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An extensive exploration of generalist microbotryum species through host interactions.

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An Extensive Exploration of generalist *Microbotryum* Species Through Host Interactions

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Abstract

The *Microbotryum* species complex is a group of fungi, commonly known as anther smuts. Anther smuts are plant parasites that must invade a host plant in order to fulfill their life cycle. As the *Microbotryum* fungus grows, it sterilizes the host plant, such that infected plants produce fungal teliospores instead of pollen on the male reproductive organs and female reproductive structures do not mature. *Microbotryum* usually exists as a specialist species, however, there is evidence that several species of anther smuts can exist as generalist species. Comparisons of the infection process and the resulting response of both parasite and host can help elucidate the behavior of generalist species of *Microbotryum* in comparison with their specialist counterparts. This was pursued through seed germination and fungal spore germination studies. The *Microbotryum* life cycle was also investigated through microscopy and RNA isolation for host plant gene expression analyses, to provide insights into the role of host response to infection to this interaction.

Lay Summary

This study explores how two types of fungi, *Microbotryum superbum* and *Microbotryum lagerheimii*, are able to infect the reproductive structures of plants. The *Microbotryum* life cycle is composed of pathogenic and non-pathogenic phases, but host plant infection is essential for the completion of the life cycle. Most species of *Microbotryum* fungi can only infect one plant species, but *M. superbum* and *M. lagerheimii* exhibit characteristics suggesting that they can infect more than one species of plant, thus displaying generalist behaviors. This study examined the maturation process of *M. superbum* and *M. lagerheimii* in different stages of their life cycles through RNA sequencing and microscopy. By comparing generalist and specialist species, this study helps explain how generalist and specialist fungi differ in how they affect their host plants and how successful they are in their life cycles.

Introduction

Pathogens are currently a massive topic of interest. As technology and genetic sequencing methods have evolved, so has the ability to understand the ways in which pathogenicity works. One such pathogen or parasite, the *Microbotryum* genus, is found in the Basidiomycota division of fungi; the spores from such fungi develop in specific reproductive structures known as basidia. The *Microbotryum violaceum* species complex consists of many species, all of which are commonly known anther smuts. These are obligate parasites meaning that to accomplish their entire life cycle, they must invade a host plant (Abbate et al, 2018). Since the fungus does not kill the host plant but may reduce its overall fitness, and, in fact, requires that the plant remain alive, anther smuts are referred to as parasites. They most commonly infect the Caryophyllaceae species which are widely known as part of the carnation family (Bruns et al., 2017). *Microbotryum* can invade the plant host through multiple means such as hitching rides on pollinators and through wind dispersal. As the fungus continues to grow, it sterilizes the plant in an organ-specific process (Van der Linde et al., 2021). This sterilization consists of the *Microbotryum* co-opting the plant's reproductive system such that infected flowers contain the reproductive form of the fungus, i.e., thicker-walled spores known as fungal teliospores, instead of pollen on the male reproductive organs; female reproductive structures do not mature. There is an exception in dioecious plants which only produce male or female reproductive structures, not both. In dioecious plants, upon fungal infection, the female reproductive organs abort and, instead, have spore-bearing pseudo anthers (Abbate et al, 2018). The teliospores produced during the plant sterilization process are vital to the life cycle of *Microbotryum* because teliospores are the primary mode of dispersal.

The species of investigation for this study, *Microbotryum superbum* and *Microbotryum lagerheimii*, exhibit a dimorphic life cycle, meaning that the fungus can be found in yeast-like and filamentous forms (Schaefer et al., 2010). As seen in Figure 1, in the yeast-like phase, the fungus is haploid- it only has one copy of each chromosome- and is saprophytic- it obtains sustenance through the decaying of organic matter, or, for example, from nectar in flowers. However, the spore phase of *Microbotryum*, in infected flowers, can effectively spread to other potential hosts via pollinators, as mentioned above.

Upon landing on a suitable flower or other viable surface, the spores will germinate and undergo meiosis. The products of meiosis, haploid yeast-like cells, are capable of mating with each other if they are of different mating-types. There are two mating-type classifications: a1 and a2. If the haploid cells are of different mating-types, they will form a bridge between them, fuse, and then differentiate into a filamentous form that is infectious to host plants. This process constitutes fungal sexual reproduction. The filamentation produces a dikaryotic fungus meaning that the fungal cell contains two separate nuclei, one from each partner. After this phase, *Microbotryum* can successfully invade its host.

Recently, this process has been carefully studied as it has been discovered that some *Microbotryum violaceum* species exhibit a mating-type bias. This bias is caused by a deleterious recessive allele that is inherited with the a2 mating-type. Thus, the majority of *Microbotryum violaceum* that present this bias are of the a1 mating-type due to the lethal nature of the allele inherited with a2 (Hood and Antonovics, 2000). As a result, the mating system favors intratetrad mating: the mechanism in which mating occurs between haploid sibling cells within the same tetrad- a cluster of four spores produced in meiosis. If intratetrad mating occurs frequently, these recessive mutations may continue to exist, especially in regions of the genome that do not

recombine. When mating happens within the same tetrad, harmful recessive mutations near centromeres can accumulate without being exposed, as they stay masked by heterozygosity. This situation resembles the permanent heterozygosity seen in other genomic regions where recombination is suppressed, allowing the mutations to persist across generations. If mating occurs between different tetrads, half of the centromeres could become homozygous, revealing these mutations and reducing fitness. Studies show that mating between tetrads is rare, supporting the idea that *Microbotryum violaceum* populations maintain genetic similarity through intratetrad mating (Hood and Antonovics, 2000). This process keeps centromeres heterozygous while pushing other parts of the genome toward homozygosity, leading to genetically uniform populations.

After successful sexual reproduction and during the winter, the *Microbotryum* parasite remains in the plant's roots where it grows and establishes an adequate population which will optimize the hijacking of the plant in the warmer months (Schaefer et al., 2010). As time passes, the fungus is drawn to mesophyll in the plant. This is due to the role the mesophyll tissue plays in plant function; most plant photosynthesis occurs in this tissue. This action reveals the opportunistic parasitic nature of *Microbotryum* as the mesophyll cells in plants contain many chloroplasts which are sources of large amounts of energy. Eventually, the fungus will take over the plant’s reproductive organs as previously mentioned and the process repeats.

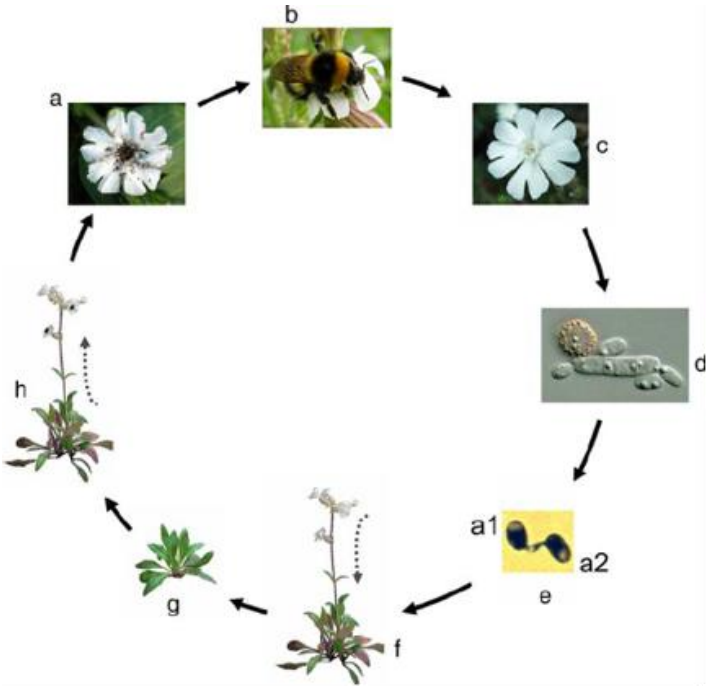

Figure 1: This graphic depicts the life cycle of a similar subspecies of *Microbotryum*, *Microbotryum lychnidis-dioicae*. In (a), diploid teliospores are generated in the anthers or pseudoanthers of infected plants; the teliospores are dispersed to potential plant hosts (b). The teliospores undergo meiosis when they reach a compatible plant host (c) and (d). Haploid cells are produced and different mating types form bridges for sexual reproduction (e). The fungus enters a filamentous phase and begins to invade the plant (f). The plant continues to experience the consequences of infection and eventually the cycle restarts. Pictures are sourced from (Lopez-Villavicencio et al. 2007)

The disease caused by anther-smut fungi presents a model system for studying sexually transmitted and vector transmitted diseases. Sexually transmitted diseases are predominantly transferred through sexual contact while vector transmitted diseases are transmitted by one organism to another wherein the organism transferring the disease is unaffected. This is presented in the takeover of the sexual reproductive organs of the plant host by the teliospores and the transmission of the teliospores by pollinators (Bruns et al., 2017). Other applications in studying the interactions between *Microbotryum* and host plants in the Caryophyllaceae family

are the characteristics of the *Microbotryum*'s host range. By observing and establishing what drives the changing evolution of host range in pathogens, the potential for host shifts and an overall understanding of disease dynamics can be enlightened (Bruns et al., 2021). Moreover, it is this aspect of host "shifts" that makes the respective *Microbotryum* host systems excellent models for predicting emerging infectious diseases across all organismal domains.

Host range is a defining trait in parasites/pathogens and can be loosely described as the number of specific host species that a given pathogen can successfully infect. Parasites such as *Microbotryum* can also have a *realized* host range which is the host species that it has been observed to infect in nature. Conversely, the *potential* host range is the number of different hosts the pathogen can infect under simulated conditions (Bruns et al., 2021). Another factor that prompts further investigation is the evolutionary benefits of being a specialist pathogen or a generalist pathogen. Specialist pathogens can only successfully infect one host species while a generalist pathogen can infect multiple hosts. It has been hypothesized that an evolutionary trade-off mechanism exists, where specialist species tend to have a higher abundance in hosts shared with generalists than the generalists themselves. This was observed in aphid parasites after data were published from multiple field surveys, thus promoting the idea that although generalists can infect a larger range of species, specialists are more abundant in their host (Straub, 2011). Despite some evidence supporting this hypothesis, no conclusion about the subject has been determined as conflicting results have also been observed.

The specificity mechanisms in *Microbotryum* prove to be a precarious topic. The paradigm for many years has been that each *Microbotryum* species is limited to one specific plant host species. However, this view has arisen from extensive work with *Microbotryum* species primarily that infect hosts of the flowering plants of the Caryophyllaceae (Pinks or

carnations) family. However, some *Microbotryum* species are so diverse in terms of having both specialist and generalist strains that they have been divided into subspecies, based on this characteristic. It is believed that host divergence, ecological similarities, and other morphological similarities have impacts on the degree of specialization observed in certain strains of *Microbotryum* (Kemler et al., 2020).

Anther smuts were first recorded in the 17th century and have since been used to investigate many genetic processes including evolutionary disease resistance, modes of transmission, host shifts, and pathogen evolution (Antonovics and Alexander, 2024). One species under study, *Microbotryum superbum*, has been more frequently studied in the past fifteen years. *M. superbum* primarily infects the *Dianthus* species of the Caryophyllaceae family. Although *M. superbum* primarily infects this host, it is contrary to what is normally seen in *Microbotryum* species. While as mentioned earlier, most species of *Microbotryum* only infect one species of host, *M. superbum*, however, has demonstrated an ability to infect multiple types of *Dianthus* species (Lucotte et. al, 2024). *Microbotryum lagerheimii* was discovered roughly 15 years ago and was distinctly separated into its own subspecies due to its lower color intensity on host species *Lychnis alpina, Lychnis viscaria,* and *Silene vulgaris*. In this study, this new species was also proposed because the other spores observed in infected plants were known to be from *Microbotryum silenes-inflatae,* which only infected *Silene vulgaris* and had a high color intensity (Denchev, 2007). This suggests that *M. lagerheimii* could be a generalist, unlike the paradigm of specialists (Perlin et al., 2015); thus, *M. lagerheimii* may be even more intriguing as a subject for investigation (Kemler et al., 2020).

Some possible characteristics that could have a bearing on the degree of specialization seen in *Microbotryum* are genetic relationship, similar floral character (in terms of infection),

shared pollinator guilds (the types of pollinators that are attracted to the plant), and similar flowering times (Tang et al., 2019). Flowering time may seem like a trivial feature of the infection process, but it is indeed an indicator of pathogen presence. Infected plants flower up to one month earlier than uninfected plants (Tang et al., 2019).

M. lagerheimii and *M. superbum* appear to be generalist species that are capable of infecting multiple host plant species. This ability may manifest itself in characteristics of the infected plants as they develop, along with the parasitic fungus; furthermore, there would be observable differences in the generalist *M. lagerheimii/ M. superbum* infections compared to specialist *Microbotryum* species infection such as the severity of anther takeover and range of viable host plants. It was the goal of this thesis research to investigate these differences.

Materials and Methods

Seed Germination Studies

Before seed germination, the seeds were sterilized in a solution composed of 40% bleach, 0.1% Triton-X, and 59.99% sterile water. After the seeds were submerged in this solution for 10 minutes, the solution was pipetted off. The seeds were then rinsed with sterile water five times consecutively for 1 minute each time. Next, the seeds were placed under a fume hood to dry for ten minutes before plating onto 0.8% water agar plates. The seeds to be infected and the non-infected seeds were placed on different plates and parafilm was placed over them. The plates were then covered with aluminum foil and placed in a 4°C refrigerator for 48 hours.

After 48 hours, the aluminum foil was removed from the plates and the seeds were placed in an environmental growth chamber at 16-20 ◦C. After 7-14 days (each plant species had a different amount of time before appropriate germination was reached) the seeds were planted. After planting, the plants were watered approximately every other day with careful consideration not to overwater them. *D. segueiri* and *S. vulgaris* seeds were surface sterilized as described above. In seed-infection experiments, this process was repeated, but after 48 hours, the seeds were infected with 20 microliters each of the *Microbotryum* solution described below.

Infection of *Dianthus seguieri/ Silene vulgaris*

For seed infection, two strains of *Microbotryum* (mating-types indicated in their names) were grown on agar plates: LXT1(a1) and LXT2(a2) for *M. lagerheimii* and 6P(a1) and 6D(a2) for *M. superbum*. Two days before seed infection, the plates were restreaked so that the freshest fungal colonies could be obtained. *Microbotryum* strains were resuspended in sterile water and the cell density was adjusted to an OD600 of 1. Next, an equal amount of adjusted a1 and a2 cell

suspensions of the respective *Microbotryum* species were mixed together. After 2 days, to confirm the mating interactions, ten microliters of a mated solution (as prepared in host plant infection) was pipetted onto a microscope slide. The mated *M. superbum* or *M. lagerheimii* was then viewed under the light microscope. The first infection began after seed sterilization in one collection of seeds wherein 20 microliters of the *Microbotryum* solution was put on top of each seed in the collection set aside for infection on 0.8% water agar plates. Two sets of plants were grown for each parasite-host interaction: Collection 1 contained uninfected host seeds and Collection 2 contained infected host seeds. Then, the plates were placed in a 14℃ incubator for 48 hours to promote the mating of the *Microbotryum* in the infected seeds. Next, the plates were placed in an environmental growth chamber at 16-20 ◦C. Once appropriate germination occurred, the seeds were planted in soil with the infected seeds in a different box than the non-infected. The infected seeds were re-infected as described above only with 10 microliters of the solution.

After roughly 8 days of germination time in an environmental growth chamber, the seeds (infected and uninfected) were planted in soil, but with separation based on infection status. The second “booster” infection occurred on the infected plants that were transplanted to soil; a solution of *M. superbum* or *M. lagerheimii* was prepared and applied to germinated seedlings in the same way as above.

Teliospore isolation and Mating-type Pairing Test

After following the plants' growth for 4 weeks, the infected *D. seguieri* plants had visible teliospores that were removed and isolated. Anthers containing teliospores were collected and shaken in distilled water containing 0.025% Tween 20. The solution containing teliospores was obtained and the concentration was adjusted to 4 $x10^6$ teliospores per mL. To obtain haploid

colonies, teliospores were germinated by plating 100 mL of the adjusted teliospore solution on water agar plates and incubating at room temperature for 24 hours. For the pairing test, the isolates and the tester 6P(a1) and 6D(a2) were resuspended in distilled water and the concentration was adjusted to 1 at OD600. Each isolate was mixed with each tester, and 20 mL of the mixture was plated on 0.8% water agar, which was incubated at 14 °C for 48 hours. Mated cells were confirmed by microscopic visualization of a "bridge" between two or more cells. For the pairings that could not be confirmed by microscopic visualization, PCR was performed to amplify the a1genomic region using primers a1Forward (5'-TGGCATCCCTCAATGTTTCC-3') and a1Reverse (5'-ACCTTCCTTCTCAACAGTG-3'). Genomic DNA was isolated. The PCR reaction was performed using TaKaRa ExTaq (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's instructions. Cycling conditions were denaturation at 94 °C for 4 minutes; 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 90 seconds; and final extension at 72 °C for 5 minutes.

Scanning Electron Microscopy

Anthers were collected and fixed in a modified Karnovsky solution (2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.05M sodium cacodylate buffer, $CaCl_2$ 0.001 M, pH 7.2). Fixed tissues were washed three times for 10 min each in 0.05M sodium cacodylate buffer. Then they were dehydrated in ethanol gradients of 30, 50, 70, and 90% for 10 min, and 100% for 10 min repeated three times, and critical points dried in CO_2. The samples were mounted on aluminum stubs and sputter-coated with gold. Images were acquired using a Thermo Fisher Apreo Scanning Electron Microscope, ETD detector under working conditions of 5 Kv and a working distance of 10 mm.

RNA Isolation from *Dianthus seguieri* and *Silene vulgaris* leaves for RNA Sequencing

Seedling fragments of infected and non-infected *D. seguieri* and *S. vulgaris* plants were collected at 6-leaf and 12-leaf stages. The samples were placed into a sterilized cold mortar and liquid Nitrogen was poured over top. The seedling fragments were then crushed into a fine powder. The RNA extraction was performed using the QIAGEN RNeasy Plant Mini Kit (Qiagen, Germantown, Maryland) following the manufacturer's instructions. The remaining DNA was removed using the Invitrogen DNA-free™ DNA Removal kit and confirmed by PCR using primers specific for the genomic DNA. Sample quality was confirmed by BioAnalyzer and sent to CD Genomics (Shirley, NY) for library construction and sequencing. Raw RNA-seq data for infected and non-infected samples were uploaded in Galaxy (Batut et al., 2021) and quality control was performed using fastqc. HISAT2 was used for alignments and DESeq2 was used for the pairwise comparison.

Results and Discussion

Seed Sterilization Studies

At the beginning of this investigation, seed germination studies were conducted to test the viability and appearance of different potential host species. A wide variety of Caryophyllaceae seeds had been collected in the lab over a long time period; it was necessary to determine the suitability of these seed types for infection and further experimentation. This approach also helped pinpoint seed types that were more adaptable to environmental stressors, improving the chances of successful cultivation in laboratory conditions. Moreover, testing multiple seeds also allowed for additional seed collection after appropriate growth.

As indicated in Table 1, a variety of seeds from species of Caryophyllaceae plants were tested in the germination studies; some of these seeds had been in the seed library for over 30 years and did not yield any or many viable seedlings, including *S. alba, S. latifolia, S. latifolia x S. alba 1, S. latifolia x S. alba 2,* double pollinated *S. alba, S. noctiflora,* and *D. armeria* seeds. Two other seed types tested: *Silene chalcedonica* and *Petrorhagia prolifera* also had poor results, however, their failure to germinate was likely due to issues in replicating environmental conditions in the lab that would have been required for positive germination. *S. chalcedonica* is a member of the Caryophyllaceae family native to Eurasia where the average temperature during prime growth season exceeds 39°C. *Petrorhagia prolifera* is native to northern Africa and the Middle East and also grows in its natural habitat at higher temperatures than could easily and reproducibly be replicated under lab conditions.

One species tested had an extremely high germination rate. All of the sterilized *Agrostemma githago* seeds germinated. Although the characteristics of the flower were not very different from the stereotypical Caryophyllaceae plants used for lab study (*Silene* and *Dianthus*

species), the seeds were much larger than those from most Caryophyllaceae plants. Apart from having a larger size, the *Agrostemma githago* seeds also had extremely thick seed coverings and germinated much faster than any of the other seeds tested in the germination studies. This species could potentially be used in the future for lab studies as it grew exceptionally well under laboratory conditions. Of course, this would necessitate identifying and isolating species of *Microbotryum* capable of infecting and parasitizing this host species.

Table 1: Seed germination studies were conducted to determine potential host plant viability. All seeds were part of the Caryophyllaceae family and could work as potential hosts for *Microbotryum* infection. Many of these seeds had been stored at 4℃ in some cases, for up to 30+ years, thus their ability to grow and sustain a pathogen was questionable.

Seed Type	Result
Silene alba	No germination
Silene latifolia	No germination
Silene latifolia x Silene alba 1	No germination
Industry *Silene alba* “White campion”	No germination
Silene latifolia x Silene alba 2	10% germination
Double pollinated *Silene alba*	10% germination
Silene noctiflora	No germination
Agrostemma githago	100% germianted
Dianthus armeria	Limited growth
Silene chalcedonica	Limited growth

Petrorhagia prolifera	No growth

Infection of *Dianthus seguieri/ Silene vulgaris*

The gene expression of *M. superbum*-infected *Dianthus pavonius* and *Dianthus seguieri* was investigated through RNA sequencing. Results demonstrated in Figure 2 show that *D. pavonius* showed more differentially expressed genes (DEGs) at the 6-leaf cotyledon stage (6,938) than at the 12-leaf cotyledon stage (3,442). Meanwhile, *D. seguieri* showed more DEGs at the 12-leaf cotyledon stage (5,719) than at the 6-leaf cotyledon stage (4,946). The total *Dianthus* plant genomes for *D. segueri* and *D. pavonius* analyzed included 56,139 predicted genes. Appressorium formation, the organ used for host infection, of mated cells was observed on plant tissue of *D. pavonius* and *D. segueri* seedling roots. Infected *D. segueri* plants flowered, but not *D. pavonius*, which requires the induction of its flowering process by exposure to winter climate conditions, also known as vernalization. Thus, limited conclusions can be drawn from *D. pavonius* as they have not yet completed their life cycle.

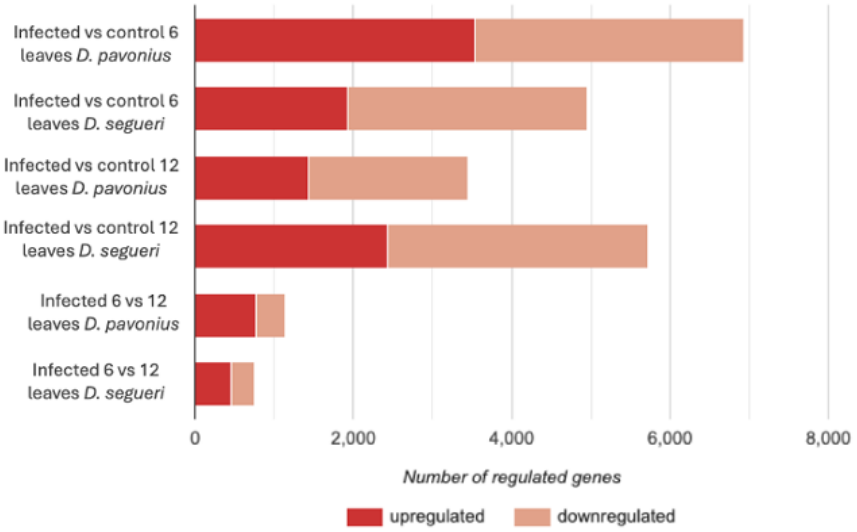

Figure 2: Summary of differentially expressed genes in 6 and 12-leaf cotyledon stages of *D. pavonius* and *D. seguieri* seedlings.

Teliospore Isolation and Mating-type Pairing Test

The life cycle of *M. superbum* and *M. lagerheimii* was diligently examined. Figure 3 illustrates the life cycle of *M. superbum* after host infection. 3(b) demonstrates the visible evidence of pathogen infection during *M. superbum's* takeover of the host plant reproductive structures. After *Microbotryum superbum* hijacked its host plant, replacing the pollen of *Dianthus seguieri*, the *M. superbum*'s teliospores as seen in 3(b) were collected and isolated. The teliospores demonstrated the linear tetrad character after germination, under light microscopy in 3(e). The teliospores were also viewed using SEM as shown in 3(d) and (f).

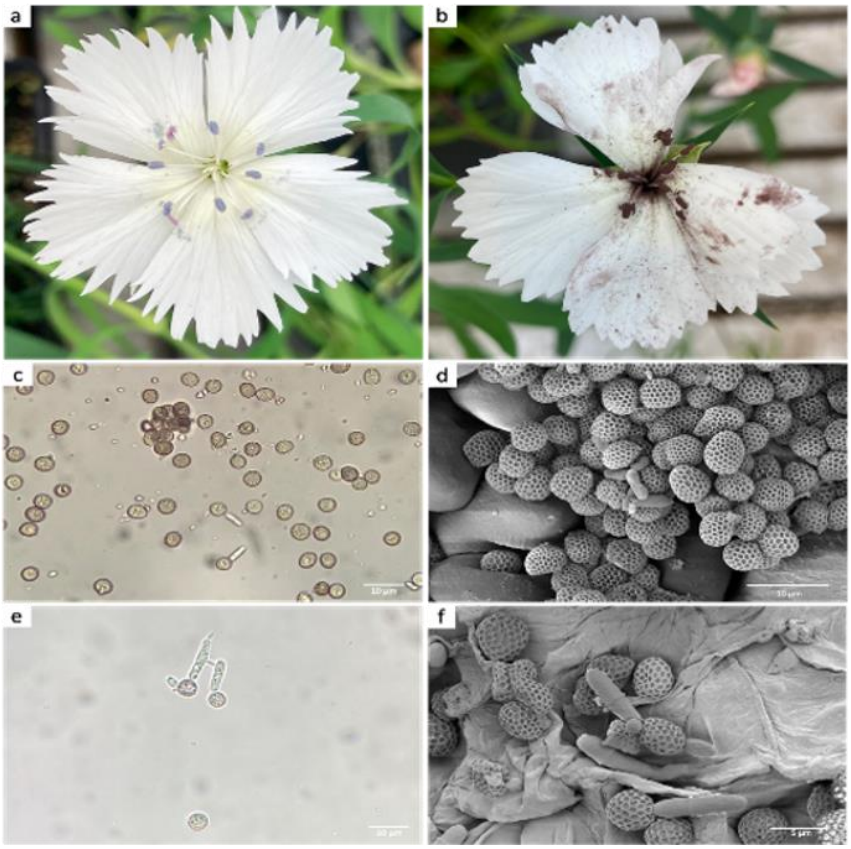

Figure 3: Uninfected and infected *Dianthus seguieri* flowers and *Microbtroyum superbum*-infected *Dianthus pavonius* teliospores. Uninfected flower (a) of *D. seguieri* showing anthers with pollen. Infected flower (b) of *D. seguieri* showing diploid *M. superbum* teliospores replacing pollen in the anthers. *M. superbum* teliospores from infected *D. seguieri* flower light microscopy (c, e) and Scanning Electron Microscopy-SEM (d, f).

The fungal spore characteristics of *Microbotryum superbum* and *Microbotryum lagerheimii* were examined through light microscopy and growth on YPD media. As shown in Figure 4 (a), the two mating types of *M. superbum*(6P and 6D) came together to form a bridge structure, indicating that the species was sufficiently undergoing mating as a prelude to sexual reproduction. This was also seen in (b) as the two mating types of *M. lagerheimii* came together to form bridge structures. It is through this mode of reproduction that the pathogen can develop

into a filamentous phase and then infect a host plant, thus the bridge formation was crucial to this study. Figure 4 also demonstrates physiological differences between *M. superbum* and *M. lagerheimii*. *M. lagerheimii* haploid sporidial cells are visually much smaller than *M. superbum* to the point that regular light microscopy barely allowed for the mating ability to be determined i*n M. lagerheimii*. The size of *M. superbum* haploid cells averaged around 9.5-10 μm while the size of *M. lagerheimii* haploid cells only averaged to be around 6-6.7μm.

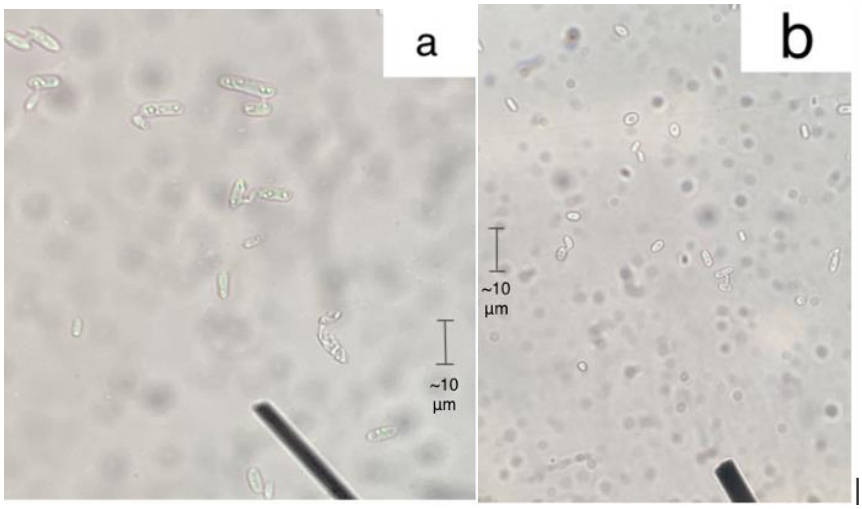

Figure 4: *M. superbum* and *M. lagerheimii* mated cells under light microscopy. The two mating types of *M. superbum* can be seen: 6P (a1) and 6D (a2) (a). This is demonstrated by the conjugal bridge formation between two of the *M. superbum* cells. Cells of the same mating type cannot form this bridge structure for mating. Similarly, the two mating types of *M. lagerheimii*: LXT1 and LXT2 can be seen in (b).

Mating-type analysis was performed on *M. superbum* teliospores to determine which mating-type was inherited. Forty isolated teliospores were placed under conditions with 6P and 6D such that mating could occur. Table 2 depicts the results of the prospective mating under light microscopy. As shown, the majority of the teliospores were able to mate with 6D haploid cells. For this to be the case, the segregants in the colony obtained after teliospore germination would have to have an a1 mating-type. Several of the interactions were difficult to decipher, so

further testing was performed. PCR and gel electrophoresis were performed on teliospore isolates 1, 2, 5, 6, 11, 18, 19, 22, 24, 26, 28, and 39. As demonstrated in Figure 5, only three of the bands matched the 6D band, the others matched the 6P band. Thus, the majority of teliospore isolates inherited the 6P (a1) mating type.

This provided an interesting insight into *Microbotryum* mating characteristics as the persistence of one mating-type in teliospores increases inbreeding as haploid colonies are forced to mate within tetrads. These findings support the idea that some *Microbotryum violaceum* species or isolates exhibit a mating-type bias in their progeny; this allows for the spread of deleterious mutations and lethal alleles while still protecting the organism from the harmful mutations by maintaining heterozygosity. (Hood and Antonovics, 2000).

Table 2: Mating type segregation in *M. superbum* teliospores was determined. The two mating types were viewed under the light microscope with known 6D (a2) or 6P (a1) cells. A "+" indicates that successful bridge formation occurred; " -" indicates mating did not occur. Combinations with a "?" indicate that results were inconclusive. Cell isolates with a "?" in the results were viewed multiple times under the microscope.

6D Haploid Cells	Mating (+/-)	6P Haploid Cells	Mating (+/-)
6D + 1	+	6P + 1	-?
6D + 2	+	6P + 2	-
6D + 3	+	6P + 3	-
6D + 4	+	6P + 4	-
6D + 5	-?	6P + 5	+
6D + 6	-?	6P + 6	+

6D + 7	+	6P + 7	-
6D + 8	+	6P + 8	-
6D + 9	+	6P + 9	-
6D + 10	+	6P + 10	-
6D + 11	+?	6P + 11	+
6D + 12	+	6P + 12	-
6D + 13	+	6P + 13	-
6D + 14	+	6P + 14	-
6D + 15	+	6P + 15	-
6D + 16	+	6P + 16	-
6D + 17	+	6P + 17	-
6D + 18	+?	6P + 18	+
6D + 19	-?	6P + 19	+
6D + 20	+	6P + 20	-
6D + 21	+	6P + 21	-
6D + 22	-?	6P + 22	+
6D + 23	+	6P + 23	-
6D + 24	-	6P + 24	+
6D + 25	+	6P + 25	-

6D + 26	+	6P + 26	+?
6D + 27	+	6P + 27	-
6D + 28	+	6P + 28	+?
6D + 29	+	6P + 29	-
6D + 30	+	6P + 30	-
6D + 31	+	6P + 31	-
6D + 32	+	6P + 32	-
6D + 33	+	6P + 33	-
6D + 34	+	6P + 34	-
6D + 35	+	6P + 35	-
6D + 36	+	6P + 36	-
6D + 37	+	6P + 37	-
6D + 38	+	6P + 38	-
6D +39	+	6P +39	+?
6D +40	+	6P + 40	-

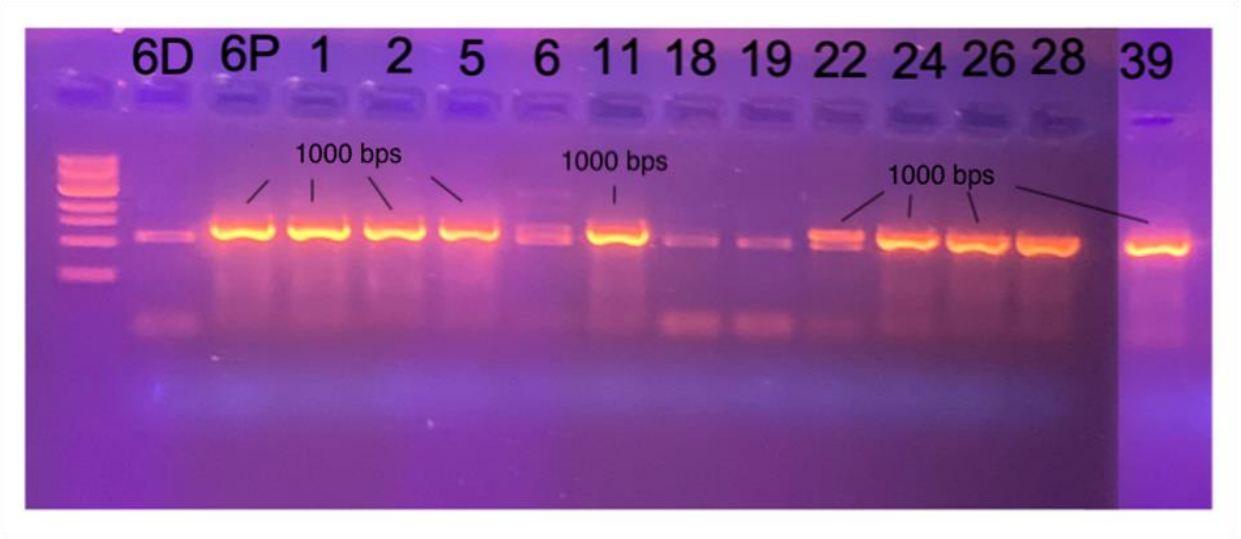

Figure 5: PCR of interactions from Table 2 that had inconclusive results was performed. After PCR, gel electrophoresis was performed on an agarose gel and confirmed the mating-types of those cells. Lane 1 used a 1 kb size standard (New England Biolabs). All of the bands matching the 6P band were 1000 bps.

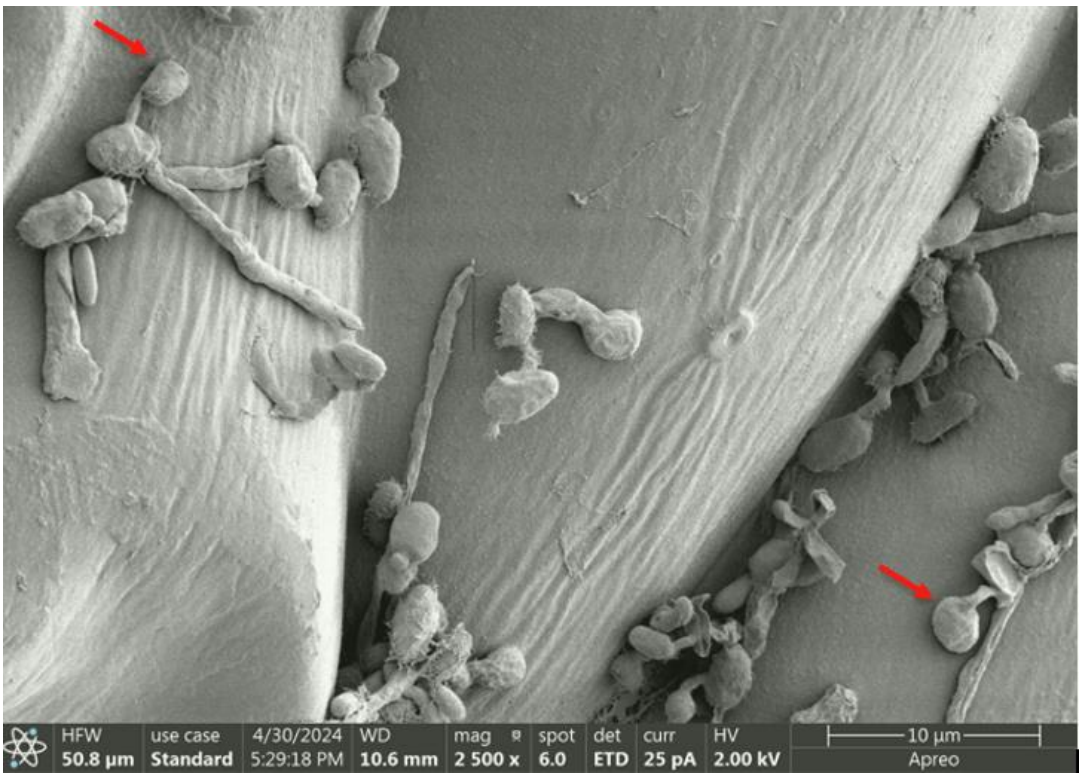

Figure 6: SEM image of *M. lagerheimii* presence on *S. noctiflora* seeds. The arrow indicates appressoria.

Conclusions & Limitations

The results of this study provide insights into host specificity and the infection mechanisms of *Microbotryum superbum* and *Microbotryum lagerheimii*. Characteristics of *Microbotryum violaceum* infection were investigated by the mating-type bias observed in *Microbotryum* teliospores. Most teliospores inherited the 6P (a1) mating type, a characteristic that encourages intratetrad mating and preservation of harmful, recessive alleles. The intratetrad mating, however, allows for heterozygosity to be maintained, thus the recessive mutation is not exposed and the fitness of the *Microbotryum* is not reduced.

Additionally, the gene expression analysis and reproductive behavior of the infected host plants demonstrate distinct host responses. The difference in DEGs between *Dianthus pavonius* and *Dianthus seguieri* suggests that host species react uniquely to infection, further shaping the specificity of *Microbotryum* pathogens. The ability of *D. seguieri* to flower under our laboratory conditions allows us to observe the completion of the fungal life cycle during infection. In contrast, *D. pavonius*, which requires vernalization prior to flowering, also demonstrates the complexity of host-pathogen interactions and the importance of environmental conditions in determining host suitability. Such host species provide an additional challenge for the investigator using such a model organism.

Thus, this investigation highlights the intricate factors that contribute to host specificity in *Microbotryum* species. These findings provide a framework for understanding how these pathogens exploit specific hosts for their reproductive cycles and how host characteristics can either facilitate or impede infection.

Some obstacles occurred in this study regarding the life cycle of *Microbotryum lagerheimii*. Three collections of *Silene vulgaris* were used with existing seeds found in the lab, and these did not germinate. These results prompted seed acquisition from a commercial source.

This source had been used in other studies, but unfortunately, the seeds acquired were not *Silene vulgaris* as they were marketed. The appearance of the seeds did not significantly differ from *S. vulgaris*, thus nothing out of the ordinary was suspected during seed germination. It was not until after 2-3 weeks of plant growth that it became obvious that the plants were not *S. vulgaris*. After consulting with multiple academic colleagues, it was determined that the plants were actually *Silene noctiflora*. *S. noctiflora* is characterized by small hair-like projections on its stems that secrete a sticky substance allowing it to catch insects. None of the other host plants in this study had this ability. Although these results were not anticipated, some interesting discoveries were made. *S. noctiflora* is not traditionally used as a host for *Microbotryum* infection. When infected with *M. lagerheimii*, the host anthers kept their function and were not taken over by the pathogen and no observable difference occurred in infected plants over the non-infected plants. However, when viewed under SEM, *M. lagerheimii* was observed on the *S. noctiflora* seedlings (Fig. 6), suggesting that the infection process was partially successful. The fungus also remained present in infected plants after performing PCR. Perhaps this infection process could be replicated in the future to establish a baseline for *M. lagerheimii's* ability to infect this plant.

The life cycle of *M. lagerheimii*-infected *S. vulgaris* is currently being observed, however, it has not yet reached a viable stage for RNA isolation and sequencing. In the future, further microscopic analysis of *M. lagerheimii*-infected *S. vulgaris* will be performed, as will RNA sequencing throughout different stages of the parasite life cycle. The ability of *M. lagerheimii* to partially infect the *S. noctiflora* contributes to the understanding of its host specificity characteristics and future infections with a variety of host plants will be undertaken.

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