ETIOLOGY AND EPIDEMIOLOGY: INTERACTIONS OF
SYZYGITES MEGALOCARPUS AND AGARICUS BISPORUS

A Thesis in
Plant Pathology

by
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Submitted in Partial Fulfillment
of the Requirements
for the Degree of
Master of Science

December 2018
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ABSTRACT

Syzygites megalocarpus is a Mucoromycotan fungal pathogen that infects numerous fungi, including Agaricus bisporus, the button mushroom. This pathogen was first reported on a commercial Pennsylvania mushroom farm in 2011 and has since become a recurring problem. Controls for the S. megalocarpus/A. bisporus system have been little studied. The objectives of this research were as follows: 1) Determine the efficacy of commonly used fungicides on S. megalocarpus mycelial growth and investigate the potential inhibition of sporangiospore germination by commonly used disinfectants. 2) Determine the thermal death point of sporangiospores of S. megalocarpus. 3) Determine whether mycelia and/or sporangiospores cause infection. 4) Determine the susceptibility of various A. bisporus strains to S. megalocarpus infections. 5) Identify the bacteria often associated with S. megalocarpus cultures. Results were as follows: 1) Three fungicides, two currently approved for use on mushrooms, chlorothalonil (Bravo Weatherstik®) and thiabendazole (Mertect®), and one not yet approved for use on mushrooms, metrafenone (Vivando®), were tested for efficacy. S. megalocarpus growth on PDA plates containing chlorothalonil and metrafenone reached an EC50 at 1000 ppm. Thiabendazole reduced growth, though it did not reach an EC50 based on the concentrations tested. Additionally, three commercially available disinfectants labeled for use on mushroom farms, sodium hypochlorite bleach (Pure Bright®), iodine (Rapidyne®), and phenol (1-Stroke Environ®), were tested for sporangiospore control; only phenol inhibited sporangiospore germination. 2) The sporangiospore thermal death point was determined to be 36.6-37.2 °C with a 30 minute exposure time, indicating that Phase II mushroom composting
parameters are adequate to kill the pathogen. 3) Both mycelia and sporangiospores caused infection. 4) Four strains of *A. bisporus* were tested for their susceptibility to *S. megalocarpus* infections: two recently released hybrid brown strains (Amycel ‘Brawn’ and ‘Heirloom’), one traditional brown strain (‘Lambert 805’), and one off-white strain (‘Lambert 901’). Mushrooms were inoculated with either mycelial plugs or spore suspensions to determine susceptibility, as well as to determine whether either inoculum source initiated more disease development in the four strains tested. Strains were also tested for susceptibility to infection if wounded by stabbing with a sterile toothpick prior to pathogen inoculation. The unwounded traditional brown strain, ‘Lambert 805’, took on average twice as long to exhibit signs of the pathogen (p=.029). The recently released brown strain, ‘Heirloom’, demonstrated both a consistent level of infection whether wounded or unwounded and a susceptibility of its stumps to infection by spore suspension. 5) Multiple bacterial species were associated with *S. megalocarpus* cultures, with *Serratia marcescens*, previously shown to be pathogenic on zygomycetes, being the most common.

The *in vitro* results of these experiments suggested that the fungicide chlorothalonil (Bravo Weatherstik®) and the disinfectant phenol (1-Stroke Environ®) may be efficacious *in vivo*, that composting and room steaming procedures are at a high enough temperature to control sporangiospores, and that the pathogenic bacterium *S. marcescens* might bear further investigation as a biocontrol. Also, when comparing the traditional ‘Lambert 805’ brown strain and the newly released ‘Heirloom’ hybrid brown strain ‘Lambert 805’ tended to demonstrate less susceptibility compared to ‘Heirloom’ based on infection development by *S. megalocarpus*. 
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ACKNOWLEDGEMENTS

Thank you to my PI Dr. John Pecchia and my other committee members, Dr. David Beyer, Dr. Michael Fidanza, and Dr. David Geiser for their advice, help, and guidance during this process. They are all great. Thank you to the Giorgi Mushroom Co. (Temple, PA), to the Bunton-Waller program, and to the Department of Plant Pathology and Environmental Microbiology for their financial support. Thank you to my family for their support. I would also like to thank the PI of the first lab I worked in, Dr. Richard Bostock, for piquing my interest in plant pathology and always being willing to help. Also, thanks to everybody at the Pecchia Lab and the Mushroom Research Center for teaching me so much and for creating and offering a place where I always felt welcomed and happy to belong.
Chapter 1

Introduction

Crop and pathogen identity and significance

*Agaricus bisporus* (Lange) Imbach button mushrooms are a crop of major economic importance in Pennsylvania. In 2011, a fungus was reported on a brown hybrid strain, first on two commercial farms in Chester County and subsequently on additional farms. Based on its appearance, microscopy, and genetics the pathogen was identified as *Syzygites megalocarpus*, which had not previously been reported in the literature as a problem on a mushroom farm (Beyer et al. 2013). The infection of the crop with this pathogen has since become chronic. Previously, another fungal pathogen, *Trichoderma aggressivum f. aggressivum*, the causal agent of “green mold,” was not a problem for mushroom growers, appeared in a limited range, was not controlled at the time of emergence, and ultimately spread and became a serious mushroom pathogen that still causes significant losses. This pathogen could follow a similar trajectory. Systems where both pathogen and host are fungi are more difficult to work with since both have similar environmental preferences. Since *S. megalocarpus* may have the potential to spread further and cause large losses to industry, one purpose of this thesis was to investigate the efficacy of potential controls.

The button mushroom belongs to Kingdom Fungi, Phylum Basidiomycota, Class Agaricomycetes, Order Agaricales, Family Agaricaceae, Genus *Agaricus*, and Species *A.*
Agaricus bisporus has a pileus and stipe, is generally light brown with a closed hemispherical cap that opens with maturity to planar morphology. Its gills, pink to start, brown at maturity, are crowded and free from the stipe. A. bisporus, literally “two spore,” basidia generally produce two spores with occasional 4-spored variants, 4-spored basidia being more common in the phylum Basidiomycota (Agrios 2005; Kuo 2018). First described in the 1800’s, the nomenclature has included more than thirty name changes (Kew 2015).

A. bisporus grows wild in Europe and California and is also grown as a food crop including small brown (crimini), large mature brown (portabella), and white (button) mushrooms (Kerrigan et al. 1993; Kuo 2018), and the crop is generally referred to as “button” mushrooms. The cultivated button mushroom originated in Europe where it was collected and eaten for centuries, but it was not cultivated until the 1600’s during the reign of Louis XIV (Kligman 1950). The production of these mushrooms was then moved into limestone caves, particularly in Paris, France, and cultivation was later adopted by other European nations. Mushroom cultivation was then perpetuated in the United States by gardeners who were familiar with button mushroom cultivation in Europe. Mushrooms were cultivated in many locales, for example in houses, barns, root cellars, and under the benches in greenhouses. During the late 1800’s specialized structures were built to produce button mushrooms; one of the primary locations for production was Kennett Square, Pennsylvania (Kligman 1950).

According to the American Mushroom Institute (Avondale, PA), mushroom sales in the United States accounted for 1.22 billion dollars in 2016-2017, and 1.13 billion dollars of this total was from button mushrooms. In the United States, Pennsylvania
produced 64% of all button mushrooms, and 23% of those sales were the brown button mushrooms (crimini/portabella) that *S. megalocarpus* preferentially infects (Beyer et al. 2013; NASS 2017).

*S. megalocarpus* is currently classified in Kingdom Fungi, Phylum Mucoromycota, Class Mucoromycetes, Order Mucorales, Family Mucoraceae, Genus *Syzygites*, and Species *S. megalocarpus* Ehrenb.: Fr. This species is the only member of the genus. On hosts, *S. megalocarpus* displays a mold-like, fuzzy, and hair-like appearance, starting yellow and turning bluish-gray as it matures. It is a recently emerging pathogen of *A. bisporus* in Pennsylvania (Beyer et al. 2013). *S. megalocarpus* is a mycoparasitic member of the Mucorales and, in nature, is parasitic on a broad range of Basidiomycetes, and occasionally parasitizes Ascomycetes (Kovacs and Sundberg 1999).

Our understanding of the biology and ecology of *S. megalocarpus*, as a pathogen of *A. bisporus*, is vague and nascent. One goal of this research was to better understand the biology of this fungal pathogen that parasitizes a broad range of fungal fruiting bodies. It parasitizes commercial button mushrooms, mycorrhizal fungi, and the fungal decomposers of organic matter that are critical to ecosystem health and regulate carbon cycling (Gougoulias et al. 2014).

**Significance**

Fungi themselves are of great interest and are a ubiquitous group that plays a key role in many environmental ecosystems. Fungi interact with many other organisms in
relationships ranging from pathogenic to symbiotic. The phylum Mucoromycota, to which *S. megalocarpus* belongs, is an ancient group in which some basic traits of fungi evolved though it has been studied very little. Mycoparasitism, where both host and parasite are fungi, is widespread and also little studied. Significant amounts of research exist on the relationships of fungi as they interact with plants, insects, animals, and humans, but little research exists about mycoparasites or how fungi interact with other fungi.

This research topic is also particularly timely because *S. megalocarpus* was first reported on button mushroom farms in 2011 and growers have been seeing increased incidence of this pathogen on their crops (Beyer et al. 2013). This pathogen arose temporally in conjunction with the release and subsequent adoption of a recently released hybrid brown strain of button mushroom. The reasons for concern about the increasing prevalence of this pathogen are that it causes both yield and fresh quality loss, is fast growing and quick spreading, requires constant monitoring, and increases the cost of labor due to the need for increased levels of sanitation. *S. megalocarpus* can also cause post-cropping issues, appearing quickly in packages of mushrooms once they are already at the supermarket or in a consumer’s refrigerator (J Pecchia, The Pennsylvania State University, personal communication 2016). A significant control challenge is the lack of knowledge about this pathogen. Therefore, a more thorough understanding of the etiology and epidemiology of this fungal pathogen will assist in the development of improved control practices.

The mushroom industry is one of Pennsylvania’s largest cash crops (NASS 2017), and this pathogen could seriously impact Pennsylvania’s economy. It is important to
proactively study a potentially devastating pathogen before it becomes an established and major problem for commercial mushroom farmers like *Trichoderma aggressivum*, “green mold,” which first appeared on mushroom farms in Ireland in the 1980’s, was mostly ignored, and has since caused massive annual crop losses around the world (Doyle 1991; Fletcher 1990). It would be an error to follow the same path of inattention with *S. megalocarpus*.

Additionally, the mushroom crop represents an acceptable protein source in various cultures and can be grown in developing regions of the world that have limited readily available nutritive protein sources. Since mushrooms are a good protein source that can grow on common agricultural wastes such as horse manure, poultry manure, and corn stalks, and since mushrooms are a good source of protein, Vitamin D, and trace minerals (Beyer 2003), the use of mushrooms as a nutritional food source may increase in importance due to reduced cropping space and arable land, and limited water availability globally.

This crop, *A. bisporus*, the button mushroom, is of major economic importance to Pennsylvania. The recently observed pathogen, *S. megalocarpus*, which is becoming increasingly problematic on mushroom farms, has the potential to be a serious threat to the crop and ultimately to the livelihood of Pennsylvania mushroom farmers.

**Review of literature**

*Syzygites megalocarpus*
Little has been published or researched about *S. megalocarpus* as an agricultural pathogen. Most of the existing publications are dated and focus on its homothallic nature and zygospores, with little if any consideration of its agricultural significance. However, the peer review literature does provide basic groundwork for some aspects of control by assisting in clarifying some of the pathogen’s reproductive aspects, habitat preferences, and potential vectors.

*Syzygites megalocarpus* (Ehrenberg 1820), previous scientific name *Sporodinia grandis*, is the sole member of the monotypic genus *Syzygites*. In 1957, an exhaustive historical review of the scientific names of *S. megalocarpus* was published (Hesseltine 1957). This genus was previously classified in the phylum Zygomycota. However, as of 2016 this phylum no longer exists, and it has been split into two new phyla, Mucoromycota and Zoopagomycota, and *S. megalocarpus* is currently assigned to the Mucoromycota (Spatafora et al. 2016).

*S. megalocarpus* is a mycoparasite, a fungal parasite of other fungi, particularly of the large fruiting bodies of the Basidiomycota. Like many other necotrophic parasites, *S. megalocarpus* has quite a broad host range. It has been found to parasitize 65 genera across multiple phyla of fungi including Ascomycota, and its range in Illinois has been described in 23 species in 18 genera (Kovacs and Sundberg 1999). In nature, this mycoparasite is generally found on older/senescing fruiting bodies. *S. megalocarpus* was identified and described as a necrotrophic parasite on *Tricholoma matsutake* mushrooms in Korea (Ka et al. 1999). *S. megalocarpus* was first reported on a North American cultivated button mushroom (*Agaricus bisporus (Lange) Imbach*) crop in 2011 (Beyer et
It was first observed and isolated from a recently available hybrid brown strain of *A. bisporus* on a Pennsylvania mushroom farm.

Previous research on *S. megalocarpus* primarily focused on its sexual reproduction strategy. It was the first fungus to have sexual reproduction described, an important step in the research that led to the recognition that fungi could reproduce sexually. The first mention of *S. megalocarpus* in the literature was made by Ehrenberg when he observed zygospores and proposed that zygospores were fungal sexual structures (Ehrenberg 1820). De Bary is credited with assigning the term “zygospore” to the sexual spores of *S. megalocarpus* (1864). Blakeslee concluded that zygospore formation was a sexual process and that homothallic mycelium was bisexual and heterothallic mycelium was unisexual (Blakeslee 1904). Keene (1914) described the cytology of the sexual reproduction between two branches of hyphae that are morphologically the same. The cytology included retraction of protoplasm of one branch and presence of small nuclei in both gametangia that stain the same color as mycelium, followed by dissolution of the wall between the gametangia, fusing of the gametes, flowing of the protoplasm, and a second wall arising in the zygospore. Then suspensors and unfused nuclei degrade, with large cytoplasmic bodies containing numerous characteristic nuclei postulated to serve as food reserves for the fungus.

A preponderance of the research on *S. megalocarpus* was carried out between the early 1900’s and the 1970’s, with a primary focus on its homothallic nature and sexual spores. Focusing on the sexuality of this fungus, both Falck (1901) and Blakesee (1904) chose *S. megalocarpus* as a model for homothallic fungi. Falck showed that the fungal zygospores gave rise to homothallic progeny, and Blakesee’s research focused on
differentiating fungi into two main mating categories: those that are homothallic (self-fertile) and those that are heterothallic (not self-fertile). Blakesee (1906) demonstrated that germinating zygospores of *S. megalocarpus* were homothallic in nature. One focus of research during the early 1900’s was determining how to consistently produce zygospores of *S. megalocarpus* in culture. Blakesee’s media included carrot slices and dilute prune decoction. Falck also suggested that there might be physiological differences between cultures germinated from zygospores, but Blakesee was unable to find such permanent differences. Additionally, in the 1940’s it was hypothesized that *S. megalocarpus* produced zygospores by apomixis as opposed to meiosis, which means that the sexual spores would be clones of the fungus that produced the spores (Cutter 1942).

More recently, in the continuing study of the homothallic nature of *S. megalocarpus*, Idnurm (2011) described *S. megalocarpus* as having the loci for *sexM* and *sexP* at different locations; *sexM* and *sexP* occurring at different locations is unlike most Mucorales where *sexM* and *sexP* genes are found at the same locus but code for different proteins (Wetzel et al. 2012). Idnurm focused on the theory that the two MAT loci found in *S. megalocarpus* make it homothallic and determined that, unlike other previously investigated examples, *S. megalocarpus* individuals contain both the *sexM* and *sexP* proteins in the HMG domain of the genome in two different locations. He also determined that flanking genes found adjacent to the mating type loci have started to decay into pseudogenes. Furthermore, the chromosomal rearrangement that switches the alleles into two different loci and results in heterothallism has also occurred in other
ascomycetes. Based on these findings, Idnurm (2011) postulated that a heterothallic individual gave rise to the homothallic *S. megalocarpus*.

The morphology of the sporangiophores, sporangiospores, and zygospores of *S. megalocarpus* has been described in detail by Hesseltine (1957). Sporangiophores grow out of the substrate, are branched dichotomously up to four times, and terminate in a globose column-shaped sporangium with a dissolving wall and few spores. The sporangiospores are shaped like globes and have spines on them. The zygospores are melanized and ornamented, produced on opposite suspensors on zygophores that branch dichotomously and end in spines that are sterile (Hesseltine 1957). Benny and O’Donnell using light microscopy (1978) and O’Donnell using scanning electron microscopy (1979) described sporangia as globose and columnate, sporangiophores as dichotomously divided with all terminal branches ending in sporangia, and zygospores as homothallic with zygosporangia between opposed suspensors. Ekpo and Young noted the unusual formation of spinose walls in sporangiospores of *S. megalocarpus* (1979). This trait, the formation of spinose walls, is shared by *Sporodiniella umbellata*, a fungus that is pathogenic on insects (Ekpo and Young 1979). Using light microscopy and scanning electron microscopy, the spines were determined to be 4-6 micrometers wide and 5-8 micrometers long (Kovacs and Sundberg 1999).

Baker (1931) reported on conditions that influenced growth rate, speed of spore formation, and type of spore formed. He reported that zygospore and sporangia production were influenced by the following: 1) the variety of nutrients in the media, 2) the concentration of these nutrients in the media, 3) the humidity, and 4) the temperature. Low temperatures favored zygospore formation, and high temperatures
favored sporangia, but both zygospores and sporangia developed over a wide temperature range. Higher nutrient concentrations favored zygospore formation, and low nutrient concentrations favored sporangia formation, and when sporangia were produced, zygospores were generally produced afterwards. Humidity did not markedly affect production, but high humidity seemed to favor zygospore formation and lower humidity sporangia formation, though both were produced at all humidity levels tested. Media composition also affected zygospore production, which required an adequate and correct balance of mineral salts, carbohydrate, and nitrogen.

In the 1950’s and 1960’s researchers continued testing what conditions would give rise to consistent and plentiful *S. megalocarpus* zygospores in culture. Hesseltine (1957) focused on the following: the difficulties propagating and maintaining *S. megalocarpus* in culture and lyophilization; how different temperatures and growth media affected zygospore production; and how light affected the production of zygospores. Hesseltine observed various growth characteristics on different media: PDA produced lush growth with many asexual sporulating structures; on corn steep and hay agar the growth was scant but with marked asexual reproduction; on Czapek’s solution growth was again thin with little sporangial formation. *S. megalocarpus* propagated well on malt agar and produced zygospores on a media containing 20 % glucose. *S. megalocarpus* sporulated occasionally and unpredictably with zygospores on malt, SMA, and PDA media. It appeared to produce more zygospores at lower temperatures and did not grow above 32 °C. Failure to grow above 32 °C was confirmed by Ka et al. in 1999. Hesseltine also concluded that light exposure did not affect zygospore production. However, Davis (1967) incubated two different strains on Difco yeast malt agar at seven
constant temperatures from 5-32 °C and suggested that continuous darkness at 10 °C for 10-14 days would promote the production of zygospores. He noted that any exposure to light irreversibly altered spore production from sexual to asexual spores.

Additional research focused on the impact of other factors on zygospore production, such as the effects of light and carotenogenesis, which were investigated by Wenger and Lilly (1966). They concluded that *S. megalocarpus* produced several pigments in culture, tentatively identified as beta- and gamma- carotene and lycopene. Wenger and Lilly also determined that pigment production was increased by continual light exposure, though the growth rate of *S. megalocarpus* decreased. Zygospores formed when *S. megalocarpus* was exposed to both light and darkness with media sugar levels of either 80 or 160 g/L, and zygospores formed in darkness with media sugar levels of 40 g/L. Sporangia only formed when exposed to darkness after previous light exposure. Sporangia did not form in light following darkness or in either constant light or constant darkness. Poff (1965) proposed a similar hypothesis but had studied the effect of carbohydrate concentrations on the production of asexual spores (sporangiospores).

Kaplan and Goos (1982) noted the degree of conflicting results on the various factors impacting zygospore formation: low temperatures (Baker 1931; Hesseltine 1957) or light and temperature (Davis 1967), carbohydrate composition (Wegner and Lilly 1966) or carbohydrate composition and the asexual spore quantity (Poff 1965). Kaplan and Goos further investigated zygospore formation and attempted to determine if a single primary factor strongly affected zygospore formation. They evaluated the number of zygospores produced on Petri dishes at 19 °C on GYP media with 0.2 M KCl. Kaplan and Goos concluded that the formation of sexual spores was related to water potential,
not to the various other factors investigated and postulated by previous researchers such as carbohydrate concentration and/or light/dark exposure. Kaplan and Goos concluded that reduced water availability results in S. megalocarpus changing its reproductive strategy from asexual sporangiospores to sexual zygospores.

S. megalocarpus isolates are limited in culture collections and have previously been difficult to maintain on media as the cultures tend to weaken when subcultured. Robinson (1925), while investigating growth and reproduction, noted that S. megalocarpus grew well on media with abundant sugar levels, and used malt-extract agar (a liquid medium with either 5% or 10% malt extract). On malt-extract agar, the fungus was described as rapidly spreading to the dish’s edges followed by the development of aerial branches producing sporangia or zygospores, at which time the growth rate diminished, possibly due to nutrient depletion of the media. Robinson also referred to prior work by Klebs (1898) in which it was stated that a medium with moderate nitrogen levels and abundant sugar would promote sporangia production at 75-80% humidity, and only developed zygospores at 90-100% humidity, thereby concluding that the concentration of carbohydrates in the medium, as well as humidity, affected viability.

Hesseltine noted that S. megalocarpus, except in one case, died after being lyophilized (1957). Indurm (2011) also noted difficulties with the viability of spores produced from lyophilized cultures.

However, Hwang (1966) stated that, although S. megalocarpus could not be successfully lyophilized, in his experiments freezing a wide variety of fungi in nitrogen refrigerators, S. megalocarpus was preserved and still viable at 54 months of storage if processed as follows. Samples were frozen in 10% glycerol-water menstruum in a heat-
sealed ampoule which was cooled from ambient to -35 °C at 1 °C/minute and then cooled to -165 to -195 °C in an accelerated and uncontrolled manner. The test for viability involved thawing the frozen ampoule in a water bath at 38-40 °C prior to culturing on media (Hwang 1966). Goos, Davis, and Butterfield also noted that although S. megalocarpus sustained some damage from freezing, rapid warming to 38 °C appeared beneficial and yielded germination percentages only slightly less than observed with unfrozen controls. It was postulated that viable spore loss was likely due to heat injury, not the warming rate (Goos et al. 1967).

Additionally, Weete et al. (1998), studying fungi that could potentially produce high quantities of gamma-linolenic acid (GLA), looked at a wide variety of zygomycetous fungi. They determined that some zygomycetes had gamma-linolenic acid levels that were 35-62% of total fatty acids, including S. megalocarpus. It was also determined that the level of GLA in S. megalocarpus could be significantly increased based on the nutritional composition of the media. A GLA dry biomass percentage of 9.8% could be increased up to 20-25% by growing the fungus on a medium with a high carbon/nitrogen ratio.

As a thorough review of the literature shows, S. megalocarpus research is sparse and much of what has been published focused on the homothallic nature of the fungus, often to the exclusion of other aspects. With its emergence as a threat to a major Pennsylvania crop, it is apparent that the amount unknown about S. megalocarpus far exceeds the amount known and much research remains to be done. One purpose of this master’s thesis is to address some of the unknown etiology and epidemiology of S.
megalocarpus and A. bisporus interaction, specifically control management issues, which have not been reported in previous research and literature.
Chapter 2

Effects of \textit{in vitro} chemical and heat treatments on \textit{S. megalocarpus}, an emerging pathogen of \textit{A. bisporus}

Abstract

The fungus \textit{Syzygites megalocarpus}, a known mycoparasite of senescing wild mushroom fruiting bodies, is an emerging pathogen of the cultivated mushroom \textit{Agaricus bisporus}. This pathogen was first reported in 2011 on two commercial farms in Pennsylvania and is sometimes called “beard mold” by growers. The effectiveness of fungicides, disinfectants, and heat treatment to control and management this disease has not been studied. Three fungicides were tested \textit{in vitro} for their ability to influence the mycelial growth of \textit{S. megalocarpus}: chlorothalonil (Bravo Weatherstik\textsuperscript{®}) and thiabendazole (Mertect\textsuperscript{®} 340-F), which are both approved for use on the button mushroom crop in the United States, and metrafenone (Vivando\textsuperscript{®}), which is not yet approved. Of the three, chlorothalonil and metrafenone reached an EC\textsubscript{50} at 1000 ppm. To determine which available disinfectants were effective against \textit{S. megalocarpus}, sporangiospores were exposed to three disinfectants labeled for use in the mushroom industry: phenol (1-Stroke Environ\textsuperscript{®}), sodium hypochlorite (Pure Bright\textsuperscript{®}), and iodine (Rapidyne\textsuperscript{®}). Of the three, phenol showed inhibition of \textit{S. megalocarpus} \textit{in vitro}. The thermal death point (TDP) of sporangiospores of \textit{S. megalocarpus} was determined to be 36.6-37.2 °C for 30 minutes, well below temperatures reached during composting and post crop steaming.
Introduction

*Syzygites megalocarpus* was reported as a new pathogen of the cultivated mushroom *Agaricus bisporus*, the button mushroom, first observed and collected in 2011 on two commercial farms in Chester County, Pennsylvania (Beyer et al. 2013). The causative agent was determined to be *Syzygites megalocarpus* (Mucorales, Mucoromycota; formerly Zygomycota (Spatafora et al. 2016)), a known mycoparasite of senescing wild mushroom fruiting bodies. By the third harvest of the cropping cycle, *S. megalocarpus* covered approximately 25% of the growing bed surface (Beyer et al. 2013). The sign of this pathogen is a hair-like mycelium that changes color from white to golden, and later to gray (Figure 2.1).

![Image 1](image1.png)

**Figure 2.1:** A) *S. megalocarpus* growing on *A. bisporus* in the laboratory and B) *S. megalocarpus* growing on an *A. bisporus* crop in a growing room

Symptoms include pitting, discoloration, and necrosis of the mushroom caps (Beyer et al. 2013). Since the initial outbreak, other growers have reported the pathogen, and it has become a persistent problem at many farms in Pennsylvania (D Beyer and J Pecchia, The Pennsylvania State University, personal communication 2016). This disease was first observed on farms growing a recently released hybrid brown strain of *A. bisporus* called
‘Heirloom’. This new hybrid strain included genetic material from a brown wild-type
strain parent (Beyer et al. 2013). However, the disease has also been found on other
brown strains and on some white strains (D Beyer and J Pecchia, The Pennsylvania State
University, personal communication 2016). With brown (‘portabella’ and ‘crimini’)
varieties representing 23 % of sales in a 1.13 billion US industry (NASS 2017), the
emergence and potential expansion of this disease could be very costly. This pathogen
causes yield and fresh quality losses and results in increased labor costs associated with
additional sanitation and cleaning of dead mushroom tissue from the surface of the beds
after harvesting.

*S. megalocarpus*, the only described species in the genus *Syzygites*, is commonly
found on senescing fruiting bodies of a wide range of fungal taxa, including both
Basidiomycota and Ascomycota (Kovacs and Sundberg 1999), and has been reported in
natural settings in Europe, Korea, and in North America (Ka et al. 1999; Kovacs and
Sundberg 1999). *S. megalocarpus* produces mitotic sporangiospores and a homothallic
zygospore stage (Baker 1931; Blakeslee 1904). While the pathogen has been reported to
occur on wild edible mushrooms (Ka et al. 1999), prior to 2011 *S. megalocarpus* had
never been reported as a pathogen in commercial button mushroom cropping systems
(Beyer et al. 2013). Little is known about the etiology or epidemiology of this fungus
either in nature or in agricultural systems.

Several fungal pathogens have been previously reported associated with
commercial button mushroom production, including *Hypomyces pernicious* (“wet
bubble”), *Lecanicillium fungicola* (“dry bubble”), *Hypomyces rosellus* (“cobweb”), and
*Trichoderma aggressivum f. aggressivum* (“green mold”) (Kew 2015; van Zaayen and
Green mold was first reported in the 1980’s on a mushroom farm in Europe and subsequently appeared in the United States in the 1990’s. It was at first a minor problem but has since developed into the most prevalent fungal pathogen of button mushrooms worldwide (D Beyer, The Pennsylvania State University, personal communication 2017). Trichoderma green mold is now an ongoing problem that requires constant management and causes massive crop losses annually (Doyle 1991, Fletcher 1990). This rapid development of the Trichoderma green mold epidemic suggests the importance of understanding the pathogen’s biology to rapidly develop control and management strategies.

Mycoparasitism, where pathogen and host are both fungi, inherently makes control difficult because the same environmental factors that are beneficial for the crop may also benefit the fungal pathogen. Cultural controls such as adjusting temperature or humidity are therefore unlikely to be successful avenues of control (Beyer 2003). A limited number of fungicides are known to kill the pathogen without influencing the host. Breeding of new strains is a challenging and expensive undertaking (Sonnenberg et al. 2008). Current methods to control other fungal diseases of cultivated mushrooms include fungicides, disinfectants, sanitation, and insect vector management.

It is still unknown how or where S. megalocarpus originated in mushroom cultivation and how it may persist during cropping. Fungus gnats, such as Lycoriella ingenua (Cloonan et al. 2016), occur in Pennsylvania and may vector S. megalocarpus into mushroom houses when and where they are active, and within and between growing rooms. Some fungal pathogens may infect the composted substrate used for mushroom production. They may also survive the phase II pasteurization process where substrate
temperatures reach 60 °C and post-crop steaming (routine steam sanitization of growing rooms after harvest completion) that also reaches at least 60 °C.

The three objectives of this research were: 1) Determine what available fungicides influence the growth of *S. megalocarpus* and determine EC\textsubscript{50}'s (50% reduction in mycelial growth). The fungicides chlorothalonil (Bravo Weatherstik\textsuperscript{®}) and thiabendazole (Mertect\textsuperscript{®} 340-F), both approved for use on the *A. bisporus* crop in the United States, and metrafenone (Vivando\textsuperscript{®}), not yet approved for *A. bisporus*, were tested *in vitro*. 2) Determine the efficacy of currently available commercial disinfectants to inhibit germination or growth of *S. megalocarpus* sporangiospores. Sporangiospores of *S. megalocarpus* were exposed to three disinfectants labelled for use in the mushroom industry: phenol (1-Stroke Environ\textsuperscript{®}), sodium hypochlorite (Pure Bright\textsuperscript{®}), and iodine (Rapidyne\textsuperscript{®}). 3) Determine the thermal death point of sporangiospores of *S. megalocarpus*.

Materials and Methods

Fungicide efficacy

*S. megalocarpus* (NRRL accession # 66833 used for all experiments) was subcultured from potato dextrose agar (PDA) onto oatmeal agar (HiMedia\textsuperscript{®} 72.5 g/L) two days before the fungicide screening experiments were conducted since younger growth allowed for easier handling of the culture. Mature hyphae grew very long, were sticky, and were difficult to cut cleanly and transfer on a plug. After two days on oatmeal agar,
the edges of the colonies were cut using a 7 mm diameter cork-borer to make identical-sized plugs. Plugs of agar colonized by *S. megalocarpus* mycelia were placed centrally onto 100 mm x15 mm PDA (Difco™ 39 g/L) plates amended with fungicides in concentrations of 1000, 100, 10, 1.0, 0.1, .01, and 0 parts per million (ppm) of the active ingredient. The plates were wrapped with Parafilm® M, incubated at 25 °C, left to grow for 24 hours, and then the colony diameter was measured to determine the influence on mycelial growth.

Each experiment contained five replicates per treatment. After 24 hours, the mycelial growth was measured twice, at 90-degree angles. Because mycelial growth rates varied between the repeated experiments, absolute measurements were not used to compare data. Instead, a ratio of the growth rates (treatment mycelial growth divided by control mycelial growth for each repeated experiment) was used and the ratios were then compared across repeated experiments. The ratios allowed determination of EC$_{50}$'s (50 % reduction in mycelial growth) by simple division.

**Disinfectant efficacy**

One hundred microliters of a 3-5 x 10$^5$ spores/ml spore suspension of *S. megalocarpus* was placed onto a PDA plate and then spread with a glass rod. Three disinfectants, phenol (1-Stroke Environ®), sodium hypochlorite (Pure Bright®), and iodine (Rapidyne®), were poured into separate sterilized beakers (Pyrex® 500 ml) at recommended label rates: (½ fluid ounce/gallon for 1-Stroke Environ®, 500 ppm for Pure Bright®, and 50 ppm for Rapidyne®), and then 2.1 cm diameter pieces of filter paper
(Millipore Corporation, Massachusetts, USA) were soaked in the disinfectant for three minutes. Additionally, filter paper pieces were soaked in a beaker containing only deionized water and used as a negative control. A 2.1 cm filter paper soaked in a treatment solution and a 2.1 cm filter paper soaked in the negative control were then placed onto the center of each half of the plate, sealed with Parafilm® M, and left at room temperature, 21 to 22 °C. After 24 hours the zone of inhibition for each plate was visually inspected and assessed based on a binary rating system of either positive (presence of inhibition) or negative (lack of inhibition). This experiment was repeated three times with three replicates for each disinfectant.

**Thermal death point**

A pure culture of *S. megalocarpus* was subcultured onto three PDA Petri dishes and then incubated at 15 °C for 10 days. Fifty milliliters of sterile water was placed in a 150 ml beaker and used to moisten 15 cm cotton tipped applicators (swabs). The moistened swabs were brushed across the cultures of *S. megalocarpus* and placed back into the beaker containing autoclaved water. This was repeated until most of the spores from the culture were dislodged and transferred into the water. The 50 ml of water containing spores was then filtered through sterilized cheesecloth into another beaker to remove larger pieces of hyphae and then filtered through sterilized cheesecloth once more as it was poured into an Erlenmeyer flask. After the spore suspension was made, it was quantified using a Bright-Line™ Hemocytometer from Hausser Scientific (Horsham, PA) following manufacturer’s instructions.
Nine 100 µl samples were prepared containing 3-5 x 10^4 spores each, which were then loaded into sterile PCR tubes. A negative control was also prepared, consisting of an uninoculated 100 µl sterile water. Eight of the nine samples containing sporangiospores were placed into a gradient thermocycler (BIORAD T100™, Hercules, CA) for 30 minutes, each exposed to a different temperature between 34-38 °C: 34.0 °C, 34.2 °C, 34.7 °C, 35.6 °C, 36.6 °C, 37.2 °C, 37.7 °C, 38.0 °C. The remaining sporangiospore sample (positive control) was kept at room temperature, along with the negative control. After the heat treatment, samples were plated onto PDA plates, incubated at 25 °C, and observed daily to determine survival. The experiment was replicated eight times. The cultures were evaluated daily for one week using a binary positive or negative visual inspection of viable spores on the plate. The observation of any visual spore germination received a positive rating and no germination a negative rating.

Results

Fungicide efficacy

*S. megalocarpus* growth *in vitro* containing chlorothalonil and metrafenone had an EC₅₀ of 1000 ppm (Figure 2.2). Thiabendazole did not reach an EC₅₀ at the concentrations tested, although a reduction in growth was demonstrated. Based on the slope of the lines of chlorothalonil and metrafenone, chlorothalonil appeared to reduce growth at a concentration of 1 ppm, while metrafenone and thiabendazole did not inhibit
growth until 100 ppm.

Figure 2.2: *S. megalocarpus* mycelial growth on PDA amended with differing concentrations of chlorothalonil (Bravo Weatherstik®), thiabendazole (Mertect® 340-F), and metrafenone (Vivando®). The data represents the means of two trials. The horizontal line at .50 is the EC₅₀ (50% reduction in mycelial growth).

**Statistical analysis: fungicide experiments**

The adjusted R-squared values for the fungicide trials, shown in Table 2.1, demonstrate the degree to which the input variable explained variation of the output/predicted variable, i.e. the goodness of fit (greater is better). A linear regression with a log transformation was run on the data. Statistical analysis (Table 2.1) was carried out using the statistical program R version 3.4.4.
Table 2.1: Adjusted R-squared values showing goodness of fit and validity of model for active ingredients/fungicides: chlorothalonil, thiabendazole, and metrafenone

<table>
<thead>
<tr>
<th>Fungicide active ingredient</th>
<th>Adjusted R-squared value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorothalonil</td>
<td>0.7722*</td>
</tr>
<tr>
<td>Thiabendazole</td>
<td>0.8413*</td>
</tr>
<tr>
<td>Metrafenone</td>
<td>0.9698*</td>
</tr>
</tbody>
</table>

Disinfectant efficacy

Three disinfectants were tested for efficacy on sporangiospores: phenol (1-Stroke Environ®), sodium hypochlorite (Pure Bright®), and iodine (Rapidyne®). At recommended label rates (1-Stroke Environ®: ½ fluid oz/gallon, Pure Bright®: 500 ppm, Rapidyne®: 50 ppm), only phenol (1-Stroke Environ®) inhibited *S. megalocarpus* germination or mycelial growth which was indicated by the presence of a zone of inhibition (Figure 2.3). The two other disinfectants displayed no zone of inhibition on visual inspection indicating that they did not inhibit germination or growth. Results were consistent for all nine plates (Table 2.2).

Figure 2.3: *S. megalocarpus* sporangiospore germination and subsequent growth on PDA plates containing filter paper impregnated with disinfectants (A. phenol (1-Stroke Environ®), B. sodium hypochlorite (Pure Bright®), and C. iodine (Rapidyne®)). The filter paper on the right in each plate is a negative control (water).
Table 2.2: Inhibition of *S. megalocarpus* plated on PDA containing filter paper impregnated with disinfectants (A. phenol (1-Stroke Environ®), B. sodium hypochlorite (Pure Bright®), and C. iodine (Rapidyne®))

<table>
<thead>
<tr>
<th>Disinfectant</th>
<th>Percentage and number of plates demonstrating inhibition of <em>S. megalocarpus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Phenol</td>
<td>100% (9/9)</td>
</tr>
<tr>
<td>B. Bleach</td>
<td>0 % (0/9)</td>
</tr>
<tr>
<td>C. Iodine</td>
<td>0 % (0/9)</td>
</tr>
</tbody>
</table>

**Thermal death point (TDP)**

Results from preliminary TDP tests (data not shown) indicated the thermal death point range was between 30.0 °C and 40.0 °C. Therefore, subsequent testing included the following temperature set points: 34.0 °C, 34.2 °C, 34.7 °C, 35.6 °C, 36.6 °C, 37.2 °C, 37.7 °C, and 38.0 °C. Germinating spores were observed on the plates after exposure to 34.0 °C, 34.2 °C, 34.7 °C, 35.6 °C, and 36.6 °C for 30 minutes. No germination was observed on the plates with spores exposed to 37.2 °C, 37.7 °C, and 38.0 °C. Therefore, the thermal death point of sporangiospores of *S. megalocarpus* was determined to be between 36.6 to 37.2 °C (Figure 2.4). This experiment was replicated eight times with the same results.
Discussion

Fungicide and disinfectant efficacy

The results of the fungicide experiments demonstrated that chlorothalonil and metrafenone had an EC$_{50}$ of 1,000 ppm showing growth was inhibited by at least 50%. At 1,000 ppm, thiabendazole approached but did not reach an EC$_{50}$. The adjusted R-squared values also demonstrated goodness of fit and validity of model for the active ingredients/fungicide experiments.

Commercially, chlorothalonil can be applied twice during cropping: the first application at 2,467 ppm and the second at 1,233 ppm. Following label rates
thiabendazole can be applied commercially at 981 ppm. Metrafenone is not registered for use on mushrooms at this time.

The results of these experiments demonstrated that in vitro the growth of *S. megalocarpus* was inhibited by commercially available fungicides. However, it is difficult to predict how these results will perform on a commercial mushroom farm without further testing to determine if the fungicides will have any efficacy in a cropping system as well as determining optimal application timing. Chlorothalonil may be effective at its registered rates and application timing. However, it currently has a required pre-harvest interval of five days, which would prohibit its use during cropping due to the short interval of harvest timing between mushroom flushes. Additionally, sporangiospores were found to cause infection (Beyer et. al 2013), and since spores are typically considered more resilient due to being survival structures and not being as biologically active as the growing hyphae (Agrios 2005), sporangiospores may not be as susceptible to these fungicides.

The results of the disinfectant experiments demonstrated that sporangiospores of *S. megalocarpus* are not susceptible to sodium hypochlorite and iodine, at the concentrations tested. However, since phenol caused a zone of inhibition, either sporangiospore germination or subsequent growth was inhibited, and *S. megalocarpus* appears more susceptible to phenol. The results suggest that the choice of disinfectant may have a role in the control of *S. megalocarpus*; however, phenols are known to be toxic to aquatic wildlife and its use is of environmental concern (Babich and Davis 1981). Phenols are also not a food contact disinfectant and, therefore, cannot be applied directly onto the mushroom beds and its use around a mushroom farm is limited.
Thermal death point

Since temperatures during phase II composting (pasteurization) reach 60 °C, a pathogen with a thermal death point between 36.6 and 37.2 °C would not survive temperatures reached during phase II composting. Furthermore, it was reported by Hesseltine (1957) that no growth of *S. megalocarpus* mycelia occurs above 32 °C, so it also seems unlikely that hyphae would survive and become a source of inoculum. Therefore, substrate at spawning would likely not be a source of inoculum for infection by *S. megalocarpus* during cropping. Additionally, during post-crop steaming of growing rooms, the compost temperatures generally exceed 60 °C, which would also eliminate *S. megalocarpus* inoculum. Therefore, *S. megalocarpus* spores and mycelium would not be able to survive and be present as a source of inoculum for subsequent crops that will be grown in the same room. The experiments and control methods investigated in these experiments were only tested on sporangiospores, zygospores, sexually reproduced spores that are difficult to obtain consistently in the lab (Baker 1931), were not tested and may yield different results.

Summary

The results of these experiments suggest that *S. megalocarpus*, an emerging disease of the cultivated mushroom *A. bisporus*, may at least be partially managed with fungicides. Fungicides that reached an EC$_{50}$ in these experiments included chlorothalonil, approved for use on *A. bisporus* but not during the five-day pre-harvest interval, and metrafenone which is not currently approved for use on mushrooms. Thiabendazole,
though it never reached an EC₅₀ at the rates tested, appeared to work nearly as well as the other two fungicides. The evaluation of disinfectants demonstrated that neither sodium hypochlorite bleach nor iodine products demonstrated efficacy, but phenol appeared effective in inhibiting germination or mycelial growth. Though the temperatures reached during composting exceed the TDP, composting alone has not proved adequate to control the pathogen, which indicates that subsequent infection is probably occurring on commercial mushroom farms after compost pasteurization. The TDP results suggest that post-crop steaming, as a non-chemical means of control, should be included as part of an integrated pest management program on a commercial mushroom farm.
Literature Cited


http://www.speciesfungorum.org/GSD/GSDspecies.asp?RecordID=531546


Weil JD, Cutter CN, Beelman RB, LaBorde LF. 2013. Inactivation of Human Pathogens during Phase II Composting of Manure-Based Mushroom Growth Substrate. J Food Prot. 76:1393-1400.

Chapter 3

Strain susceptibility of \textit{A. bisporus} to \textit{S. megalocarpus} and pathogenicity and virulence of sporangiospores and mycelia of \textit{S. megalocarpus}

Introduction

The emergence of \textit{S. megalocarpus} as a problem in mushroom farming followed the introduction of a recently released hybrid strain of the brown button mushroom. It is known that the recently released strain resulted from crossing a commercial white strain of \textit{A. bisporus} with a brown wild type \textit{A. bisporus} (Beyer et al. 2013). Other information about genetics and parentage is not available due to the proprietary nature of the information. Observations by mushroom growers were that the recently released strain, ‘Heirloom’ (Amycel Spawn Company, San Juan Bautista, CA) was more susceptible to \textit{S. megalocarpus} than previous brown strains (D Beyer and J Pecchia, The Pennsylvania State University, personal communication 2017). Therefore, one research objective was to determine whether laboratory experimentation comparing the susceptibility of a number of strains would support this observation.

An experiment testing four commercially available strains of \textit{A. bisporus} for comparative susceptibility was designed to provide information on the relative susceptibility of the different strains as well as the pathogenicity and virulence of sporangiospores of \textit{S. megalocarpus} versus its hyphae. Spores may be more challenging to control than mycelia since they are generally more resistant to external treatments such
as fungicides and disinfectants, and spores may also become windborne or be vectored by other organisms such as insects (Agrios 2005).

Documentation of strain susceptibility could help growers limit disease on their farms by choosing less susceptible strains. Determination of susceptibility to infection caused by mycelia versus spores may help in the development of future control practices. The *S. megalocarpus* isolate used for all experiments was NRRL # 66833.

**Materials and Methods**

**Pathogenicity and virulence of spores and mycelia**

*S. megalocarpus* was grown on three PDA plates at room temperature (21 to 22 °C) for one week and on three PDA plates at 15 °C for 10 days. Zygospore formation has been reported to vary based on temperature (Hesseltine 1957); therefore, two different temperatures were selected to produce spores for cropping trials. The resultant cultures were then used to make a spore suspension according to the following spore suspension protocol. Fifty milliliters of sterile water was placed in a 150 ml beaker and used to moisten 15 cm cotton tipped applicators (swabs). The moistened swabs were brushed across the cultures of *S. megalocarpus* and placed back into the beaker containing autoclaved water. This was repeated until most of the spores from the culture were dislodged and transferred into the water. The 50 ml of water containing spores was then filtered through sterilized cheesecloth into another beaker to remove larger pieces of hyphae and then filtered through sterilized cheesecloth once more as it was poured into
an Erlenmeyer flask. After the spore suspension was made, it was quantified using a Bright-Line™ Hemocytometer from Hausser Scientific (Horsham, PA) following manufacturer’s instructions. The same protocol was followed for both the room temperature incubated plates and the 15 °C plates. Both spore suspensions were quantified via hemocytometry. One milliliter of sterile Milli-Q® water was then added to each glass container (Pyrex® 100x80 mm Crystallizing Dish, No. 3250) containing a piece of filter paper (Whatman® 42 ASHLESS 90 mm) to provide a moist environment for the fungal pathogen.

To make the mycelial plugs, *S. megalocarpus* was subcultured from PDA onto oatmeal agar (HiMedia® 72.5 g/L). At two days of age the edges of the colonies were cut using a 10 mm diameter cork-borer to make identical-sized plugs.

All mushrooms used were commercial strain ‘Heirloom’. Dish #1 was used as the negative control and contained two mushrooms with 1000 µl of sterile water, 500 µl added to each mushroom. The room temperature (21 to 22 °C) spore suspension concentration was 6 x 10³ spores/ml, and the 15 °C spore suspension concentration was 5 x 10⁵ spores/ml. Dish #2 was used as the positive control and contained two mushrooms each inoculated with a mycelial plug colonized by *S. megalocarpus*. Dish #3 contained two mushrooms each inoculated with 500 µl of room temperature spore suspension from the 21 to 22 °C spore suspension. Dish #4 contained two mushrooms each inoculated with 500 µl of suspension from the 15 °C spore suspension. This experiment was repeated twice.
Strain susceptibility

Environmentally controlled growing rooms at the Mushroom Research Center (MRC) were used to test strain susceptibility. Each growing room contained four racks, each with nine tubs. Within each tub, two mushrooms were knocked over during first break (first week of harvest) and one mushroom was cut at the base of the stipe. Each tub contained two knocked over caps (dead tissue) and one stump (cap cut off with a knife, leaving behind the stalk) to be used as inoculation points.

Four commercially available strains of *A. bisporus* were tested. The strains used were as follows: ‘Lambert 805’ (traditional brown strain), Amycel ‘Brawn’ and ‘Heirloom’ (two recently released hybrid brown strains), and ‘Lambert 901’ (a commercial hybrid off-white strain). The 12 controls (three tubs of each strain) were placed on top shelves to prevent contamination by potential runoff of inoculum during watering. The other 24 tubs (six tubs of each strain) were spawned and assigned to a position on a shelf at random. Details of the Mushroom Research Center cropping procedures can be found in Appendix G.

Inoculation with *S. megalocarpus* was initiated during the beginning of first break, and each tub received one of two different treatments, either a mycelial plug or spore suspension. For the mycelial plug treatments, a 1x1 cm plug of agar colonized with *S. megalocarpus* was placed on each knock over (mushroom knocked over by a sterile toothpick and left on the bed) or stump. For the spore suspension treatments, 500 µl of a 5.5 x 10⁵ spores/ml suspension of *S. megalocarpus* was placed onto the knock overs and stumps.
Growth and disease progression of *S. megalocarpus* was measured daily. Pathogen growth was measured daily, in two directions, with the two measurements at 90-degree angles to each other.

An additional set of experiments with one variable was also performed to determine the effect of wounding on susceptibility by staking each stump and mushroom with a sterilized wooden toothpick. Pathogen growth was measured daily, in two directions, with the two measurements at 90-degree angles to each other.

**Results**

**Pathogenicity and virulence of spores and mycelia**

Infection only occurred on mushrooms inoculated with the spore suspension produced from the 15 °C culture and the positive control. The spore suspension incubated at 15 °C contained spores on the order of two to three magnitudes greater than the number of spores in the 21 to 22 °C spore suspension. The culture maintained at room temperature, with fewer spores, was unable to induce infection.

**Strain susceptibility**

‘Lambert 805’ (traditional brown) displayed signs of infection on average after 7.5 days when unwounded and 3.5 days when wounded (Table 3.1). ‘Lambert 901’ (hybrid off white) knock overs took 3.5 days, on average, to develop signs of infection on
either unwounded or wounded mushrooms, and both ‘Heirloom’ and ‘Brawn’ knock overs developed visible signs of infection after three days, on average. This experiment was repeated twice for wounded and twice for unwounded mushrooms.

Table 3.1: Average days until signs of visible infection for the four strains tested: ‘Lambert 805’ demonstrated a difference in time to infection for wounded versus unwounded (p=.029); the other strains exhibited no difference.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Average days until signs of visible infection wounded</th>
<th>Average days until signs of visible infection unwounded</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Lambert 901’</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>‘Lambert 805’</td>
<td>3.5</td>
<td>7.5</td>
</tr>
<tr>
<td>‘Heirloom’</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>‘Brawn’</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

‘Lambert 805’ mushrooms, when unwounded, whether inoculated by spore suspension or mycelial plug (Table 3.3), displayed the least amount of disease development of the various strains tested. ‘Heirloom’ displayed consistent levels of infection, whether wounded or unwounded.

Table 3.2: Percent coverage by *S. megalocarpus* on tubs with wounded mushrooms; differences were not statistically significant.

<table>
<thead>
<tr>
<th>Strain/Inoculum method</th>
<th>Number of data points</th>
<th>Average % tub coverage</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Lambert 901’/mycelia</td>
<td>N=6</td>
<td>25.0</td>
<td>27.6</td>
</tr>
<tr>
<td>‘Lambert 901’/spores</td>
<td>N=6</td>
<td>14.3</td>
<td>8.91</td>
</tr>
<tr>
<td>‘Lambert 805’/mycelia</td>
<td>N=6</td>
<td>8.67</td>
<td>10.2</td>
</tr>
<tr>
<td>‘Lambert 805’/spores</td>
<td>N=4</td>
<td>15.0</td>
<td>16.5</td>
</tr>
<tr>
<td>‘Heirloom’/mycelia</td>
<td>N=5</td>
<td>17.7</td>
<td>21.3</td>
</tr>
<tr>
<td>‘Heirloom’/spores</td>
<td>N=6</td>
<td>15.7</td>
<td>16.3</td>
</tr>
<tr>
<td>‘Brawn’/mycelia</td>
<td>N=6</td>
<td>22.9</td>
<td>15.5</td>
</tr>
<tr>
<td>‘Brawn’/spores</td>
<td>N=6</td>
<td>20.3</td>
<td>17.6</td>
</tr>
</tbody>
</table>
Table 3.3: Percent coverage by *S. megalocarpus* on tubs with unwounded mushrooms; differences were not statistically significant.

<table>
<thead>
<tr>
<th>Strain/Inoculum method</th>
<th>Number of data points</th>
<th>Average % tub coverage</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Lambert 901’/mycelia</td>
<td>N=3</td>
<td>7.03</td>
<td>6.77</td>
</tr>
<tr>
<td>‘Lambert 901’/spores</td>
<td>N=3</td>
<td>18.0</td>
<td>8.68</td>
</tr>
<tr>
<td>‘Lambert 805’/mycelia</td>
<td>N=1</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>‘Lambert 805’/spores</td>
<td>N=2</td>
<td>1.63</td>
<td>2.30</td>
</tr>
<tr>
<td>‘Heirloom’/mycelia</td>
<td>N=3</td>
<td>11.3</td>
<td>7.92</td>
</tr>
<tr>
<td>‘Heirloom’/spores</td>
<td>N=4</td>
<td>11.3</td>
<td>10.2</td>
</tr>
<tr>
<td>‘Brawn’/mycelia</td>
<td>N=2</td>
<td>8.56</td>
<td>10.7</td>
</tr>
<tr>
<td>‘Brawn’/spores</td>
<td>N=3</td>
<td>9.72</td>
<td>14.7</td>
</tr>
</tbody>
</table>

*Average and standard deviation cannot be calculated for a single data point.

Of the total stumps that were inoculated with either a spore suspension or mycelial plug, 20.8 % (20/96) displayed infection. Of the 8.3 % (8/96) of stumps that became infected when inoculated with a spore suspension, 75 % (6/8) of them were ‘Heirloom’. 12.5% (12/96) of the stumps inoculated with a mycelial plug displayed infection, with no differences between strains observed.

**Statistical analysis of strain susceptibility**

All statistical analyses (Tables 3.1, 3.2, 3.3) were carried out using the statistical program R version 3.4.4. A Welch two-sample T-test was used to compare the data points. The initial data for wounding by strain and inoculation method was shown to be non-normal when a Shapiro-Wilk test for normality was used (Tables 3.2 and 3.3). The data was transformed using a square root transformation, after which the data was shown to be normal using a Shapiro-Wilk test for normality. Subsequently, an ANOVA test was run comparing the strain, inoculation method, and interactions between the two factors. A
Tukey’s range test was run to make a pair-wise comparison between all variables, and a two-sample t-test was run to compare the effects of unwounded versus wounded on infection severity. Wounding caused a statistical difference in time to infection for only one of the strains, ‘Lambert 805’.

Discussion

Pathogenicity and virulence of spores and mycelia

These results indicate that both mycelia and sporangiospores of *S. megalocarpus* are able to cause infection of button mushrooms when knocked over (damaged). It is also notable that an observation was made that the mycelia tend to stick to lab equipment. Being sticky could aid in spreading the pathogen vectored by workers, insect vectors, and/or mechanically. Additional preliminary results on possible methods of dispersion may be found in Appendix B: Experiment: Epidemiology, movement of sporangiospores of *S. megalocarpus* in the growing room.

Previous research (Baker 1931; Hesseltine 1957) reported that *S. megalocarpus* produced more zygospores at lower temperatures, and Baker found that higher temperatures favored sporangiospore production, though both zygospores and sporangia could develop over a wide temperature range. Therefore, two temperatures were used to produce spore suspensions: 15 °C and 21 to 22 °C. The fact that cultures incubated at 15 °C produced two orders of magnitude more sporangiospores compared to cultures incubated at 21 to 22 °C adds to the findings of Hesseltine (1957) that more zygospores
are produced at lower temperatures. Difficulties subculturing *S. megalocarpus* and keeping it viable confirmed previous reports (Robinson 1925; T Bruns, University of California, Berkeley, personal communication 2017; K Paley, The Pennsylvania State University, personal communication 2017). Additional information on the challenges of culturing this pathogen can be found in the first two paragraphs of the introduction to Chapter 4: Bacteria associated with *S. megalocarpus* culture, and in Appendix F: Methods of keeping *S. megalocarpus* alive in the lab.

**Strain susceptibility**

The one statistically significant difference in strain susceptibility was that time to display signs of infection was similar among all strains tested whether wounded or unwounded, except for unwounded traditional ‘Lambert 805’. ‘Lambert 805’ took twice as long to display signs of infection (p=.029). These results suggest that the traditional brown strain ‘Lambert 805’ has a mechanism of protection that makes it less susceptible to infection, until the fruiting body is wounded. This difference appears to be related to some aspect of the outer layer of the fruiting body, since when it was breached its susceptibility to infection became similar to the other strains. It is also noteworthy that 75% (6/8) of stumps that displayed signs of infection, from stump inoculation with a spore suspension, were from the recently released hybrid brown strain ‘Heirloom’.

On a commercial farm, it is typical to find dead mushrooms on the bed surface knocked over during the picking process. Many growers make a concerted effort to remove this tissue to reduce the risks associated with *S. megalocarpus* as well as other
possible pathogens. However, leaving damaged stumps and tissue is also a common occurrence from picking and these stumps are often left behind due to the labor intensive and time demanding process of removing them without damaging the bed surface. The differential susceptibility to stump inoculation between the ‘Heirloom’ strain compared to the other strains tested could possibly account for the development of this new disease on commercial farms. If this is true, it is possible that *S. megalocarpus* had previously been present on commercial mushroom farms, though the inoculum level might have been at too low a concentration to initiate disease. The development and use of a recently released ‘Heirloom’ strain that has demonstrated more susceptible stumps may have led to higher inoculum levels on farms, potentially leading to infection of other strains of *A. bisporus* not previously affected. These experiments suggest that increased attention to sanitation of beds of ‘Heirloom’ is quite important due to the susceptibility of its stumps to infection.

**Summary**

Mycelia and sporangiospores are both capable of causing infection, and differences between the two types of inoculum lacked statistical significance. Regarding strain differences, only the traditional brown ‘Lambert 805’ was more susceptible to infection when its mushrooms were wounded compared with when its mushrooms were unwounded, and ‘Lambert 805’ exhibited disease signs and symptoms more slowly than all other strains tested. The difference between the time to development of signs of infection for ‘Lambert 805’ with wounding versus without wounding suggests that
‘Lambert 805’\’s tolerance is possibly due to some aspect of the outer layer of the fruiting body, perhaps useful information for future breeding programs. Also, 75\% (6/8) of the stumps infected by inoculation with a spore suspension were the recently released brown strain, ‘Heirloom’. It appears that ‘Heirloom’ stumps are more susceptible and that unwounded ‘Lambert 805’ mushrooms are less susceptible compared with other tested strains, to *S. megalocarpus*. The data from these experiments suggests that, for the newer hybrid brown ‘Heirloom’ strain, increased attention to bed sanitation after “breaks” (multiples harvests), particularly the careful removal of remaining dead tissue such as stumps, is important to manage this disease.
Chapter 4

Bacteria associated with S. megalocarpus culture

Introduction

During experiments, isolates of S. megalocarpus consistently became “contaminated” with bacteria in culture. While working with this fungus, it was noted by the author as well as other scientists (K Paley, The Pennsylvania State University, personal communication 2017; ME Smith, University of Florida, personal communication 2018) that bacterial “contamination” also routinely appeared on plates that previously appeared to be pure cultures.

Preliminary testing showed that bacteria frequently persisted through subculturing, even when grown on media containing various antibiotics (ampicillin, chloramphenicol, tetracycline), and on acidified media, which typically controls bacteria. The ampicillin and chloramphenicol used to prevent bacterial growth also noticeably reduced the growth of S. megalocarpus in vitro. Acidified PDA media had no apparent effect.

The original hypothesis was that a fungal bacterial symbiotic relationship was occurring, perhaps one affecting virulence. Other studies have reported that many fungi contain endosymbiotic bacteria, including fungal species in the Ascomycota, Basidiomycota, and particularly in older lineages such as Mucoromycota and Zoopagomycota (Hoffman and Arnold 2008, 2010; Spatafora et al. 2016). For example,
*Rhizopus microsporus* contains the toxin rhizoxin, but does not produce the toxin itself; instead it has an endosymbiotic bacterium, *Burkholderia rhizoxinica*, that produces the toxin which is necessary for both infection of host plants and for sporulation of the host fungus (Partida-Martinez and Hertweck 2005; Partida-Martinez et al. 2007). Another example of an endosymbiotic bacteria and a zygomycete is *Mortiella elongata* (same phyla as *S. megalocarpus*) and *Mycoavidus cysteinexigens*, a highly specific symbiotic relationship hypothesized to have originated approximately 350 million years ago (Uehling et al. 2017).

Another possible relationship was an antagonistic interaction in which the bacterium acted as a pathogen, which might explain some of the difficulties reported with keeping *S. megalocarpus* alive in culture. For example, in 2016, Hover et al. showed that *Serratia marcescens*, a common gram-negative bacterium, could migrate along the hyphae of fungi and is pathogenic on zygomycetes and some basidiomycetes, though it does not seem to affect ascomycetes. The proposed mode of action by *S. marcescens* is that it migrates along the hyphae-forming microcolonies that then coalesces and forms a biofilm that kills the fungus. Hover et al. (2016) also reported that the chitin of the zygomyceteous molds is more susceptible to this bacterium than other fungi. If a bacterium present with the *S. megalocarpus* is antagonistic, then using it as a biocontrol might be a possibility. A first step was to identify the bacteria associated with *S. megalocarpus* in vitro.
Materials and Methods

Five isolates of *S. megalocarpus* were collected from several different mushroom farms in Pennsylvania. The cultures were originally contaminated with fungi and bacteria since they were isolated from mushrooms on farms. Due to the consistent presence of bacteria in the isolates, various control methods were attempted.

A mixture of 10 ml of 10 ppm benomyl and 1 ml of tetracycline (12.5mg/ml in 95 % ethanol) per 100 ml of media was tested to control fungal and bacterial contaminants. The combination displayed limited and sometimes temporary control but was the most efficacious of the various fungicide and/or antibiotic combinations tested. Chloramphenicol was tested as a control, at 147 µl (stock solution concentration 34 mg/ml) per 100 ml of PDA media, but it was not effective. Ampicillin was tested as a control at 50 µl (stock solution concentration 100 mg/ml) per 100 ml of PDA media and was also ineffective. Acidified PDA was tested as a control, 1 ml of 1 % lactic acid per 100 liters of PDA media, and it was also ineffective.

The isolates used in this experiment were cultured on potato dextrose media (PDA) amended with benomyl and tetracycline to best isolate *S. megalocarpus* from other organisms. After the cultures were visually clear of contaminants, they were subcultured onto PDA plates. After two days at room temperature (21 to 22 °C), the mycelia were harvested by scraping the plate with a sterilized scalpel and placed into a 1.5 ml Eppendorf tube.

DNA extraction was performed by two different methods due to the ease of extraction from bacteria versus fungi. DNA was extracted from a *S. megalocarpus* culture
using a PowerLyzer® UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories Inc., USA). Bacteria were also isolated from each *S. megalocarpus* isolate by subculturing the isolates on PDA and then isolating visibly growing bacteria in association with *S. megalocarpus* on each plate. The DNA of these bacteria was not extracted using an extraction kit.

The second method of DNA extraction was as follows: 100 ml of sterile water were micropipetted into a 1.5 ml Eppendorf tube and then each bacterial sample was transferred from the PDA plate into each individual Eppendorf tube and then vortexed (Scientific Industries Vortex-Genie 2, USA) for five seconds. The bacterial suspensions were then placed into a water bath at 60 °C for 15 minutes in order to lyse the cells and release DNA.

The PCR products of four *S. megalocarpus* isolates that showed a strong band for bacterial presence (Figure 4.1) were combined and then cloned. Four bacterial species were identified (Invitrogen TOPO TA Cloning® Version U, CA, USA) from a combination of PCR products from the four *S. megalocarpus* isolates that showed a strong band, with plasmid purification (OMEGA Bio-tek E.Z.N.A.® Plasmid DNA Minikit 1, USA) and subsequent sequencing (Table 4.1).

PCR was then performed (Applied Biosystems Gene AMP PCR System 9700, USA) on the DNA extractions from *S. megalocarpus* and on the bacterial suspensions using universal bacterial primers 27F (5’-AGAGTTTGATCMTGGCTCAG-3’) and 1492R (5-GGTTACCTTG TTACGACTT-3’) (Lane 1991). The PCR products were then quantified spectrophotometrically using a Thermo Scientific NanoDrop 2000 (USA), and the quality of the PCR product was determined by gel electrophoresis (FisherBiotech®)
Mini-Horizontal Unit, Pittsburgh, USA) and then imaged under a transilluminator (AlphaImager®, USA) (Figure 4.1). The PCR product was then purified using an OMEGA Bio-tek E.Z.N.A.® Cycle Pure Kit (USA) and then sequenced by the Pennsylvania State University Genomic Core Facility. The resulting sequences were analyzed using Vector NTI and run through both the Ribosomal Database Project (Cole et al. 2014) and the National Center for Biotechnology Information (NCBI) website BLAST (Basic Local Alignment Search Tool) program (Altschul et al. 1997). These bioinformatics algorithms were used to compare sequence information against previously identified endosymbiotic bacteria.

Figure 4.1: Gel showing bands with universal bacterial primers 27F and 1492R; 1-5 (from left to right, NRRL #'s: 66833, 66835, 66836, 66834, 66832) are isolates of S. megalocarpus collected from different farms; + and – are controls; four of five isolates showed bands of the correct size, approximately 1500 base pairs for the primers used.
**Results**

*S. megalocarpus* strains are identified by their assigned numbers in the ARS Culture Collection (NRRL), a culture collection of the Agricultural Research Service (ARS), United States Department of Agriculture (USDA) (Figure 4.1, Table 4.2). Three bacterial species were identified from the PCR product that was made from a combination of the four *S. megalocarpus* isolates with strong bacterial bands (Figure 4.1, Table 4.1). Five bacterial species were identified from the four different isolates of *S. megalocarpus*, (NRRL #’s 66832, 66834, 66835, 66836), obtained directly from cultures without using a DNA extraction kit (labeled “plate” in Table 4.2), and from DNA extracted directly from mycelia of *S. megalocarpus* with DNA extraction kits (labeled “DNA extraction” in Table 4.2) and sent for sequencing (Table 4.2).

Table 4.1: Identity of bacteria from combined PCR product by cloning

<table>
<thead>
<tr>
<th>Clone #</th>
<th>Bacterial species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 1</td>
<td>Lelliottia amnigena</td>
</tr>
<tr>
<td>Clone 2</td>
<td>Lelliottia amnigena</td>
</tr>
<tr>
<td>Clone 3</td>
<td>Serratia liquefaciens</td>
</tr>
<tr>
<td>Clone 4</td>
<td>Ewingella americana</td>
</tr>
</tbody>
</table>

Table 4.2: Identity of bacteria isolated from four different isolates of *S. megalocarpus* (NRRL #’s 66832, 66834, 66835, 66836) both from media on plates without a DNA extraction kit (“plate”), and from DNA extracted from mycelia of *S. megalocarpus* using DNA extraction kits (“DNA extraction”)

<table>
<thead>
<tr>
<th>Isolate of <em>S. megalocarpus</em>/technique</th>
<th>Bacterial species</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRRL # 66832/plate</td>
<td>Serratia marcescens</td>
</tr>
<tr>
<td>NRRL # 66832/DNA extraction</td>
<td>Serratia marcescens</td>
</tr>
<tr>
<td>NRRL # 66834/plate</td>
<td>Serratia marcescens</td>
</tr>
<tr>
<td>NRRL # 66834/DNA extraction</td>
<td>Serratia liquefaciens</td>
</tr>
<tr>
<td>NRRL # 66835/plate</td>
<td>Serratia marcescens</td>
</tr>
<tr>
<td>NRRL # 66835F/DNA extraction</td>
<td>Ewingella americana</td>
</tr>
<tr>
<td>NRRL # 66836/plate</td>
<td>Lelliottia amnigena</td>
</tr>
<tr>
<td>NRRL # 66836/DNA extraction</td>
<td>Achromobacter spanius</td>
</tr>
</tbody>
</table>
Discussion

The most commonly isolated bacterium associated with *S. megalocarpus* cultures was *Serratia marcescens*, present on 75% of the culture plates. *S. marcescens* was recently reported to be pathogenic on zygomycetes (Hover et al. 2016), and therefore, the possibility of using *S. marcescens* as a biocontrol may exist. Of the four other bacterial species isolated and identified, *Achromibacter spanius, Ewingella americana, Lettiottia amnigena*, and *Serratia liquefaciens*, none are known bacterial endosymbionts of the Mucoromycota.

The occurrence of bacteria associated with *S. megalocarpus* had not been noted in the literature. However, two scientists who have worked with this fungus communicated about the frequency of visible bacterial “contamination” when culturing *S. megalocarpus* (K Paley, The Pennsylvania State University, personal communication 2017; ME Smith, University of Florida, personal communication 2018), and these experiments support their findings. The relationship between these bacteria and *S. megalocarpus* remains unknown, though it may not be a true symbiotic relationship due to the number and apparent randomness of the bacterial species found to be associated with *S. megalocarpus* isolates. If only one bacterium was repeatedly found associated with the *S. megalocarpus* isolates, it would seem more likely to be a true symbiotic relationship. Further research into the ecological role that these bacteria play in this pathogenic system is needed.
Summary

*Serratia marcescens* was present on 75 % of the culture plates and was the bacterium most commonly associated with *S. megalocarpus*. Four other bacterial species were isolated and identified: *Achromibacter spanius, Ewingella americana, Lettiottia amnigena*, and *Serratia liquefaciens*, though the relationship of these bacteria and *S. megalocarpus* is currently unknown. None of the bacteria identified are known endosymbionts of fungi.
Chapter 5

Future research

Very little has been studied about S. megalocarpus as an agricultural pathogen. Therefore, there are many avenues that may be useful in future research, both for basic research and for developing a control management strategy for growers.

Phylogenetics and genetics

Phylogenetics

S. megalocarpus is a widespread organism associated with wild mushrooms in Europe, the United States, and Korea, among other locations and has a large host range. The occurrence of different lineages of S. megalocarpus is a distinct possibility due to the wide geographic range that S. megalocarpus can be found. This raises a question on the degree of genetic variability of S. megalocarpus in nature. It brings up basic phylogenetic questions such as to what extent genetic variation would be present if isolates found on mushroom farms in Pennsylvania were compared with wild S. megalocarpus, even locally. It is possible that the temporal coincidence of the emergence of S. megalocarpus and the recently released strain of mushroom, ‘Heirloom’, is not the sole cause of the sudden increased virulence of S. megalocarpus. It is also possible that a new strain of S. megalocarpus is involved.
Genetics

Though not all aspects of the data were statistically significant in this set of experiments, the results suggest that further research may demonstrate even more conclusively that the recently introduced *A. bisporus* hybrid brown strain ‘Heirloom’, particularly its dead tissue (knock overs), is more susceptible to infection by *S. megalocarpus* and that the traditional brown strain ‘Lambert 805’ is less susceptible.

The existing data, which would be strengthened by further studies, already raises the issue of what genetic and physiochemical changes may have led to some strains being more susceptible, noting also that the recently introduced strains were produced by backcrossing a traditional commercially available strain with a wild *A. bisporus*.

As recently released strains are popular among growers due to the potential for increased yield and additional positive differences in growth characteristics and phenotypical appearance of the fruiting body, it is unlikely that commercial problems caused by *S. megalocarpus* will be solved by growers returning to a traditional strain. If the spawn companies would release the genetics of the strains involved, including the parentage and individual strain of ‘Heirloom,’ this would allow comparison with traditional strains such as ‘Lambert 805’, and then through comparative genomics it may be possible to discover the gene or combination of genes that leads to increased susceptibility. This information could potentially be applied to future breeding programs focused on developing *S. megalocarpus* resistant varieties as well as potentially developing green mold resistant varieties if the defense mechanisms are similar.
Ecology, epidemiology, and etiology of *S. megalocarpus*

**Ecology: vectors**

The origin of *S. megalocarpus* and how it is perpetuated on a farm is unknown. The most logical reservoir is that inoculum originates naturally around the farms, where the fungus is commonly found on senescing wild mushroom fruiting bodies in the fall. Sporangiospores of *S. megalocarpus* are possibly vectored by fungus gnats, due to the sporangiophores being covered in spinose projectiles. A close relative, *Sporodiniella umbellata*, produces similar projectiles and is a facultative insect pathogen (Ekpo and Young 1979), plus fungus gnats are commonly found on senescing fungal fruiting bodies in the wild. In nature, the known niche of *S. megalocarpus* is as a necrotroph/decayer of senescing fungal fruiting bodies. It is likely that sporangiospores are of primary importance in the lifecycle and are the main mode of overwintering and vectoring to new hosts, likely spread by fungus gnats found around decaying plant and fungal material. Since fruiting of mushrooms can be sporadic and wildly variable, it seems less likely that wind is the main vector, as a reliance on chance and timing is perhaps an unlikely evolutionary strategy. This issue also relates to how long the sexual and asexual spores are viable; spores may rest in the same location, infecting fruiting bodies in subsequent years. Fungus gnats should be examined for their ability to vector this pathogen. If shown to be a vector, additional controls for fungus gnats could be investigated.
Epidemiology

How *S. megalocarpus* is moved and/or vectored both in nature and in commercial growing operations is also unknown. This issue can be tested in several different ways, including the following: sampling mushroom beds at different time points throughout the cropping cycle of button mushrooms, testing whether fungus gnats are able to vector this pathogen, and using air samplers to detect the levels of *in vivo* sporangiospores throughout a mushroom house.

Etiology

It is also unknown how long sporangiospores of *S. megalocarpus* can survive on compost or casing, and to what extent spores deposited on the casing layer can cause additional infection. These questions can be tested with the use of mycelial plugs and spore suspensions. A methodology would be using small tubs for a cropping experiment at the MRC, filling them with spawned compost, then applying a casing layer, and finally inoculating the casing layer with spores or a mycelial plug and then placing a mushroom on or near the inoculum points at different points in time. This would demonstrate whether the inoculum can cause infection if it lands on the casing in the absence of dead or dying mushroom tissue and how long inoculum is viable during the cropping cycle.
Fungal-bacterial interactions

Bacterial blotch and synergism with *S. megalocarpus*

In experiments performed at the Mushroom Research Center (MRC), an association was noted that tubs spawned with an off-white hybrid (‘Lambert 901’) and inoculated with *S. megalocarpus* demonstrated an increased incidence of bacterial blotch symptoms. This was noted in three different trials. The level of bacterial blotch was noticeably more pronounced in the experimental tubs compared to the controls. This leads to various further areas to study: whether a synergistic interaction exists between the bacteria and fungal pathogen and whether the appearance of bacterial blotch might be an indicator of pending *S. megalocarpus* infection.

Potential bacterial biocontrol: *Serratia marcescens*

Hover et al. 2016, stated that the bacterium *Serratia marcescens* is pathogenic on zygomycetes, and it was present in most of the cultures in these experiments. *S. marcescens* could be tested in the lab for pathogenicity against *S. megalocarpus* isolates from mushroom farms. If it demonstrates control in the lab, a cropping experiment could then be developed to test efficacy *in vitro*. However, a potential problem is that the bacterium can be a potential human pathogen, so it would require testing to determine whether the bacterium could cause infections in animals or if the bacterium persists in any measurable amount on marketed mushroom caps. Since the mushroom industry is currently interested in biocontrol measures, this might be a timely research project.
bacterium proves ineffective as a biocontrol, other closely related bacteria could also be tested, or commercially available biocontrol bacteria-based products could also be tested.

**Loss of vigor in cultivation**

*S. megalocarpus* typically loses vigor in culture, but the reason is unknown. The loss of vigor was typically associated with extensive growth of bacterial colonies in subcultured isolates and a subsequent reduction in mycelial growth.

Issues cultivating *S. megalocarpus* have been a recurring theme with researchers who have worked with it in culture (Robinson (1925); T Bruns, University of California, Berkeley, personal communication 2017; K Paley, The Pennsylvania State University, personal communication 2017). These difficulties suggest that the organism is failing to acquire something necessary to remain viable at some point in its life cycle, possibly a bacterial symbiont, temperature variation, or nutrient. Future experiments to explore this question would include maintaining the same isolate under different environmental conditions and nutrient conditions to narrow down a possible growth limiting factor. Additionally, it might be helpful to inoculate a wild mushroom with *S. megalocarpus* and compare all results of spore formation, growth, and vigor when placed back into the artificial environment of a Petri plate and media.
**Habit**

The morphology and growth characteristics of *S. megalocarpus* vary considerably based on many factors: temperature, weather, season, age, media, and the duration or presence of light intensity and darkness. When the cultures originate from the same isolate and are grown on the same media and in the same location, then variation in color, shape, growth rate, sporulation, and longevity are frequently observed. The observed differences in growth characteristics may be why the production of zygospores has been difficult to consistently produce in culture; this inherent variability might also explain why researchers have disagreed as to what conditions give rise to sexual spores (Baker 1931; Blakeslee 1904; Davis; 1967; Kaplan and Goos 1982; Poff 1965).

**Summary**

To summarize, little is known about the relationship between *A. bisporus* and *S. megalocarpus*, an interesting mycoparasitic system that has now emerged as an agricultural pathogen affecting a crop of major importance to Pennsylvania. A great deal of work, both basic and applied, remains to be done on this system.
Appendix A

Experiment: Presence or absence of specialized penetration structures as determined by scanning electron microscopy (SEM)

Introduction

The presence or absence of penetration structures, like an appressorium, may give information relevant to future strain breeding of button mushrooms for control of S. megalocarpus. Mycoparasitism penetration structures have not been widely researched, so it is not well known what structures, if any, are formed during the preliminary penetration of the host fungus cuticle. The original hypothesis was that S. megalocarpus might produce a specialized structure for penetration of the host mushroom as many fungi do produce specialized penetrations structures such as appressoria for the infection of plant hosts (Agrios 2005). SEM provided a means to investigate this. Two samples were evaluated with SEM to look for specialized penetration structures.

Materials and Methods

A. bisporus ‘Heirloom’ strain mushrooms were bought from Weis Markets, State College, PA. Two mushrooms were placed into crystallizing dishes with a filter paper with 1 ml of sterile Milli-Q® water and inoculated with a plug of S. megalocarpus grown on PDA. This container was kept at room temperature, 21 to 22 °C, for three days and
then transported to the Microscopy and Cytometry Facility where the sample was fixed for scanning electron microscopy (SEM).

SEM was performed at the Microscopy and Cytometry Facility, Huck Institute of the Life Sciences. The sample preparation for SEM protocol was provided by John Cantolina from the Microscopy and Cytometry Facility at Pennsylvania State University, University Park (Dykstra 1992 and Hall and Hawes 1991). The samples were cut and then placed into a solution of 2.5 % glutaraldehyde and 0.1 molar sodium cacodylate; the volume to samples ratio was 10:1. The samples were held overnight at 4 °C. These fixed samples were then washed three times with the previously used buffer (0.1 molar sodium cacodylate) for five minutes each; next they were placed into a 1 % osmium tetroxide buffer solution (0.1 molar sodium cacodylate with 1 % osmium tetroxide) and placed in the dark for an hour. The samples were then washed three times with the buffer, which completed the preparation for the alcohol dehydration process. The alcohol dehydration process used gradients of: 25 %, 50 %, 70 %, 85 %, 95 %, and 100 %, starting with low concentrations and proceeding to higher concentrations. The samples were completely immersed in the solution for five minutes before the liquid was decanted; the samples were then washed once with each of the alcohol gradients except for the 100 % solution, which was used three times for five minutes each. The original protocol was slightly altered due to the sample being entirely fungal in nature: before the alcohol dehydration process, 1 % osmium solution was added to the samples for an hour, followed by washing three times with a buffer. The alcohol-dehydrated samples were placed into a critical point dryer (LEICA EM CPD300, Germany). The protocol used was a preset program called “Black Mold.” After drying, these samples were mounted onto aluminum
stubs via conductive tape and placed in the SEM where images were taken. The scanning electron microscope used was a Zeiss Sigma VP-FESEM.

Results

No specialized penetration structures were observed when viewing the SEM (Figure A1).

Discussion

No specialized structures, such as an appressorium, were observed in this sample. Since no structures were observed, the current hypothesis is that *S. megalocarpus* secretes enzymes to break down the fruiting body and mycelial tissue for nutrient absorption. This
seems logical for a necrotrophic pathogen that targets tissues that are already decaying and relatively easy to penetrate and infect. However, it is not definitive that no specialized structures are ever present, only that the imagery obtained through SEM did not capture any images of such structures.
Appendix B

Experiment: Epidemiology, movement of sporangiospores of *S. megalocarpus* in the growing room

Introduction

Discovering how *S. megalocarpus* moves within growing rooms is clearly important to developing practices for the management of this disease. A preliminary experiment tested for the presence of *S. megalocarpus* in growing rooms both before and after inoculation of the crop with *S. megalocarpus*.

Materials and Methods

Rodac plates containing benomyl-treated PDA were used to sample different locations of two growing rooms before the inoculation of the pathogen, and then approximately two weeks post inoculation. The experiment included two trials in two different growing rooms at the MRC. Following a research crop not specifically inoculated with *S. megalocarpus*, and after steaming, two growing rooms were sampled. The rooms were additionally sampled after a new crop was inoculated with *S. megalocarpus*. For each trial, to determine preexisting infestation before inoculation, Rodac plates were used to test the following surfaces in the growing rooms for *S. megalocarpus*: the door handle, doorjamb, floor drain, tubs, lower air return vent, and
hose nozzle. Then, after inoculation and subsequent infection, the previously described surfaces were sampled again during harvesting. In addition, after inoculation, the following surfaces were tested: the knife used for harvesting, the picking box, the picker’s shoes, sleeves, and gloves, as well as the upper air exhaust vent. After infection had occurred, a Rodac plate was used to sample a symptomatic mushroom and used as a positive control, and an unopened plate was used as a negative control.

Results

None of the samples taken from the growing room after steaming and before inoculation tested positive for *S. megalocarpus*. Two weeks after inoculation, none (0/6) of the negative controls were positive, and all (6/6) of the positive controls were positive. Two weeks after inoculation, many locations in the growing room tested positive for *S. megalocarpus*: door handle 4/6, doorjamb 1/6, lower air return vent 6/6, upper air exhaust vent 1/1, floor drain 6/6, hose nozzle 1/6, tubs 5/6, picking box 6/6, picker’s knife 2/6, gloves 0/6, sleeves 3/6, and shoes 2/3.

Discussion

Surfaces tested in the growing rooms after steaming and before inoculation with *S. megalocarpus* tested negative for *S. megalocarpus*, after which surfaces frequently tested positive. Based on results obtained post-inoculation, it appears that this pathogen can be vectored by wind and water and also by workers and cropping equipment in the
growing room. *S. megalocarpus* was found in all instances on both the lower air return vent (6/6) and also on the upper air exhaust vent (1/1). The upper air exhaust is located in the ceiling and would probably not be splashed with water during watering, indicating that *S. megalocarpus* was probably spread by air currents. The floor drains tested positive in all instances (6/6), which makes it likely that this pathogen is also moved by water.

The picking boxes hang on the growing tubs, and the tubs were predominantly positive (5/6). The picker harvests infected mushrooms, placing them into the picking boxes which all tested positive (6/6). *S. megalocarpus* was also found on the workers’ sleeves (3/6) and on the knives used during the harvest (2/6). Since *S. megalocarpus* was found on the harvester’s sleeves, picking box, and knife, this suggests that *S. megalocarpus* can be vectored mechanically. Overall, infestation was widespread after inoculation and during cropping.
Appendix C

Raw data from Chapter 2 fungicide experiment

The following tables (Table C1, Table C2, and Table C3) display raw data of growth of *S. megalocarpus* during the Chapter 2 fungicide experiment.

Table C1: Raw data, Chapter 2 fungicide experiment, growth of *S. megalocarpus* with chlorothalonil (Bravo Weatherstik®), two trials; average of 2 radial measurements (cm) taken at 90° per plate.

**Trial 1**

<table>
<thead>
<tr>
<th>Chlorothalonil concentration</th>
<th>Plate 1</th>
<th>Plate 2</th>
<th>Plate 3</th>
<th>Plate 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ppm (PDA)</td>
<td>5.45</td>
<td>5.45</td>
<td>5.65</td>
<td>5.80</td>
</tr>
<tr>
<td>.01 ppm</td>
<td>5.00</td>
<td>5.25</td>
<td>5.15</td>
<td>5.10</td>
</tr>
<tr>
<td>0.1 ppm</td>
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<td>5.30</td>
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<td>5.10</td>
</tr>
<tr>
<td>1.0 ppm</td>
<td>4.60</td>
<td>4.70</td>
<td>4.85</td>
<td>5.10</td>
</tr>
<tr>
<td>10 ppm</td>
<td>4.20</td>
<td>4.30</td>
<td>4.25</td>
<td>4.30</td>
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<tr>
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<tr>
<td>1000 ppm</td>
<td>2.95</td>
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</table>

**Trial 2**

<table>
<thead>
<tr>
<th>Chlorothalonil concentration</th>
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<th>Plate 2</th>
<th>Plate 3</th>
<th>Plate 4</th>
<th>Plate 5</th>
</tr>
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<tbody>
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<td>0 ppm (PDA)</td>
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<td>5.85</td>
<td>5.95</td>
<td>5.80</td>
<td>5.70</td>
</tr>
<tr>
<td>.01 ppm</td>
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<td>5.65</td>
<td>5.05</td>
<td>5.30</td>
<td>5.90</td>
</tr>
<tr>
<td>0.1 ppm</td>
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<td>5.70</td>
<td>5.55</td>
<td>5.65</td>
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<td>4.75</td>
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<td>3.70</td>
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<td>3.80</td>
</tr>
<tr>
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<td>2.70</td>
<td>2.55</td>
<td>2.75</td>
<td>2.80</td>
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</table>
Table C2: Raw data, Chapter 2 fungicide experiment, growth of *S. megalocarpus* with thiabendazole (Mertect® 340-F), two trials; average of 2 radial measurements (cm) taken at 90° per plate

**Trial 1**

<table>
<thead>
<tr>
<th>Thiabendazole concentration</th>
<th>Plate 1</th>
<th>Plate 2</th>
<th>Plate 3</th>
<th>Plate 4</th>
<th>Plate 5</th>
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<td>5.15</td>
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**Trial 2**

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<th>Plate 3</th>
<th>Plate 4</th>
<th>Plate 5</th>
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<td>5.65</td>
<td>5.65</td>
<td>5.60</td>
</tr>
<tr>
<td>0.1 ppm</td>
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<td>5.45</td>
<td>5.65</td>
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Table C3: Raw data, Chapter 2 fungicide experiment, growth of *S. megalocarpus* with metrafenone (Vivando®), two trials; average of 2 radial measurements (cm) taken at 90º per plate

**Trial 1**

<table>
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<tr>
<th>Metrafenone concentration</th>
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<th>Plate 2</th>
<th>Plate 3</th>
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<th>Plate 5</th>
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**Trial 2**

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<th>Plate 5</th>
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<td>5.65</td>
<td>5.60</td>
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<tr>
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<td>1.85</td>
<td>1.75</td>
<td>1.90</td>
<td>1.80</td>
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</table>
Appendix D

Linear regression (Adjusted $R^2$) data from Chapter 2 fungicide experiment

Table D1: Linear regression data, Chapter 2 fungicide experiment, chlorothalonil

|                | Estimate  | Std. Error | t value | Pr(>|t|)   |
|----------------|-----------|------------|---------|------------|
| (Intercept)    | -0.1788580| 0.0187115  | -9.559  | 4.82e-13 ***|
| Bravo.XY$X     | -0.0006129| 0.0000456  | -13.440 | < 2e-16 ***|

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.1227 on 52 degrees of freedom
Multiple R-squared: 0.7765, Adjusted R-squared: 0.7722
F-statistic: 180.6 on 1 and 52 DF, p-value: < 2.2e-16

Table D2: Linear regression data, Chapter 2 fungicide experiment, thiabendazole

|                | Estimate  | Std. Error | t value | Pr(>|t|)   |
|----------------|-----------|------------|---------|------------|
| (Intercept)    | -4.466e-02| 1.334e-02  | -3.348  | 0.00143 ** |
| Mertect.XY$X   | -5.759e-04| 3.251e-05  | -17.716 | < 2e-16 ***|

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.09219 on 58 degrees of freedom
Multiple R-squared: 0.844, Adjusted R-squared: 0.8413
F-statistic: 313.9 on 1 and 58 DF, p-value: < 2.2e-16

Table D3: Linear regression data, Chapter 2 fungicide experiment, metrafenone

|                | Estimate  | Std. Error | t value | Pr(>|t|)   |
|----------------|-----------|------------|---------|------------|
| (Intercept)    | -2.300e-02| 1.006e-02  | -2.286  | 0.026 *    |
| Vivando.XY$X   | -1.050e-03| 2.433e-05  | -43.152 | <2e-16 *** |

---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 0.06898 on 57 degrees of freedom
Multiple R-squared: 0.9703, Adjusted R-squared: 0.9698
F-statistic: 1862 on 1 and 57 DF, p-value: < 2.2e-16
Appendix E

Raw data from Chapter 3 strain susceptibility experiment

Tables E1 and E2 show combined measurements of three strain susceptibility replicates for inoculated wounded and inoculated unwounded mushrooms.

Table E1: Raw data, Chapter 3 strain susceptibility experiment, strain, inoculum method, and percent coverage of infection on inoculated wounded mushrooms

<table>
<thead>
<tr>
<th>Strain/inoculum method</th>
<th>Percent coverage of tub by <em>S. megalocarpus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Lambert 901’/mycelia</td>
<td>12.27 .097 15 70.6 5.71 46.5</td>
</tr>
<tr>
<td>‘Lambert 901’/spores</td>
<td>11.3 13.7 18.5 13.7 28.0 .802</td>
</tr>
<tr>
<td>‘Lambert 805’/mycelia</td>
<td>2.07 .053 .47 26.3 10.3 12.8</td>
</tr>
<tr>
<td>‘Lambert 805’/spores</td>
<td>5.35 38.3 14.7 1.64 --- ---</td>
</tr>
<tr>
<td>‘Heirloom’/mycelia</td>
<td>2.11 13.1 2.50 54.1 16.42 ---</td>
</tr>
<tr>
<td>‘Heirloom’/spores</td>
<td>42.8 8.56 28.4 1.27 8.41 4.77</td>
</tr>
<tr>
<td>‘Brawn’/mycelia</td>
<td>17.7 11.9 3.46 20.3 26.2 23.2</td>
</tr>
<tr>
<td>‘Brawn’/spores</td>
<td>2.3 11.4 5.82 20.7 33.6 47.9</td>
</tr>
</tbody>
</table>

Table E2: Raw data, Chapter 3 strain susceptibility experiment, strain, inoculum method, and percent coverage of infection on inoculated unwounded mushrooms

<table>
<thead>
<tr>
<th>Strain/inoculum method</th>
<th>Percent coverage of tub by <em>S. megalocarpus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Lambert 901’/mycelia</td>
<td>.479 14.0 6.60 ---</td>
</tr>
<tr>
<td>‘Lambert 901’/spores</td>
<td>8.0 22.5 23.5 ---</td>
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<tr>
<td>‘Lambert 805’/mycelia</td>
<td>4.47 --- ---</td>
</tr>
<tr>
<td>‘Lambert 805’/spores</td>
<td>0 3.25 --- ---</td>
</tr>
<tr>
<td>‘Heirloom’/mycelia</td>
<td>20.1 4.78 8.93 ---</td>
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<tr>
<td>‘Heirloom’/spores</td>
<td>6.02 7.40 26.5 5.34</td>
</tr>
<tr>
<td>‘Brawn’/mycelia</td>
<td>16.1 1.02 --- ---</td>
</tr>
<tr>
<td>‘Brawn’/spores</td>
<td>26.7 2.0 .47 ---</td>
</tr>
</tbody>
</table>
Appendix F

Methods of keeping isolates of *S. megalocarpus* alive in the lab

As discussed by Robinson (1925), isolates of *S. megalocarpus* are difficult to keep alive in the lab. The precise difficulty remains unknown, though chronic bacterial contamination may play a role. The best methods found to keep isolates of *S. megalocarpus* alive and vigorous for extended periods of time were as follows: 1) Keeping them in a 4 °C refrigerator appeared to hinder both senescence and bacterial contamination. 2) Redundancy of cultures was necessary, and the minimum number of cultures of each strain that provided reliable redundancy in these experiments was three. 3) Making spore suspensions and refrigerating them helped keep the spores alive. Spore suspensions were generally viable for one to two months if they were kept in a refrigerator at 4 °C.

Spore suspensions were made by plating an agar plug of uncontaminated *S. megalocarpus* onto three PDA plates, then placing these in an incubator at 10 to 15 °C for 10 days, and then following the spore suspension protocol described in Chapters 2 and 3 of this thesis.
Appendix G

Button mushroom production methods

Button mushroom production contains six distinct chronological steps: Phase I composting, Phase II composting, spawning, casing, pinning, and cropping (Beyer 2003).

Phase I composting

The raw materials for the mushroom compost at the MRC were wheat straw-bedded horse manure, dried, pelletized poultry manure, dried distiller’s grain, and gypsum. The objectives of Phase I composting are to begin the breakdown of the organic matter into a form that is accessible to button mushrooms and make a uniform, high moisture substrate. It starts with mixing all of the raw materials except the dried distiller’s grain with water on day 0. The gypsum is added to promote flocculation, which helps to prevent anaerobic fermentation. The mixed raw materials were then placed in an aerated bunker to compost at 80 °C for three days at which point the compost was removed, placed into a mixer and the dried distiller’s grain and additional water was added. The remixed compost was then placed back into the aerated bunker for three more days to finish Phase I. Phase I composting was complete when it appeared chocolate in color, had flexible/supple straw, moisture levels were approximately 72 to 74 %, and the compost had a distinct smell of ammonia. Phase I composting took six days to complete for each cropping trial.
Phase II composting

The objectives of Phase II are to pasteurize the compost and remove the ammonia that was formed in phase I (conditioning). Pasteurization is needed to eliminate fly eggs, nematodes, fungi, and other pathogens and pests from the compost. The Phase I compost was placed into a Phase II tunnel (a large tunnel with an aerated floor). Pasteurization took place at 60 °C for four hours, one day after filling the tunnel. After pasteurization, the compost was cooled to 48 °C for the remainder of the Phase II composting until ammonia was removed (as determined by smell). Phase II composting took six days to complete for each cropping trial.

Spawning

Spawning is the act of introducing mycelium on a carrier, such as rye or millet, that has been colonized by the fungus after the completion of Phase II composting. The spawn is added to the compost at a rate of 2 % spawn weight to dry compost weight, when the substrate temperature is below 30 °C. It has become common practice to add a delayed-release nutrient supplement at spawning to increase yield. For each cropping trial a commercially available supplement (Promycel Gold) was added to the compost at the manufacturer’s recommended rates, along with the spawn. Subsequent colonization of the compost is called the spawn run and took approximately 15 to 17 days for each trial.
Casing

Casing is applied to the colonized spawn-run compost and is a combination of peat moss buffered with crushed limestone. Without this step rhizomorphs and therefore fruiting bodies will never be formed and mushrooms will not be produced. Casehold is the period between casing the crop and harvest which typically takes approximately 15 to 16 days. For these experiments commercial casing inoculum was not used. Instead, CAC (colonized compost mixed into the peat moss) was used to speed up the case hold and produce a more uniform pinset.

Pinning

The next procedure is pinning and pin (primordial) formation, initiated by dropping the level of carbon dioxide to or below 0.08 %. In these conditions, the rhizomorphs give rise to primordia that will be future fruiting bodies.

Cropping

The final stage is cropping or harvesting of the mushrooms. They are picked in what are called “flushes” or “breaks,” typically repeated for three cycles of harvesting. On a commercial mushroom farm cropping is commonly 35 to 42 days but can be as long 150 days in developing countries. Air temperature is kept in the range of 14 to 17 °C; this temperature is optimal for the growth of button mushrooms and helps to decrease pest levels and to slow down the life cycle of many pathogens and pests.
Appendix H

Protocol: Bright-Line\textsuperscript{TM} Hemocytometer (Hausser Scientific, Horsham, PA)

The chamber was placed under the microscope at 10x and the central 1 mm square located. The central square of the hemocytometer was further divided into 25 smaller squares, and further into 16 even smaller squares. The 25 square grids are separated by a triple line, each .2 mm in area. The surface below the glass cover was .10 mm, and the volume of liquid for 1 mm\textsuperscript{2} was 0.1 mm. Spores were counted in the smallest sixteen squares using magnification of 20x by counting the corner squares and the center square. The spore concentration was calculated by inputting the total number of spores counted into the following formula: \#spores counted \times 50 = \#spores/mm\textsuperscript{3}, then \#spores/mm\textsuperscript{3} \times 1000 = \#spores/ml.
Literature Cited


Ekpo EJA, Young, TWK. 1979. Fine structure of the dormant and germinating sporangiospore of *Syzygites megalocarpus* (Mucorales) with notes on *Sporodiniella umbellata*. Microbios Letters.10:63-68.


http://www.speciesfungorum.org/GSD/GSDspecies.asp?RecordID=531546


Weil JD, Cutter CN, Beelman RB, LaBorde LF. 2013. Inactivation of Human Pathogens during Phase II Composting of Manure-Based Mushroom Growth Substrate. J Food Prot. 76:1393-1400.
