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ASSESSING THE ROLE OF MATING ON *MORCHELLA IMPORTUNA* AND *MORCHELLA RUFOBRUNNEA* ON YIELD POTENTIAL

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ABSTRACT

Morels, *Morchella spp*., are edible mushrooms collected worldwide, including in the Northeastern United States. They are highly prized for their culinary, nutritional, and medicinal value. Dried morels sell for as much as $415.68 per pound in US grocery stores and many of these morels are imported from China. Since 2012, a successful outdoor cultivation strategy that supports vegetative mycelial growth using sterilized plant polysaccharides such as sawdust, wheat, and corn kernels contained in a plastic bag launched the Chinese morel cultivation industry then rapidly expanded. The Chinese growers call it the nutrient bag. This Chinese nutrient bag technique is the only cost-effective production model for morel cultivation. In 2017, the Pecchia Lab at Penn State imported this cultivation strategy and successfully cultivated *M. rufobrunnea* indoors in 2019. Building on this, this work tested the effect of mating on yield potential by replicating the Chinese nutrient bag technique outdoors in a shaded structure (a high tunnel) and indoors in grow rooms with a controlled environment. The experiments were done with two species with historic fruiting success - *M. rufobrunnea* and *M. importuna* and demonstrated that *M. importuna* with either *MAT 1-1-1* or *MAT 1-2-1* strains were capable of fruiting as well as mixed. *M. importuna* strains appeared heterothallic, while *M. rufobrunnea* strains appeared homothallic. These data will help growers establish morel cultivation sites and manage the cropping procedure to produce consistent yields, making morel farming profitable. The specific aims of this project are: 1. to grow morels indoors in a growing room and outdoors in a high tunnel in the Northeastern United States (State

College, PA), and 2. to determine the role of mating on fruiting body formation of *M. importuna* and *M. rufobrunnea*.

Keywords: *Morchella*, morel cultivation, mating-type, ascocarp, fruiting body, conidiation, nutrient bag

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CHAPTER 1 Literature Review

Morel's value around the world

Why are morels so loved and expensive? Morels, *Morchella* spp., are edible mushrooms with high nutrient, culinary, and medicinal values. Wild morels' short fruiting season and desirable flavor made them the world's most prized edible fungi (Du & Zhao., 2015).

As delicious, nutritious, and medicinal mushrooms, wild morels are constantly in shortage on the market (Du & Zhao., 2015). Dried export-grade morels are sold for $160 USD per kg on average (Du & Zhao., 2015), and can reach $915.59 per kg ($415.68 per pound).

Morels have a distinct taste, aroma, and texture that give them a special culinary value. Their distinct intense meaty and nutty tastes are desired by chefs and gourmet enthusiasts worldwide. These distinct tastes are from the rich monosodium-glutamate-like (umami) compounds, quantified by Equivalent Umami Concentration (EUC) (Tsai *et al*., 2006). *Morchella* spp. are rich in EUC. For example, *M. esculenta* contains 351.7 - 374.5g EUC / 100g dry weight (Tsai *et al*., 2006), compared to *Agaricus bisporus* (button mushrooms) with 207 - 284g EUC / 100g dry weight (Tsai *et al*., 2006). In comparison, soy sauce, a benchmark for EUC, has 15.34 – 285.83 g EUC / 100ml (Lin *et al*., 2015).

As delicious mushrooms, morels are also nutritious. *M. crassipes*, *M. esculenta*, *M. hortensis*, *M. conica* (later identified as *M. deliciosa*, *M. purpurascens*, *M. tridentina* and *M. vulgaris*) (Richard *et al*., 2015), and *M. elata* were analyzed for

nutritional values and reported to contain 7.5 - 11.52g protein, 2.2 - 3.9g fat, 6.7 - 14.6g ash, and 74.55 - 80.5g carbohydrates / 100g dry weight. In comparison, *A. bisporus* has 1.57 - 1.69g protein, 0.32 - 0.34g fat, 0.77 - 0.87g ash, and 5.18 - 5.30g carbohydrates on a dry weight basis (Manzi *et al*., 2001). *Morchella spp*. are also rich in amino acids. Wu *et al*. 2011 analyzed wild and cultivated morels and found that the average amino acids in fresh morel ascocarps are 16.19 - 19.50g / 100g, with no significant difference between morel species, wild or cultivated (Wu *et al.*, 2011). In contrast, total amino acid concentrations in fresh button mushrooms are 18.02 g / 100 g and 71.39 - 81.17g / 100 g in flageolet beans (Jaworska *et al.*, 2013). However, the mechanism or active ingredient was unknown.

Morels are not only considered delicious and nutritional mushrooms but also highly desired for their medicinal value, especially in Asia (Tsai *et al*., 2006). In traditional medicine, morels are used for rapid healing, antiseptic, digestive system symptoms, immunostimulant, and as a general tonic. (Mahmood *et al*., 2011). Today, morels and their mycelium or mycelium extract are consumed as a nutraceutical, or functional food with anti-hepatotoxic, nephroprotective, anti-inflammatory, anti-oxidative, and antitumor properties in mice (Tietel & Masaphy, 2018). Nitha *et al.* 2013 induced hepatotoxicity with carbon tetrachloride and ethanol, which drastically elevated the enzyme activity of liver functions of mice. The treatment with aqueous-ethanolic extract of cultured mycelia of *M. esculenta* decreased such levels of liver enzyme activity in a dose-dependent manner. Therefore, the authors reported that morels have demonstrated anti-hepatotoxic and hepatoprotective activity from an

aqueous-ethanolic extract of cultured mycelia of wild *M. esculenta* against carbon tetrachloride and ethanol-induced chronic hepatotoxicity in mice (Nitha *et al*., 2013). Nitha and Janardhanan 2008 induced renal failure in mice with cisplatin and gentamicin. These mice had a significant increase in serum urea and creatinine. When the mice were treated with an aqueous-ethanolic extract of *M. esculenta* mycelia, their serum urea and creatinine levels decreased, and their antioxidant defense system was restored. This experiment suggested that morel mycelial extract have nephroprotective effects against cisplatin and gentamicin-induced nephrotoxicity in mice (Nitha & Janardhanan, 2008). Nitha *et al*. 2007 treated mice with acute and chronic inflammation with ethanolic extract of cultured *M. esculenta* mycelium and reported that on a dose-dependent usage, morels demonstrated anti-inflammatory effects for both acute and chronic inflammation (Nitha *et al*., 2007). The same inhibitory effects are also reported on intracellular oxidation status in HT-29 colon cancer cells (Kim *et al*., 2011). An extract of cultured mycelia of *M. esculenta* was used to treat mice with Dalton's lymphoma ascites (DLA) cell line-induced tumor and Ehrlich's ascites carcinoma (EAC) cell line-induced tumor and showed significant anti-tumor activity against both ascites and solid tumors (Nitha et al., 2007).

Morchella **taxonomy and global distribution**

Morel is the common name for *Morchella spp*. The genus *Morchella* is in the Morchellaceae family, order Pezizales, class Pezizomycetes, and division Ascomycota (Du & Yang 2021). Wild morel ascocarps were previously identified based on

morphological traits such as color, size, and shape. These traits can vary even within the same species at different stages of growth (Volk & Leonard 1989). With the advancement of molecular biology, accurate identification of cultivatable species was developed through DNA sequencing. Taylor *et al*., 2000 suggested that genealogical concordance phylogenetic species recognition, or GCPSR, a multi-locus phylogenetic recognition method, should be adopted when identifying *Morchella spp*. (Taylor et al., 2000). O'Donnell *et al*., 2011 first applied GCPSR of internal transcribed spacer (ITS), the largest and second-largest subunits of RNA polymerase, RNA polymerase I (RPB1) and RNA polymerase II (RPB2), or translation elongation factor 1α (EF-1α) for *Morchella* identification (O'Donnell *et al*., 2011), and this method was extensively used later for *Morchella* identification (Du & Yang 2021). Molecular sequencing of *Morchella spp*. through GCPSR revealed three discrete clades: black morels (Elata clade), yellow morels (Esculenta clade (O'Donnell *et al*., 2011), and blushing morels (Rufobrunnea clade) (Richard *et al*., 2015). Phylospecies that have not been formally named are assigned Mes (Esculenta clade) and Mel (Elata clade) followed by a unique number (O'Donnell *et al*. 2011; Richard *et al*. 2015). To date, as shown in Figure 1-1, 75 *Morchella spp*. were identified with GCPSR, and 348 *Morchella* spp. registered on Index Fungorum, the largest fungi database (indexfungorum.org/Names/Names.asp).

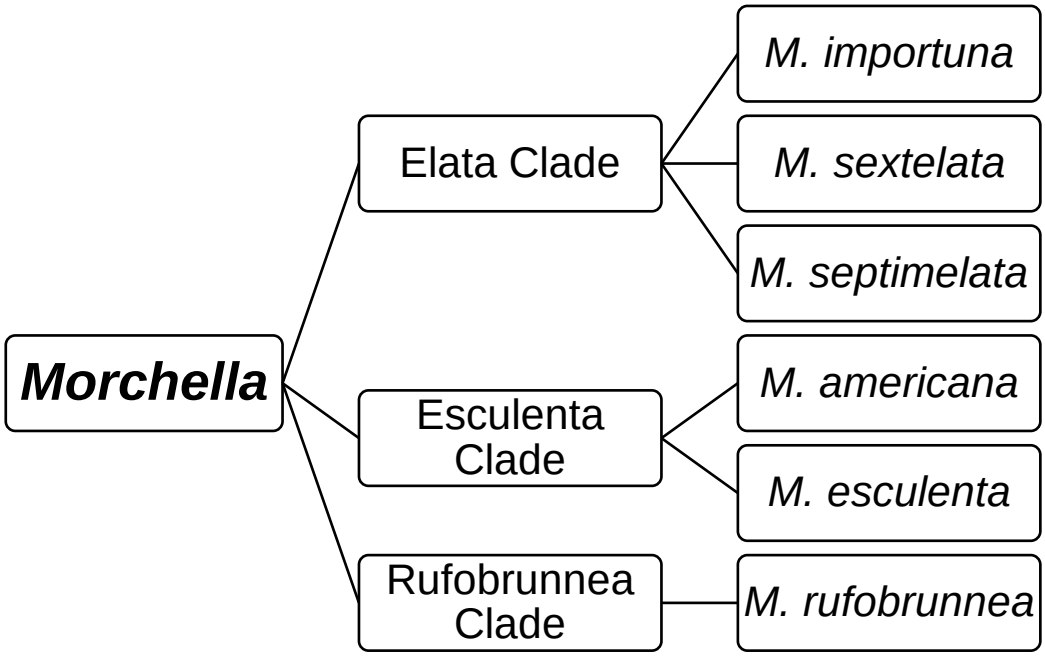


Figure 1-1. Distinct phylogenetic clades and common species harvested or foraged of the genus Morchella

While identifying *Morchella* spp., they were thought to exhibit continental endemism and provincialism (O'Donnell *et al*., 2011). Of the *Morchella spp*., 77.6% are specific to continents, while 22.4% were found on multiple continents. For example, *M. importuna* and *M. septimelata*, two fire-adapted *Morchella* spp. of the Elata clade, were found in Europe, East Asia, and North America, yet *M. tomentosa* was found only in Western North America (O'Donnell *et al*., 2011).

Sexual reproduction and asexual stage in *Morchella* spp.

Morchella fruiting bodies, or ascocarps, are usually the final products that consumers buy to consume as food. The fruiting body formation of *Morchella* spp. is controlled by the mating-type (*MAT*) locus, which has two mating-types, the *MAT 1-1* and *MAT 1-2* idiomorphs (Du *et al*., 2017). Individuals with either mating type are

morphologically identical for most of their life cycle, so mating-type idiomorphs are used for only identifying the mating-type identity of individuals (Debuchy & Turgeon 2006). The *MAT 1-1* and *MAT 1-2* are highly divergent idiomorphs, not true alleles (Chai *et al*., 2019). The *MAT 1-1* and *MAT 1-2* idiomorphs each have a major *MAT* gene, *MAT 1-1-1* and *MAT 1-2-1*. Both genes encode proteins that act as transcription factors that regulate sex-related genes, such as pheromone receptors (Du & Yang, 2021). The *MAT 1-1-1* gene encodes a protein with an α-box domain, and the *MAT 1-2-1* gene encodes a protein with a high mobility group (HMG) domain (Debuchy & Turgeon, 2006). The two genes are also found in other heterothallic ascomycetes such as *Podospora anserina*, *Neurospora crassa*, and *Aspergillus nidulans* (Ferreira *et al*., 1998; Paoletti *et al*., 2007; Staben *et al*., 1990). In contrast, homothallic species also have both mating-type idiomorphs, but are contained in the same individual (Debuchy and Turgeon, 2006).

Chai *et al*., 2019 successfully amplified *MAT 1-1-1* and *MAT 1-2-1* genes from 15 Elata clade species, one Rufobrunnea clade species, and 22 Esculenta clade species (Chai *et al*., 2019). Based on this, Du *et al*., 2022 hypothesized three mating strategies of *Morchella* that could form mature fruiting bodies: heterothallism (self-incompatible), pseudo-homothallism (self-compatible and capable of outcrossing), and homothallism (self-compatible) (Chai *et al*., 2022). Heterothallic sexual reproduction requires mating between two individuals of opposite mating-types (either *MAT 1-1* or *MAT 1-2* idiomorph) (Chai *et al*., 2017). Pseudo-homothallic species can form fruiting bodies similar to heterothallic species but carries both

mating-type idiomorphs in the same individual and can therefore fruit with a single-ascospore (Chai *et al.,* 2022). Lastly, homothallism utilizes a single ascospore packaging two mating-types in the haploid genome. Du *et al*., 2017 assigned three types of fruiting bodies with different distributions of mating type genes among cultured morels according to the three hypothesized mating strategies. Type I ascocarp has both *MAT 1-1-1* and *MAT 1-2-1* genes in the hymenia (fertile tissue) and stipes (mushroom stalk, sterile tissue). Type II ascocarp is the most dominant type; it has both mating-type genes at the hymenia but only one mating-type gene in the stipe (either *MAT 1-1-1* or *MAT 1-2-1*). Type III ascocarp has *MAT 1-1-1* gene in the stipe, but no ascospores are formed (Du *et. al*, 2017). To date, *M. importuna* is the only species with both heterothallism and pseudo-homothallism mating strategies, and *M. rufobrunnea* is homothallic (Du & Yang 2021; Chai *et al*., 2022).

Alternative to sexual reproduction, an asexual stage in *Morchella spp*. was observed from the wild and cultivation sites repeatedly (Alvarado-Castillo *et al*., 2014). The asexual stage of *Morchella* was reported as chlamydospores by Alvarado-Castillo *et al*., 2014, but was reported as conidia by Volk and Leonard 1990 (Alvarado-Castillo *et al*., 2014; Volk & Leonard 1990). Volk and Leonard 1990 suggested that conidium can be formed via vegetative mycelium. Although the conidial stage is often observed at cultivation sites, it is unknown whether this stage is crucial for fruiting body formation (Du & Yang 2021).

The life cycle of *Morchella* spp.

An understanding of the life cycle of *Morchella* spp. is crucial for morel cultivation. Saprophytic (feeding, absorbing, or growing on decaying organic matter) morels, such as *M. importuna* have a proposed life cycle, while the life cycles of mycorrhizal morels remain entirely unknown (Tan *et al*., 2019). Researchers have attempted to outline a proposed life cycle of *Morchella spp*. (Volk & Leonard 1990; Alvarado-Castillo *et al*., 2014). Du and Yang 2021 proposed a hypothetical life cycle of black morels based on the premise that they are saprotrophic. This proposed life cycle of *Morchella spp*. begins with the mature ascocarp (Figure 1-3). An ascocarp can form via three pathways, heterothallism, pseudo-homothallism, and sterile ascocarp formation (unisexual reproduction) (Du & Yang, 2021). Du and Yang 2021 speculated that Type I ascocarps are formed from a heterokaryotic ascospore that independently performed sexual reproduction via the pseudo-homothallism pathway; Type II ascocarps are formed by mating two *Morchella* individuals via the heterothallism pathway; Type III ascocarps are formed from an ascospore with either mating-type via the unisexual reproduction pathway, but no ascospores formed in the asci of the ascocarp and therefore it produces a sterile ascocarp (Fig. 1-2).

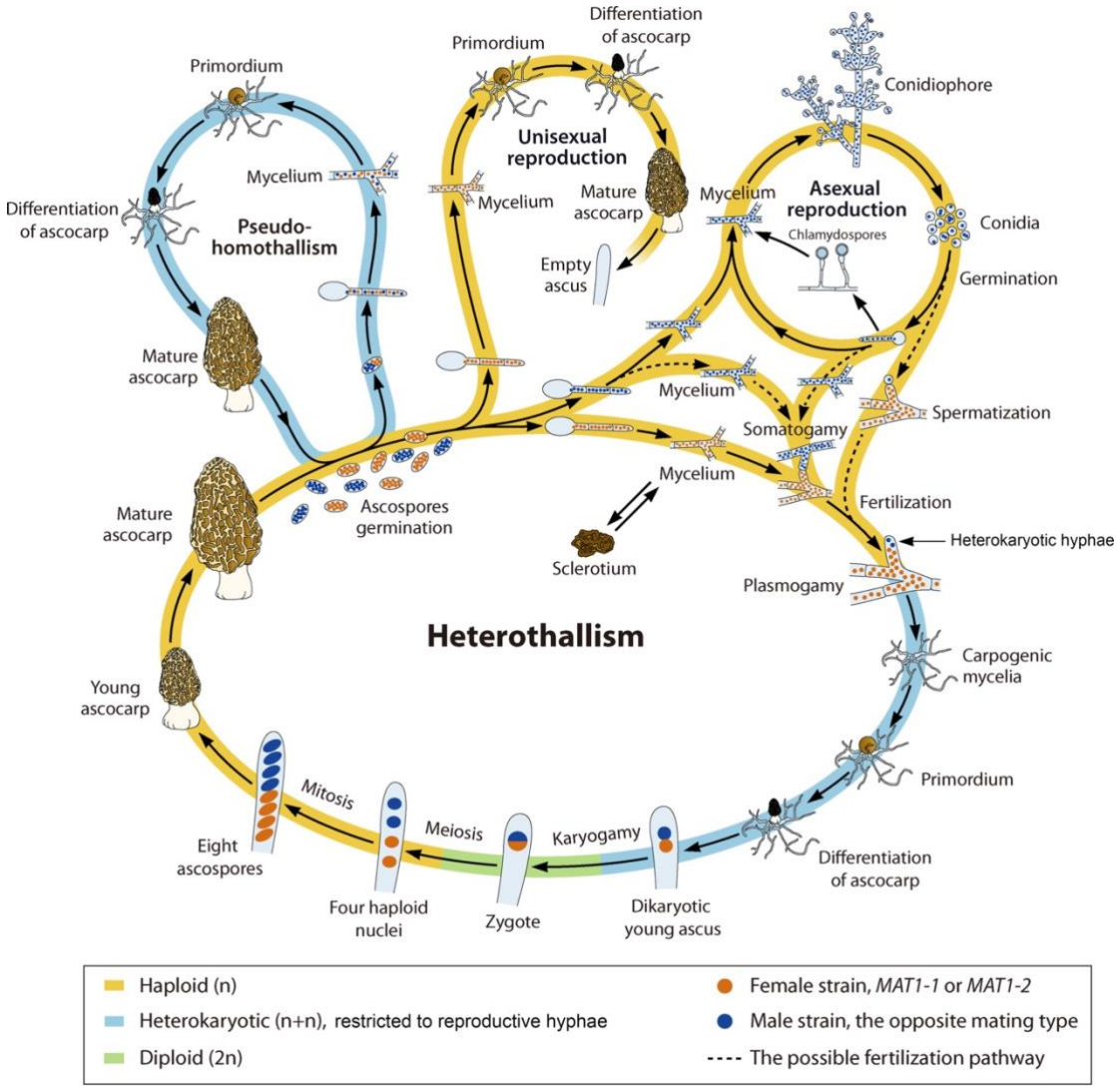


Figure 1-2 Generalized life cycle of Morchella, with heterothallism, pseudo-homothallism, unisexual reproduction, and asexual reproduction included (Du & Yang, 2021)

On the surface of each ascocarp, each ascus of this ascocarp contains eight ascospores (Miles & Chang, 1997). Ascospores are formed via meiosis and are haploid, containing either the *MAT1-1* or *MAT 1-2* idiomorph (Du *et al.*, 2017). Ascospores germinate to produce haploid hyphae, germinative tubes that elongate and thicken. Later, multiple hyphae bundle into an interconnected multi-karyotic mycelium that provides structural support and a nutrient absorption unit (Pilz *et al.*,

2007). All or parts of this mycelium with either *MAT 1-1* or *MAT 1-2* idiomorph can form conidia or asexual spores. Du and Yang 2021 hypothesized three conidial growth pathways depending on whether they germinate (Du & Yang, 2021). In pathway I, conidia do not germinate and act as spermatia (Nelson, 1996). In pathway II, conidia germinate into hyphae and later grow into reproductive propagules (Yuan *et al*., 2021). In pathway III, hyphae germinated from conidia form chlamydospores (Yuan *et al*., 2021). If no conidium is formed, the mycelium will continue to grow, twist, and harden to form pseudo-sclerotia, which allows *Morchella* spp. to remain dormant in adverse environments (Volk & Leonard, 1989). *Morchella* sclerotia are referred to as "pseudo-sclerotia" because they lack the medulla and rind (Volk & Leonard, 1990). However, they are often referred to as "sclerotia" (Ower, 1982; Pilz *et al*., 2007; Alvarado-Castillo *et al*., 2014). Volk and Leonard 1989 proposed that under specific environments, usually adverse environmental stimuli such as fire, drought, heavy rains, flooding, snow, or lack of nutrition, the pseudo-sclerotia can enter sexual growth by twisting secondary mycelia into pinheads or nodes, which will later form primordia (Volk and Leonard, 1989). However, Pilz *et al*., 2007 proposed that this is unlikely to happen due to the haploid nature of mycelium grown from pseudo-sclerotia (Pilz *et al*., 2007). In a study on black morel sclerotia, Du and colleagues analyzed mating-type genes of hundreds of sclerotia. They only found one idiomorph in most of the samples, with very few containing *MAT 1-1* and *MAT 1-2* (Du, X.H., Li, T., Xu, N., unpublished data).

Du and Yang 2021 proposed that heterothallism, or the formation of the type II ascocarp, is the dominant strategy (Du & Yang, 2021). Through anastomosis (hyphal fusion) and plasmogamy (union of cytoplasmic content of cells), hyphae from the vegetative mycelium can fuse with hyphae from another vegetative mycelium to form a heterokaryotic secondary mycelium (n + n). Through a combination of environmental and genetic factors, this mycelium forms primordium, which then differentiates into an ascocarp containing recombinant meiotic ascospores (Pilz *et al.*, 2007).

Morchella ecological role

After mapping out the proposed life cycle of *Morchella* spp., it is essential to understand their ecological roles: saprophytic or mycorrhizal, or both facultatively, to simulate the preferred growth environment of each species fully. Both saprophytic and mycorrhizal species have been recorded (Pilz *et al*., 2007). Hobbie *et al*., 2017 investigated the nutritional strategy of morels in Oregon and Alaska one or two years following forest fires. By comparing radiocarbon analysis, Hobbie *et al*., 2017 found that post-fire morels were assimilating old radiocarbon instead of current-year photosynthate and therefore concluded that these post-fire *Morchella spp*. are saprophytic (Hobbie *et al*., 2017). In addition, Hao *et al*., 2019 sequenced the genes responsible for decomposition in *M. importuna*. As a result, the expression of carbohydrate-active enzymes, or CAZymes, oxidoreductase (SOD), and catalase (CAT), are capable of rapidly decomposing starch and cellulose (Hao *et al*., 2019). In

contrast, no significant amount of nitrogen was consumed (Tan *et al*., 2019). Although no such study was done on *M. rufobrunnea* and other *Morchella* spp., cultivation (without mycorrhizal hosts) success with *M. sextelata*, and *M. rufobrunnea* suggests these species are saprophytic (Masaphy, 2010; He *et al*., 2019; Ge, 2019).

The history of morel cultivation

As early as 1883, scientists from France tried to cultivate morels outdoors with apples, pumpkins, Jerusalem artichoke, and various substrates (Constantin, 1936). One hundred years after the first recorded outdoor morel cultivation attempt, Ronald D. Ower successfully formed fruiting bodies while completing his Ph.D. thesis *Cultural Studies of Morels*, at the San Francisco State University (Ower, 1980). Ower stressed the importance of culturing *Morchella* pseudo-sclerotia, which store energy for fruiting. He used a jar filled with soil and soaked wheat grains to culture sclerotia, which can be later used for fruiting body formation (Ower, 1982). Ower's findings have three significant contributions to the understanding of morel cultivation: 1. vegetative mycelial growth should be maintained at a relatively low temperature (10 – 22 ℃) and high humidity (75 – 95% air humidity); 2. depleting nutrient source switches *Morchella spp*. vegetative growth into sexual growth; 3. maintaining 90 – 100% soil moisture triggers *Morchella* spp. fruiting body formation. These findings are still critical steps in morel cultivation today. With the help of Gary L. Mills, Ower filed three patents on these findings in 1986, 1988, and 1989 (US Patent 4594809, 4757640, and 4866878).

Soon after Ower's finding, in 1983, Ding (1983) conducted a study on wild morel distribution in Guizhou, China. The morels he found were believed to be predominantly *M. conica* (without molecular evidence). The fruiting bodies appeared in April and May on mountains that faced the sun, away from the wind, and in moist areas, with or without plants nearby (Ding, 1983). This finding led to the belief that *Morchella* spp. are saprophytic fungi and can be cultivated without plant hosts (Ding, 1983). The same year, Gu (1983) found *M. angusticeps* are abundant in leaf litter and dead plant organic matter near spruce and fir woods in southern Gansu, China (Gu, 1983). This finding led to the belief that *Morchella* spp. can be cultivated in the woods where dead plant organic materials are ample (Gu, 1983). Therefore, Gu hung an *M. angusticeps* ascocarp in a flask to collect ascospores for production. The substrate was prepared with 75% sawdust, 15% wheat bran, 5% humus, 0.4% superphosphate, 1% glucose, 0.5% ammonium sulfate, 0.05% monopotassium phosphate, 0.05% magnesium sulfate, 1% urea, and 1% calcium carbonate. This substrate was inoculated and kept in a jar for 35 days with a temperature of 16 - 20 ℃. In 55 days, primordia formed. However, after 65 days post-inoculation, primordia never develop into mature fruiting bodies (Gu, 1983).

Yao 1991 isolated ascospores from *M. conica*, cultured mycelia on PDA media, and used 38% cotton seed hull, 39% sawdust, 20% wheat bran, 1% calcium carbonate, and 1% cane sugar as a substrate (Yao, 1991). Yao then made raised beds in the field with garden soil and dug a strip 10 cm deep in a pear orchard. The inoculated substrate was then buried in the strip, covered with 3 cm of soil, and then

covered with a plastic sheet. Sixty-five days after spawning, the plots developed multiple fruiting bodies 2.5 – 3 cm tall. Seventy-eight days after spawning, the same fruiting bodies grew into 9 – 16 cm tall mature fruiting bodies (Yao, 1991). Following the fruiting success of Yao 1991 and before the discovery of nutrient bags in the 2000s, researchers tried to cultivate morels with various substrate formulas. However, none of the methods yielded consistently with unidentified or misidentified species (Zhu, 1994; Deng *et al*., 1997), high material cost (Yi *et al*., 1994), and wrong fruiting stimulation timing (Li *et al*., 2008).

Inspired by Ower's idea of nutrient management in morel production, an outdoor cultivation method was found by serendipity in the 2000s by Dr. Fang He Tan in Sichuan, China (Xu *et al*., 2022). In the early 2000s, *M. importuna*, *M. sextelata*, and *M. septimelata* were considered saprotrophic, so Chinese growers and researchers started to domesticate these *Morchella spp*. While testing whether fusing field *Morchella* mycelia with *Morchella* mycelia grown in nutrient bags would enhance yield, Tan found that laying uninoculated nutrient bags in the field alone would increase yield (Tan, 2016). These nutrient bags are autoclavable plastic bags that contain plant polysaccharides, such as wheat grains, rice husks, and sawdust (Liu, S. *et al*., 2016). The sterilized nutrient bags are cut open on the bottom, facing the soil to allow mycelial colonization. This method supports vegetative mycelial growth with various nutrient bag formulas consisting of carbon and nitrogen sources and is repeatable (Tan, 2016). Removal of these nutrient bags triggers fruiting body formation. The belief was that a nutrient-deficient environment stresses the fungus

and stimulates sexual reproduction. Although this technique is now widely used in the morel cultivation industry in China, its mechanism remains a mystery (Tan, 2016; Liu, S. *et al*., 2016).

Several other outdoor production techniques have been developed in China but eventually abandoned due to inconsistent yield or high costs, such as log cultivation, wheat intercropping cultivation, and forest cultivation. Log cultivation was discovered by farmers in Yunnan, China. The local farmers successfully formed fruiting bodies by placing *M. conica* fruiting bodies between wood with a diameter of 10 – 15 cm like a "sandwich" and covering it with soil (Zhao *et al*., 2007). However, four cubic meters of wood were needed per hectare for production, so log cultivation was eventually terminated due to high costs and environmental protection laws (Zhao *et al*., 2007). Wheat intercropping cultivation is a morel cultivation plan adopted by growers in the Sichuan province, China, with a total of about 66,700 square meters (Fan *et al*., 2013). Seventy-five to one hundred and fifty kilograms of morel spawn was applied to 667 square meters, and 3 – 4 kg wheat was applied 1- 10 days after spawning. After a 38 – 45 days spawn run, a nutrient solution was applied at 200 mL per square meter (Fan *et al*., 2013). From 2009 to 2011, this cultivation technique yielded 120 kg, 135 kg, and 142 kg of fresh ascocarps per 667 square meters. However, the overall yield from 2008 to 2012 was volatile and this cultivation plan was not adopted (Fan *et al*., 2013).

Forest cultivation takes advantage of the rich organic matter under trees, which can be a great nutrient source for *Morchella spp*. The trees can also be used as

structural support for shading layers. Growers would till the soil lightly before production. Then, the inoculated substrate was buried in the soil, covered with 1 – 3 cm hummus and broad tree leaves of 2 cm. However, this technique yielded poorly potentially due to uncontrollable nutrient removal stimulation (Suo, 2015).

Masaphy 2010 cultivated *M. rufobrunnea* with the cultivation techniques of Ower 1982 and successfully formed fruiting bodies indoors (Masaphy, 2010). Masaphy first cultured pseudo-sclerotia by inoculating a mixed wheat, vermiculite, and garden soil substrate for two weeks under 18 – 25 ℃. Cultured pseudo-sclerotia were soaked in water for 5 – 20 hours and used as "seeds" for production. Three to six days after soaking the pseudo-sclerotia, mycelia emerged. When the mycelia colonized the substrate's surface, a dense layer of conidia formed. Later, the conidial layer diminished, and primordia formed. Two to three weeks after primordial formation, mature fruiting bodies formed with a 7 – 15 cm height. Although fruiting success was observed, production could not scale up due to the difficulty of repeating Ower's technique (Masaphy, 2010).

Current global morel cultivation status

With the application of nutrient bags, the current Chinese nutrient bag technique has been practiced for over ten years, and the production is booming in Southern Chinese provinces such as Sichuan and Yunnan. The story of successful morel cultivation attracted farmers, scientists, mushroom industry producers, and even amateurs to enter the morel cultivation industry in China. With provincial

governments subsidizing this industry, the area of Chinese outdoor morel production increased from 300 hectares in 2011 to over 10,000 hectares in 2018, yielding 15,000 kg/ hectare of fresh morels (Du *et al*., 2017). However, the Chinese morel cultivation industry has been severely destabilized by faulty practices such as continuous cropping and *Morchella* strain aging (Liu, Q. *et al*., 2017; He *et al*., 2019). These faulty practices may result in the premature death of primordia, fruiting body deformation, and low yields (Liu, Q. *et al*., 2017). He *et al*., 2019 found that successive sub-culturing increased *Morchella* senescence. Senesced *Morchella* cultures grew slim mycelia with lower viability and formed smaller, defective shapes, sometimes without fruiting bodies (He *et al*., 2019). The need for more understanding in *Morchella spp.* Biology also contributes to yield inconsistency. Yield inconsistency is still a problem in the Chinese morel cultivation industry, causing the price of cultivated morels to fluctuate. In 2019, China's total area under production declined by 30-50% compared to 2018, to 5000 - 6000 hectares (He *et al.*, 2019). In the Western hemisphere, although American and Israeli scientists had fruiting success indoors from 1982 to 2010, production could not scale up due to the difficulty repeating Ower's technique (Masaphy, 2010).

Current knowledge gap and general objective of this study

Although heterothallism is the dominant reproductive mode observed among black morels (Chai *et al*., 2019), sterile fruiting body formation (unisexual reproduction) occasionally occurs without a compatible mating-type partner, and the fruiting body

has no ascospores (Du *et al*., 2017). Due to the lack of understanding of *Morchella spp*. Biology, Chinese cultivation sites have frequently experienced low yield or failure to fruit. Therefore, understanding the role of mating is essential for a consistent yield and profitable morel cultivation.

This study aims to cultivate *M. importuna* and *M. rufobrunnea* in the Northeastern United States (State College, PA) by replicating the Chinese nutrient bag technique indoors and outdoors and to determine the role of mating on fruiting body formation of *M. importuna and M. rufobrunnea*. This study hypothesized that the yield of *M. importuna* will be significantly higher when using mixed mating-type spawn than spawn with individual mating-type, and no significant differences between mixed and individual spawn of *M. rufobrunnea*.

CHAPTER 2 Mating-type Identification

Introduction

In ascomycetes, *MAT 1-1-1* and *MAT 1-2-1* are the two primary *MAT* genes responsible for sexual reproduction (Chai *et al*., 2017). *MAT 1-1-1* and *MAT 1-2-1* genes have been identified in 38 *Morchella spp*. in the Rufobrunnea, Elata, and Esculenta clades (Chai *et al*., 2017; Chai *et al*., 2019), including the species of interest, *M. importuna* and *M. rufobrunnea*. Although *M. importuna* and *M. rufobrunnea* harbor both *MAT 1-1-1* and *MAT 1-2-1* genes, their modes of sexual reproductive are different. Three types of reproductive modes are present in *M. importuna*: sterile fruiting body formation, heterothallism, and pseudo-homothallism (Du *et al*., 2022). On the other hand, the sexual reproductive mode of *M. rufobrunnea* is homothallic (Du *et al*., 2022). This chapter aims to identify the mating-type identity of each isolate of interest in the Penn State culture collection as well as isolates ordered from the United States Department of Agriculture Agricultural Research Service National Center for Agricultural Utilization Research Culture Collection (USDA ARS NRRL). Chai and colleagues sequenced the genome and identified the size of the *MAT 1-1-1* and *MAT 1-2-1* idiomorphs for *M. importuna* and *M. rufobrunnea* (Chai *et al*., 2019). For *M. importuna* isolates, the expected band size for *MAT 1-1-1* is about 1.5 kb, and the expected band size for *MAT 1-2-1* is about 0.85 kb (Chai *et al*., 2017; Chai *et al*., 2019). For *M. rufobrunnea* isolates, the expected band size for *MAT 1-1-1* is about 1.760 kb, and the expected band size for *MAT 1-2-1* is about 1.342 kb (Chai *et al*., 2019). The

isolates with identified mating-types will be used individually or paired for production.

Materials and Methods

Culture preparation

Seven *M. importuna* strains and six *M. rufobrunnea* strains were cultured for mating-type identification as shown in Table 2-1.

Table 2-1 Strain information

Strain ID	Species	Location	Culture Source
WC 172	*M. importuna*	unknown	Kept in the Penn State Culture Collection
WC 178	*M. importuna*	unknown	Kept in the Penn State Culture Collection
WC 1021	*M. importuna*	culture collected from China	Kept in the Penn State Culture Collection
WC 1022	*M. importuna*	culture collected from China	Kept in the Penn State Culture Collection

NRRL 22311	*M. importuna*	Oregon, USA	Identified and isolated by K. O'Donnell
NRRL 36693	*M. importuna*	Washington, USA	Identified and isolated by K. O'Donnell
NRRL 36694	*M. importuna*	Washington, USA	Identified and isolated by K. O'Donnell
WC 833	*M. rufobrunnea*	unknown	Kept in the Penn State Culture Collection
NRRL 28462	*M. rufobrunnea*	California, USA	Identified and isolated by Kerry O'Donnell; Spore print made by Gary Mills
NRRL 28465	*M. rufobrunnea*	East Lansing, Michigan, USA	Identified and isolated by Kerry O'Donnell; Spore print made by

NRRL 28466	*M. rufobrunnea*	East Lansing, Michigan, USA	Gary Mills Identified and isolated by Kerry O'Donnell; Spore print made by Gary Mills
NRRL 36664	*M. rufobrunnea*	California, USA	Identified and isolated by Kerry O'Donnell
NRRL 36872	*M. rufobrunnea*	USA	Isolated by Kerry O'Donnell

Preparations before DNA extraction

Two 100 mm x15 mm Potato Dextrose Yeast Agar (PDYA) (Potato Dextrose Agar (Difco™ 39 g/L) + Yeast Extract (Difco™ 1.5 g/L)) plates were prepared for each *Morchella* isolate and incubated at room temperature until the mycelial growth reached the edge of the plate. Two mycelial plugs were taken from the edges of the plate for each strain using a heat-sterilized and cooled 5 mm cork borer. The two mycelial plugs were transferred to 50 ml of Potato Dextrose Broth (PDB) Media (Difco™ 24 g/L) in a 250 mL flask and incubated at room temperature. When the colony diameter reached approximately 2 cm, the liquid mycelial cultures were placed in a refrigerator at 4 ℃ until all cultures had completed growth (7 days).

Mycelium from the flasks was removed using a sterilized inoculation needle and placed in an Eppendorf tube (1.8 – 2.0 ml) at room temperature. The sterilized needle was then used to squeeze the liquid from the mycelia in the Eppendorf tube. Mycelia samples were lyophilized for 48 hours, and the freeze-dried samples were stored at -20 ℃ until DNA extraction.

DNA extraction

Before DNA extraction, lyophilized mycelial tissue was ground into a powder using a mortar and pestle. DNA extraction was done using a Qiagen QIAamp® DNA Mini Kit (Qiagen**,** Qiagen GmbH, Dusseldorf, Germany**)** following the manufacturer's instructions.

Polymerase chain reaction (PCR)

Four sets of PCR were used to amplify the *MAT 1-1-1* and *MAT 1-2-1* segments of *Morchella* isolates' DNA. Four sets of PCR were performed to amplify *MAT 1-1*-1 and *MAT 1-2-1* genes in *M. importuna* as well as in *M. rufobrunnea*. Each PCR reaction used OneTaq 2X Master Mix with Standard Buffer's protocol by New England Biolabs (protocol: www.neb.com/protocols/2012/09/06/protocol-for-onetaq-2x-master-mix-with-standard-buffer-m0482, New England Biolabs, MA, USA). Each PCR consisted of 1µl (10ng/µl) genomic *Morchella* DNA, 10.5 µl sterile MilliQ H_2O, 2.5 µl New England Biolabs One*Taq* 2X Master Mix with standard Buffer (M0482), 0.5 µl forward primer and 0.5 µl reverse primer.

In PCR set 1, the goal was to amplify *MAT 1-1-1* genes from *M. importuna* strains. The extracted genomic DNA of seven *M. importuna* strains along with the master mix, MQ water, forward and reverse primers were loaded into seven PCR tubes. The forward primer sequence was TTACCTTACTGGACTGGTTCGTGAG (P8-5f, Chai *et al*., 2019) and the reverse primer sequence was TGGAATGTCTGTGATTGAGGCTGTG (P8-5r, Chai *et al*., 2019). The PCR started with 94 ºC for 30s, then went into 30 cycles of 94 ºC 30s, 56 ºC 30s, 68 ºC 120s, and finished with 68 ºC 5 min and 4 ºC hold.

In PCR set 2, the goal was to amplify *MAT 1-2-1* genes from *M. importuna* strains. The extracted genomic DNA of seven *M. importuna* strains along with the master mix, MQ water, forward and reverse primers were loaded into seven PCR tubes. The forward primer sequence was GGCCAGAACAGATGCTCGAAGAAGC (P10-2f, Chai *et al*., 2019) and the reverse primer sequence was GTGGCAACTCCCAAAGCATGATCAA (P10-2r, Chai *et al*., 2019). The PCR started with 94 ºC for 30s, then went into 30 cycles of 94 ºC 30s, 58 ºC 30s, 68 ºC 120s, and finished with 68 ºC 5 min and 4 ºC hold.

In PCR set 3, the goal was to amplify *MAT 1-1-1* genes from *M. rufobrunnea* strains. The extracted genomic DNA of six *M. rufobrunnea* strains along with the master mix, MQ water, forward and reverse primers were loaded into seven PCR tubes. The forward primer sequence was CTCTTCAGTAACCCCTTCAATGGAC (P1-3f, Chai *et al*., 2019) and the reverse primer sequence was TGTGGAGAAACAGGGAAAACTAAAC (P1-3r, Chai *et al*., 2019). The PCR

started with 94 ºC for 30s, then went into 30 cycles of 94 ºC 30s, 52 ºC 30s, 68 ºC 120s, and finished with 68 ºC 5 min and 4 ºC hold.

In PCR set 4, the goal was to amplify *MAT 1-2-1* genes from *M. rufobrunnea* strains. The extracted genomic DNA of six *M. rufobrunnea* strains along with the master mix, MQ water, forward and reverse primers were loaded into seven PCR tubes. The forward primer sequence was CTCTTCAGTAACCCCTTCAATGGAC (P1-3f, Chai *et al*., 2019) and the reverse primer sequence was TGTGGAGAAACAGGGAAAACTAAAC (P1-3r, Chai *et al*., 2019). The PCR started with 94 ºC for 30s, then went into 30 cycles of 94 ºC 30s, 52 ºC 30s, 68 ºC 120s, and finished with 68 ºC 5 min and 4 ºC hold.

Agarose Electrophoresis

PCR amplicons were separated in a 1% agarose gel with 50x TAE (Tris-Acetate-EDTA) buffer (AccuGene®, Lonza USA, Rockland, ME) containing ethidium bromide.

Meanwhile, the DNA ladder (Quick-Load® New England Biolabs Inc., MA, USA) and loading dye were defrosted. 1.5 uL dye was mixed with 5 uL PCR product on a Parafilm strip, and the mixture was gently added to the gel wells. Five uL of DNA ladder was loaded into the gel well. Lastly, the top cover was attached, and the power was turned on at 100 volts for 1 hour and 10 minutes.

Results and Discussion

The PCR results indicate that each *M. importuna* isolate had amplicons of either *MAT 1-1-1* or *MAT 1-2-1* gene, with the *MAT 1-1-1* amplicon around 1.6 kb (Fig. 2-1a) and the *MAT 1-2-1* amplicon around 1.1 kb (Fig. 2-1b). Each *M. rufobrunnea* isolate had amplicons of both *MAT 1-1-1* and *MAT 1-2-1* genes (Fig. 2-2a; Fig. 2-2b). The bands on the gel were close to 2.0 kb and 1.6 kb for *MAT 1-1-1* and *MAT 1-2-1* genes, respectively. The amplicons were larger than that of Chai *et al*., 2019, and it could be caused by the way DNA resolved in the gel. The finding of either mating-types in *M. importuna* individuals indicated that this species is heterothallic, and the presence of both mating-types in a single individual of *M. rufobrunnea* suggests that this species is homothallic, which is similar to the finding of Du *et al*., 2022.

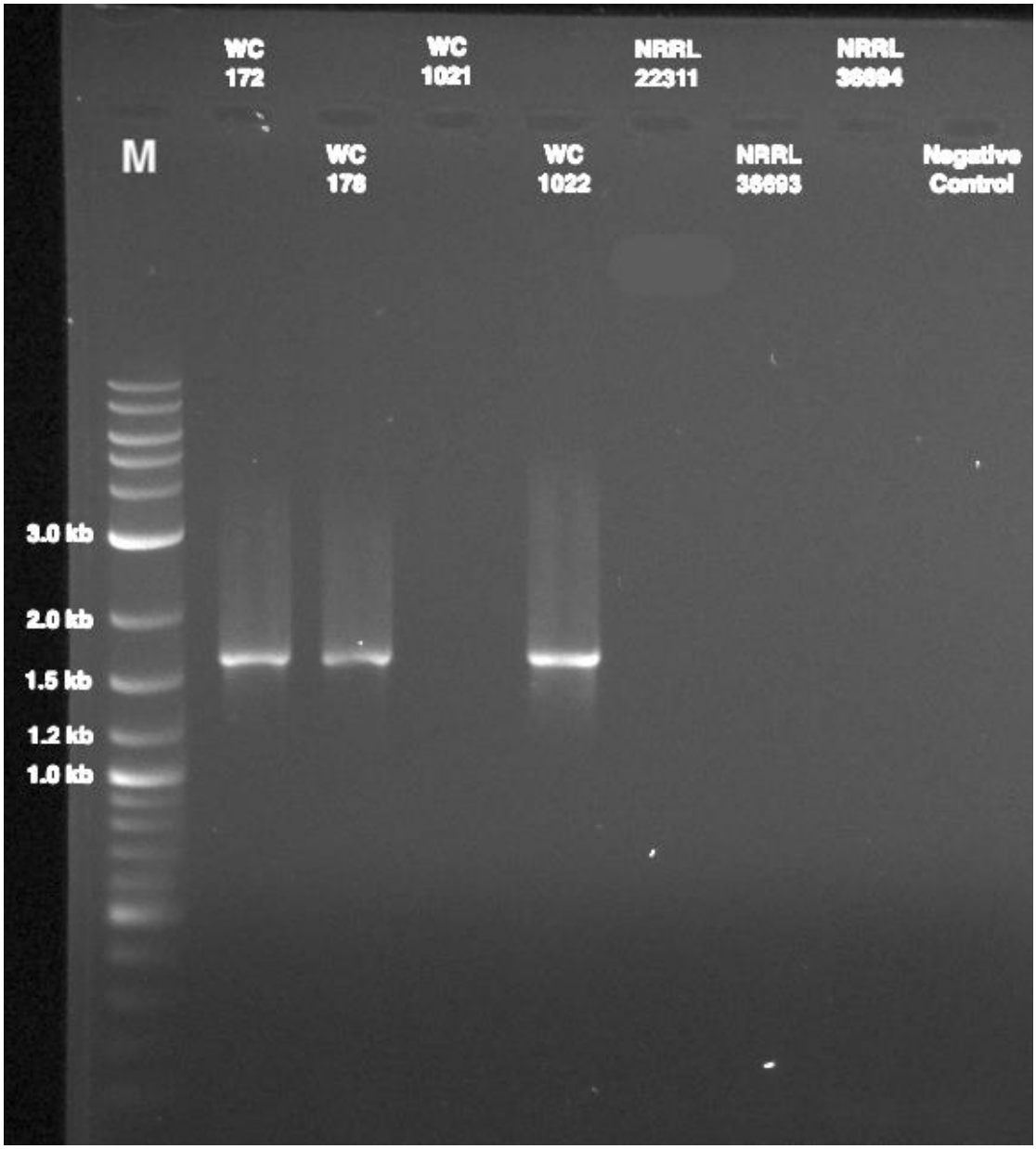


Figure 2-1a Agarose gel showing PCR amplicons of *MAT 1-1-1* idiomorphs of *M. importuna* isolates.

The letters each represent – "M": DNA size marker lane (0.1 – 10.0 kb), "NC": negative control. The first lane is DNA ladders. The WC or NRRL + number line represents the isolate, such as WC 172, or NRRL 22311.

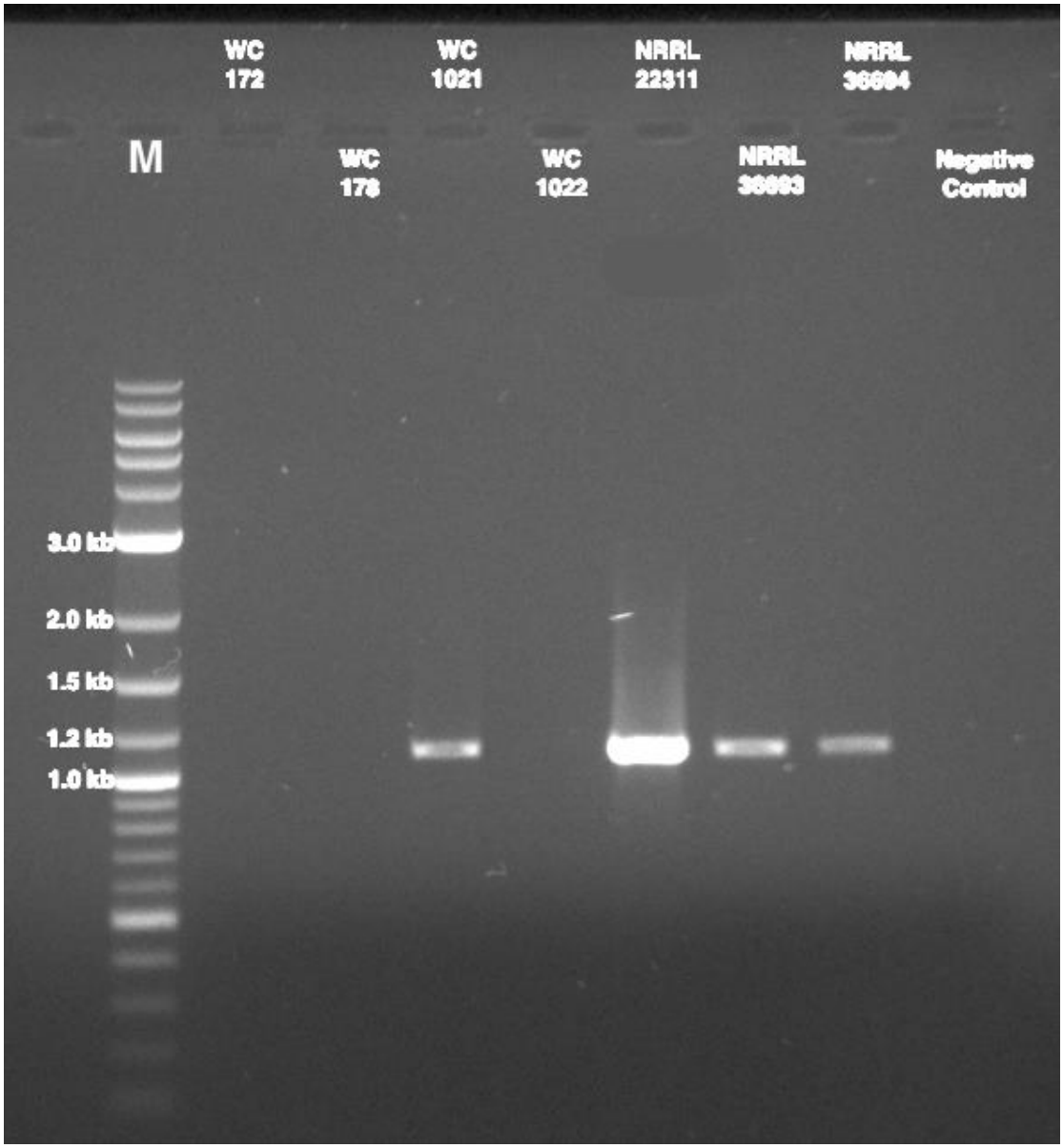


Figure 2-1b. Agarose gel showing PCR amplicons of MAT1-2-1 idiomorphs of M. importuna isolate.

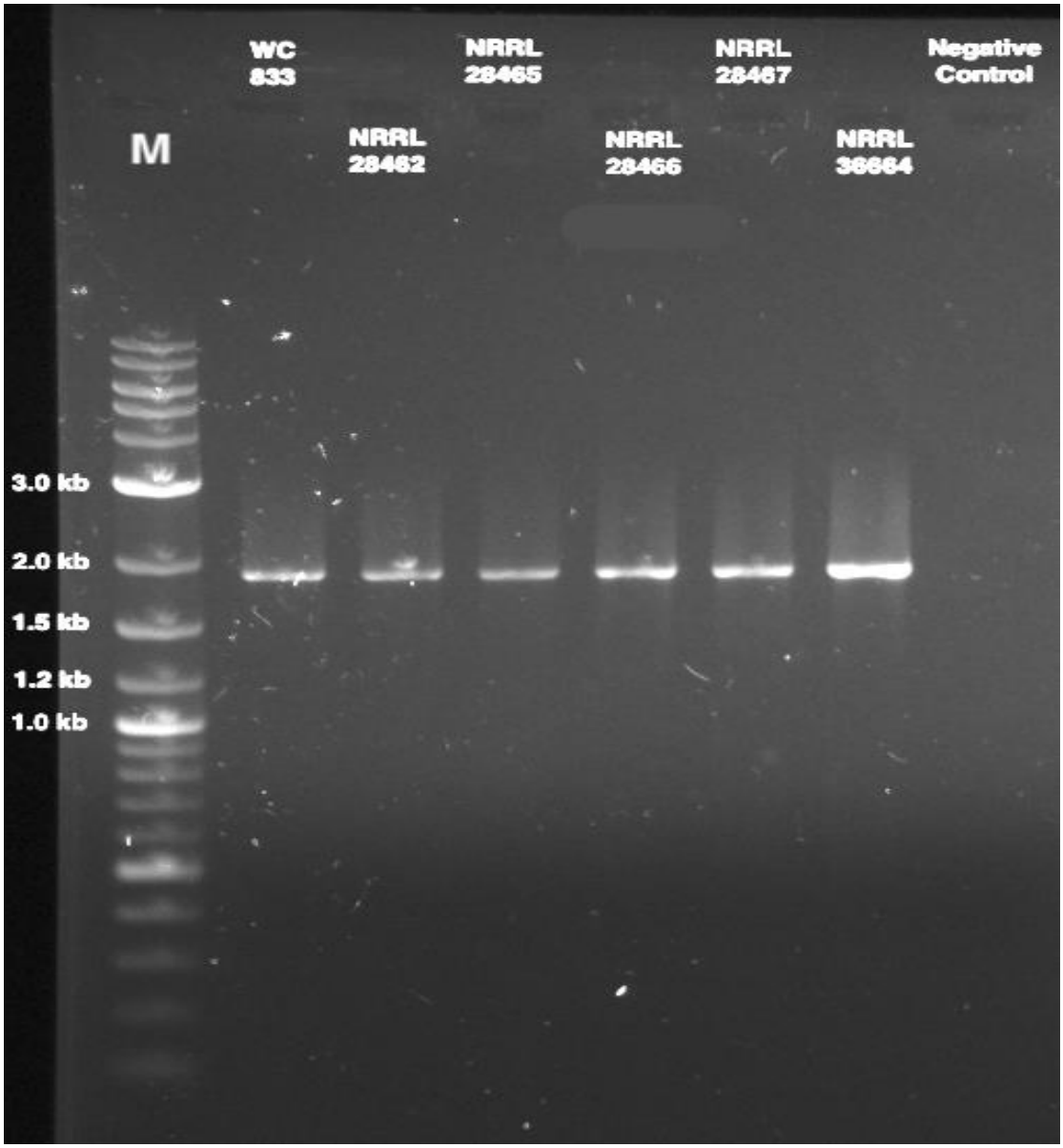


Figure 2-2a. Agarose gel showing PCR amplicons of MAT 1-1-1 idiomorphs of M. rufobrunnea isolates.

The letters are the same as Fig. 2-1. The first and tenth lane were DNA ladders (0.1 – 10.0 kb). The last lane is negative control. The WC or NRRL + number line represents the isolate, such as WC 833, or NRRL 28462.

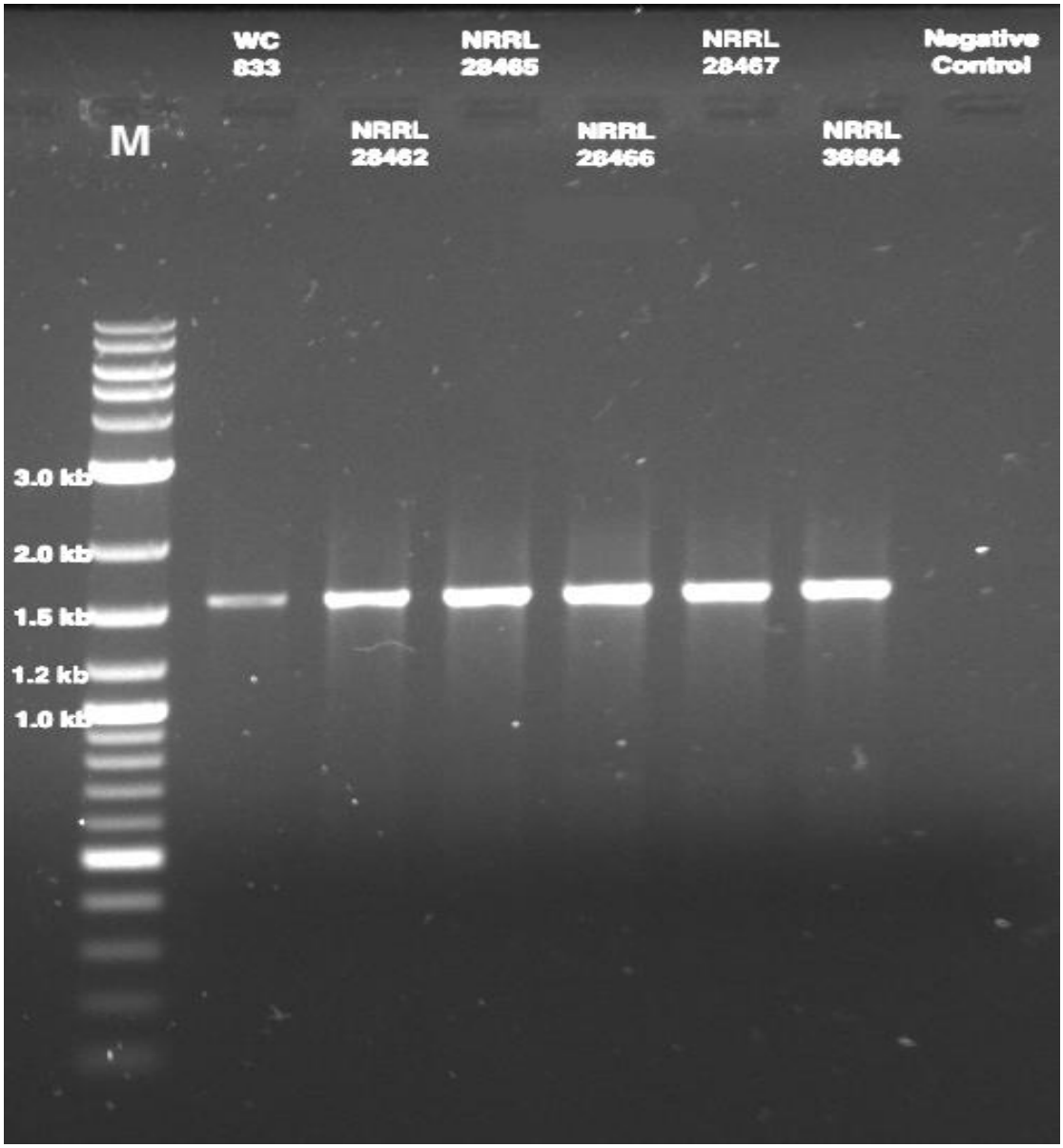


Figure 2-2b. Agarose gel showing PCR amplicons of MAT 1-2-1 idiomorphs of M. rufobrunnea isolates.

The agarose gel mating-type testing result is summarized in Table 2-2.

Table 2-2 Strain mating-type identification results

M. importuna			
Strain	*MAT 1-1-1*	*MAT 1-2-1*	Mating-type
WC 172	+	-	*MAT 1-1-1*

WC 178	+	-	*MAT 1-1-1*
WC 1021	-	+	*MAT 1-2-1*
WC 1022	+	-	*MAT 1-1-1*
NRRL 22311	-	+	*MAT 1-2-1*
NRRL 36693	-	+	*MAT 1-2-1*
NRRL36694	-	+	*MAT 1-2-1*
M. rufobrunnea			
Strains	*MAT 1-1-1*	*MAT 1-2-1*	Mating-type
WC 833	+	+	Both
NRRL 28462	+	+	Both
NRRL 28465	+	+	Both
NRRL 28466	+	+	Both
NRRL 28467	+	+	Both
NRRL 36664	+	+	Both

Sequencing and analysis

Since all *M. rufobrunnea* samples had both *MAT 1-1-1* and *MAT 1-2-1* DNA segments amplified, they were sent to the Nucleic Acid Facility at Penn State for sequencing analysis. The sequencing results were aligned on Geneious Prime (Biomatters Ltd). For each isolate, the *MAT 1-1-1* or *MAT 1-2-1* forward sequence and

reverse sequence were aligned, and the sequences' ends, including poorly identified bases, were trimmed off. The aligned sequences were compared with the draft *M. rufobrunnea* genomes that Chai *et al*., 2017; Chai *et al*., 2019 uploaded on GenBank (Chai *et al*., 2017; Chai *et al*., 2019).

After comparing the sequenced *MAT 1-1-1* and *MAT 1-2-1* genes of six *M. rufobrunnea* strains to the *M. rufobrunnea* genome on GenBank, the mating-type identity are summarized in the list below:

WC 833 *M. rufobrunnea*: *MAT 1-1-1* sequence was 100% identical (1760/1760 bp) to "*Morchella rufobrunnea* mating-type protein *MAT 1-1-1* gene, complete cds" (GenBank Sequence ID: MG680986.1, Chai *et al*., 2019) and 99.8% identical (1331/1334 bp) to "*Morchella rufobrunnea* mating-type protein *MAT 1-2-1* gene, complete cds" (GenBank Sequence ID: MG681025.1, Chai *et al*., 2019).

NRRL 28462 *M. rufobrunnea*: *MAT 1-1-1* sequence was 100% identical (1721/1721 bp) to "*Morchella rufobrunnea* mating-type protein *MAT 1-1-1* gene, complete cds" (GenBank Sequence ID: MG680986.1, Chai *et al*., 2019) and 99.8% identical (1331/1334 bp) to "*Morchella rufobrunnea* mating-type protein *MAT 1-2-1* gene, complete cds" (GenBank Sequence ID: MG681025.1, Chai *et al*., 2019).

NRRL 28465 *M. rufobrunnea*: *MAT 1-1-1* sequence was 100% identical (1721/1721 bp) to "*Morchella rufobrunnea* mating-type protein *MAT 1-1-1* gene, complete cds" (GenBank Sequence ID: MG680986.1, Chai *et al*., 2019) and 99.8% identical (1281/1283 bp) to "*Morchella rufobrunnea* mating-type protein *MAT 1-2-1* gene, complete cds" (GenBank Sequence ID: MG681025.1, Chai *et al*., 2019).

NRRL 28466 *M. rufobrunnea*: *MAT 1-1-1* sequence was 99.9% identical (1663/1664 bp) to "*Morchella rufobrunnea* mating-type protein *MAT 1-1-1* gene, complete cds" (GenBank Sequence ID: MG680986.1, Chai *et al*., 2019). However, due to poor sequence data, the *MAT 1-2-1* consensus sequence could not be compared to the one on GenBank.

NRRL 28467 *M. rufobrunnea*: *MAT 1-1-1* sequence was 100% identical (1721/1721 bp) to "*Morchella rufobrunnea* mating-type protein *MAT 1-1-1* gene, complete cds" (GenBank Sequence ID: MG680986.1, Chai *et al*., 2019) and 99.8% identical (1331/1334 bp) to "*Morchella rufobrunnea* mating-type protein *MAT 1-2-1* gene, complete cds" (GenBank Sequence ID: MG681025.1, Chai *et al*., 2019).

NRRL 36664 *M. rufobrunnea*: *MAT 1-1-1* sequence was 100% identical (1663/1663 bp) to "*Morchella rufobrunnea* mating-type protein *MAT 1-1-1* gene, complete cds" (GenBank Sequence ID: MG680986.1, Chai *et al*., 2019) and 99.9% identical (1332/1334 bp) to "*Morchella rufobrunnea* mating-type protein *MAT 1-2-1* gene, complete cds" (GenBank Sequence ID: MG681025.1, Chai *et al*., 2019).

CHAPTER 3 Outdoor Cultivation

Introduction

Morels are prized mushrooms with short fruiting seasons and are highly sought after for their unique flavor. Morel cultivation has been explored in attempts to supply morels out of the fruiting season since 1883 (Constantin, 1936). Consistent morel cultivation was successful only in the last decade in Southern China. This success would not be possible without utilization of the nutrient bag, which was a technique that applied plant polysaccharides, in an autoclavable bag, to support mycelial growth. With this technique, Chinese growers successfully cultivated black morels, such as *M. importuna*, *M. sextelata*, and *M. septimelata*, and China became the largest morel exporter in the world (Liu, Q. *et al*., 2017). Although the total yield from outdoor cultivation exceeds 900,000 kg in China, 70% of farmers produce little to no yield (Liu, W. *et al*., 2018). The yield is inconsistent which may be due to a poor understanding of the *Morchella* life cycle, mating mechanism (Du *et al*., 2021), knowledge of optimal growing conditions and or poor spawn quality (Liu, Q. *et al*., 2017).

To explore the feasibility of morel cultivation in the Northeastern United States, Siyi Ge, a master's student at Penn State, tested the optimal pH, temperature, and light conditions for mycelial growth of *M. importuna* and *M. rufobrunnea* and simulated outdoor cultivation of *M. rufobrunnea* in a controlled environment indoors. Ge reported that *M. rufobrunnea* had the highest level of mycelial growth under a pH of 6 in darkness (0 lux) and between 20 to 25 ℃; *M. importuna* had the highest level of

mycelial growth under pH of 5 to 6 in darkness (0 lux) and low light (400 lux), and between 20 to 25 ℃. Ge also concluded that the different tested nutrient bag formulas had no effect on fruiting body formation. From extrapolated fruiting data, Ge concluded that outdoor cultivation might be feasible.

Based on soil and air temperature analysis, this study concluded that State College, PA and Orono, ME had suitable temperatures for outdoor morel cultivation. Therefore, the two sites could conduct experiments on growing morels by replicating the Chinese morel outdoor cultivation technique. With the success of outdoor cultivation techniques for *M. importuna*, *M. sextelata*, and *M. septimelata* in China and the Ge, 2019 indoor fruiting success with *M. rufobrunnea*, this study aimed to take a step further to test the yield potential of *M. importuna* and *M. rufobrunnea* outdoors in Pennsylvania by replicating the Chinese cultivation technique with or without compatible mating-types.

Materials and methods

Soil temperature analysis

Soil temperature is one of the most critical factors that affect *Morchella* growth. Unlike the other factors that affect *Morchella* growth, outdoor temperatures cannot be controlled. Therefore, it was crucial to know if the soil temperature of State College, PA, and Orono, ME (the cultivation site of a collaborator) is conducive for outdoor morel cultivation. This study aimed to compare the 2019 daily soil temperature data of the two Northeastern US outdoor cultivation sites with the coldest two Chinese morel

outdoor cultivation reported sites: Xinzhou and Yushu. Xinzhou is China's northernmost morel outdoor cultivation site, with a latitude of 38.4167° N. Yushu was chosen for this study because it is the coldest outdoor morel cultivation site in China. Although State College, PA (40.7934°N) and Orono, ME (44.8831° N) are located north of Xinzhou, which could mean colder soil temperatures, a statistical analysis on the comparison between the cultivation sites can give conclusive results on whether State College, PA, and Orono, ME are too cold for morel outdoor cultivation. However, the soil temperature data of the two Chinese sites are probably not available to the public. This study then worked around this problem by using air temperature to predict soil temperature.

First, the available soil temperature data and air temperature data of State College, PA, were analyzed to assess if a positive correlation exists. If the such a positive correlation was strong, then it can be assumed that the same correlation was present in the Chinese cultivation sites because the cultivation practices are almost identical without other factors affecting soil or air temperature. Based on this assumption, the available air temperature data of Xinzhou and Yushu, the coldest Chinese sites, can be compared with that of State College, PA, and Orono, ME. If the average, extreme high, extreme low air temperature data of State College, PA and Orono, ME were not significantly colder than Xinzhou and Yushu; then we can conclude that it is not too cold to cultivate morels outdoors in State College, PA and Orono, ME.

The statistical analysis was done using the R statistical computing software (R Core Team 2022). The correlation between air temperature and soil temperature of State

College, PA, was determined by comparing 768 hourly air temperatures and 768 hourly soil temperatures from March 20th to April 20th, 2020.

There were three websites on which air temperature data were collected: Centre County Weather Stem, Chinese Historical Weather Database, and US Climate Data (centre.weatherstem.com; lishi.tianqi.com; usclimatedata.com). Soil temperature data were collected by 12 HOBOware Onset® temperature data loggers (Onset Computer Corporation, 470 MacArthur Blvd. Bourne, MA 02532) inserted in the soil at the Rock Springs Farm in State College, PA. The temperature data was plotted to determine trends. Correlation analysis and simple linear regression were conducted on the hourly air and soil temperature data of State College, PA. T-tests were conducted to compare if air temperatures significantly differ between the cultivation sites. Using a two-sided test, T-tests were done at the significance level of $\alpha = 0.05$.

2020 – 2021 Season 1

Morchella isolates

Two strains with previous fruiting success were chosen for this experiment, *M. importuna* WC 1021 and *M. rufobrunnea* WC 833. They were identified and recorded by the Penn State Spawn Lab. The strain information is recorded in Table 3-1.

Grain spawn preparation, nutrient bag formula, high tunnel, soil, and air temperature measurement

The materials and methods for these sections were identical to that of Season 2

written in detail below.

Watering schedule

After spawning, the plots were watered until saturation. During the vegetative growth phase, the plots were watered when needed via drip lines based on visual observation, about once every five to seven days. On December 23rd, 2021, soil temperature dropped below 4°C, and watering was stopped. On April 6th, 2021, 186 days after spawning, the nutrient bags and plastic sheets were removed to trigger fruiting. The plots were watered heavily for two days beyond saturation to stimulate sexual growth further.

Outdoor cultivation experimental design for season 1 (2020 – 2021)

We hypothesized that yield with three soil amendments will be in the following order due to soil bulk density differences, therefore, mycelial soil colonization differences – straw > sawdust > control. Twenty-four plots were randomly assigned to a field in Rock Springs, PA. There was a total of six treatments (two strains: *M. rufobrunnea* WC 833, *M. importuna* WC 1021, and three soil amendments: sawdust, straw, and control). Each treatment had four replications. The plots were tilled and formed into raised beds, and 1115 g standard spawn rate (scaled from 0.5 kg / m^2) (He *et al*., 2019) spawn was applied to the top of the raised beds, each had an area of about 2.23 m^2. The raised beds were separated by a furrow about 61 cm wide. Spawning was performed on October 2nd, 2020. Thirty days after spawning, seven

nutrient bags were cut open and applied to each plot facing the soil. Drip irrigation lines were established for watering.

2021 – 2022 Season 2

***Morchella* isolates**

Based on visual observation of PDYA medium colonization, *M. importuna* WC 1021, WC 1022, and *M. rufobrunnea* NRRL 28465, NRRL 28466 colonized PDYA media most rapidly within seven days in room temperature (23℃) and were selected for this study among seven *M. importuna* strains and six *M. rufobrunnea* strains as shown in Table 3-1. They were identified and recorded by the Penn State Spawn Lab.

Table 3-1 Strain information

Strain ID	Species	Substrate location	Comment
WC 1021	*M. importuna*	culture collected from China	Kept in the Penn State Culture Collection
WC 1022	*M. importuna*	culture collected from China	Kept in the Penn State Culture Collection
WC 833	*M. rufobrunnea*	unknown	Kept in the Penn State Culture

NRRL 28465	*M. rufobrunnea*	East Lansing, Michigan, USA	Collection Identified and isolated by Kerry O'Donnell; Spore print made by Gary Mills
NRRL 28466	*M. rufobrunnea*	East Lansing, Michigan, USA	Identified and isolated by Kerry O'Donnell; Spore print made by Gary Mills

Grain spawn preparation

Grain spawn was prepared by combining 1337g rye grains, 38 g calcium carbonate, 16 g calcium sulfate, and 995 ml of warm tap water in an autoclavable 20 x 50 cm Unicorn® (1005 N. Ave., Plano, Tx, 75074, USA) plastic spawn bag with a filter patch pore size of 0.2 microns and was autoclaved twice at 121℃ for 40 minutes then cooled overnight. These grains were inoculated with five 4 mm plugs of agar from a PDYA medium at the edge of actively growing *Morchella* mycelium. Grain bags were heat-sealed above the filter patches. The grain bags were incubated for 42 days at room temperature (23℃), and the bag was shaken by hand every other day to distribute the mycelium within the bag.

Nutrient bag formula

Autoclavable Unicorn® 20 x 50 cm plastic bags with a filter patch were used as nutrient bags. Each bag contained 209.56 g of corn (wet weight at about 5.5% moisture), 140.6 g of sawdust (wet weight at about 8% moisture), and 157.4 g of added water to maintain about 55% moisture. The total weight was about 350 g. The nutrient bags were autoclaved twice at 121℃ for 40 minutes and cooled overnight before applying to the field. The nutrient bags were prepared one day prior to application to minimize contamination in the bags.

High tunnel and field treatment before spawning

A high tunnel was erected over the field plot for shading and protection against heavy rain/hail. The field was treated with Roundup® PowerMax™ (Bayer AG, 51368 Leverkusen, Germany) at 0.23 ml per m^2 for weed control 119 days prior to spawning, and a dark blue plastic cover was applied over the field to minimize weed seed germination (Figure 3-1).

Figure 3-1 Field treatment before spawning.
The field was covered with a plastic sheet before spawning in the summer to deter weeds from growing in the field.

Outdoor cultivation experimental design for season 2 (2021 – 2022)

We hypothesized that the yield of *M. importuna* would be significantly higher when using mixed mating-type spawn than spawn with individual mating-type, and no significant differences between mixed and individual spawn of *M. rufobrunnea*. However, due to contamination in spawn bags, there was only enough spawn for two replications, and statistical comparison could not be conducted. Twelve plots were randomly assigned to a field within a high tunnel in Rock Springs, PA. There were six treatments (*M. rufobrunnea* NRRL 28465, 28466, 28465 + 28466, *M. importuna* WC 1021, 1022, 1021 + 1022). Each treatment had two replications. The plots were tilled and formed into raised beds, and 2386 g spawn was applied to the top of the raised beds;

each had an area of about 2.2 m^2 and then covered with 3 – 4 cm of topsoil. The raised beds were separated by a furrow about 61 cm wide. In the case of two strains in one plot, for example, WC 1021 mixed with 1022, 1193 g of spawn of each isolate was used. Spawning was performed on September 29th, 2021. Seven days after spawning, seven nutrient bags were cut open and applied to each plot facing the soil. Drip irrigation lines were installed, and a black plastic cover was then added to shade the plots and retain moisture (Figure 3-2a; 3-2b). There were several critical steps in outdoor cultivation: spawning, applying nutrient bags, applying plastic cover sheet, removing nutrient bags and the plastic sheet, heavy watering, and finally, harvesting. The DNAs of harvested fruiting bodies were extracted, and their mating-type loci were amplified via PCR.

Figure 3-2a Drip irrigation lines established for watering the plots.

Figure 3-2b Plastic sheet applied for shading and retaining moisture.

Soil and air temperature measurement

Soil temperature was measured and recorded by HOBOware® (Onset Computer Corporation, 470 MacArthur Blvd. Bourne, MA 02532) dataloggers (Figure 3-3). They were buried in Rock Springs, PA at 5 cm deep.

Figure 3-3 HOBOware dataloggers

Watering schedule, fruiting management, and harvesting

After spawning, the plots were watered, and the plastic cover sheet was applied. During the vegetative growth phase, with the nutrient bags on and plastic sheets covered, the plots were watered via drip lines based on visual observation about once every five to seven days. On December 20th, 2021, soil temperature dropped below 4°C, and watering was stopped. On April 4th, 2022, 160 days after spawning, the nutrient bags and plastic sheets were removed to trigger fruiting. The plots were watered heavily for two days to saturation to stimulate sexual growth further. During the fruiting phase, the plots were watered more often, daily, if there were no rain events. On day 203 after spawning, the matured fruiting bodies were harvested with a sharp sterilized knife in separate paper bags. Later in the lab, the fruiting bodies were measured for length and weight, and the data was recorded.

Revenue calculation standard

Malone *et al*. surveyed and assessed the economics of foraging and selling morels in Michigan, USA, and found that the average in-season price at $36 / lbs. and $120+ / lbs. out of season (Malone *et al*., 2022). The revenue of the extrapolated yield data (projected weight if no ascocarp was missing or damaged) was calculated according to this standard.

Results

Soil temperature analysis

The soil and air temperature data of State College, PA, were plotted and the trend appears (Figure 3-4). The correlation coefficient was 0.8220307, which was strong, positive, and linear.

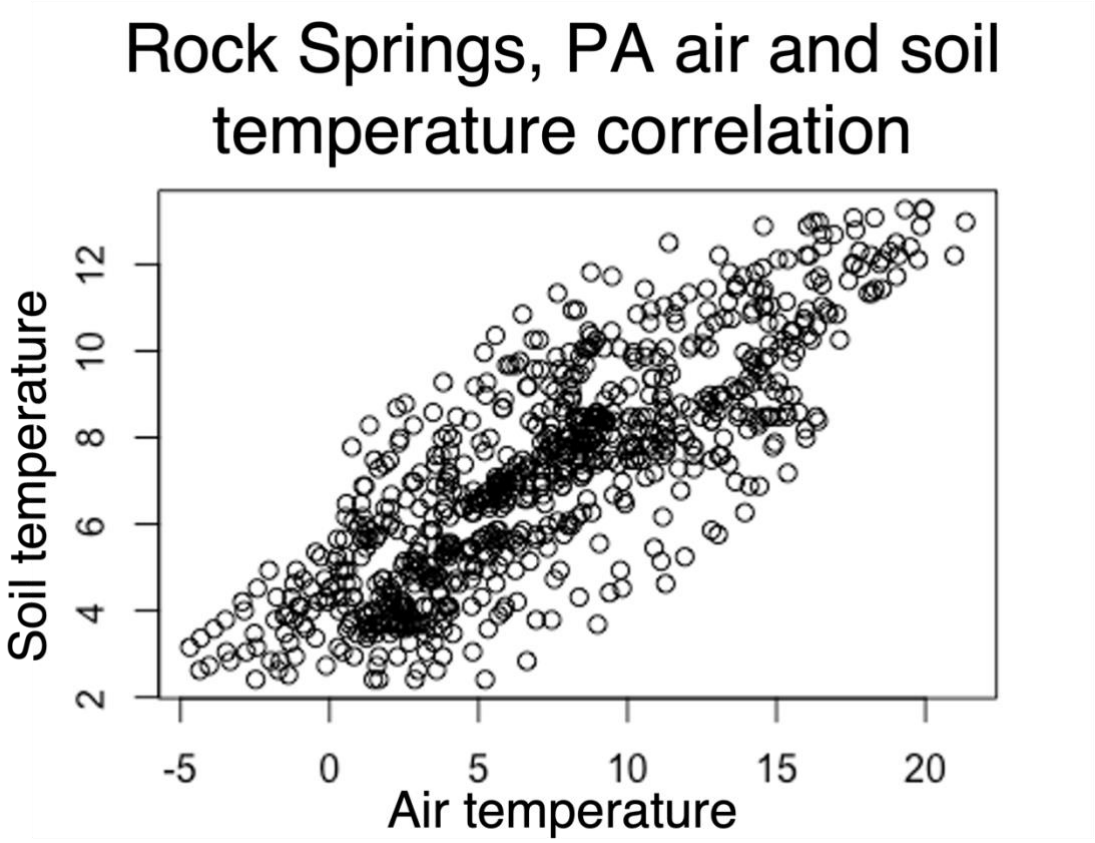


Figure 3-4 Air and soil temperature data correlation. The correlation coefficient being 0.822.

Knowing that the correlation between air and soil temperature in State College, PA is strong, linear, and positive, T-tests were done between the cultivation sites. In each T-test, the null hypothesis, H_0, is that either State College or Orono air temperature = Xinzhou or Yushu air temp. The alternative hypothesis, H_a, is either State College or Orono air temperature $\neq$ Xinzhou or Yushu air temperature.

The T-test results indicated that the average/extreme high/extreme low air

temperature of Orono, ME is not significantly different from that of Xinzhou and Yushu China. The average/extreme high/extreme low air temperatures of State College, PA, were not significantly different from that of Xinzhou. Although the extreme low air temperature of State College was not significantly different from Yushu, its average and extreme high temperatures were significantly different from but warmer than Yushu. Based on our assumption, the soil temperatures of State College, PA, and Orono, ME were expected to have a similar correlation with air temperature, and therefore not significantly colder than the coldest two Chinese outdoor morel cultivation sites, and therefore suitable to grow morels outdoors.

Mycelial growth assessment on PDYA media

M. importuna WC 1021 and 1022, *M. rufobrunnea* NRRL 28465 and 28466 grew thick mycelium on PDYA media and were the most rapidly growing strains among all strains. Both *M. importuna* and *M. rufobrunnea* mycelium appeared white/light grey.

2020 – 2021 Season 1

Mycelial growth assessment seven days and three weeks after spawning

Little *Morchella* mycelium growth was observed seven days after spawning on each plot. There was no difference observed between *M. importuna* and *M. rufobrunnea* plots. Three weeks after spawning, or seven days after nutrient bags were applied, mycelial growth was observed around and in the nutrient bags on each plot, binding the nutrient bags to the soil.

Conidial growth assessment

Light conidia growth was observed 52 days after spawning. On two *M. importuna* plots, both with straw soil amendment. However, no conidium was observed on the *M. rufobrunnea* plots.

Fruiting body formation

No fruiting body formation was observed in Season 1.

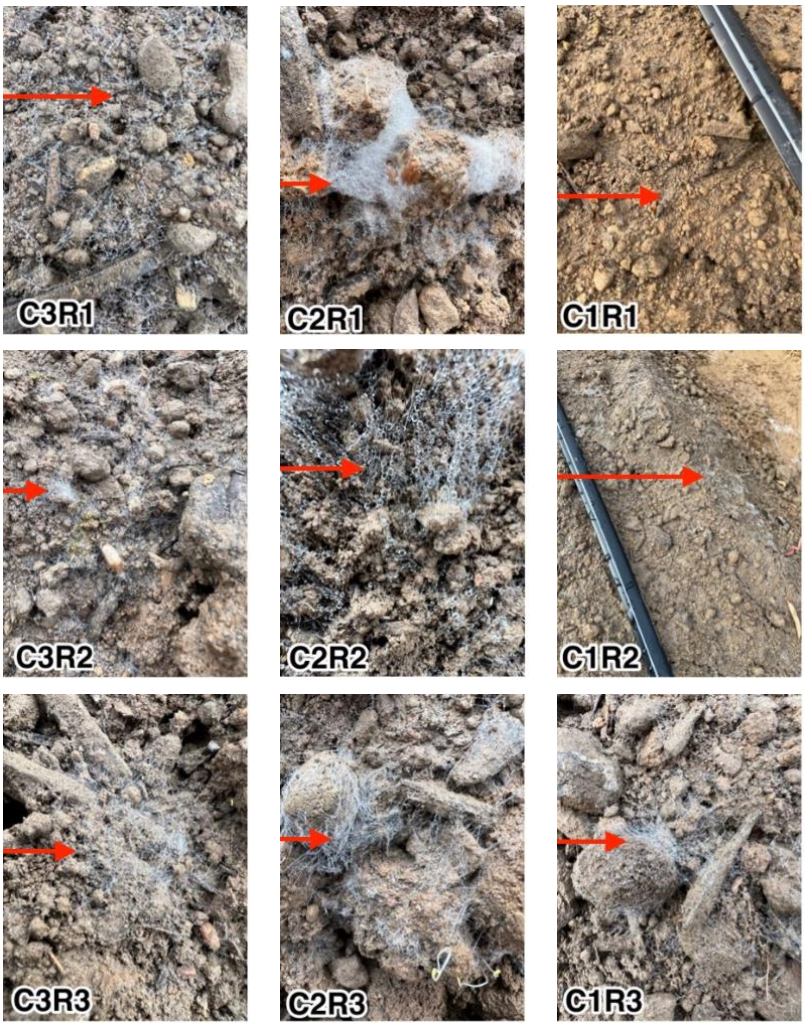
Table 3-2. The timeline of M. importuna and M. rufobrunnea outdoor cultivation 2020 – 2021

Days after spawning	Key steps	Average soil temperature 5 cm deep	Average air temperature
0	Spawning	13 ℃	14℃
30	Nutrient bags added	7 ℃	4 ℃
186	Nutrient bags and plastic cover removed/plot heavy watered	12 ℃	23 ℃

2021 – 2022 Season 2

Mycelial growth assessment seven days after spawning

Seven days after spawning, mycelium grew horizontally across the plot and vertically above the topsoil. Heavy mycelial growth was observed on each plot except C1R1, *M. rufobrunnea* NRRL 28465 seven days after spawning (Figure 3-5). From visual observation, the mycelium in the field appeared similar to the color of *Morchella* mycelium on agar medium, and no difference was observed between *M. importuna* and *M. rufobrunnea* plots.



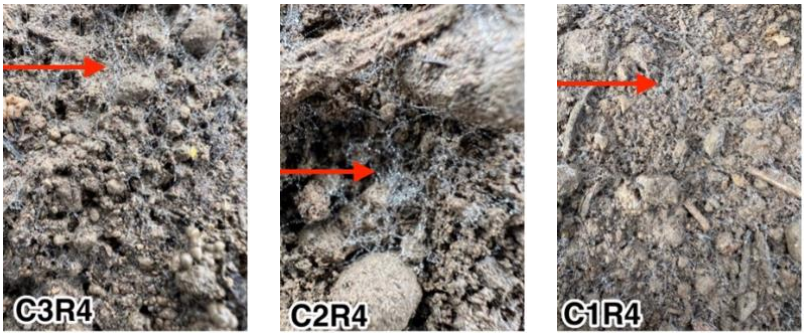


Figure 3-5 Mycelial growth was observed 7 days after spawning.

In each photo, "C" is column "R" is row. The red arrows pointed to where mycelium was grown. *M. rufobrunnea* and *M. importuna* were abbreviated as MI and MR in the following plot labels. C1R1: MR NRRL 28466, C1R2: MI WC 1022, C1R3: MR NRRL 28465 + 28466, C1R4: MI WC 1021 + 1022, C2R1: MR NRRL 28465, C2R2: MI WC 1021 + 1022, MI WC 1022, C2R4: MR NRRL 28466, C3R1: MR NRRL 28465 + 28466, C3R2: MR NRRL 28465, C3R3: MI WC 1021, C3R4: MI WC 1021.

Mycelial growth assessment 3 weeks after spawning (2 weeks after nutrient bags applied)

Seven days after nutrient bags were applied, heavy mycelial growth was observed around and in the nutrient bags on each plot (Figure 3-6). Fourteen days after nutrient bags were applied, heavy conidial growth was observed on each plot, especially on the edge of each plot. However, little mycelial growth was observed on the edge of the plots (areas farther from the nutrient bags).

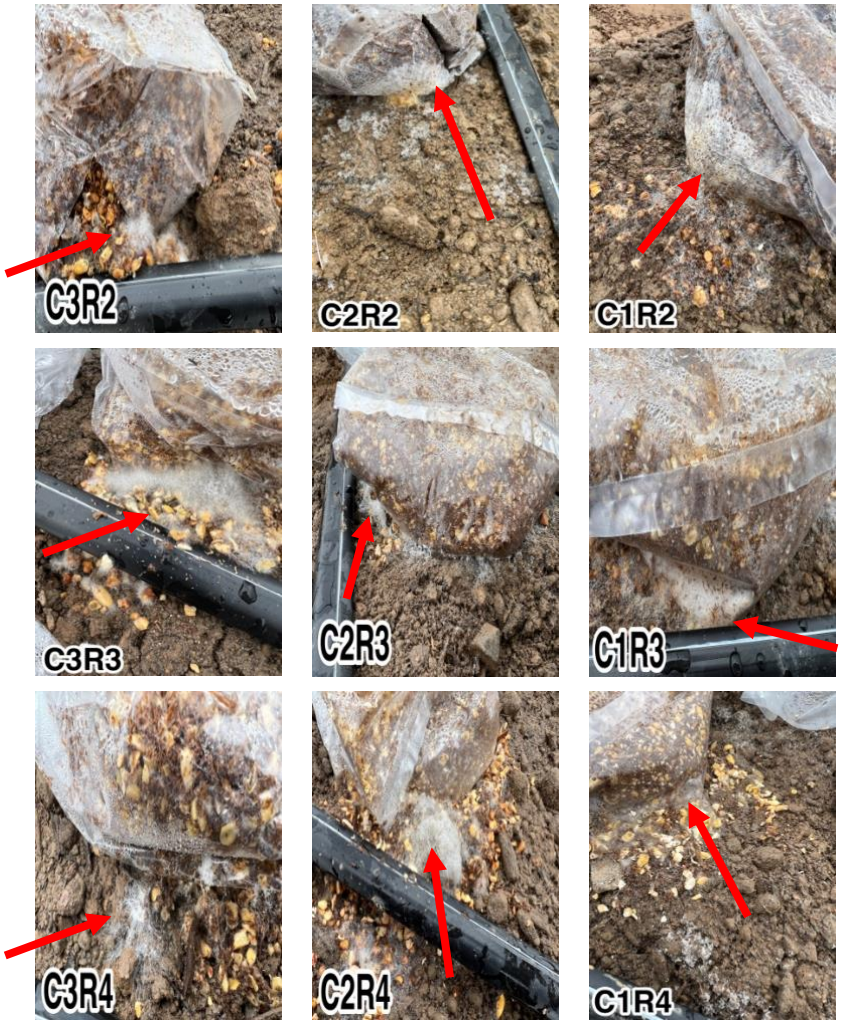


Figure 3-6 Mycelial growth was observed 21 days after spawning, 14 days after nutrient bags applied.

The red arrows pointed to where mycelium was grown.

Conidial assessment 3 weeks after spawning

Heavy conidial growth was observed on the raised beds and the sides of the raised beds. The texture was similar to chalk, and a white powder-like fume appeared once the water was dropped on the conidia (Figure 3-7a, 3-7b, 3-7c).

Figure 3-7a Conidial growth was observed 21 days after spawning, 14 days after nutrient bags applied.

Photo was taken on plot C3R3, *M. importuna* WC 1021. The red arrow points to the conidia.

Figure 3-7b Conidial growth on plot C2R2, M. importuna WC 1021 + 1022. The red arrow pointed the conidia.

Figure 3-7c Conidial growth on plot C1R2, M. importuna WC 1022.
The red arrow pointed to the conidia.

There were several critical steps in outdoor cultivation: spawn, apply nutrient bags, apply plastic cover sheet, remove the nutrient bags and plastic sheet, heavy watering, and harvest. These key steps were performed and documented in Table 3-3.

Table 3-3 The timeline of M. importuna and M. rufobrunnea outdoor cultivation in Season 2 2021 – 2022

Days after spawning	Key steps	Average soil temperature 5 cm deep	Average air temperature
0	Spawning	18 ℃	20 ℃
7	Nutrient bags added	11 ℃	5 ℃
160	Nutrient bags and plastic cover removed/plot heavy watered	5 ℃	5 ℃
203	Harvest	15 ℃	16 ℃

The whole days from spawning were approximately 160 days, and it took approximately 40 days for fruiting bodies to grow to maturity to be harvested.

The outdoor cultivation timeline is summarized in Table 3-4. On day 160 after spawning, primordia were observed on plot column 2, row 3 with WC 1022 *M. importuna*. Primorida were observed on plot column 2 row 3 *M. importuna* 160 days after spawning (Fig. 3-8a). Within 21 days after emerging, the fruiting bodies appeared to stop growing (Figure 3-9b). Most fruiting bodies had spherical caps and short stipes, and only a few had conical caps and longer stipes (Figure 3-9a; Figure 3-9b).

Table 3-4 2021 – 2022 M. importuna and M. rufobrunnea outdoor cultivation timeline

Days after spawning	0 – 28	28 – 133	133 – 168	168	168 - 203
Spawning (day 0)	active mycelial growth	overwinter	active mycelial growth	primordia observed	fruiting bodies mature/harvest

Figure 3-8a Primordia observed on plot column 2 row 3 with WC 1022 M. importuna 160 days after spawning.

The white dot which the red arrow pointed to was the primordium.

Figure 3-8b Fruiting body formation on plot Column 2 Row 3.

Figure 3-9a Conical fruiting body on plot C2R2

Figure 3-9b Spherical fruiting body on plot C2R3

The plastic sheet, as well as the nutrient bags, were removed on day 160 after spawning. About half of the nutrient bags were chewed open and some were completely consumed by rodents and maybe other pests. Most nutrient bags had green/black/red colored microbial growth. Primordia was not observed on any other plot, but fruiting bodies emerged on day 182 after spawning on plots column 1 row 4 with WC 1021 + 1022 *M. importuna* as well as column 2 row 2 with WC 1021 + 1022

M. importuna. The harvested fruiting bodies ranged from 3 g to 121.6 g each. In total, column 1 row 4 had 4 fruiting bodies formed with 1 damaged or missing, column 2 row 2 had 43 fruiting bodies formed with 12 damaged or missing, column 2 row 3 had 57 fruiting bodies formed with 22 damaged or missing. The harvested fruiting body data is summarized in Table 3-5. Despite having heavy mycelial growth seven days after spawning, none of the six *M. rufobrunnea* plots formed fruiting bodies, nor did the two plots with WC 1021 *M. importuna* and one WC 1022 plot.

Table 3-5 2021 – 2022 outdoor morel cultivation yield data

Plot #	**C1R4**	**C2R2**	**C2R3**
Treatment	WC 1021 + 1022	WC 1021 + 1022	WC 1022
Fruiting bodies formed	4	43	57
Fruiting bodies harvested	3	31	35
Cap tissue damaged or missing	1	8	10
Fruiting body missing	0	4	2
Total weight (g)	13	538	985
Ascocarp mean	4	17	28

weight (g)			
Projected weight if no ascocarp missing or damaged (g)	18	747	1604
Projected revenue ($) based on in-season price produced in a 500 ft^2 farm at ($36 / lbs.)	12	493	1058
Projected revenue ($) based on out-of-season price produced in a 500 ft^2 farm at ($120+ / lbs.)	39	1642	3527

Although profit margin could not be calculated because the exact cost could not be determined, revenue both in and out of season could be calculated based on the average in and out of season prices sold in Michigan, surveyed by Malone *et al*., at $36 / lbs or $120+ / lbs accordingly (Malone *et al*., 2022). The projected revenue was calculated based on multiplying extrapolated yield data ("projected weight if no ascocarp missing or damaged" row in Table 3-5) by in or out-of-season prices.

The mating-type identity of cap and stipe tissue was tested via PCR to observe if any pattern occurred. Of the fruiting bodies on plots with mixed WC 1021 and 1022 spawn, two fruiting bodies' stipe tissue tested *MAT 1-2-1*, and one tested *MAT 1-1-1*, while others had no band (Figure 3-10a; 3-10c). Of the fruiting bodies on plots with WC 1022 spawn (*MAT 1-1-1*), stipe tissue only tested *MAT 1-1-1*. The *MAT 1-1-1* amplicons were ~1.6 kb amplicons, and the *MAT 1-2-1* amplicons were ~ 1.1 kb, as shown in Figure 3-10b and 3-10d. The mating-type testing result of the outdoor fruiting bodies is listed in Table 3-6.

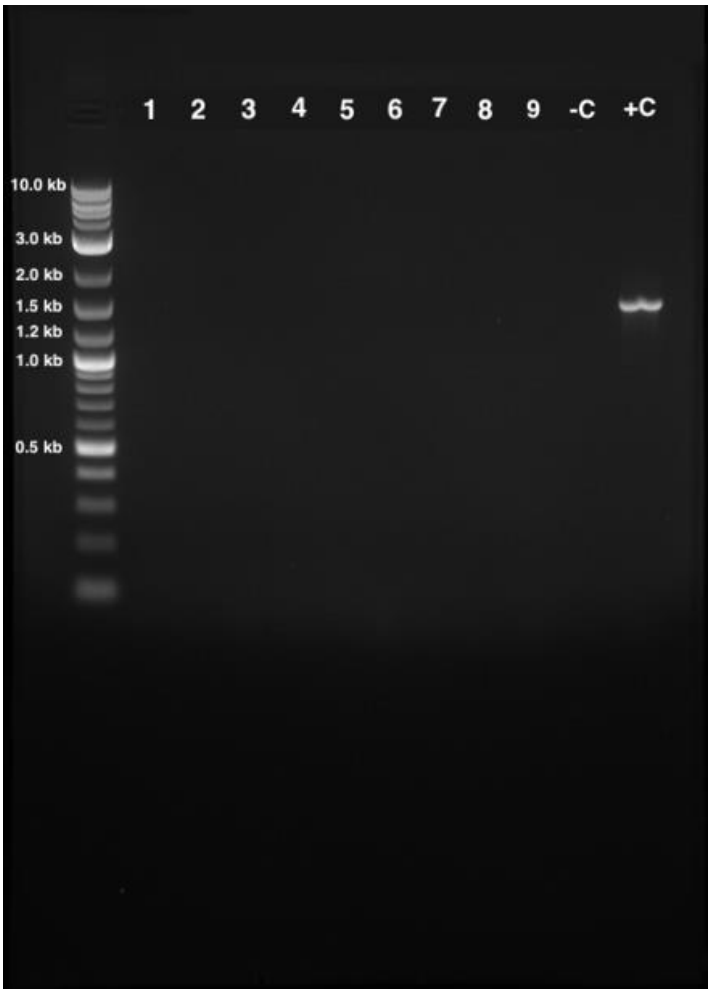


Figure 3-10a Outdoor cap tissue MAT 1-1-1 test results.

The numbers on the top each represents – 1: fruiting body #1 in plot C1R4 (column 1 row 4), 2: fruiting body #2, C1R4, 3: fruiting body #3, C1R4, 4: fruiting body #1, C2R2, 5: fruiting body #2, C2R2, 6: fruiting body #28, C2R2, 7: fruiting body #1, C2R3, 8: fruiting body #2, C2R3, 9: fruiting body #35, C2R3, The letters each represent – "-C": negative control, "+C": positive control.

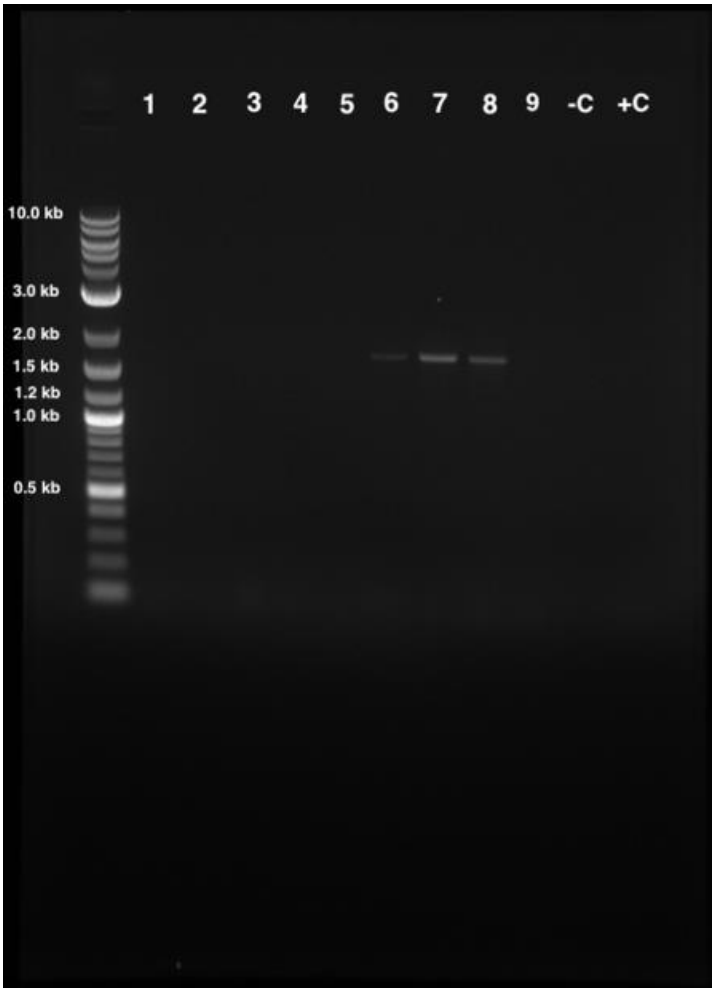


Figure 3-10b Outdoor stipe tissue MAT 1-1-1 test results.

The numbers and letters are the same as Figure 3-10a.

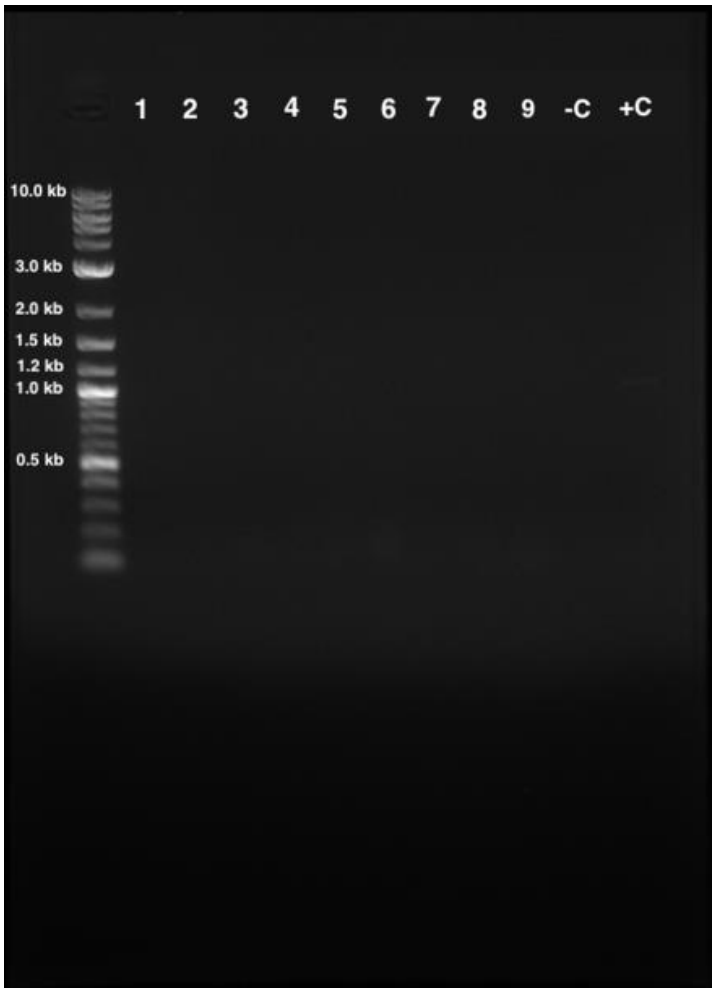


Figure 3-10c Outdoor cap tissue MAT 1-2-1 test results.

The numbers and letters are the same as Figure 3-10a.

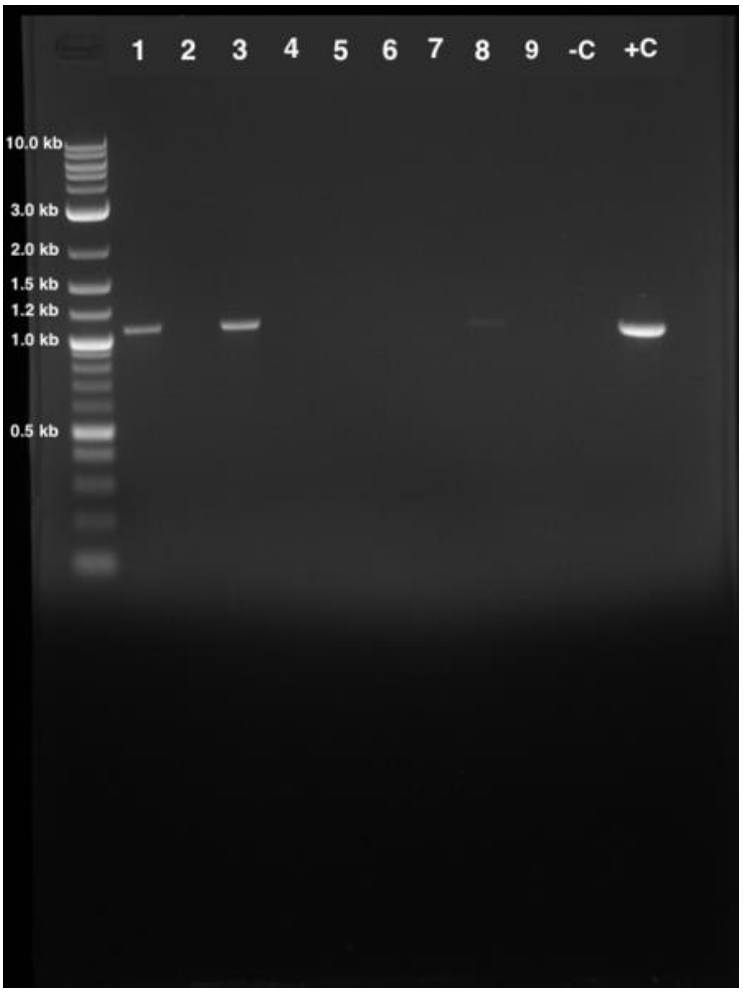


Figure 3-10d Outdoor stipe tissue MAT 1-2-1 test results.

The numbers and letters are the same as Figure 3-10a.

Table 3-6. Outdoor fruiting bodies mating-type testing results

Field # - Fruiting body	Cap tissue mating type	Stipe tissue mating type	Spawn mycelium mating type
C1R4-1	No band	*MAT 1-2-1*	Mixed
C1R4-2	No band	No band	Mixed
C1R4-3	No band	*MAT 1-2-1*	Mixed

C2R2-1	No band	No band	Mixed
C2R2-2	No band	No band	Mixed
C2R2-28	No band	*MAT 1-1-1*	Mixed
C2R3-1	No band	*MAT 1-1-1*	*MAT 1-1-1*
C2R3-2	No band	*MAT 1-1-1/1-2-1*	*MAT 1-1-1*
C2R3-35	No band	No band	*MAT 1-1-1*

Discussion

The soil temperature when spawning was 13 ℃ in Season 1 and 18 ℃ in Season 2. Both were below the < 20 ℃ standard set by Liu *et al*., 2017 for black morel mycelial growth (Liu, Q. *et al*., 2017) and were in the suitable temperature range (10 – 22 ℃) set by Ower 1982 for *M. rufobrunnea* mycelial growth (Ower, 1982).

In Season 1, all plots had little mycelial growth seven days after spawning. This result could indicate that the spawn rate of 1115 g on a 2.23 m^2 plot was insufficient to sustain mycelial growth. However, the plots were not covered with 3 – 4 cm of topsoil or plastic like in season 2, so a conclusion cannot be drawn on a sufficient spawn rate. When nutrient bags were applied 30 days after spawning, all plots had signs of *Morchella* mycelium grown into the nutrient bags. The *Morchella* mycelium was expected to absorb nutrients from the nutrient bags and store energy for future sexual growth when stimulated. Removing the plastic sheet and nutrient bags and heavy watering are all thought to promote fruit body formation in morels via light exposure,

nutrient deficiency, and oxygen deficiency (Liu, Q. *et al*., 2017). However, no fruiting body was formed. The lack of cover plastic and topsoil may have prematurely exposed the *Morchella* mycelium to light and allowed desiccation.

In Season 2, all plots displayed heavy mycelial growth seven days following spawning. This result could indicate that the spawn rate of 2386 g on a 2.23 m^2 plot was sufficient as the nutrient from grains sustained mycelial growth. Later, when nutrient bags were applied seven days after spawning, all plots showed signs of *Morchella* mycelium grown into the nutrient bags. The nutrient bags adhered to the plots making them challenging to remove. Later in the spring, when the cover plastic sheet was removed, about half of the nutrient bags were found chewed open and consumed by pests. The lack of nutrients could be a reason for the low yield. The plastic sheet provided a protected environment from unwanted pests such as slugs and rodents. All of the above pests were observed in the field. Fruiting bodies without cap tissue and damaged fruiting bodies were observed, which were possibly caused by these pests.

When the cover plastic sheet was removed in early April, when the soil temperature was only 5 ℃, plot C2R2 already have primordia forming. This result could indicate that nutrient bags on this plot were chewed open and partially consumed, causing nutrient deficiency that triggered fruiting. In addition to a lack of nutrients, none of the *M. rufobrunnea* plots fruited. This could be a result of *M. rufobrunnea* being more adapted to warmer weather to its origin in Western North America (Du *et al*., 2012). The primordia and later fruiting bodies were grown on the

side of the trapezoidal raised beds, and none formed on top of the plots. This fruiting result contradicted the fruiting distribution in Chinese outdoor cultivation and could result from better water retention near the base of the raised beds.

Even though the fruiting bodies were carefully rinsed with water before performing DNA extraction, they could still contain other microbes and soil preventing the reagents from extracting DNA from the fruiting bodies. This could explain why none of the cap tissue had any amplicons. Although the spawn had a mix of both mating-types, the stipe tissue of C1R4-1 (fruiting body #1 from plot C1R4), C1R4-3 (fruiting body #3 from plot C1R4) tested *MAT 1-2-1* while the stipe tissue of C2R2-28 (fruiting body # 28 from plot C2R2) tested *MAT 1-1-1*. This observation was consistent to that of Du *et al*., 2021, both *MAT 1-1-1* and *MAT 1-2-1* mycelium can be maternal tissue for stipe formation (Du *et al*., 2021).

With limited replications, yield from plot C2R3 with a *MAT 1-1-1* only spawn was 33% higher and weighed 83% more than C2R2, a plot with mixed mating-type spawn. Plot C2R3 yielded 1325% higher and weighed 7249% more than C1R4, a plot with mixed mating-type spawn. The difference in yield between C1R4 and C2R2, two plots with the exact spawn, was unclear. These results could suggest that using spawn with *MAT 1-1-1* may produce better than spawn with mixed mating-types. With a pest management plan (keeping nutrient bags intact), the yield of *MAT 1-2-1* only spawn could be tested and compared to spawn with mixed mating-types and spawn with *MAT 1-1-1* only.

Conclusion

The soil temperatures of State College, PA, and Orono, ME, were found to be suitable for morel outdoor cultivation based on comparison with morel cultivation sites in China. Based on this, *M. importuna* and *M. rufobrunnea* were cultivated with the Chinese nutrient bag technique in two seasons, and *M. importuna* successfully fruited in Season 2. In season 2, with limited replications, plots with *MAT 1-1-1* spawn yielded higher than plots with mixed mating-type spawn. With this result, the hypothesis of "*M. importuna* will have a higher yield with mixed mating-type spawn than spawn with individual mating-type" was rejected. The main difference between Seasons 1 and 2 was spawn rate, use of cover plastic, and topsoil. The fruiting success observed in season 2 may have attributed to a sufficient spawn rate at 2386 g on a 2.23 m^2 plot, sufficient moisture, and shading provided by the cover plastic and topsoil.

The extrapolated *M. importuna* yield data from Season 2 suggested that growers could expect to make a revenue as high as $3527 with a 1604 g projected yield from a standard 500 ft^2 farm if WC 1022 spawn was used in one crop cycle (in 203 days). Neither mating-type was found to be the predominant stipe tissue, and no mating-type information was acquired on the cap tissue.

Although the harvested fruiting bodies from Season 2 appeared to be free of pathogen infection, nutrient bags being consumed prematurely as well as pests foraging on fruiting bodies were a significant issue to overcome if growers aim to be profitable.

Chapter 4 Indoor Cultivation

Introduction

There have been attempts in the history of morel cultivation to grow morels indoors. Indoor cultivation aims to supply the desirable mushroom year-round as well as save the time and labor required to find them outdoors. By growing morels indoors, growers could avoid the seasonality, weather fluctuation, intense labor, and pests from outdoor cultivation.

As early as 1982, suggested environmental parameters for morel cultivation were described, such as maintaining soil temperature below 20 ℃ and high air humidity for indoor cultivation of *M. rufobrunnea* (Ower, 1982). These findings were patented and adopted by Neogen Corporation, Terry Farms, and Diversified Natural Products (now Gourmet Mushrooms, Inc.). Terry Farms, for example, reported growing 3000 lbs. of *M. esculenta* (speculated *M. rufobrunnea* by Kuo, 2006) per week at an unknown production area (Pilz 2007; Kuo 2006). However, there were complaints about the lack of taste and aroma in the cultivated morel crops. In addition, a speculated bacterial disease was found, and the yield numbers decreased sharply. As a result, production was shut down in 2006 (Pilz, 2007).

An indoor controlled environment helps protect the morel crops from pest pressure and harsh outdoor environments. This controlled environment requires active air exchange, temperature and humidity control, and lighting to simulate the adequate growing conditions found outdoors. These simulated environmental parameters can be adjusted when switching from vegetative growth to sexual reproduction. Ower's

patent describes using a black plastic sheet to reduce light exposure, prevent weed growth, and retain moisture. From 1982 to 2010, this indoor cultivation setup emphasized the formation of sclerotia and had fruiting successes in research and commercial production (Ower, 1982; Masaphy, 2010; Pilz 2007). In 2012, the cultivation technique with nutrient bags was developed and widely practiced in China, which was based on Ower's jar cultivation method described in Chapter 1 (Tan 2016). Within a decade, the total yield numbers set a record high of 900,000 kg in China, but 70% of morel farmers had little to no yield (Liu, W. *et al*., 2018). This yield inconsistency may be due to a poor understanding of the *Morchella* life cycle, the mating mechanism (Du *et al*., 2021), and poor spawn quality (Liu, Q. *et al*., 2017).

This study aimed to take a step further to explore the effect of mating with two species with historic fruiting success – *M. importuna* and *M. rufobrunnea* as well as spawn rate on fruiting body formation indoors by replicating the Chinese nutrient bag technique with or without compatible mating-types and spawn rates.

Materials and Methods

Morchella isolates

The same strains used in outdoor cultivation in Chapter 3 were used for this study indoors.

Grain spawn preparation

First, 191 g sterilized rye grains were mixed with 3.4 g calcium carbonate, 5.3 g

calcium sulfate, and 220 ml warm tap water in a 250 ml flask and were autoclaved twice at 121℃ for 40 minutes. The sterilized grains were cooled overnight and inoculated with five 4 mm agar discs from a PDYA medium at the edge of actively growing *Morchella* mycelium with a sterilized cork borer and a needle under a biosafety cabinet. The rye grains were incubated for 14 days at room temperature, approximately 23 ℃, and the flask was shaken by hand every other day to distribute the *Morchella* mycelium. After 14 days, the grains in the flasks were well-colonized, and the grains were covered with actively growing *Morchella* mycelium. An autoclavable Unicorn® plastic spawn bag with 1337g sterilized grains, 38 g calcium carbonate, 16 g calcium sulfate, and 995 ml of warm tap water was autoclaved twice at 121℃ for 40 minutes and cooled overnight. Then, the grains with actively growing *Morchella* mycelium from the 250 ml flask were transferred into the autoclaved Unicorn® spawn bags under a biosafety cabinet. The grain bags were heat-sealed above the filter patches. The inoculated grains were incubated for an additional 28 days for a combined 42 days of mycelial growth at room temperature of about 23 ℃. However, all of the *M. rufobrunnea* spawn bags and some of the *M. importuna* spawn bags were contaminated and could not be used in this study. Therefore, this study only experimented with *M. importuna* spawn.

Nutrient bag formula

An autoclavable 19 x 48 cm Unicorn® plastic bag with a filter patch was used as a nutrient bag. Each bag contained 209.6 g of corn (wet weight at about 5.5%

moisture), 140.6 g of sawdust (wet weight at about 8% moisture), and 157.4 g of added water to maintain about 55% moisture for a total weight of 350 g. The nutrient bags were autoclaved twice at 121°C for 40 minutes and cooled overnight before applying to the tubs. After autoclaving, on day nine after spawning, two nutrient bags were cut on the side, facing the soil, and placed near the center of each tub (Figure 4-1).

Figure 4-1 Nutrient bag placed near the center of each tub.

Tub and grow room preparation before spawning.

The indoor cultivation used soil collected from Rock Springs, PA. The soil was pasteurized for 24 hours at 65°C in the Rubbermaid® tubs in the grow room. Each tub had a width of 45 cm, a length of 60 cm, and a depth of 18 cm. Three holes with

diameters of approximately 2 cm were drilled on one side of each tub for water percolation, and a plastic lining was placed in each tub. Twenty-three kg of soil was added to each tub 72 hours before spawning. The grow room was filled with four racks on the sides (two on each side), and tubs were evenly spread out on the racks.

Indoor cultivation experimental design

We hypothesized that the yield of *M. importuna* would be significantly higher when using mixed mating-type spawn than spawn with individual mating-type, and no significant difference between mixed and the use of 3x spawn rate would yield significantly higher than 1x spawn rate. There were six treatments (*M. importuna* WC 1021, 1022, 1021 + 1022) with either 1x or 3x standard spawn rate (scaled from 0.5 kg / m^2) (He *et al*., 2019). Each treatment had five replications for a total of 30 tubs. The tubs were spawned by placing the grain spawn in a row near the center of each tub with either 1x spawn rate: 122 g inoculated grains or 3x spawn rate: 366 g inoculated grains near the center of each tub and were then covered with approximately 3 cm of steam pasteurized topsoil. The spawned area was about 1/4 of the tub surface area. Each tub was lightly watered and covered with black plastic sheets. In the case of two strains mixed in one tub, for example, WC 1021 mixed with 1022, 61 g of each isolate was used. The room temperature was set to 16 ℃, and the probes in the soil confirmed that the soil temperature was the same as the room air temperature. Nutrient bags were made one day prior to use (eight days after spawning) to minimize contamination and retain moisture in the bags. Seven days

after spawning, two nutrient bags were cut open longitudinally for about 19 cm and applied 3 cm apart near the center of each tub, under the plastic cover sheet, to supply nutrients to *Morchella* mycelium (scaled from 1.2 kg / m^2) (He *et al*., 2019). Forty-nine days after spawning, the cover plastic cover was removed, and the lighting system was turned on to simulate the natural light cycle. Lighting was set to follow a diurnal schedule during the sexual growth phase, 12 hours on and 12 hours off, by Intermatic® (1950 Innovation Way, Suite 300 Libertyville, IL 60048). Room temperature and humidity were measured and adjusted by J. Mähr Electronic® controls (J. Mähr Electronic GmbH, Föhrenweg 7, AT 6973 Höchst). The nutrient bags were also removed to stimulate sexual reproduction. Harvesting of mature fruit bodies occurred from 100 to 124 days after spawning and was counted and weighed when harvested.

Watering schedule, fruiting management, and harvesting

During the vegetative growth phase, with the nutrient bags on and plastic sheets covering the substrate, the tubs were watered based on visual observation about once every five to seven days as needed (plastic sheets were removed, water added, and plastic sheets placed back on tubs). Later, when the nutrient bags and plastic sheets were removed, the tubs were watered heavily until saturation three times to stimulate sexual growth based on the Chinese cultivation technique (Du and Zhao, 2015). After heavy watering to induce fruiting, the plastic liner was cut at each opening. Excess water drained out from the holes drilled in the tubs during the saturated watering

sessions. During the sexual reproduction phase, the tubs were watered more often, about once every three to five days, to maintain a moist soil surface but not so much to cause water ponding on the surface. Primordia formed 80 days after spawning. On day 124 after spawning, the mature fruiting bodies were harvested with a sharp sterilized knife, placed in paper bags, and weighed. Harvesting of mature fruit bodies started 44 days after the first primordia emergence.

Table 4-1 The timeline of M. importuna indoor cultivation

Days after spawning	Key steps	Soil temperature	Humidity %	Light cycle
0	Spawning	16 ℃	94	Dark
9	Nutrient bags added	16 ℃	94	Dark
49	Nutrient bags and plastic cover removed/plot heavy watering	14 ℃	94	12 hours on, 12 hours off
124	Harvest	14 ℃	94	12 hours on, 12 hours off

DNA extraction and mating-type testing

The harvested fruiting bodies were rinsed with water, and a small piece of the cap and stipe tissue was cut off and lyophilized in Eppendorf tubes for 48 hours. The freeze-dried cap and stipe tissue samples were stored at -20°C until DNA extraction to test for mating-type identity. The DNA extraction, PCR, and gel electrophoresis protocols were the same as described in Chapter 2.

Results

Heavy mycelial growth was observed on the soil surface of each tub seven days after spawning (Fig. 4-2). Sixteen days after spawning, (seven days after nutrient bags application), little mycelial growth was observed around and in the nutrient bags in each tub (Fig. 4-1).

Figure 4-2 Mycelial growth seven days after spawning.
The red arrow pointed to the mycelium on the soil surface.

Thirty-four days after spawning, twenty-five days after nutrient bags were applied, conidial growth was observed in each tub. The conidia appeared as white,

chalk-like powder and fumed when watered. Conidial samples were taken to the lab and observed under the microscope (Fig. 4-3).

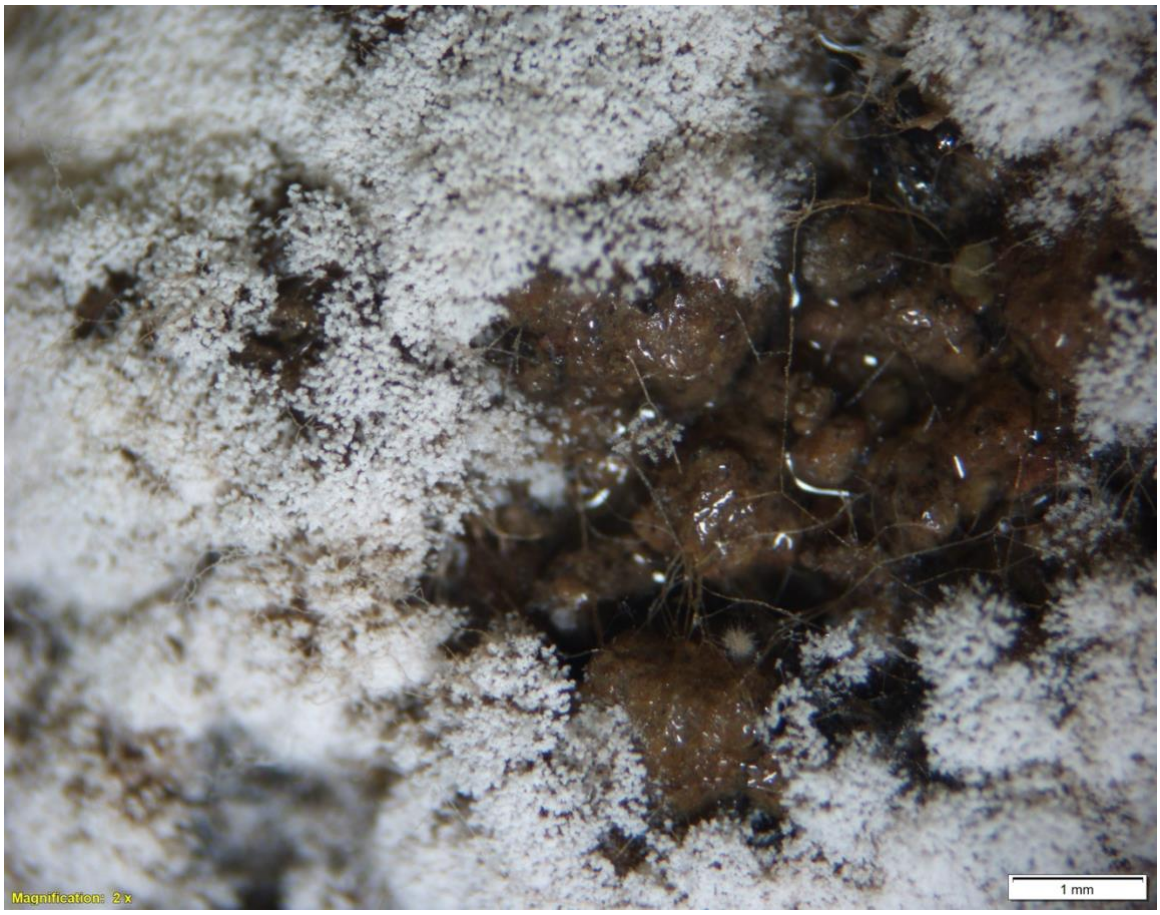


Figure 4-3 Conidiation on soil observed under dissecting microscope with 20x magnification.

Three days after nutrient bag removal, heavy mycelial growth was observed on the soil surface (Fig. 4-4), and heavy conidial growth was observed ten days after the nutrient bag removal (Fig. 4-5).

Figure 4-4 Heavy mycelial growth three days after nutrient bag removal.

Figure 4-5 Heavy conidial growth ten days after nutrient bag removal.

Seventeen days after nutrient bag removal, conidial growth reached its highest level, and light yellow peziza-like fungi were observed in some of the tubs (Fig. 4-6). Seventy-one days after spawning, conidia started receding, and nine days later, primordia formed.

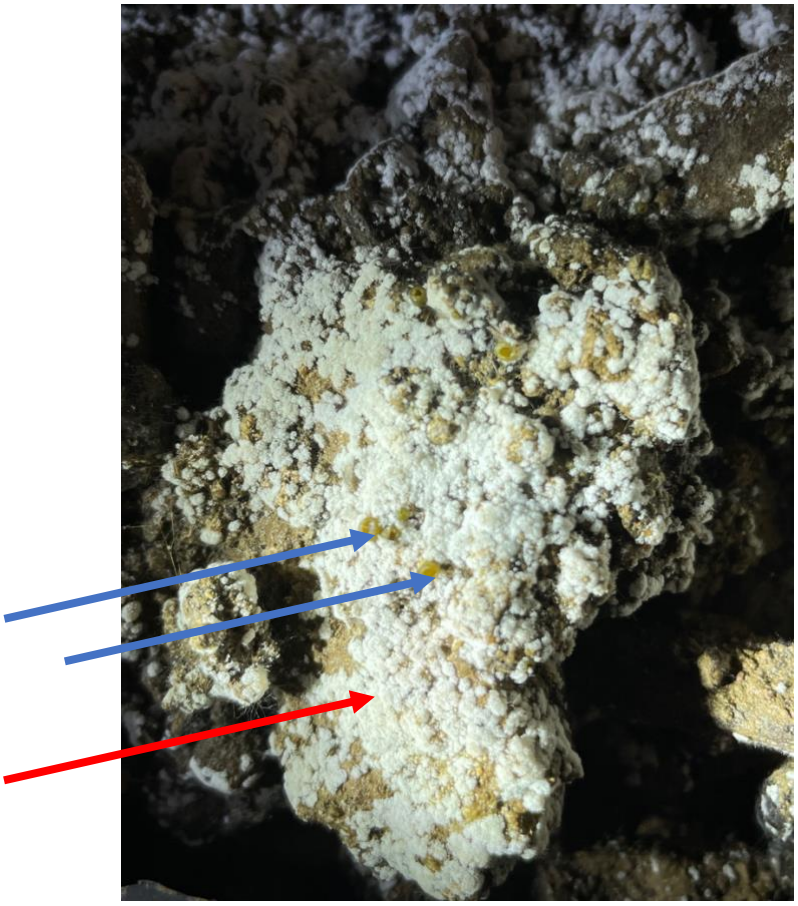


Figure 4-6 Conidial growth reached its highest level and dark brown peziza-like fungi were observed 17 days after nutrient bag removal.

The blue arrows indicate the peziza-like fungi, and the red arrow points to the conidia.

Mycelium grew and colonized the soil for approximately 49 days after spawning, and mature fruit bodies started to form 80 days after sexual growth stimulation. The indoor cultivation timeline is summarized in Table 4-3. Most of the removed nutrient bags had green-colored contamination (Fig. 4-7), and some tubs had green fungal growth on the soil surface of the soil.

Figure 4-7 Contaminated nutrient bags with speculated Penicillium spp., Trichoderma spp., or both.

Table 4-3 M. importuna indoor growth observations

Days after spawning	**0-49**	**49**	**80**	**124**
Spawning (day 0)	active mycelial growth	Sexual growth stimulation	primordia observed	fruiting bodies mature/harvest started

On day 80 after spawning, the first primordia were observed in tub 26 with WC 1021, 1x spawn rate *M. importuna*. Primordia were observed in 17 tubs, which included 5 tubs of 1x WC 1021 spawn, 3 tubs of the 3x WC 1021 spawn, 4 tubs of the 1x mixed spawn, and 5 tubs of the 3x mixed spawn. Seventy-five days after heavy watering/nutrient bag removal/light exposure (124 days after spawning), the tubs had fruiting bodies that appeared elongated with white stipe and dark/light brown caps

(Figure 4-8 a, b). The fruiting bodies from indoor cultivation had longer stipe and lighter colored cap than outdoors (Fig. 4-9 a, b). The harvested fruit bodies ranged from 3 g to 76.8 g and showed mild or no disease symptoms. These fruiting bodies had dark or light grey conical caps, and long white stipes, and were hollow inside. Only a few fruiting bodies had spherical caps and short stipes. Despite having heavy mycelial and conidial growth, none of the WC 1022 tubs had primordia form.

Figure 4-8 Two types of fruiting bodies were observed on harvesting day.
a. a dark grey/light yellow cap with elongated beige stipe tissue with 1x WC 1021 spawn; b. a light grey cap with elongated white stipe tissue with 3x WC 1021 spawn.

Figure 4-9 Morphological difference between outdoor and indoor fruiting bodies.
a: fruiting bodies grown outdoors with short beige stipe and spherical brown/black cap.
b: fruiting body grown indoors with long white stipe and conical light yellow/grey cap.

Fruiting body senescence

In the 1x WC 1021 tubs 89 out of 105 fruiting bodies started to senesce and displayed softening, and reddening symptoms, ultimately resulting in the death of the fruiting body before maturation (Fig. 4-10); 3x WC 1021 tubs had 38 out of 47 fruiting bodies displaying symptoms and dying; 1x mixed tubs had 27 out of 34 fruiting bodies dying; 3x mixed tubs had 8 out of 13 fruiting bodies dying as described in (Table 4-4). The disease rate was calculated by taking fruiting bodies formed minus fruiting bodies harvested and dividing by fruiting bodies formed.

Figure 4-10 Diseased primordia.
The dying primordia had reddening, softening symptoms throughout the stipe and cap tissue, and stopped growing once displaying symptoms.

The healthy fruiting bodies were weighed and the ascocarp mean weight was calculated by dividing total weight by the number of fruiting bodies harvested. Based on this ascocarp mean weight, a projected weight (if no ascocarp death) was calculated by multiplying fruiting bodies formed and the ascocarp mean weight. A projected revenue was calculated by converting the projected weight into pounds and multiplying the in-season price of $36 (Malone *et al*., 2022) and finally multiplying by (500 ft^2 divided by 3 ft^2 per tub) = 166.67 times to fit a 500 ft^2 growing space indoor farm (walking space not counted). Projected revenue for out-of-season of $120+ (Malone *et al*., 2022) was calculated with the same formula above but with $120 as a per-pound price.

Table 4-4 2021 – 2022 indoor morel cultivation yield data

Treatment	1x WC 1021	3x WC 1021	1x mixed	3x mixed
Fruiting bodies formed	105	47	34	13
Fruiting bodies harvested	16	9	7	5
Fruiting bodies senesced	89	38	27	8
Senescence rate (%)	85	81	79	62
Total weight (g)	593	191	191	60
Ascocarp mean weight (g)	37	21	27	12
Projected weight if no ascocarp diseased (g)	3892	996	930	155
Projected revenue ($) based on in-season price produced in a 500 ft^2 growing area at ($36 / lbs.)	44240	11450	15096	2111

Projected revenue ($) based on out-of-season price produced in a 500 ft^2 growing area at ($120 / lbs.)	147319	38128	50270	7030

The increasing growth of the first fruiting body was measured and recorded as the next photos ordered from a to i (Figure 4-10). On day 80 after spawning, the primordium first appeared as a white pin with a light grey cap and a broader base than the stipe (Fig. 4-10 a.). Then, on day 82 after spawning, the cap and the stipe elongated, with the cap ridges becoming clearer and the base widening (Fig. 4-10 b.). As the stipe grew longer from day 85 to 87 after spawning, the stipe darkened into a beige color with a powdery/thread-like bright white base (Fig. 4-10 c.; d.; e.). The fruiting body started bending when grown over 8.5 cm and was harvested on day 100 after spawning (Fig. 4-10 d.; i.).

a.

b.

c.

d.

e.

f.

g.

h.

i.

Figure 4-10 Fruiting body formation in tub 26 in 20 days (a-i).

a. Day 80 after spawning, ~ 3 cm; b. Day 82 after spawning, ~ 5 cm; c. Day 85 after spawning, ~ 7.5 cm; d. Day 86 after spawning, ~ 8.2 cm; e. Day 87 after spawning, ~ 8.2 cm (bent); f. Day 88 after spawning, ~ 8.5 cm (bent); g. Day 90 after spawning, ~ 10 cm (bent); h. Day 92 after spawning, ~ 14 cm (bent); i. Day 100 after spawning, harvested, ~ 10 cm, tissue adjacent to soil was removed.

Results from PCR demonstrated that the cap tissue had a mix of *MAT* bands amplified, while the stipe tissue consisted of *MAT 1-2-1* only. The *MAT 1-1-1* amplicons were ~1.6 kb, and the *MAT 1-2-1* amplicons were ~ 1.2 kb (Figure 4-11a; Figure 4-11b). Mating-type testing results of the caps and stipes are summarized in Table 4-5.

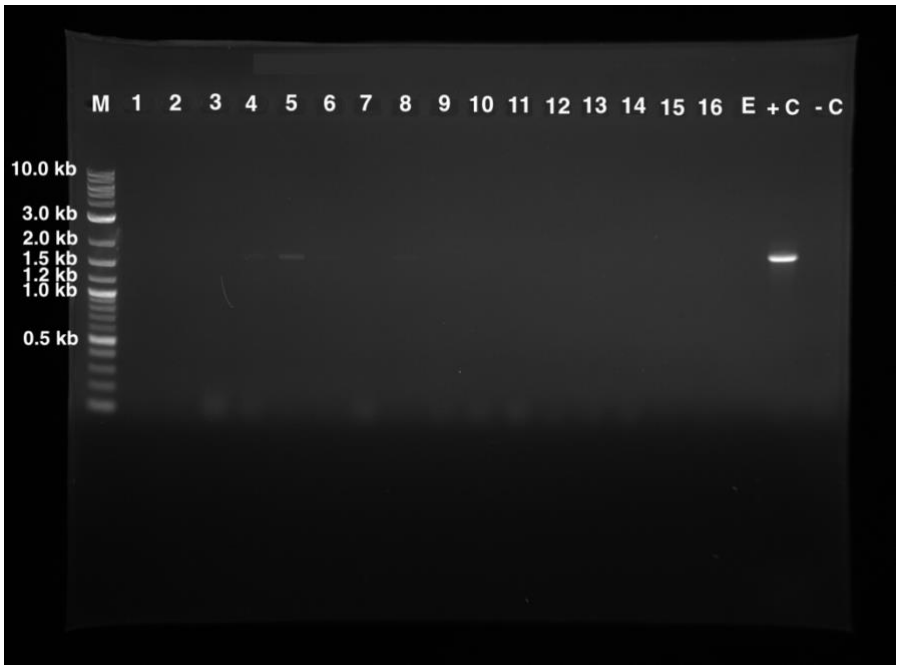


Figure 4-11a Agarose gel showing PCR amplicons of MAT 1-1-1 from cap and stipe tissue.

Samples in lanes are as follows: Lane M) DNA size makers, 1) cap tissue, tub 2, 2) cap tissue, tub 4, 3) cap tissue, tub 5, 4) cap tissue, tub 8, 5) cap tissue, tub 14, 6) cap tissue, tub 20, 7) cap tissue, tub 24, 8) cap tissue, tub 25, 9) stipe tissue, tub 2, 10) stipe tissue, tub 4, 11) stipe tissue, tub 5, 12) stipe tissue, tub 8, 13) stipe tissue, tub 14, 14) stipe tissue, tub 20, 15) stipe tissue, tub 24, 16) stipe tissue, tub 25, E) empty lane, +C) positive control, -C) negative control. The *MAT 1-1-1* amplicons were ~1.6 kb.

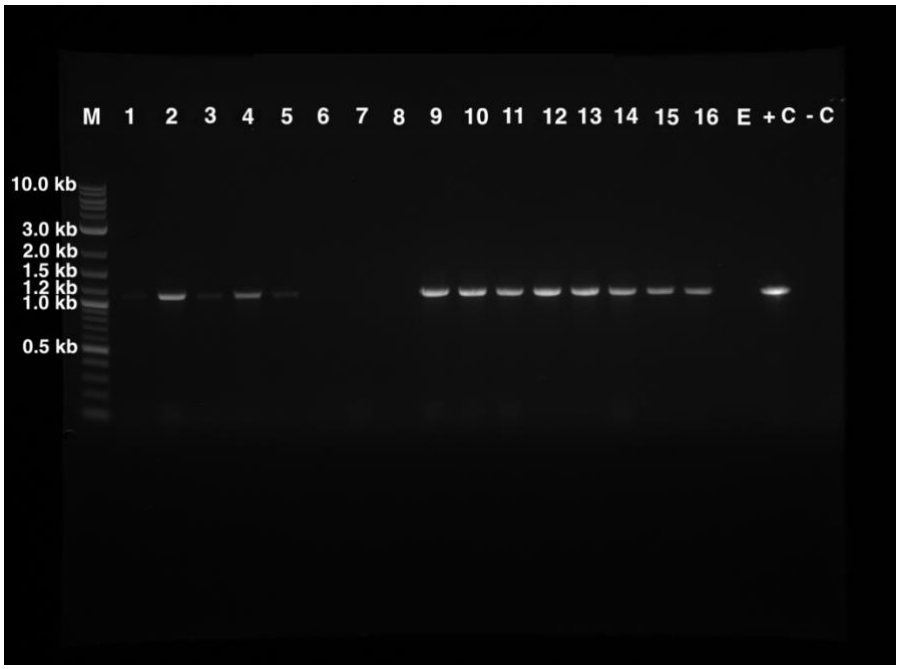


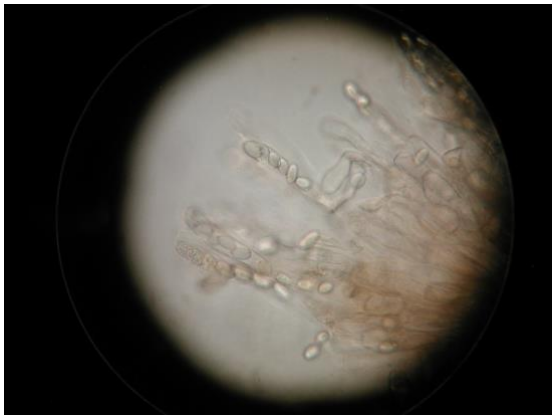
Figure 4-11b Agarose gel showing PCR amplicons of MAT 1-2-1 from cap and stipe tissue.

The numbers and letters are the same as Figure 4-4a. The *MAT 1-2-1* amplicons were ~ 1.2 kb.

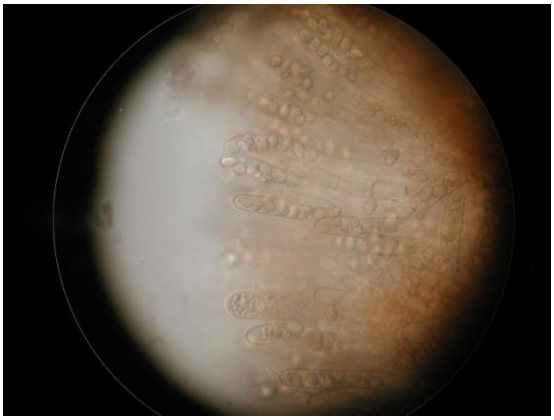
Table 4-5 Indoor fruiting bodies mating-type test results

Tub #	Cap tissue mating-type	Stipe tissue mating-type	Spawn mycelium mating-type
2	*MAT 1-2-1*	*MAT 1-2-1*	Mixed
4	*MAT 1-2-1*	*MAT 1-2-1*	Mixed
5	*MAT 1-2-1*	*MAT 1-2-1*	Mixed
8	*MAT 1-1-1/1-2-1*	*MAT 1-2-1*	*MAT 1-2-1*
14	*MAT 1-1-1/1-2-1*	*MAT 1-2-1*	*MAT 1-2-1*
20	*MAT 1-1-1/1-2-1*	*MAT 1-2-1*	Mixed
24	No band	*MAT 1-2-1*	*MAT 1-2-1*
25	*MAT 1-1-1*	*MAT 1-2-1*	Mixed

The cap tissue of the harvested fruiting bodies was dissected and observed under a compound microscope with 400x magnification (Figure 4-12). All except fruiting bodies from tub #20 had ascospores in the asci. However, the fruiting body from tub #20 still had both mating-types amplified. This could indicate that ascospores with *MAT 1-1-1* landed on the cap tissue, considering that the stipe tissue tested *MAT 1-2-1*, in which the cap tissue was grown.

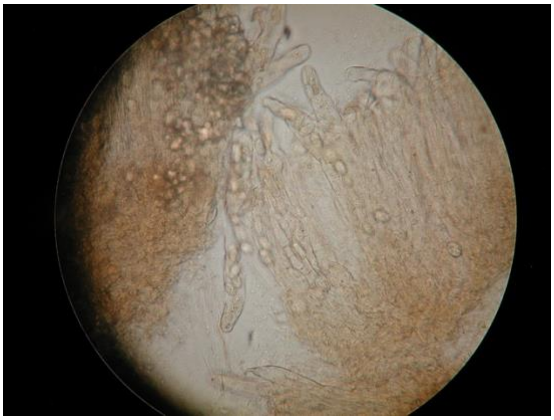


a.



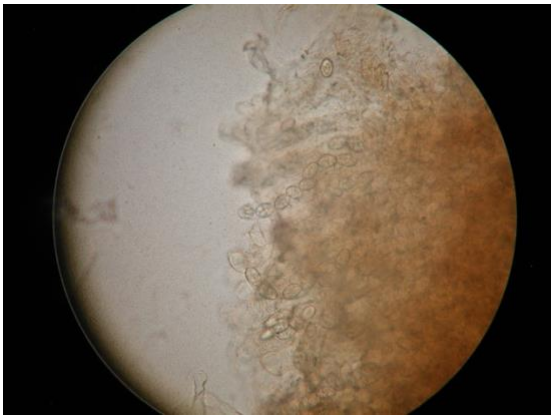
b.

c.



d.

e.



f.

g.

Figure 4-12 Indoor fruiting bodies microscopic photos of ascospores in asci with 400x magnification.

a: a fruiting body from tub #4 with ascospores in the asci. b: a fruiting body from tub #5 with ascospores in the asci. c: a fruiting body from tub #8 with ascospores in the asci. d: a fruiting body from tub #14 with ascospores in the asci. e: a fruiting body from tub #20 without ascospores in the asci. f: a fruiting body from tub #24 with ascospores in the asci. g: a fruiting body from tub #25 with ascospores in the asci.

Discussion

All tubs had heavy mycelial growth seven days following spawning. This result could indicate that the 1x spawn rate of 122 g in a 0.27 m^2 tub was sufficient as the nutrient from grains sustained mycelial growth prior to nutrient bag application. Later, when nutrient bags were applied nine days after spawning, all tubs appeared to have *Morchella* mycelium grown into the nutrient bags. It is proposed that the *Morchella* mycelium absorbs nutrients from the nutrient bags and store energy for sexual reproduction when stimulated (Tan *et al*., 2019). When the plastic sheet was removed to trigger sexual growth, most of the nutrient bags had green/red/black contamination. This could be a result of incomplete sterilization of the nutrient bags during the double-autoclaving process. Although the nutrient bags were double-autoclaved, they

were stacked in autoclavable trays. Without stacking, it would take much longer to autoclave. Another possible source for the contamination in the nutrient bags could be organisms from the soil. Although the soil was exposed to elevated temperatures during steam pasteurization, incomplete pasteurization may have occurred due to incomplete heating of the soil within the tubs. Four autoclaved nutrient bags were not utilized; and kept in the lab after autoclaving, and all four nutrient bags had green colored microbial growth. This growth could have been *Trichoderma* or *Penicillium spp.* indicating that the nutrient bags were not sufficiently sterilized during the autoclave procedure. A high disease rate on fruiting bodies was observed, as shown in Table 4-4 (Table 4-4). The diseased fruiting bodies appeared dark red and softened, and the growth of diseased fruiting bodies slowed significantly compared to healthy ones (Fig. 4-10). These symptoms appear similar to symptoms observed in China and are described as “red body disease” (Xu et al., 2022) and are suggested to be caused by pathogenic bacteria. Masaphy 2022 reported on *Purpureocillium lilacinum* pathogenic growth on primordia when growing *M. rufobrunnea* indoors (Masaphy 2022). These diseased *M. rufobrunnea* primordia turned brown, had dense white foreign mycelium cover, and finally disintegrated (Masaphy 2022). Another disease reported by Guo *et al*., 2016 indicated that more than 30% of cultivated *M. importuna* in Nanyang, China, had a rot disease caused by the *Fusarium incarnatum – F. equiseti species complex.* This disease started as a watery round scar on the stipe and eventually developed into a dark brown sunken patch with foreign white hyphae growing on the surface of the diseased fruiting body (Guo *et al*., 2016). Although the

browning and rotting symptoms found in this study were similar to that of Masaphy 2022 and Guo *et al*., 2016 observed, no dense white foreign mycelium was growing over the diseased primordia. Therefore, the diseased primordia and fruiting bodies were unlikely to be infected by a fungal pathogen due to the lack of foreign hypha or mycelium covering the primordia or fruiting bodies. The symptoms of diseased primordia and fruiting bodies observed in this study were similar to a severe *Pseudomonas spp*. infection in *Agaricus bisporus* – brown lesions with sunken surfaces, often referred as "bacterial blotch," "brown blotch," or "blotch" (Soler-Rivas *et al*., 1999). Although this evidence pointed to *Pseudomonas spp*. as the pathogen, this study did not identify the disease-causing microbe. This bacterial pathogen could be from insufficiently sterilized grain bags/nutrient bags/soil. Since the grain bags and nutrient bags were autoclaved at 121℃, the pasteurized soil was the likely source of the bacterial pathogen. Bacteria in the order of *Bacillales*, *Pseudomonadales*, *Xanthomonadales*, and others could survive pasteurization at 57 ℃, 60 ℃, and 68 ℃ (Vieira & Pecchia, 2018).

The primordia and later fruiting bodies formed mainly near the corners and edges of the tubs, and none formed in the middle. This could be a result of microbial competition in the nutrient-rich area in the middle of the tubs where the nutrient bags were laid. This was similar to the fruiting distribution from outdoor cultivation, where the fruiting bodies appeared on the edge of the trapezoidal raised beds and away from the center where the nutrient bags were laid.

Kuo 2006 described the cap morphology of *M. importuna* found in the wild in

North America as “3 – 15 cm tall, conical, but occasionally egg-shaped or nearly round (Kuo, 2006). Both conical and “nearly round” caps were observed indoors and outdoors in this study. Kuo 2006 also indicated that the cap is “grayish to brownish” when young and will turn into dark brown, black, or yellowish brown. These colors were all observed either indoors or outdoors in this study (Kuo 2006). Kuo 2006 described the stem of wild *M. importuna* found in North America as 3 – 10 cm high and 2 – 6 cm wide, white to pale brown (Kuo 2006). The stipe of fruiting bodies in this study was in the range that Kuo 2006 described, and the color was also similar. Although almost all morphological traits described by Kuo 2006 was found in this study, more fruiting bodies from indoors appeared conical than spherical compared to outdoors. This could be a result of environmental influence, such as lack of ultraviolet light.

Since all mating-type-tested fruiting bodies had stipe tissue with *MAT 1-2-1* regardless of mixed or *MAT 1-2-1* only spawn mycelium, this result could indicate that strains with *MAT 1-2-1* were more adequate as “maternal tissue” than *MAT 1-1-1*. However, a conclusion cannot be drawn that *MAT 1-1-1* could not form “maternal tissue” or stipe tissue due to fruiting success outdoors on one WC 1022 plot. In contrast, Du *et al*., 2017 found *MAT 1-1-1* to be the predominant maternal tissue (Du *et al*., 2017). The reasoning behind this disparity was not understood.

Even though the fruiting bodies were carefully rinsed before performing DNA extraction, they could still contain ascospores or conidia from fruiting bodies in other tubs. This could be a reason to explain some of the cap tissue had both *MAT* genes

amplified. The experiment could be conducted with three different rooms separating spawn of different mating-types: *MAT 1-1-1* only, *MAT 1-2-1* only, and mixed mating-types, in order to minimize unwanted spores or conidia landing on other tubs.

The 1x WC 1021 treatment had a 210% higher yield than the 3x WC 1021 treatment. The 1x mixed treatment had a 220% higher yield than the 3x mixed strain treatment. This directly tied to the projected revenue, as the 1x WC 1021 treatment was 290% higher than the 3x WC 1021, and the 1x mixed treatment was 490% higher than that of the 3x mixed treatment. Although the 3x treatments had lower disease rates than the 1x treatments, a disease management plan would be better than using 3x treatments to lower disease rates. This result suggests that using spawn with only the *MAT 1-2-1* mating type may provide higher yields than mixed mating-type spawn, and the 1x treatment had a higher yield than the 3x treatment. However, additional spawn rates were not tested to determine optimal ones based on yield results and cropping costs.

Conclusion

The extrapolated yield data from this indoor cultivation experiment with *M. importuna* suggested that with a disease management plan that minimizes loss due to premature death, and earlier harvesting, growers could expect to make a revenue as high as $147,319 with 3892 g projected yield from a standard 500 ft^2 growing area farm based on $120+ per pound. The yield was in the following order: 1x WC 1021 spawn > 3x WC 1021 spawn > 1x mixed spawn > 3x mixed spawn. *MAT 1-2-1* was

found to be the predominant stipe tissue, and both mating-types could become the cap tissue. With this result, the hypothesis that the yield of "*M. importuna* will be significantly higher when using mixed mating-type spawn than spawn with individual mating-type" was rejected.

Although fruiting bodies were successfully formed, disease pressure will be a significant issue to overcome if growers aim to be profitable. There was also another phenomenon that this experiment could not explain – *MAT 1-2-1* was the predominant mating-type in the stipe tissue of indoor crops. Yet, both mating-types were found in the stipe tissue of outdoor crops.

Chapter 5 Conclusion and future work

The goals of this thesis were to 1) grow morels indoors in a grow room and outdoors in a high tunnel in the Northeastern United States (State College, PA) and 2) determine the role of mating on fruiting body formation of *M. importuna* and *M. rufobrunnea*. As a result, the goals were achieved with *M. importuna* successfully since this species fruited both indoors and outdoors at a profitable yield (data extrapolated to fit a 500 ft^2 grow room/field), and the role of mating was partially understood.

The mating-type of *M. importuna* and *M. rufobrunnea* strains from the Penn State culture collection and those obtained from the USDA ARS were tested before the cultivation experiments. As a result, the *M. importuna* strains appeared heterothallic. Each strain was determined to be either a *MAT 1-1-1* or *MAT 1-2-1* idiomorph. The *M. rufobrunnea* strains, on the other hand, appeared homothallic. Each strain harbored both *MAT 1-1-1* and *MAT 1-2-1* idiomorphs. These mating-type results were consistent with Du and Yang 2021 (Du and Yang 2021).

This study replicated the most practiced Chinese nutrient bag technique and demonstrated that both *MAT 1-1-1* and *MAT 1-2-1* strains were capable of fruiting individually and mixed. The yield was higher indoors with *MAT 1-2-1* individually and outdoors with *MAT 1-1-1* individually, which growers could use for profitable cultivation. The indoor crops displayed a high rate of disease-causing senescing and ultimate death, possibly caused by a bacterial pathogen (disease rates were as high as 65 to 85%). This disease is a significant issue to overcome if growers aim to be

profitable. With no yields in the outdoor cropping experiment, the sexual reproductive mode of *M. rufobrunnea* could not be determined.

The difference in outdoor cultivation results between Season 1 and Season 2 indicated that either 3x spawn rate, using 3 – 4 cm topsoil, using the plastic cover sheet during spawn run, or slight differences in watering or environmental conditions contributed to fruiting. A higher spawn rate theoretically improves the speed of soil colonization by *Morchella*. The topsoil shades *Morchella* mycelium from the sun, and the cover plastic further shades the field and maintains moisture which may have contributed to the successful fruiting observed during the second season.

Aside from the fruiting success and mating discoveries, this study had a few limitations. Limited by the size of the grow room, it was unclear whether spawn with *MAT 1-1-1* or *MAT 1-2-1* could fruit individually or whether conidiation is required to complete the life cycle. With a limited experiment size in the outdoor experiment, it was unclear why the *M. rufobrunnea* spawned plots had no yield. Ge 2019, however, had fruiting success with *M. rufobrunnea* indoors. It is speculated that M. rufobrunnea, a species native to warmer climates, did not overwinter well as *M. importuna*. The presence of mycelium before overwintering and the absence of primordia in spring on the *M. rufobrunnea* plots could be evidence of this speculation.

Although *M. rufobrunnea* is not a commonly traded/consumed morel species, understanding its biology could contribute to the greater field of morel research and cultivation. It was also unclear whether having nutrient bags intact (protected against pests such as rodents) would have a higher yield outdoors.

The results and their interpretation of them lead to challenges for future research. The challenges for future research on indoor cultivation are: 1. to investigate the reason for the morphological difference between indoor and outdoor fruiting bodies; 2. to examine the yield potential of <1x spawn rate; 3. to explore the effect of mating in three grow rooms – one with *MAT 1-1-1* spawn only (to see if a strain with a single mating-type could individually complete the life cycle), one with *MAT 1-2-1* spawn only, and if the two rooms do not form any fruiting bodies, then use a third room with *MAT 1-1-1* only, *MAT 1-2-1* only, and mixed spawn (to see if conidiation is necessary to complete the life cycle); 4. to identify the pathogen and explore methods of disease control; 5. To investigate environmental conditions such as humidity and temperature that are optimal for fruiting and minimizing loss to disease.

The challenges for future research outdoors are: 1. to have a pest management plan to prevent nutrient bags from partially or completely consumed before the appropriate removal time; 2. to investigate the yield potential of *M. importuna* with only *MAT 1-2-1* spawn; 3. to investigate watering, moisture retention to optimize fruiting and yield.

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APPENDIX

Appendix A Rock Springs soil analysis

Table A-1 Rock Springs soil analysis

			lbs/acre		
Field	pH	phosphate	potash	Mg	Ca
1	5.9	92	310	418	3391
2	6.2	92	427	535	4580
3	6.1	87	398	601	4981
topsoil	7.5	165	526	461	8892

Appendix B Indoor contamination and growth assessment

Table A-2 Indoor cultivation contamination and mycelial/conidial growth assessment

Tub number	spawn rate	strain and spawn rate	relative position to light source	conidial and mycelial growth (1 little, 3 heavy)	contamination severity (0 none, 3 worst)
1	1.00	1x 1022	near	2	2
2	1.00	1x 1021+1022	away	1	3
3	3.00	3x 1022	away	3	0
4	3.00	3x 1021+1022	near	2	1
5	3.00	3x 1021+1022	near	2	0
6	3.00	3x 1021	away	2	0
7	3.00	3x 1022	away	3	0

8	1.00	1x 1021	away	3	2
9	3.00	3x 1021+1022	near	3	1
10	1.00	1x 1022	near	3	3
11	1.00	1x 1021+1022	away	3	0
12	3.00	3x 1021	away	3	5
13	1.00	1x 1022	away	1	0
14	1.00	1x 1021	near	2	1
15	1.00	1x 1021	near	3	5
16	1.00	1x 1021	near	1	4
17	3.00	3x 1021	near	1	4
18	3.00	3x 1021+1022	away	2	3
19	3.00	3x 1021+1022	away	3	3
20	3.00	3x 1021	near	1	0
21	3.00	3x 1022	near	2	1
22	1.00	1x 1021+1022	away	2	0
23	1.00	1x 1022	away	3	5
24	1.00	1x 1021	away	2	2

25	1.00	1x 1021+1022	near	3	3
26	1.00	1x 1021	near	1	1
27	3.00	3x 1022	away	3	0
28	3.00	3x 1022	away	3	1
29	3.00	3x 1021	near	3	0
30	1.00	1x 1022	near	3	0

Appendix C Soil temperature analysis

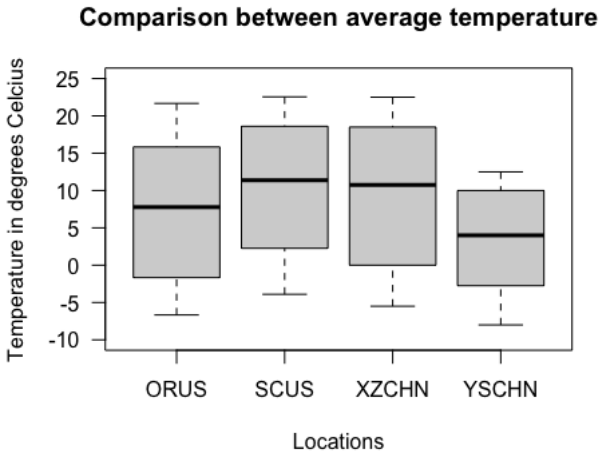

Figure A-1 2018 – 2019 Average temperature comparison.

ORUS: Orono, US; SCUS: State College, US; XZCHN: Xinzhou, China; YSCHN: Yushu, China

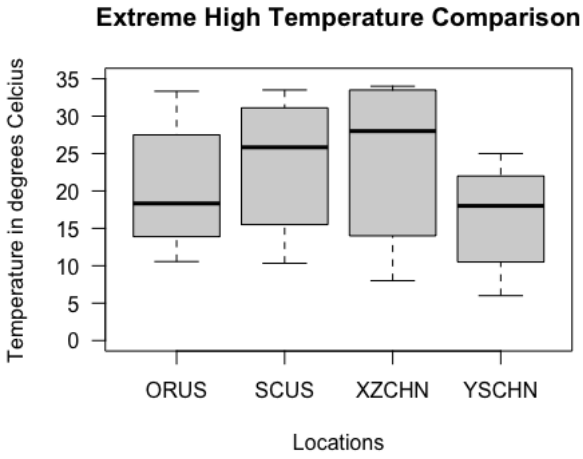

Figure A-2 2018 – 2019 Extreme high temperature comparison.

The letters are the same as Figure A-1.

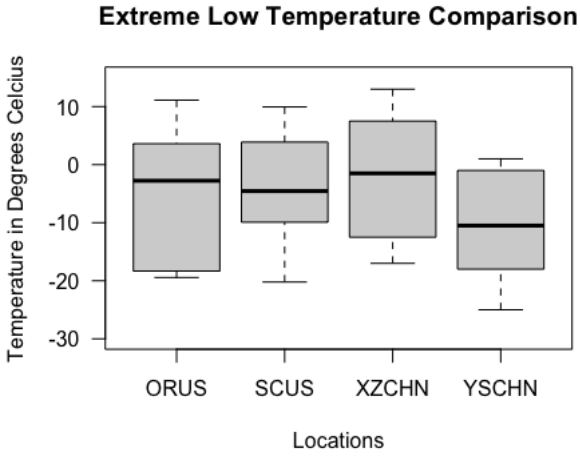

Figure A-3 2018 – 2019 Extreme low temperature comparison.

The letters are the same as Figure A-1.

Appendix D R statistical software code for soil temperature analysis

> Rock.Springs.320to420.hourly.weather.data <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/Rock Springs 320to420 hourly weather data.csv", header=FALSE)

> View(Rock.Springs.320to420.hourly.weather.data)

> Soil.temperature.data.320to420.hourly <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/Soil temperature data 320to420

hourly.csv", header=FALSE)

> View(Soil.temperature.data.320to420.hourly)

> cor.test(Soil.temperature.data.320to420.hourly$V2,Rock.Springs.320to420.hourly.weather.data$V3)

Pearson's product-moment correlation

data: Soil.temperature.data.320to420.hourly$V2 and Rock.Springs.320to420.hourly.weather.data$V3

t = 39.953, df = 766, p-value < 2.2e-16

alternative hypothesis: true correlation is not equal to 0

95 percent confidence interval:

0.7976743 0.8437099

sample estimates:

cor

0.8220307

> t.test(Soil.temperature.data.320to420.hourly$V2,Rock.Springs.320to420.hourly.weather.data$V3, alternative = "two.sided", paired = T)

Paired t-test

data: Soil.temperature.data.320to420.hourly$V2 and Rock.Springs.320to420.hourly.weather.data$V3

t = -1.0214, df = 767, p-value = 0.3074

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-0.3758087 0.1185782

sample estimates:

mean of the differences

-0.1286152

> t.test(Soil.temperature.data.320to420.hourly$V2 - Rock.Springs.320to420.hourly.weather.data$V3, alternative = "two.sided")

One Sample t-test

data: Soil.temperature.data.320to420.hourly$V2 - Rock.Springs.320to420.hourly.weather.data$V3

t = -1.0214, df = 767, p-value = 0.3074

```
alternative hypothesis: true mean is not equal to 0
95 percent confidence interval:
 -0.3758087  0.1185782
sample estimates:
 mean of x
-0.1286152

> #establish a simple linear regression
> #soil temperature is the response variable
> #air temperature is the predictor
> #visualize data
> plot(Soil.temperature.data.320to420.hourly$V2,Rock.Springs.320to420.hourly.weather.data$V3)
> plot(Rock.Springs.320to420.hourly.weather.data$V3, Soil.temperature.data.320to420.hourly$V2)
> m = lm(Soil.temperature.data.320to420.hourly$V2~Rock.Springs.320to420.hourly.weather.data$V3)
> summary(m)

Call:
lm(formula = Soil.temperature.data.320to420.hourly$V2 ~
Rock.Springs.320to420.hourly.weather.data$V3)

Residuals:
     Min       1Q   Median       3Q      Max
-4.1636 -0.9394 -0.0077  0.8847  4.0620

Coefficients:
                                                  Estimate Std. Error t value
(Intercept)                                       4.24698    0.09022   47.07
Rock.Springs.320to420.hourly.weather.data$V3      0.40028    0.01002   39.95
                                                  Pr(>|t|)
(Intercept)                                       <2e-16 ***
Rock.Springs.320to420.hourly.weather.data$V3      <2e-16 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 1.465 on 766 degrees of freedom
Multiple R-squared:  0.6757,  Adjusted R-squared:  0.6753
```

F-statistic: 1596 on 1 and 766 DF, p-value: < 2.2e-16

> #Residual = observed value - fitted value

> #beta 0 hat = 4.24698

> #beta 1 hat = 0.40028

> #std s = 0.01002

>

Boxplot code:

> rm(list=ls())

> Orono.ave.temp.2019 <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/SC ME XZ YS data/CSV files/Orono ave temp 2019.csv", header=FALSE)

> View(Orono.ave.temp.2019)

> SC.ave.temp.2019 <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/SC ME XZ YS data/CSV files/SC ave temp 2019.csv", header=FALSE)

> View(SC.ave.temp.2019)

> XZ.ave.temp.2019 <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/SC ME XZ YS data/CSV files/XZ ave temp 2019.csv", header=FALSE)

> View(XZ.ave.temp.2019)

> YS.ave.temp.2019 <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/SC ME XZ YS data/CSV files/YS ave temp 2019.csv", header=FALSE)

> View(YS.ave.temp.2019)

boxplot(Orono.ave.temp.2019$V2,SC.ave.temp.2019$V2,XZ.ave.temp.2019$V2,YS.ave.temp.2019$V2, xlab = "Locations", ylab = "Temperature in degrees Celcius", main = "Comparison between average temperature", ylim = c(-10,25), names = c("ORUS", "SCUS", "XZCHN", "YSCHN"), las = 1)

t-test code:

> sd(Orono.ave.temp.2019$V2)

[1] 9.882738

> sd(SC.ave.temp.2019$V2)

[1] 9.226226

> sd(XZ.ave.temp.2019$V2)

[1] 10.24575

> sd(YS.ave.temp.2019$V2)

[1] 7.356197

###Orono vs XZ temp

t.test(Orono.ave.temp.2019$V2,XZ.ave.temp.2019$V2,alternative = "two.sided")

Welch Two Sample t-test

data: Orono.ave.temp.2019$V2 and XZ.ave.temp.2019$V2
t = -0.64994, df = 21.971, p-value = 0.5225
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
-11.193805 5.852138
sample estimates:
mean of x mean of y
7.037500 9.708333

> t.test(Orono.ave.temp.2019$V2,XZ.ave.temp.2019$V2,var.equal = T,alternative = "two.sided")

Two Sample t-test

data: Orono.ave.temp.2019$V2 and XZ.ave.temp.2019$V2
t = -0.64994, df = 22, p-value = 0.5225
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
-11.193163 5.851496
sample estimates:
mean of x mean of y
7.037500 9.708333

###Orono vs YS temp

t.test(Orono.ave.temp.2019$V2,YS.ave.temp.2019$V2,alternative = "two.sided")

Welch Two Sample t-test

data: Orono.ave.temp.2019$V2 and YS.ave.temp.2019$V2

t = 1.065, df = 20.326, p-value = 0.2994

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-3.62355 11.19855

sample estimates:

mean of x mean of y

7.0375 3.2500

```
t.test(Orono.ave.temp.2019$V2,YS.ave.temp.2019$V2,var.equal = T,alternative = "two.sided")
```

Two Sample t-test

data: Orono.ave.temp.2019$V2 and YS.ave.temp.2019$V2

t = 1.065, df = 22, p-value = 0.2984

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-3.588177 11.163177

sample estimates:

mean of x mean of y

7.0375 3.2500

###SC vs. XZ temp

```
t.test(SC.ave.temp.2019$V2,XZ.ave.temp.2019$V2,alternative = "two.sided")
```

Welch Two Sample t-test

data: SC.ave.temp.2019$V2 and XZ.ave.temp.2019$V2

t = 0.1765, df = 21.763, p-value = 0.8615

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-7.557037 8.962037

sample estimates:

mean of x mean of y

10.410833 9.708333

t.test(SC.ave.temp.2019$V2,YS.ave.temp.2019$V2,alternative = "two.sided")

###SC vs YS temp

Welch Two Sample t-test

data: SC.ave.temp.2019$V2 and YS.ave.temp.2019$V2

t = 2.1022, df = 20.96, p-value = 0.0478

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

0.07616974 14.24549692

sample estimates:

mean of x mean of y

10.41083 3.25000

> Orono.max.temp.2019 <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/SC ME XZ YS data/CSV files/Orono max temp 2019.csv", header=FALSE)

> View(Orono.max.temp.2019)

> SC.max.temp.2019 <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/SC ME XZ YS data/CSV files/SC max temp 2019.csv", header=FALSE)

> View(SC.max.temp.2019)

> XZ.max.temp.2019 <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/SC ME XZ YS data/CSV files/XZ max temp 2019.csv", header=FALSE)

There were 30 warnings (use warnings() to see them)

> View(XZ.max.temp.2019)

> YS.max.temp.2019 <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/SC ME XZ YS data/CSV files/YS max temp 2019.csv", header=FALSE)

> View(YS.max.temp.2019)

> # test to see if there are differences in std

> sd(Orono.max.temp.2019)

Error in is.data.frame(x) :

'list' object cannot be coerced to type 'double'

```
> sd(Orono.max.temp.2019$V2)
[1] 7.681299
> sd(SC.max.temp.2019$V2)
[1] 8.411565
> sd(XZ.max.temp.2019$V2)
[1] 10.08149
> sd(YS.max.temp.2019$V2)
[1] 6.675941
> #Orono vs XZ max temp comparison
> t.test(Orono.max.temp.2019$V2,XZ.max.temp.2019$V2,alternative = "two.sided")

        Welch Two Sample t-test

data:    Orono.max.temp.2019$V2 and XZ.max.temp.2019$V2
t = -0.96686, df = 20.552, p-value = 0.3449
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  -11.156422      4.081422
sample estimates:
mean of x mean of y
    20.4625      24.0000

> #Orono vs YS max temp comparison
> t.test(Orono.max.temp.2019$V2,YS.max.temp.2019$V2,alternative = "two.sided")

        Welch Two Sample t-test

data:    Orono.max.temp.2019$V2 and YS.max.temp.2019$V2
t = 1.4339, df = 21.581, p-value = 0.1659
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  -1.887066 10.312066
sample estimates:
mean of x mean of y
    20.4625      16.2500
```

```
> #SC vs XZ max temp comparison
> t.test(SC.max.temp.2019$V2,XZ.max.temp.2019$V2,alternative = "two.sided")

        Welch Two Sample t-test

data:  SC.max.temp.2019$V2 and XZ.max.temp.2019$V2
t = -0.070796, df = 21.316, p-value = 0.9442
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -8.143456  7.606789
sample estimates:
mean of x mean of y
 23.73167  24.00000

> #SC vs YS max temp comparison
> t.test(SC.max.temp.2019$V2,YS.max.temp.2019$V2,alternative = "two.sided")

        Welch Two Sample t-test

data:  SC.max.temp.2019$V2 and YS.max.temp.2019$V2
t = 2.4134, df = 20.921, p-value = 0.02506
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  1.033313 13.930020
sample estimates:
mean of x mean of y
 23.73167  16.25000

boxplot(Orono.max.temp.2019$V2,SC.max.temp.2019$V2,XZ.max.temp.2019$V2,YS.max.temp.201
9$V2, xlab = "Locations", ylab = "Temperature in degrees Celcius", main = "Comparison between
average temperature", ylim = c(-10,25), names = c("ORUS", "SCUS", "XZCHN", "YSCHN"), las = 1)
boxplot(Orono.max.temp.2019$V2,SC.max.temp.2019$V2,XZ.max.temp.2019$V2,YS.max.temp.201
9$V2, xlab = "Locations", ylab = "Temperature in degrees Celcius", main = "Extreme High Temperature
Comparison", ylim = c(0,35), names = c("ORUS", "SCUS", "XZCHN", "YSCHN"), las = 1)
```

> Orono.min.temp.2019 <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/SC ME XZ YS data/CSV files/Orono min temp 2019.csv", header=FALSE)

> View(Orono.min.temp.2019)

> SC.min.temp.2019 <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/SC ME XZ YS data/CSV files/SC min temp 2019.csv", header=FALSE)

> View(SC.min.temp.2019)

> XZ.min.temp.2019 <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/SC ME XZ YS data/CSV files/XZ min temp 2019.csv", header=FALSE)

> View(XZ.min.temp.2019)

> YS.min.temp.2019 <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/SC ME XZ YS data/CSV files/YS min temp 2019.csv", header=FALSE)

> View(YS.min.temp.2019)

>boxplot(Orono.min.temp.2019$V2,SC.min.temp.2019$V2,XZ.min.temp.2019$V2,YS.min.temp.2019$V2, xlab = "Locations", ylab = "Temperature in degrees Celcius", main = "Extreme Low Temperature Comparison", ylim = c(-30,15), names = c("ORUS", "SCUS", "XZCHN", "YSCHN"), las = 1)

> sd(Orono.min.temp.2019$V2)

[1] 11.63409

> sd(SC.min.temp.2019$V2)

[1] 9.880644

> sd(XZ.min.temp.2019$V2)

[1] 10.76189

> sd(YS.min.temp.2019$V2)

[1] 8.959082

>#Orono vs XZ min temp comparison

> t.test(Orono.min.temp.2019$V2,XZ.min.temp.2019$V2,alternative = "two.sided")

Welch Two Sample t-test

data: Orono.min.temp.2019$V2 and XZ.min.temp.2019$V2

t = -0.77686, df = 21.868, p-value = 0.4456

```
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  -13.045514      5.937181
sample estimates:
mean of x mean of y
-5.554167 -2.000000

>#Orono vs YS temp comparison
> t.test(Orono.min.temp.2019$V2,YS.min.temp.2019$V2,alternative = "two.sided")

        Welch Two Sample t-test

data:    Orono.min.temp.2019$V2 and YS.min.temp.2019$V2
t = 1.0292, df = 20.652, p-value = 0.3153
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  -4.461785 13.186785
sample estimates:
mean of x mean of y
-5.554167 -9.916667

>#SC vs XZ min temp comparison
> t.test(SC.min.temp.2019$V2,XZ.min.temp.2019$V2,alternative = "two.sided")

        Welch Two Sample t-test

data:    SC.min.temp.2019$V2 and XZ.min.temp.2019$V2
t = -0.43253, df = 21.841, p-value = 0.6696
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  -10.574363      6.926029
sample estimates:
mean of x mean of y
-3.824167 -2.000000
```

>#SC vs YS min temp comparison

> t.test(SC.min.temp.2019$V2,YS.min.temp.2019$V2,alternative = "two.sided")

Welch Two Sample t-test

data: SC.min.temp.2019$V2 and YS.min.temp.2019$V2

t = 1.5824, df = 21.792, p-value = 0.128

alternative hypothesis: true difference in means is not equal to 0

95 percent confidence interval:

-1.896823 14.081823

sample estimates:

mean of x mean of y

-3.824167 -9.916667

> rm(list = ls())

> YS.ave.temp.2019 <- read.csv("~/Documents/Ryan's Master's Degree Classwork/Fall 2020 Classes/STAT 500 Dr. Lin/STAT 500 Final Project/SC ME XZ YS data/CSV files/YS ave temp 2019.csv", header=FALSE)

> View(YS.ave.temp.2019)

> YS.max.temp.2019 <- read.csv("/Volumes/Extreme SSD/CSV files/YS max temp 2019.csv", header=FALSE)

> View(YS.max.temp.2019)

> YS.min.temp.2019 <- read.csv("/Volumes/Extreme SSD/CSV files/YS min temp 2019.csv", header=FALSE)

> View(YS.min.temp.2019)

> XZ.ave.temp.2019 <- read.csv("/Volumes/Extreme SSD/CSV files/XZ ave temp 2019.csv", header=FALSE)

> View(XZ.ave.temp.2019)

> XZ.max.temp.2019 <- read.csv("/Volumes/Extreme SSD/CSV files/XZ max temp 2019.csv", header=FALSE)

> View(XZ.max.temp.2019)

> XZ.min.temp.2019 <- read.csv("/Volumes/Extreme SSD/CSV files/XZ min temp 2019.csv", header=FALSE)

> View(XZ.min.temp.2019)

> t.test(XZ.ave.temp.2019$V2,YS.ave.temp.2019$V2, alternative = "two.sided")

Welch Two Sample t-test

```
data:  XZ.ave.temp.2019$V2 and YS.ave.temp.2019$V2
t = 1.7737, df = 19.96, p-value = 0.09136
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -1.137793 14.054459
sample estimates:
mean of x mean of y
 9.708333  3.250000

> t.test(XZ.max.temp.2019$V2,YS.max.temp.2019$V2, alternative = "two.sided")

        Welch Two Sample t-test

data:  XZ.max.temp.2019$V2 and YS.max.temp.2019$V2
t = 2.2203, df = 19.091, p-value = 0.0387
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
  0.4466266 15.0533734
sample estimates:
mean of x mean of y
       24.00       16.25

> t.test(XZ.min.temp.2019$V2,YS.min.temp.2019$V2, alternative = "two.sided")

        Welch Two Sample t-test

data:  XZ.min.temp.2019$V2 and YS.min.temp.2019$V2
t = 1.9585, df = 21.3, p-value = 0.0634
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 -0.4825812 16.3159146
sample estimates:
mean of x mean of y
-2.000000 -9.916667
```