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Discovery of Truffles (Tuber species) in North Mississippi Pecan Orchards

Scout Hodges

DISCOVERY OF TRUFFLES (TUBER SPECIES) IN NORTH MISSISSIPPI PECAN ORCHARDS

By

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A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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DEDICATION

This thesis is dedicated to my amazing parents who allowed their five-year old daughter to order truffle pasta all those years ago. Thank you for teaching me to have a curious mind and a hunger to try new things in this amazing world God gave us.

ACKNOWLEDGEMENTS

This thesis would not have been possible without my advisor, Dr. Jason Hoeksema. Dr. Hoeksema led me through this process, every step of the way. I am so grateful to him for his patience, intelligence, and willingness to help me along the way. I want to express my immense gratitude to him, the rest of the Department of Biology at Ole Miss, and the Sally McDonnell Barksdale Honors College for their leadership, funding, and providing me with the resources necessary to accomplish this study. I would like to thank Giaccomo Boccacini for sharing his immense wisdom of truffle hunting, and my aunt and grandmother who blessed me with the amazing opportunity to go truffle hunting with him. Thank you to all of my friends and family who have encouraged me along the way; I could not have done this without your constant support and love. A special thanks to Gabriel Frasca of Straight Wharf in Nantucket for introducing me to truffles in the first place. Lastly, I thank my Holy Father for this marvelous world He has given us, filled with wonderful treasures such as truffles, and the ability He has given me to delight in them.

ABSTRACT

VIVIAN SCOUT HODGES: Discovery of truffles (*Tuber* species) in north Mississippi pecan orchards
(Under the direction of Jason Hoeksema)

This thesis describes a study conducted in the years 2019-2022, which aimed to discover a new species of truffle in the state of Mississippi. *Tuber lyonii*, more commonly referred to as the "pecan truffle," is an ectomycorrhizal fungus found on the roots of pecan trees in much of the southeastern region of the United States. Though there are many truffles native to the United States, the pecan truffle is one of the few that has been found to have high culinary value. Given the plethora of pecan orchards in the state of Mississippi, I decided to search for evidence of *Tuber lyonii* in Mississippi soil, and also attempted to use dogs as a means to truffle hunt in the pecan orchards. While truffle hunting with the lab's truffle dog, I took soil samples from several pecan trees in the different orchards. These samples were then investigated for ectomycorrhizal root tips, and tested through polymerase chain reaction to determine the DNA of the ectomycorrhizal fungi present. Additionally, I went truffle hunting in Tuscany, Italy with a truffle hunting expert, Giacomo Boccacini. While there I learned useful training methods to be applied to hunting with dogs in Mississippi. Through all of our efforts, we found evidence of several multiple truffle species in the pecan orchards, including *Tuber lyonii* in the Bounds Pecan Orchard. Though we were not successful in hunting the truffles with the dogs, the evidence shows that *Tuber* species are present in Mississippi. With more training of the dogs, and perhaps more knowledge of the fruiting season of *Tuber lyonii*, I think it very possible for dogs to be trained to hunt truffles in Mississippi.

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INTRODUCTION:

The genus *Tuber* of fungi, more commonly known as the "truffle," is a type of fungus that grows on the roots of many tree species in central-southern Europe, Asia, and America. The most notorious truffles are those grown in Italy, *Tuber magnatum,* and France, *Tuber melanosporum.* Often referred to by chefs as the "Diamonds of the Kitchen," these truffles are extremely lucrative for the Italian and French economies, given their rarity and culinary value (Mello et al. 2006). Chefs around the world import truffles from Italy and France in order to garnish their dishes with the exotic and expensive aroma of truffles. Those that are most desirable typically only grow within a four-month time span, giving harvesters of truffles an intense time limit to their work. They grow on the roots of oak, chestnut, hazelnut, and other hardwood trees (Hall et al. 2016). Hunters typically train dogs or pigs to find the truffles, given their incredible sense of smell that can detect the aromas of truffles that penetrate through the forest floor, in order to sniff out the potent truffle beneath. Truffles do not grow in abundance, and it is difficult to cultivate and artificially grow them, which contributes to their high cost (Hall et al. 2016). Until recent years, it had been thought that truffles of such high culinary value did not grow in eastern North America. However, there have been recent studies showing that pecan trees in the southeastern United States might support the growth of a different, yet culinarily valuable, species of truffle than those in Europe (Bonito et al. 2011). Discovery of this truffle species in Mississippi was the goal of my thesis project.

Truffles, like many other mushroom species, grow in the soil via ectomycorrhizal (ECM) relationships with trees. ECM symbioses are typically a mutually beneficial relationship between the two organisms (Pacioni et al. 1989). The fungus absorbs

nutrients and water from the soil and shares with the tree, while the tree creates carbohydrates via photosynthesis and shares with the fungus (Hoeksema et al. 2010). Through this relationship, fungi can prosperously grow and fruit, i.e., produce mushrooms or fruiting bodies, which are the sexual reproductive structures of these fungi. Some of these ECM fruiting bodies are edible, such as the truffle, and can be very lucrative for those who can find them.

The pecan (*Carya illinoinensis,* Juglandaceae) is a tree native to the region along the Mississippi River and the main tributaries in the American South and is a very important source of economic wealth to North America. The United States is the largest producer of pecans in the world. The annual gross value of 2015 for pecans produced was $560.2 million (Thomas et al. 2017). Pecans are commercially grown in Alabama, Arizona, Arkansas, California, Florida, Georgia, Kansas, Louisiana, Mississippi, Missouri, New Mexico, North Carolina, Oklahoma, South Carolina, and Texas (Thomas et al. 2017). Pecans are known to form ectomycorrhizal (ECM) relationships on their roots, which was discovered by Woodroof in 1933 (Gruppe II et al. 2018). Though the pecan tree is not used as a source of truffles in Europe, its characteristics are similar to the host trees of Europe where truffles are found, both in wild habitats and orchards (Ge et al. 2017). The truffle species *Tuber lyonii* has been found on the roots of pecan trees in New Mexico, Oklahoma, Kansas, Minnesota, Indiana, Missouri, Arkansas, Louisiana, Illinois, Wisconsin, Iowa, Ohio, West Virginia, Maryland, Massachusetts, Florida, Georgia, and Texas (see **Figure 1**, Grupe II et al. 2019).

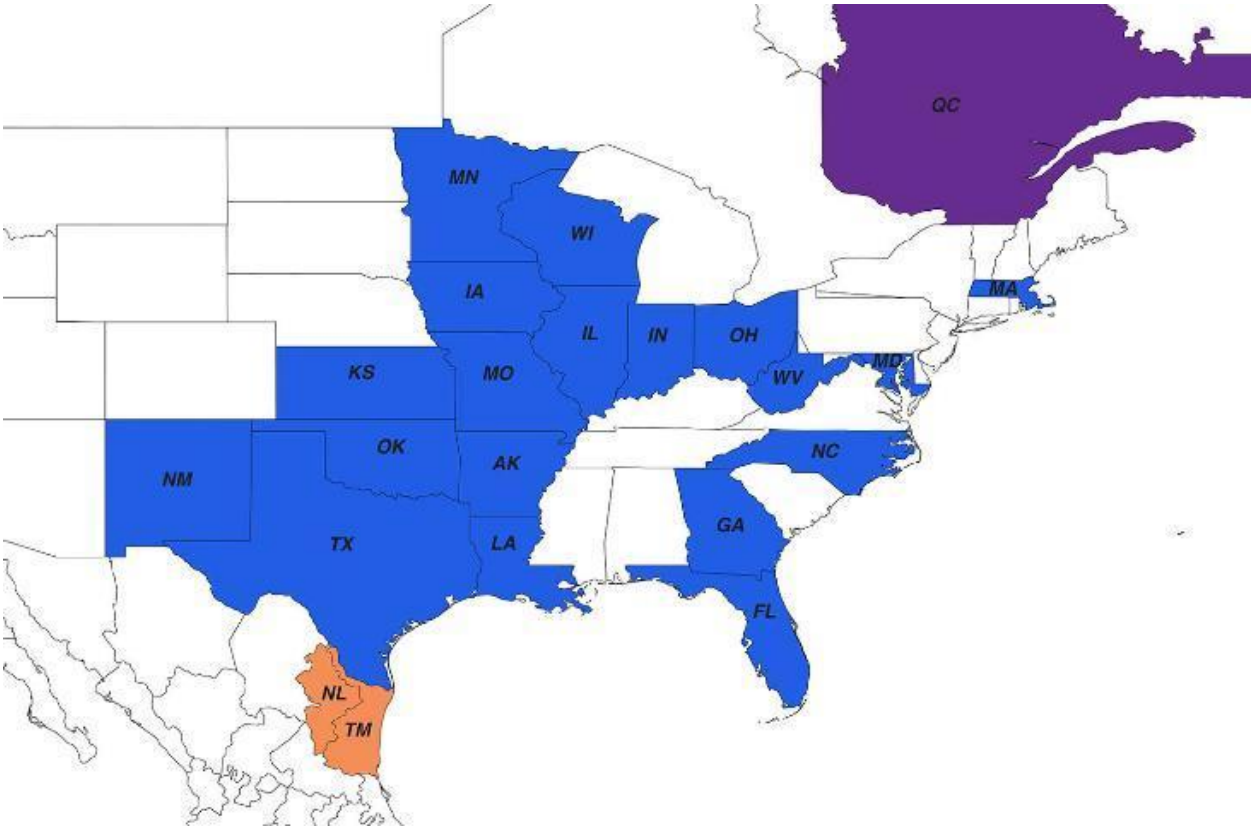

Figure 1. Map of the known distribution of *Tuber lyonii* in North America. The blue represents US states with known *T. lyonii* presence. The purple and orange indicate regions outside the United States with pecan truffle presence as well. (Grupe II et al. 2019).

Tuber lyonii, also known as the "pecan truffle," is a brownish truffle, typically smaller in size than white or black truffles, averaging between one to two inches (Trappe 1996, Gruppe II et al. 2019). *T. lyonii* belongs to the rufum clade of the genus tuber, according to a study done in 2010 by Gregory Bonito (Bonito et al. 2010). It has proven to hold some culinary value, as quite a few chefs across the southeastern United States have started to use it (Smith et al. 2012). *T. lyonii* is known for its rather earthy aroma and flavor, with more nutty undertones (Portman J 2015). So far, compared to its

European relatives, the pecan truffle has a much lower cost. Prices of pecan truffles today range from $10-20 per ounce (Gruppe II et al. 2019), whereas the European truffles range anywhere from $22/oz (Truffle Farm) to $375/oz (RobbReport). It is not clear, however, whether current prices of pecan truffles in the United States reflect a lower inherent culinary value, or perhaps result from a general lack of awareness of its potential value among restaurateurs and consumers in the region. Such awareness can only come with increased discovery of the pecan truffle in the region.

In order to optimize the discovery and potential production of pecan truffles, it is important to study the nature of the soil of pecan orchards, along with the production of ECM relationships, specifically those that produce truffles. There are still holes in the understanding of ECM fungi, particularly *T. lyonii*, in pecan orchards. Pecan orchards are often limed to bring the soil pH to a higher level (Sparks 1976). According to a previous study of ECM fungi of pecan orchards in southern Georgia, pecan orchards with higher pH had a greater presence of *T. lyonii* (Bonito et al. 2011). Other factors that could vary depending on the orchard, and may influence the abundance of pecan truffles, are additional soil characteristics beyond pH (such as soil texture), the age of the pecan trees, and additional plant species growing in or near the orchards (Healy et al. 2016).

While understanding what is going on in the soil is crucial to determining how truffles might grow under pecan trees, it is also important to understand how to find the truffles once they have grown. Europeans have relied on mammals such as pigs and dogs to hunt truffles for centuries. In fact, it has become a unique part of European culture to become a “truffle hunter.” In recent years, dogs in particular have been trained to find

truffles in North America, especially in the Pacific Northwest. The question remains if the same techniques could be used for the pecan truffles as well.

This research study was performed for the purpose of discovering whether or not *Tuber lyonii* mycorrhizae are present on the roots of pecan trees within the state of Mississippi, specifically in the northwestern region, and whether the truffles themselves are present. Pecan plantations are a staple to the economy of Mississippi, and it is possible, perhaps even likely, that *Tuber lyonii* grows across the state, unnoticed. Being that pecan orchards are already successful and abundant in Mississippi, it would be arguably easy to develop a system of harvesting truffles as well, should they be present. In an attempt to find *Tuber lyonii* in northwestern Mississippi, I sought to analyze the soil and roots of *Carya illinoinensis* orchards, and whilst doing so, I also studied whether trained dogs could be used to assist in the hunt and harvest of pecan truffles.

So, my project was designed to answer these two questions:

Question 1: Do the roots of *Carya illinoinensis* trees in northwestern Mississippi sustain an ECM relationship with *Tuber lyonii?*

Question 2: Can we use trained dogs to hunt pecan truffles that grow in Mississippi?

METHODS:

Overview:

During my research, I traveled to three different pecan orchards in northwestern Mississippi. At each site, I collected soil and root samples from multiple different trees. The samples were taken back to the lab, where I performed soil analyses and PCR and Sanger sequencing on the ECM root tips. Whilst collecting soil samples in the field, we also took out our lab dog, Charlie, to develop his ability to hunt truffles, and attempt to discover the truffles themselves. I also had the opportunity to spend a day in the field with a truffle hunter and his dog in northern Italy, to learn methods of training and hunting truffles, which can hopefully be applied here in Mississippi.

Field Sites and Field Collection of Soil:

On my first trip, I visited the Bounds Pecan Orchard (34.688749, -90.391730) in Tunica County, MS on September 15, 2019. It was a sunny day, with average temperatures around 77 F. The Bounds Orchard was very well kept, and rather dry. I haphazardly selected five different trees and took soil samples from the soil underneath the drip line (outer perimeter) of the branches. At first, I took samples in a 15cm diameter, about 10cm deep. However, at the third tree, I noticed that most of the roots were towards the surface of the soil, so I started to take samples 5cm deep, 15cm wide, and 30cm long. The second orchard visited was one in Yalobusha County near Water Valley, MS (34.2338889, -89.5911111). I traveled to the orchard on February 19th, 2020; the weather was partly cloudy and 43 F. The Water Valley orchard was unkempt, with several different species of trees present besides *C. illinoinensis.* Weather conditions were fairly rainy in the weeks leading up to the hunt. I took samples from five different

trees, each of which were 5cm deep, 15cm wide, and 30cm long. Finally, on October 2nd, 2020, I traveled to the Moon Lake Orchards (34.4177778, -90.5588889) in Coahoma County, MS, just east of the Mississippi River. Moon Lake Orchards has two sections to its land, one of which mostly contains pecan trees planted in the 19th century, the youngest being planted in 1860's. The other side of the orchard is all new trees, planted in 1967. For my study I took samples from the "Old" Moon Lake Orchard, under the older trees. The weather was sunny and 61 F. I took samples from 10 different trees. Under the first 6 trees I took samples that were 10cm deep, 10cm wide, and 20cm long. However, I noticed that there were more pecan roots along the surface of the soil around 4ft from the trunk of the tree, and that pecan roots were fairly sparse in the samples. So, for samples 7-10, I collected roots (not soil) near the top of the soil, one sample from each cardinal direction from the tree.

Soil Processing in the Laboratory:

All soil samples collected in the field were placed in plastic bags and transported in a cooler back to the Hoeksema lab at the University of Mississippi. The soil samples were kept refrigerated at 4 C in between the time they were collected and analyzed. The first step of the analysis was to extract roots from the soil, saving loose soil for analysis and washing remaining roots free of additional soil. Then I analyzed the roots underneath a microscope, looking for unique morphotypes of ECM fungi. As I did so, I kept notes and descriptions of the way that the tips looked and removed root tips from each root for molecular analysis. I selected root tips based on the different morphotypes and took at least two samples of each type of morphotype found in each sample, based on color and texture, which were then placed in separate wells of a 96-well plate, for molecular

analysis. For Bounds, I took 2 root tips of each morphotype per sample. For each of the Water Valley and Old Moon Lake samples, I collected three root tips per morphotype per sample.

Root tip DNA was extracted using the Sigma Extract-N-Amp Tissue Kit, with the following protocol: 10 ul of the Sigma Extraction Buffer was added to each well (each root tip), each plate was heated in a thermocycler at 65°C for 10 minutes, then 95°C for 10 minutes, and then 30 ul of Neutralization Solution was added to each well. The wells were then stored in a freezer at -20°C. I then performed polymerase chain reaction (PCR) for amplification of the fungal DNA. In doing this I used primers specific to fungi: ITS1-F and ITS4 (Henrion et al. 1994). The PCR reaction was 8 ul, including 2.2 ul water + 4 ul of 2X Red Taq Premix + 0.4 ul ITS1-F (10uM concentration) + 0.4 ul ITS4 (10uM concentration) + 1 ul DNA extract. Thermocycling for PCR included these conditions: Initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 53°C for 45 seconds, and extension at 72°C for 60 seconds, ending with the final extension at 72°C for 10 minutes. Once the plate was removed from the thermocycler, it was centrifuged briefly and stored at -20°C. The PCR products were then tested for success in a 1% agarose gel with SYBR® Safe DNA gel stain. The PCR products were then enzymatically cleaned of unincorporated nucleotides and excess primer using ExoSAP-IT, as follows: 0.25ul of ExoSap-IT was diluted with 4.75ul of PCR-grade water and then 5ml of PCR product was added. Every sample was then placed in the thermocycler and heated for 30 minutes at 37°C, 20 minutes at 80°C, and 5 minutes at 4°C. Sanger sequencing was performed using ABI Big Dye Terminator Sequencing Kit (v3:1). The Big Dye reactions each consisted of 0.4ul Big Dye Reaction

Pre-Mix, 1.8 ul Big Dye 5x sequencing buffer, 0.5 ul of the primer (10mM stock concentration), 6.3 ul of sterile PCR-grade water, and 1 ul of the cleaned PCR product. For the Water Valley and Old Moon samples, Sanger sequencing was performed using both ITS4 and ITS1-F, with the same protocol as listed above. For Bounds, only ITS1-F was used for the initial Sanger sequencing process, but unidentified samples (after examination of results from the initial round of sequencing) for both Bounds and Old Moon were subsequently sequenced with both the forward and reverse primers. For the Sanger sequencing, the samples were placed in the thermocycler for 1 minute at 96°C, then placed through 35 cycles of denaturation at 95°C for 20 seconds, annealing at 51°C for ITS4 for 20 seconds or annealing at 52°C for ITS1-F, and extension at 60°C for 4 minutes. The reactions were all then air dried at 4°C and then shipped to the DNA Lab in the School of Life Science at Arizona State University (Tempe, AZ, USA) or to Functional Biosciences (Madison, WI, USA) where they were purified and read on a capillary genetic analyzer.

Computer methods:

Raw sequences were imported into Geneious software, where individual bases were edited as much as possible, then the low-quality ends were trimmed off. Samples with successful sequences in both directions (i.e., with both ITS1-F and ITS4 primers) allowed for *de novo* assembly, which compared the complementary strands to each other, to improve the accuracy of the sequences, given that it often provided solutions to ambiguities. Sequences with fewer than 150 bases or more than 3% ambiguities were removed from the sample dataset. The sequences passing these criteria were then assembled into operational taxonomic units (OTUs) with the CAP3 software package

(Hung and Madan, 1999), using default parameters with the exception of the following changes: h = 60 (max. % overhang length), m = 6 (match score factor), p = 97 (overlap % identity cutoff), y = 6 (clipping range). This stage of analysis was performed to sort the various sequences into operational taxonomic units (OTUs), including contigs (OTUs that appeared more than once) and singlets (OTUs that appeared only one time). After this sorting, the best sequence representatives of each OTU were submitted to the UNITE fungal sequence database using the BLAST operation (Abarenkov, Kessy, et al., 2010). At this point in the research it was discovered that the Water Valley soil samples had been contaminated, and those data were removed from further analysis.

The results from the UNITE comparison were then reviewed with several criteria. Sequences that matched at 99% or higher were assigned likely taxonomic identities at the species level. The sequences with matches between 95%-98% accuracy were assigned the genus level identity. And lastly, sequences that matched between 90%-94% were assigned family level identities. The queries with matches less than 90% accuracy were removed from further analyses.

Soil processing:

There were two different tests I performed on the soil samples that I collected for this study. I first tested the pH of the soil samples. I did this by mixing ~15.0 g of soil with 25ml of dI water. I then took the pH of that sample and repeated this process 3 times for each soil sample. The average of the three pH measurements was the estimated pH I analyzed for the study. The second soil analysis taken was the soil texture. This test was performed using the LaMotte Soil Test Kit (Code 1067). The soil sample was placed in the provided testing tube up to the 15mL mark. I then added 1mL of Texture Dispersing

Reagent (5644PS) to the sample and diluted it with water up until the 45 mL mark. The mixture was then mixed thoroughly for 2 minutes. I then allowed the tube to sit undisturbed for 30 seconds, and a measurement of the soil level was taken, resulting in the sand level. The remaining liquid was poured into a second provided tube. This tube was allowed to stand undisturbed for 30 minutes. The reading after this session resulted in the sand level. The remaining calculation was to subtract the sand and silt content from total grams of soil (15g) in order to determine the clay content.

These tests were performed only on the soil samples form which I found roots to analyze. For example, though soil samples were taken at 5 trees throughout the Old Moon orchard, I only found roots in Old Moon tree 3 and 4. Therefore, samples 1, 2, and 5 do not have soil analyses. Every sample taken at Bounds was analyzed for pH and soil texture.

Observations on dogs hunting truffles in the wild:

Mississippi. Charlie is a chocolate lab and pit-bull mix, approximately 4 years old. Since the summer of 2019, Charlie had been undergoing training to hunt truffles in the woods. During our excursions to pecan orchards, *Tuber lyonii* specimens (purchased from a vendor in North Carolina) were placed in small plastic containers (film canisters) with holes in the top. Charlie was then kept out of view, while the canister of *T. lyonii* was hidden somewhere amongst the dirt beneath the trees, typically in a shallow hole with a small amount of soil placed over the canister. Several "decoy holes" were dug throughout the terrain, to encourage Charlie to truly use his ability to sniff out the truffles based on their unique aromas. Charlie would be released into the orchards, and was commanded to "Find the truffle, Charlie!" These efforts with Charlie were made at the

Bounds pecan orchard on September 15, 2019 and at the Old Moon River orchard on October 2nd, 2020 and November 14, 2021.

Italy. In order to better understand the practice of hunting truffles with a dog, I met with Giacomo Boccaccini, a trained truffle hunter and chef in Tuscany, for a truffle hunt lesson, on 21st of December 2021. Alongside his trained cocker spaniel, Lupo, we hunted for truffles in the Chianti region of Tuscany. Boccaccini led me through the ins and outs of using a trained hunting dog to hunt for truffles. Through centuries of tradition and years of practice with Lupo, Boccaccini has found a successful way by which to lead a dog on a truffle hunt. During this experience, I kept careful notes on the methods used, and conversed with Mr. Boccaccini in Italian when possible.

RESULTS:

Question 1: Do the roots of *Carya illinoinensis* trees in northwestern Mississippi sustain an ECM relationship with *Tuber lyonii?*

There were a total of 38 fungal OTUs found across the sites. There were 21 fungal OTUs at Bounds, including *T. lyonii*. In sample 1, the OTUs identified were *Inocybe radiata*, Heliotales, Coprinellus, and Dothideomycetes. In sample 2, the OTUs identified were Tomentella, Periconia, and three different OTUs of Thelephoraceae. At sample 3, *Tuber lyonii* was found along with two other Tuber OTUs. At sample 4, Omphalotaceae and three OTUs of Thelephoraceae were identified. In sample 5, Thelephoraceae was found in two OTUs, along with *Rhodotorula mucilaginosa*, a basidiomycete yeast. Additionally, samples of Inocybaceae and Inocybe were present. Thelephoraceae was the most frequent fungus identified throughout Bounds, given its presence at ⅗ of the sample trees. As seen in **Figure 2A**, every OTU was found only once throughout the entire site. There were 8 different Thelephoraceae OTUs present within the Bounds site. The soil characteristics were relatively the same throughout the 5 tree sites, averaging 63.3% sand (SE= 0.01498), 30.9% silt (SE= 0.01183), and 5.8% clay (SE= 0.01492). The pH across the sample trees averaged 6.49 (SE= 0.163).

At the Old Moon site, there were 18 fungal OTUs. Samples at Old Moon 1, 2, and 5 did not have any identifiable fungi. At sample 3, *Inocybe radiata*, *Rheubarbariboletus persicolor*, *Rhodotorula mucilaginosa*, and Atraciellales were found. At sample 4, the OTUs identified were Tuberaceae and *Rheubarbariboletus persicolor*. The OTUs found in sample 6 were two different Scleroderma OTUs and *Tuber mexiusanum.* At sample 7, the OTUs identified were two different OTUs of Moriterella, two different OTUs of

Scleroderma, Tuberaceae, Inocybe, and Tuber. At sample 8, the OTUs identified were *Tuber mexiusanum, Rheubarbariboletus persicolor*, two OTUs of Boletaceae, and Mortierella. Within sample 9, the OTUs identified were Tuberaceae, Inocybe, *Rheubarbariboletus persicolor*, two different identifications of Dactylonectria, and Auriculariales. At sample 10, the OTUs found were *Rheubarbariboletus persicolor, Tuber mexiusanum,* Tuberaceae, and Inocybe. As seen in **Figure 2B**, the OTU with highest frequency was *Rhubarbiboletus persicolor.* The soil content was consistent throughout the sample sites, averaging at 55% sand (SE= 0.05), 40.1% silt (SE= 0.0335), and 4.9% clay (SE= 0.0165). The pH at the different sample trees averaged 5.52 (SE= 0.082).

The average OTU count per sample at Bounds was 4.2. The average OTU count per sample at Old Moon was 2.9. Only one OTU, *Inocybe radiata,* was found at both Old Moon and Bounds. The average OTU per sample throughout the study was 3.5. Truffle ECM (i.e., belonging to the genus *Tuber* or the family Tuberaceae) were found in 6 of the 7 samples at Old Moon, and in 7/12 total samples within my study. *Tuber lyonii* was found in only one sample, in the Bounds site. *Tuber mexiusanum* was found within three different samples at Old Moon. There were also three different Tuber OTUs and a Tuberaceae OTU that were not specified to species. *Rheubarbariboletus persicolor* was identified in 5 different samples. 8 different OTUs of Thelephoraceae were identified throughout the study.

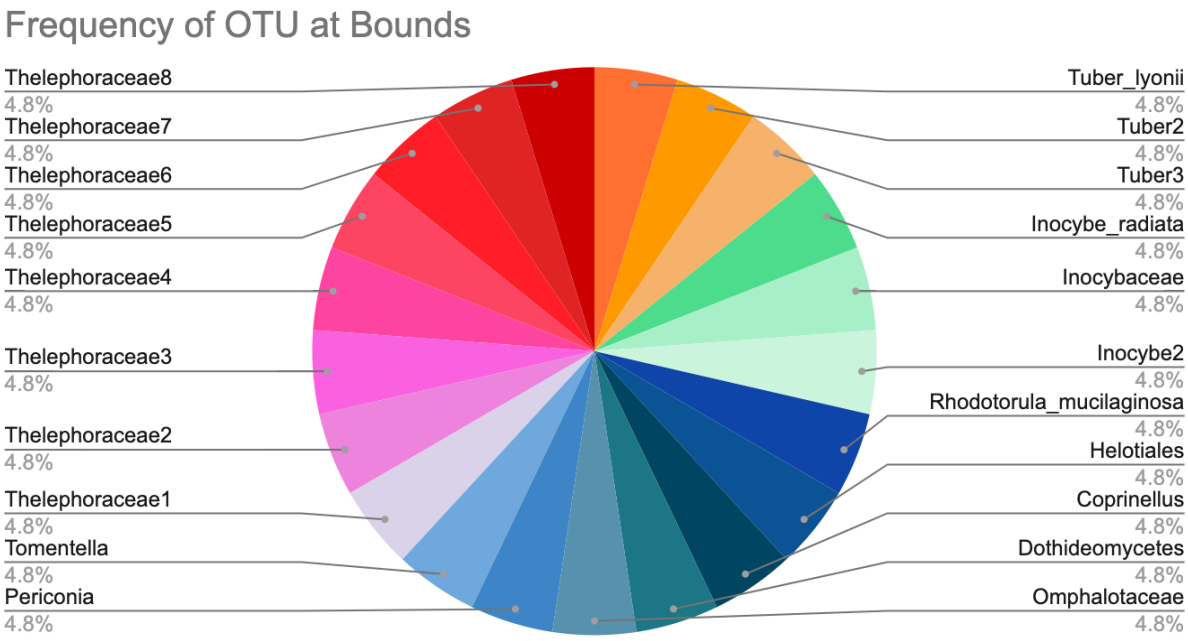

Figure 2A. This pie chart shows the frequency of OTUs at the Bounds site.

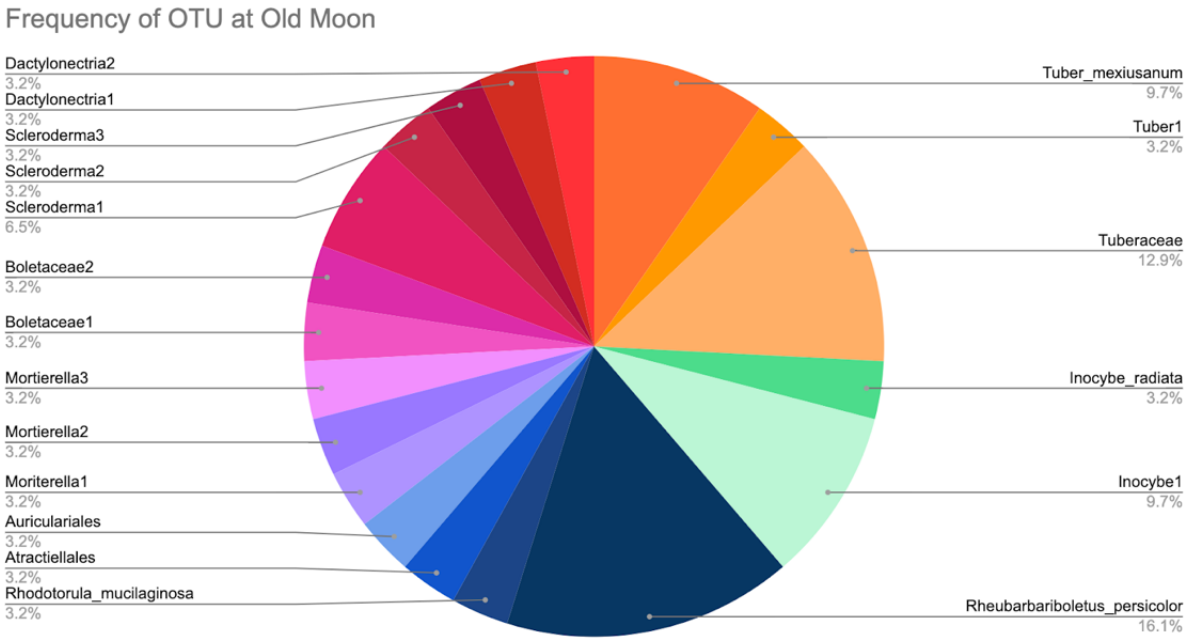

Figure 2B. This pie chart shows the frequency of OTUs at the Old Moon site.

Question 2: Can we use trained dogs to hunt pecan truffles that grow in Mississippi?

Mississippi. We trained Charlie consistently at the sample sites, with immense success on his practice runs. However, when it came to the field studies themselves, Charlie was unable to find a truffle in the wild. When we took Charlie to the field sites to hunt for truffles, we also trained him throughout the fields. We were unsuccessful in leading Charlie to hunt out a wild truffle. We released Charlie into the orchard, commanding him to "Find the truffle!" Charlie often ran around smelling the ground, checking around trees and out in the open grass of the orchard. We waited for him to stick to one spot, or perhaps begin digging, but to no avail.

However, we used the fields as opportunities to train him in hunting out *T. lyonii*. We found that Charlie was able to find the practice truffle successfully nearly every time. When we held Charlie back, we hid him from view of where the truffle canisters were being hidden. This seemed successful given that when he was released, he relied on his nose to determine where we had hidden the truffle (**Figure 3**). Our command to "Find the truffle!" worked well to guide him on what he was being commanded to do. Charlie would wander around the orchard smelling out the truffles we had hidden. He often found the truffle canisters within 5 minutes or less of being released into the orchard. We did not reward him with pieces of truffle; however, he was often rewarded with traditional dog treats.

Figure 3. Scout allowing Charlie to sniff out the *Tuber lyonii* that would then be placed in a canister and hidden in the fields at Old Moon River Orchards.

Italy. Studying under Giaccomo Boccacini, I learned several things that are crucial to the process of truffling with a dog. Alongside Mr. Boccacini's trained cocker spaniel, Lupo, we hunted for truffles in the Chianti region of Tuscany. Lupo has been hunting truffles for several years now, though he has not reached expert level. Boccaccini led us through several hills and trails all while Lupo bounced from tree to tree sniffing for the truffle. Rather than let Lupo run off and search alone, Boccacini kept him on a tight leash, leading him through the woods. Should Lupo hesitate at a specific spot or stick to one area, Boccacini would lead him away and bring him back several times, beckoning him to check the spot over and over again. According to him, this helped Lupo focus on one scent he caught, rather than get overwhelmed, and move on without investigating further. Because the truffle is buried under ground, the dog often moves on if the scent is

not strong enough, so bringing it back to the same spot again and again helped Lupo focus. If Lupo became agitated or started to dig, Boccacini would call him off and use a small shovel and rake to sift through the dirt himself (**Figure 4A**).

Several factors were considered when choosing the location of our hunt. It was mid-December, which is the prime season for *Tuber uncinatum chatin*. *T. uncinatum*, often referred to as the "burgundy truffle" or "autumn truffle," is similar in composition and morphology to *Tuber aestivum* (Paolocci et al. 2004, Benucci et al. 2012). It often grows at the base of oak trees in Italy. When hunting for truffles, Boccacini does not simply guide Lupo from tree to tree. He often searches for other plants that indicate an ecosystem in which *T. aestivum* would flourish. These plant species include *Laurus nobilis* (Patrakar et al. 2012), Rosa canina l. (Ilyasoğlu et al. 2014), Ruscus aculeatus (Hadžifejzović et al.2013). These plants flourish around healthy oak trees, which to truffle hunters often indicates the presence of truffles. Boccacini was careful to guide his dog to the trees around which he saw these indicator plants.

Lupo was successful in his hunt twice that day. Both times, he was rewarded with a piece of truffle (either a rotten portion of one we found, or one that Boccacini packed along). After extracting the truffle from the ground, Boccacini replaced the soil he had dug up (**Figure 4B)**, explaining that this helped more truffles to grow in the same place in the future, and was an important part of the process of legal truffle hunting in Italy.

Figure 4A. Giacomo Boccacini and dog, Lupo, dig through soil for truffle.

Figure 4B. *Tuber aestivum* in ground where Lupo hunted it.

DISCUSSION:

Overview. Multiple ECM fungi (38 OTUs altogether, and at least 18 at each site) were found across the sites. Multiple species of *Tuber* were present as well, though some could not be identified at the species level. *Tuber lyonii*, the pecan truffle and the main target of our study, was clearly present in the form of mycorrhiza at the Bounds location. However, we did not find any actual truffles when searching with a trained dog at any of these sites. Truffle hunting with Giaccomo Boccacini was extremely informative in the habits and practices of truffle hunting with dogs. Several of the techniques I learned on the field trip in Tuscany will be helpful to use when training truffle dogs in the state of Mississippi. Though we were unsuccessful hunting for truffles in the wild with Charlie, we have the evidence to show that they are out there.

Question 1: Do the roots of *Carya illinoinensis* trees in northwestern Mississippi sustain an ECM relationship with *Tuber lyonii?*

The most significant result of this project was finding the presence of *Tuber lyonii* at one of the sites. This was the hard evidence needed to conclude that it is possible for pecan trees in northwestern Mississippi to sustain an ectomycorrhizal relationship with *Tuber lyonii.* The average pH at bounds was 6.49. This was nearly an entire point higher than the average pH of Old Moon, which was 5.52. According to the study done by Ge et al. (2017), there is correlation between pH and presence of *T. lyonii.* That study found that in orchards of higher pH, *T. lyonii* were more common and successful. This observation aligns with the evidence found within this study. The low pH of the Old Moon site could help explain the absence of *T. lyonii* in our samples collected there.

The Bounds site not only had a higher average pH than Old Moon, but it also had a greater number of OTU identifications, with Bounds resulting in 21 (from only 5 samples) and Old Moon in 18 (from 10 samples). There was a consistent presence of Thelephoraceae, Inocybe, and *Rheubarbariboletus persicolor.* This result confirmed that the soil of pecan trees can sustain diverse ECM relationships. One thing I found surprising was the presence of six different *Tuber* OTUs throughout the study. *T. lyonii* was present at only one sample within Bounds, though there were two OTUs at that same sample, identified only to the genus of tuber. Truffle OTUs (likely representing five additional *Tuber* species) were present in 6/7 samples at the Old Moon site. This high diversity of Tuberaceae fungi could be due to a number of reasons, be it soil texture, soil pH, or the old age of the trees. Within Bounds, there were 8 different OTUs of Thelephoraceae, as well. This contributed to 38.1% of the OTUs at Bounds. Bounds was interesting because each OTU had the same frequency throughout the site.

Old Moon is a site near the Mississippi River. This location allows for potential interaction with more ecosystems than the Bounds site, which was deep inland. For example, the proximity to the Mississippi River could explain the presence of *Tuber mexiusanum* at Old Moon. *T. mexiusanum* is a recently discovered species (Guevara et al. 2013) that has been found throughout a wide geographic range, namely, Mexico, eastern United States, Minnesota and Iowa, and it is possible that the orchards proximity to the Mississippi River provides more biodiversity, leading it to possess truffles such as *T. mexiusanum*. The Mississippi River could have picked up the spores of truffle species and carried them to Old Moon. Old Moon's age could also contribute to the presence of diverse Tuberaceae ECM fungi, given that some of the pecan trees have been there for

over one hundred and fifty years; perhaps Tuberaceae fungi thrive on the roots of older pecan trees.

One primary limitation to the study was time. The interruption of COVID-19 was disrupting; however, it did not alter any of my data that I know of. Another limitation of the study was our lack of knowledge of *T. lyonii* at the beginning of the project. There is still so much to learn about the fungus that it was sometimes hard to follow a strict protocol. The project was a learning process, in which I altered methods and procedures according to how best to continue and learn more about the target organisms. For example, rather than collect large soil samples at Old Moon, I altered the method to collect root from the topsoil. This seemed successful due to the fact that so many OTU's were found on the roots selected. Another caveat to the study was the disposal of the Water Valley data due to contamination; addition of those samples would have provided information about the potential presence of Tuberaceae fungi in a small upland pecan orchard, different from the two larger orchards we studied in the Delta.

Question 2: Can we use trained dogs to hunt pecan truffles that grow in Mississippi?

Charlie consistently found truffles hidden, throughout our many tests and exercises, including when the lures were buried, suggesting that he would be successful in finding real wild truffles if they were present. However, when it came to hunting the truffles in the wild, we were unsuccessful with Charlie. There are many factors that could be impacting the success of Charlie's hunts. First and foremost, it has not been determined when the peak season for hunting truffles is in the state of Mississippi. We went truffle hunting on three different days, ranging from mid-September to mid-

November. Although we found evidence of *Tuber lyonii* in Bounds on September 15, we were unsuccessful in finding the actual truffles. In recent studies in Georgia and Florida, it has been observed that the peak pecan truffle season is in August and September, lasting throughout the fall and into January (Smith et al. 2012). This suggests that our hunting excursions were performed at appropriate times according to studies in Georgia and Florida. However, it could be that *T. lyonii* in Mississippi fruit in later months than in Georgia and Florida. In the future, I would suggest trying dates later into the fall and winter, given that we found the root tips of *T. lyonii* in September.

The mere lack of experience hunting wild truffles could also impact the success of the hunts, as we may not have optimally directed him during the hunts. For example, there may have been distractions that could take Charlie's focus away from the hunt itself, which could be better managed in future attempts. There are several techniques that Boccacini used with Lupo that I think would help contribute to the success of truffle hunts with Charlie.

Boccacini and Lupo taught me several techniques/practices that seemed to ring out as the most important to be applied to the practices here. First, Boccacini limited distractions for Lupo on the hunt. Though we had a hunting party of around 15 people, Lupo and Boccacini were often alone or with me, at least 20 yards away from the rest of the group. This was to limit the distractions of smells for Lupo, so that he could focus on sniffing out the truffle alone. Most wild environments that one can find truffles in are bounteous with biodiversity. This alone can contribute to distracting smells for the dog truffle hunting. For this reason, Boccacini attempts to limit scents that would pique his dog’s curiosity when hunting.

The second practice of Boccacini was his identification of what he called "friendly plants." These were plants that in his experience were often found with *Tuber uncinatum chatin*. In his experience, he had often found the autumn truffle near trees with plants such as the bay leaf (*Laurus nobilis)*, rose hip (*Rosa canina l.*), butcher's broom (*Ruscus aculeatus*), and wild asparagus (*Asparagus officinalis*) at the base. His understanding of these botanical associations was based on habitual practice rather than science, though I suspect there is scientific evidence to support his hypothesis. This practice could hypothetically very easily be applied to Mississippi. However, studies would need to be done to understand what exact plants grow in areas with *Tuber lyonii.*

The third technique that stood out to me was the way in which Boccacini would lead Lupo through the field and guide him to spots that could have truffles. Rather than releasing Lupo into the forest to search at his own will, Boccacini led him through the terrain, on a leash. By doing this, Boccacini was able to lead Lupo over the specific spots where truffles were most likely to be growing. Boccacini closely monitored Lupo's behaviors and reacted to any behavioral change by returning to the spot where that occurred. This relationship between master and dog was key, according to Boccacini. His ability to identify when Lupo might have smelt something only comes through years of practice and a dynamic relationship. Boccacini would lead Lupo back to the spot where he smelt something repeatedly, forcing Lupo to really get a sense of what the smell could have been. This method was successful twice, and Lupo began digging rapidly to try to reach the truffle.

Lastly, was the aftermath of finding the truffle. Boccacini would reward Lupo with a piece of truffle, either from one he had packed along with him or from the

ground. This was a crucial step, because it taught the dog that it would be rewarded with the exact scent it found.

All of these techniques could be applied to the practice of truffle hunting with dogs in Mississippi. The methods and practices of Italian truffle hunters have been tried and true through centuries of tradition and history. These techniques are not necessarily specific to *Tuber uncinatum chatin* and could be applied to the practice of hunting *Tuber lyonii* in the state of Mississippi.

CONCLUSIONS:

Given the discovery of *Tuber lyonii* mycorrhiza on the roots of pecan trees at Bounds, our hypothesis that *Carya illinoinensis* in Northwestern Mississippi could sustain an ectomycorrhizal relationship with *T. lyonii* is not rejected. This was extremely exciting given that we now can confirm there is evidence of pecan truffles in the state of Mississippi, but now we must determine how to find them when they are ripe. For further study, I would certainly recommend visiting more sites. Taking soil samples from the sites was beneficial given the study of pH, but I would take more soil samples at each site, as well, to allow correlation analyses with the presence and abundance of particular OTUs. Additionally, I would check the same sites (and the same trees if possible) at different times throughout the year. One difficult aspect of studying *T. lyonii* is the lack of knowledge possessed about the fungus. Particularly, it is unclear when the best season of fruiting is for the species.

Which leads to the other question, whether or not dogs can be used to hunt pecan truffles in the state of Mississippi. Given the lack of success in hunting the pecan truffle, I can make no conclusion regarding this topic. However, I think that with more training of the dogs and more knowledge acquired regarding the truffle, and especially the timing of its fruiting, it could be possible. For example, I think dogs should be taken out to the orchards and used to hunt with the methods of Giacomo. However, one key aspect to the Italian hunt is that they are certain of when their truffles fruit. Therefore, more studies are needed.

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