Studying Arbuscular Mycorrhizal Fungi, Their Endosymbiotic Bacteria, and Their Spores: Challenges and Solutions

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Abstract

Arbuscular mycorrhizal fungi (AMF) have broad applications in agriculture and land restoration due to their significance in forming symbiotic relationships with most terrestrial plants. However, the field of AMF is understudied, often extending results from a handful of AMF species to all AMF. Spores are a highly significant but often overlooked aspect of the AMF life cycle. In this honors thesis, I summarize the current knowledge surrounding the significance of spores in the AMF life cycle. Great strides have been made in understanding AMF spore germination, dormancy, and life strategies, but there are also gaps in the knowledge that, if filled, would further advance the various applications and studies of AMF. In particular, past research done on AMF must be revisited in order to: (1) redefine terms and standardize experiments; (2) reinforce genetic work in AMF; and (3) use knowledge from plant and seed experiments to better inform AMF experiments. I then empirically demonstrate that the endobacterium '*Candidatus* Moeniiplasma glomeromycotorum' can be lost or gained during serial culturing practices, although the mechanisms by which endobacteria are lost or gained. I also show that routine spore processing methods, such as sonication and chemical washes during surface decontamination, can be detrimental to spore survival in some species, and therefore the interpretation of spore viability studies should be carefully considered.

General Introduction

Arbuscular mycorrhizal fungi (AMF) are mutualists that can colonize most terrestrial plants to form symbiotic relationships and exchange nutrients, improve pathogen or drought resistance, and much more. The symbiotic relationship between AMF and plants suggest that AMF have broad applications in agriculture and land restoration. However, the field of AMF is understudied, often extending results from a handful of AMF species to all AMF. Spores are a highly significant but often overlooked aspect of the AMF life cycle, as are the endobacteria that live in their cytoplasm. Great strides have been made in understanding AMF spore germination, dormancy, and life strategies as well as the role of their endobacteria, but there are also gaps in the knowledge that, if filled, would further advance the various applications and studies of AMF. In this honors thesis, I aim to summarize what is known about AMF spore biology, examine endobacteria populations in AMF, and investigate how experimental procedures impact AMF on a species level to better understand AMF spore biology.

In chapter 1, I review the current knowledge on AMF spore biology and their significance in the AMF life cycle. In chapter 2, I empirically demonstrate that the endobacterium '*Candidatus* Moeniiplasma glomeromycotorum' can be lost or gained during culturing practices and that a routine spore surface decontamination protocol was detrimental to *Rhizophagus clarus* spore survival. A general conclusion follows that past AMF research must be revisited to: (1) redefine terms and standardize experiments, (2) reinforce genetic work in AMF, and (3) use plant and seed experiments to further substantiate that of AMF.

Chapter 1: Spores of Arbuscular Mycorrhizal Fungi: Form and Function

Introduction

Spores, though not unique to fungi, are one of the defining features that typify this group of eukaryotes in addition to filamentous (hyphae) or isodiametric (yeast) somatic structures[1]. Spores are essential to the life cycles of most fungi. For these immobile and often ephemeral organisms, spores provide the means of long-distance travel (dispersal in space) and persistence during unfavorable conditions (dispersal in time). Spores can be formed as the result of sexual mating followed by meiosis or through asexual means[1], both of which have their own advantages and disadvantages. Depending on the lifestyle, fungi differ in their ability to use spores to disperse in space and time and their dependence on sexual versus asexual spore production[1].

The lifestyle of arbuscular mycorrhizal fungi (AMF; Mucoromycota: Glomeromycotina) are dominated by spores. These fungi are mutualists that colonize the roots of the vast majority of lands plants[2] and are credited with facilitating the origin of land plants in the early Devonian[3]. AMF provision their hosts with mineral nutrients translocated from the soil in return for the products of photosynthesis. Because the biotrophic lifestyle of AMF magnifies the significance of successful proliferation and dispersal, spores play an outsized role in AMF biology. These features are also important for practical applications of AMF, such as boosting crop production by capitalizing on AMF's ability to increase plant pathogen protection[4], aid in nutrient acquisition[2], and to improve plant tolerance to drought[5] and heavy metal contaminants[6] among many other desirable benefits. More broadly, AMF are found worldwide in a variety of environments, and some taxa show global distribution[7]. AMF facilitate the benefits by producing

structures called arbuscules that act as an interface with plants to exchange nutrients and other benefits[1], although this could not occur without the formation and dispersion of spores. This pattern reflects the importance of AMF species to a wide range of plant hosts that rely on AMF in a symbiotic relationship.

In this minireview, I synthesize what is known about AMF spores: their development, dormancy, and germination. I also suggest research directions aimed at successful harnessing of AMF in the sustainable agriculture for the future.

Spore Fundamentals

Due to knowledge gaps in AMF biology and the extrapolation of experimental results from a limited handful of model AMF species to all AMF species[8,9], the value of spores both in real-world and experimental applications is easily underappreciated and overlooked. To better understand how spores can be used agriculturally as a natural fertilizer, experimentally as a model organism for symbiosis, as well as other applications, there must be a return to the fundamental biology of AMF spores. In this section, the knowledge surrounding the biology of spores, from development to germination to dispersal, is summarized.

Spore Form and Development

The development of AMF symbiosis can be briefly summarized by spore germination, hyphal growth, and subsequent penetration and colonization of roots, development of a mutually beneficial symbiotic relationship with roots, and spore production[10]. Formation of spores begins in the apical hypha[11]. Hyphae play a large role in the formation and development of spores,

which then go on to form symbiotic relationships with host plants after dispersal and germination.

In early divergent fungi, of which AMF are representatives, there are two types of fertile hyphae: (1) sporangiophores which bear asexual spores (sporangiospores) in sporangia and (2) specialized hyphal branches differing from somatic hyphae known as zygophores which give rise to the sexual counterparts, namely progametangia that fuse and form zygosporangia[1]. Whether AMF spores, which appear to be strictly asexual propagules, are homologous structures to either type of spore is still uncertain. Briefly, AMF spore ontogeny begins when a septum forms near the tip of a sporogenic hypha, after which the terminal hyphal tip swells to become a new spore[11]. Cytoplasm carrying nuclei, organelles, nutrients, and other cytoplasmic contents travels through the hyphae to fill the new spore[12]. The swelling of the hyphae is followed by the accumulation of large amounts of lipids in the developing, and eventually maturing, spore[11].

Although AMF spores are asexual, they have been classified as both azygospores and chlamydospores based on differing forms of development[1,2] and are sometimes called secondary spores. Most spores are produced from symbiotic hyphae next to plant roots[1], but spores can also be produced from other resting spores[13]. AMF spore diversity can be described differently based on how and when they form[1,2].

Secondary Spores

Spores of AMF are nearly always produced during the symbiotic phase while the AMF has established arbuscules and is actively deriving nutrients from the host plant. However, spore formation can still occur in the nonsymbiotic phase (secondary spores), but spores that do not establish symbiosis have been observed to senesce[11]. In other words, AMF can make spores

without symbiotic relationship with a plant, but AMF have a limited reserve of nutrients without symbiosis. Without eventually forming a successful symbiotic relationship, AMF do not have sufficient nutrients to produce new and mature spores indefinitely. Thus, plant hosts play a critical role in the AMF life cycle because they facilitate spore formation and development, and therefore the continued survival of AMF generations.

AMF are known to produce secondary spores (also called vegetative or pre-symbiotic spores), which are spores that grow from germ tubes stemming from resting spores[13], or pre-symbiotic spores. Unfortunately, the research on secondary spores is difficult to navigate, as these spores have been referred to as “secondary” and “auxiliary” cells interchangeably and it is not always clear that these terms refer to the same type of structures.

The research around the biology of secondary spores remains limited. Fatty acids, including methyl branched-chain fatty acids isolated from bacteria and palmitoleic acid, have been demonstrated to stimulate the development of secondary spores capable of infecting plant hosts at the asymbiotic stage[14]. Secondary spores have been suggested to have a sympodial form of growth[15].

The ecological significance of secondary spores is rarely addressed, but secondary spores are able to form symbiotic relationships with plant hosts and generate progeny[14]. Presumably, secondary spores can act as a form of repackaging resources when conditions become unfavorable, development of symbiotic relationships fail, or when germination fails. This repackaging could then allow detachment from the mycelia or hyphae and dispersal to a new location for better chances of survival. However, further research is required to clarify their role.

Auxiliary Cells

"Auxiliary cells" of unknown function are produced on extraradical mycelium by two genera, *Scutellospora* and *Gigaspora* in place of vesicles[2]. The function of these auxiliary cells remains elusive and they are even speculated to be the remnants of former functional structures[16]. Confusing the issue, the terms "secondary" and "auxiliary" seem to be used interchangeably at times in the literature.

It has been reported that auxiliary cells can exist individually or in clusters, and 8 to 20 nuclei have been observed in the auxiliary cells of *Dentiscutata erythropus* (previously *Scutellospora erythropa*) and *G. gigantea*[17]. In *Racocetra persica* (*S. persica*), more nuclei were typically observed and up to 26 nuclei have been seen[17].

Spore Lipids

Lipid bodies are structures that act as the main form of lipid storage in eukaryotic cells[18]. AMF lipids are an area of intense study, as the accumulation of large, conspicuous, energy-dense lipid bodies commonly seen in AMF spores is an important adaptation for spore persistence and survival. Although trehalose is the major storage carbohydrate in AMF, its low concentrations suggest that lipids are instead nearly the exclusive source of energy during spore germination and hyphal growth[19]. Lipid bodies are translocated to and consumed during hyphal growth in AMF[20]. In addition to acting as an energy source, lipids may play a role in stress responses in AMF. Fungal triacylglycerol (TAG) metabolism has been suggested to counter oxidative stress by not only providing carbon skeletons and energy for membrane regeneration but also activating metabolic processes involved in cellular stress defense[21]. However, the role of lipids in the stress response of AMF requires further clarification and research. From these experiments, lipids

appear to be critical for AMF spore growth and survival by providing energy and may also activate molecular pathways for counteracting stress.

Spore Nuclei

Compared to most fungi and their spores, AMF spores have exceptionally large numbers of nuclei[12]. Hundreds of nuclei can coexist in an AMF spore with hundreds of thousands of coexisting nuclei in the surrounding mycelium serving to buffer against the accumulation of deleterious mutations in these apparently-strictly-asexual fungi[12]. This multiplicity of nuclei acts as a simple selection system through asynchronous nuclear replication, where nuclei that replicate faster may have an advantage over nuclei that replicate at a slower pace[12]. AMF can be homokaryons or dikaryons and work has been done to show that differences appear between dikaryons and homokaryons. Dikaryons can influence their plant partner as co-existing alleles in dikaryons can be transcribed simultaneously[22]. In addition, interactions between cooperating nuclei, including those of different histories, in dikaryons have been shown to have compensatory effects for mutations and suggest a heterozygote advantage[23]. Multiple nuclei are thus a powerful adaptation for AMF spores, serving as a buffer against deleterious mutations and allowing selective genetic exchange.

Furthermore, these coexisting nuclei can differ in number, shape, and frequency among and within species[16]. Bidirectional cytoplasmic movement of nuclei has been observed in hyphae[24]. The multiple nuclei also have high genomic diversity and some strains have been observed to have different nuclear types co-existing (i.e. heterokaryotic)[25,26]. Together with its diverse range of physical and mechanical features, the multiple nuclei of AMF are a powerful adaptation for maintaining genetic diversity and responding to stress.

Spore Dormancy

Similar to plant seeds, AMF spores enter dormancy to allow them to survive in diverse environments by preventing germination from occurring immediately after development. Newly formed spores are innately dormant[27] and dormancy is maintained until germination is possible. In seed dormancy in plants, primary dormancy is defined as innate dormancy upon release from the parent plant, whereas secondary dormancy is the onset of blocked germination in mature seeds based on environmental conditions[28]. Like that of plant seeds, AMF spores may experience differences in dormancy and types of dormancy.

Arguably, there is uncertainty in the degree of metabolic activity in the cytoplasm when defining spore dormancy. Most of AMF research has focused on breaking dormancy, and the biology of AMF dormancy is not well-studied, especially when defining dormancy can be difficult because dormancy is measured by the absence of germination[29]. Experimentally, dormancy has been defined by the failure to germinate in conditions that would otherwise support germination of a non-dormant spore, and quiescence has been defined by the failure to germinate when physical and chemical conditions are met but the environment is otherwise unfavorable[27]. Dormancy prevents germination through stopped metabolism, while quiescence prevents an organism capable of germinating from doing so due to unfavorable conditions.

Dormancy is a critical step of spore development and survival, and numerous factors influence AMF dormancy.

Breaking Spore Dormancy

Physiological aspects of a spore restrict its ability to transition from dormancy to quiescence to beginning germination[27]. A dormant spore must transition to quiescence before germination can be achieved. Dormancy ultimately allows AMF to synchronize their development with plant development. However, the uniformity of spore age (i.e. near synchrony of spore germination across many spores) and innate dormancy of newly formed spores has been noted to contribute to developmental synchrony[27].

In order for AMF spores to begin forming a symbiotic relationship with plant hosts, spore dormancy must be broken. It has been suggested that spore dormancy is regulated by a basic internal biological clock[30]. The length of dormancy varies from species to species and is linked to the geographical distribution of the species[30], suggesting that conditions that mimic a species' natural ecological environment are optimal for breaking dormancy. For example, cold exposure imitates natural ecological conditions such as winter[31]. Passing spores through cycles of cold and non-cold treatments over a number of months is a common protocol for initiating germination in the laboratory, and this method can be likened to imitating the natural ecological environment that would break dormancy.

In addition to cold temperatures, other factors have been observed to influence the dormancy period. Although newly formed spores are unable to germinate and are innately dormant when first formed, wet soil and dry soil have been observed to influence the dormancy period of *Funneliformis caledonius* (*Glomus caledonium*), *Funneliformis monosporus* (*Glomus monosporum*), and *Scutellospora calospora* (*Gigaspora calospora*)[27]. However, other species such as *Acaulospora laevis* had a consistent dormancy period under all conditions[27]. The author speculates that the large differences observed in dormancy periods was likely related to spore age

in wet soil, while dry soil caused the spores to change quickly to quiescence and weigh spore age less significantly. However, the mechanisms of this or how soil and spore age are related remains unclear. Although, most incubation temperatures do not influence the dormancy period, incubation temperatures of 37° Celsius in wet soil can kill hydrated, dormant cells[27]. In addition to mimicking the cold, other factors such as soil can play a role in imitating the natural ecological environment to break or maintain dormancy. Whether spores can re-enter a state of dormancy if unfavorable germination conditions are experienced is unknown, but the primary and secondary dormancy of seeds[28] suggest that AMF might also have the ability to reenter dormancy.

Plant Factors Breaking Spore Dormancy

The significance of the symbiosis between AMF and its plant host hints that plants may influence dormancy, though this remains unclear. One way to investigate dormancy is to identify germination or the absence thereof. Non-specific root exudates influencing AMF spore germination have been observed[32], although volatile compounds originating from non-host plants have also been observed to inhibit AMF germination[33]. This research suggests that plant hosts can release compounds impacting AMF germination, and therefore, break or support spore dormancy. However, the role of plant hosts in influencing dormancy requires further clarification and research as it currently remains unclear if the presence of host plants or growing roots activates or inhibits germination[27,32].

Initiating Germination

Environmental Factors Promoting Spore Germination

The best conditions for AMF to germinate appear to be linked to the best conditions for host plants to grow optimally[34]. Germination (emergence of a hypha to eventually grow and develop a hyphal network) in AMF spores can occur through germination shields, through spore walls, or through regrowth through hyphal attachment[2]. Factors that influence germination in lab environments are well documented, although the optimal conditions for certain factors will differ among various species.

Factors where the optimum conditions can vary based on the species of AMF can include soil pH[34–36], storage duration[37], temperatures that reflect the environment the fungi were isolated from[34,38], and other various compounds and chemicals. Temperature requires further distinctions as incubation temperatures of spores in water agar, soil, and so on do not modify the onset of quiescence[27], but cold storage has been demonstrated to influence dormancy[30,31,39–41] and temperatures can influence the time between quiescence and germination[38]. Similar to factors that influence dormancy, mimicking the natural ecological environment of AMF can promote germination.

More generally for all AMF, carbon dioxide improves spore germination [42,43] along with other factors that induce germination such as: water potential[41,44,45], root exudates and volatiles[32,33], low nutrient (particularly phosphorus) concentrations[46–49], microbial metabolies[34,50–52]. Experimental and growth conditions that match the needs of the species of AMF as well as their natural ecological environment is important.

Documented compounds and chemicals that induce germination include: dialysates and various commercial agars[47]; Tween 20, antibiotics, or high levels of surface sterility[40]; small amounts of vitamins and some organic acids[35]; phosphorus and NPK salts[35]; thiamine[35]; calcium hydrogen phosphate[35]; nitrogen[35]; and sucrose[53]. Appropriate nutrients and chemical compounds are critical for germinating AMF in addition to having the optimal abiotic conditions.

Inhibitors of Spore Germination

Among environmental factors that influence spore germination, numerous inhibitors have been observed experimentally. Morphological factors include the presence of the peridium[48] and spore age[54]. Spore germination can be inhibited by organic compounds such as glucose, fructose, sucrose, L-arabinose, aspartic acid, succinic acid, malic acid, and pyruvic acid[35] and phosphorus[55]. Spore germination can also be inhibited by heavy metals such a manganese[56], zinc[56], and copper[57], and sterile media or soil[34,50]. Inorganic compounds that inhibit germination include ethidium bromide[57], benomyl[10,58], isothiocyanates[59], Rose Bengal/streptomycin[60], chlorine anions and sodium cations[61], as well as cycloheximide, actinomycin D, proflavine, hemisulphate, and 5-fluorouracil[57]. These observations demonstrate again that it is important to understand the experimental and growth conditions conducive to germination of the AMF species being studied.

Repeated Germination

Spores are capable of germinating multiple times (i.e. reinitiating new hyphal growth after the original germ tubes were severed)[60,62], allowing them to survive unfavorable conditions. In particular, spores of *Gigaspora gigantea* with severed germ tubes have been observed to successfully germinate up to ten times[62]. Similarly, an unidentified AMF species was reported to

initiate the same amount of hyphal growth after germinating a second or third time[60]. The authors of this study concluded that the limitation to how many times a spore can germinate is regulated by inhibitory factors from the roots, plant exudates, or the fungus itself as opposed to exhausted spore reserves[60]. However, there does not seem to be much research reproducing this conclusion or establishing why there is a limit to repeated germination. Though further research is required to clarify the biology of repeated germination, I would speculate that spores are unable to germinate repeatedly because the lipid stores become depleted, and the spore no longer has the necessary energy reserves to germinate again, thus explaining the large lipid stores inside of them. Koske (1981) notes that the implications of spore germination are that reserves within the spore itself are depleted and germ tubes must make contact a host for additional resources. The ability to germinate multiple times allows spores to survive the absence of host plants, seasonal fluctuations, and mycophagous soil animals while ensuring that germination occurs during favorable conditions without mycorrhizal investments that drain soil inoculum[62]. Spores have the adaptation and ability to germinate multiple times, but further research is required to clarify the limitations of multiple germination.

Spore Dispersal

In addition to having the ability to germinate multiple times or re-enter dormancy to survive unfavorable conditions, spores have additional adaptations enabling them to move to new locations through dispersal. Genera of AMF spores show distinct patterns of abundance in various biomes[63] as others prevail in early successional sites[64]. Altogether, studies suggest that AMF exhibit various mechanisms of dispersal and life history strategies.

Biotic Vectors

AMF spores have been demonstrated to survive ingestion and transit through a digestive tract without loss of viability[65,66], thus allowing above ground dispersal through animals. Numerous animal vectors are known to disperse AMF spores by intentionally including spores in their diet or incidentally ingesting spores. Earthworms[66]; migratory birds such as geese[67]; terrestrial and arboreal small mammals such as rodents and marsupials[65,68]; bison[69]; and Collembola[70] are a few examples among animal vectors known to assist AMF in above ground dispersal. AMF dispersal through biotic vectors is thus primarily through ingestion by animals.

Abiotic Factors Promoting Spore Dispersal

In addition to animal vectors, spores are also known to disperse through abiotic factors promoting spore dispersal. Spores are capable of dispersing aerially[63,67,71,72], where factors such as spore size and other potential traits such as spore shape and wall thickness influence the ability to aerially disperse[72]. Spores can also be dispersed through water, such as floods that disturb fine sediments[73].

Life Strategy Classification Under the Competitor, Stress-Tolerator, and Ruderal (CSR) Framework

The CSR framework (competitor, stress-tolerator, and ruderal framework) was designed by J. Philip Grime in an effort to map the life strategies of various plants[74]. His framework classifies organisms as competitors, ruderals, and stress-tolerators defined on the basis of stress, disturbance, and competition. This framework demonstrates that plant life strategies are incredibly diverse, and plants often fit intermediates of the classification as opposed to any one

classification. Although Grime's CSR framework was initially used in the context of plants, the CSR framework appears to be a powerful and predictive tool for AMF in topics not limited to observing the effect of disturbance[75], predicting preferred AMF hosts[76], and influencing macroorganism-centric ecology[77]. Under the CSR framework, spore formation and germination features can also be classified to life strategy classification. In the context of agricultural purposes, the CSR framework could prove to be a powerful tool in matching appropriate AMF species to crops.

Grime's CSR framework broadly defines competitors as organisms that thrive in conditions of low stress and low disturbance, stress-tolerators as those adapted to conditions of high stress and low disturbance, and ruderals as those specialized to conditions of low stress and high disturbance[74]. In the context of AMF spores, competitors are expected to have higher soil hyphal densities, stronger carbon-sink strength, and late production of asexual spores in the growing season while stress tolerators are expected to have low growth rates, long-lived mycelium, and resistance to abiotic stressors[76]. In addition, ruderals are expected to have high growth rates, early production of many asexual spores, high hyphal turnover rate, and more efficient hyphal healing[76].

Spore Development Life Strategies

The family Gigasporaceae can be described as competitors under the CSR framework. In particular, Gigasporaceae spores sporulates later in the growing season in temperate ecosystems[78,79]. Research has also shown that Gigasporaceae often occur in terrestrial ecosystems at low spores densities compared to that of Glomaceae and Acaulosporaceae[80,81]. Gigasporaceae rely on spores as their dominant propagule[79,82], which are typically large in size[77].

The family Glomaceae can be described as ruderal. Glomaceae colonize roots faster than other isolates[83], while being adapted for growth in disturbed ecosystems rich in available resources[82], suggesting that they sporulate early in the season. Glomaceae can regenerate from hyphal fragments[84], though they also rely on spores as propagules that are larger than many other fungi in Glomeromycota and with a higher inoculum load[75]. Glomaceae also has the largest range of spore sizes[77].

Spore Germination Life Strategies

Finally, the family Acaulosporaceae can be described as stress tolerators. Acaulosporaceae is more tolerant to mechanical disturbances and the least tolerant to chemical disturbance, suggesting a primarily nutritional role in AMF symbiosis[75] and a stress tolerant life strategy. In addition, Acaulosporaceae establish an external mycelium that makes up a small fraction of the total mycelium, which may allow them to experience less loss, while spore dormancy allow them to persist through episodic soil disturbance[75].

Life Strategy Framework Utility

Despite its predictive power, the CSR framework is currently limited in AMF for various reasons: (1) the number of tested species across a few genera and families is limited[75,83], (2) there is a need for replication among species[75], (3) there is a lack of comparative study[75] or standardized method for comparison, and (4) a better understanding of AMF in areas such as their role in pathogen protection and the function of arbuscular mycorrhizal symbiosis is required[75]. Furthermore, the power of the CSR framework also lies in identifying possible features of spore form, development, dormancy, and dispersal. One of the most important

takeaways from this new predictive tool may be the urgency to better understand the fundamental biology surrounding AMF and to look further into the genetics of AMF.

Applications and the Future of Arbuscular Mycorrhizal Fungi

Sustainable Agriculture

One of the rising motivations to pursue research in AMF and their spore biology lies in crop protection against environmental stresses[4,5] and the movement towards eliminating chemical fertilizers using microbial inoculants including AMF[85]. AMF can boost crop production by capitalizing on their ability to increase phosphorus, nitrogen, and other nutrient acquisition[2], plant pathogen protection[4], improve plant tolerance to drought[5] and heavy metal contaminants[6], and protect plants against salinity[86] among other benefits after inoculating plant hosts. By taking advantage of the symbiotic relationship between AMF and plants and the benefits exchanged, AMF may also be used as microbial inoculants that could eventually eliminate chemical fertilizers[85]. At this time, research has demonstrated that combining AMF with fertilizers can increase fertilizer efficiency and reduce fertilizer use[87]. Not all AMF inoculum is composed solely of spores, but AMF spores are appealing in the agricultural context because they are small, and a better understanding of their biology will permit larger and controlled production of healthy spores for large-scale agricultural use. Altogether, the formation of symbiotic relationships and exchange of nutrients and benefits with plant hosts suggest that AMF spores and other forms of AMF inoculum have great potential in improving human agriculture.

More broadly, AMF also have great potential in land restoration by improving soil aggregation[88,89], suggesting further possible applications in agriculture. Research has

demonstrated that soil aggregation can be improved through the fungal-plant symbiosis[89] and is influenced by the combination of various species of earthworms and arbuscular mycorrhizal fungi[88]. However, studies report varying degrees of positive effects of AMF on soil aggregation. Thus, even though AMF show significant promise in land restoration, further research is required to better understand the complex relationship between fungi, plant hosts, and other soil organisms.

AMF have great potential in agricultural and land restoration applications, and spores are an important, although not the only form, of AMF inoculum. Hyphal fragments can also serve as inoculum for new colonization[90]. To better understand how AMF can be used in practical applications, further research is required.

Experimental Advantages

Arbuscular mycorrhizal fungi colonize the roots of the vast majority of lands plants[91], presenting a worldwide presence that could allow them to become a model organism to study symbiosis and mutualisms. AMF spores are large and easy to manipulate and separate, thus allowing single spore genetics and other experiments to be done on single genotypes, even directly from field material without intermediate subculture. In addition to the study of fungal-plant symbiosis, endobacteria within arbuscular mycorrhizal fungi themselves provide yet another layer of study and complexity.

However, barriers such as obligate biotrophy, poor understanding of AMF life cycles, and the lack of efficient genetic transformation technology in AMF have led AMF to not only be severely understudied in comparison to other fungi but also created challenges for experiments using AMF. There is already a depth of knowledge surrounding arbuscular mycorrhizae and their

spores, but there are also numerous fundamental gaps in knowledge of many AMF that limit the use of their spores in experimental settings. As the biology of spores is better understood, the applications of spores will undoubtedly expand.

Outlook: Future of AMF Research

AMF have great potential in agriculture[4,5,85], land restoration[88,89], and basic biological research and must not be overlooked. However, the field of AMF research has large gaps in knowledge, and future progress requires revisiting older data on fundamental aspects of their biology and life history. One of the limitations of the current knowledge surrounding these fungi is that the majority of research been focused on a very small number of select taxa, leading to assumptions being made for all AMF, including the biology of their spores, based on this research on a limited number of taxa[8,9]. Several future avenues of research include but are not limited to: (1) the compatibility between plants and AMF depending on their developmental states respectively as well as co-existence with multiple AMF species[9], (2) general investigations in species outside those commonly used[9], and (3) case-by-case studies of AMF response to various environments[8]. Current knowledge on AMF is in no way irrelevant. Rather, it has created a foundation that acknowledges that previous assumptions and other "dogmas," as coined by Albornoz et al. 2020, of these fungi need to be revisited.

Future Research: AMF Spore Dormancy

Unfortunately, not much is known about AMF dormancy - factors influencing spore germination and hyphal growth are better understood than factors affecting dormancy. Dormancy is undoubtedly an important characteristic of AMF spores and requires a deeper understanding. Consider the similar goal of dormancy in both seeds and AMF spores: the prevention of

germination in unfavorable conditions. As such, the symbiotic relationship between AMF and host plants suggests the evolution of similar adaptations, which may allow seed dormancy to be a starting point for new insights on AMF dormancy.

The first step is to define dormancy for AMF spores, particularly the metabolic aspects of AMF dormancy. Similar to how taxonomy and species names have been updated and improved overtime, the terms used to describe AMF spore life cycles, including dormancy, also need review. For example, Finch-Savage and Leubner-Metzger (2006) describe seed dormancy as "an innate seed property that defines the environmental conditions in which the seed is able to germinate," which in turn, may be a starting point for the standard definition of spore dormancy. A widely accepted definition of spore dormancy will create a baseline for experiments and comparisons, though new questions will be raised. For example, defining dormancy will call into question the definition of germination - as Finch-Savage and Leubner-Mertzger (2006) noted, defining dormancy is difficult because dormancy is measured by the absence of germination. In addition, the lines drawn between dormancy, quiescence, and germination must also be carefully dictated, particularly through metabolic changes in the cytoplasm. These events can be seen as a continuum of connected processes that do not have clear boundaries, but definitions clearly defining each could provide a way for standardizing and interpreting experimental results. However, before these distinctions can be made, experimentalists must consider the processes that make up and differentiate dormancy and germination as Bewley (1997) suggests. Furthermore, the definition of dormancy further begs the question of whether there are various classes of dormancy. For example, seed dormancy classes include physiological dormancy, morphological dormancy, morphophysiological dormancy, physical dormancy, and combination dormancy[29,92]. Undoubtedly, there will be biases and experimental results may be influenced by

standard definitions. However, clarifying definitions of dormancy will be vital to improving upon past knowledge.

Genetics also plays an imminent role in learning more about AMF spore dormancy. The genetic work surrounding seed dormancy suggests that there may be numerous important dormancy-specific genes at work within AMF. General mechanisms of seed dormancy have been observed to be highly conserved among plant species[93], implying that perhaps dormancy-specific genes and dormancy mechanisms of AMF spores could also be conserved across AMF species. Beyond genes, cytoplasmic factors, chromatin organization, and post translational modifications are only a few of other genetic aspects that should be considered. As genetic experimental protocols for AMF continue to be improved, there will also be new revelations about genes influencing AMF dormancy.

Furthermore, seed dormancy reveals new factors that should be considered. Mechanical constraints, such as seed coats, are known to influence seed dormancy. Additional factors such as the temporal or spatial window and circadian clock should be explored. Most importantly, factors previously considered to prevent germination may instead promote or break dormancy, which would then require past papers to be revisited. Environmental factors, including but not limited to temperature, nitrate, light, water, oxygen, smoke, and allelochemicals, influence seed dormancy levels and development[93], suggesting that AMF dormancy may be similarly influenced by the same factors.

Several factors regulating seed dormancy have parallels in AMF, suggesting that perhaps seed dormancy and AMF dormancy share similarities. Abscisic acid (ABA) and gibberellin (GA) are known regulators of seed dormancy[93]. For example, decreasing levels of ABA was observed in the seeds of various species when cold stratification was used to break dormancy[94–96]. This

suggests that the cold stratification of AMF also influences unknown factor(s), which may include ABA or GA, within the spore itself. However, there does not seem to be any current research on whether these fungi are able to produce ABA, GA, or some other molecule similar to these plant hormones. In plants, strigolactones have been observed to influence ABA and GA in secondary dormancy control[93]. In AMF, strigolactones induce hyphal growth[46], suggesting that strigolactones may also play a role in AMF dormancy. Bykova et al (2011) demonstrated that the antioxidant defense pathway of wheat influences the maintenance of dormancy. Although the antioxidant defense pathway in AMF is known to combat oxidative damage caused by plants impacted by drought[5,97], it is possible the pathway may also play a role in AMF dormancy.

There is no doubt that AMF biology is heavily understudied. However, the dependency of AMF on plant hosts and the field of seed dormancy provides a starting point for creating a baseline framework for future research on AMF. With more research and understanding of AMF biology, experimental procedures for AMF and fundamental aspects of their biology can be better clarified to improve applications of AMF in human agriculture and for land restoration purposes.

Conclusion

The value of AMF extends from its worldwide ecological significance in forming valuable symbiotic relationships with plant hosts to that of applications in human agriculture. The field of AMF biology cannot be overlooked, but the work that needs to be done cannot be ignored. Great strides have been made in understanding spore germination, dormancy, and AMF life strategies. However, there are also gaps in the knowledge that if filled, would further advance the various applications of AMF.

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Chapter 2: Towards Understanding the Role of Endosymbiotic Bacteria in the Biology of AMF

Introduction

Arbuscular mycorrhizal fungi (AMF; phylum Mucoromycota, subphylum Glomeromycotina) are mutualists colonizing the roots of the vast majority of land plants[1]. These fungi provide their plant hosts with mineral nutrients derived from the soil and receive photosynthetic products in exchange. In addition to forming symbioses with plants, many AMF themselves harbor endosymbiotic bacteria (EB). One of these endobacteria is '*Candidatus* Moeniiplasma glomeromycotorum' (*Ca*Mg). Despite metabolic dependance of *Ca*Mg on its host[2], the role of *Ca*Mg in AMF biology is unknown. Given that AMF are obligate biotrophs, elucidating their relationships with *Ca*Mg is of great biological significance because of the role of AMF in ecosystem functioning and potential practical applications in agriculture and land restoration. One of the ways of assessing the impact of *Ca*Mg on AMF fitness is exploring its effects on AMF spore germination. However, achieving spore germination is challenging.

*Ca*Mg possesses a reduced genome[2]. The reduced genome is capable of primarily basic cell maintenance and has unknown mechanisms of energy production, indicating that *Ca*Mg rely on their fungal hosts for metabolic purposes[2]. Genes encoding the proteins that interact with their eukaryotic hosts and other orphan genes without known function have also been identified[2]. *Ca*Mg have been demonstrated to exist in the cytoplasm of AMF spores, and have not been detected as free-living bacteria[3]. As such, EB are obligate symbionts whereas the fungi are facultative symbionts that can survive without EB. *Ca*Mg are evolutionarily related to bacteria

distinguishable by the lack of a cell wall, known as *Mollicutes.*[3] Despite having shared a common ancestor and more currently sharing exploitative traits, cells of *Ca*Mg are enveloped by a cell wall-like structure, setting them apart from other *Mollicutes*[3]. The function of these EB as well as the mode of transfer between AMF strains in nature are unknown.

Despite the gaps in knowledge, AMF and their spores have great potential in agricultural applications and land restoration. There is a growing interest in using these fungi as a natural fertilizer in sustainable agriculture to replace synthetic fertilizers. AMF provide their plant hosts with mineral nutrients derived from the soil, but by using spores to establish fungal-plant symbioses, a host of benefits including but not limited to increased plant pathogen protection[4], plant tolerance to drought[5] and heavy metal contaminants[6], and protection of plants against salinity[7] can also be leveraged. Fungi-plant symbiosis can improve soil aggregation[8], a benefit that is influenced by the interactions between earthworm species and AMF[9]. By better understanding AMF biology and more specifically, how endobacteria impacts AMF fitness, it can be better understood how to optimize AMF for practical applications.

Preliminary data (Yogi, Pawlowska, et al., unpublished) indicated that the presence of *Ca*Mg increases germination success and germ tube branching in *Rhizophagus clarus*, suggesting that *Ca*Mg acted more as a mutualist than an antagonist to AMF. Further research is therefore required to investigate and confirm the impact of *Ca*Mg presence on AMF spore germination. The present study originally aimed to expand on this preliminary work to further investigate whether *Ca*Mg presence improved spore germination and germ tube branching. However, experiments to examine the influence of *Ca*Mg on *R. clarus* fitness by assessing germ tube growth and spore germination rate were cut short due to the pandemic and unexpected difficulty with cultures and novel methods, so this study shifted to investigate AMF protocols and the

spore genetically. To better understand the relationship between *Ca*Mg and AMF, I aimed to identify accessions of the AMF fungi species *R. clarus* host *Ca*Mg endobacteria within them. AMF spores were unable to form symbiotic relationships with host plants due to issues with unhealthy root cultures. AMF spores also had high mortality prior to germination due to issues with a decontamination protocol. In this chapter, I empirically demonstrate that the endobacteria '*Candidatus* Moeniiplasma glomeromycotorum' can be lost or gained during culturing practices and that the spore surface decontamination protocol was detrimental to *Rhizophagus clarus* spore survival.

Materials and Methods:

Identifying Endobacteria in AMF Accessions

Isolate selection and spore extraction: All AMF samples were received from the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) at West Virginia University. INVAM cultures vesicular-arbuscular mycorrhizal fungi and makes them available to researchers. To detect the presence of *Ca*Mg in AMF hosts, I surveyed pairs of INVAM accessions sourced from different geographical origins (Table 1). Surveying pairs of accessions from nearby locations distributed around the globe would allow me to identify fungi harboring *Ca*Mg (EB+) and fungi free of *Ca*Mg (EB-) for my future experiments. All spores of *Rhizophagus clarus* were extracted from INVAM potting medium in which the spores of AMF were stored in at low temperature. I extracted spores using the sieving methods of Lastovetsky et al. 2018 followed by sucrose density separation.

Hydrogen peroxide spore sterilization protocol for *in vitro* experiments: In order to remove surface contaminating bacteria, I surface-sterilized spores using the methods of Mondo et al. 2012. In brief, spores were rinsed in 0.05% Tween 20, soaked in successive baths of hydrogen peroxide at 1 mM, then at 50 mM, then 4% chloramine T, and finally rinsed with sterile nanopure water.

PCR and amplicon sequencing: I crushed surface-sterilized spores in microtubes under a dissecting scope using a pipette tip. Crushed AMF spores were subjected to whole genome amplification using the GenomiPhi V2 DNA Amplification Kit (Cytiva, Marlborough, MA) following manufacturer's instructions with an extended incubation time of 5 hours.

To confirm fungal identity of spores, 20× diluted Genomiphi products[10] were 28S DNA genotyped using LR0R and LR primers for amplification and LR0R and LR3 primers for sequencing[11,12]. Fungal amplification used the following PCR conditions: 94°C for 95 s; 35 cycles of 94°C for 35 s, 49°C for 1 m, 72°C for 3 m; 72°C for 15 m; and 12°C on hold.

*Ca*Mg were identified by amplification and sequencing of their 16S DNA gene. To detect the presence of *Ca*Mg in AMF accessions, 20× diluted Genomiphi products[10] were 16S DNA genotyped using *Ca*Mg specific primers 109F1+2 and 1184R1+2+3[2] under the following PCR conditions: 94°C for 2 m; 10 touchdown cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 1 m; 25 touchdown cycles of 94°C for 30 s, 49°C for 30 s, 72°C for 1 m; 72°C for 7 m; and 12°C on hold. Samples that yielded bacterial PCR product were sequenced to confirm bacterial identity. Sanger sequencing was performed at Cornell's Biotechnology Resource Center. Although absence or presence of *Ca*Mg was typically determined by the absence or presence of amplified PCR product using species-specific primers, representative samples were sequenced.

Electropherograms were examined in Geneious v.2021.0.1 (Biomatters Ltd., Auckland, New Zealand) and then forward and reverse reads were assembled into consensus contigs, which were then used as a query for NCBI BLASTn.

Identifying the Influence of Endobacteria on AMF Fitness

Spore surface decontamination protocol for *Ca*Mg survey: I surface decontaminated spores using the methods of Bécard and Fortin 1988[13] and Chabot et al. 1992[14]. Spores were sterilized within 24 hours of extraction from INVAM samples, and all steps were performed in a laminar flow hood under sterile conditions. Spores were sterilized by successive soaking in 0.05% Tween 20 solution, 2% chloramine T, 2% streptomycin sulfate and, 1% gentamycin sulfate[14]. The rotations were repeated twice over the course of two days. Spores were originally sonicated before these sterilization steps to clean debris from their surfaces in accordance with the original protocol. However, this sonication step was later omitted after encountering a negative effect on viability, as detailed below.

Single spore transfer: To generate isogenic lines of *Ca*Mg(+) and *Ca*Mg(-) of *R. clarus* needed for spore germination experiments, I used serial single spore transfers to cure accessions known to host endobacteria. Cultures were grown on *Agrobacterium*-transformed chicory roots following the protocol of Bécard and Fortin (1988)[13] and Chabot et al. (1992)[14] except on MSR media[15] rather than M media. For root inoculation, *R. clarus* spores were extracted from INVAM samples, surface sterilized, and placed individually or in small clusters or five to ten spores proximal to a growing root tip, then root cultures were incubated in the dark at 26° Celsius. Newly produced spores were serially transferred to new single-spore cultures as above until cured of endobacteria.

Viability staining: To diagnose the cause behind the lack of expected spore germination, I conducted spore viability assays using the yellow tetrazolium salt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) stain following the protocol as listed in Walley and Germida (1995)[16]. The percentage of spores that stained purple after 24h incubation in MTT at 37° C, indicating viability, were counted under a dissecting microscope. Autoclaved spores were used as a negative control.

Statistical testing: I performed Shapiro Wilke tests to test if data sets were normally distributed. T-tests were then performed to test for significance between two conditions. To test for significance between three conditions, ANOVA was performed and followed by a TukeyHSD post-hoc analysis.

Results

The first objective was to identify which accessions of *Rhizophagus clarus* host '*Candidatus* Moeniiplasma glomeromycotorum' (*Ca*Mg) endobacteria. The accessions (experimental populations) NB112A, AU402B, WV112, WV238, and WV236 of *Rhizophagus clarus* were surveyed by PCR using *Ca*Mg-specific primers for incidence of *Ca*Mg endobacteria and compared to *Ca*Mg incidences of the same accessions reported by Naito et al. 2017[17] (Table 1). Unexpectedly, AU402B was reported to host endobacteria in 2017 but tested negative in the present study. Heterogeneity of EB was also apparent in spores WV236, which were found to both harbor *Ca*Mg and be *Ca*Mg free despite being from the same accessions.

After assessing EB incidence in various accessions, the influence of *Ca*Mg on the fitness of *R. clarus* was examined by examining germ tube growth and spore germination rate. Based on

preliminary data (Yogi, unpublished), the presence of endobacteria was hypothesized to improve spore germination rate and hyphal elongation.

However, this objective could not be fully completed due to problems with spore germination and the COVID 19-related interruption of lab activities. Spores inoculated onto plates with roots failed to germinate after 3 – 5 days despite prior success in the referenced extraction, sterilization, and culturing methods. Although the difference in spore viability between freshly extracted spores and fully-sterilized spores was non-significant ($p= 0.07365$), there is a trend towards the loss of spore viability due to surface sterilization (Figure 1). MTT viability staining of spores at multiple stages of the decontamination protocol revealed that the majority of spores were dead after spore surface decontamination and prior to inoculation, suggesting that spore surface decontamination protocol affected spore viability (Figure 1).

To identify which component of the spore surface decontamination protocol affected spore viability, MTT staining was performed under different modified sterilization conditions (Figure 2). “Fresh extraction” refers to spores that were extracted from INVAM potting medium and did not undergo further spore surface sterilization. “Post-sonication” refers to extracted spores that had undergone sonication to clean debris from their surface but no further steps in the spore surface decontamination protocol, including chemical baths. “Fully sterilized” refers to extracted spores that have completed the full spore surface decontamination protocol, including sonication and chemical baths in Tween 20 solution, chloramine T, and antibiotics. The MTT stain revealed that among spores that were freshly extracted, sonicated, and fully sterilized, there was a significant difference in spore viability between post-sonicated and fully sterilized spores. It appears that chemical washes between sonication and the completion of the decontamination protocol were the most detrimental to spore viability (post-sonication – fresh extraction

p=0.8386320; fully sterilized – fresh extraction p=0.208658; fully sterilized – post-sonication p=0.0110948; Figure 2), although sonication may play a role in spore viability as later discussed.

Although sonication was not found to be the detrimental step to spore viability, I speculated that the spores were being weakened by sonication and therefore, became more susceptible to later chemical and antibiotic soaks after sonication. The sonication step was removed, and gentle rotation on a LabQuake was extended to last the entirety of the sterilization protocol before another MTT staining was performed (Figure 3). The difference in the viability of freshly extracted spores and fully sterilized spores was not found to be significant, suggesting again that the chemical baths were detrimental to spore viability (p= 0.4537; Figure 3).

Examination of factors affecting spore germination through MTT viability staining revealed that spore viability was most affected during the sonication step in the spore surface sterilization condition. However, why the decontamination protocol may have been detrimental remains unclear.

Discussion

The significance of this project lies in that it captures the difficulty of achieving germination of AMF spores for experimental purposes and elucidating the knowledge gaps surrounding consistent and broadly applicable methods for working with AMF spores. The cumulative empirical data collection suggests future directions in research of AMF, such as reviewing the impact of routine procedures on different AMF species.

*Ca*Mg Adaptations Explaining Variable Endobacteria Populations and Occurrence

The accessions NB112A, AU402B, WV112, WV238, and WV236 of *Rhizophagus clarus* by PCR were surveyed by PCR using *Ca*Mg-specific primers for incidence of *Ca*Mg endobacteria and compared to *Ca*Mg incidences of the same accessions reported by Naito et al. 2017[17] (Table 1). AU402B was reported to host endobacteria in 2017 but tested negative in the present study. Variability of EB populations were also apparent in spores WV236, which were found to both harbor *Ca*Mg or be *Ca*Mg free despite being from the same accessions. I demonstrate variable endobacteria populations and occurrence in *Rhizophagus clarus* in this study.

The differences of *Ca*Mg presence between accessions tested in this study and those tested by Naito et al. 2017[17] as well as within the same accession tested in this study suggest variable occurrence of endobacteria within AMF populations (WV236) and that endobacteria can be lost within an accession during storage (AU402B). However, how endobacteria are lost or gained or transferred to progeny is unknown. The heterogeneity observed in *Rhizophagus clarus* could perhaps be an adaption of the endobacteria to its fungal host to balance pressures on its host and surviving environmental changes where endobacteria may be lost from its host. Specifically, *R. clarus* that host *Ca*Mg may lose their endobacteria due to environmental changes and *Ca*Mg may be able to recolonize their hosts through horizontal transmission under specific conditions[18]. *Ca*Mg may be lost or gained through serial transfer at INVAM due to bottlenecks (i.e. small sample sizes of spores between generations).

Mondo et al. 2012 tested *Ca.* Glomeribacter gigasporarum (*Ca*Gg), another nonessential endosymbiont of AMF like *Ca*Mg, and provided insight into potential host-endobacterial interactions. Like Yogi et al. 2019's preliminary observations of how *Ca*Mg improves *R. clarus*

germination and germ tube branching, *Ca*Gg improves fungal hyphal growth after spore germination[19], although they do so by modifying the metabolism of fungal spore lipid stores[20]. The genome sequence of *Ca*Gg demonstrates energy dependence on their fungal hosts[21], as does *Ca*Mg on their fungal hosts. Mondo et al. 2012 note that vertical transmission, recombination in endosymbiont populations, and host switching allow the maintenance of AMF and endobacterial interaction. Mondo et al. 2012 also concluded that despite a recombination frequency that may be sufficient to regulate deleterious mutation accumulation in the genome[22] and host switching, *Ca*Gg populations were likely largely influenced by transmission bottlenecks. Considering the similarities between *Ca*Mg and *Ca*Gg, *Ca*Mg may share many of the features of *Ca*Gg or have similar adaptations in how *Ca*Mg interacts with fungal hosts such as *R. clarus*.

In terms of genomes, *Ca*Mg (formerly referred to as mycoplasma-related endobacteria, MRE) populations tested by Naito et al. 2017[17] reveal further insight into the nature of the facultative relationship between endobacteria and fungal hosts as well as what genes are facilitating endobacterial-fungal host interactions. MRE populations tested by Naito et al. 2017[17] were found to have extensive chromosomal rearrangements and highly reduced gene content that indicate metabolic dependence on the host. Some genes of note are small ubiquitin-like modifier proteases, which were speculated to be a likely mechanism of how MRE manipulate their host and a potential mechanism for how *Ca*Mg might influence fungal hosts[2]. Contrary to the conclusion of Yogi et al. 2019's preliminary data that *Ca*Mg acts mutualistically rather than antagonistically to its AMF host, MRE have been suggested to be antagonistic by imposing a fitness cost on their fungal hosts[23]. Desirò et al. 2018 demonstrated that MRE negatively impacts biomass production of their host, Mortierellomycotina, through a non-lethal parasitic lifestyle. Desirò et al. 2018 also conclude that extent of MRE impact depends on temperature and media

type, suggesting further unknowns about endobacteria biology. If *Ca*Mg is similarly antagonistic, it would likely have adaptations to balance the pressure it exerts on its fungal hosts and its ability to infect a fungal host that has lost endobacteria.

I have demonstrated variable occurrence of *Ca*Mg in AMF populations and I speculate this variability in EB presence reflects the EB selective pressures on its host and surviving environmental changes where EB may be lost from its host. Altogether, further research is required to clarify *Ca*Mg biology and how it interacts with fungal hosts. In order to better understand the role of endobacteria in AMF biology, it is also necessary to develop experimental methods to work with AMF spores without affecting their viability.

Impact of Spore Surface Decontamination Protocol on AMF Spores

Sonication does not appear to have a direct detrimental effect on *R. clarus* spore viability, whereas the chemical baths following sonication appeared to be detrimental to *R. clarus* spore viability. I speculate that the settings for the sonication step were too harsh and disrupted the cell wall of the particular species used in the experiments, *R. clarus*, which made its spores more susceptible to the chemical treatments.

Considering that fungal spores can survive sonication[28], and *R. clarus* spores were still viable after sonication but before chemical treatment in this study, it seems unlikely that the physical damage from sonication directly killed the experimental spores. However, sonication can break up fungal spores[29], suggesting that whether spores survive sonication or not is dependent on many factors, including but not limited to sonication speed and time.

Generally, sonication is a procedure that sends sonic waves through a sample to break cell walls and disrupt cell membranes to then release cell contents, such as fungal genomic

DNA[30]. Sonication has a wide range of applications for spores, including that of AMF, to achieve spore inactivation[31], sterilized spores for inoculation[13,14], improvement of attachment of sonicated spores to their hosts[28], and more. There are many examples in which sonication is successfully used to induce spore attachment. However, it remains unclear how sonication impacts AMF spore biology. It appears that sonication may not be killing spores directly but may damage their spore wall so that spores are more susceptible to being killed by sterilization chemicals.

The spore decontamination protocol using the methods of Bécard and Fortin 1988[13] and Chabot et al. 1992[14] were designed for *Glomus intraradix* and *Gigaspora margarita*. Considering that that the chemicals used for surface sterilization in this study (Tween 20 solution, chloramine T, streptomycin sulfate, and gentamycin sulfate) are routinely used in AMF spore surface decontamination[24–27], it was not expected that the chemical wash would be so detrimental to the viability of *R. clarus* spores. However, not all AMF species are studied equally and cannot be assumed that one protocol that worked for one species would work equally well for another.

G. intraradix and *G. margarita* may have different morphological properties from that of *R. clarus*. For example, AMF spores vary greatly in the number of spore wall layers and composition[32], and thick, pigmented spore walls have been suggested to shield spores from chemical and physical treatments[33]. Pigmentation in fungal cell walls allow fungi to survive harsh environmental conditions[34], but *R. clarus* spore walls are thin enough that they are translucent under the microscope and the lipids are visible. This suggests that *R. clarus* may have weaker spore walls that are more susceptible to sonication and needs a gentler process, such as the gentle rotations on the LabQuake. Other avenues of decontaminating spores without altering their viability, such as ultra violet radiation and oxidizing agents, can also be considered[33]. This

further suggests that the production of AMF aseptic spores and their viability after chemical and physical stress, including that of sterilization chemicals and sonication, differ from species to species. Rather than entirely removing the sonication step, the settings used and the model of the sonicator may need review to better understand how sonication is impacting AMF spores specifically and if they are indirectly affecting spore viability by damaging spore walls and making spores more susceptible to sterilization chemicals. Further research is needed to clarify how chemical solutions affect spore viability if they enter the spore.

General Conclusion

AMF biology is a rapidly growing field with broad applications in agriculture and land restoration. Spores are a critical stage in AMF life cycle and both general spore biology, such as dormancy, as well as specifics, such as endobacteria residing in AMF spores, require further research. In this honors thesis, the significance of spores in the AMF life cycle was summarized. It was demonstrated that the endobacterium '*Candidatus* Moeniiplasma glomeromycotorum' can be lost or gained during culturing practices and that the spore surface decontamination protocol was detrimental to *Rhizophagus clarus* spore viability.

The biology of AMF is vastly understudied. The observations from a handful of AMF species are frequently extended to all AMF both in literature and experiments. Specifically, the mechanisms of how endobacteria is lost or gained as well as how sonication affects different AMF species remain unclear. To fill these and many other gaps in AMF knowledge as well as further advance the applications of AMF, past research done on AMF must be revisited to (1) redefine terms and standardize experiments, (2) reinforce genetic work in AMF, and (3) use knowledge from plant and seed experiments to further inform AMF research.

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Tables and Figures

Table 1. *Ca*Mg presence/absence in accessions NB112A, AU402B, WV112, WV238, and WV236 of *Rhizophagus clarus*. This data is compared against accessions tested for endobacteria by Naito et al. 2017. Bolded text represent differences in *Ca*Mg presence within accessions between 2017 and 2021 and within accessions tested in 2021.

Accession	Geographic Origin	Spore ID (2021, this study)	*Ca*Mg Presence or Absence[1]	Spore ID (Naito et al. 2017)	*Ca*Mg Presence or Absence[1]
NB112A	Namibia	NB112A E-40	present	NB112A A-1	present
		NB112A E-42	present	NB112A B-1	present
				NB112A C-1	present
AU402B	Australia	**AU402B E-65**	**absent**	**AU402B 1-7**	**present**
		AU402B E-67	**absent**	**AU402B 2-15**	**present**
		AU402B E-68	absent		
		AU402B E-69	absent		
WV112	West Virginia	WV112 E-32	absent		
		WV112 E-88	absent		
		WV112 E-89	absent		
WV238	West Virginia	WV238 E-74	absent		
		WV238 E-76	absent		
		WV238 E-77	absent		
		WV238 E-92	absent		
		WV238 E-93	absent		
		WV238 E-95	absent		
		WV238 E-96	absent		
WV236	West Virginia	**WV236 E-58**	**present**		
		WV236 E-59	**present**		
		WV236 E-60	**present**		
		WV236 E-61	**present**		
		WV236 E-62	**present**		
		WV236 E-79	**absent**		
		WV236 E-82	**absent**		
		WV236 E-83	**absent**		

[1] Not all spores were sequenced and therefore, quantitative data reporting percent 16S DNA similarity to *Ca*Mg is not available. *Ca*Mg presence and absence was inferred by the presence of a band on the gel[2].

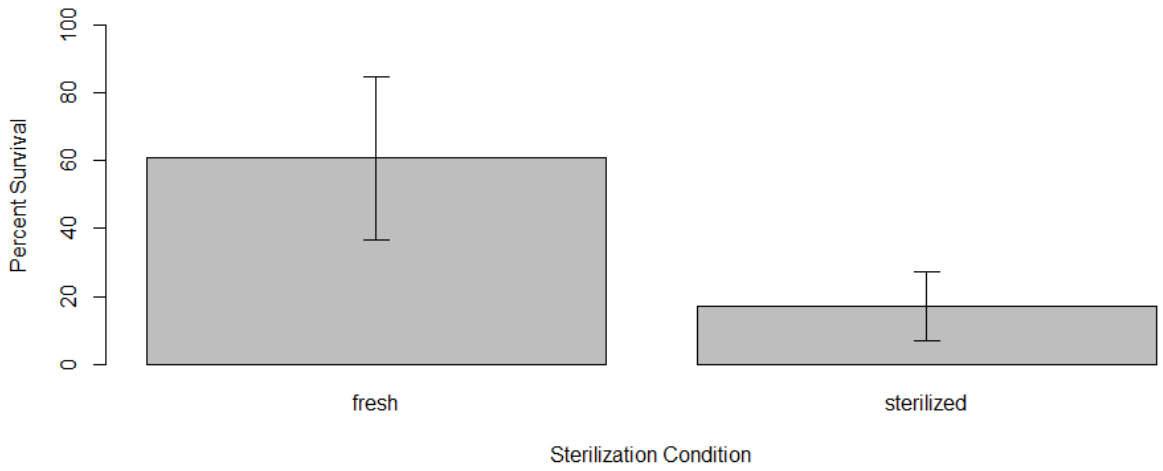

Figure 1. Comparison of spore viability, as assessed by MTT staining, between freshly extracted versus surface-sterilized spores. (Fresh, spores freshly extracted from soil, no sterilization; sterilized, full gentle sterilization protocol performed). A Shapiro Wilke test indicated ($p=0.1082$) that data sets are normally distributed. A t-test indicated that while the difference is non-significant ($p= 0.07365$), there is a trend towards the loss of spore viability due to surface sterilization. Error bars represent one standard deviation (SD) of the mean. The fresh extraction condition consisted of 2 replicates of between 9 and 14 individual spores each. The fully sterilized condition consisted of 2 replicates of between 7 and 11 individual spores each.

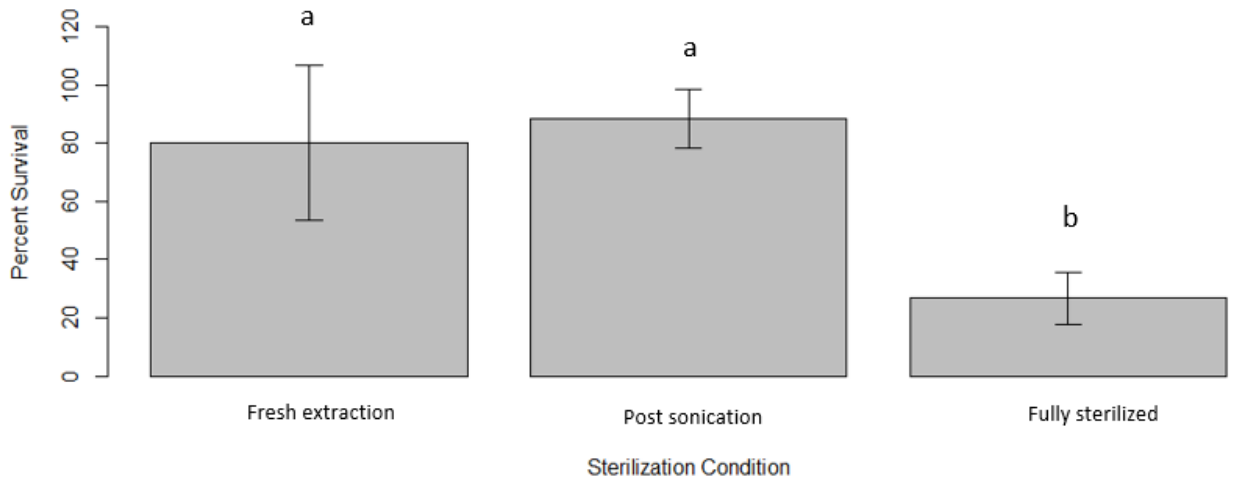

Figure 2. Comparison of spore viability among freshly extracted, sonicated and fully surface-sterilized spores. (Fresh, spores freshly extracted from soil, no sterilization; post sonication, sterilization stopped after sonication; sterilized, full gentle sterilization protocol performed). An ANOVA was performed on MTT stain data from 11-8-2019 at different sterilization (p-value>0.00919), indicating that there was some significant difference between the data sets. A post-hoc analysis (TukeyHSD) was performed (post-sonication – fresh extraction p=0.8386320; fully sterilized – fresh extraction p=0.208658; fully sterilized – post-sonication p=0.0110948). a and b indicate significant difference between the two sterilization conditions. Error bars indicate one SD of the mean. The fresh extraction condition consisted of 3 replicates with between 4 and 9 individual spores each. The post sonication condition consisted of 3 replicates with between 6 and 15 individual spores each. The fully sterilized condition consisted of 3 replicates with between 6 and 23 individual spores each.

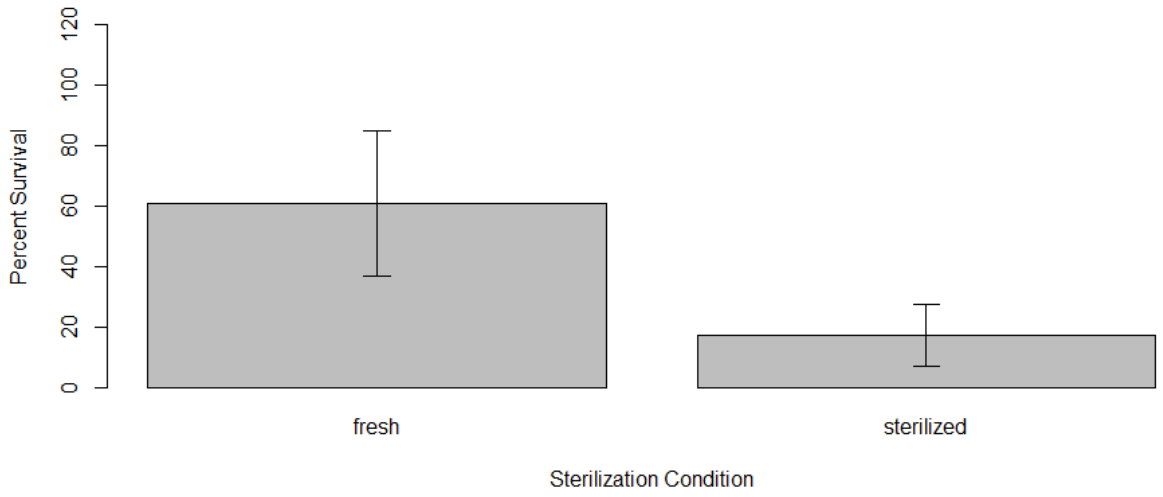

Figure 3. Comparison of viability between spores that were freshly extracted vs surface-sterilized with gentle agitation but no sonication. (Fresh, spores freshly extracted from soil, no sterilization; sterilized, full gentle sterilization protocol performed). A Shapiro Wilke test was performed on MTT stain data from 12-9-2019 ($p=0.2742$), indicating that data sets are normally distributed. A t-test indicated that the difference is non-significant ($p= 0.4537$). Error bars represent one SD of the mean. The fresh extraction condition consisted of 2 replicates with between 9 and 14 individual spores each. The fully sterilized condition consisted of 2 replicates with between 7 and 11 individual spores each.