.0 mL/L, 1.0 mL/L, 10.0 mL/L, and 40.0 mL/L. *Benniella erionia, L. elongata, M. americana,* and *M. importuna* was plated with both herbicide brands (n=2) for each concentration (n=6) with three replicates totaling 144 Petri dishes. Additionally, two isolates of *T. borchii* and one isolate of *T. gibbosum* were plated with both herbicide brands with all six concentrations and plated in five replicates totaling 180 Petri dishes. Petri dishes were incubated at room temperature (23 C) in a dark location. Plates were randomly distributed within a dark box to prevent any block effects.

To measure fungal growth, four quadrants on each Petri dish were marked and daily measurements of the longest hyphal growth within each quadrant were collected for 14 days amongst the fast growing *Morchella, Benniella* and *Linnemannia* isolates. For the slower growing *Tuber* isolates, hyphal growth within each quadrant was collected weekly for an 8 week duration.

Experiment 2 Liquid Media

Liquid Media preparation

Two liquid media were formulated for the second experiment. The first media, referred to as *Tuber* media (TM) in this experiment, was chosen because it generally supports growth of ectomycorrhizal *Tuber* species. *Tuber* media consisted of 5.0g potato dextrose broth, 5.0mL glycerol, and 0.82g calcium nitrate then filled with double distilled H_2O to 1000mL. Prior to autoclaving the TM, pH was brought up to 7.5 with 5M sodium hydroxide. Once the temperature of the media after autoclaving fell below 45°C, 1mL/L biotin (0.5g/L stock) was added. The

second medium, malt extract broth (MEB), was prepared using 10.0g malt extract and 1.0g yeast extract, and double distilled water filled to 1000.0mL, then autoclaved. Once the TM and MEB temperature reached room temperature (23° C), glyphosate herbicide was filter sterilized at 0.2 microns and added at the following concentrations: 0.0mL/L, 0.0456 mL/L, 0.456 mL/L, and 4.65 mL/L. These concentrations were chosen based upon herbicide label coverage information adjusted to the surface area of the liquid media within the 125.0 mL Erlenmeyer flasks used for this experiment. The label on the herbicide bottle recommends a coverage of 73.32cm^2/mL. The surface area of the liquid within the flasks is 33.44cm^2. Thus a ratio of 33.44cm^2/73.32cm^2/mL (836 : 1833) gives a working concentration of 0.456mL, which was set at the middle strength concentration for this experiment. At each concentration of herbicide 50mL of TM or MEB was aliquoted into Erlenmeyer flasks. Using a 7.5mm cork borer, a single piece of the following isolates was added to each flask. Five species of truffles were screened in the TM: *Tuber floridanum* (BR61c), *Tuber rugosum* (BR64a), *Tuber canaliculatum* (BR7), *T. borchii* (BR25), and *Tuber lyonii* (GB17). Four isolates of *Morchella* and other soil fungi were screened in MEB including: *Morchella americana* (BR3), *Morchella importuna* (GB772G), *B. erionia* (GB_AUS_27b) and *L. elongata* (GB_AUS24). Five replicates for each *Tuber* isolate and ten replicates each of *Benniella, Linnemannia,* and both *Morchella* isolates at each herbicide concentration (4) were carried out for a total of 260 flasks in this experiment. As in the aforementioned agar experiment, the agar removed from the mother culture was such that the front of the colony's growth was centered across the diameter of the plug cut by the cork borer. All replications for each isolate came from the same mother culture from the same Petri dish such that the mass of the agar plug would be consistent. Flasks were plugged with sterile cotton that had been wrapped in gauze and covered with aluminum foil to allow gas exchange while

preventing contamination. These flasks were stored randomized in a dark room on a shelf at room temperature (23°C) and were gently swirled and re-randomized every two days.

After 7 days of growth, each flask was vacuum filtered with a Büchner funnel whereby the medium was poured through pre-massed sections of miracloth leaving the mycelial mass and agar plug behind. Prior to use, miracloth (EMD Millipore Corp., Burlington MA, USA) sections were fully desiccated by running them through a lyophilizer and stored in a bag with silica beads. These desiccated miracloth sections were massed for later use. The pre-massed miracloth containing the fungal tissue was stored in a 15mL centrifuge tube and placed in a lyophilizer at -40°C and -0.01kPa for 48 hours. After lyophilization, tissue was stored in a bag with silica beads to maintain the low moisture content. Miracloth containing fungal tissue was massed and the mass of the miracloth was subtracted from the total mass such that the only mass used in subsequent calculations was the fungal growth and the initial agar plug.

Statistical Analysis

Data were analyzed and visualized in RStudio (Version 2021.09.1+372 "Ghost Orchid") running R (version 4.1.2), using ggplot 2 (version 3.3.5), ggpubr (version 0.4.0) plyr (version 1.8.6), and readxl (version 1.3.1). Means of treatment groups were compared using a t-test with the reference set as the negative control group. Means were compared by one-way analysis of variance based on a completely randomized design.

Results

Experiment 1 Solid Media

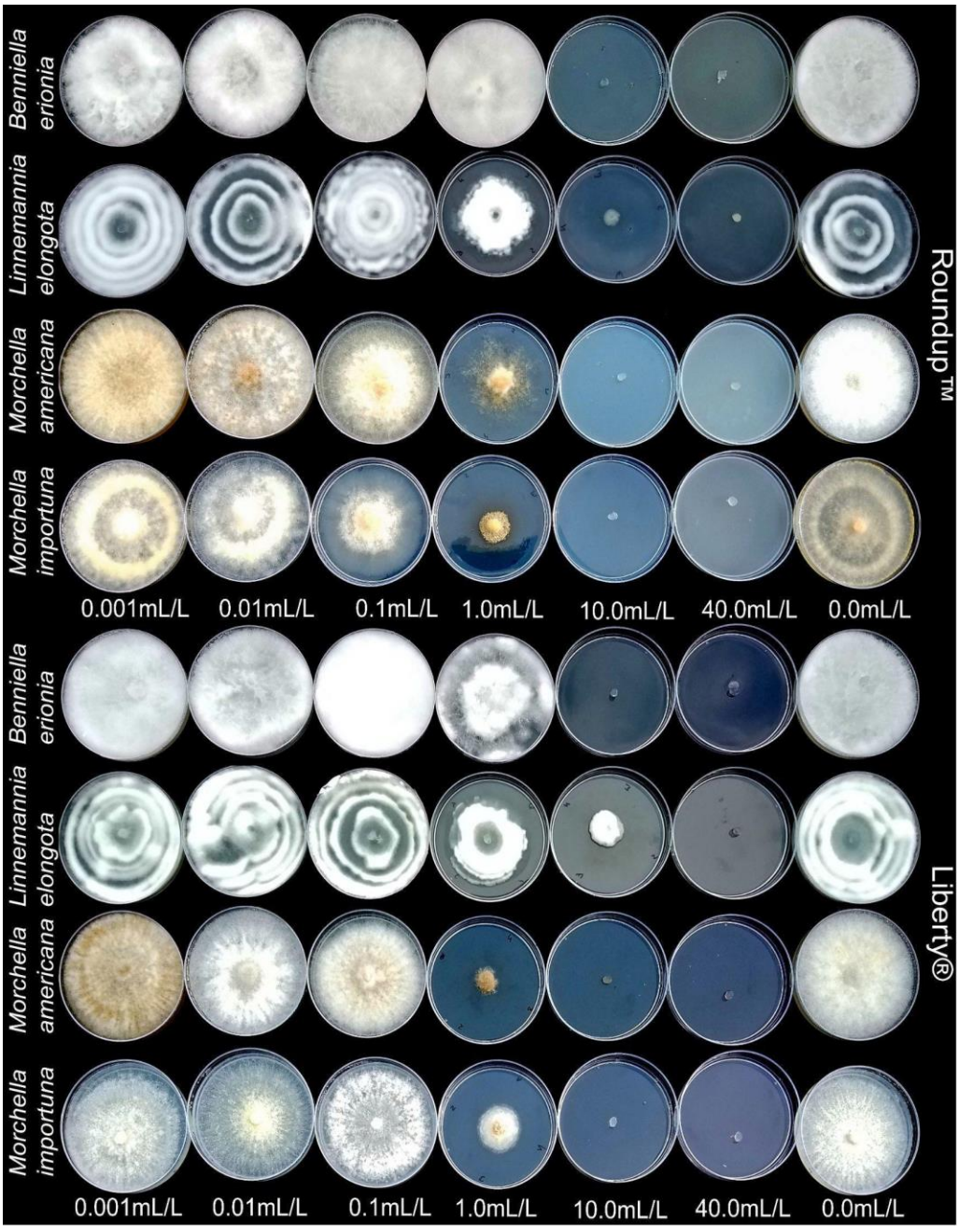

FIGURE 2.1 - REPRESENTATIVE SELECTIONS OF ISOLATES. Here, *Benniella eronia* (GB_AUS_27b), *Linnemannia elongata* (GB_AUS_24), *Morchella americana* (BR3), and *Morchella importuna* (GB772G) were grown on solid media at seven different concentrations of

glyphosate-based herbicide or glufosinate-based herbicide. Hyphal length measurements were collected at four marked poles of each plate.

In the first experiment, impacts of the glufosinate and glyphosate herbicides on fungal growth in solid media were tested for seven isolates from four genera. Between 0.1mL/L and 1.0mL/L concentrations, hyphal extension length was similar but mycelial density was reduced as the herbicide concentration increases. There was no hyphal growth for any isolate at herbicide concentrations of 40.0 ml/L (Fig 2.1). FIGURE 2.1 illustrates fungal growth response along the range of colonization from the lowest concentrations to the highest concentrations of the herbicide. FIGURE 2.1 shows a representative for each treatment, however, FIGURE 2.7 and FIGURE 2.8 show all repetitions. While the data collected highlight growth in terms of length from inoculation point, they do not capture mycelial density, hyphal diameter, or aerial growth. However, the reoccurring measurements shown in FIGURE 2.2 and FIGURE 2.3 illustrate the delay in growth and the threshold concentration of herbicide that suppressed the growth of the truffle fungal isolates. were The highest concentration of glufosinate that supported growth of *Tuber borchii* (BR11 and GMNB230) and *T. gibbosum* (GMNB46) was 0.1mL/L , and growth was delayed or reduced as herbicide concentration increased as seen in FIGURE 2.2. Among the concentrations tested, *T. borchii* (BR11) and *T. gibbosum* (GMNB46) were unable to grow vegetatively above 0.01mL/L glyphosate.

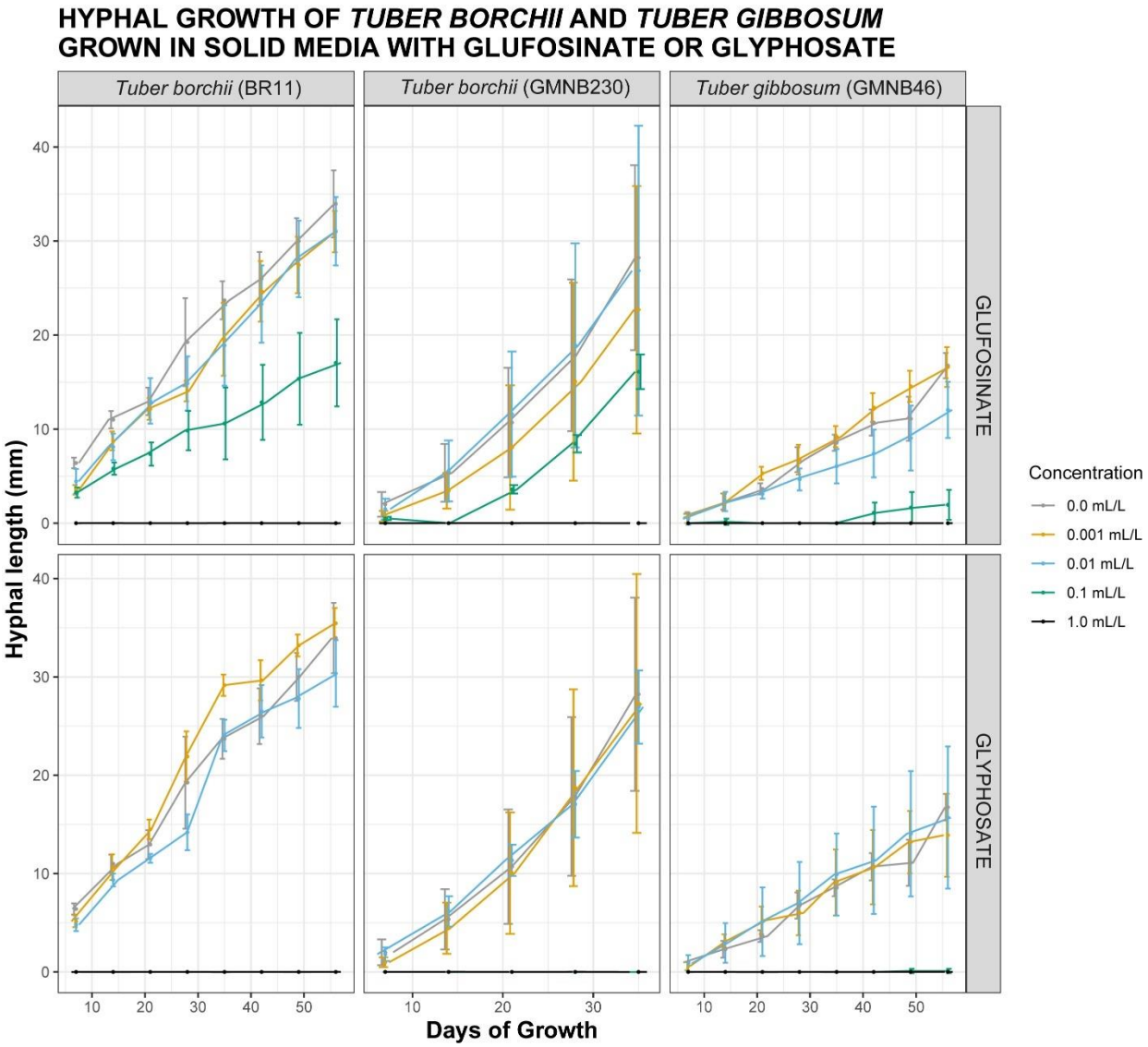

FIGURE 2.2 – DAILY GROWTH OF *TUBER BOCHII* AND *TUBER GIBBOSUM*.

Average hyphal growth of *Tuber borchii* (BR11 and GMNB230)) and *Tuber gibbosum* (GMNB46) grown in solid media at five concentrations of either glufosinate or glyphosate herbicides. At 1.0mL/L concentrations of either herbicide there was no measurable growth for both *Tuber* isolates. At 0.1mL/L concentrations of glyphosate, there was no measurable growth for either *Tuber* isolate.

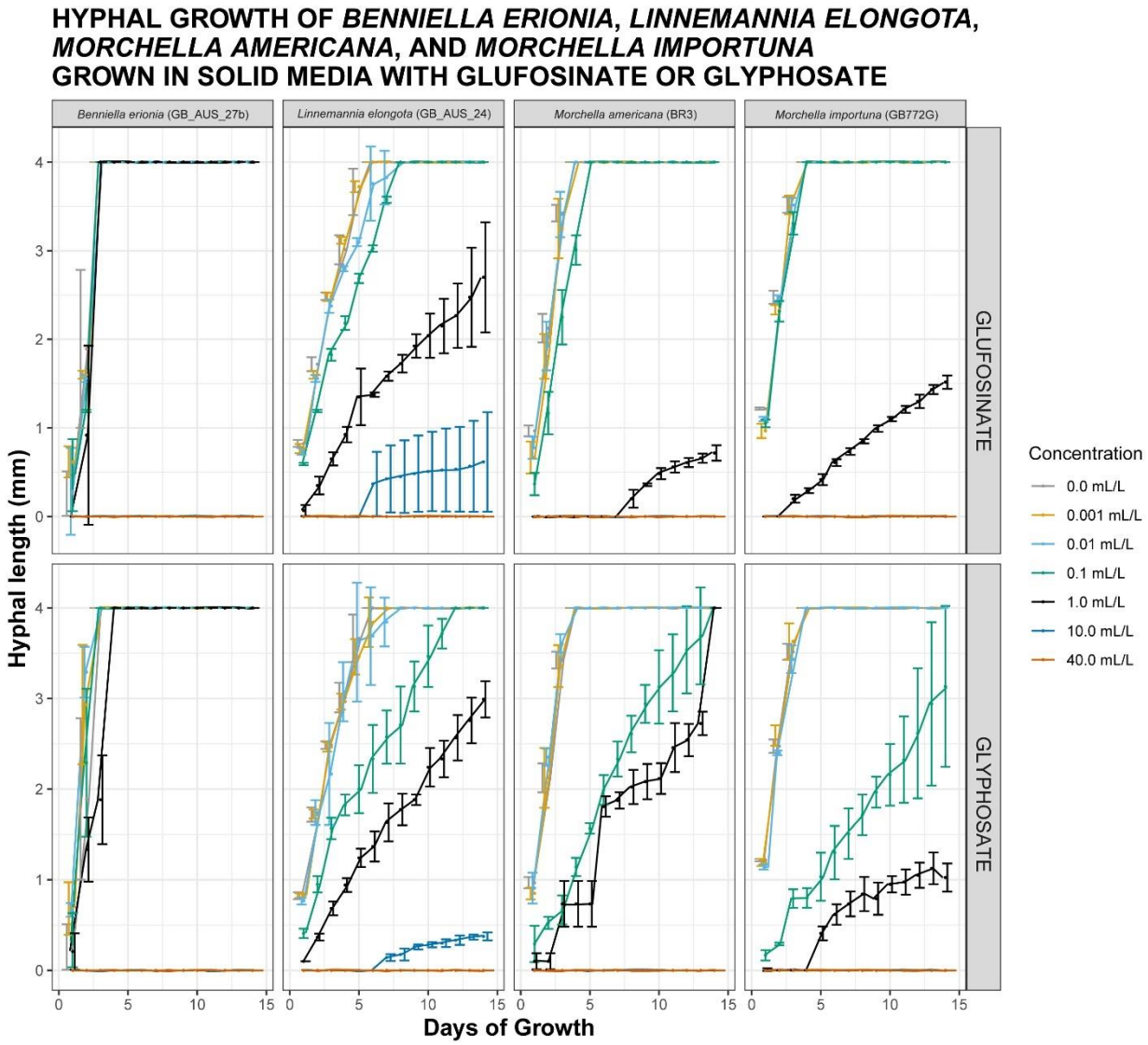

FIGURE 2.3 - DAILY GROWTH OF SAPROBIC FUNGI. Hyphal growth of *Benniella eronia* (GB_AUS_27b), *Linnemannia elongata* (GB_AUS_24), *Morchella americana* (BR3), and *Morchella importuna* (GB772G) grown on solid media at seven different concentrations of either glufosinate or glyphosate brand herbicides. No isolate grew on media containing 40.0mL/L of either herbicide. Neither *Benniella* nor *Morchella* isolates grew on media containing 10.0mL/L of either herbicide.

Linnemannia elongata was the most tolerant of the saprotrophic fungi assayed, with growth in 10.0mL/L of both glufosinate or glyphosate herbicide (FIGURE 2.3), whereas, neither *Morchella*

species grew on media with herbicide concentrations above 1.0mL/L. None of the *Morchella* species nor *L. elongata* grew at 40.0mL/L, and all isolates appeared to grow equally fast at herbicide concentrations below 0.01mL/L independent of herbicide brand.

Experiment 2 Liquid Media

In the second experiment, the impacts of glyphosate herbicide on fungal growth in liquid media was tested for nine isolates from nine genera. Among the nine fungal isolates tested, no discernable mycelium was visible at the highest concentration of glyphosate herbicide (4.561mL/L). There was a relatively steady decrease in visible growth from the lowest concentration to the highest concentration for isolates grown in MEB (Fig. 2.9). *Linnemannia elongata* (GB_AUS_24) grew on the surface in the negative control flask (Fig. 2.9). This fluffy growth is typical and was seen in every replicate, however, no surface growth was observed in any flask containing glyphosate herbicide. Similar growth was observed in all saprotrophic fungi (FIGURE 2.4) but was not observed uniformly amongst the mycorrhizal fungi. The biomass (FIGURE 2.5) produced in the working concentration of 0.451mL/L was significantly less than that of the negative control for *B. erionia* ($P<0.001$), *L. elongata* ($P<0.001$), *M. americana* ($P=0.0019$), *M. importuna* ($P<0.001$), *T. rugosum* ($P<0.001$), and *T. lyonii* ($P=0.0047$).

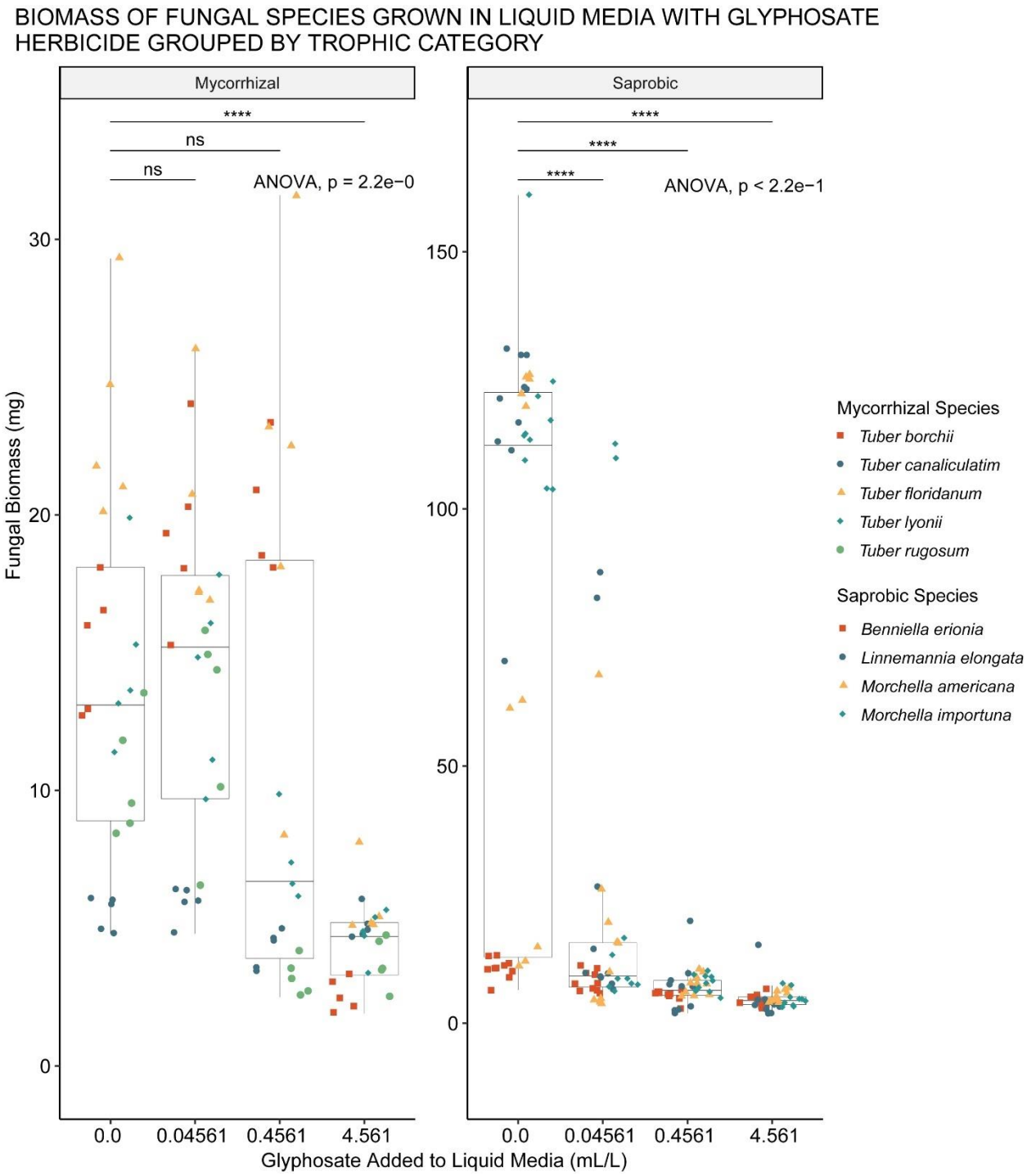

FIGURE 2.4 – BIOMASS OF FUNGI GROUPED BY TROPHIC ECOLOGIES. Dosage responses of *T. borchii, T. canaliculatum, T. floridanum, T. lyonii, T. rugosum, B. erionia, L. elongata, M. americana,* and *M. importuna* across four concentrations of glyphosate herbicide. Saprotrophic species (*B. erionia, L. elongata, M. americana*, and *M. importuna*) exhibit more

FIGURE 2.4 (cont'd) – predictable growth declines as concentration increases whereas the *Tuber* species show variable reactions to the three lower concentrations.

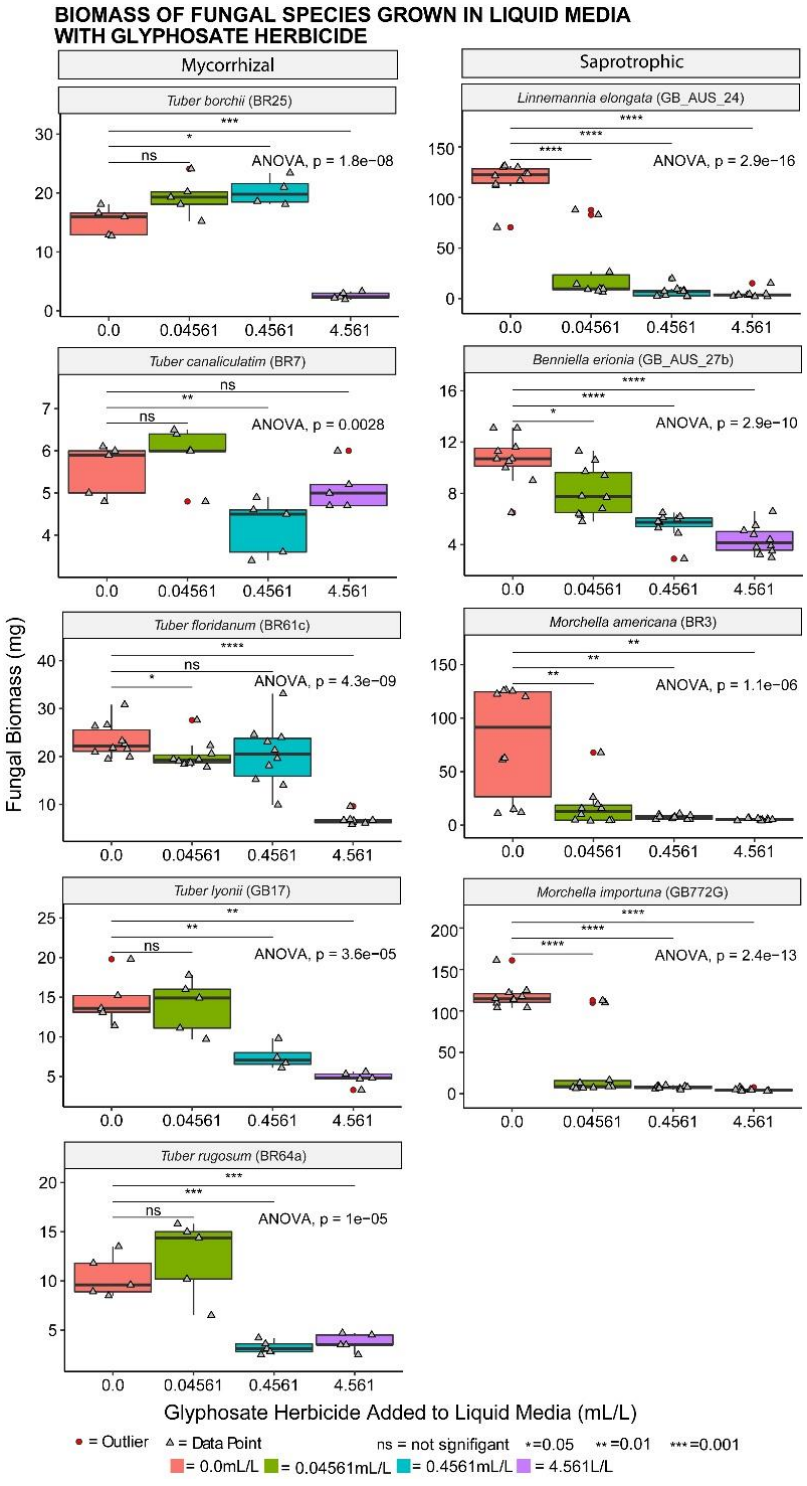

FIGURE 2.5 - BIOMASS OF FUNGAL ISOLATES. Each plot shows the biomass of *Tuber borchii* (BR25), *Tuber canaliculatum* (BR7), *Tuber floridanum* BR61c), *Tuber lyonii* (GB17), *Tuber rugosum* (BR64a), *Benniella erionia* GB_AUS_27b), *Linnemannia elongata* **FIGURE 2.5**

FIGURE 2.5 (cont'd) - (GB_AUS_24), *Morchella americana* (BR3), and *Morchella importuna* (GB772G) when grown in four concentrations of glyphosate herbicide in liquid media. The mycorrhizal species were grown on a medium referred to as *Tuber* media (TM) whereas the saprotrophic species were grown in malt extract agar.

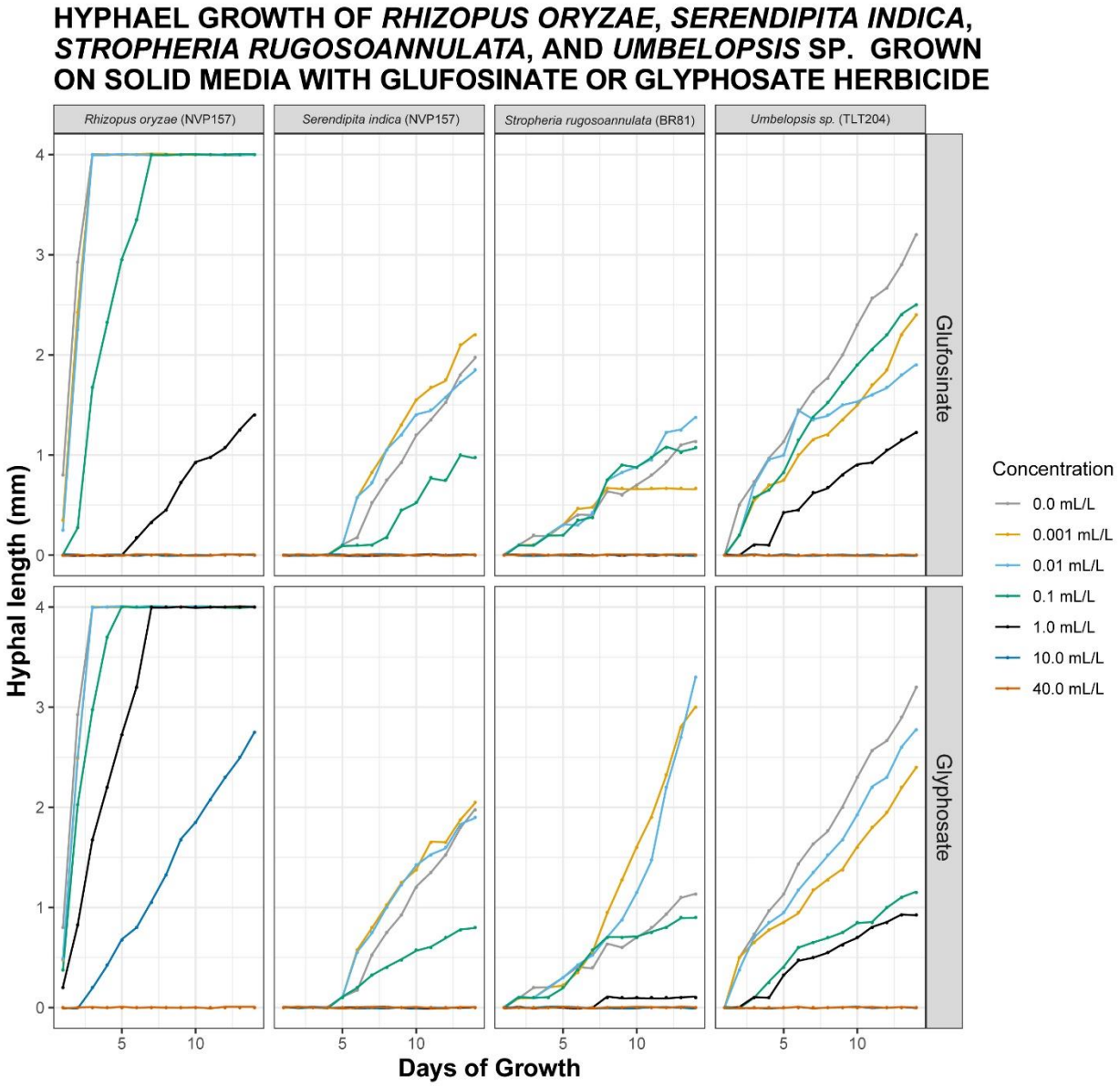

FIGURE 2.6 – AVERAGE HYPHAL LENGTH MEASUREMENTS OVER A 14-DAY PERIOD OF *RHIZOPUS ORYZAE, SERENDIPITA INDICA, STROPHARIA RUGOSOANNULATA*, AND *UMBELOPSIS* SP. ON SOLID MEDIA CONSISTING OF

FIGURE 2.6 (cont'd) - SEVEN CONCENTRATIONS OF EITHER GLUFOSINATE OR GLYPHOSATE BRAND HERBICIDE. These were not replicated and therefore do not hold statistically significant information, however, the trends amongst these soil inhabiting fungi mirror those seen in FIGURE 2.2 and FIGURE 2.3. *Rhizopus oryzae* was the only taxa in this study to grow on media containing 10.0 mL/L glyphosate herbicide.

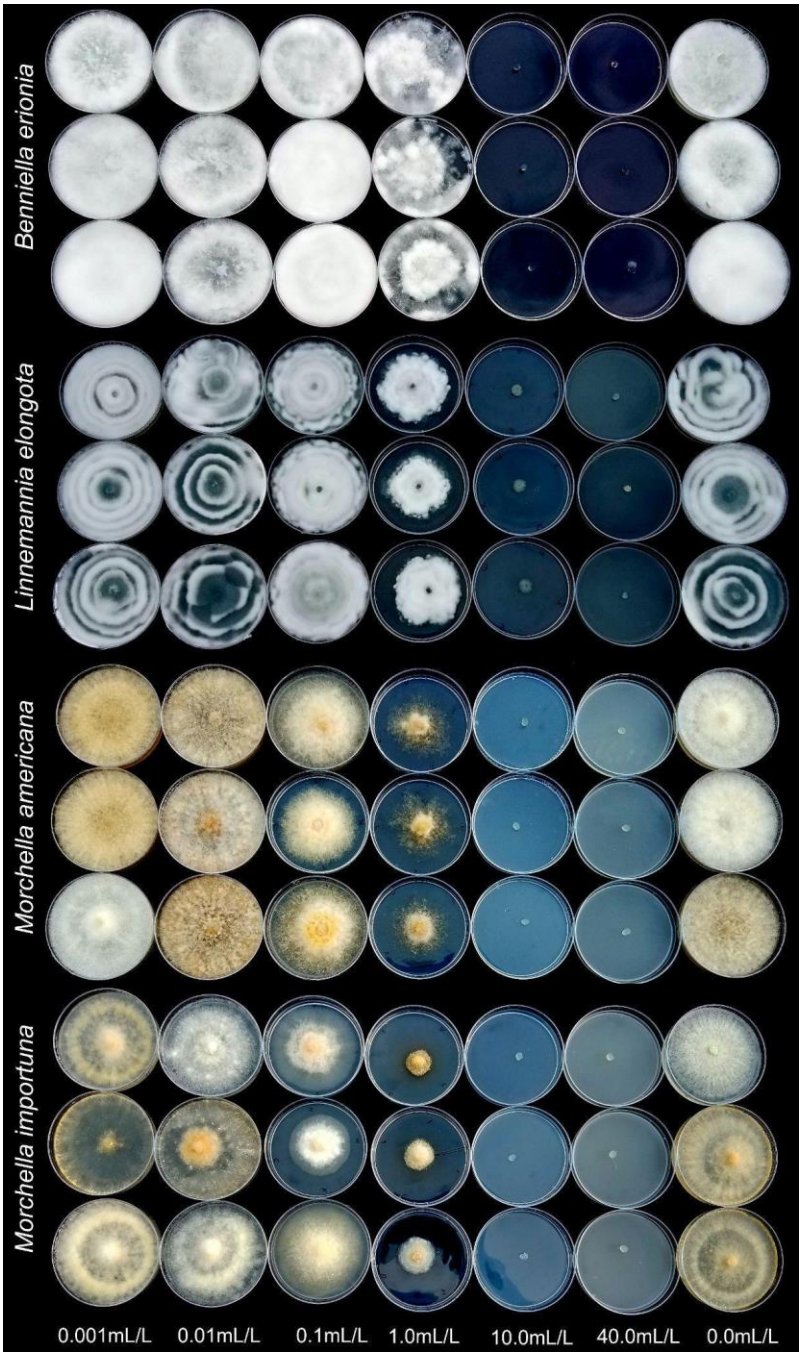

FIGURE 2.7 - REPLICATIONS OF *BENNIELLA ERIONIA*, *LINNEMANNIA ELONGATA*, *MORCHELLA AMERICANA*, AND *MORCHELLA IMPORTUNA* GROWING ON MEDIA WITH GLYPHOSATE HERBICIDE AT 7 DIFFERENT CONCENTRATIONS.

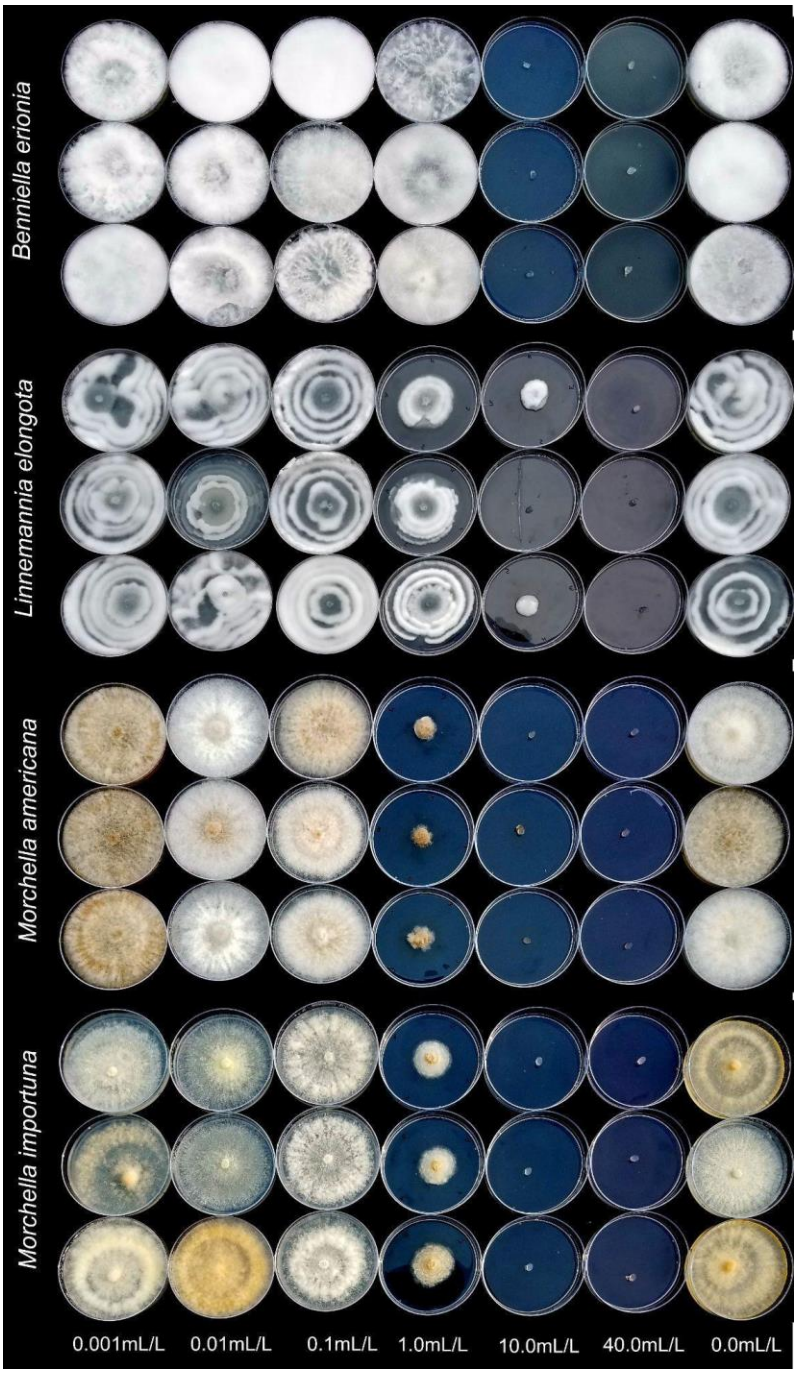

FIGURE 2.8 - REPLICATIONS OF *BENNIELLA ERIONIA*, *LINNEMANNIA ELONGATA*, *MORCHELLA AMERICANA*, AND *MORCHELLA IMPORTUNA* GROWING ON MEDIA WITH GLUFOSINATE HERBICIDE AT 7 DIFFERENT CONCENTRATIONS.

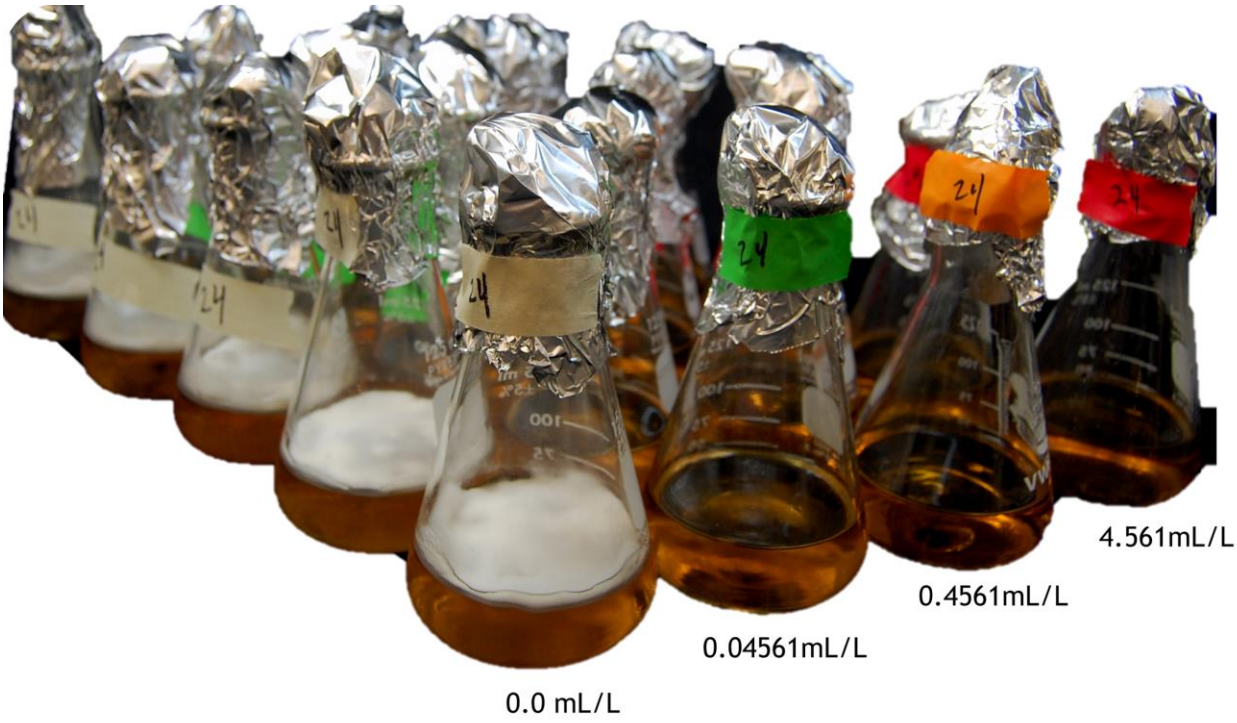

FIGURE 2.9 – GROWTH OF LINNEMANNIA ELONGOTA IN BROTH WITH HERBICIDE AT FOUR CONCENTRATIONS. Only the media without any herbicide displayed thick characteristic growth above the liquid surface. Media with herbicide at 0.04561mL/L did have growth, but it remained solely within the broth. Media at 0.4561mL/L and 4.561mL/L had little if any observable hyphal growth.

Discussion

Globally, an estimated 950,000 tons of chemical herbicides are applied to land annually (Sharma et al., 2019). In fact, glyphosate is one of the most common herbicides used in agriculture. It is a broad-spectrum systemic herbicide, a phosphonate, which interrupts the shikimate pathway. Although the impacts of this herbicide on plants and animals is fairly well studied, impacts of these chemicals on non-target soil biota is less well understood. To address this, in this study we tested the impact of glyphosate and glufosinate on pure culture isolates of

diverse soil fungi including saprotrophic morels (*Morchella*), ectomycorrhizal truffles (*Tuber*), and beneficial root symbionts (Mortierellaceae; Sebacinales) of agricultural plants.

In this study the impacts of herbicides were tested on different fungal guilds including plant growth-promoting fungi, ectomycorrhizal fungi, and soil saprotrophs. Among isolates tested, ectomycorrhizal fungi were the least sensitive to these herbicides, and only showed detrimental growth effects at concentrations at or above the rate of application (0.456mL/L) as per the herbicide label suggestions. This finding supports other studies that found little to no effect in mycorrhizal status or extraradical hyphae density with moderate applications of glyphosate (Bonet et al., 2006; Chakravarty & Chatarpaul, 1990; Gómez-Molina et al., 2020; Olivera et al., 2011). Interestingly, the saprotrophic fungi assayed here were more sensitive to glyphosate and showed deleterious effects at concentrations lower than the recommended application rate. One possible explanation is that ectomycorrhizal species grow slowly in vitro and therefore have more time to either process the herbicide or more time to produce the amino acids disrupted by the disruption of the shikimate pathway. Future work focusing on the effects of glyphosate on the fungal shikimate pathway may yield more clues. In non-agricultural settings where hand applications of glyphosate are commonplace, it is therefore important to prevent overapplication of this herbicide to prevent concentrations where fungistatic or fungicidal outcomes are observed.

Our results demonstrate that the herbicide glyphosate can impact fungal vegetative growth when applied at concentrations above the recommended concentration. Glyphosate has also been shown to influence other organisms, such as by decreasing melanization and increasing infection rates among the moth *Galleria mellonella* L. and mosquito *Anopheles gambiae* Giles (Smith et al., 2021). There have been numerous studies outlining varied outcomes of glyphosate

applications on earthworms such as weight loss (Correia & Moreira, 2010), negative reproductive influences (Domínguez et al., 2016), avoidance response driven migration (Verrell & Van Buskirk, 2004), and decrease mycorrhization by arbuscular mycorrhizal fungi in mesocosms with *Trifolium repens* L. and earthworms (Zaller et al., 2014). Despite the growing breadth of knowledge into non-target herbicide interactions, there is still clearly more that needs to be studied.

Outdoor morel cultivation has garnered much interest recently, particularly since the first successful artificial outdoor cultivation of morels in 2012 in Sichuan, China (Xu et al., 2022). One appealing facet of morel cultivation are improvements to the soil and given morels' short growing period they fit neatly into certain rotating cropping systems (Su et al., 2022). As morel cultivation further expands into more crop rotations, further work is needed to identify interactions with the pesticides used with the preceding crop. The use of herbicides, such as those containing glyphosate, decrease fungal biomass in the soil as well as cultivable fungi species richness (Vázquez et al., 2021). However, there is still much work to be done regarding pesticides and their implications on plant growth-promoting fungi.

In conclusion, we used pure fungal cultures to assess the impact of glyphosate and glufosinate on fungal growth in solid and liquid media. We found that as concentration of glyphosate or glufosinate increased in the growth media, the herbicide decreased fungal network density, branching and biomass. Interestingly, we found that the saprotrophic fungi tested were more susceptible to glyphosate compared to ectomycorrhizal species. Taken together, these results indicate that glyphosate herbicides can impact fungal populations if used at concentrations higher than recommended by the producers. Glyphosate is sometimes used in truffle orchards to help control weed establishment around planted seedlings. It is also used in

agricultural systems where plant growth-promoting fungi or other edible fungi may be of interest. Thus, care should be taken if applying glyphosate herbicides in agricultural systems where fungal populations are of interest to limit overapplication. If applied at recommended concentrations, our results indicate that commercial blends of glyphosate or glufosinate may not negatively impact the vegetative growth of the fungal mycelium growing in treated soils in the short-term. However, further research will be needed to determine the fate of herbicides in complex real world environmental ecosystems.

Chapter 3

Does ectomycorrhizal inoculum after methyl bromide field applications in bare-root conifer nursery seedlings establish and persist?

Abstract

Conifer seedlings are widely grown in the midwestern United States for Christmas tree plantations, landscape nurseries and reforestation. Seedlings are typically grown for two years prior to lifting and replanting. Many of these bare-root nurseries have been in business for many decades and typically sow seed in the same soil cycle after cycle. In part to reduce disease pressure, but also to reduce weed seed banks, some nurseries apply methyl bromide with chloropicrin as a fumigant to eradicate these pests. With concern about this fumigant destroying not only pests but also beneficial mycorrhizal fungi, there is interest in whether the application of mycorrhizal spores following fumigation is worthwhile. We used this fumigation-cropping cycle to investigate how introducing local, exotic, or commercial inoculants may influence health, height, and biomass of the seedlings grown in these fumigated soils. Based on investigating two nurseries over two cropping cycles, our findings indicate that there are no disease reduction or growth increases gained regardless of any mycorrhizal inoculants tested. Rather, we found a robust diversity of mycorrhizae, beyond our inputs, infesting the roots of *Pseudotsuga menziesii, Picea abies,* and *Pinus sylvestris* irrespective of whether the inoculum was from local, exotic, or commercial sources. These findings suggest that there are ectomycorrhizal fungi either surviving fumigation or blowing in from nearby tree stands. The major finding of this study is that Norway spruce, Douglas-fir, or Scots pine grown in a bare-root nursery do not benefit from the addition of additional ectomycorrhizal spores.

Introduction

Currently, there are approximately 300,000 acres of cultivated conifer trees grown on 15,000 farms in the United States (USDA, 2017). Combined with Europe, over 80 million trees are grown every year, many of which begin life in bare root nurseries (Chastagner & Benson,

2000). As with any high-density cropping system, pests and pathogens can become a major issue in conifer nursery beds. The most concerning pathogens to conifer tree nurseries cause root rots and damping off diseases. These diseases reduce production yields and quality of seedlings and are often seen as chlorosis, stunting, and necrosis (Weiland et al., 2016). Fungal species of *Fusarium* Link, *Cylindrocladium* Morgan, and *Rhizoctonia* DC. and Oomycete species of *Phytophthora* de Bary and *Pythium* Pringsh. are the most commonly reported causal agents of seedling death amongst nurseries (Cram, 2015)**.**

Oomycetes such as *Pythium* and *Phytophthora*, once thought to be fungi, are in the kingdom Chromista. Unlike fungi, oomycetes lack chitin in their cell walls and live primarily as diploid organisms. This group hosts some of the most important pathogens whose motile zoospores cause several damping-off diseases in nurseries. *Pythium irregulare* Buism. for example, has been noted to kill *Pinus patula* (Linde et al., 1994) and *Picea abies* (Kozlowski & Métraux, 1998), and *Pseudotsuga menziesii* (Weiland et al., 2013). *Pythium ultimum* var. *ultimum* and *P. cinnamomi* were also found virulent on *Pinus sylvestris* (Chavarriaga et al., 2007). However, pathogens are not the only challenge nurseries must contend with.

Weed plants are a concern as they compete for water, nutrients, and sunlight, especially at the nursery stage, but also at each subsequent stage of the conifer tree production cycle. Growers have many options to control weeds. Non-chemical means include mowing, pulling weeds by hand, and even employing sheep to graze on the non-target crop (Saha et al., 2020). With over 100,000 people employed in the conifer tree industry within the United States alone (Saha et al., 2020), growers have incentive to reduce labor expenditures and chemical weed reduction is often chosen. In bare-root tree nurseries, preplant soil fumigation is a common method employed to reduce both weeds and pathogens in the soil (Shrestha et al., 2008; Weiland

et al., 2016). A popular choice among growers for preplant fumigation is methyl bromide with chloropicrin as it has proven effective at controlling pathogens as well as weed seed germination (Weiland et al., 2016).

Methyl bromide is naturally produced in marine environments by many phytoplankton species (Sœmundsdóttir & Matrai, 1998) although it remains unclear what role it plays. It has been suggested that halogenated metabolites act as chemical defense or as antifouling agents (Paul & Pohnert, 2011). However, methyl bromide is also a powerful broad-spectrum pesticide produced commercially for use in food crops such as peppers, strawberries, grapes and structural fumigation applications for treating pests such as termites (Piccirillo & Piccirillo, 2010). Typical agricultural application of methyl bromide is injection into the soil followed by covering the ground with plastic for several days to seal in the fumigant (Piccirillo & Piccirillo, 2010). Since methyl bromide is a colorless and odorless gas, chloropicrin is often added to help applicators detect exposure since methyl bromide is neurotoxic in humans (Piccirillo & Piccirillo, 2010). Used as a chemical warfare agent in World War I (Sciuto & Kodavanti, 2015), chloropicrin also has biocidal and fungicidal applications (Wilhelm et al., 1996) similar to methyl bromide. Additionally, as with methyl bromide the exact mode of action is unclear (O'Malley, 2010; Sparks et al., 1997). In unison, these two fungicides are effective in controlling nematodes, fungi, oomycetes, and many more soil-borne pathogens though methyl bromide is being phased out and restricted due to its role in ozone depletion (Weiland et al., 2011; Zasada et al., 2010).

Another unintended consequence of methyl bromide used is the impact it has on non-target organisms. Beneficial soil microbiota are known to significantly influence plant fitness and broad spectrum fumigants reduce this critical facet of soil health in our agricultural systems (Astudillo-García et al., 2019; Castellano-Hinojosa et al., 2022). Broad-spectrum fungicidal soil

fumigants have the potential to reduce and effectively reset the community structure leading to unique opportunities for studying priority effects. An ever broadening assemblage of studies provide strong evidence as to the importance of priority effects on microbiome assembly, diversity, host and ecosystem function (Debray et al., 2022). However, some edible ectomycorrhizal fungi cultivation systems aim to restrict mycorrhizal diversity.

Early dominating establishment is critical for cultivating targeted ectomycorrhizal fungi, such as truffles, whereby seedlings are raised to be well-colonized by select *Tuber* species before planting out into prepared fields. These highly prized hypogeous fruiting bodies of *Tuber* spp. P. Micheli form in association with various hosts including *Quercus* L., *Pinus* L., *Picea* A. Dietr., and *Pseudotsuga* Carrière. It is estimated that over 500,000 trees inoculated with truffle spores are produced annually in nurseries where they are grown in individual pots for later sales and establishment by truffle plantation owners (Murat, 2014). Beyond marketing edible mycorrhizal fungi, many companies choose to focus on growth and health aspects of various crops in an ever growing industry**.**

Research strongly implicates the importance of fungi in the rhizosphere of plants. However, despite this growing field of interest and an ever expanding commercial presence, the results have been largely lackluster as there are numerous reports of little to no growth or health benefits observed (Duell et al., 2022). There are a multitude of challenges these products must overcome. For example, one study found that *Sphagnum* peat moss, commonly used in container nurseries, contains many ectomycorrhizal species which are still viable and were able to colonize the roots of *Pinus montezumae* (Ángeles-Argáiz et al., 2016). However, most mycorrhizal products sold primarily contain arbuscular mycorrhizae; though some recent research suggests that the advertised arbuscular inocula is rarely accurate. One study found that the products they

sampled had unaccounted for taxa and were nearly all missing taxa claimed on the product label (Vahter et al., 2023). Two of these products contained none of the taxa listed on the packaging and five products only contained one of the listed inoculants (Vahter et al., 2023).

This study was conducted to investigate whether introducing ectomycorrhizal inoculum increases tree growth and reduces disease incidence in three species of conifers at two bare-root nurseries. We utilized locally acquired ectomycorrhizal fungi, exotic *Tuber* species, and readily available commercial blends of mycorrhizal inoculum in this field experiment. Specifically, we wanted to investigate the following: can roots of *Pseudotsuga menziesii, Picea abies,* and *Pinus sylvestris* grown in methyl bromide fumigated soils be colonized by targeted ectomycorrhizal fungi. We also wanted to assess how well locally sourced, exotic (*Tuber spp.),* and commercial inoculum establishes and persists in methyl-bromide fumigated soils, and similarly how host species, inoculum treatment, and location impact fungal and oomycete diversity in the roots of conifer tree seedlings.

Methods

Two variations of an experimental design were carried out over two complete bare-root conifer nursery cropping cycles. They will be referred to in this manuscript based on the year of inoculation as either experiment 1 or experiment 2 for inoculation 2018 or inoculation 2019 respectively. Tables 2-4 provide the complete experimental design for both farms in both years.

Site Selection

The two bare-root conifer tree nurseries 20 miles apart in Michigan were selected for this field experiment. These sites share a temperate continental climate with cold winters. The average low-temperature in the coldest month is -7.2 C, and the average high-temperature in the warmest month is 26.4 C. There are an average of 100.8 rainfall days and 186 mm of

precipitation annually. Both nurseries utilize Tri-Brom 80-20 soil fumigant at 240lb/ac and are composed of the following active ingredients: 80% Methyl Bromide, 19.9% Chloropicrin. These two sites are referred to as Allegan and Gobles. Soil at Allegan is a Chelsea loamy fine sand with a pH of 7.0 and the soil at Gobles is Ottokee loamy fine sand with an average pH of 6.8. The pH of homogenized soil was collected from 5 points approximately 12.0 cm deep for each tree species at each farm. Each was prepared by mixing two parts soil into one part double distilled water by mass and letting it rest for 30 min. Readings were collected using a calibrated pH Meter (AB15 Accumet Basic, Fisher Scientific).

Soil Preparation

Both farms fumigated their fields early September in the year prior to planting. See Supplementary table 3.1 for the complete pesticide application schedule. For experiment 1, seeds were sown on May 15th, 2018 at Allegan and May 22nd, 2018 at Gobles. For experiment 2, seeds were sown on May 16th, 2019 at Allegan and June 5th, 2019 at Gobles. Prior to sowing, grower-cooperators prepared seedbeds using their standard practices (FIGURE 2.1A). Once seedbeds were formed (1.0 m wide), the cooperator sowed seeds at the following rates: Norway spruce 650 / m^2; Scots pine 550 / m^2; Douglas-fir 370 / m^2.

Inoculum Preparation

Application rates for all inoculum are listed in Table 2.2. *Tuber indicum* Cooke & Massee, *Tuber aestivum* Vittad., and *Tuber borchii* Vittad. ascocarps were weighed and surface sterilized using 3% hydrogen peroxide for 2 minutes and rinsed with double distilled water. *Tuber indicum a*scocarps were pulverized using a Waring commercial blender (Model 4324X) on the high setting for 15 minutes; *T. borchii* and *Tuber aestivum* for 10 minutes. Ascocarps, crushed ice, and double distilled water were added to the blender and after running for the

aforementioned times, a compound light microscope (Leica DM750) was used to verify that approximately 50% of ascospores were released from their asci. This spore suspension was brought to a concentration of 0.2g/mL using additional double distilled water and then stored at 4C overnight for application the following day. While in transport to the field sites, inocula remained in a cooler on ice until application to preserve spore viability.

Laccaria bicolor (Maire) P.D. Orton, and *Scleroderma citrinum* Pers. basidiocarps were each also prepared the same as with *Tuber,* however blended for only 5 minutes. *L. bicolor,* and *S. citrinum* suspensions were each brought to 0.1g/mL.

Field inoculation 2018

At both Allegan and Gobles, beds were formed in the morning just prior to inoculation. At Allegan the study was installed as a fully randomized block design (Table 3.3). However, Gobles did not have a randomized design to reduce treatment blending by farm equipment. Allegan spanned 85 treatment blocks and Gobles included 75 treatment blocks. Each treatment block was 1.0 m^2 and a PVC frame was utilized to ensure even application over each block. Specific locations for each block were identified using an iron spike with an identification number such that locating exact locations for subsequent sampling during the fall of the following year could be made precisely where spore application was made. Spore suspensions were further diluted to facilitate even spraying using a 4.0 L portable pump sprayer (Chapin brand). Each inoculum was applied at three concentrations; *T. aestivum*, *T. borchii,* and *T. indicum* were applied at 0.2g/m^2, 2.0g/m^2, and 20g/m^2. Similarly, *L. bicolor,* and *S. citrina* were each applied at 0.1g/m^2, 1.0g/m^2, and 10g/m^2. Negative control blocks were sprayed with water from the same distilled water as was used to make the fungal spore slurries. Each treatment block was replicated five times at both farms.

At Allegan and Gobles, *Picea abies* (Norway spruce), *Pinus sylvestris* (Scots pine), and *Pseudotsuga menziesii* (Douglas-fir) were subjected to these inoculation treatments. Treatment blocks at Allegan were spread evenly across the total length of the bed for each tree species; *P. abies* 274.32 meters, *P. sylvestris* and *P. menziesii* each 122 meters. At Gobles, treatment blocks were grouped (supplementary table 4) to reduce treatment mixing effects.

Spore slurries were applied at both sites between bed formation and seed sowing by spraying the slurry directly onto the soil surface. After seeds were sowed into the soil, a roller press finished the formation of the bed.

Field inoculation 2019

The inoculation in 2019 was carried out in a similar manner as the 2018 inoculation with key differences being inoculum type and timing. Here, spore application was performed before bed formation such that spores could be rototilled into the soil. Allegan spanned 40 treatment blocks and Gobles included 20 treatment blocks. In 2019 *T. borchii, T. indicum*, and a commercial mycorrhizal fungi (MycoApply® Endo/Ecto, Mycorrhizal Applications, Grants Pass, Oregon USA) blend were used. The commercial product used is a mix of endomycorrhizal and ectomycorrhizal fungal spores homogenized in a fine powder designed for water suspension. According to the label the commercial inoculum includes spores of *Glomus intraradices, G. mosseae, G. aggregatum, G. etunicatum* as the included endomycorrhizal inoculants and *Rhizopogon villosulus, R. luteolus, R. amylopogon, R. fulvigleba, Pisolithus tinctorius, Scleroderma cepa, and S. citrinum* as the included ectomycorrhizal fungal inoculants.

Collection dates

Trees inoculated in the Spring of 2018 were collected from Allegan and Gobles on the 9th and 13th of September 2019, respectively. Trees inoculated in the Spring of 2019 were collected from Allegan and Gobles on the 11th and 15th of June 2020, respectively.

Collection Methodology

At both sites for both years the collection methodology was the same. A point-intercept frame was utilized for each block such that trees closest to points on the frame were selected in a non-biased way. This frame was placed over the treatment block being sampled and the tree nearest to a point was carefully dug out of the soil using a hand trowel. Five trees from each block were collected and placed into a plastic bag which was stored on ice in a cooler while in the field. The samples were stored that day in a 4°C walk-in cooler until further processing the following day.

Plant growth metrics

Tree height was measured from the root collar to the apical tip using a ruler for all trees, both years. We measured root collar diameters of trees collected in 2020 from both sites using digital calipers. Overall tree health was assessed based on chlorosis and necrosis on a scale of 1-4, where 1 indicated more than 75% necrosis/chlorosis; 2 indicates 50-75% necrosis/chlorosis. Each rating level represents a 25% rating, for example, a rating of 2 indicates 50-75 necrosis/chlorosis; 3 indicates 25 to 50 necrosis/chlorosis whereas a 4 indicates less than 25% necrosis/chlorosis. Root colonization levels were determined by analysis conducted under a compound light microscope (Leica Model S9i,1700 Leider Lane, Buffalo Grove, IL 60089 United States) (FIGURE 3.1C).

Plant Tissue Preparation

After morphological analysis concluded, roots were separated from the above ground growth at the root collar. Root sections were then soaked briefly in a 0.01% solution of Tween20 then rinsed with tap water until any loose soils had been removed. Surface cleaned roots were then wrapped in paper towels and allowed to air dry overnight on. The following day, roots in paper towels were stored in a plastic bag with silica desiccant beads until further processing. Process controls consisting of the same paper towels were prepared with water and dried but without roots inside.

DNA extraction

Dried roots placed in paper towel bundles were rolled against a hard surface such that root tips were dislodged and freed from the larger root sections from which they arose. Loosened root pieces were then collected and placed into a 2.0 mL microcentrifuge tube with two stainless steel ball bearings. Each sample was ground up and homogenized utilizing the Qiagen Tissuelyser II bead mill for 30 RPS for one minute (QIAGEN Sciences Inc, 19300 Germantown Rd, Germantown, MD 20874). DNA was extracted from the plant tissue using the Mag-Bind Plant DNA Plus Kit (Omega Bio-tek, United States). Process controls were included with each extraction and also included the paper towel controls used in the drying process.

Illumina MiSeq Sequencing and Library Preparation

Amplicon libraries for MiSeq sequencing were produced based on the same three step PCR protocols used in (Benucci, et al., 2019; Lundberg et al., 2013). The ITS1F and ITS2 primer sets were used for the Fungal library and the ITS-6 (5’ GAAGGTGAAGTCGTAACAAGG 3’) and ITS-7 (5’ AGCGTTCTTCATCGATGTGC 3’) primer sets were used for the Oomycetes library. The products of the PCR were visualized utilizing a QIAxcel Advanced capillary

electrophoresis device (QIAGEN Sciences Inc, 19300 Germantown Rd, Germantown, MD 20874). PCR products from successful amplifications were normalized using the Invitrogen SequalPrep™ Normalization Plate (96) kit (Fisher Scientific International L.L.C., Wyman Street, Waltham, Massachusetts 02454-9046). Normalized amplicons were then pooled using the Amicon® Ultra 0.5 mL 50 K filters (EMD Millipore, Germany) then purified to remove primer dimers as well as remaining fragments using the Agencourt AMPure XP magnetic beads kit (Beckman Coulter, USA). The purified amplicons were then sequenced at the Research Technology Support Facility (RTSF) Genomics Core at Michigan State University utilizing the Illumina MiSeq V3 analyzer with the 600 cycles kit (Illumina Inc., 5200 Illumina Way, San Diego, CA).

Bioinformatic and Statistical analyses

Sequences were initially processed using the same pipeline as in (Benucci, et al., 2019b) up to the taxonomic assignments. Here, CONSTAX2 taxonomic classifier was utilized (Liber et al., 2021) as well as FUNGuild (Nguyen et al., 2016) for ecological classification. Operational taxonomic unit (OTU) tables and metadata files were imported to R Studio (build 372) running R version 4.1.2 (Bunn & Korpela, 2015). The scripts used in this pipeline are available at https://github.com/rennickb.

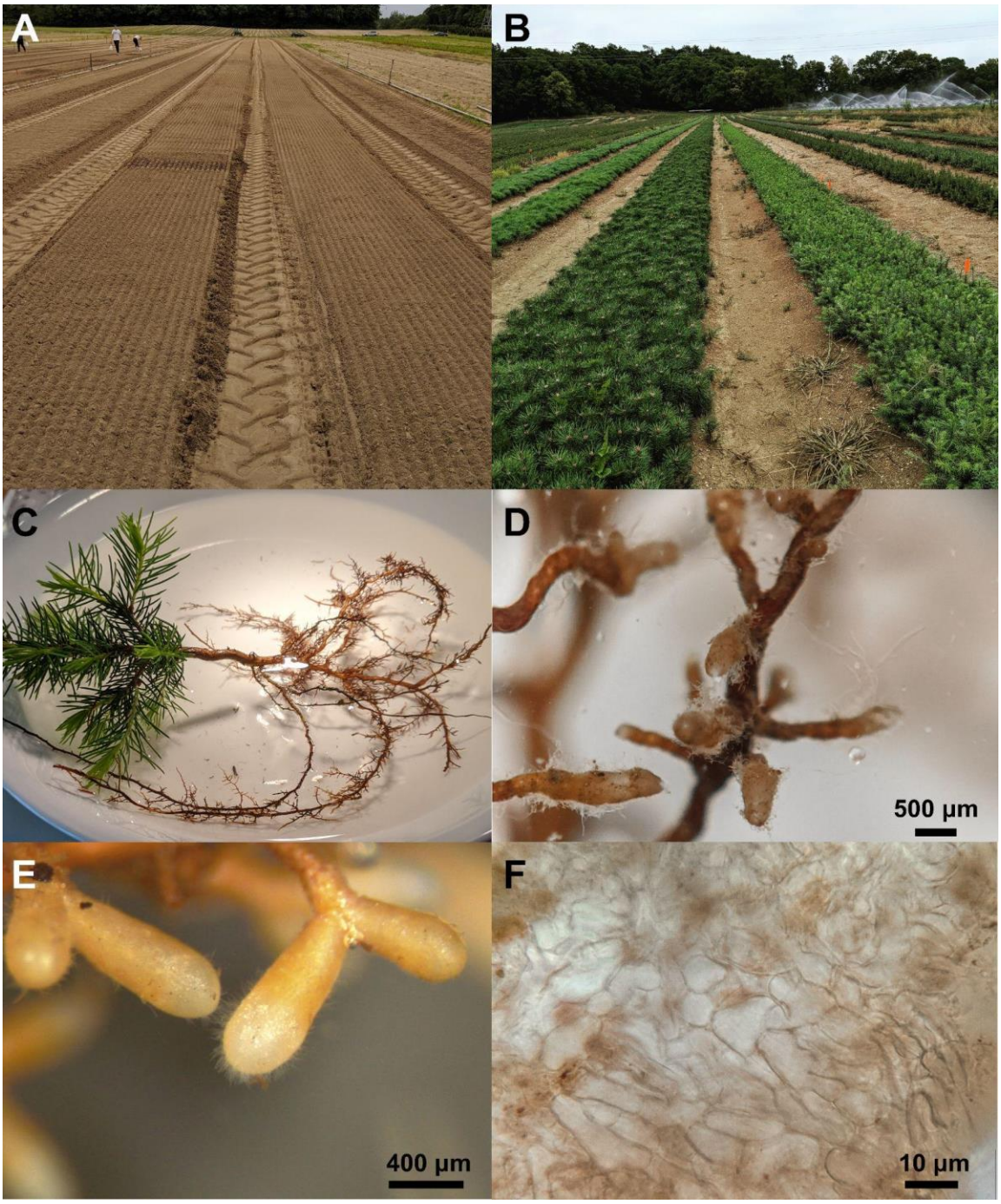

FIGURE 3.1 - FIELD SITES, ROOT INSPECTION, AND COLONIZED ROOT TIP. A) Gobles seed bed formation. B) Allegan conifer seedlings before lifting. C) Norway spruce under compound light microscope with root system suspended in water. D) Extramatrical

FIGURE 3.1 (cont'd) - hyphae on Douglas-fir typical of Basidiomycota ectomycorrhizae. E) Example of *Tuber borchii* mycorrhizae on *Pinus*. F) Ectomycorrhizal mantle on *Pinus* roots.

Tree growth

Inoculation did not affect ($p>0.05$) tree height (FIGURE 3.3), biomass (FIGURE 3.4), or root collar diameter (FIGURE 3.5 and 3.6) regardless of tree species or field location. With an average health rating of 3.9, overall tree health was high and similarly, there was very little to no disease noted for any inoculated or uninoculated blocks. Visual inspection of the roots for exotic mycorrhizae did not reveal any roots colonized by *Tuber* species (FIGURE 3.1). Irrespective to the field location, tree species, or inocula there was a great diversity of mycorrhizal fungi on the roots of conifer trees in soils previously fumigated with Tri-Brom 80-20 (Fig 3.1).

High-throughput sequencing results

The total reads after filtering for quality were 20,975,165 for the fungal library and 19,839,935 reads for the oomycete library. Across 314 samples, the average read depth in the fungal library was 66,800 and 63,185 in the oomycete library. There were 1,680 OTU's in the fungal library and 117 OTU's in the oomycete library.

Fungal Library Community Composition

The fungal library was dominated by two taxa, *Sphaerosporella* (Svrček) Svrček & Kubička (33.4% relative abundance across dataset) and *Wilcoxina* Chin S. Yang & Korf. (32% relative abundance across dataset) Other notable taxa included *Suillus* Gray, *Peziza* Dill. ex Fr., *Hebeloma* (Fr.) P. Kumm., and *Geopora* Harkn. *Suillus* spp. (14.3% relative abundance) are well represented across the roots of *P. sylvestris* at Gobles during the first experiment. Successful *Tuber borchii* inoculation seems to have occurred on the roots of Douglas-fir trees at Gobles within experiment 1 (12.7% relative abundance), though read counts were low, *T. borchii* was

detected on Norway spruce and Scots pine here as well. Interestingly, *Laccaria* Berk. & Broome was found in the control blocks placed with Douglas-fir at Allegan, experiment 1 (1.2% relative abundance) and 2 (1.0% relative abundance), however, it was also found on Scots pine at Gobles in low abundances as well. Scleroderma Pers.was not found in any treatment, tree, location, or experiment.

Pathogenic taxa varied greatly between field locations and the community structure (FIGURE 3.7). With experiment 1, the pathogen fungal library at Allegan was dominated by *Fusarium*, *Trichoderma* Pers., *Diaporthe* Fuckel, *Clonostachys* Corda, and a Ceratobasidiaceae G.W. Martin (FIGURE 3.7). However, *Paraphoma* Morgan-Jones & J.F. White, *Phoma* Sacc., Nectriaceae Tul. & C. Tul., *Diplodia* Fr., and *Rhizoctonia* DC. had the most reads from the Gobles roots during the first experiment. However, experiment 2 resulted in higher *Macrophomina* Petr. and *Paraphoma* reads from Allegan and *Nectria* (Fr.) Fr., *Clonostachys*, and similarly, *Paraphoma* at Gobles (FIGURE 3.7).

Oomycete Library Community Composition

Many other important pathogenic taxa in the Oomycetes lineage were also present at both locations. One *Pythium* OTU dominated the oomycete library at Allegan during the first experiment (FIGURE 3.3). As a genus, *Pythium* comprises nearly all reads in the dataset (92.6% relative abundance). Notably tied to damping-off disease in nursery seedlings, *P. dissotocum* (9.1% relative abundance), *P. irregulare* (11.1% relative abundance), *P. rostratifingens* (11.2% relative abundance), and *P. ultimum var ultimum* (5% relative abundance) were each widespread across the dataset. The *Phytophthora* genus only represents 1.3% relative abundance in the dataset.

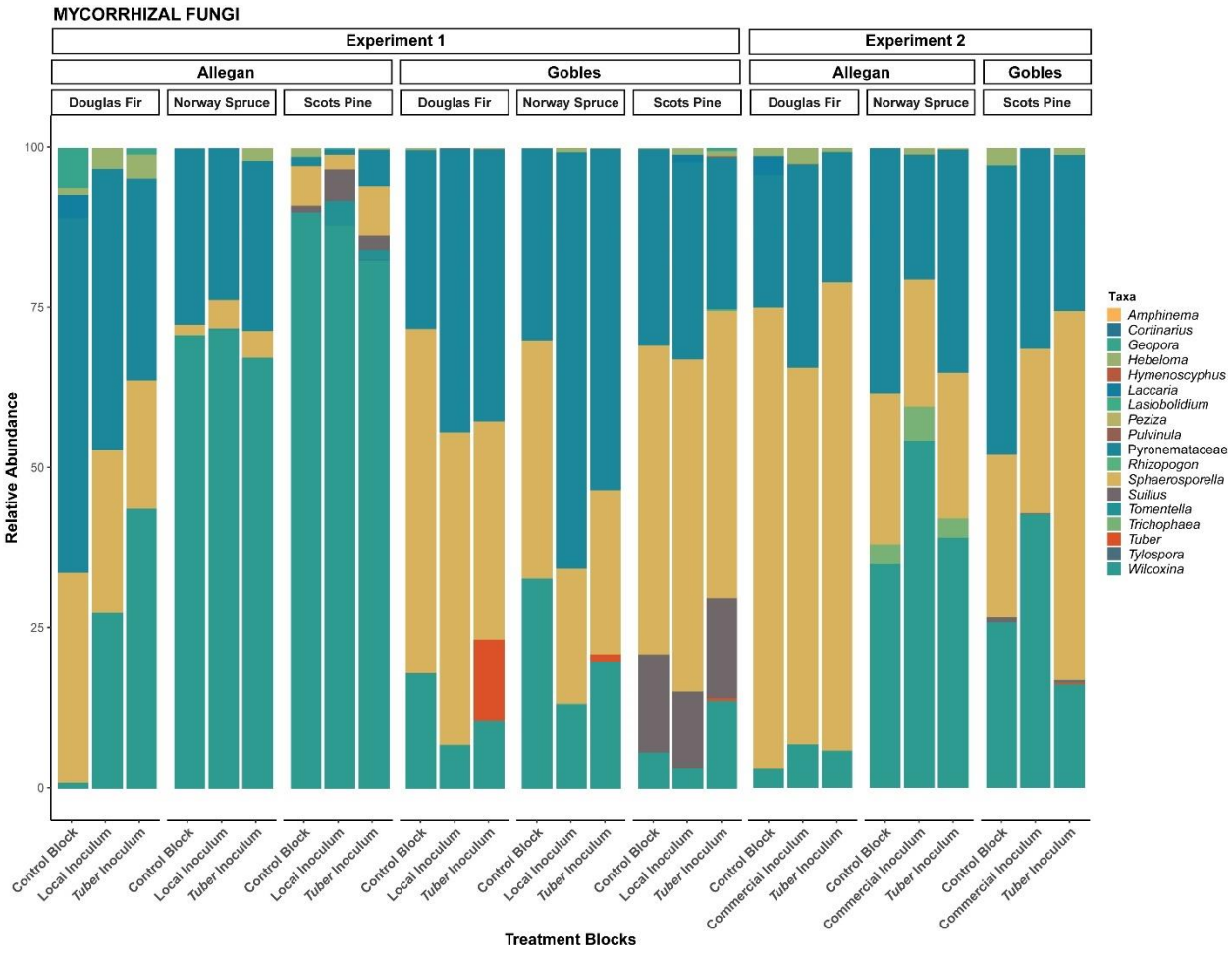

FIGURE 3.1 – MYCORRHIZAL FUNGAL TAXA STACKED BAR PLOT. Mycorrhizal fungi in roots of *Picea abies* (L.) H.Karst. (Norway Spruce), *Pinus sylvestris* L. (Scots pine), and *Pseudotsuga menziesii* (Mirb.) Franco (Douglas-fir) after one year of growth in soils inoculated with either a commercial mycorrhizal blend (MycoApply®), local inocula (*Laccaria* Berk. & Broome and *Scleroderma* Pers.), *Tuber* P. Micheli inocula (*T. aestivum* Vittad., *T. borchii* Vittad., and *T. indicum* Cooke & Massee), or no inocula (control). Experiment 1 at Gobles has the strongest *Tuber borchii* reads at 12.7% relative abundance within *Tuber* inocula blocks in *P. menziesii* across both experiments. *Suillus* species (14.3% relative abundance) are well represented across the roots of *P. sylvestris* at Gobles during the first experiment. Nearly all tree

FIGURE 3.1 (cont'd) – species were largely dominated by *Wilcoxina* Chin S. Yang & Korf, *Sphaerosporella* (Svrček) Svrček & Kubička, and unclassified Pyronemataceae taxa.

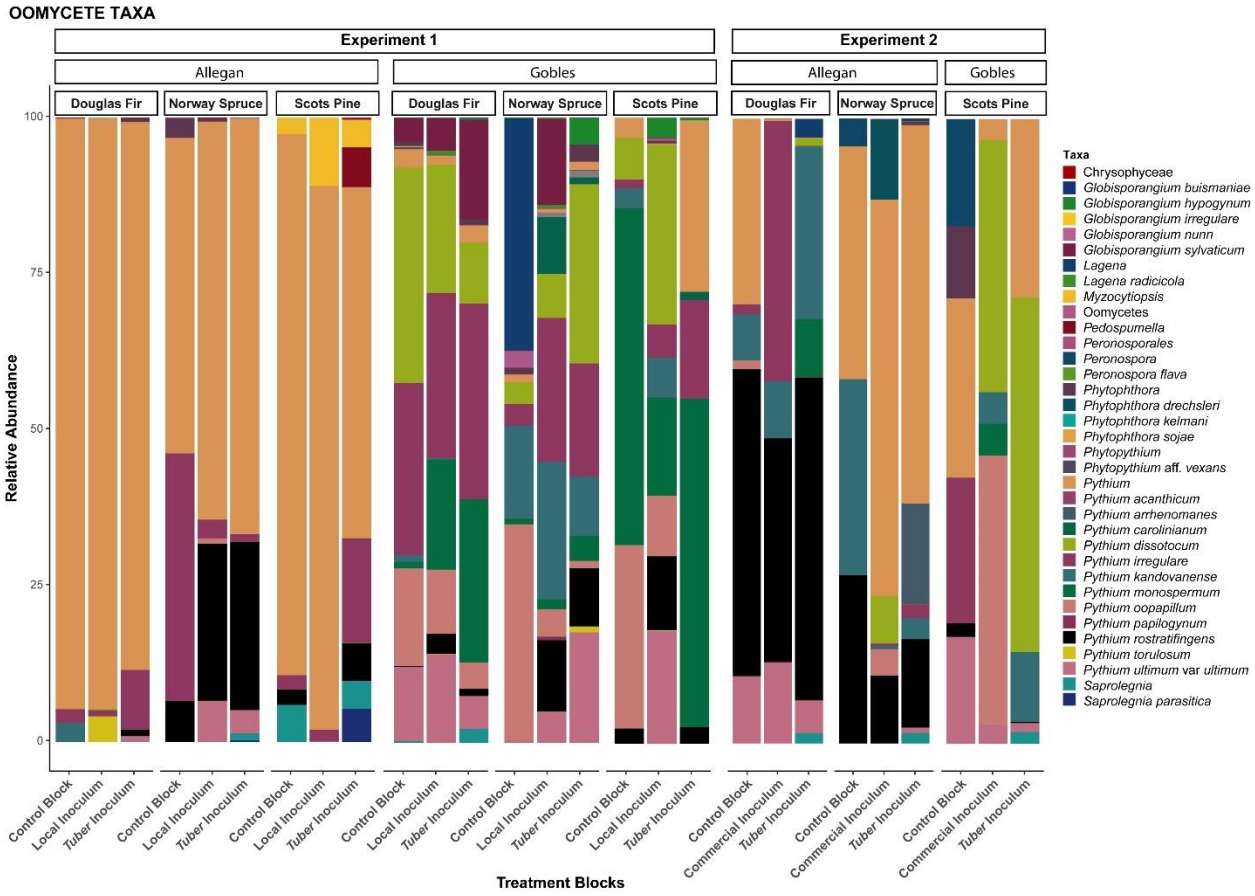

FIGURE 3.2 – OOMYCETE TAXA STACKED BAR PLOTS. Oomycete taxa in roots of *Picea abies* (L.) H.Karst., *Pinus sylvestris* L., and *Pseudotsuga menziesii* (Mirb.) Franco after one year of growth in soils inoculated with either a commercial mycorrhizal blend (MycoApply®), local inocula (*Laccaria* Berk. & Broome and *Scleroderma* Pers.), *Tuber* P. Micheli inocula (*T. aestivum* Vittad., *T. borchii* Vittad., and *T. indicum* Cooke & Massee), or no inocula (control). *Pythium dissotocum* Drechsler, *P. irregulare* Buisman, *P. rostratifingens* De Cock & Lévesque, and *P. ultimum var ultimum* Trow are notable pathogens linked to damping-off disease and comprise a significant portion of all reads in this dataset.

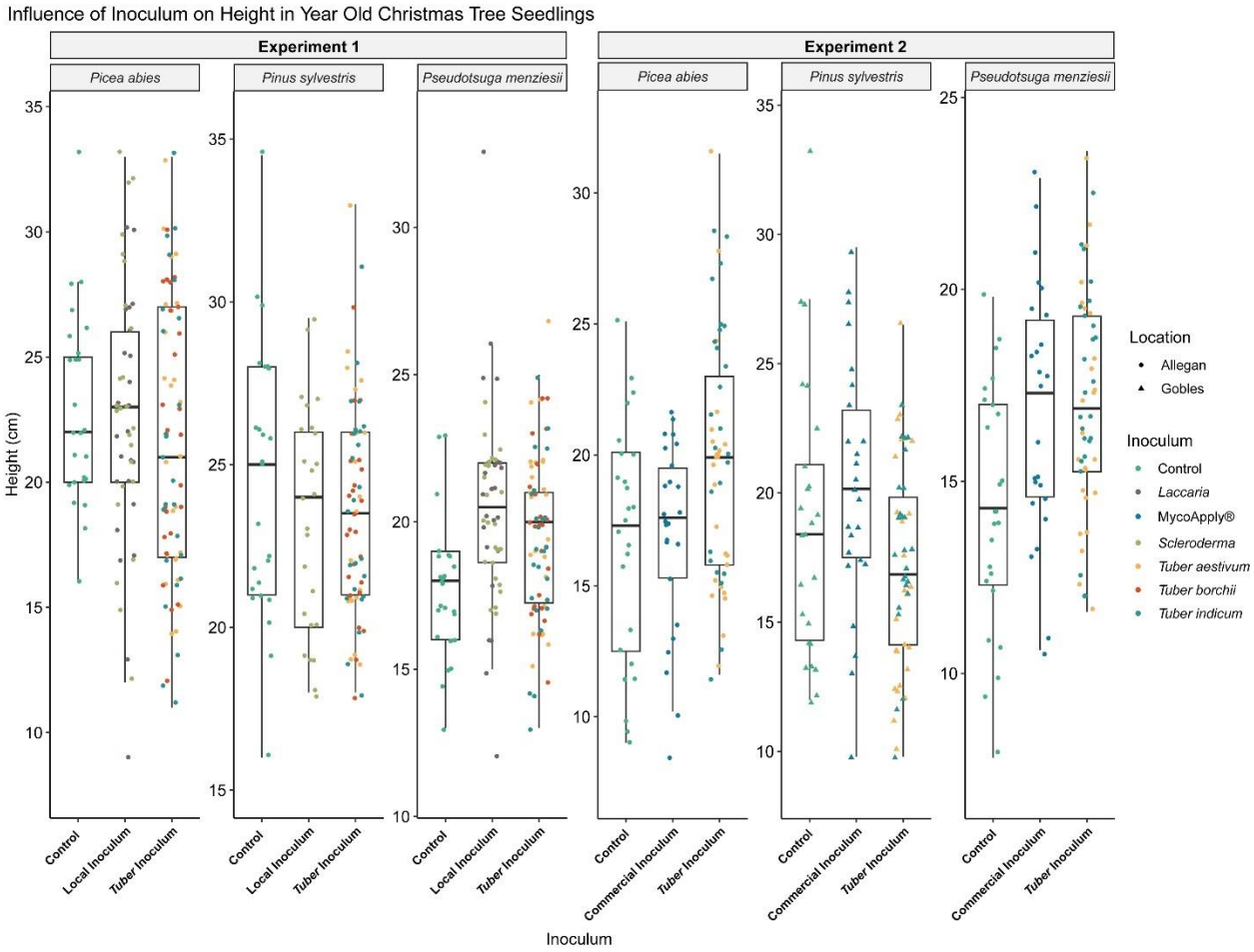

FIGURE 3.3 – INFLUENCE OF INOCULUM ON HEIGHT. *Picea abies* (L.) H.Karst., *Pinus sylvestris* L., and *Pseudotsuga menziesii* (Mirb.) Franco height after one year of growth in soils inoculated with either a commercial mycorrhizal blend (MycoApply®, local inocula (*Laccaria* Berk. & Broome and *Scleroderma* Pers.), *Tuber* P. Micheli inocula (*T. aestivum* Vittad., *T. borchii* Vittad., and *T. indicum* Cooke & Massee), or no inocula (control). There is no significant difference in height among trees grown in control blocks (no inoculation) and those grown with any of the inocula.

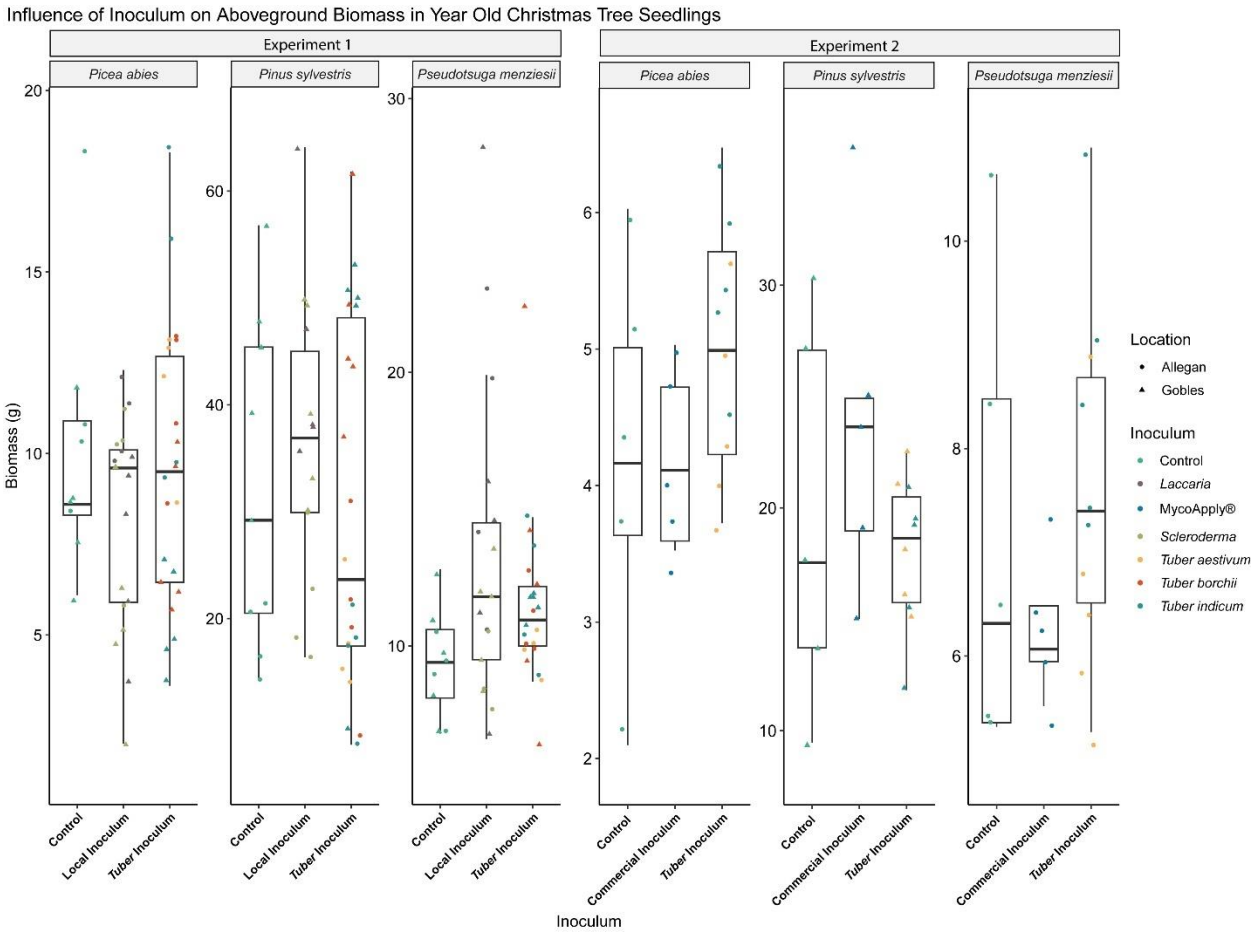

FIGURE 3.4 – INFLUENCE OF INOCULUM ON BIOMASS. *Picea abies* (L.) H.Karst., *Pinus sylvestris* L., and *Pseudotsuga menziesii* (Mirb.) Franco biomass after one year of growth in soils inoculated with either a commercial mycorrhizal blend (MycoApply®, local inocula (*Laccaria* Berk. & Broome and *Scleroderma* Pers.), *Tuber* P. Micheli inocula (*T. aestivum* Vittad., *T. borchii* Vittad., and *T. indicum* Cooke & Massee), or no inocula (control). There is no significant difference in biomass among trees grown in control blocks (no inoculation) and those grown with any of the inocula.

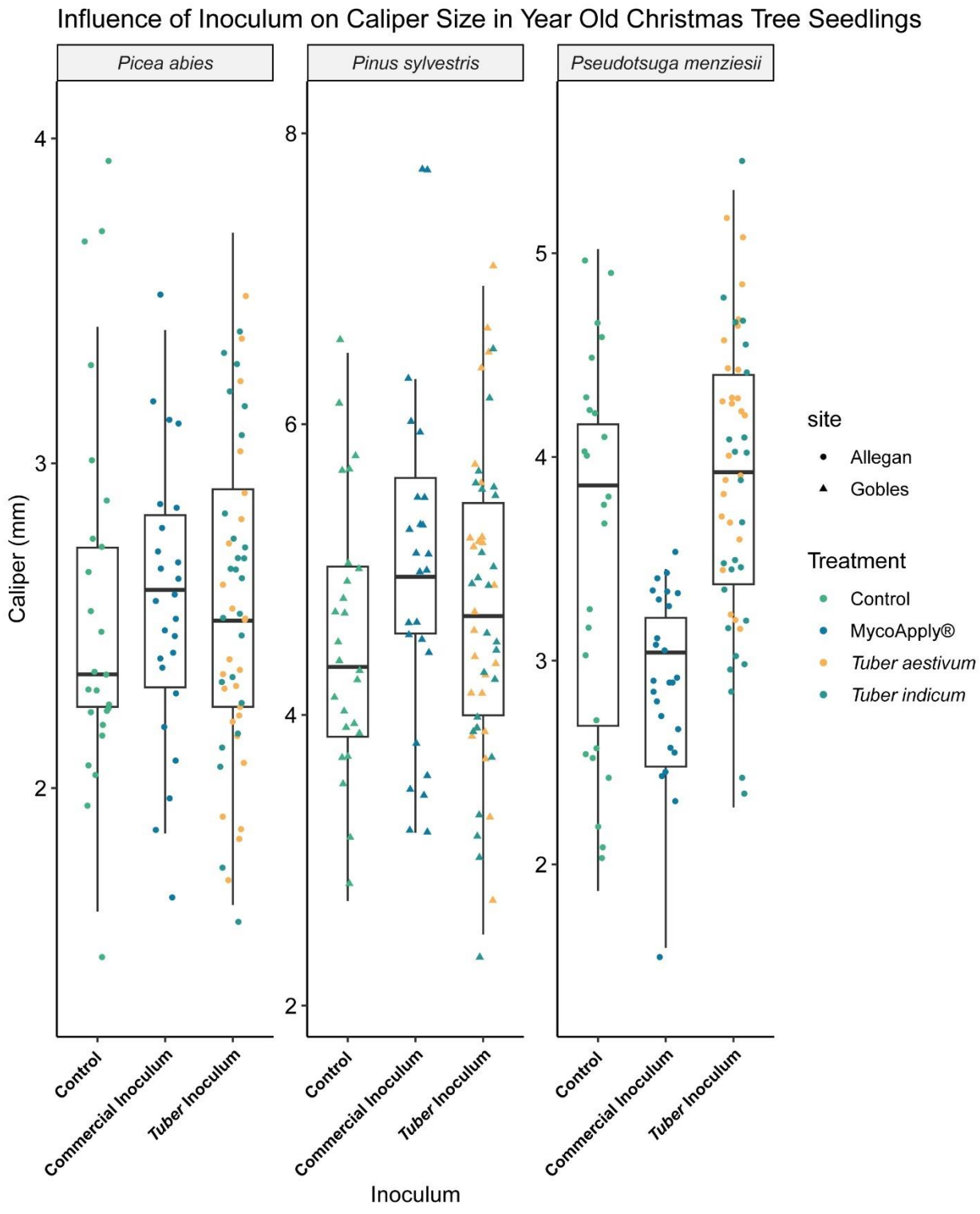

FIGURE 3.5 – INFLUENCE OF INOCULUM ON ROOT COLLAR DIAMETER. *Picea abies* (L.) H.Karst., *Pinus sylvestris* L., and *Pseudotsuga menziesii* (Mirb.) Franco root collar

FIGURE 3.5 (cont'd) – diameter after one year of growth in soils inoculated with either a commercial mycorrhizal blend (MycoApply®), *Tuber* P. Micheli inocula *(T. borchii* Vittad., and *T. indicum* Cooke & Massee), or no inocula (control). There is no significant difference in root collar diameter among trees grown in control blocks (no inoculation) and those grown with any of the inocula.

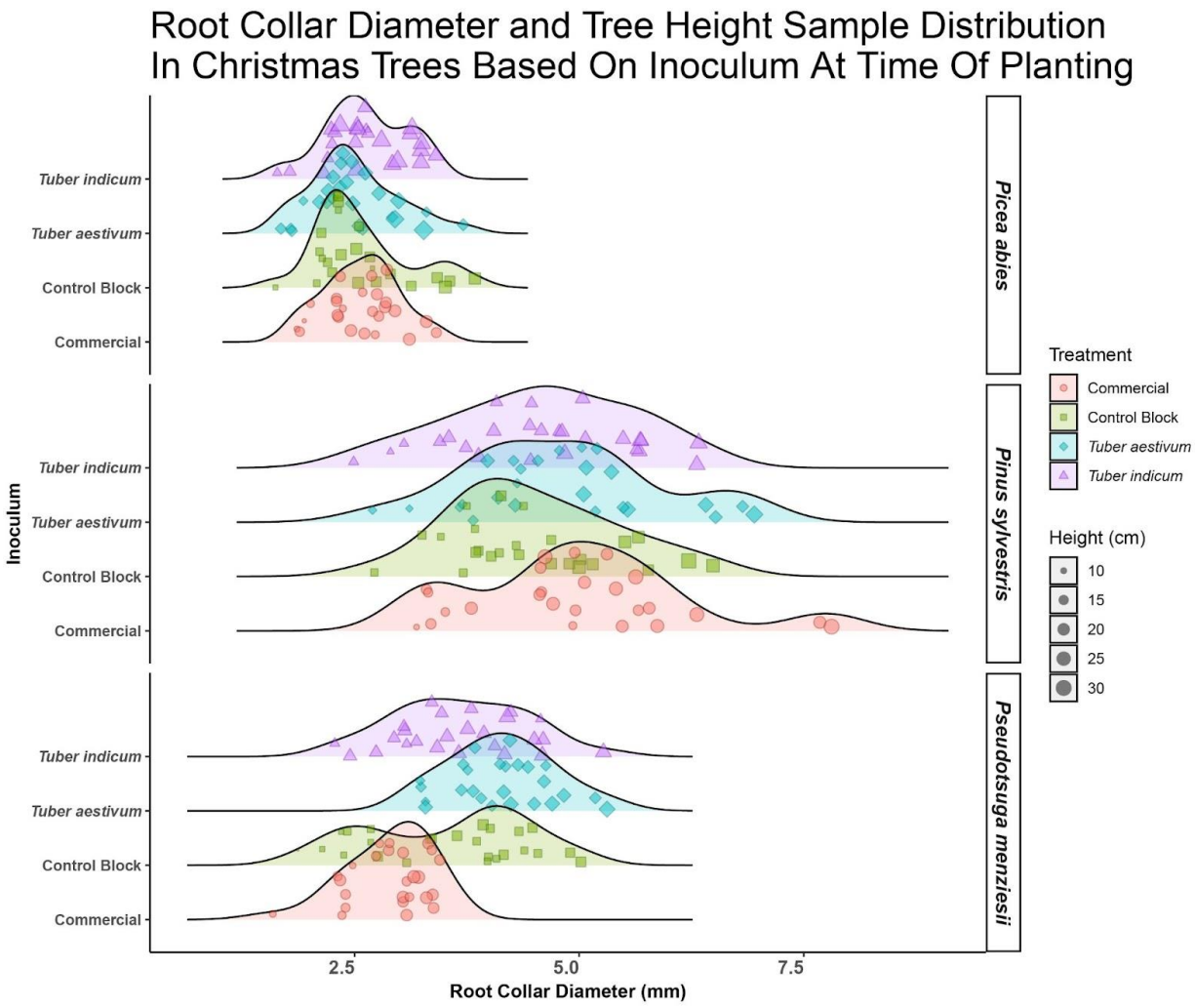

FIGURE 3.6 – ROOT COLLAR DIAMETER AND TREE HEIGHT SAMPLE DISTRIBUTION. Distribution of root collar diameters of *Picea abies* (L.) H.Karst., *Pinus sylvestris* L., and *Pseudotsuga menziesii* (Mirb.) Franco grown in soils inoculated with either a commercial mycorrhizal blend (MycoApply®), *Tuber* P. Micheli inocula (*T. borchii* Vittad., and *T. indicum* Cooke & Massee), or no inocula (control), or in soils that received no inoculation.

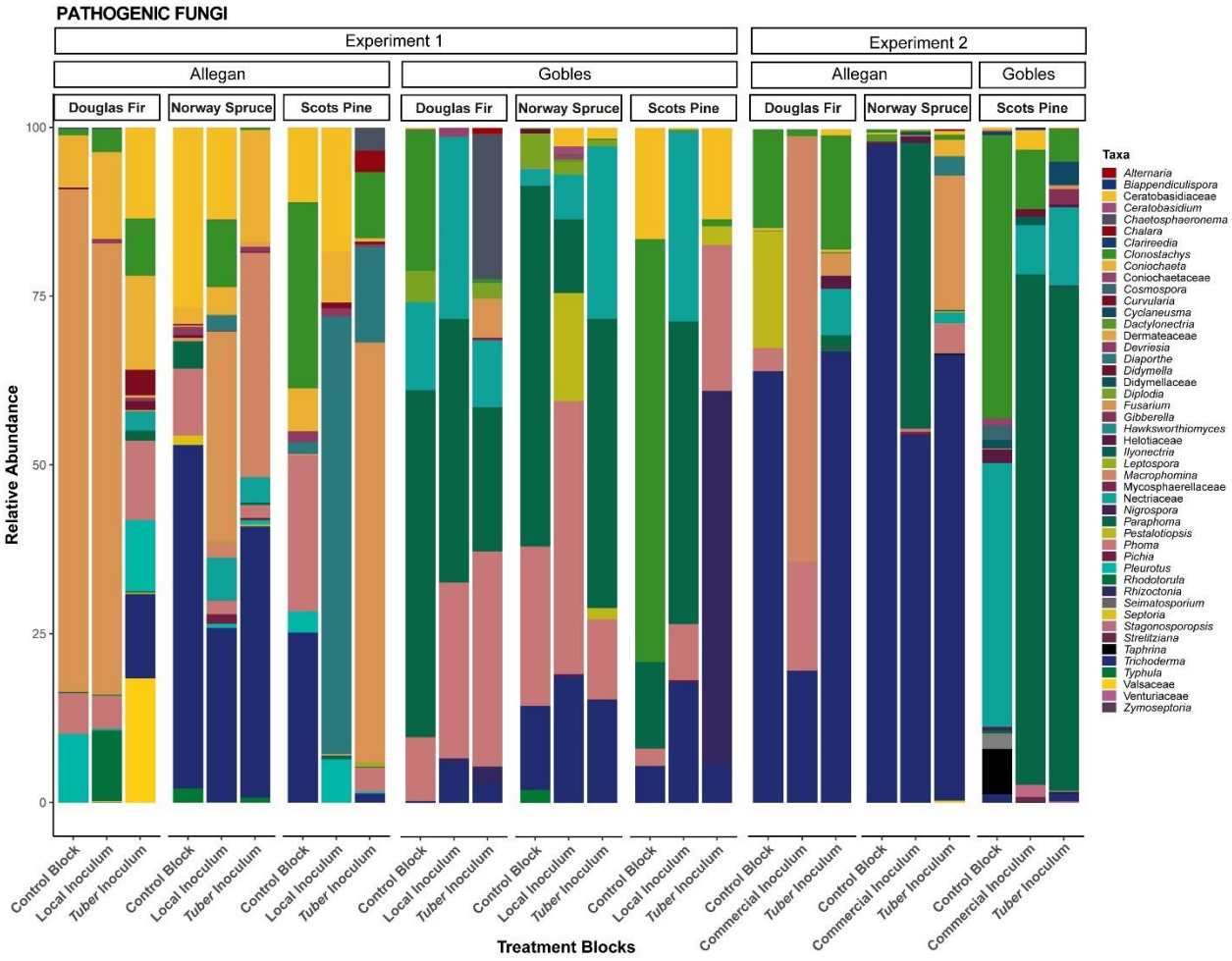

FIGURE 3.7 – PATHOGENIC FUNGAL TAXA STACKED BAR PLOT. Pathogenic fungi in roots of *Picea abies* (L.) H.Karst., *Pinus sylvestris* L., and *Pseudotsuga menziesii* (Mirb.) Franco after one year of growth in soils inoculated with either a commercial mycorrhizal blend (MycoApply®), local inocula (*Laccaria* Berk. & Broome and *Scleroderma* Pers.), *Tuber* P. Micheli inocula (*T. aestivum* Vittad., *T. borchii* Vittad., and *T. indicum* Cooke & Massee), or no inocula (control). Experiment 1 Allegan is dominated by *Fusarium* Link, *Trichoderma* Pers., *Diaporthe* Fr., *Clonostachys* Corda, and a Ceratobasidiaceae G.W. Martin operational taxonomic unit, whereas Gobles was dominated by *Paraphoma* Morgan-Jones & J.F. White, *Phoma* Sacc., Nectriaceae (Fr.) Fr., *Diplodia* Fr., and *Rhizoctonia* DC. Experiment 2 Allegan trees were dominated by *Trichoderma* Pers., *Macrophomina* Petr., and *Paraphoma* Morgan-Jones & J.F.

FIGURE 3.7 (cont'd) – White, whereas Gobles was dominated by *Nectria* (Fr.) Fr., *Clonostachys* Corda, and similarly, *Paraphoma.*

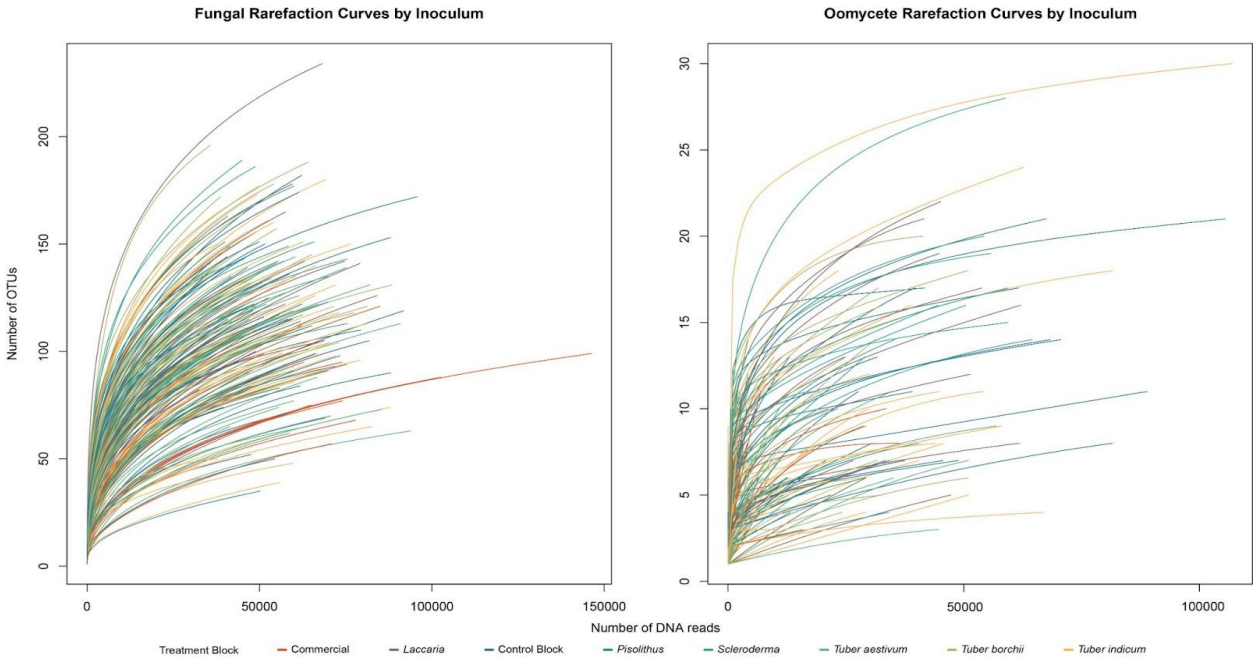

FIGURE 3.8 – FUNGAL AND OOMYCETE RAREFACTION CURVES.

Table 3.1 – Pesticide application rates.

Supplemental Table 1. Pesticide applications and rates

Experiment	Site	Date of application	Pesticide Name	Application Rate
1	A	7-Sep-2017	Tri Brom 80-20	240 lbs / acre
1	A	19-May-2018	Goal 2XL Herbicide	2 pints / acre
1	A	13-Oct-2018	Goal 2XL Herbicide	2 pints / acre
1	A	21-Apr-2019	Goal 2XL Herbicide	2 pints / acre
1	A	17-Jun-2019	Imidicloropid Insecticide	1 pint / acre
1	A	24-Jun-2019	Bravo Fungicide	1 quart / acre
1	B	10-Sep-2017	Tri Brom 80-20	240 lbs / acre
1	B	26-May-2018	Goal 2XL Herbicide	4 pints / acre
1	B	27-Jun-2018	Goal 2XL Herbicide	2 pints / acre
1	B	15-Jun-2018	Captan Fungicide	4 lb / acre
1	B	25-Jun-2018	Topsin M	1 quart / acre
1	B	5-Jul-2018	Bravo Weather Stik	1 quart / acre
1	B	16-Jul-2018	Topsin M	1 quart / acre
1	B	26-Jul-2018	Bravo Weather Stik	1 quart / acre
1	B	30-Aug-2018	Topsin M	1 quart / acre
1	B	9-Aug-2018	Bravo Weather Stik	1 quart / acre
1	B	20-Aug-2018	Topsin M	1 quart / acre
1	B	30-Aug-2018	Bravo Weather Stik	1 quart / acre
1	B	11-Sep-2018	Topsin M	1 quart / acre
1	B	21-Sep-2018	Bravo Weather Stik	1 quart / acre
1	B	1-Oct-2018	Topsin M	1 quart / acre
1	B	11-Oct-2018	Bravo Weather Stik	1 quart / acre
1	B	22-Oct-2018	Topsin M	1 quart / acre
1	B	1-Nov-2018	Bravo Weather Stik	1 quart / acre
2	A	8-Sep-2018	Tri Brom 80-20	240 lbs / acre
2	A	24-May-2019	Goal 2XL Herbicide	2 pints / acre
2	A	30-Jul-2019	Fusilade Herbicide	1 pint / acre
2	A	18-Oct-2019	Goal 2XL Herbicide	2 pints / acre
2	A	17-Apr-2020	Goal 2XL Herbicide	2 pints / acre
2	A	16-Jun-2020	Imidicloropid Insecticide	1 pint / acre
2	A	6-Jul-2020	Fusilade Herbicide	1 pint / acre
2	B	11-Sep-2020	Tri Brom 80-20	240 lbs / acre
2	B	28-May-2019	Goal 2XL Herbicide	4 pints / acre
2	B	29-Jun-2019	Goal 2XL Herbicide	2 pints / acre
2	B	17-Jun-2019	Captan Fungicide	4 lb / acre
1, 2	B	27-Jun-2019	Topsin M	1 quart / acre
1, 2	B	7-Jul-2019	Bravo Weather Stik	1 quart / acre
1, 2	B	18-Jul-2019	Topsin M	1 quart / acre
1, 2	B	28-Jul-2019	Bravo Weather Stik	1 quart / acre
1, 2	B	2-Sep-2019	Topsin M	1 quart / acre
1, 2	B	11-Aug-2019	Bravo Weather Stik	1 quart / acre
1, 2	B	22-Aug-2019	Topsin M	1 quart / acre
2	B	2-Sep-2019	Bravo Weather Stik	1 quart / acre
2	B	13-Sep-2019	Topsin M	1 quart / acre
2	B	23-Sep-2019	Bravo Weather Stik	1 quart / acre
2	B	3-Oct-2019	Topsin M	1 quart / acre
2	B	13-Oct-2019	Bravo Weather Stik	1 quart / acre
2	B	25-Oct-2019	Topsin M	1 quart / acre
2	B	3-Nov-2019	Bravo Weather Stik	1 quart / acre
2	B	26-Jun-2020	Topsin M	1 quart / acre
2	B	6-Jul-2020	Bravo Weather Stik	1 quart / acre
2	B	17-Jul-2020	Topsin M	1 quart / acre
2	B	27-Jul-2020	Bravo Weather Stik	1 quart / acre
2	B	1-Sep-2020	Topsin M	1 quart / acre
2	B	10-Aug-2020	Bravo Weather Stik	1 quart / acre
2	B	21-Aug-2020	Topsin M	1 quart / acre

Table 3.2 – Inoculum application rates.

Supplemental Table 2. Inoculum application rates at two locations over two separate experiments.

Experiment 1						
	Site A			Site B		
Inoculum	Picea abies	Pseudotsuga menziesii	Pinus sylvestris	Picea abies	Pseudotsuga menziesii	Pinus sylvestris
Tuber aestivum	20.0	20.0	20.0	N/A	N/A	N/A
Tuber borchii	20.0	20.0	20.0	20.0	20.0	20.0
Tuber indicum	20.0	20.0	20.0	20.0	20.0	20.0
Scleroderma	10.0	10.0	10.0	10.0	10.0	10.0
Laccaria	10.0	10.0	N/A	10.0	10.0	10.0
Negative Control	0.0	0.0	0.0	0.0	0.0	0.0

Experiment 2			
	Site A		Site B
	Picea abies	Pseudotsuga menziesii	Pinus sylvestris
Tuber aestivum	20.0	20.0	20.0
Tuber indicum	20.0	20.0	20.0
MycoApply®	12.0	12.0	12.0
Negative Control	0.0	0.0	0.0

Note: All application rates are in grams per meter

Table 3.3 – Experiment 1 inoculation block design.

Supplemental Table 3. Experiment 1 Inoculation Block Design

Site A			Site B		
Picea abies	*Pinus sylvestris*	*Pseudotsuga menziesii*	*Picea abies*	*Pinus sylvestris*	*Pseudotsuga menziesii*
Tuber indicum	Laccaria bicolor	Tuber indicum	Tuber indicum	Control	Control
Scleroderma citrinum	Tuber indicum	Tuber aestivum	Tuber indicum	Control	Tuber borchii
Tuber aestivum	Tuber borchii	Control	Tuber indicum	Control	Tuber borchii
Control	Scleroderma citrinum	Tuber borchii	Tuber indicum	Control	Tuber borchii
Tuber borchii	Tuber aestivum	Control	Tuber indicum	Control	Tuber borchii
Laccaria bicolor	Laccaria bicolor	Scleroderma citrinum	Tuber borchii	Laccaria bicolor	Tuber borchii
Laccaria bicolor	Control	Tuber aestivum	Tuber borchii	Laccaria bicolor	Control
Control	Laccaria bicolor	Tuber borchii	Tuber borchii	Laccaria bicolor	Tuber indicum
Tuber borchii	Control	Tuber indicum	Tuber borchii	Laccaria bicolor	Tuber indicum
Scleroderma citrinum	Scleroderma citrinum	Tuber borchii	Tuber borchii	Laccaria bicolor	Tuber indicum
Tuber aestivum	Tuber aestivum	Tuber indicum	Scleroderma citrinum	Scleroderma citrinum	Tuber indicum
Tuber indicum	Tuber borchii	Scleroderma citrinum	Scleroderma citrinum	Scleroderma citrinum	Tuber indicum
Control	Tuber indicum	Control	Scleroderma citrinum	Scleroderma citrinum	Control
Laccaria bicolor	Control	Tuber aestivum	Scleroderma citrinum	Scleroderma citrinum	Laccaria bicolor
Tuber aestivum	Scleroderma citrinum	Control	Scleroderma citrinum	Scleroderma citrinum	Laccaria bicolor
Tuber indicum	Tuber indicum	Scleroderma citrinum	Laccaria bicolor	Tuber borchii	Laccaria bicolor
Scleroderma citrinum	Tuber borchii	Tuber aestivum	Laccaria bicolor	Tuber borchii	Laccaria bicolor
Laccaria bicolor	Laccaria bicolor	Tuber indicum	Laccaria bicolor	Tuber borchii	Laccaria bicolor
Tuber indicum	Tuber aestivum	Tuber borchii	Laccaria bicolor	Tuber borchii	Control
Control	Laccaria bicolor	Tuber borchii	Laccaria bicolor	Tuber borchii	Scleroderma citrinum
Scleroderma citrinum	Tuber borchii	Tuber aestivum	Control	Tuber aestivum	Scleroderma citrinum
Tuber borchii	Control	Scleroderma citrinum	Control	Tuber aestivum	Scleroderma citrinum
Tuber aestivum	Tuber indicum	Scleroderma citrinum	Control	Tuber aestivum	Scleroderma citrinum
Control	Scleroderma citrinum	Tuber indicum	Control	Tuber aestivum	Scleroderma citrinum
Laccaria bicolor	Tuber aestivum	Control	Control	Tuber aestivum	Control
Tuber aestivum	Control				
Tuber borchii	Scleroderma citrinum				
Scleroderma citrinum	Tuber borchii				
Tuber indicum	Tuber aestivum				
Tuber borchii	Tuber indicum				

Notes: All of site B and *P. abies* at site A maintained a minumum of a 1.0 m gap between treatment blocks. *P. sylvestris* and *P. menziesii* at site A maintained a minumum of a 0.2 m gap between treatments.

Table 3.4 – Experiment 2 inoculation block design.

Supplemental Table 4. Experiment 2 Inouclation Block Design

Site A		Site B
Picea abies	*Pseudotsuga menziesii*	*Pinus sylvestris*
2.0	2.0	2.0
Control	Control	Control
Tuber aestivum	Tuber indicum	Tuber indicum
Tuber aestivum	Tuber indicum	Tuber indicum
Tuber aestivum	Tuber indicum	Tuber indicum
Tuber aestivum	Tuber indicum	Tuber indicum
Tuber aestivum	Tuber indicum	Tuber indicum
Control	Control	Control
Tuber indicum	Tuber aestivum	Tuber aestivum
Tuber indicum	Tuber aestivum	Tuber aestivum
Tuber indicum	Tuber aestivum	Tuber aestivum
Tuber indicum	Tuber aestivum	Tuber aestivum
Tuber indicum	Tuber aestivum	Tuber aestivum
Control	Control	Control
MycoApply®	MycoApply®	MycoApply®
MycoApply®	MycoApply®	MycoApply®
MycoApply®	MycoApply®	MycoApply®
MycoApply®	MycoApply®	MycoApply®
MycoApply®	MycoApply®	MycoApply®
Control	Control	Control
Control	Control	Control

Note: A minumum of 2.0 meters between treatment blocks was maintainted

Discussion

This study was designed to assess whether mycorrhizal inoculum is effective or necessary in bare-root conifer tree nurseries that manage seedling beds with methyl bromide and chloropicrin. Our field experiments were carried out during two growing seasons and indicate that in-field ectomycorrhizal inoculum supplements are not effective or necessary. Despite the use of local, exotic, or commercial inocula, there were no significant increases in the growth or health of trees observed in this study. While there may be cropping systems that benefit from these sorts of inputs, the robustness of the mycorrhizae either persisting through fumigation or blowing in from nearby tree stands may be all that is needed to meet the requirements of these tree species. However, it is likely that wind dispersed spores are the primary source as one study found *Glomus intraradices* spores were no longer viable after fumigation by methyl bromide (Bendavid-Val et al., 1997). Yet, more work is needed to determine the effects of methyl bromide on spores of ectomycorrhizal fungal species.

Fungal community

In fact, native infield inoculum is sufficiently high enough that it displaces most of the ectomycorrhizal spore inoculum provided in our treatments. Certain ectomycorrhizal groups including *Suillus* were particularly pervasive (Fig 3.1). *Suillus* is strongly associated with *Pseudotsuga, Pinus,* and *Abies* (Smith & Thiers, 1964) it is therefore not surprising that the locations this study took place within would be inundated with spores blowing in from nearby conifer tree stands. Additionally, there is some evidence that suggests certain ectomycorrhizal species such as *Laccaria laccata* (Scop.) Cooke produce methyl bromide raising questions about natural tolerance to this compound when used as a fumigant (Redeker et al., 2004). Potential production and tolerances to methyl bromide by ectomycorrhizal fungi have not been widely

studied and may yield fruitful insights given future research. The wide diversity of ectomycorrhizal species found on the roots within these nurseries also suggests that interest in applying inoculants may not be necessary or beneficial. There were differences in fungal community composition among treatments, however, these differences were not reflected in growth metrics or overall tree health, hinting at the importance of robust and diverse fungal communities.

Oomycete community

The other community investigated in this study, oomycetes, are among the most concerning pathogen groups within our dataset. Unlike mushrooms, oomycetes produce motile spores that travel in water and not in the air. Oomycete spores spread largely through irrigation and splashing. Methyl bromide application typically reaches 20 to 25 cm in depth, however, soil composition, tilth, moisture, and temperature each factor into the efficacy and penetration of this fumigant (Dunlap, 2009). Some *Pythium* species, such *P. splendens* Hans Braun are most abundant at soil depth of 15 to 30 cm, others have been recorded at depths of up to 355 cm (Plaats-Niterink, 1981). Our analysis of the oomycetes found *Pythium ultimum* var. *ultimum* and *Pythium irregulare*, two of many concerning pathogens amongst Pinaceae (Kozlowski & Métraux, 1998; Chavarriaga et al., 2007; Weiland et al., 2013). While methyl bromide may be effective at controlling *Pythium* populations near the surface at the time of application, deeper populations combined with irrigation or other mass water flow events can easily return species to the fumigated zone.

Persistence of introduced taxa

Our work here demonstrates that the fungal community dynamics of these fumigated bare-root nurseries are not conducive to addition of inoculants at the time of planting. By using

exotic truffle inocula easily discernible from local mycorrhizal species, we aimed to have both an easily identifiable mycorrhizal morphology for visual inspection and a unique genetic sequence for identification within the MiSeq library. Though the visual inspection did not reveal any *Tuber* mycorrhizae, we did uncover *Tuber borchii* in the MiSeq library. While this does indicate the presence of the exotic inocula both establishing and persisting on trees grown in methyl bromide fumigated soils, the relative abundance was rather low. One of the goals of this work was to investigate priority effects in a real-world dynamic system, and the persistence *Tuber borchii* indicates that introduced fungi can persist even if relatively rare. This is not very surprising given what is known about how well fungi have adapted spore dispersal.

There are many challenges to calculating spore dispersal among mushrooms (Dam, 2013), however, there is sufficient evidence that conducive conditions may allow spores to travel hundreds of kilometers in the wind (Viljanen-Rollinson et al., 2007). Open-field bare-root nurseries surrounded by mature trees of the same species being cultivated such as those utilized in this study are no doubt showered in an abundant siege of mutualistic fungal spores, but with the inflow of beneficial species, so too come pathogens even trans-continentally (Brown & Hovmøller, 2002).

Incomplete picture

Biases inherent to amplicon sequencing mean one must use caution when relating read counts to actual environmental abundance or diversity. The metabarcoding in this study was applied to surface-cleaned roots from living trees, however, this does not guarantee the amplicons belong to active living organisms. As an additional limitation, we know that every locus, primer set, and reference database have their own intrinsic biases as well so it was no surprise that the detection of *Tuber aestivum*, for example, did not occur as the primer set used

does not select for this species. Similarly, as made evident by the dominating *Pythium* OTU not identified to species level (FIGURE 3.3), there are yet many challenges faced when metabarcoding Oomycetes. A known limitation of the ITS6 and ITS7 primers often used in oomycete community amplification (Cooke et al., 2000), are their propensity to bind and amplify fungal and plant sequences (Coince et al., 2013). Additionally, these primers have been shown to preferentially amplify *Pythium* over *Aphanomyces* de Bary (Taheri et al., 2017) leading to unbalanced abundancies.

Summary

In summary, through looking at two bare-root nurseries over two cropping cycles and three conifer tree species (*Pseudotsuga menziesii, Picea abies,* and *Pinus sylvestris*), and using local (*Laccaria bicolor* or *Scleroderma citrinum*), exotic (*Tuber aestivum*, *Tuber borchii,* or *Tuber indicum*), or a commercial blend (MycoApply®) of mycorrhizal inoculants, we did not observe statistically significant reduction in disease or increase in tree height, root collar diameter, or biomass. We did not see the exotic mycorrhizae during a visual inspection but did find *T. borchii* in low abundance on the roots of *P. menziesii* during the first experiment. We also observed over 12 species of *Pythium* and four species of *Phytophthora* in the oomycete MiSeq library dataset we generated.

Conclusions

Based on the lack of disease reduction, the lack of growth increases, and the diversity of ectomycorrhizal fungi on the roots of *P. menziesii, P. abies,* and *P. sylvestris* in these bare-root nurseries, we cannot recommend the use of mycorrhizae inoculants. Should bare-root nurseries decide to continue using methyl bromide with chloropicrin, spores blowing in from fungi

growing in nearby tree stands will likely be sufficient to provide the health and growth benefits that mycorrhizal fungi like *Suillus*, *Sphaerosporella*, and *Wilcoxina* are known to provide.

Chapter 4

Tuber rugosum, a new species from Northeastern North America: Slug mycophagy aides in electron microscopy of ascospores

Source:

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Abstract

Species in the genus *Tuber* are ascomycetous fungi that produce hypogeous fruiting bodies commonly called truffles. These fungi are ecologically relevant owing to the ectomycorrhizal symbiosis they establish with plants. One of the most speciose lineages within *Tuber* is the Rufum clade, which is widely distributed throughout Asia, Europe, and North America and is estimated to include more than 43 species. Most species in this clade have spiny spores, and many still have not been formally described. Here, we describe *Tuber rugosum* based on multigene phylogenetic analysis and its unique morphological characters. *Tuber rugosum* (previously designated in literature as *Tuber* sp. 69) has been collected throughout the Midwest, USA, and Quebec, Canada, and is an ectomycorrhizal symbiont of *Quercus* trees, as confirmed through morphological and molecular analyses of root tips presented here. We also present a novel method for preparing *Tuber* ascospores for scanning electron microscope imaging that includes feeding, digestion, and spore excretion by the slug *Arion subfuscus*. Following this method, spores become free from ascus and other mycelial debris that could obscure morphological traits during their passage through the snail gut while maintaining ornamentation. Finally, we report the fatty acid analysis, a fungicolous species association, and we provide an updated taxonomic key of the Rufum clade.

Introduction

Although first described in 1780, truffles in the genus *Tuber* have a much longer history of being sought after due to their culinary value (Wang and Marcone, 2011), which can be attributed to their unique aroma (Martin et al., 2010). The white truffle *T. magnatum* Picco and the black truffle *T. melanosporum* Vittad, for example, are amongst the most well-known and highly prized fungal species with unique aromas (Pelusio et al., 1995). Beyond their aroma,

truffles are rich in carbohydrates, proteins, and unsaturated fatty acids (Bouatia et al., 2018; Yan et al., 2017), but it is still unknown how variable these traits are between *Tuber* species. In addition, the ecology and microbiology few *Tuber* species has been extensively studied (Splivallo et al., 2011), often linking fungivory by animals to the aromatic lure produced by a mature ascocarp (Hochberg et al., 2003; Maser et al., 2008).

Successful truffle spore dispersal relies on mycophagous animals detecting, consuming, and defecating mature sporocarps, as hypogeous fungi are not able to actively discharge their spores. Numerous studies have highlighted *Tuber* spores found in animal scat, including that of pika (Cázares and Trappe, 1994), northern flying squirrels (Gabel et al., 2010), crested porcupines (Ori et al., 2018), and wild boar (Piattoni et al., 2013). Passage of spores through animal digestive tracts such as the crested porcupine removes asci, may lead to some degradation of the ornamentation, and often will promote spore germination (Ori et al., 2018). Observations made while collecting specimens for this study indicated slugs, which are known to be mycophagous (Beyer and Saari, 1978; McGraw et al., 2002), regularly consume truffles and other fungi that grow beneath the leaf litter of the forest floor. These observations led to the question of whether slugs could be used as an alternative to chemical preparations (e.g., Puliga et al., 2020) to obtain clean ascospores for unobstructed and improved scanning electron imaging.

Further field observations of truffles as they matured in situ led to observations of fungal infections. In 2017, Leonardi et al. reported on fungi living within eight species of *Tuber* where they found 58.6% were infected by *Clonostachys rosea* (Link) Schroers, Samuels, Seifert & W. Gams (= *Bionectria ochroleuca* (Schwein.) Schroers & Samuels) (Leonardi et al., 2018). Other studies have indicated that yeasts including *Candida saitoana* Nakase & M. Suzuki, *Rhodotorula mucilaginosa* (A. Jörg.) F.C. Harrison, and *Trichosporon moniliiforme* E. Guého & M.T. Sm.

isolated from *Tuber melanosporum* and *Tuber magnatum* ascomata produce, and may contribute to, the characteristic aroma profile of the truffles in which they are found (Buzzini et al., 2005). Mycelial fungi such as *Trichopezizella nidulus* (J.C. Schmidt & Kunze) Raitv., *Absidia cylindrospora* Hagem, and *Peniophora cinerea* (Pers.) Cooke are also known to be associated with truffle fruiting bodies (Pacioni et al., 2007). Additionally, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Trichoderma* have been isolated from other truffle taxa, such as *Tuber aestivum* Vittad. and *Tuber melanosporum* ascomata (Rivera et al., 2010). Some mycoparasites are known to produce mycotoxins; thus, precautionary care should be taken to avoid consuming parasitized truffles. It has been suggested that truffles exposed on the surface of the soil are more prone to disease, although moisture levels in the environment likely play an equally important role in completing this disease triangle (Eslick, 2012). In concurrence with Eslick (2012), ascomata of the species that we describe here, *T. rugosum*, sp. nov., observed with disease was subhypogeous.

The genus *Tuber* contains over 200 species, with most species diversity residing within the Rufum, Puberulum, and Maculatum clades (Bonito et al., 2010; Healy et al., 2016). Many species within these clades have yet to be formally described (Bonito et al., 2010; Healy et al., 2016). Truffles in the Rufum clade can be distinguished from those in other lineages by their smooth to slightly verrucose pale to reddish-colored peridium, stemmed ascus, and the often spiny or spinose-reticulate ornamentation of their ascospores (Healy et al., 2016). Another unique facet of the Rufum clade is the absence of cystidia on the mycorrhizal mantle they form (Healy et al., 2016). Spore ornamentation, size, shape, and dimension remain the cornerstone morphological characteristics used to describe *Tuber* species.

From 2009 through 2021, we collected truffles with morphological characteristics of those in the Rufum clade. An internal transcribed spacer (ITS) meta-analysis of *Tuber* has provided a framework by which many new species have been described (Bonito et al., 2010). Sequences of the ITS region from our specimens matched with the sequence Bonito et al. (2010) designated in the literature as *Tuber* sp. 69 (GenBank HM485428), which we formally describe here as *Tuber rugosum*, sp. nov. In support of this new species to science, we provide (i) multigene phylogenies based on the ITS, the elongation factor 1α (EF1α), and the second-largest subunit of RNA polymerase II (RPB2) genes; (ii) a morphological comparison of its peridium, gleba, and spores; and (iii) a characterization of its fatty acid profile. Further, we describe an improved method for preparing spores for scanning electron microscopy (SEM) study, we identify a fungicolous species found on *T. rugosum*, sp. nov., and present a dichotomous key for the Rufum clade.

Materials and Methods

Collection and isolation

Truffles were collected with the aid of a hand-held four-pronged garden cultivator to remove leaf litter and explore within the upper 10 cm of forest soils. Photographs and field notes, including date, location, habitat, and fresh attributes, were made for each specimen. Specimens were stored at 4 C for a maximum of 24 h prior to morphological observations and pure culture isolation. Using forceps and sterile technique, small pieces of freshly exposed internal gleba hyphae from younger specimen were sampled and submerged into an agar medium composed of 8.0 g/L agar, 5.0 g/L potato dextrose broth, 1.5 g/L malt extract, 5.0 mL/L glycerol, and 0.82 g/L calcium nitrate. Prior to autoclaving, the pH was adjusted to 7.5 with 5.0 M sodium hydroxide. Once the postautoclave temperature fell below 50 C, 1.0 mL/L biotin (0.5 g/L stock), 1.0 mL/L

chloramphenicol (60.0 mg/mL stock), and 1.0 mL/L ampicillin (50.0 mg/mL stock) were added. After initial growth on antibiotic-containing medium, a subculture was made on the same medium lacking antibiotics. These cultures were incubated at room temperature (20–22 C).

Morphological analyses

Analysis of truffle micromorphological characters was conducted under a compound light microscope (Leica model DM750; Buffalo Grove, Illinois). Ascospores were collected by scraping a razor blade across the gleba and mounting the fungal tissue collected on the blade on a microscope slide with 3% KOH. In total, 85 spores from 33 asci were measured and imaged at 400× magnification against the long and short axes, excluding ornamentation (Leica Application Suite 4.0). Length, width, and Q (length:width) measurements of the spores were then calculated, as these metrics have been informative in distinguishing species of Oregon white truffles (Bonito et al., 2010).

One immature *Tuber* sp. 69 (RH999) ascocarp used for recording developmental characters was sectioned in quarters and fixed for 2 h (4 C) in 2% glutaraldehyde + 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2); rinsed three times in 0.1 M sodium cacodylate buffer for 20 min each; postfixed for 1 h (4 C) in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer; rinsed in fresh buffer followed by three changes of deionized water for 10 min each; dehydrated in a graded ethanol series (25%, 50%, 75%, 95%, and 100%, 3× for 1 h each); infiltrated in Spurr's resin (Spurr, 1969) and embedded in an aluminum dish; and polymerized for 2 days at 74 C. Sections 2 µm thick were cut with a glass knife and placed on a drop of water on a clean glass microscope slide on a warming tray. After drying, sections were stained with 0.5% toluidine blue O and preserved with a drop of per mount and a drop of xylene and a cover glass was placed on

top. Images were digitally captured on a Nikon Optiphot compound microscope (Tokyo, Japan) mounted with a QImaging MicroPublisher 3.3 RTV camera (British Columbia, Canada). Hollowed out ascocarps in close proximity to abundant slug populations of the dusky *Arion* slug, *Arion subfuscus*, were observed. To test whether the slugs would consume truffles, we placed a single slug in a plastic container with a fresh truffle sporocarp for two sessions each lasting 4 h prior to observation. Frass contents collected from slugs that had consumed truffles were observed with a compound light microscope (Leica model DM750, Buffalo Grove, Illinois) to assess ascospore morphology. To prepare ascospores for scanning electron microscopy (SEM), slugs were maintained in a plastic box for 24 h and frass was observed after the complete *T. rugosum*, sp. nov., ascocarp had been consumed (SUPPLEMENTARY FIG. 1). The *Arion* frass was collected and visualized under a compound microscope. The ascospore-containing frass was dried at room temperature and then rinsed with phosphate-buffered saline (PBS). Ascospores not subjected to slug digestion were collected by scraping a scalpel blade across dried and rehydrated gleba and rinsed with PBS. Both sets of samples were fixed in a 4% (v/v) glutaraldehyde solution, dried with a critical point dryer (Balzers model 010; Balzers Union), and then mounted on aluminum stubs using high-vacuum carbon tabs (SPI Supplies, West Chester, PA). After the samples were coated with osmium using NEOC-AT osmium coater (Meiwafosis, Osaka, Japa), the samples were observed using a JSM-7500 F scanning electron microscope (Japan Electron Optics Laboratories (JEOL) USA, Peabody, Massachusetts).

After primary character data collection commenced, specimens were cut into sections and dried with activated silica beads. Curated holotype and paratype collections have been deposited in the Michigan State University (MSU) Herbarium, with the MSU collection accession numbers MSC408482–MSC408486. These data have also been deposited into Mycobank MB838884.

Fungicolous species isolation

Tissue supporting orange conidia growing from infected specimens of *T. rugosum*, sp. nov., was photographed with a Canon EOS Rebel T6 camera (Canon Inc., Tokyo, Japan) with the Laowa 24 mm f/14 2× Macro Probe lens (Venus Optics, Hefei, China) in the field prior to further processing. Photographs were taken with a shallow depth of field and were imported, aligned, and blended using Adobe Photoshop (Adobe Inc, 2019b) for FIG. 3A. The fungal growth supporting the orange conidia was then placed in malt extract agar (MEA) medium composed of 10.0 g/L agar, 10.0 g/L malt extract, and 1.0 g/L yeast extract with 1.0 mL/L chloramphenicol (60.0 mg/mL stock), 1.0 mL/L streptomycin (100.0 mg/mL stock), and rifampicin (50.0 mg/mL stock). This isolate (BR428b) was incubated at room temperatures (20–22 C) and maintained on MEA with no antibiotics.

Confocal microscopy

Confocal microscopy was performed to visualize lipid droplets within the ascocarp. Samples were sliced with a surgical scalpel and stained with 10.0 μg mL−1 BODIPY 493/503 (Thermo Fisher Scientific, Pittsburgh, USA) in a phosphate-buffered saline (PBS) buffer for 2 days at 23 C. After two washes with a PBS buffer, the samples were then observed using an Olympus FV10i microscope (Olympus Scientific Solutions Americas, Waltham, Massachusetts). An argon (488 nm) laser was used for BODIPY (emission: 510–530 nm).

Lipid extraction and analysis

Mycelium was incubated on the agar medium described above until 40.0 mm (50% colonization of Petri dish) of growth from the inoculation point was reached. The total lipid fraction was extracted from the mycelium by placing methanol-chloroform-88% formic acid (1:2:0.1 by volume) in glass tubes, followed by a wash with half volume of 1.0 M KCl and 0.2 M

H3PO4. After phase separation by centrifugation (2000 × g for 3 min), total lipids were collected to prepare fatty acid methyl esters (FAMEs) with 1.0 M methanolic HCl at 80 C for 25 min. FAMEs were then extracted with hexane and analyzed by gas chromatography and flame ionization detection (Agilent, CA, USA).

Molecular analyses

DNA was extracted from all specimens and isolated using a rapid alkaline extraction method (Liber et al., 2022), as previously described. Ascocarp DNA was extracted by removing a small amount of the peridium and placing a 1.0-mm2 piece of sterile gleba into 40.0 µL extraction solution (ES). Colonized root tips were imaged, rinsed with deionized (DI) H2O, and placed into 20 µL ES and crushed using a pipette tip. Samples were then placed into a thermocycler set to 95 C for 10 min to lyse the cells. Following lysis, bovine serum albumin (BSA) was added at a rate of 3 times the volume of ES to help neutralize and suspend the DNA extraction. One microliter of the extracted DNA was used as template for subsequent polymerase chain reaction (PCR) amplification reactions.

Fungal rDNA was amplified with universal fungal primers ITS1F and LR3 (TABLE 1). *Tuber*-specific primers were used to amplify protein-coding genes, including the second-largest subunit of RNA polymerase II (RPB2_*Tuber*_f, RPB2_*Tuber*_r) and elongation factor 1α (EF1α_*Tuber*_f, EF1α_*Tuber*_r) (TABLE 1) following methods of Bonito et al. (Citation2010, 2013).

Table 4.1 - List of primers and sequences used in this study for phylogenetic analyses.

Primer Name	Sequence (5′ to 3′)	First Reported
ITS1-F	CTTGGTCATTTAGAGGAAGTAA	Gardes & Bruns, 1993
LR3	CCGTGTTTCAAGACGGG	Vilgalys & Hester, 1990
EF1α Tuber_f	AGCGTGAGCGTGGTATCAC	Bonito et al., 2013
EF1α Tuber_r	GAGACGTTCTTGACGTTGAAG	Bonito et al., 2013
RPB2 Tuber_f	YAAYCTGACYTTRGCYGTYAA	Bonito et al., 2013
RPB2 Tuber_r	CRGTTTCCTGYTCAATCTCA	Bonito et al., 2013

Amplicon products were Sanger sequenced bidirectionally at the Research Technology Support Facility (RTSF) Genomics Core at Michigan State University on the Applied Biosystems 3730XL capillary sequencer (Waltham, Massachusetts). Sequences were trimmed with SnapGene 4.3.7 to remove low-quality regions (GSL Biotech, Chicago, Illinois). Sequences were then compared with others in the National Center for Biotechnology Information (NCBI) database with the BLASTn algorithm to verify that they were *Tuber* and to identify other entries of this taxon in the database.

Phylogenetic analyses

Sequence alignments of taxa in the Rufum clade were made with the MUSCLE alignment algorithm (Edgar, 2004) within Mesquite (Maddison and Maddison, 2019). Sequence ends and highly ambiguous regions of ITS1 were excluded to eliminate ambiguous regions in the alignment. Aligned sequences were used to infer the phylogeny with maximum likelihood (ML) and Bayesian inference (BI). All ML searches were generated with Randomized Axelerated Maximum Likelihood (RAxML), and 1000 bootstrap replicates were carried out with the GTRGAMMA nucleotide substitution model on the CIPRES Science Gateway (Miller et al., 2010; Stamatakis, 2014). All BI searches were generated utilizing MrBayes on the CIPRES Science Gateway (Huelsenbeck and Ronquis, 2001; Ronquist and Huelsenbeck, 2003). The Markov chain Monte Carlo (MCMC) ran for 40 000 000 generations with the Metropolis-

coupled Markov Chain Monte Carlo (MCMCMC) set to run four chains in parallel, sampled every 1000 cycles, and had a burn-in rate of 25% for each BI search (Geyer, 1991). The model for among-site rate variation was set to INVGAMMA (inverse gamma distribution). Character sets for the ITS BI search were based on an alignment made to an annotated *Tuber brumale* Vittad. (GenBank AF106880) sequence extending from the 18S ribosomal RNA gene to the 28S ribosomal gene. The quality of the BI search was verified using MCMC files viewed with Tracer 1.7 to ensure parallel runs converge and to quantify the effective sample size (Rambaut et al., 2018). Visualization of the phylogenetic trees was performed using FigTree 1.4.4 (FigTree, 2018) updated to reflect the ML bootstrap support value and BI posterior probability within Adobe Illustrator (Adobe Inc, 2019a).

Dichotomous key

A dichotomous key of described species in the Rufum clade was generated based on available species descriptions used in TABLE 3 (Butters, 1903; Cao et al., 2011; Vittadini, 1831; Chen et al., 2005; Deng et al., 2009; Eberhart et al., 2020; Elliott et al., 2016; Lancellotti et al., 2016; Fan et al., 2012, 2013; Frank et al., 2006; Granetti et al., 1988; Grunow and Rabenhorst, 1884; Harkness, 1899; Hu and Wang, 2005; Leonardi et al., 2019; Suwannarach et al., 2016; Trappe et al., 1996; Uecker and Burdsall, 1977; Wang, 1988; Yan et al., 2018).

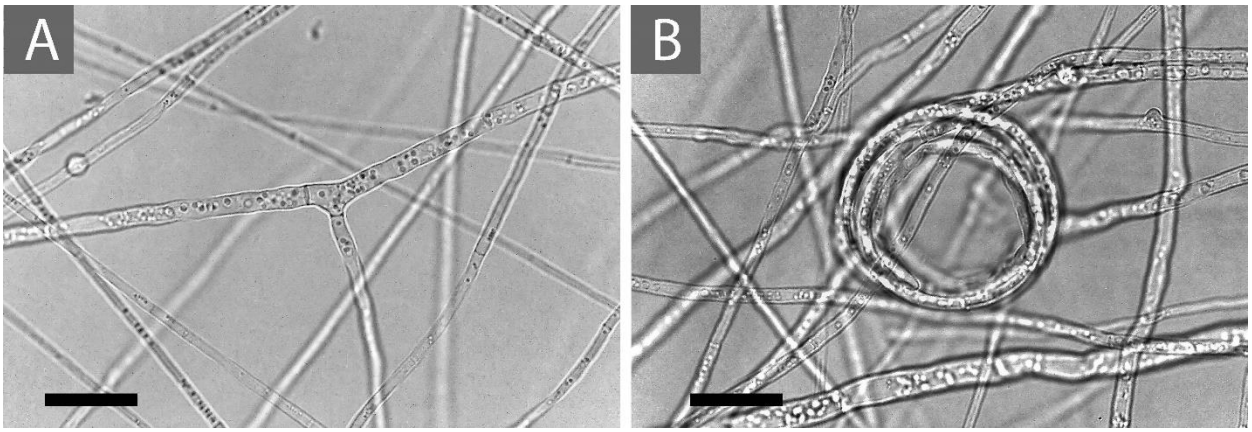

FIGURE 4.1 - *TUBER RUGOSUM* GROWTH IN VITRO. Growth shown on the pH 7.0 adjusted medium containing 1.0 mL/L biotin as described in Materials and Methods. A. A typical right-angled hyphal branch with simple septa. B. A hyphal coil that is seen regularly on surface growth in cultures as they age. Bars = 20.0 μm.

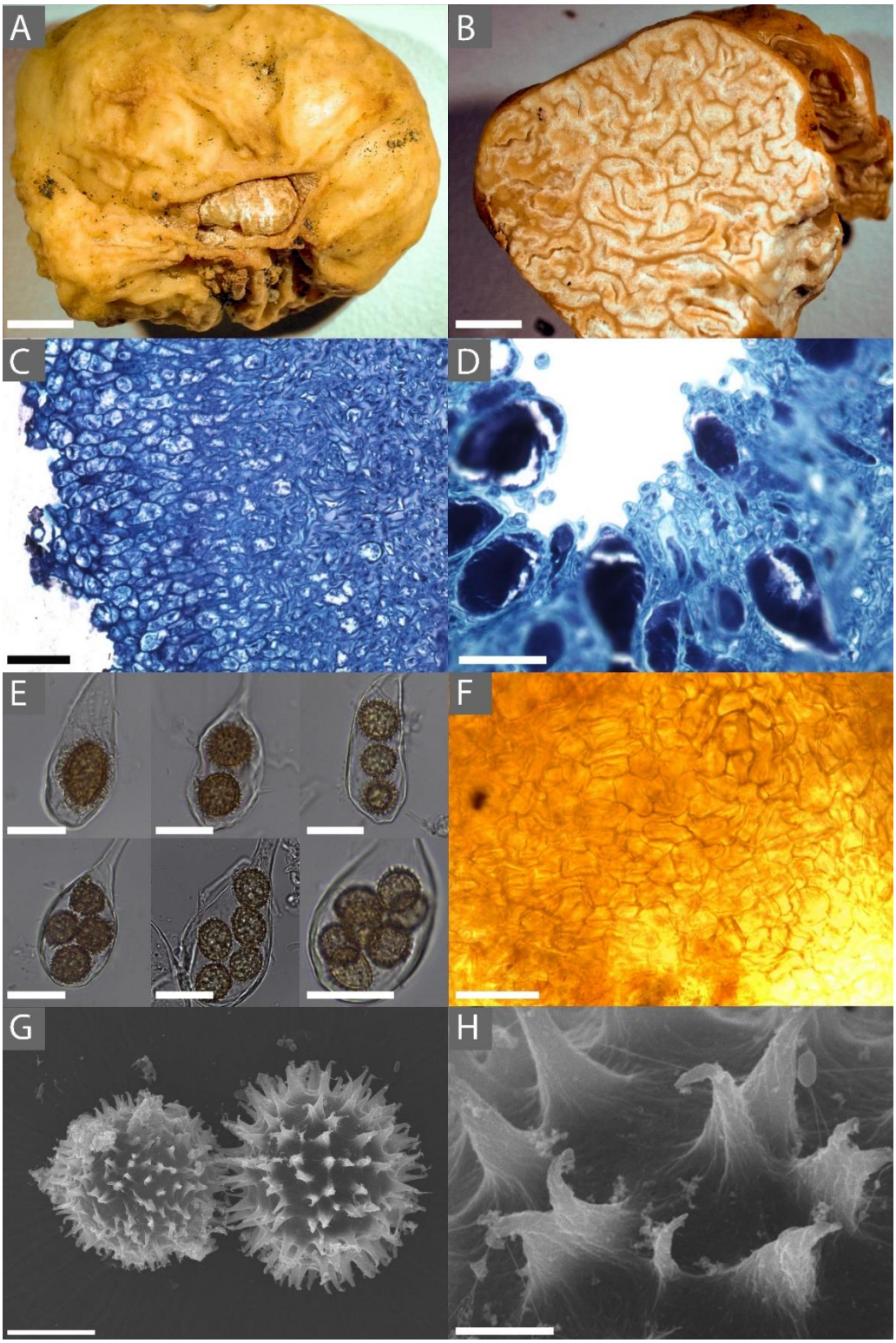

FIGURE 4.2 - *TUBER RUGOSUM* DESCRIPTION PLATE. A. *Tuber rugosum* (BR64, holotype) ascocarp showing both the rugose peridium and exposed sterile gleba. B. A cross-

FIGURE 4.2 (cont'd) – section of *T. rugosum* (BR64) showing the gleba and its marbling. C. A cross-section (stained with toluidine blue O) of *T. rugosum* (RH999) showing the distinctive long, narrow cells of the immature peridium. D. Hymenium (stained with toluidine blue O) of an immature specimen showing the developing asci and paraphyses. E. Ascii of *T. rugosum* (BR64) containing 1, 2, 3, 4, 5, and 7 ascospores. F. Mantle of *T. rugosum* (GenBank MW579340) on the root tip of a red oak (Quercus rubra). G. SEM image of a *T. rugosum* ascospore (BR64) showing the echinate surface. H. SEM image showing details of the uncinulate spines on the ascospore of *T. rugosum* (BR64, holotype; GenBank MW579336). Bars: A, B = 6.0 mm; C, F = 50.0 μm; D = 25.0 μm; E = 30.0 μm; G = 10.0 μm; H = 2.0 μm.

Scanning Electron Microscopy.

Spores that had passed through the digestive tract of the Dusky slug (*Arion* sp.) were free of nearly all of the asci remnants, leaving relatively clean and intact ascospores for imaging. SUPPLEMENTARY FIG. 1 shows clean, ascus-free *T. rugosum*, sp. nov., spores after passage through the slug digestive tract. As seen in FIG. 5, the delicate uncinulate spines are well preserved through this digestive process, which is an improved process for obtaining high-quality SEM opportunities.

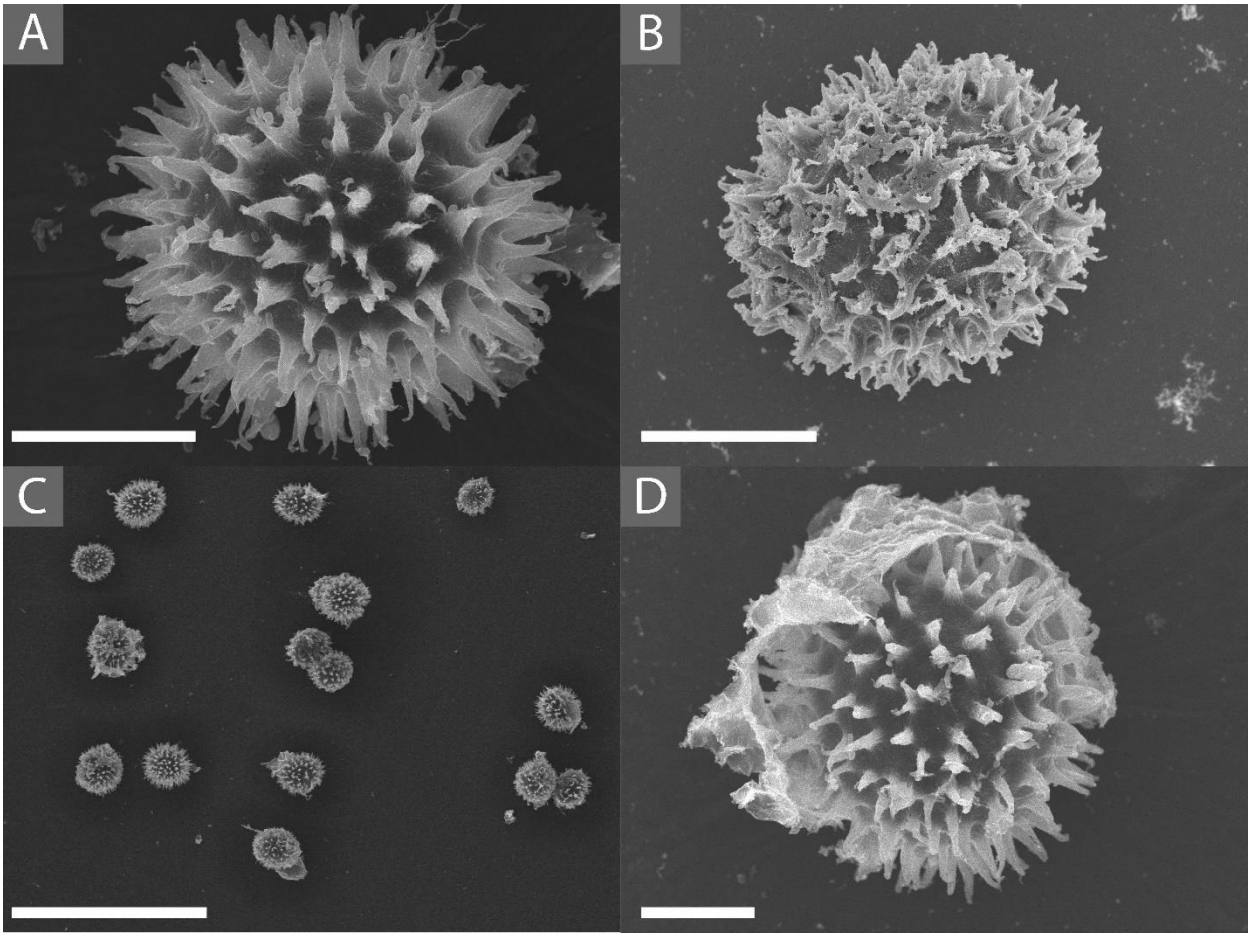

FIGURE 4.5 - SCANNING ELECTRON MICROSCOPY OF *TUBER RUGOSUM* ASCOSPORES. A. SEM image of *Tuber rugosum* ascospore having been passed through the digestive system of the dusky *Arion* slug. B. SEM image of *T. rugosum* ascospore cleared from ascus by drying down ascocarp and scraping gleba. C. Representative cluster of *T. rugosum* ascospores subjected to the slug digestive system. D. Example of remaining ascus, digestive remnants, or other unknown tissue observed on a few of the *T. rugosum* ascospores SEM-imaged after slug digestion. Bars: A, B, D = 10.0 μm; C = 100.0 μm.

Confocal microscopy, Lipid extraction, and analysis

Confocal microscopy revealed that *Tuber rugosum*, sp. nov., BR64 and *T. floridanum* A. Grupe, Sulzbacher & M.E. Sm. contain spores that are rich in lipid droplets (green fluorescence by BODIPY staining, FIG. 4A, B). This staining made visualizing the high lipid content of the spores respective to the surrounding hyphae of the gleba evident. Further fatty acid analyses of in vitro mycelial growth showed that *T. rugosum*, sp. nov. (BR64, holotype), has about 60% of polyunsaturated fatty acids (18:2 and 18:3; FIG. 4C), whereas T. lyonii Butters has the highest content of unsaturated fatty acids, including 29% 18:1 (oleic acid), 34% 18:2, and 10% 20:4 (arachidonic acid) (FIG. 4D).

Molecular analyses

Tuber rugosum, sp. nov., DNA sequences, including for ITS, elongation factor 1α, and RNA polymerase II have been deposited into NCBI GenBank; see TABLE 4 for accession numbers.

FIGURE 4.3 - *TUBER RUGOSUM* ASCOCARP FOUND IN 2021 INFECTED WITH *CLONOSTACHYS ROSEA*. A. The centermost ascocarp showing the darker red brown peridium seen when infected by *C. rosea* compared with the peridium color of noninfected ascocarps. B. Orange conidia from *C. rosea* growing from the gleba of a *T. rugosum* ascocarp. Bars: A = 20.0 mm; B = 2.0 mm.

Phylogenetic analyses.

The ITS (FIG. 6), elongation factor 1α (FIG. 7A), and RNA polymerase II (FIG. 7B) phylogenetic trees all place *T. rugosum*, sp. nov., within the Rufum clade as one of the more early-divergent species. The ITS rDNA data suggest that *T. rugosum*, sp. nov., is a sister species to *T. spinoreticulatum* Uecker & Burds. with a maximum likelihood score of 91 and a Bayesian posterior probability score of 98.9 (FIG. 6). Both the elongation factor 1α and the RNA polymerase II phylogenetic trees show the distinct placement of *Tuber rugosum*, sp. nov., near *T. spinoreticulatum* and more basal to the other taxa in the Rufum clade (FIG. 7). The elongation factor 1α phylogenetic data provide a maximum likelihood score of 75 for the placement of *T. rugosum*, sp. nov. (FIG. 7A), and the RNA polymerase II data fall below the threshold of significance with a score of 69 (FIG. 7B).

TAXONOMY

Tuber rugosum Rennick B., Benucci G.M.N., Du Z., Healy & Bonito, sp. nov. FIG. 2

MycoBank MB838884

Diagnosis

Unique to *T. rugosum* are highly rugose zones across the peridium frequently with tight peridial folds revealing exposed gleba, characteristic echinate ascospores (mean Q = 1.1) that variably have hooked apices with an occasional subreticuate framework sloping gently away basipetally from the spines, and gene sequences.

Typification

USA. MICHIGAN: Ingham County, Onondaga township, elevation 277 m, found in soil between *Quercus rubra* and *Q. alba* in a mixed hardwood forest, 27 Aug 2018, Bryan Rennick

BR64 (holotype MSC408483, designated here). GenBank: ITS = MW579343; EF1a = MW584660; RPB2 = MW584657.

Etymology

The proposed species name references the wrinkly, or rugose, appearance of the ascocarp.

Morphology

Ascomata irregular to subglobose, irregular or lobate, 7.0–28.1 ($\bar{x}$= 12.7) × 10.0–29.7 ($\bar{x}$ = 16.8) mm diam, from opaque to translucent beige to whitish, with faint mottled gleba in mature specimen. Glebal marbling, white sterile veins and melanized yellowish beige fertile tissue. Odor mild and almost nutty; flavor mild.

Table 4.2 - Ascospore length, width, and shape (Q) measurements based on the number of spores per asci.

Spores per asci	Spore count (n)	Length µm	Width µm	Q ($\bar{x}$)
1	11	24–32.5(–37)	(19.5–)20.5–25(–26.5)	1.1–1.5 (1.2)
2	12	(19–)20.5–25(–25.5)	(17–)18.5–21.5(–22)	1.0–1.4 (1.2)
3	20	(19.5–)20–23(–24.5)	(18–)18.5–20.5(–22)	1.0–1.2 (1.1)
4	20	(16–)18–22(–23)	(15.5–)16–20(–22)	1.0–1.2 (1.1)
5	22	(18–)18.5–21.5(–22.5)	(16.5–)17–18.5(–19)	1.0–1.3 (1.1)

Peridium 267.6 ± 41.6 µm thick, glabrous, large zones of rugose pellis. Pellis 139.9 ± 7.5 µm thick. Outermost layer 39.5 ± 6.1 µm thick-walled beige cells subtended by hyaline cells, isodiametric to pseudoparenchymal. Subpellis 97.3 ± 18.9 µm thick. In immature specimen, subpellis distinct from pellis with long, narrow, interwoven cells running perpendicular to the peridial surface. Clavate hyaline asci, 1–7 yellow-brown ascospores, most frequently with 4 ascospores. The main ovoid section of the asci 63.3–89.2 × 28.6–62.86 µm ($\bar{X}$ = 75.7 × 42.7 µm), Q = 1.2–2.8 ($\bar{X}$ = 1.9); peduncle from which the main ovoid section of the ascus arises 39.1–83.9 µm ($\bar{X}$ = 50.3 µm) in length.

Ascospores high in lipids (FIG. 4A, B), subglobose, covered in well-spaced, uncinulate to corniform spines, having a low-sloping ridge extending away from the base and variably fusing with the ridge of adjacent spines, rarely reticulated. Ascospores in 1-spored asci are 24–32.5 × 20.5–26.5 µm with a shape (Q) of 1.2 and in 4-spored asci spores are 18.0–22.0 × 16.0–20.0 µm (Q = 1.1). Additional ascospore size and shape data are in TABLE 2. While growing in vitro, hyphae with simple septae, common branching at right angles (FIG. 1A), rarely producing hyphal coils (FIG. 1B). Hyphae have a high content of unsaturated fats (FIG. 4B, C).

Distribution

Northeastern North America: Quebec (GenBank HM485428), Minnesota, and Michigan (see TABLE 4). The holotype of *Tuber rugosum*, sp. nov., was found within meters of an expansive collection of *T. floridanum* and *T. brennemanii* A. Grupe, Healy & M.E. Sm.

Habitat, Distribution, and Phenology

Northeastern North America from Michigan and Minnesota, USA, through Quebec, Canada. Collected Jul–Nov; hypogeous to subhypogeous in previously disturbed soil of mixed hardwood forest dominated by *Quercus rubra* and *Q. alba*.

Additional specimens examined

USA. MINNESOTA: Stearns County, found hypogeous in mixed conifer and deciduous forest, 17 Oct, 2009, Rosanne Healy RH1030 (FLAS-F-61987); MICHIGAN: Ingham County, found hypogeous in mixed hardwood dominated by *Q. rubra* and *Q. alba*, 17 Jul 2017, Bryan Rennick BR48 (MSC408482); ibid., found subhypogeous in mixed hardwood dominated by *Q. rubra* and *Q. alba*, 7 Aug, 2019, Bryan Rennick BR145 (MSC408484); ibid., found hypogeous in mixed hardwood dominated by *Q. rubra* and *Q. alba*, 18 Aug 2019, Bryan Rennick BR159

(MSC408485); ibid., found subhypogeous in mixed hardwood dominated by *Q. rubra* and *Q. alba*, 11 Sep 2020, Bryan Rennick BR378 (MSC408486); ibid., found subhypogeous in mixed hardwood dominated by *Q. rubra* and *Q. alba*, 4 Sep 2021, Bryan Rennick BR428a (MSC409443).

Notes

Both *T. rugosum* and *T. spinoreticulatum* share a distinctive small cavity revealing gleba on most ascoma, as seen in FIG. 2A. Additionally, they share similar habitats among oak trees, found in northeastern North America, with pseudoparenchyma cells forming the pellis and interwoven cells forming the subpellis. However, *T. rugosum* has a smooth, white to tan peridium surface, whereas *T. spinoreticulatum* has a leathery, brownish gray peridium. Their aroma also differs in that *T. rugosum* has a nutty aroma but *T. spinoreticulatum* smells of rotten cabbage (Uecker and Burdsall, 1977). Finally, *T. rugosum* has small, spiny ascospores at 16.1–25.7 × 15.4–22.0 μm, but *T. spinoreticulatum* has larger spiny-reticulate ascospores at 30–35 × 22–25 μm.

On the same agar medium described in Materials and Methods, *Tuber rugosum* grows vegetatively with right-angled branching and simple septae, as shown in FIG. 1A. In older cultures (FIG. 1B), hyphal coils can be seen once the culture becomes stressed.

We found some collections of *T. rugosum* that were infected by a fungicolous species that we identified as *Clonostachys rosea*. When infected by *C. rosea*, the aroma was particularly smoky and the peridium turned a darker shade with more pronounced red-brown hues, as shown in FIG. 3. The *Clonostachys rosea* isolate (BR428b) maintained a faint smoky aroma from the pure isolate after more than two transfers beyond the initial isolation plate, but directed

experiments will be needed to test the impact of this mycoparasite on truffle aroma. Cultures of the holotype (BR64) as well as *C. rosea* (BR428b) are available upon request.

Table 4.3 - Characteristics of morphologically similar *Tuber* species within the Rufum clade as reported in primary literature.

Species	Peridium surface	Peridium color	Peridium Thickness (µm)	Pellis Cell Type	Pellis Thickness (µm)	Subpellis Cell Type
Tuber lyonii	Smooth, Slightly Pruinose	Light Chestnut Brown	300–500	Interwoven	20–40	Interwoven
Tuber liaotongense	Verrucose	Brownish Yellow	Not Reported	Pseudoparenchyma	Not Reported	Prosenchyma
Tuber microspiculatum	Glabrous	White Yellow, Pale Yellow or Light Brown, Reddish Brown	200–250	Pseudoparenchyma	50–100	Interwoven
Tuber quercicola	Verrucose	Dark Red to Brownish Red	250–500	Interwoven	20–100	Interwoven
Tuber candidum	Smooth	Light Yellowish Brown to Reddish Brown	100–300	Irregularly Compact to Elongated Cells	30–100	Interwoven
Tuber ferrugineum	Papillose	Reddish	215–390	Interwoven	15–50	Interwoven
Tuber nitidum	Glabrous	Reddish Yellow	Not Reported	Not Reported	Not Reported	Not Reported
Tuber rufum	Minutely Warty to Smooth	Reddish Brown	Not Reported	Pseudoparenchyma	Not Reported	Interwoven
Tuber melosporum	Small Warts	Reddish Brown to Reddish Black	Not Reported	Pseudoparenchyma	Not Reported	Interwoven
Tuber wenchuanense	Smooth	Grey Brown	200–250	Pseudoparenchyma	50–100	Interwoven
Tuber malacodermum	Smooth	Light Brown	300–450	Pseudoparenchyma	Not Reported	Pseudoparenchyma to Interwoven
Tuber piceatum	Smooth and Glabrous	Pale Yellow Brown to Yellow Brown	200–350	Pseudoparenchyma	Not Reported	Interwoven
Tuber crassitunicatum	Smooth	Brown to Yellow Brown	250–300	Interwoven To Pseudoparenchyma	200–250	Interwoven
Tuber spinoreticulatum	Leathery	Brownish Gray	Not Reported	Pseudoparenchyma	150–600	Interwoven
Tuber theleascum	Smooth	Reddish	160–250	Pseudoparenchyma	45–150	Pseudoparenchyma to Interwoven
Tuber taiyuanense	Smooth	Pale Yellow, Yellow Brown or Brown	150–300	Pseudoparenchyma	Not Reported	Interwoven
Tuber pustulatum	Low Pyramidal Warts	Reddish	370–550	Pseudoparenchyma	160–220	Prosenchyma
Tuber lishanense	Smooth	Yellow White to Yellow Brownish	250–350	Pseudoparenchyma	150–200	Interwoven
Tuber luomae	Verrucose	Light Orange Brown	± 500	Subglobose or Subpolyhedral	± 150	Interwoven
Tuber rugosum	Smooth	White to Tan	267.6 ± 41.6	Pseudoparenchyma	139.9µm ± 7.5µm	Interwoven
Tuber umbilicatum	Smooth to Minute Papillae	Pale Yellow, Yellow Brown or Brown	320–500	Pseudoparenchyma	90–250	Interwoven
Tuber furfuraceum	Smooth	Brown	340–480	Pseudoparenchyma	170–270	Interwoven
Tuber huidongense	Verrucose, Slightly Furfuraceous, Pubescent	Yellow-Brown to Red-Brown	150–300	Pseudoparenchyma	80–150	Interwoven
Tuber lannaense	Smooth	Yellow-Brown to Dark-Brown	130−260	Pseudoparenchyma	35–80	Interwoven
Tuber wanglangense	Smooth and Glabrous	Yellow White	200–250	Pseudoparenchyma	50–100	Interwoven

Table 4.3 - (cont'd)

Species	Subpellis Thickness (µm)	Average Spore Size (µm)	Spore Shape (Q or Description)	Spores per Ascus	Ornamentation	Aroma	Source
Tuber lyonii	Not Reported	21–31 x 17–21	Ellipsoid	1–5	Spinose To Subreticulate	Pungent, Nutty, Malted Milk	(Butters, 1903; Trappe et al., 1996)
Tuber liaotongense	Not Reported	29–40 x 26–35	Ellipsoid	2–4	Alveolate Reticulate	Not Reported	(Wang, 1988)
Tuber microspiculatum	Not Reported	22.5–35 x 17.5–22.5	Ellipsoid	1–4	Alveolate Reticulate	Slight, Not Pungent	(Fan et al., 2012)
Tuber quercicola	130–400	20–45 x 15–35	Ellipsoid	1–5	Curved Spines	Earthy, Fresh Green Beans	(Frank et al., 2006)
Tuber candidum	70–200	19–42 x 14–34	Globos to Ovoid	1–5	Curved Spines	Mild to Slightly Earthy	(Harkness, 1899; Frank et al., 2006)
Tuber ferrugineum	200–350	28.2 x 20.5	Ellipsoid	2–4	Spiny	Silkworm / Pleasant	(Carlo Vittadini, 1831; Elliott et al., 2016)
Tuber nitidum	Not Reported	Not Reported	Ellipsoid	1–4	Echinulate	Nauseous	(Carlo Vittadini, 1831; Granetti et al., 1988)
Tuber rufum	Not Reported	28–42 x 18–28	Ellipsoid	4–5	Spiny	Insipid	(Carlo Vittadini, 1831; Grunow & Rabenhorst, 1884)
Tuber melosporum	Not Reported	40–45 x 20–25	1.7	1–6	Smooth	Garlic	(Enrico et al., 2016)
Tuber wenchuanense	150–200	25–45 x 17.5–30	Ellipsoid	1–5	Spiny-Reticulate	Not Reported	(Fan et al., 2013)
Tuber malacodermum	Not Reported	26.0 ± 4.6 x 21.1 ± 3.5	1.23 ± 0.12	1–4	Spinose	Celery	(Leonardi et al., 2019)
Tuber piceatum	Not Reported	26.7 ± 2.7 x 19.2 ± 1.4	1.4 ± 0.1	1–6	Spinose	Not Reported	(Yan et al., 2018)
Tuber crassitunicatum	Not Reported	29.1 ± 4.8 x 18.8 ± 2.5	1.5 ± 0.1	1–5	Spiny-Reticulate	Mild	(Yan et al., 2018)
Tuber spinoreticulatum	30–125	30–35 x 22–25	1.4	1–5	Spiny-Reticulate	Rotting Cabbage	(Uecker & Burdsall, 1977)
Tuber theleascum	85–120	26.6 ± 5.18 x 16.8 ± 2.63	1.59 ± 0.19	1–6	Spinose To Subreticulate	Not Reported	(Leonardi et al., 2019)
Tuber taiyuanense	Not Reported	20–45 x 18–30	Ellipsoid	1–5	Spiny-Reticulate	Light	(Cao et al., 2011)
Tuber pustulatum	210–350	27.4 ± 5.7 x 22.6 ± 4.55	1.22 ± 0.3	1–6	Spiny-Reticulate	Acidulous To Rancid	(Leonardi et al., 2019)
Tuber lishanense	100–150	25.5 ± 3.8 x 21.6 ± 3.7	1.2 ± 0.2	1–5	Spiny	Inconspicuous	(Yan et al., 2018)
Tuber luomae	± 350	23–30 x 18.5–23	1.21–1.32	1–5	Spiny	Mildly Acrid	(Eberhart et al., 2020)
Tuber rugosum	97.3 ± 18.9	21.5 x 19.4	1.2	1–7	Spiny	Nutty	
Tuber umbilicatum	150–400	23–33 x 17–23	1.4 ±0.13	1–6	Spiny Alveolate Reticulum	Not Reported	(Chen et al., 2005)
Tuber furfuraceum	170–210	25–46 x 14–27	1.7	2–5	Spiny-Reticulate	Slight, Not Distinctive	(Hu & Wang, 2005)
Tuber huidongense	90–150	27–35 x 18–22	1.50 ± 0.18	1–5	Spiny-Reticulate	Not Reported	(Deng et al., 2009)
Tuber lannaense	100–175	25–29 x 17–21	1.17 ±0.14	1–5	Spiny-Reticulate	Not Reported	(Suwannarach et al., 2016)
Tuber wanglangense	Not Reported	29.9 ± 3.6 x 24.5 ± 2.6	1.2 ± 0.1	1–5	Spiny-Reticulate	Not Reported	(Yan et al., 2018)

Table 4.4 - *Tuber rugosum* collections used in this study with their herbarium and GenBank accession numbers and collection dates.

					Accession Number			
Species	Collection	Source	Locale	Date Collected	Herbarium	ITS1-F	EF1α	RPB2
Tuber rugosum	RH999	Ascoma	USA:MN	8 Aug 2009		MW584702		
Tuber rugosum	RH1030	Ascoma	USA:MN	17 Oct 2009	FLAS-F-61987	MW584701		
Tuber rugosum	RH1330	Ascoma	USA:MN	3 Sep 2011		MW584700		
Tuber rugosum	BR48	Ascoma	USA:MI	17 Jul 2017	MSC408482	MW579335		
Tuber rugosum	**BR64**	Ascoma	USA:MI	27 Aug 2018	MSC408483	MW579343	MW584660	MW584657
Tuber rugosum	BR145	Ascoma	USA:MI	7 Aug 2019	MSC408484	MW579344	MW584661	MW584658
Tuber rugosum	BR159	Ascoma	USA:MI	18 Aug 2019	MSC408485	MW579345	MW584662	MW584659
Tuber rugosum	BR339	Root tip	USA:MI	5 Aug 2020		MW579346		
Tuber rugosum	BR340	Root tip	USA:MI	5 Aug 2020		MW579347		
Tuber rugosum	BR342	Root tip	USA:MI	5 Aug 2020		MW579348		
Tuber rugosum	BR343	Root tip	USA:MI	5 Aug 2020		MW579349		
Tuber rugosum	BR378	Ascoma	USA:MI	11 Sep 2020	MSC408486	MW579975		
Tuber rugosum	BR428a	Ascoma	USA:MI	4 Sep 2021	MSC409443	OL438889		
Clonostachys rosea	BR428b	Anamorph	USA:MI	4 Sep 2021	MSC409443	OL438890		

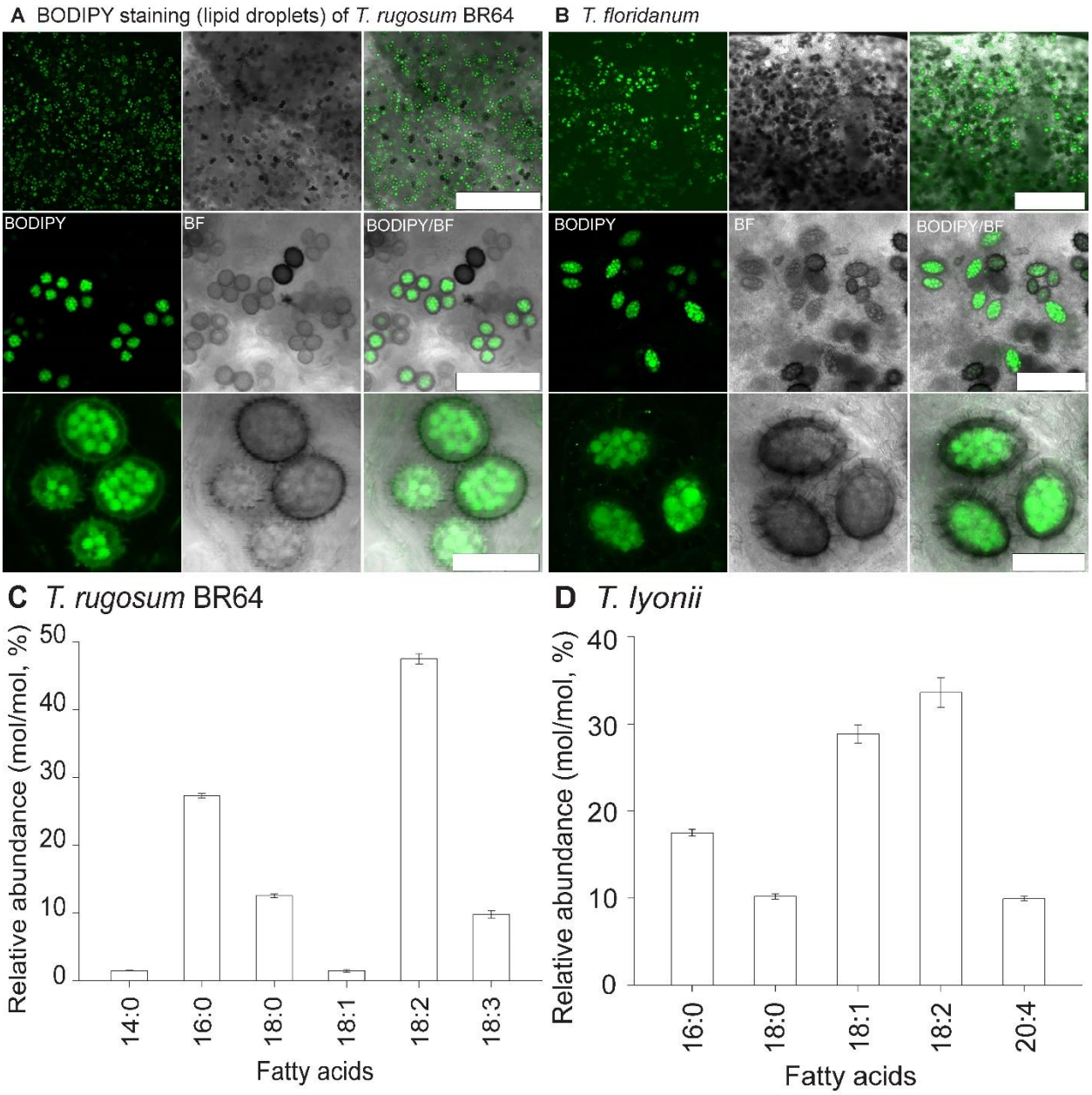

FIGURE 4.4 - LIPID ANALYSIS OF TRUFFLE SPORES. A, B. Confocal microscopy of BODIPY-stained (A) *Tuber rugosum* (BR64) and (B) *Tuber floridanum* ascospores revealing lipid content as green. C, D. FAME analysis of (C) *T. rugosum* and (D) *T. lyonii* in vitro mycelial growth showing distinct variation in fatty acid concentrations. Bars: A = 0.5 mm, 100.0 µm, 25.0 µm, respectively; B = 0.5 mm, 100.0 µm, and 30.0 µm, top to bottom.

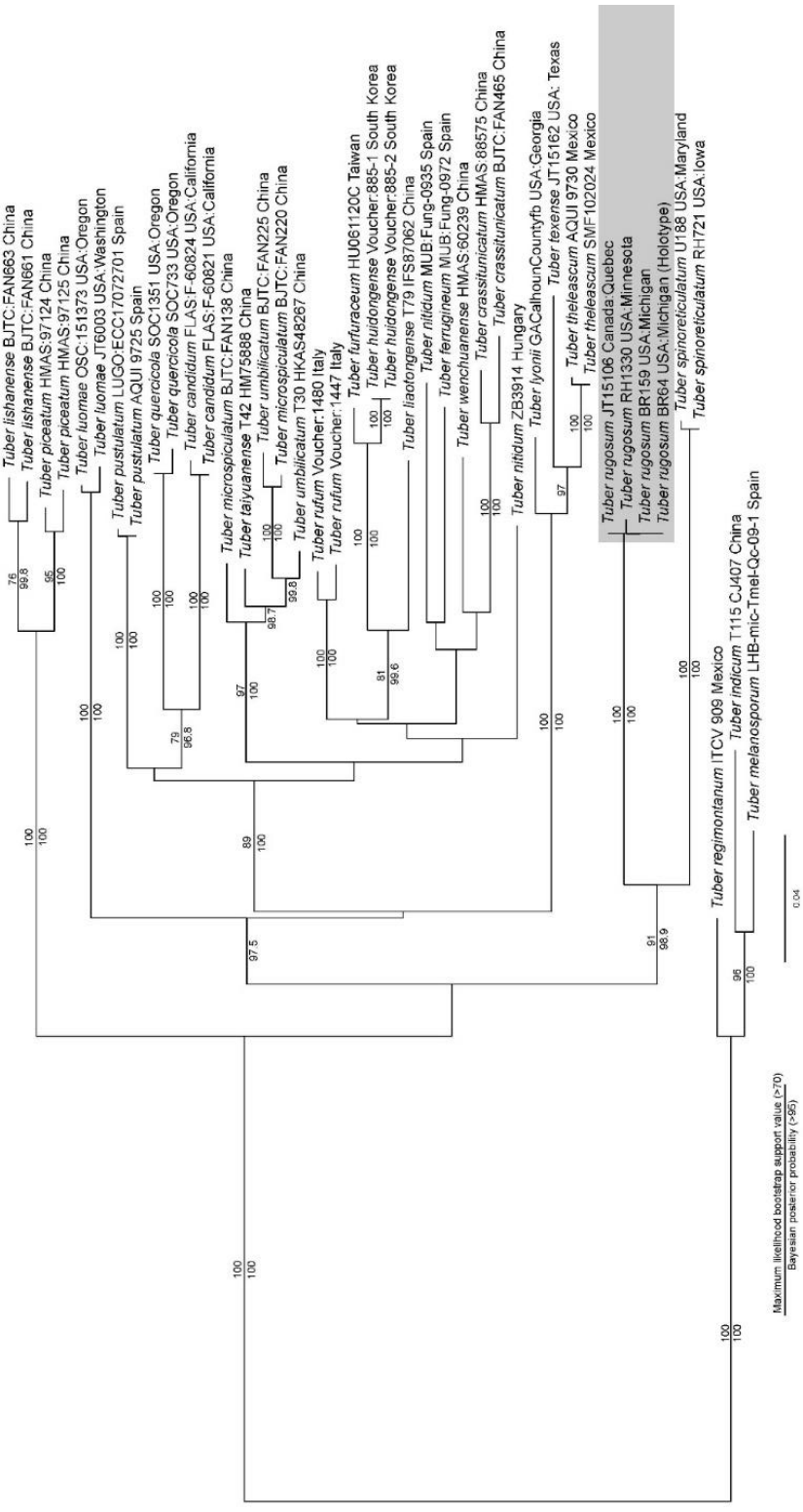

FIGURE 4.6 - ITS RDNA PHYLOGENY OF THE RUFUM CLADE. This most likely phylogenetic tree reconstructed from ITS rDNA data shows that *T. rugosum* is basal in the

FIGURE 4.6 (cont'd) – Rufum clade and supported as sister species to *T. spinoreticulatum* . Maximum likelihood bootstrap support values over 70 are shown above the nodes, whereas Bayesian posterior probabilities above 95 are shown below nodes. *Tuber regimontanum*, *T. indicum*, and *T. melanosporum* were included as outgroups as identified by Bonito et al. (2010). Taxa are shown with specimen, isolate, or collection number as listed in the NCBI database.

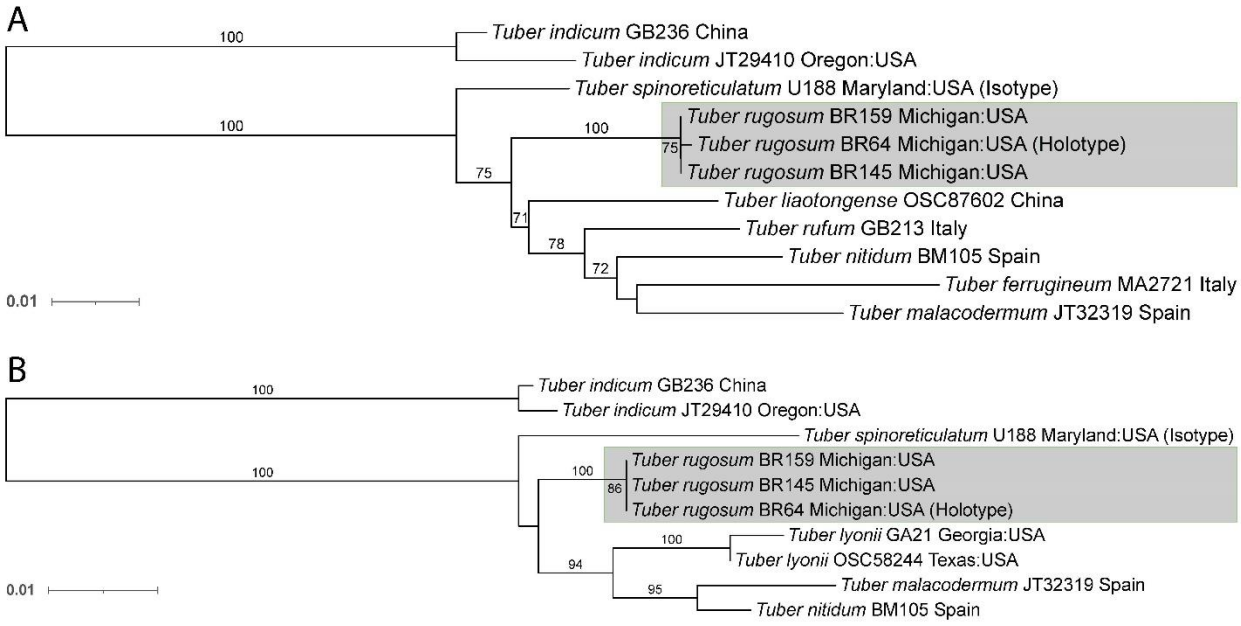

FIGURE 4.7 - ELONGATION FACTOR 1A (A) AND RNA POLYMERASE II GENE (B) PHYLOGENETIC TREES. Both show maximum likelihood bootstrap support values over 70. *Tuber* indicum was chosen as an outgroup as identified by Bonito et al. (2010). Taxa are shown with specimen, isolate, or collection number as listed in the NCBI database.

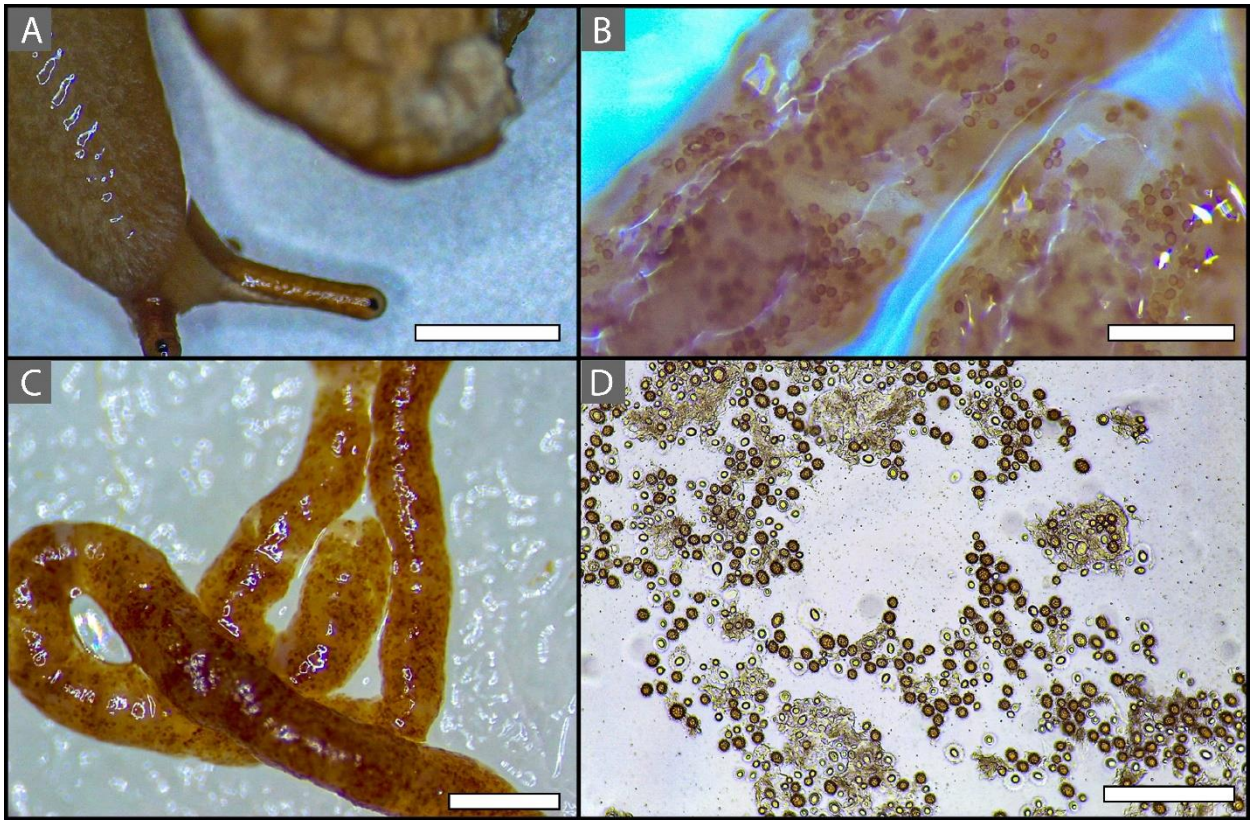

FIGURE 4.8 - TRUFFLE SPORES IN SLUG FRASS. A) Dusky *Arion* slug near *T. rugosum* ascoma. B, C) Slug frass showing high amount of *T. rugosum* ascospores. D) Slug frass mounted on slide with 3% KOH showing *T. rugosum* ascospores free of their ascus. Bars: A = 20.0 mm, B = 200.0 µm, C = 500.0 µm, D = 200.0 µm

Discussion

Here, we described a new truffle species, *T. rugosum*, as supported by three independent phylogenetic markers, as well as by morphological characters. Phylogenetic reconstructions demonstrate this species as a novel North American species in the Rufum clade. *Tuber rugosum* is most closely related to the North American species *T. spinoreticulatum*, and as seen in TABLE 3, the two species differ macroscopically in the color and texture of their peridium and microscopically by the size, shape, and ornamentation of their ascospores. The two species also

have a different aroma, with *Tuber spinoreticulatum* being particularly unpleasant and originally described as smelling like rotten cabbage (Uecker and Burdsall, 1977).

Animals play an important role in the spore dispersal of hypogeous fungi. This has been well documented in the case of small mammals, which consume truffles as food (Cázares and Trappe, 1994; Gabel et al., 2010). Other animals including *Diptera* and *Stylommatophora* have fungivorous members that have been shown to enhance mycorrhizal spore dispersal, including that of truffles (Kitabayashi and Tuno, 2018; McGraw et al., 2002). As documented previously, slugs in the genus *Arion* are mycophagous (Beyer and Saari, 1978); however, their role in spore dispersal has not been investigated. Although this paper did not set out to determine truffle spore viability in slug excreta, we did demonstrate that *T. rugosum* is consumed by *Arion* slugs and that spores that pass through the slug digestive tract are released from the asci in good condition for visualization. In fact, we found this to be a useful pretreatment for cleansing spores prior to preparation for SEM. Recent work by Ori et al. (2021) demonstrated that slug digestion of *T. aestivum* ascospores exhibit an altered episporial texture and increased mycorrhization of *Quercus robur* roots compared with those ascospores consumed by mice or uningested spores. Our observations that *T. rugosum* is found just beneath leaf litter and is often partially consumed raise questions on the role of *Arion* slugs and other gastropods in truffle spore dispersal in nature.

Truffles damaged by small animals or other mechanical means may also become more susceptible to infection by fungicolous organisms, which may alter its aromatic profile (Eslick, 2012). It is still unknown whether *C. rosea* is a primary pathogen of truffles, although the literature suggests that *C. rosea* can be a mycoparasite of many fungal species and thus may have potential use as a biocontrol against fungal crop pathogens such as *Fusarium graminearum* Schwabe (Gimeno et al., 2021). In a prior report, *C. rosea* was isolated from *Tuber magnatum*

but was not found to be chitinolytic (Pavic et al., 2013). We were unable to demonstrate *C. rosea* pathogenicity on *T. rugosum*, as our in vitro assays were inconclusive. While growing *C. rosea* on agar, we noted that it maintained a faint smoky aroma, which supports the hypothesis that it contributed to the odor profile of the ascocarps it was found growing on. Other truffle-inhabiting organisms, particularly α- and β-proteobacteria, have been found to be principal contributors to sulfur-containing volatiles characteristic of *T. borchii* Vittad. ascocarp aroma (Splivallo et al., 2011). Further work is needed to ascertain the involvement of other fungi and bacteria in the aroma development and profiles of *Tuber* ascoma, as well as truffle development and disease.

Fatty acid profiles have been used when trying to distinguish *Tuber* species within species complexes and provide some insight into their physiology and nutrition (Angelini et al., 2015). However, *Tuber* fatty acid profiles may vary across geographic regions and under different environmental and growth conditions (Shah et al., 2020). To attempt to control for these environmental variances, we obtained pure culture isolates of *T. rugosum* and the closely related species *T. lyonii* (FIG. 4), to assess and compare FAME profiles from similarly aged mycelium grown in the same medium and environment. Truffle species had distinct fatty acid profiles from one another, with *T. rugosum* being particularly reduced in 18:1 (oleic acid) and *T. lyonii* enriched in 20:4 (arachidonic acid). Fatty acid profiles are not available for most *Tuber* species, but they could provide insights into variation in *Tuber* physiology and nutrition.

Similar to previously published ITS phylogenies (Bonito et al., 2010), *T. rugosum* is found on an early-divergent branch of the Rufum clade and appears to be sister to *T. spinoreticulatum* (FIG. 6). Our ITS ML- and BI-based phylogenies also conform to more recently published *Tuber* phylogenies (Yan et al., 2018), which placed *T. lishanense* L. Fan & X.Y. Yan and *T. piceatum* L. Fan, X.Y. Yan & M.S. Song basal to *T. spinoreticulatum* within the

Rufum clade. The general structure of the phylogenetic trees analyzed from the two protein-encoding loci (FIG. 7) also agree with earlier studies (Bonito et al., 2013) but with stronger support for *T. rugosum* within the Rufum clade and its close relationship to *T. spinoreticulatum* . However, it should be noted that there are relatively few sequences available within the Rufum clade for both the RPB2 and EF1α protein-encoding regions. The lack of species representation within these protein-encoding regions is such that concatenating and condensing the phylogenetic trees into a single, better-supported phylogram is not possible at this time. Further work is needed to generate these protein-encoding sequences in other Rufum clade species in order to reconstruct a more comprehensive phylogeny.

Conclusion

In conclusion, we have described *Tuber rugosum*, a pale, wrinkly, spiny-spored truffle endemic to northeastern North America, supported by morphological and phylogenetic analyses of multiple loci. We provide the fatty acid profile of this species, describe a fungicolous species association, and present a method that involves the use of slugs to assist in cleaning ascospores prior to SEM imaging. Finally, a dichotomous key for truffles in the Rufum clade is provided below. Together, these results expand on the knowledge base of *Tuber* biodiversity and microbiome diversity in North America.

A key to Tuber spp. in the Rufum clade

1. Ascospore smooth with no ornamentation	*T. melosporum*
1’. Ascospore with ornamentation	2
2. European species	3
2’. Not European species	7

3. Peridium smooth	4
3’. Peridium not smooth	5
4. Peridium color reddish yellow	*T. nitidum*
4’. Peridium color light brown	*T. malacodermum*
5. Ascospore globose in shape	*T. pustulatum*
5’. Ascospore ellipsoid in shape	6
6. Pellis cells pseudoparenchyma	*T. rufum*
6’. Pellis cells interwoven	*T. ferrugineum*
7. North American species	8
7’. Asian species	14
8. Peridium smooth	9
8’. Peridium verrucose or leathery	12
9. Ascospore spines commonly curved or hooked	10
9’. Ascospore spines not commonly curved or hooked	11
10. Found west of the Rocky Mountains	*T. candidum*
10’. Found east of the Rocky Mountains	*T. rugosum*
11. Pellis 20–40 μm thick	*T. lyonii*
11’. Pellis 45–150 μm thick	*T. theleascum*
12. Outer pellis of pseudoparenchyma or globos cells	13
12’. Outer pellis of longer interwoven cells	*T. quercicola*
13. Ascospore globose in shape	*T. luomae*
13’. Ascospore ellipsoid in shape	*T. spinoreticulatum*

14. Peridium verrucose or with small papillae	15
14’. Peridium smooth	17
15. Ascospore reticulated but with distinct curved spines	*T. umbilicatum*
15’. Ascospore not as above	16
16. Ascospore alveolate reticulate	*T. liaotongense*
16’. Ascospore spiny-reticulate	*T. huidongense*
17. Pellis consisting of pseudoparenchyma	18
17’. Pellis consisting of interwoven cells	*T. crassitunicatum*
18. Ascospore reticulate	19
18’. Ascospore not reticulate	24
19. Ascospore alveolate reticulate	*T. microspiculatum*
19’. Ascospore spiny-reticulate or spiny	20
20. Ascospore globose in shape	21
20’. Ascospore ellipsoid in shape	22
21. Peridium color yellow brown to dark brown; 130–260μm thick	*T. lannaense*
21’. Peridium color yellow white; 200–25 μm thick	*T. wanglangense*
22. Peridium thickness > 300 μm	*T. furfuraceum*
22’. Peridium thickness < 300 μm	23
23. Ascoma deeply and densely furrowed	*T. taiyuanense*
23’. Ascoma mostly smooth with few furrows	*T. wenchuanense*
24. Ascospore globose in shape	*T. lishanense*
24’. Ascospore ellipsoid in shape	*T. piceatum*

Chapter 5

Synthesis

Synthesis

Objectives

The three primary goals of this thesis were to: 1) to investigate how glyphosate and glufosinate herbicides interact with truffles, morels, and plant growth promoting fungi in-vitro to inform use in fungal cultivation, 2) to investigate methyl bromide fumigated bare-root conifer tree nurseries as a potential candidate for targeted inoculation of local, exotic, or commercial ectomycorrhizal inoculants, and if these inoculants improve seedling growth, and 3) to confirm and describe a new truffle species utilizing morphology, a unique genetic sequence, a unique fatty acid profile, a novel electron microscopy spore cleaning method, and to build a new taxonomic key for Rufum clade. Efforts toward each of these goals are detailed through chapters 2, 3, and 4. Each of the major results and future directions of these chapters are synthesized here.

Herbicide interactions

In Chapter 2, I investigated the growth implications of several fungi cultured with media made with serial dilutions of glyphosate or glufosinate herbicides. I focused on *Benniella erionia, Linnemannia elongota, Morchella americana, M. importuna, Tuber borchii, T. canaliculatum, T. floridanum, T. lyonii,* and *T. rugosum*. Additionally, I assessed impacts of *Rhizopus oryzae, Serendipita indica, Stropharia rugosoannulata,* and an *Umbeloposis* species to the same growth assay with fewer replications. The results of this work revealed that as both herbicides increased in concentration to levels near or above the manufacturers recommended field application rate, fungal growth diminished. It also showed that ectomycorrhizal species were less sensitive to herbicides than saprotrophic fungi. This study demonstrated fungistatic properties of herbicides commonly used in truffle orchards and other environments. These effects were evident when overapplied; something easy to do when applying by hand.

Additional work may aim to investigate a wider array of pesticides including bactericides, insecticides, and oomyticides. Beyond investigating more pesticides, pesticide residuals and adjuvants may interfere with more than just mycelial growth. Future work elucidating how pesticides impact development of overwintering structures, fruiting bodies, and mitospores should be carried out. Additionally, as there are many truffle plantations using herbicides and morels cultivated between agronomic crops, we should investigate which herbicide residuals end up in these food items and how concentrated these pesticides may become.

Added inoculants to bare-root nurseries

In Chapter 3, I assessed how adding various inoculants to methyl bromide fumigated bare-root Christmas tree nurseries may impact seedling health and growth. Specifically, through two experiments at two nurseries over two years, *Pseudotsuga menziesii*, *Picea abies*, and *Pinus sylvestris* were inoculated with local (*Laccaria bicolor* or *Scleroderma citrinum*), exotic truffle (*Tuber aestivum*, *Tuber borchii*, or *Tuber indicum*), or a commercial inoculant (MycoApply®) at the time of sowing. Prior to the nursery's harvest the following year, seedlings from each treatment block were collected. Seedling height, root collar diameter, above ground biomass were collected. Additionally, fungal and oomycete MiSeq libraries were constructed for community analysis. The results of this work show no increases in height, diameter, or biomass over uninoculated control blocks. I did find low levels of *Tuber borchii* reads in the community analysis, but they were well below a threshold that would interest truffle plantations. Additionally, the oomycete library revealed over 12 species of *Pythium* and four species of *Phytophthora*, highlighting how well these motile pathogens can persist in or recolonize methyl bromide fumigated soils. Overall, it seems that fungi either persisting or blowing in from nearby

tree stands dominate the roots of these Christmas trees. The fungal diversity found on these roots combined with the lack of growth improvements suggest that adding inoculants may not be beneficial or necessary.

This work could be expanded by looking at other tree nursery species and other inoculant sources. Additionally, investigating the community of spores introduced from nearby tree stands as they relate to living mycorrhizae in nursery seedlings may reveal one avenue by which these robust communities establish. Christmas trees are grown for two years at the bare-root nursery before getting transplanted into a larger space where it will grow to harvestable size. It would also be beneficial to look at how the fungal community adapts to transplanting and any treatments which may occur at the finishing plantation as there is interest in adding inoculants at this phase as well.

A new truffle species

Chapter 4 highlights my work done in describing a new truffle species found in Michigan, and the greater north eastern portion of North America. *Tuber rugosum*, was described using a multigene phylogeny, a fatty acid profile, and morphological characters. Assisting with the morphological analysis, observations of a slug (*Arion subfuscus*) eating subhypogeous truffles led to the development of a novel ascus clearing technique for scanning electron microscopy. Slug fungivory removes asci from ascospores with delicate ornamentation, such as with spiny-spored *T. rugosum*, without damaging spore morphology. Additionally, I produced an updated taxonomic key for the Rufum clade. This work describing rare truffle taxa removes ambiguity in future taxonomic efforts and aids in expanding our knowledge of truffle ranges, diversity, and ecology.

Further work is still needed to describe other rare truffle species as they directly interact with tree species in truffle cultivation systems. Beyond their ecological roles, some yet to be described truffle species may also have important culinary value. Additionally, there is very little in the literature about slug fungivory and how it relates to ascospore viability. Developing this knowledge could yield significant insights to both truffle and slug ecologies as well as having the potential to improve truffle tree colonization.

Conclusions

Truffles have captivated our attention for centuries and with recent progress in truffle cultivation, these rare gems of the earth are more available and are gaining even greater interest as they make their way to restaurants around the world. There are still many questions on how best to manage their plantations such as how using herbicides may impact truffle development. I have shown that truffles, but also morels and plant beneficial fungi, have reduced mycelial growth at herbicide application rates above the recommended dosage. If truffières decide to use herbicides, they should not apply glyphosate or glufosinate carelessly or at rates above the manufacturers label. Additionally, at least in terms of outdoor bare-root conifer trees, adding inoculants at the time of sowing does not improve growth and therefore not recommended. Adding inoculants not only requires the purchase of costly products, but also consumes valuable labor during an already tight schedule. Finally, as was the case with *Tuber rugosum*, there may be new species of truffle in ones backyard. Careful observations of this new species led to the development of a new technique for clean electron microscopy. This highlights how serendipitous discoveries can assist in the broadening of our ecological and taxonomic knowledge of rare fungal taxa. Collectively, I have contributed to knowledge of fungal herbicide

interactions, priority effects of added inoculants in bare-root Christmas tree nurseries, and the ecology, taxonomy, and ascus clearing of truffle species.

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APPENDIX

CURRENT CURRICULUM VITAE

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Education

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Michigan State University, East Lansing, Michigan — 2018

- Bachelor of Science in Horticulture; Horticultural Science concentration.
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Lansing Community College, Lansing, Michigan — 2016

- General Studies

Community College of the Air Force, Maxwell Air Force Base, Alabama — 2009

- General Studies, Applied Science with focus in Ecological Controls

United States Air Force, Sheppard AFB, Wichita Falls, Texas — 2004

- Utility Systems Maintenance Technical Training

PUBLICATIONS

Peer reviewed

- **Rennick B**, Benucci GMN, Du Zhi-Yan1, Healy R, Bonito G. (*2023) Tuber rugosum* , a new species from Northeastern North America: Slug mycophagy aides in electron microscopy of ascospores. *Mycologia*

- Dissanayake A, Mills G, Bonito G, **Rennick B**, Naira M. (2021) Chemical Composition, Anti-inflammatory and Antioxidant Activities of Extracts from Cultivated Morel Mushrooms, Species of Genus Morchella (Ascomycota). *International Journal of Medicinal Mushrooms.*
- Benucci GMN, **Rennick B**, Bonito G (*2020*). Patient propagules: Do soil archives preserve the legacy of fungal and prokaryotic communities? *PLoS ONE.*

In Preparation for Peer Review Journals

- **Rennick B**, Benucci GMN, Bonito G. Impacts of Liberty and Roundup herbicides on mycelial growth of truffles, morels, and plant growth promoting fungi.
- **Rennick B,** Benucci GMN, Bonito G. Does ectomycorrhizal inoculum after methyl bromide field applications in bare-root conifer nursery seedlings establish and persist?

Trade Journal

- **Rennick B,** Benucci GMN, Bonito G. (2022) How do methyl bromide field applications and mycorrhizal inoculants impact bare-root conifer nursery seedlings ectomycorrhizal development? *Great Lakes Christmas Tree Journal.*

PROFESSIONAL PRESENTATIONS

Guest Lectures

- **Rennick, B.**, (February 20, 2023) *Growing Small Scale Farms by Growing Gourmet Mushrooms.* Guest Lecture for HRT 341: Vegetable Production and Management, MSU, East Lansing, MI, United States
- **Rennick, B.**, (February 21, 2022) *Growing Small Scale Farms by Growing Gourmet Mushrooms.* Guest Lecture for HRT 341: Vegetable Production and Management, MSU, East Lansing, MI, United States

- **Rennick, B.**, (March 1, 2021) *Growing Small Scale Farms by Growing Gourmet Mushrooms.* Guest Lecture for HRT 341: Vegetable Production and Management, MSU, East Lansing, MI, United States
- **Rennick, B.**, (January 21, 2020) *Cultivating the Mushroom of Immortality.* Guest Lecture for PLP 405: Plant Pathology, MSU, East Lansing, MI, United States
- **Rennick, B.**, (February 25, 2019) *Growing Small Scale Farms by Growing Gourmet Mushrooms.* Guest Lecture for HRT 341: Vegetable Production and Management, MSU, East Lansing, MI, United States

Poster Presentations

- **Rennick, B.,** Bennuci, G. N., Zhi-Yan Du, Bonito GM (October 23, 2019*) Tuber rugosum sp. nov.: A new spiny-spored truffle species from North America.* Presented poster at IWEMM10, Nagano, Japan
- **Rennick, B.,** Bennuci, G. N., Bonito GM (October 23, 2019*) Impact of the herbicide Roundup® on mycelial growth of truffles, morels, and mold.* Presented poster at IWEMM10, Nagano, Japan
- **Rennick, B.,** Bennuci, G. N., Bonito GM (July 18, 2018*) Impact of glyphosate and glufosinate on mycelial growth of truffles, morels, and molds*. Presented poster at MSA annual meeting, San Juan, PR, United States.

Professional Talks

- **Rennick, B.,** (2018) *Target Mycorrhizal Fungi in Nursery and Forestry Cropping Systems.* Department of Plant, Soil, and Microbial Sciences Plant Pathology Seminar, MSU, East Lansing, MI, United States
- Benucci, G., **Rennick, B.,** (2017*) Strategies for inoculating chestnut seedlings with truffles*.

Midwest Nut Producers Council. Clarksville, MI, United States

AWARDS AND ACHIEVEMENTS

- Plant, Soil, and Microbial Sciences Endowed Graduate Assistantship
- Mycological Society of America James M. Trappe Travel Award
- American Society for Horticultural Science 2018 Outstanding Undergraduate Student Award
- 8 Semesters on Dean's List
- Merrill S. Fuller Scholarship
- Victor Ray Gardner Scholarship
- Terry L. Schlichter Endowed Scholarship
- Carl J. Sellner Scholarship
- Air Force Longevity Service Award
- Twice awarded the Air Force Achievement Medal
- Twice awarded the Air Force Good Conduct Award
- Air Force Expeditionary Service Ribbon with Gold Border
- Global War on Terrorism Expeditionary Medal